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Cover photo: The shells and egg cases of, clockwise from top left, *Pomacea insularum*, *P. gnyanensis*, *P. diffusa*, and *P. haustrum*. Apple snails are an excellent system to address questions in evolution and biodiversity, see Hayes *et al.* 47–58.

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From Poe to Ponder...and Lindberg: Introduction to the symposium "Molluscs as models in evolutionary biology"*

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Known to students of our profession and concisely summarized most recently in Ponder and Lindberg (2008), Mollusca are, with an estimated 200,000 living species, one of the largest animal phyla, second only to the arthropods. The remarkably rich fossil record of molluscs throws light back into the earliest Cambrian revolution 543 million years ago, and ever since then we find them in nearly every ecosystem on Earth. The classes of living and fossil molluscs comprise an array of diverse animals with the most varied body plans, ranging from minute worm-like animals dwelling between sand grains on the beach to giant squids in the deep sea, and from microscopic snails in leaf-litter to giant clams in coral reefs. As objects of fascination, function, and food, molluscs play important roles in many cultures and societies. They include many taxa of immense economic significance, such as oysters, scallops, and squids; some bivalves produce precious pearls, and some snails carry diseases that infect millions of people, especially in the tropics.

Yet we feel that it is not only a curious fact in the history of science, but, unfortunately enough, much more a symptomatic indication of our discipline that it was not a professional naturalist or scientist with an interest in malacology, but the poet Edgar Allen Poe (1809-1849), who formulated an idea with much future. Poe was among the first to recognize and explicitly recommend that the study of molluscs requires a combined analysis, which in his times meant reconciling a classification based on hard shells with evidence from soft body anatomy (see details on this in the opening remarks to the symposium by Glaubrecht (2009)). This synthetic idea was long ignored by conchologists, who continued to classify molluscs almost exclusively based on features of their shell, while neglecting the soft body and the biological information that it holds. As a consequence, for a long time we knew few hard facts, for example, about the evolution and phylogeny of these soft-bodied animals but instead had much speculation by self-proclaimed authorities in the field.

In addition, most contributions in malacology long centered around morphology, anatomy, and in particular phylogenetic relationships within and among constituent taxa. Only rarely have molluscs been utilized explicitly as models for the study of the general aspects of evolutionary biology. However, molluscs, with their many features and facets, are highly suitable for providing some fundamental insights into the mechanisms of the genesis of biodiversity, its pattern in historical biogeography, and the underlying processes of speciation and radiation. An increasing number of recent studies and publications on molluscs reveal this rich potential.

Therefore, it was the aim of this symposium on molluscs as models in evolutionary biology, held during the World Congress of Malacology (WCM) in Antwerp from the 15th to 20th July 2007 (jointly organized by Unitas Malacologia and the American Malacological Society), to bring together experts and their expertise to provide—based on molluscs—some of those fundamental studies, and to show avenues for using data that are of relevance for evolutionary biology. With 43 talks over more than two full days of sessions (plus several posters), this symposium was the largest at the Antwerp WCM. Following the introduction, two invited keynotes or plenary lectures were given, one by Suzanne Williams and David Reid (on global pattern of diversity and speciation) and one by Thomas Wilke and Christian Albrecht (on genesis of biodiversity, focusing on ancient lakes). Other lectures covered a wide array of topics ranging from biogeography, shell morphology and evolution, molecular phylogenetics, radiations and extinctions as documented in the fossil record, to mitogenomics, and aspects of development and reproduction. From all these presentations, a selection of eleven contributions were made, and we invited the authors to work out their main subject as exemplars for their specific area of research, viewed from their individual perspective. Subsequently, eight of the original speakers have been able to provide manuscripts for the *American Malacological Bulletin*.

* From the symposium "Molluscs as models in evolutionary biology: from local speciation to global radiation" presented at the World Congress of Malacology, held from 15 to 20 July 2007 in Antwerp, Belgium.

Three other papers of those originally invited have been published elsewhere in the meantime, viz. Lindberg (2007) on a case study from the limpet *Scutellastra flexuosa* (Quoy and Gaimard, 1834) from Moorea, contrasting the respective roles of deep phylogenetic history with recent adaptations in shaping current ecological and life history characteristics, Hershler and Liu (2008) on vicariance and dispersal of hydrobiid springsnails in the southwestern United States, and Williams and Duda (2008) on biogeography and speciation.

We feel that together with these papers, the studies presented here dealing with phenomena from local speciation to global radiations, and including both the paleontological as well as neonotological perspective, underline the potential that molluscs have as models in evolutionary biology. The first contribution by Wilke *et al.* ("As time goes by...") provides a concise review of molecular clock methods and is at the same time a hands-on manual for molecular clock analyses. This contribution specifically is aimed at researchers in malacology, giving external clock rates for the cytochrome oxidase subunit I which may be applied for most aquatic molluscan taxa and even other invertebrates. Molecular clocks have become a standard tool in evolutionary biology and this review provides a sound basis for extending the use of molluscs as models. The next two papers by Hayes *et al.* on "Apple snails as a system for addressing fundamental questions" and by Holland and Cowie on "Land snail models in island biogeography" exemplarily highlight the use of taxa like freshwater and terrestrial gastropods for addressing major issues in evolutionary research. A common focus of both studies is on exploring patterns of biogeography, and in particular island colonization for the Hawaiian Succineidae and Achatinellinae. In addition, Hayes *et al.* also discuss the role of ampullariids in advancing knowledge of speciation and adaptation processes. Subsequently, Elejalde *et al.* (on the "land snail genus *Pyrenaearia*") uses these Iberian helicids to investigate the link between molecular data (DNA taxonomy) and classic taxonomy on one hand, and the role of climate in diversification on the other hand. While all papers so far rely heavily on molecular data, the last three papers in this symposium cover other important aspects of molluscs also relevant in evolutionary biology. Wesselingh and Renema ("Molluscan evolution from ancient long-lived lakes") offer a paleontological perspective on snail diversification in Miocene Amazonian Lake Pebas. Their focus is on the tempo and mode of evolutionary change in what we like to term a 'natural laboratory', as it has model characteristics also for interpreting evidence from modern long-lived lakes. The paper by Sigwart on "cladistic analysis in Polyplacophora" again focuses on a recent group, the living chitons, and aims at elucidating the role and value of morphological characters in tracking the phylogenetic relationships and evolution. In a nutshell, this paper questions the value of morphological characters in a molecular world. The final contribution, by Smirthwaite *et al.* on "the use of developmental sequences", offers a glimpse of molluscs in developmental biology, which is a renaissance issue in evolutionary research. Using freshwater pulmonates, the authors discuss the potential of molluscs to offer mechanistic explanations of ontogeny, including heterochrony.

We hope that these studies and the avenues they suggest will further facilitate the influence of malacology within evolutionary biology, even more so in the future than it was the case in the past. It is a pleasure to thank all who participated in this symposium during the Antwerp WCM meeting and, in particular, the speakers who contributed to what we felt to be a stimulating session, as reflected in the many discussions. We are most grateful to the authors that have contributed to this symposium volume and to Ken Brown, who kindly offered to publish these contributions as an outcome of the symposium. Finally, we would also like to thank Thierry Backeljau from the Royal Belgian Institute of Natural Sciences in Brussels, and at that time president of Unitas Malacologica, for inviting us (shortly after the Perth meeting in 2004) and then helping to organize this symposium in 2007.

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On "Darwinian Mysteries" or molluscs as models in evolutionary biology: From local speciation to global radiation*

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Abstract: Evolutionary biology is not only a biological subdiscipline but also a synthetic theory based on comprehensive scientific achievements. However, to date biodiversity, which is far from being fully documented, and the evolutionary processes leading to it are two of the least understood phenomena in evolutionary biology. Surprisingly, decades after the Modern Synthesis and centuries after the commencement of research in biological systematics, we are still unable to satisfyingly answer apparently simple yet fundamental questions. Here termed "Darwinian mysteries", these are for example, how many species inhabit Earth today, what are species, where are they distributed, and how did biodiversity originate. While many contributions in malacology center around morphology, anatomy, and phylogenetic relationships within and among constituent taxa, molluscs only rarely have been utilized explicitly as models for the study of general aspects in evolutionary biology. However, this particular group, with its many features and facets, is highly suitable for providing fundamental insights into the mechanisms that generate biodiversity, pattern in historical biogeography, and the underlying processes of speciation and radiation. Here, I discuss some aspects of these fundamental questions that are of relevance for evolutionary biology, hoping that the influence of malacology within evolutionary biology will increase in the future.

Key words: biodiversity, evolution, species, species numbers, species concepts

> *"Science is built of facts as a house is built of bricks; but an accumulation of facts is no more science than a pile of bricks is a house."*
>
> Henri Poincare, *La Science et l'hypothèse* (1902: 101)

Molluscs are not only one of the most spectacular animal phyla with great taxonomic diversity and morphological disparity but also have the potential to provide us with some of the most remarkable models in evolutionary biology. Actually, it has escaped many (and not only) malacologists' attention that molluscs were at the forefront of evolutionary theory in the first place. For example, they were instrumental in Jean-Baptiste de Lamarck's (1744-1829) first evolutionary views. When Lamarck (1801) introduced his then novel classification of invertebrates and included Mollusca as a class on its own, based on his study of the rich molluscan collection in the Paris museum, he also proposed in his introductory "*discours préliminaire*" a brief exposition of his later, much-debated evolutionary theory (Mayr 1982, Schilling 1989, Burkhardt 1995, Laurent 1997). Although Lamarck suggested an incorrect mechanism for evolution, in comparing fossil molluscs with recent species, he for the first time realized divergent evolution over geological time scales.

Molluscs were also the trigger, albeit not the focus, of one of the most famous controversies in the history of science, as it was the debate on cuttlefish anatomy that started the epochal debate in the Paris Academy of Sciences in 1830. Here again a group of molluscs was at center stage when Georges Cuvier's functionalism opposed Étienne Geoffroy Saint-Hilaire's "philosophical" morphology (Appel 1987, Le Guyader 2004).

Nevertheless, after this crucial epoch, malacology did not remain at the forefront of zoological discoveries and evolutionary biology. Although being little more than a marginal note in the history of science, to my view the following episode is not only a curious incidence but also a symptomatic indication for our field. Remarkably, not a professional naturalist or scientist with a keen interest in malacology, but the American poet Edgar Allen Poe (1809-1849) was among the first to recognize and comment that a reliable classification of molluscs requires a combined analysis, which meant in his times reconciling a system based on hard shells (as suggested by Lamarck) with evidence from soft body anatomy (as provided by Cuvier). Illustrated by 215 shells of molluscs, Poe (1839) published a scholarly and most successful textbook with a telling title, viz. *The Conchologist's First Book: or, A*

* From the symposium "Molluscs as models in evolutionary biology: from local speciation to global radiation" presented at the World Congress of Malacology, held from 15 to 20 July 2007 in Antwerp, Belgium.

system of Testaceous Malacology, arranged expressly for the use of schools, in which the animals, according to Cuvier are given with the shells, a greater number of new species added and the whole brought up as accurately as possible to the present condition of the science. And he explicitly distinguished between conchology as being merely the study of shells versus malacology as being the study of molluscs, *i.e.*, the anatomy of the whole animal including its most important soft parts.

After Myra Keen (1936) and Joseph Moldenhauer (1971) had remarked on this case of "literary curiosity", the late Stephen Jay Gould (1993, 1995), both a malacologist and evolutionary biologist himself, looked into this episode in more detail. Gould developed the argument that, irrespective of the plagiarism and piracy of Thomas Brown's earlier book, and Poe's functioning as a ghost writer and straw man for Thomas Wyatt (as is true for one other case; see Heartman and Canny 1943), it was indeed Poe himself who made the above mentioned important distinction. Although published under Poe's name, the book was essentially a less-expensive edition of Thomas Wyatt's (1838) own work *Manual of Conchology*, which plagiarized most of the text from British naturalist Thomas Brown's (1833) *Conchologist's Text-book*. However, since Poe evidently wrote the preface and introduction, then used the system of classification from Wyatt's work, but substituted for each genus a paragraph description of the soft parts (maybe taken over from Cuvier), it can be reconstructed that indeed it was Poe's own novel idea of a combined analysis.

Evidently, his early insight into what malacology should be was not borrowed from either Brown and/or Wyatt. Therefore, it remains a curious fact that Poe as a poet with only a marginal interest in science formulated an explicit conceptual reform (*i.e.*, the distinction between conchology and malacology), apparently an idea with much future. Poe's *Conchologist's First Book*, therefore, should be perceived now as more than just a literary curiosity and a collage from others' texts, as his emphasis of the eminent importance of anatomical features in the distinction and classification of molluscan species reveals innovative potential long unrecognized, paving the way toward malacology as a truly biological discipline.

Unfortunately, his insight was ignored for almost another century while conchologists continued to classify molluscs exclusively based on features of their shell while neglecting the soft body and biological information that it holds. Interestingly, following Lamarck's (1792) earliest attempts on classification of invertebrates, where the systematics of molluscs was solely based on shells, Lamarck (1809) later learned from Georges Cuvier's studies on soft-body anatomy, and acknowledged explicitly the importance of these morphological data for his classification (Corsi 1988: 62). Unfortunately, although Lamarck's classification of invertebrates in his original seven-volume *Histoire Naturelle des Animaux sans Vertèbres* (Lamarck 1815-1822) was widely known and used by many 19th century naturalists, his theoretical insights and reference to Cuvier's attempt were largely ignored. Continuing in this unhappy tradition, students of molluscs for a long period missed the chance to modernize malacology as a scientific discipline. The problem inherent to the traditional study of only shells, for example, for systematic-taxonomic purposes was that no other sources of information were utilized to evaluate the diagnostic value of shell features. This stands in stark contrast to other disciplines like ornithology, where the so called "*new systematics*" provided the foundation for developing the modern theory of evolution (see Glaubrecht 2007, references therein). In the context of malacology developing as a biological science, a study of systematics vs. merely "stamp collecting" (Glaubrecht 2004: 117), I add here as surely more than coincidence that one of Britain's former leading conchologists, Francis James Stainforth (1797-1866), who was a supplier of many rare shell specimens to Lovell Reeve, also initiated in the early 1860s in London the first stamp collector's club, thus helping to found philately (Allen 2008).

However, molluscs are much more than only collectors' items. With their many features and facets, they are highly suitable for providing some fundamental insights into the mechanisms of the genesis of biodiversity, its pattern in historical biogeography, and the underlying processes of speciation and radiations. As recently summarized by Ponder and Lindberg (2008), with about 200,000 living species the Mollusca are one of the largest animal phyla, second only to the arthropods. The remarkably rich fossil record of molluscs enlightens the earliest Cambrian revolution some 540 million years ago, and ever since we find them in nearly every ecosystem on Earth. The seven or eight classes of living molluscs plus two extinct class-rank taxa comprise an array of most diverse animals with most varied body plans, ranging from minute worm-like animals dwelling between sand grains on the beach to giant squids in the deep sea, and from microscopic snails in leaf-litter to giant clams in coral reefs. As objects of fascination, function, and food, molluscs play important roles in many cultures and societies. Molluscs include many taxa of immense economic significance, such as oysters, scallops, and squids; some bivalves produce precious pearls, and some snails carry diseases that infect millions of people, especially in the tropics.

Yet, for a long time we knew few hard facts, for example, about the evolution and phylogeny of these soft-bodied animals. For the greater part of the last century, it was Johannes Thiele's (1929-1931) epochal *Handbuch der Systematischen Weichtierkunde* that long provided the standard in molluscan systematics. For his classification, Thiele evaluated characteristics from the shell but consequently used a synthetic, albeit pre-cladistic manner when he included the

most important radula features as well as other anatomical characters following a tradition started by Troschel (1856-1863). Although Thiele sometimes erred, for example when he discarded aplacophoran molluscs as "worms" belonging to the phylum Annelida (see Glaubrecht *et al.* 2005), his systematization was a highly influential masterpiece. Another curious fact was that when Thiele's handbook was translated into English and re-published by Bieler and Mikkelsen (1992), a substantial amount of new data from both morphology and molecular genetics as well as from paleontology had begun to accumulate, too diverse for a single malacologist to master.

New tools—such as fluorescence-coupled antibody staining and confocal laser-scanning microscopy, in concert with new approaches such as computer-assisted cladistics, which allow the recurrent testing of phylogenetic hypotheses under various models and assumptions—have generated a renewed interest in reconstructing evolutionary history with molluscs in a key position (Valentine 2004, Minelli 2009a). Consequently, within the last two decades our understanding of molluscan phylogeny has undergone a remarkable, if not even revolutionary, transformation that is about to change fundamentally the classification of phyla. Ponder and Lindberg (2008) provide a collation badly needed when virtually every phylogenetic tree calculated from another partial gene fragment of more or less randomly represented taxa is considered publishable.

While many contributions center on morphology, anatomy, and phylogenetic relationships within and among constituent taxa, molluscs have only rarely been utilized as general models for the study of evolutionary biology. Molluscs rarely make it into textbooks on evolutionary theory, and are highly underrepresented when it comes to discussing evolutionary concepts and/or phenomena by example (Ridley 1996, Futuyma 1997, Mayr 2001, Barton *et al.* 2007). For only one more recent example, in a very readable book on evolutionary pathways by Avise (2006), only two cases (coiled vs. uncoiled shells and land snail chirality) explicitly referred to recent studies utilizing molluscs. Undoubtedly, however, molluscs have so much more to teach us.

As evolution is the vibrant foundation for biology, evolutionary biology in this context has a double function. It is not only a biological subdiscipline but also a synthetic theory and discipline on its own, based on comprehensive scientific achievements. Unfortunately, malacologists in particular have followed one of the most prevailing methodological claims that gathering facts should be the primary role of naturalists (Johnson 2005) which often discourages explicit references to evolutionary theory. In addition, as taxonomy and systematics once marked the beginning of zoology in general, it is still among the primary interests of many malacologists. My point is that malacology has more potential to contribute to evolutionary biology than previously assumed or revealed. Taxonomy, albeit not experimental, does not have to remain merely descriptive, but should be hypothesis-driven as well as drive hypotheses. It forms an integral part of evolutionary biology, as taxonomic facts are a prerequisite to the proper formulation of evolutionary and ecological questions (May 1992, 1999).

However, although systematics continues to lay the foundation for many disciplines of the life sciences, its ultimate goals of providing an inventory of biodiversity, and reconstructing a tree of life (Glaubrecht 2007), are far from being achieved in general or in molluscs in particular. The *diversity* of organisms, and the morphological *disparity,* as well as the evolutionary processes leading to it, are the least understood phenomena in evolutionary biology. Some facts and reasons why biological diversity is far from being discovered, or its origin understood, will be briefly highlighted here.

THE SIX "DARWINIAN MYSTERIES" IN BIOSYSTEMATICS AND EVOLUTIONARY BIOLOGY

A love of science has much to do with its mysteries that drive basic scientific research; questions are often considered more important than answers in shaping the future of science and its disciplines. Fundamental questions can be used as guidelines for future, cutting-edge research and reveal opportunities to be exploited, starting from the supposition that scientists should answer these questions over the next quarter century (Kennedy and Norman 2005). In this context, the apparently simple question "What determines species diversity?" was recently ranked among the 25 "big" questions, based on how fundamental they are, how broad-ranging, and whether their solutions will impact other scientific disciplines (Pennisi 2005). Among the others were how Earth's interior works, the composition of the universe, and whether we are alone in it, or whether the laws of physics can be unified.

Highlighting our scientific ignorance, it is surprising that centuries after the commencement of research in biological systematics and decades after the Modern Synthesis of evolutionary biology, we are still unable to answer a series of simple questions linked to this big question on biological diversity. Today, 300 years after Carl Linnaeus (1707-1778) and 200 years after Charles Darwin (1809-1882), both biosystematics and evolutionary biology are still left with the following six "Darwinian mysteries" (Glaubrecht 2003, 2004, 2005, 2007). One and a half centuries after *On the Origin of Species* by Darwin (1859), these questions, all relevant to his (r)evolutionary theories, are all largely unanswered, irrespective of the many fruitful attempts to solve them.

(1) Species numbers: How many species are there?

(2) Species concepts: What are species and how do we know?
(3) Speciation: How do new species evolve?
(4) Biogeography: Where are the species and why are they distributed there?
(5) Phylogenetics: How are species (groups) related?
(6) Genetic causation and molecular processes: How do new forms come into being?

Undoubtedly, answering these questions will ultimately help to unravel some of the most important issues not only in biosystematics, but also in evolutionary biology. To accomplish modern systematists' tasks, viz. quantifying biodiversity, establishing phylogenies, and understanding the evolutionary process of speciation and radiation, several prerequisites are indispensable, albeit neglected even in many modern systematic approaches. Malacology, with one of the richest animal phyla at hand, both in terms of species numbers and biological phenomena, can have its share in solving these "Darwinian mysteries". I outline here only the first three of these Darwinian mysteries in more detail.

(1) The mystery of species numbers: A Linnean enterprise

As taxonomy and systematics provide the reference system for all biology (Wilson 1989), compiling, organizing, and updating taxonomic information has an urgent priority. After decades of de-emphasizing biosystematics (Whitehead 1990, Mikkelsen and Cracraft 2001), and recent taxonomic modernization and renaissance (*e.g.*, Godfray 2002, 2007, Mallet and Willmott 2003, Wheeler 2004, Dayrat 2005, Glaubrecht 2007), the reawakening interest in taxonomy has also led to realization that most animal species, especially invertebrates like molluscs, are far from being discovered yet.

In fact, it can be called the "Linnean shortfall" that, while knowing how many atoms are in a molecule, how many craters are on the moon, and how many stars are in the Milky Way or galaxies in the universe, we still have not learned how many species of butterflies live on tropical trees, how many buccinid gastropods are in the sea, freshwater melanopsids around the Mediterranean, or camaenids in Australia. For way too long a period of time, zoologists seemed to perceive "a number of undescribed creatures rather a nuisance", as Darwin long ago complained (Keynes 2003). Nearly three centuries after Linnaeus's first attempts to inventory nature, his task remains a daunting challenge for systematists, including malacologists.

Surprisingly, only recently has it become apparent that we lack the most fundamental data on biodiversity, viz. systematic inventories on any organismal, ecological, and geographical level (*e.g.*, Wilson 1988, 1992, Raven and Williams 1997, for molluscs see Bouchet 1997). Recognizing and describing the living species of plants and animals on Earth, however, is a major task, calling for a large-scale approach to taxonomy (Raven and Wilson 1992, Wilson 2000, 2003, Lawler 2001, Mikkelsen and Cracraft 2001, Blackmore 2002, Wheeler *et al.* 2004, Stork 2007), comparable to the Human Genome Project or the NASA next generation space telescope and Sloan Digital Sky Survey. Indeed, it remains an unfinished Linnean challenge to inventory the many species that exist on Earth.

Estimates of the number of living species in the world, given by Terry Erwin (based on neotropical insect diversity) as up to 30 million species, have triggered more specific attention, as the question 'How many species are there?' has finally been regarded as scientifically important (Erwin 1982, May 1988, 1990, 1992, 1994, 1999, Stork 1988, 1993). Estimates of the total number of species vary now from 5 to over 50 million, using various direct and indirect assessments. Over the last two decades, these global estimates dropped to a total of 5 to 15 million species (Stork 1993, Odegaard 2000). A most comprehensive compilation of species numbers has been provided by Chapman (2005), who settled on between 8 and 9 million species.

In this context, the discussion has largely centered around the question of what fraction of the insect species found on a given host-tree is likely to be effectively specialized on it, on species-size relations, or in food web structure (May 1990). For example, after having taken into account the host specificity in particular of herbivorous insects, which is essentially responsible for driving most of these species number estimates, the figure has recently been corrected to 4.8 to 6.6 million species (Novotny *et al.* 2002, 2007). The latter authors attempted to reconcile an order of magnitude discrepancy between extrapolations based on ecological samples with those based on sampling regional faunas or estimates based on taxonomic collections. However, it is doubtful that neotropical, herbivorous insect diversity is a direct function of plant species number (comprising taxonomic and architectural diversity), suggesting that additional factors like the existence of morphologically cryptic species and distributional ranges would again increase global species numbers (Dyer *et al.* 2007, Condon *et al.* 2008). Surprisingly, very little solid data has been contributed to the discussion of species richness from other invertebrates, such as molluscs or for aquatic biota, with the notable exception of Bouchet *et al.* (2002) for marine molluscs, that may account for a large fraction of all marine invertebrate species. Therefore, not even the magnitude of the world's diversity is currently known to systematists, let alone exact figures for particular groups of animals. Although it is agreed now that we desperately need a biodiversity assessment comprising a complete inventory of life, we are still challenged to develop rough estimates on the quantity of species as the units of biodiversity studies and evolution (see below).

Irrespective of a more exact estimate of the number of species, we are faced with a tremendous task, given that only

approx. 1.8 million animal species have been properly described (Stork 1988, May 1990). Amazingly, not even the number of species that have already been named and recorded is known precisely. Thus, not only does Linnaeus lag so far behind Newton (given our knowledge about the universe compared to the living world), but more importantly at the level of individual taxonomic groups we see major differences in the accumulation of recorded numbers of known species, with most invertebrate groups being largely underexplored. May (1990, 1992) has calculated the rate of discovery from the times of Linnaeus (1758) up to 1970 and 1990, respectively. In Fig. 1, vertebrates are illustrated by the most representative group, birds, versus an invertebrate group, arthropods. Expressed as a fraction of those species known in 1990, half of all known bird species were already recorded in the century after Linnaeus, by 1845. In contrast, only half of the arthropods known in 1990 were described within the preceding three decades. The "furries and featheries" are very well known by now, at least at the genus level, with only 134 bird species being added to the total of over 9000 since 1934 (Diamond 1985) and only a few mammal species. With respect to invertebrates (including, of course, molluscs, see below), we have just started to deal with the Linnean challenge of a species inventory.

We also have to face the fact that most biological assessments largely use focal groups, *i.e.*, vertebrates and vascular plants, but few invertebrate groups such as butterflies. They all comprise not more than five percent of the known diversity while major environmental players (bacteria, fungi, mites, nematode worms, and beetles) are largely unknown, underrated, and underestimated (May 1988, 1992). The major part of diversity in most invertebrates, the other 99% as it has been called (Ponder and Lunney 1999), is therefore still hidden. In Fig. 2 the most recent figures for individual animal groups used by Chapman (2005) is shown, giving the number of known and of estimated species, respectively, for Mollusca as 70,000 to 120,000 species. Other figures of the number of described species of invertebrates were in the past rightly criticized for not only being a matter of great difficulty but also of little value, and those in most textbooks of zoology were long simply copied from each other. Currently, only a few studies are available for global estimates of specific taxonomic groups, such as (1) arthropods and more specifically Hymenoptera (Odegaard 2000, Dolphin and Quicke 2001), (2) on some crustaceans trying to also quantify the underestimation (Adamowicz and Purvis 2005), or (3) on marine fishes (Mora *et al.* 2008). However, how much do we as malacologists know about the various subjects of our special interest among the marine, freshwater, and terrestrial taxa? With the world's oceans and tropical rain forests teeming with life forms, each new survey increases the window through which we see this living world. Our museum collections are already filled with nearly endless numbers of scientific hypotheses, biological tests, and questions. However, can we hope that we will eventually understand the biosphere if we do not even struggle to make comparable inventories of our knowledge on these new and old discoveries?

It is estimated that we currently add roughly 10,000 new animal species each year (May 2004), among which are well over 400 new species of Mollusca (Bouchet 1997), discounting for past synonymies (see discussion below). With this plethora of new species and taxa continuously being found, however, we do not even attempt to list how many new species *exactly* are described annually, or how many await description. Yet we lack, curiously enough, several of the most important parameters or instruments to compile a complete list of species on Earth. To begin with, we still have an incomplete taxonomic database for the known species in the world, as no

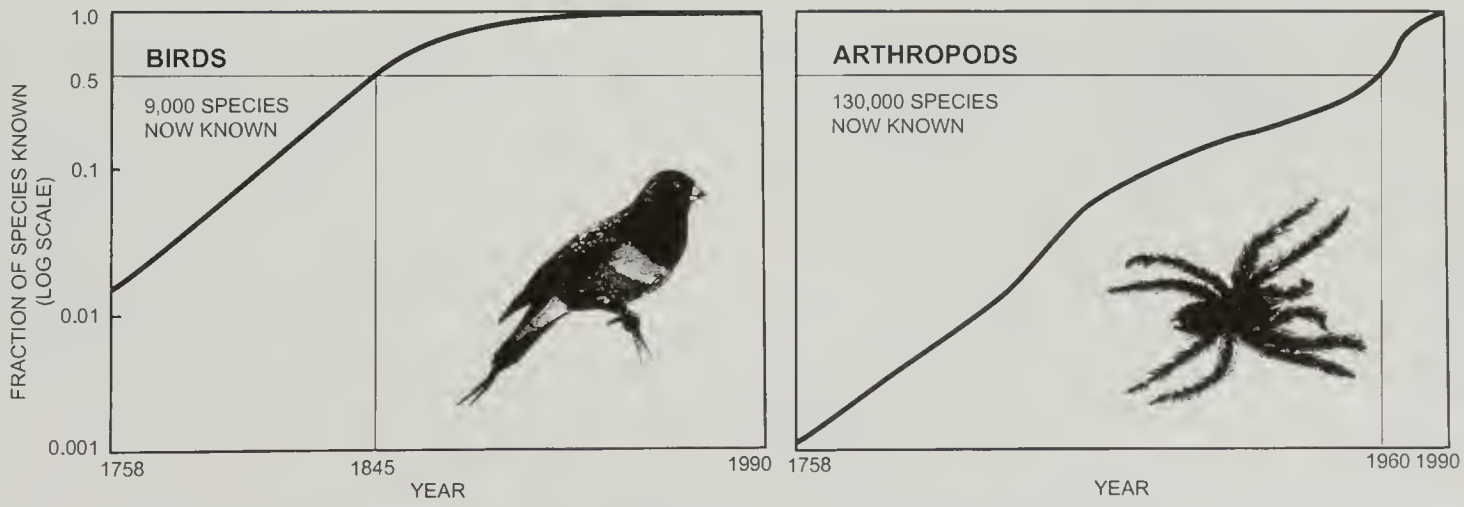

Figure 1. The rate of discovery from the time of Linnaeus (1758) up to 1990. Accumulation of species for a vertebrate representative group, birds, versus arthropods (excluding Insecta) expressed as a fraction of those known in 1990 on a logarithmic scale plotted against time. Note that half of all known birds were already described by 1845, in contrast to 1960 in arthropods. Modified from May (1992).

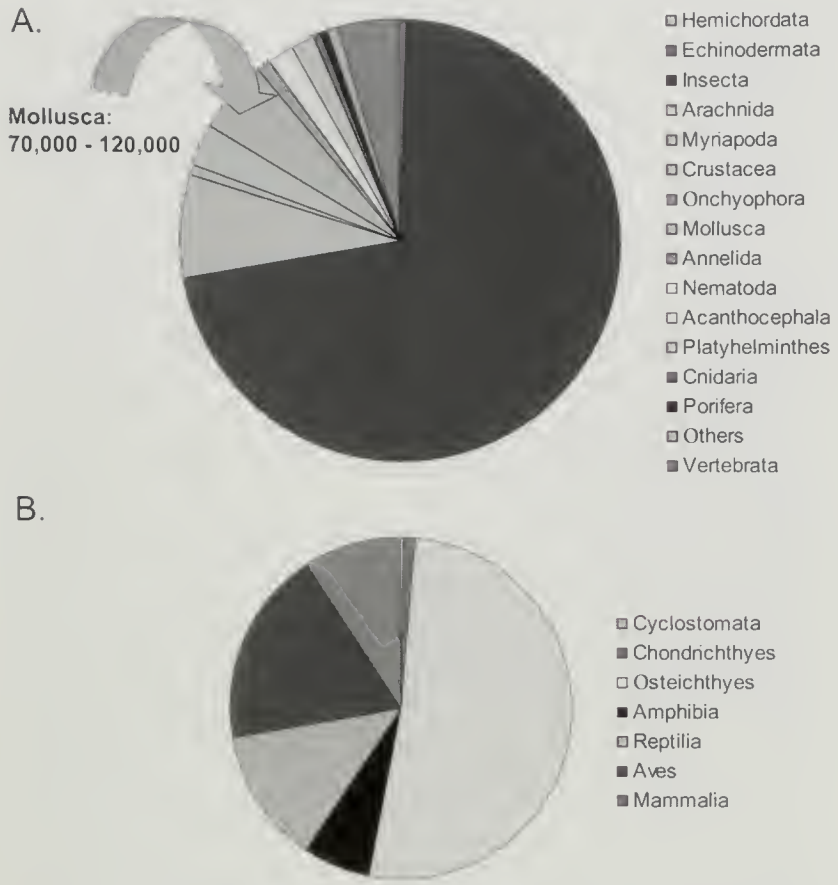

Figure 2. Numbers of known animal species: A, all major taxa; B, vertebrates. Note that for Mollusca the figures is given as 70,000 known species, with the most recent estimate of 120,000 species total. Modified from Chapman (2005).

synoptical and coordinated catalog or compilation exists, nor is any effort made to comprehensively list all named species, in particular for invertebrates. This situation basically leaves us with the *Zoological Record* as the only (albeit with an omission rate of over 20% of new molluscan names) far from complete compilation of supra-specific names (Bouchet and Rocroi 1992, 1993, Edwards and Thorne 1993). It has been suggested to separate this registration of all new names and nomenclatorial acts from the scientific content of taxonomic papers (Minelli 2003a). However, recently the ICZN has set up a new system, called ZooBank, that requires species descriptions to be registered online (Polaszek 2005). Bouchet and Rocroi (1992, 1993) also suggested that names should be registered before they are declared available.

Inventorying the malacofauna

Molluscs as the second largest animal phylum are not an exception to this Linnean challenge as outlined above, with exact figures on the number of living and/or fossil species lacking and extrapolations widely variable. Linnaeus in the 10th edition of his *Systema Naturae* in 1758 described among the 4,236 animals listed (there were 549 species in his first edition of 1735) nearly 700 species of molluscs (listed under "Testacea" that also included brachiopods). Half a century later, Lamarck assumed in his classification 135,000 species of known invertebrates (Laurent 1997).

One of the earliest attempts to more precisely extrapolate the number of living species for all classes of animals from representatives in museum collections was done by the director of the Berlin natural history museum, Karl August Möbius (1825-1908), as detailed in Glaubrecht (2008c). In 1898, using the roughly 30,000 species (with an estimated number of 300,000 specimens) then extant in the Berlin collection (albeit at that time still united with the brachiopods), Möbius arrived at the very conservative figure of 50,000 known species of molluscs (out of or among the well over 400,000 species in total that he at that time estimated). More than half a century later, using instead the available compilations of generic and subgeneric names, Franz Alfred Schilder (1896-1970) tried to estimate at least for prosobranch gastropods, one of the major classes, the number of fossil and extant families. For this subclass alone, he estimated 415 families and about 20,000 genera, a total of 150,000 species (Schilder 1947). He later extrapolated the number of all living molluscs as 250,000 species (among an estimated 3 million animal species), assuming that about half of all species were described at that time (Schilder 1948, 1949).

Later, Muller and Campbell (1954) gave the total number of known molluscs species as a much more reduced 73,000, of which over 41,000 (57%) are living species and over 31,000 (43%) fossil species. Later, Boss (1970) doubted the total of 107,000 living species, with 58,000 marine, 14,000 freshwater, and 35,000 terrestrial molluscs estimated at that time (Nicol 1969). Boss (1971) arrived at only about 35,000 species of molluscs known and, guessing the number yet to be described, he suggested a total of nearly 47,000 molluscan species (with 37,500 gastropods and 7,000 bivalves), later corrected to about 60,000 Recent species.

As noted earlier (Bouchet 1997) molluscs are a group where the number of described species is problematic, ranging from 45,000 to 150,000. For example, May (1990) gave the estimated number of species in this group recorded scientifically to 1970 as 45,000, with 1887 to 1899 being the peak for discovery of new species. According to his estimates, it has taken over 70 years prior to 1970 to record the second half of this total number of 45,000 species. However, May (1988) listed Mollusca with about 100,000 recorded species, estimating the research effort devoted to molluscs (from the average number of publications per year in the *Zoological Record* from 1978 to 1987) as comparable to Lepidoptera and Hymenoptera, but an order of magnitude less than in birds or mammals. Baillie *et al.* (2004) gave a total of 107,718 described molluscan species (75,000 gastropods, 30,000 bivalves, 768 cephalopods, and 1,950 others). These estimates are closer to the one given in Chapman (2005), who arrived at an estimated number of 120,000 species. However, Ponder and Lindberg (2008) tentatively figured that there might be 200,000 extant

molluscan species. For marine molluscs alone, Bouchet (2006) estimated 52,500 species worldwide.

However, as assessments of species richness are confronted with a plethora of difficulties, reliable figures are still wanting. Only rarely are surveys on mollusc species available to date, even for particular classes or larger subgroups. Therefore, we can only roughly extrapolate where the species in Mollusca are, *i.e.*, which molluscan taxa hold how many described species. Using various catalogues and checklists as well as the *Zoological Record* for taxonomic papers (although the later is not without its deficiencies), Bouchet and Rocroi (1992, 1993) compiled the nomenclaturally available genus-group names for the classes of all Mollusca and Recent Mollusca that were introduced since Linnaeus' time (1758) until 1989 (Fig. 3). While cephalopods dominate the fossil record with ca. 34% of all named molluscs, bivalves make up ca. 17% among the living taxa. Depending on the method, Bouchet and Rocroi (1992) found between 25,000 and 28,000 supra-specific names, with nearly half of them (all Mollusca) or two thirds (among Recent Mollusca only) for gastropods. They gave the number of genus-group names of Recent molluscs at about 12,000. Before their compilation, Schilder (1947), in his attempt to estimate the number of species in prosobranchs, suggested that there might be 20,000 genus-group names for all Molluscs, with some 5,000 taxa still being extant while Vaught (1989) arrived at 15,000 genus-group names. With an average of 224 new genus-group names per year, the annual increment has remained relatively stable for molluscs since the late 19th century, thus mirroring the same increasing rate of discovery described by May (1990, 1992) for other invertebrates (Fig. 1).

Several compilations I found in the literature exhibit this pattern. Thus, with respect to molluscs we surely continue to live in the golden age of discoveries, with a plethora of new species yet to be found and described. As only one of the many cases stated here, we continue to do this for a small group of freshwater gastropods endemic to the island of Sulawesi (Rintelen and Glaubrecht 2003, Rintelen *et al.* 2007). Bouchet (1997) figured this progressing species inventory in selected groups of Recent molluscs, among them a few groups of marine gastropods, land snails and bivalves (Fig. 4A). The same general trend can also be seen in the data compiled with a much more restricted focus, for example, by Vermeij (1999) for species with shells having a labral tooth although there are two peaks in the number of described species (Fig. 4B). Here, we have charted the progress of knowledge on two randomly picked groups of molluscs, also using Linnaeus and 1758 as starting point, figuring the cumulative number of known species over the subsequent centuries (Fig. 5). As is evident from these data compiled here for Cephalopoda and *Conus* Linnaeus, 1758, both taxa reveal the one trend already discussed above for invertebrate taxa in general versus vertebrates (Fig. 1).

In his compilation, Bouchet (1997) estimated that, on average, 1,395 new species-group molluscs are named each year (69% are fossil species, leaving 430 living species per year), with the number of new marine species described each year increasing by 68%, while the number of new non-marine species decreased by more than 15% over the past three decades. Nevertheless, Bouchet (1997: 1) also saw no sign of leveling off in the inventory of molluscan diversity, concluding that "for the foreseeable future, micromollusks, the deep-sea, and the marine and non-marine tropics will remain effectively inexhaustible reservoirs of undescribed species". Actually, there is evidence of hitherto unrecognized taxonomic groups among molluscs from all biotopes. Thus, an inescapable conclusion emerges about a largely uncharted biosphere, and that we are still far from having completed the catalogue of life. However, not only are many biotas incompletely studied, but also our lack of knowledge limits our ability to comprehend and respond urgently to the biodiversity crisis, viz. the loss of many of Earth's species. Only one of the many reasons is certainly that institutional and financial support for systematics and natural

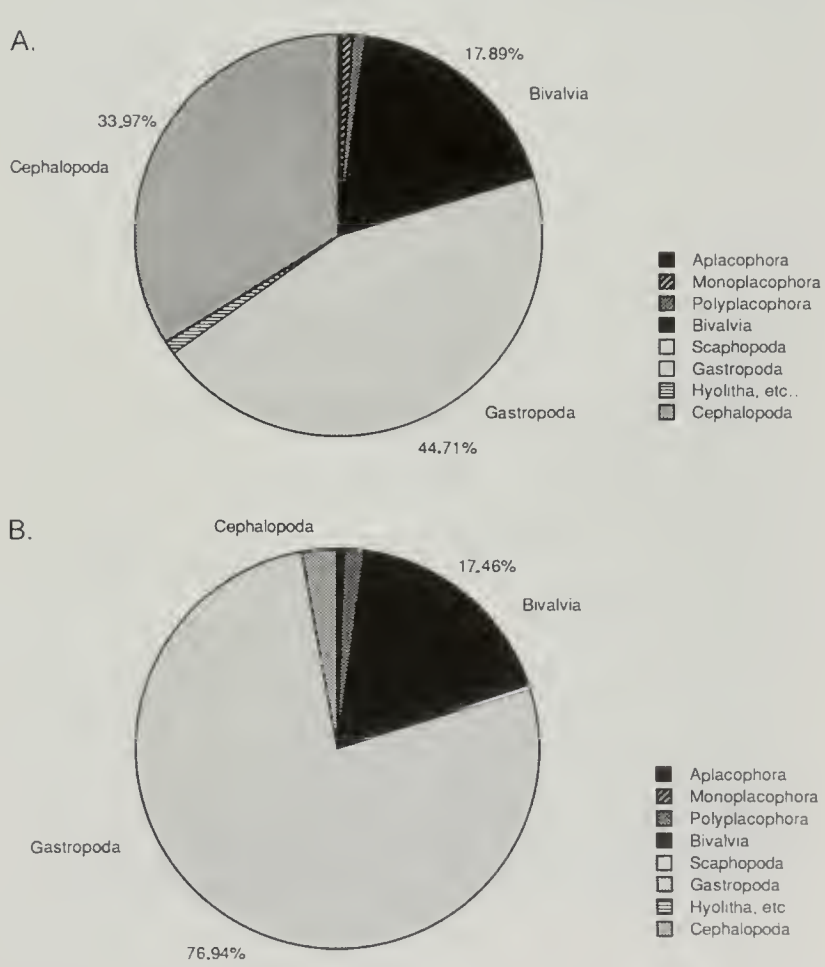

Figure 3. Where are the species in Mollusca? A, genus-group names of all Mollusca, introduced since 1758 (until 1989), partitioned by class, with percentage given for main taxa. B, genus-group names of only Recent Mollusca, introduced from 1758 to 1989, partitioned by class, with percentage given for main taxa. Both figures modified from Bouchet and Rocroi (1992).

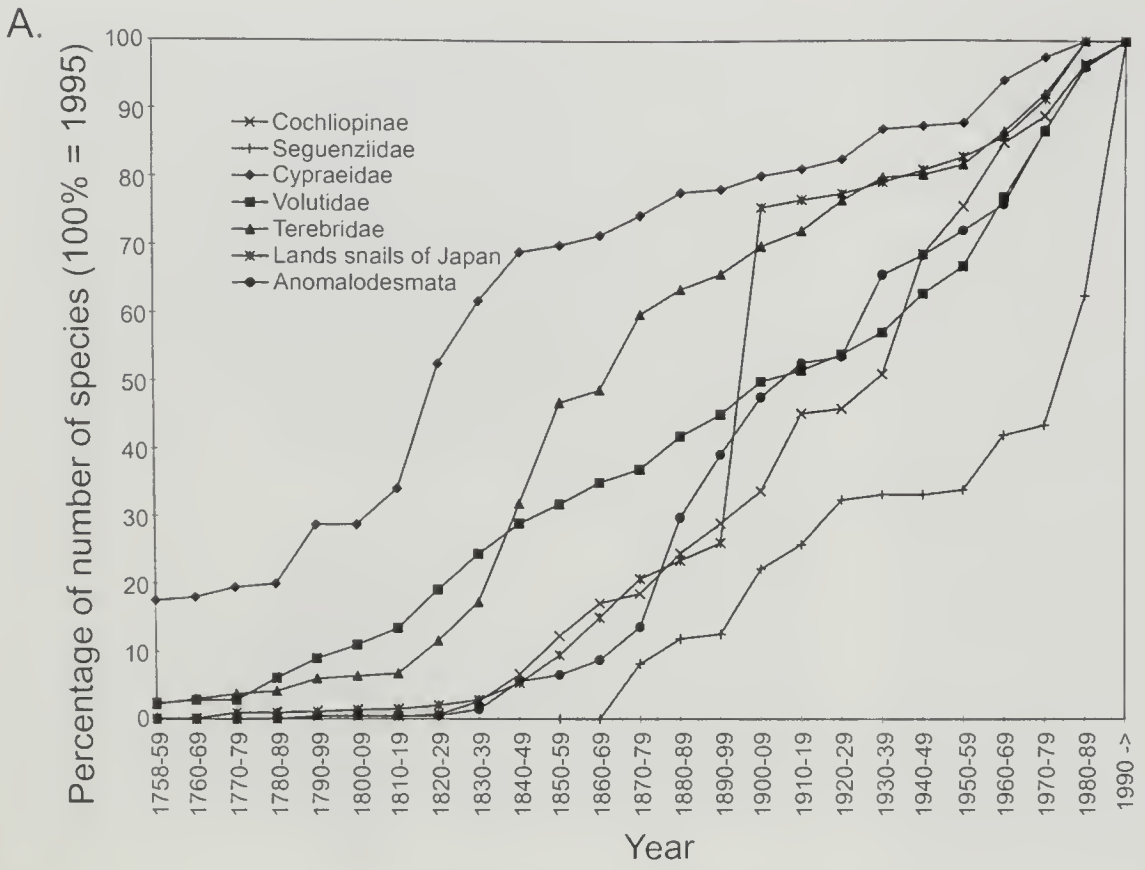

Figure 4. Inventorying the malacofauna. A, number of species of selected molluscan groups since 1758, plotted as a percentage of the known number of species in 1995 (from Bouchet 1997). B, number of gastropod species with a labral tooth described per 20-year interval from 1740 to 1999 (from Vermeij 1999).

history studies have continued to dwindle for several decades now, resulting in a situation called the "other" taxonomic impediment (Evenhuis 2007). At the same time, there have been various claims that this situation will require a fundamental restructuring of the way we do systematics (Godfray 2007), and calls for accelerated biodiversity assessments, in particular suggesting molecular taxonomy (and DNA barcoding) as the proper way out (see below). I claim that this barely faces the real and fundamental problems, as there are still other Darwinian mysteries involved that are not tackled or solved by this approach. These problems center on the question if species are real and how many are valid.

(2) The mystery of the species concept: "*Defining the undefinable*"

May (2004) noted that detecting new species in the field will remain the rate-limiting step to tomorrow's taxonomy. Species are life's mosaic across space and time. However, there is a fundamental disagreement among systematists of all kinds and expertises about what these species are and what we can regard as the functional unit in evolution on the one hand and in taxonomy on the other hand. Searching for biologically meaningful entities, *i.e.*, species as they are conventionally called, as well as how to define and delimit them has been a century-long challenge, and its full understanding needs to take into account an equally long history which cannot even be outlined here. Discussions of the species problem litter the scientific literature, hampering progress, often obscuring a clear vision of the conceptual issues and practical problems centered around species, with this distinction not being always clear-cut. However, the species problem still remains fundamental, irrespective of the common conjecture of it being merely a question of semantics.

Although the question "what is meant by a species?" cannot be pursued

here in more detail (see Bock 2004, Coyne and Orr 2004, Glaubrecht 2004, Reydon 2004, Hey 2006, Stamos 2007, Samadi and Barberousse 2006, 2009), a few remarks should be made on the understanding (and often enough misperception) of what earlier, influential systematists and evolutionary theorists have thought about the nature of species and why this is of relevance in particular today. Astonishingly, the species problem is not only regarded as being an old one, but it is often erroneously concluded from this fact that the species question is either irrelevant or problematic (Noor 2002) and, thus, should be avoided. I completely disagree and anticipate that this perception is at least as fatal as our lack of knowledge of species numbers.

Firmly convinced about the divine origin of nature and the stability of species, for example, Carl Linnaeus and his followers during the subsequent century believed that "*species tot sunt quot diversas formas (in principio) in initio produxit infinitum eus*", *i.e.*, there are so many species as God has created in the beginning (Blunt 2001: 266). Under this assumption, delimiting species was only a matter of describing the most relevant (*i.e.*, morphological) features for identification and classifcation. However, during Darwin's time it became a widely realized problem of how to define the "undefinable", as the species problem is called. It has repeatedly been erroneously referred to in the literature that Darwin, after having struggled long with the species question, applied a somewhat cynical definition of species being merely what "competent naturalists" say they are. Several statements in Darwin's work seem to prove this view on his confusion over species that many systematists and evolutionary biologists commonly hold (Mallet 1995, 2008a, 2008b, Stamos 2007). However, it seems worth noting that Darwin's species concept changed, for sound reasons, since the time when he started his early notebooks on the transmutation of species in 1837 and publishing his "*Origin of Species*" in 1859. As Kottler (1978) and Sulloway (1979) have shown in great detail, Darwin clearly subscribed to the reality of species and identified the acquisition of reproductive isolation as a mark of completed speciation, irrespective of his later statements on species as arbitrary

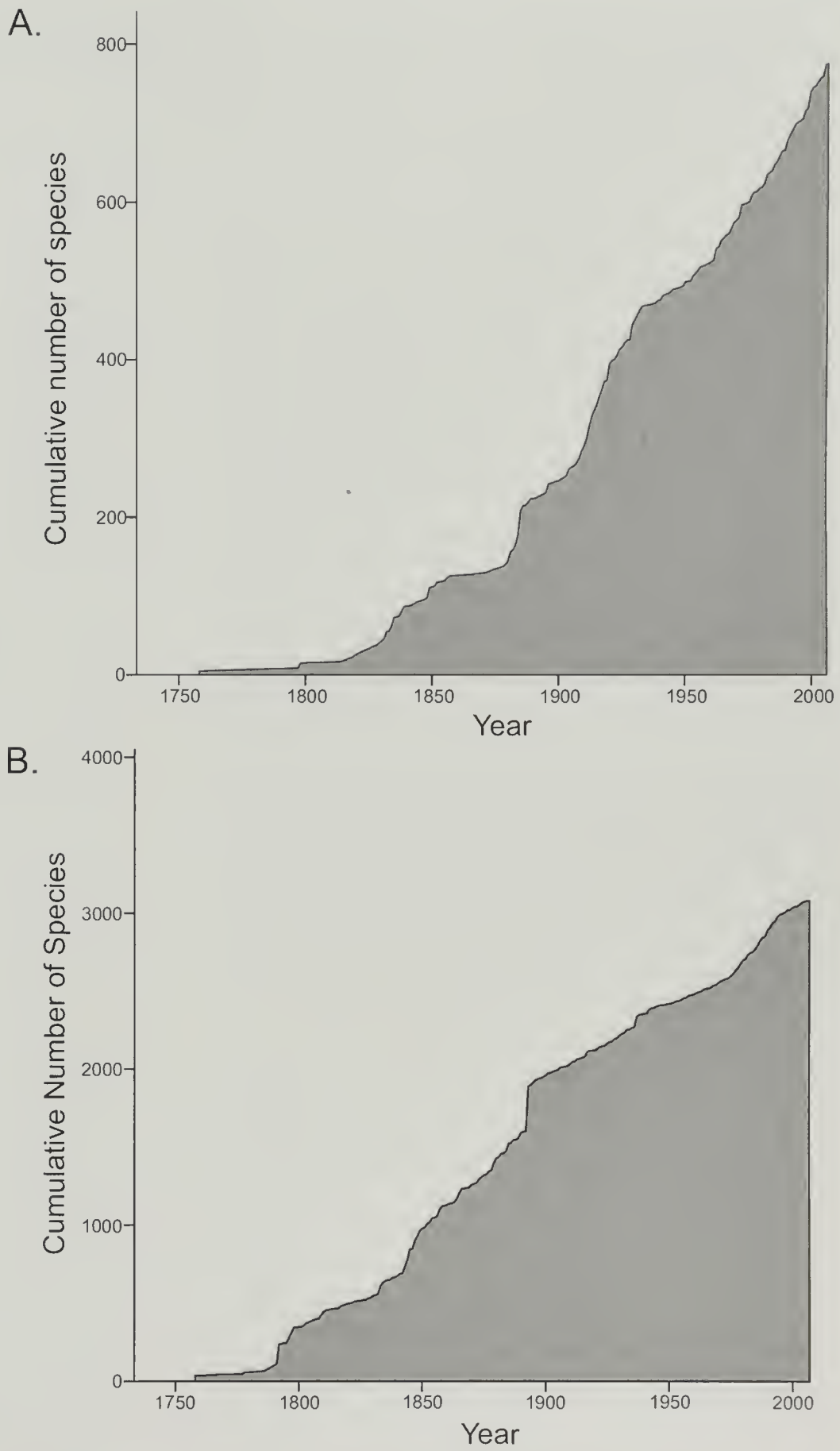

Figure 5. Increase of described species in Cephalopoda and in *Conus*, revealing one trend in two randomly chosen taxa. A, cumulative species number in Cephalopoda, 1758-2006, with species currently considered valid (modified from Wood and Day 2006, Cephbase as of July 2007). B, cumulative species number in the neogastropod *Conus*, 1758-2007, with available species names given (modified from Kohn and Anderson, 2008, *Conus* Biodiversity Website as of July 2007).

constructions of taxonomists in relation to his new evolutionary theory. This discussion and the Darwinian dilemma on the duality of species as a taxon and concept remain today, in particular with the practical procedure of how to delimit species with the availability of new tools.

The reality and duality of species

In many respects, modern research has returned to some of Darwin's original interpretations, as Hendry (2009) stated, referring to the most recent perceptions propagated by Mallet (2008a, 2008b). Ernst Mayr (1904-2005) with his 1942 synthesis a watershed in the history of the species-problem, triggered a new age of this debate when elevating several different approaches to species identification to the level of concept (Hey 2006, Glaubrecht 2007, Mallet 2008a, 2008b). Mayr's biological species concept surely remains a highly useful approach to the study of species and speciation. Evolutionary biologists, in particular those who study the pattern and processes of speciation (see below), believe that species of sexually reproducing organisms are real (*i.e.*, they exist without us to assign them) and that they exist by virtue of reproductive isolation rather than phenotypic distinctness (Mayr 1996, 2001, Coyne and Orr 2004, Glaubrecht 2004, Hendry 2009). However, as is clearly evident from the vast literature still being produced on this subject, the species problem has still not been adequately resolved from its empirical and practical aspects as well as its philosophical perspective. Persisting misconceptions as to the nature and importance of species, in particular the permanent confusion of *species taxon* with *species concept*, hampers both biodiversity research and understanding evolution, leading to pertinent problems with merely artificial delineation and naming of species.

In light of the many proposals of species concepts, which have become almost a cottage industry, Bock (1995, 2004) and Mayr (1996, 2001) have explained that there is a fundamental distinction as to this duality of species as category and concept. On the one hand, there is the practical procedure of how to delineate a described taxon as a species within a taxonomic category of the Linnean hierarchy; on the other hand, there is the theoretical concept of species using objective criteria, as *e.g.*, defining species based on reproductive isolation. As species concepts (the meaning of species in nature) and species taxa (as zoological objects to be categorized in an ordering system) are different things, we need to clearly differentiate between *describing* species and *defining* species. As pointed out before (Glaubrecht 2004), these two sets of species problems are often ignored, in particular with respect to the description and identification of species, *i.e.*, the question of the delineation of species as a practical procedure using operational and empirical criteria. Unfortunately, recently it is largely this latter, operational aspect (how to delimit species using new molecular tools, rather than what it actually represents in nature) that has been focused on (Sites and Marshall 2003, 2004, Agapow *et al.* 2004, Samadi and Barberousse 2009).

As much as these procedures are important, I would argue that having a clear idea what species are remains essential. In malacology, for example, naming nearly every conchologically distinguishable specimen or population in the typological manner of the 19th century has been misleading (Glaubrecht 2000, 2004). As argued there, our task is not to find the least distinguishable unit but to make meaningful inferences on the existence of evolutionary entities in nature. In terms of testing of the null hypothesis of typology (*i.e.*, what looks different is different), we need to strive for identifying not only arbitrarily delineated taxa but also specific entities with their own evolutionary trajectories, using all available biological data including the reproductive criterion of the biospecies concept as well as relevant morphological, molecular genetic, geographic, and other data. Irrespective of the enormous use of molecular markers in systematics, phylogeny, and phylogeography (Avise 2004, 2006), it is worth realizing that as much as morphology can only be used as a proxy to phenotype, molecular sequence differences are not more than a far less reliable proxy to the genetic history of a given taxon.

Currently, with about two dozen species concepts around and in view of the ongoing confusion of species concepts and species taxa (see above), the theoretical foundation of any biodiversity approach is weak. At the same time our understanding of the nature of species has, of course, fundamental implications for evolutionary biology and historical biogeography. Thus, it still is a relevant task to establish if and where the concept of biospecies is applicable or if we need to find and define new and improved concepts. In any case it will be necessary to implement a common language for all biodiversity assessments, *i.e.*, to specify those units that are used in individual approaches. As much as it is claimed by proponents of the practice of DNA profiling of animal taxa (see below) that species are recognizable by sequence variation as groups of close relatives, the idea of finding and applying statistical generalities for species differentiation is fallacious. Eventually, it needs to be established whether all units currently used, including MOTU ("molecular operational taxonomic units") but also, *e.g.*, chronospecies in the fossil record, carry meaningful information in the global task of a bio-inventory in space and time.

Therefore, reiterating an earlier plea (Glaubrecht 2004), we should explicitly say what we mean when dealing with the species question, for example, which species concept we want to apply and for what reason. As crucial as diagnosing and demarcating species are, only if we understand the nature of species and define more properly the various levels of evolutionary units, can we arrive at meaningful conclusions for

evolutionary biology and biological diversity. It will be the challenge of modern biosystematics to review the various approaches and develop unified strategies for the verification of these units of evolution as a major principal theoretical component, not only for biosystematics but also for any biodiversity assessment and evolutionary biology in general. In the following, I will only discuss a few aspects relevant to this task.

Taxonomic redundancy: Which of the nominal species are real?

The history of taxonomy and of malacology in particular teaches us that there have been many specimens, populations, or taxa named, thus immortalized until later often synonymized. Undoubtedly, superfluous or duplicate names for species as biological entities have been introduced repeatedly, at all times and in all taxonomic groups. Thus, in particular the taxonomic literature for invertebrates often deals with names only but not real species or biological entities. This problem of unresolved synonymies might generally be widely known, but its resolution has not been attempted systematically, hampering many scientific hypotheses ranging from biodiversity assessments to speciation theory, models for radiation, and historical biogeography.

Actually, only rarely has the degree of taxonomic redundancy that inflates species number estimates been derived from any compilation of catalogues and checklists (see, however, for insects Solow *et al.* 1995). Alroy (2002) studied this bias by comparing historical rates of invalidation and revalidation of named species using taxonomic data of unrivaled completeness for North American fossil mammal species. From analyzing how many named species remained valid, he concluded that diversity estimates are inflated by 32-44% for the taxa under study (Fig. 6), arguing that this is a conservative figure compared to other methods. Most importantly, Alroy (2002) suggested from several lines of evidence that the same bias probably affects more poorly studied, hyper-diverse living groups, such as insects and, as I anticipate here, also molluscs. As we have to expect substantial inflation of total species counts everywhere in the literature, Alroy suggested that current estimates of total global diversity should be revised downwards to as low as 3.5 to 10.5 million species, if a third of named species are a reflection created by unsettled taxonomy. Others have argued that his optimistic estimation of fossil coverage, while ignoring the vastly different techniques used to delineate species in living and fossil taxa, render such an extrapolation meaningless (Agapow *et al.* 2004: 168). Nevertheless, May and Harvey (2009) estimated that with synonyms removed, the total number of known animal species that is currently assumed to be 1.8 to 1.9 million, may be 1.6 million or fewer.

For molluscs, though, a level of taxonomic redundancy that is twice as high as this latter estimate and in the range of Alroy's figure might be likely. Schilder (1949) estimated that 34% of the names available to classify prosobranchs were synonyms. He found that 49% of the names that were established in the first half of the 19th century were synonyms, with the synonymy ratio decreasing to 37%, 34%, and 21% respectively for the next consecutive quarter-century intervals. As Bouchet and Rocroi (1992: 84) suggested, this is either the result of taxonomists doing better work since the early 19th century or indicates that it takes many decades to assess the value of a given name. That many molluscan species names are synonyms was also assumed by Boss (1970) who estimated that the synonymy ratio (*i.e.*, the number of available nomina for the number of actual species) averages 4:1 to 5:1, suggesting that a single species may have on the average four to five names in molluscs. His guess was certainly driven by extreme cases like the European swan mussel *Anodonta cygnea* Linné, 1758 with over 500 names. Bouchet (1997) found, based on a small subsample of his data, a synonymy ratio of 1:6. Extrapolating from the assumption that of the average number of 430 molluscan species described as new each year, only 265 are actually valid, we can calculate a synonymy ratio in molluscs to be as high as 38%, or that 62% of the new species described in the last few decades are indeed new species (compare this, for example, to insects with an estimated inflation rate of 28%, Alroy 2002).

It would be interesting to thoroughly investigate the influence of revised biological concepts such as that of what a species is and how we define and delimit it (see above) on our diversity estimates, as discussed by Boss (1978) for bivalves and for freshwater cerithioidean gastropods in Glaubrecht (2004). As I argued, such a study would be all the more timely,

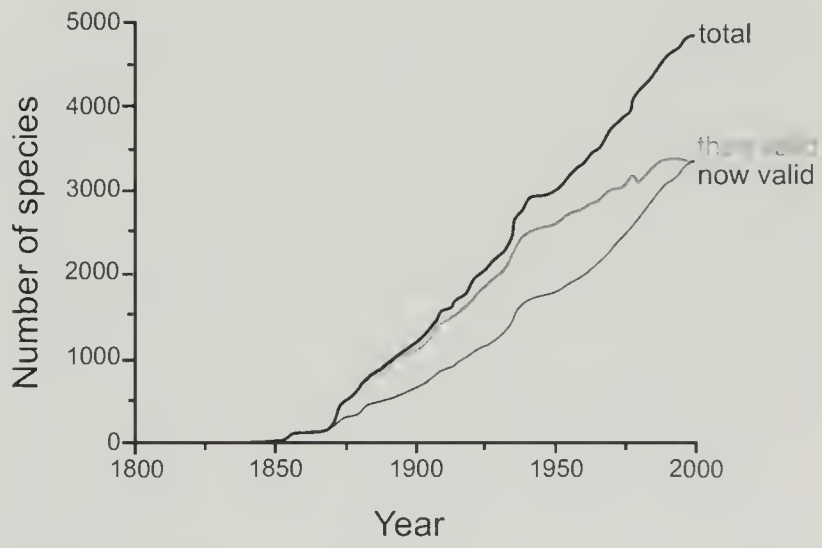

Figure 6. How many named species are valid? Growth through historical time in the number of all named species compared with those considered valid at a time and thought to be valid today. These data from North American fossil mammals suggest diversity estimates are inflated by 32-44% (from Alroy 2002).

since the increasing application of merely phylogenetic species concepts, that name least distinguishable units instead of biologically meaningful entities, will contribute to the taxonomic inflation.

The only study explicitly to review in a comparative manner the influence of, for example, the phylogenetic species concept (resting on the idea of diagnosable differences) versus the biological species concept is hopelessly flawed in respect to the only three examples stated for molluscs, given that one of the three studies cited in Agopow *et al.* (2004: 167, table 1) is actually a deep-sea shrimp, while the other two deal with only two species under the BSC (biological species concept) versus one under the PSC (phylogenetic species concept). Apart from the doubtful relevance of these cases, this reverses (as the perplexed authors stated) the commonly expected trend they otherwise found in their study, that surveys based on the PSC lead to recognition of a far greater number of much less inclusive units. Accordingly, under the PSC we would end up with 48% more "species", which has serious consequences not only for conservation and biodiversity (as it will shift identifying taxa and regions of biodiversity) but also for all biology. Again, however, for molluscs what we know is from very few samples, and what we need is a systematic survey on this taxonomic redundancy and inflation.

Taxonomic inflation: In search of the "new" species diversity

In general, biodiversity is synonymous with the number of species (at least in neonotology, as paleontologists count numbers of genera or even families, which are artificial hierarchical categories, not natural entities). Consequently, if species were used as a proxy for diversity, not only the theoretical understanding of these units becomes essential for all conclusions, but their number itself. Recurrent discoveries using more advanced molecular genetic techniques suggest a plethora of cryptic species and thus hidden diversity, implying that we might largely underrate organismal diversity (Bickford *et al.* 2006, Pfenninger and Schwenk 2007), possibly including molluscs.

Clearly, gene sequence information for many taxa has contributed to the ascendancy of phylogenetic over biological species concepts. However, any kind of taxonomic inflation, *e.g.*, when known subspecies are raised to species as a result in change of species concept rather than new discoveries, also has important influences on many fields in biology. It will, for example, result in re- and sub-classifications, heat up biodiversity hot spots, and lead to devaluation if the smallest distinctions are raised up to the level of what makes a species (Agapow *et al.* 2004, Isaac *et al.* 2004).

Next to the adoption of the PSC over the BSC as most recent development in this context, the application of routine methods in molecular taxonomy (such as DNA barcoding) have contributed immensely to the view of biodiversity being underestimated. Undoubtedly, for a long time the lack of obvious morphological differences between taxa has impeded the identification of species in many groups of animals including molluscs. Currently, molecular species identifications boost species diversity figures in all parts of the tree of life (Blaxter 2004, Lee 2004, Savolainen *et al.* 2005, Köhler 2007, Vogler and Monaghan 2007, Meier 2008). Given the increasing armamentarium of molecular genetic techniques for exploring the genetic structure of populations, species, and higher level taxa, not only do we uncover further complexities in what we mean by a species but also we are confronted with modifying and/or adopting the species concepts used to evaluate the level of taxonomic and biologically relevant diversity (May 1990). I have discussed this for freshwater cerithioidean species diversity elsewhere (Glaubrecht 2000, 2004).

Although more thorough comparative studies in concert with systematic revisions are lacking to substantiate this claim, there are cases to support the caveats. For example, Meyer and Paulay (2005), using cypraeid gastropods, showed that the much-debated barcoding approach works well only for species that are already well studied but is hardly an instrument for accelerating species inventories, as there is not a certain and universal threshold. Tackling the "barcoding gap", *i.e.*, using different means in intra- and interspecific sequence variability for congeneric COI sequences to distinguish between taxa (Meier *et al.* 2008), Köhler (2007) discussed for pachychilid gastropods the largely overlapping intra- and interspecific genetic variation. Using uncorrected p-distances in COI that vary between 0.0 and 0.11 within, and 0.0 and 0.16 between species, he also pointed out the variation even among congeners, which argues against the acceptance of threshold values to postulate species identity, as DNA taxonomy promises something that it cannot deliver. A most striking case is reported by Dillon and Robinson (2009) who found extremely high intra- and inter-population sequence divergence in "living fossil" pleurocerid gastropods from the Appalachians, ranging more than 22% within and between populations, respectively. Using any kind of threshold is a license for splitting over lumping but will not provide answer to the essential question of the genetic integrity of species in nature.

Nevertheless, DNA techniques certainly help as an identification tool, *e.g.*, in marine larvae, or where traditional techniques underestimate species diversity, such as in microscopic animals, or to identify complex phenomena such as the existence of cryptic species complexes, particularly in hyperdiverse taxa, morphologically very similar taxa, or in young radiations. Recent cases from molluscs are marine *Conus* (Duda *et al.* 2008), the vermetid *Dendropoma* Mörch, 1861 (Calvo *et al.* 2009), or species complexes in calyptraeids (Collin 2000, 2005) as well as freshwater *Bythinella* Moquin-Tandon,

1856 (Bichain *et al.* 2007, Haase *et al.* 2007, Benke *et al.* 2009), and the (presumably truly adaptive) radiation of *Tylomelania* Sarasin and Sarasin, 1897 on Sulawesi (Rintelen *et al.* 2007, Glaubrecht and Rintelen 2008).

Currently, large-scale DNA sequencing provides new opportunities for the study of species and speciation. However, single short mtDNA gene fragments, as in the barcoding approach, should be used for classification and systematics only if placed in a wider phylogenetic context. Since sequence divergence in itself is an extremely crude method for determining species limits, it is the plurality of systematics tools (including morphological, biogeographical, ecological, and ethological) that needs to be employed in concert. Molecular taxonomy such as DNA barcoding should no longer try to bypass traditional taxonomy but be integrated within and done in concert with relevant systematic knowledge and practices. However, as Isaac *et al.* (2004) predicted, as practically oriented species concepts such as the PSC gain popularity, in particular among molecular taxonomists, the resulting taxonomic inflation will affect more taxa, any biodiversity survey, and even evolutionary studies and hypotheses. It will be interesting, if we will eventually witness this current trend from splitting to lumping to reverse again. As we will only be able to understand biodiversity and evolution fully once we know what species are and how many there are, both issues are of eminent importance and not fully reflected yet in our research activities.

(3) Speciation: Darwin's "*mystery of the mysteries*"

As much as of species themselves and their roles in taxonomy and ecology, a better understanding of speciation is not just an academic exercise but crucial for evolutionary and biodiversity studies. Of course, only if species are real, does the process of speciation become a real process and the study of their formation and transformation become a meaningful scientific endeavor (Glaubrecht 2004). Speciation, as the process of how new species evolve, eventually results in the multiplication of species; thus, speciation is the major factor causing biodiversity and is also fundamental to our understanding of evolution. As much as the debate on what species are has created confusion and controversy for a long time, so has the process that brings species about been discussed. Therefore, speciation remains at the forefront of current evolutionary biology (Mallet 2008a, Nosil 2008, Schluter 2009).

Unfortunately, too often taxonomists feel that describing any assemblage of species (living as well as fossil taxa) already justifies the term "speciation" in the titles of their publications. The subject of speciation, though, is much more than just an assemblage of species names. With respect to the above-mentioned "big questions" raised for the near future of biological sciences, studying the underlying mechanisms of diversity still remains a most fascinating subject.

Toward solving Darwin's mystery

In this context, it is important to realize the two main focal points of evolutionary biology, namely anagenesis (natural selection acting to transform existing taxa) versus cladogenesis (the actual multiplication of species). While Darwin's (1859) work is acknowledged as brilliant induction, presenting insightful examples of adaptation and anagenesis, Mayr's (1942) synthesis provided an explanation for cladogenesis, grounded chiefly in geography, largely ignored before. Thus, our current understanding of how species multiply was influenced by Mayr's credo that geographic distribution and spatial isolation play a key role in allopatric speciation, as in the course of the Modern Synthesis of evolution it became firmly established that geographic separation is a major factor in speciation (Mayr 1982, 2001, Coyne and Orr 2004). Glaubrecht (2002, 2007) reviewed the historical avenues and intellectual roots, including the contributions of German systematists of the "Berlin School", built by Erwin Stresemann, Bernhard Rensch, and Ernst Mayr. Their "New Systematics" was regarded as crucial for providing, in the 1920s and 1930s, the foundation for synthetic evolutionary theory.

In the last decade, however, it became apparent that although allopatric speciation is important in many cases, it is not the only mechanism and might not even be as important in evolution as Mayr was convinced throughout most of his long life. It became evident recently that an ecological rather than geographical explanation may account for how new species come into being. A key feature of sympatric speciation (Bolnick and Fitzpatrick 2007), ecological speciation by adaptive divergence is an engine of speciation. Accordingly, as assumed implicitly by Darwin, natural selection and adaptation are the means of generating biodiversity, particularly in the context of adaptive radiation (Schluter 1996, 2000a, 2000b, 2001, 2009, Orr and Smith 1998, Sudhaus 2004, Rundle and Nosil 2005, Hendry 2009, Nosil *et al.* 2009). Instead of geography, factors such as phenotypic plasticity, intra- and interspecific competition, with character displacement and specialization, lead to divergence and eventually to speciation. Thus, with ecological speciation as a major player in the diversity of life, allopatric versus sympatric speciation remains one of the most conspicuous current battlegrounds in evolutionary biology.

This central controversy is far from being settled as it is not resolved yet what genetic chance will result in a new species, and under which conditions, thus what the true influence of genetics, geography, and adaptation on speciation is. With new genetic research and genomic techniques homing in on the molecular and cellular mechanisms that enable diversification to occur, our current understanding of the speciation process has improved considerably, allowing us to answer to the next generation of questions on the frequency and importance of different processes that cause speciation

(Noor and Feder 2006). In this context, the long debated phenomenon of interspecific hybridization, a major focus for evolutionary biologists since Darwin, has gained attention lately (Seehausen 2004, Mallet 2005, Schwenk *et al.* 2008). Hybridization and introgression, with various levels of gene flow, in the absence of strict allopatry are antagonistic to the process of speciation but, nevertheless, might play an important role in adaptive radiation (Seehausen 2004, Gavrilets and Losos 2009).

Using molluscs as models

Molluscs certainly can contribute to these highly interesting and controversial subjects of speciation mechanisms, such as hybridization and adaptive radiation. For molluscs, however, we lack textbook examples for sympatric and ecological speciation, adaptive divergence, and hybridization, such as provided by Darwin's finches, threespine sticklebacks, or hyper-diverse cichlid fishes. Yet, there are certainly suitable potential candidates, for example molluscs in ancient lakes, from oceanic islands, mountains, or other insular environments with endemic species. It is in this context that we regard the freshwater pachychilid snails *Tylomelania* Sarasin and Sarasin, 1897, endemic to the central lakes on the Indonesian island of Sulawesi (Glaubrecht and Rintelen 2008) and the thalassoid paludomid snails in East Africa's Lake Tanganyika (Glaubrecht 2008a) as being truly "Darwinian snails". Unfortunately, too few studies have been experimentally designed and/or conducted in such "natural laboratories". No attempt will be made here to review those but the papers given during the 2007 WCM meeting in Antwerp, of which some are in the present volume, will certainly indicate how much malacology currently contributes to these issues.

As was shown by Schwenk *et al.* (2008) in their introductory overview on hybridization, not only has the number of publications risen enormously during three phases (from up to the 1990s, from 1991 to 2002, and since 2003), with the third phase driven by the availability of multiple nuclear markers, *i.e.*, microsatellite DNA, AFLP, and SNP. But, again using the database of the *Zoological Record* (for 1947-2007), they found that, when corrected for taxon bias and taxonomic practice (*i.e.*, organismal preferences of research teams and number of species per taxonomic group), the rate of interspecific hybridization is relatively homogeneous among animal groups, approx. 1%, fairly low compared to predictions of about 10% on average in animals (compared to 25% in plants, Mallet 2005). Consequently, for mollusc species we have *a priori* the same chance for finding and studying this evolutionary phenomenon as in other groups where of course, birds, butterflies, and mammals again take a lead. However, as in the case of the term "adaptive radiation" (Glaubrecht 2008b), too often malacologists continue to use evolutionary issues for their paper or book section titles in lieu of a soundly designed study. Instead of perpetuating an outdated concept (inferring adaptive radiation from the taxonomic description of any speciose group), we should investigate with adequate and timely methods founded on solid theoretical background the underlying mechanisms of anagenetic and cladogenetic change.

As evolutionary biologists working with molluscs, we should test the universality of the known and discussed speciation mechanisms. To date, these are essentially applied to vertebrates (mostly to birds and fishes), but remain largely untested for various groups of invertebrates with the notable exception of butterflies and dipterans among the insects. It is with a clear focus on the above-mentioned mechanisms, however, that we should choose our molluscan models to increase the frequency with which we utilize them as a source of data for studies on speciation, adaptation, hybridization, and radiation, in order to help decipher the underlying mechanisms of biodiversity.

(4) Biogeography: Where are the species and why are they distributed there?

Since the time of Darwin and Wallace, biogeography was at the forefront and at the same time an integral part of further developments in evolutionary biology. Despite centuries of noting the occurrences of animals and plants and the establishment of biogeography as a scientific discipline in its own right (Glaubrecht 2000), we have too few data on distributional patterns for most invertebrates, with, unfortunately, the majority of molluscs being no exception. Nevertheless, it would be easy for us to change this situation. Using more molluscs as model cases would immediately result in an increase in contributions toward this discipline, as is indicated by papers published in *Journal of Biogeography* (Glaubrecht, unpubl. data). For example, in 1980-2000, a total of 345 taxa-related titles yielded the frustrating rate of only 7% being dedicated to Mollusca, with 80% on non-marine taxa (Fig. 7). On the other hand, our contributions to a symposium on the biogeography of SE Asia in 2000, explicitly employing freshwater gastropods as models, resulted in a share of 28% (out of 25) of the taxa-related papers given. Similar results have been found in a recent review of phylogeography by Beheregaray (2008: 3764).

However, aside from noting the data for comprehensive and comparative analyses are generally lacking, I will not attempt to review the existing contributions to molluscan biogeography. Instead, I will only mention the two most recent seminal analyses by Reid *et al.* (2006) and Williams and Duda (2008) as stimulating approaches for future studies of this kind. The former employed molecular analyses of littorinid snails in the central Indo-West Pacific to test phylogeographic hypotheses and showed that species from 'continental' habitats exhibited strong phylogenetic breaks

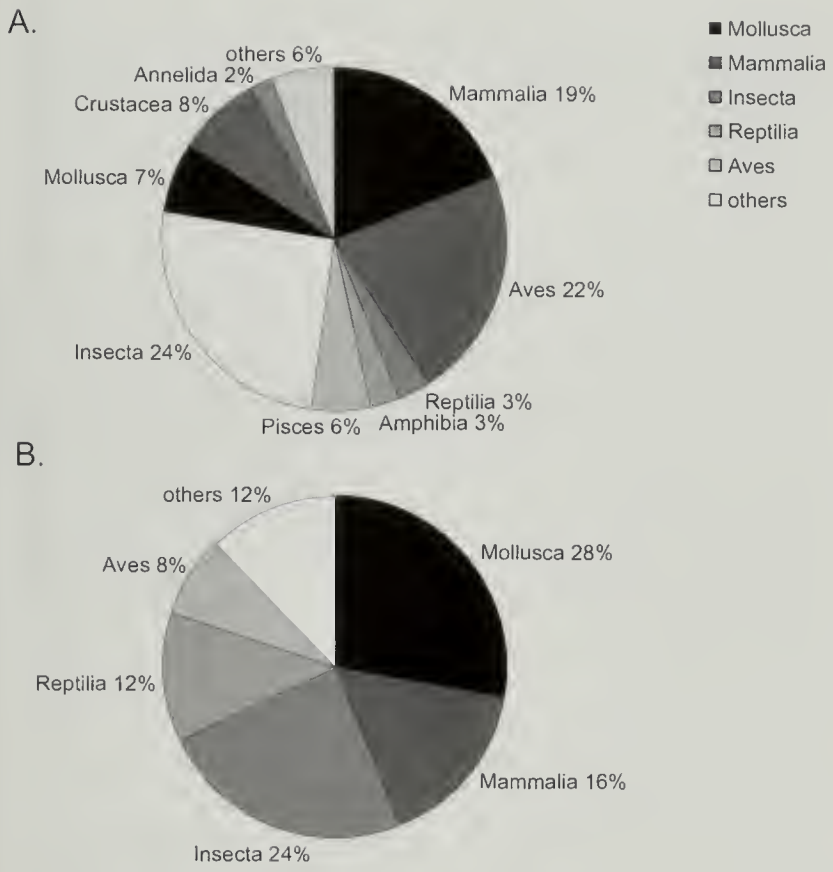

Figure 7. Which animal group is employed in biogeographical studies? A, analysis of papers published in *Journal of Biogeography* in 1980-2000, with a total of 345 taxa yielding a rate of 7% for Mollusca. B, increased percentage of contributions on non-marine molluscs given at a symposium on the biogeography of SE Asia in Leiden in 2000.

between the Indian and Pacific Ocean, in contrast to 'oceanic' species. The latter used analyses of molecular phylogenies of three unrelated tropical marine gastropods, in concert with the timing of plate tectonic events (in this case the collision of the Australia and New Guinea plate with the southeast extremity of the Eurasian plate) during the Oligo-Miocene about 25 million years ago, to show that a speciation pulse occurred in the central Indo-West Pacific at that time.

(5) Phylogenetics: How are species (groups) related?

To Darwin, although helping contemporaries and future generations embrace the common ancestry of organisms on Earth, large parts of the tree of life remained enigmatic. This holds true even today, to the extent that the phylogenetic relationships not only of major animal phyla but also of many terminal branches are still unresolved. Having replaced earlier intuitive ways of finding or subjectively postulating relationships by a clear-cut empirical methodology, it was the German entomologist Willi Henning (1913-1976) who in the 1950s and 1960s single-handedly revolutionized biosystematics. With his guidance, phylogenetic studies were transformed from an art to a truly scientific method, *i.e.*, phylogenetic systematics, creating the basis for a completely new subdiscipline, cladistics (Williams and Forey 2004). It was actually a "silent revolution", as beyond the limits of systematists this new method remained completely unrecognized, with its implications still being underestimated.

Another "silent revolution" took place when phylogenetic systematics was wedded recently with modern molecular genetic methods, first with new sequencing techniques, then with genomic approaches. Albeit those latter developments are too young to be fully applied yet, the contributions in Ponder and Lindberg (2008) provide an up-to-date overview of where we stand 150 years after Darwin in respect to the phylogeny of Mollusca. Currently, even newer advances in molecular genetic techniques (see below) open further avenues for research in systematics, phylogenetics, phylogeography and, thus, the study of evolution.

(6) Genetic causation and molecular processes: How do new forms come into being?

The question of how new forms come into being has long fascinated naturalists. An emerging discipline, called "evo-devo", currently probes how genes involved in development contribute to evolution. It tries to connect developmental and evolutionary biology and to bridge the gap between the genotype and the phenotype, thus linking DNA-focused studies and molecular evolutionary processes with the question of biological diversity and disparity (Carroll *et al.* 2001, Minelli 2003b, 2009b, Carroll 2005, Minelli and Fusco 2008). For an overview in Mollusca, see Wanninger *et al.* (2008).

At the same time, genome sequencing is no longer a pricy luxury done only by few, well-funded researchers on even fewer model organisms. With new technological developments and tools, prices are constantly falling. New methods, such as high-throughput SNP custom cDNA, microarrays, whole-genome and next-generation sequencing (*e.g.*, pyro-sequencing) that can also generate large amounts of expressed sequence tags (EST), are developing faster than ever before, making functional and comparative genomics one of the hottest fields and emerging frontiers of research (Clark 2006, Lee and Mitchell-Olds 2006, Service 2006, Schuster 2008, Wheat 2009). For the current situation in Mollusca, see Simison and Boore (2008).

As eventually malacology will go genomic, the overview in Ponder and Lindberg (2008) provides available phylogenetic knowledge from which we can proceed into this new genomic era. I anticipate that within the next decade the continued exploration of genomes and the study of the molecular underpinnings of any aspect of the phenotype of organisms will give us a better knowledge of the genetic architecture and those genes responsible for form and function in many animals. In reviewing an example of first new insights from the functional genomic study of a *Haliotis* Linnaeus, 1758

species, Medina (2009: 764) noted "that given the, until recently, unimaginable ease with which we can now develop genomic tools for non-model organisms, examining developmental processes in multiple species will be a powerful tool". She concluded that "the transition of comparing a few genes in few species to whole transcriptomes in an even wider range of species will enhance our understanding of the evolution of animal diversity" (2009: 764), as non-model organisms step into the spotlight. Utilizing these new genomic tools and integrating them with evo-devo studies, phylogenetics, ecology, and historical biogeography will greatly change and enrich biosystematics and evolutionary biology in the near future. For both evolutionary biologists and malacologists it is indeed an exciting time—and what a long way we came since Edgar Allen Poe pondered about the soft bodies of molluscs.

CONCLUSION: NEXT GENERATION BIOSYSTEMATICS—A PROGRAM FOR THE FUTURE

As I have tried to show, any biosystematic and evolutionary research on the metazoan level is based on (i) inventorying and describing the basic taxonomic components (*i.e.*, species), (ii) resolving the phylogenetics of the taxa involved (*i.e.*, the genealogical relationships), and (iii) asking questions about the mechanisms of causation, *e.g.*, modes of speciation (*i.e.*, allopatric, sympatric, ecological), including the ecological and developmental constraints, by studying the molecular underpinnings. However, given the lack of knowledge and baseline data, filling these gaps clearly remains the daunting challenge for biological sciences in general and malacology in particular. For quantifying biodiversity, establishing phylogenies, and understanding the evolutionary process of speciation and radiation as well as the taxonomic diversity and morphological disparity, several prerequisites are indispensable, albeit neglected in many modern systematic approaches.

Understanding how diversity is shaped, what species are and how they come into being, where species are distributed and how they are related, will require a major interdisciplinary effort, involving many different kinds of studies. Along the way toward clarifying the history of life we will have to eventually solve the six Darwinian mysteries, which involve inventorying and describing the basic taxonomic components, species, resolving the phylogenetics of taxa by uncovering their genealogical relationships, asking questions about mechanisms of causation on all levels, from modes of speciation to evolutionary transformations, and including ecological data as well as a study of developmental constraints and other genetic factors.

While evolutionary biologists have just started to sort out the most relevant factors intertwined in answering these questions, molluscs with their multitude of morphological, genetic, and ecological features have much to offer. They are a highly suitable group for providing fundamental insights into mechanisms of the genesis of biological diversity and disparity, into historical zoogeography, and into the underlying processes of speciation and radiation, as is also shown in the many contributions to the Antwerp symposium on this subject (Glaubrecht and Rintelen 2009). The myriad of mollusc species offer the material basis for conducting a research program along the lines sketched out above.

We still need to discover many missing bricks in the sense of Henri Poincare's statement used as an epitaph here for this article. Surely, molluscs will not only provide many of the yet missing pieces, but we should also strive to integrate new findings based on this extraordinary taxonomic group into a more comprehensive synthesis of forces and factors influencing biological diversity.

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As time goes by: A simple fool's guide to molecular clock approaches in invertebrates*

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Abstract: Biologists have used a wide range of organisms to study the origin of taxa and their subsequent evolutionary change in space and time. One commonly used tool is the molecular clock approach, relating substitution rates of nucleotide or amino acid sequences to divergence times. The accuracy of the molecular clock, however, has long been subject to controversy, and numerous papers have addressed problems associated with estimating divergence times. Some workers pointed out a striking imbalance between sophisticated software algorithms used for molecular clock analyses on the one hand, and the poor data on the other hand. Moreover, there is often unease among workers relative to molecular clocks because of the controversy surrounding the approach, the complex mathematical background of many molecular clock tools, the still limited number of available, user-friendly software packages, the often confusing terminology of molecular clock approaches, and the general lack of reliable calibration points and/or external clock rates. The current review therefore briefly provides an overview of analytical strategies, covering approaches based on calibration points and/or bounds, approaches based on external clock rates, and approaches that attempt to estimate relative divergence times in the absence of information that can be used for estimating substitution rates. It also deals with major problems and pitfalls associated with data and analyses, including potential errors of calibration points and bounds, the performance of the gene(s) used, estimation of confidence limits, and misinterpretation of the results of clock analyses due to problems with sampling design. A substantial part of the review addresses the question of "universal" molecular clock rates and summarizes important biological and life history variables that account for deviations from rate constancy both between lineages and at different times within lineages. The usefulness of these factors is discussed within the framework of "trait-specific" molecular clock rates. One such clock rate is introduced here for the cytochrome *c* oxidase subunit I (COI) gene in small dioecious, tropical and subtropical Protostomia with a generation time of approximately one year. A flow chart is provided as a "simple fool's guide" to molecular clock analyses, together with a glossary of widely used terms in molecular clock approaches. Finally, step-by-step examples are provided for calculating divergence times in the caenogastropod subfamily Pyrgulinae based on both an internal calibration point and a "trait-specific" molecular clock rate, and it is demonstrated how a relative clock approach can be used for testing evolutionary hypotheses. Our review encourages a judicious use of molecular clock analyses in evolutionary studies of invertebrates by demonstrating their great potential on the one hand and (often-manageable) problems and pitfalls on the other hand.

Key words: molecular clocks, calibrating, Protostomia, Pyrgulinae

Over several decades, evolutionary biologists have used a wide range of organisms to study the origin of taxa from a common ancestor and their subsequent change and diversification in space and time. One commonly used tool is the molecular clock approach, relating number of fixed mutations (= substitutions) in nucleotide or amino acid sequences to divergence time of taxa.

The introduction of the molecular clock concept is attributed to Zuckerkandl and Pauling (1962), who found amino acid differences in mammalian α and β chains of hemoglobin to be roughly proportional to divergence times inferred from paleontological data. In 1965, these workers published a landmark paper (Zuckerkandl and Pauling 1965), naming the molecular clock and describing its stochastic nature as a Poisson process. It was also suggested that, if a molecular clock exists, amino acid changes must be limited almost exclusively to functionally nearly neutral changes—supporting the concept of near neutrality at the molecular level (Takahata 2007). In subsequent years, several workers attributed spontaneous mutations due to replication errors as a driving force of molecular evolution and suggested evolutionarily "neutral" changes in sequences be used to measure divergence times (*e.g.*, Sarich and Wilson 1967, Kimura 1968, Wilson and Sarich 1969).

The accuracy of the molecular clock, however, has long been subject to controversy and early on, workers noted that different proteins evolve at different rates. Over the years, numerous papers addressed further problems with molecular clock approaches like rate heterogeneity in different taxonomic groups or body size effects (see Takahata 2007 and references therein, also see below). Unfortunately, the controversy surrounding the molecular clock approach has

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divided the scientific community. Although an increasing number of workers perform judicious molecular clock analyses, there are still many "believers" who almost uncritically use statistical tools to estimate divergence times, often resulting in unrealistic estimates; "disbelievers" who reject molecular clock approaches as inappropriate; and "ignorant" workers who refrain from using molecular clock approaches due to the critics (right or not) of the method. No matter to what group people belong, there often is a feeling of unease among workers relative to molecular clock approaches. This is due to the controversy of the molecular clock approach (see above), the complex mathematical background of many molecular clock tools, often making it difficult for biologists to understand the statistics involved, the still limited number of user-friendly software packages, the often confusing terminology used in molecular clock approaches, and a general lack of reliable calibration points and/or rates.

As pointed out by Takahata (2007: 4) "It is now generally accepted that, although it is uncertain and rejected for a substantial proportion of proteins and genomic regions in comparisons of main taxonomic groups, the molecular clock can put a new timescale on the history of life, thereby allowing exploration of the mechanisms and processes of organismal evolution. Similarly, a molecular clock is an irreplaceable source of information in evolutionary biology and it would be foolish to abandon it altogether". He also states that the molecular clock needs not be exact and that an approximate clock can still be very useful. However, it often is exactly this approximation of the molecular clock (*i.e.*, estimations of meaningful errors) that is difficult to conduct (*e.g.*, Ayala 1997).

Moreover, there frequently is a striking imbalance between sophisticated algorithms used for molecular clock analyses and poor data (*e.g.*, Bandelt 2007). In fact, whereas many studies deal with optimizing the performance of molecular clock tools, there are relatively few publications addressing data and model selection. This is not trivial, as problems with data and/or misinterpretation of the results can account for divergences of molecular clock estimates in one and the same taxon by >1000% (*e.g.*, Wilke 2004, Pulquerio and Nichols 2007).

In the present review, we therefore attempt to: (1) give a brief overview of molecular clock approaches, (2) discuss major problems and pitfalls associated with data and analyses, (3) address the question of universal and trait-specific molecular clocks in invertebrates, (4) provide a conservative "simple fool's guide" to molecular clock analyses, (5) provide a step-by-step example for clock estimations in the caenogastropod subfamily Pyrgulinae based on both an internal calibration point and a trait-specific molecular clock rate, and (6) give definitions of some of the most widely used terms in molecular clock approaches in a glossary.

This review is intended neither to provide a full statistical background of the molecular clock hypothesis nor to cover all relevant methods and developments. Instead, we give basic information on principal molecular clock strategies, on approaches for mitigating problems commonly associated with molecular clock analyses, and on major pitfalls in molecular clock estimations.

Thus, we specifically target the "ignorant" people mentioned above, hoping to persuade them to look into the application of molecular dating. At the same time, we hope to make "believers" aware of major pitfalls in molecular clock approaches and to convince "disbelievers" that under a specific set of circumstances, the molecular clock can be a very useful tool for evolutionary analyses.

THE MOLECULAR CLOCK APPROACH

Molecular-dating methods, the estimation of divergence times of lineages from a common ancestor based on nucleotide or amino-acid sequences, can be broadly classified into population genetic and phylogenetic (*i.e.*, molecular clock) approaches. In population genetic approaches, a coalescent framework is used to estimate the 'age' of a most recent common ancestor (MRCA) of a number of alleles. The age of the MRCA is hereby measured in number of generations. This approach works backwards in time and is based on the assumption that a pair of alleles will coalesce, *i.e.*, find their MRCA, at some point in time in the past (see Edwards and Beerli 2000). Several models were developed to describe this process with respect to various parameters such as effective population size, gene flow, and changes in population size over time. These population genetic approaches are, as the name suggests, typically only applicable to estimation of divergence time within a species.

For estimating divergence times between species or between groups of species, several phylogenetic approaches have been suggested. Whereas in early studies, genetic distance matrices were used to estimate substitution rates for molecular clock estimations (*e.g.*, Nei 1987, Li and Graur 1991), today these substitution rates are typically derived from phylogenies or from sequence data in conjunction with tree topologies (Rutschmann 2006).

Given the scope of our review, we focus on these tree-based approaches that now appear to be the most widely accepted molecular-clock methodologies in phylogenetic studies. Tree-based molecular clock approaches typically use the branching topology of a phylogeny together with branch length information to estimate the node depth (d_N in number of substitutions per site). Together with a substitution (=

"molecular clock") rate (λ in number of substitutions per site and year), divergence time (t_D in years) can be calculated as following:

$$t_D = \frac{d_N}{\lambda}.$$

Some workers, however, use genetic distances not in terms of node depth but in terms of total branch length between two taxa for estimating divergence times. As total branch length equals node depth × 2, substitution rates have to be modified accordingly and in such cases, typically divergence rates (divergence rate = substitution rate × 2) are used as molecular clock rates.

Therefore, it is crucial to state whether molecular clock rates are based on substitution or divergence rates. Wilson and Sarich's (1969) controversial universal molecular clock rate for protein-coding mitochondrial genes of 2% My^{-1}, for example, is based on divergence rates and corresponds to a substitution rate of 1% My^{-1}. This ambiguity results from the fact that in early molecular clock approaches, workers used genetic distance matrices to calculate molecular clock rates. Today, most workers use tree-based approaches where divergence times are best estimated from node depths and known substitution rates. Thus, in this review all molecular clock rates are substitution rates, unless stated otherwise.

In order to calculate divergence times from node depths, substitution rate(s) have to be estimated from externally derived dates. This can be done with information from ancestral DNA, fossils, or biogeographical events (Bromham and Penny 2003). Alternatively, external molecular clock rates (see below) could be used to estimate divergence times (Fig. 1).

A critical point in molecular clock approaches is the question of rate constancy. Originally, it was believed that mutation rates, particularly in protein-coding genes, are largely constant across loci, species, and time (see introduction). This is also reflected in the term "molecular clock", which, in a strict sense, is associated with rate constancy. Several studies, however, indicated that in many cases the assumption of rate constancy is violated (*e.g.*, Arbogast *et al.* 2002, Pulquerio and Nichols 2007).

This has proved to be a major obstacle for molecular dating as the estimation of divergence times from branch length *per se* requires rate constancies. Whereas this is particularly important for molecular clock approaches employing external clock rates (Fig. 1C), there are several attempts to deal with problems of rate heterogeneity in trees utilizing calibration points or bounds (Figs. 1A-B). These statistical approaches focus on estimating rate variations across lineages ("relaxed molecular clock") in order to obtain more realistic divergence times. Whereas a strict molecular clock tree (Fig. 2A) assumes a single rate for all lineages in the tree, relaxed clocks assume different rates for different branches (Fig. 2C). As a special case of the relaxed clock, lineages within a clade may share the same evolutionary rates ("local clocks"). Note that from a formal standpoint, the strict clock also is a special case of the relaxed clock with the number of rates being one. However, for simplicity, we here only refer to relaxed clocks when the strict clock model is rejected. In the following section of this review, we will discuss the problem of rate heterogeneity in more detail together with other major problems that might affect molecular clock analyses.

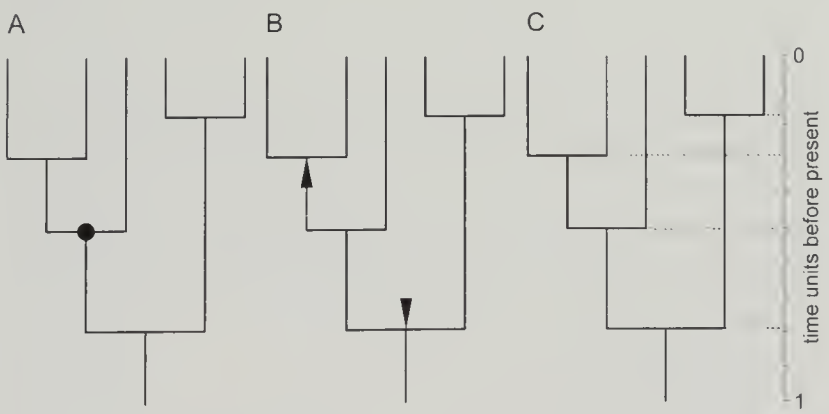

Figure 1. Methods for calibrating molecular clock trees. A, Calibration with point(s) from externally derived dates. B, Calibration with bounds from externally derived dates. C, Calibration with an external molecular clock rate.

PROBLEMS ASSOCIATED WITH MOLECULAR CLOCK ESTIMATIONS

Rate heterogeneity

Testing the global molecular clock

The most fundamental assumption for a global molecular clock is that mutations occur at a single rate along all branches

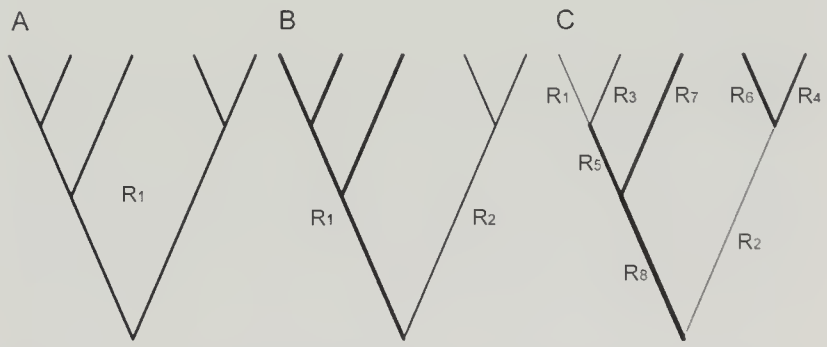

Figure 2. Sample phylogenies with different clock models based on different degrees of rate heterogeneity. A, Strict molecular clock tree with a single substitution rate (R_1). B, Molecular clock tree with two different substitution rates ("local clocks"; R_1, R_2). C, Relaxed clock tree with different substitution rates for different branches (R_1-R_8).

of a phylogenetic tree. This strict clock-like behavior, however, is rare and rates tend to vary. The question arises whether actual deviations are severe enough to bias dating approaches significantly. Several methods have been developed to test clock-like behavior in a data set. In the following paragraphs, we consider two of these approaches.

The likelihood ratio test (LRT) is a widely used approach for testing the acceptance of a global molecular clock. It is calculated as follows: LR = $2(\ln L_1 - \ln L_0)$, where $\ln L_0$ is the maximized log likelihood of the null hypothesis (*i.e.*, the clock-like tree) and $\ln L_1$ of the alternative hypothesis (*i.e.*, the non clock-like tree). Under the assumption that the L_0 model is nested, *i.e.*, that it is a special case of the L_1 model, the LRT follows approximately a chi-squared distribution with q degrees of freedom (*df*), whereby $q = n-2$ with n being the number of taxa in the phylogeny (Felsenstein 1988). If the calculated ratio exceeds the critical value of the chi-squared distribution, the difference between the two models is considered to be significant and the global molecular clock is rejected. It has to be noted, however, that the chi-squared approximation applies only if the sample size is large enough (Posada and Buckley 2004). Under a given number of *df*, the critical value of the chi-squared distribution is a constant figure. In contrast, the calculated ratio of log likelihoods is roughly proportional to sequence lengths. Thus, the clock hypothesis is more often accepted in data sets with short sequences than in data sets with long sequences and/or multiple genes. To demonstrate this, let us assume two data sets of four sequences each (1: $GATC_n$, 2: $ATCG_n$, 3: $TCGA_n$, and 4: $CGAT_n$), with n = 100 ("short sequence data set") and 1000 ("long sequence data set"). Whereas the LRT value is only 0.48 under the HKY-model for the short sequence data set, it is 8.58 for the long one. With both data sets having 2 *df*, the clock is accepted for the short sequence data set (LR = 0.48; critical value of 5.99), but rejected for the long sequence data set (LR = 8.58; critical value of 5.99).

Another bias not accounted for by the LRT is the problem of identical haplotypes. Whereas data sets with identical haplotypes may not (or only slightly) affect the overall log likelihood of the phylogeny, they directly affect the number of *df* (*i.e.*, each duplicate haplotype raises the *df* by one). Thus, the clock is more often accepted in data sets with identical haplotypes than in data sets with unique haplotypes (Wilke, unpubl. data). However, if a deviant haplotype is represented by multiple copies in a data set, the clock may be rejected, whereas it may have been accepted when using unique haplotypes only. To avoid these problems, only data sets with unique haplotypes should be used in LRTs.

Alternatively to the LRT, Akaike's information criterion (AIC; Akaike 1974) can be used for testing the molecular clock. Although widely utilized in assessing models of sequence evolution for phylogenetic estimations, the AIC is still rarely utilized for testing the applicability of a global molecular clock (but see Thomas *et al.* 2006). Akaike's information criterion estimates the distance between a given model and the "truth" the model aims to approximate. The AIC for a certain model can be calculated with the following formula: $AIC = -2\ln L + 2K$, where lnL is the maximized log likelihood of the respective model and K the number of estimable parameters. Since the AIC is a relative value, it is crucial to calculate the difference of the AICs of the two competing models: $\Delta AIC = AIC_0 - AIC_1$, where AIC_0 is the value of the null model (*i.e.*, the clock-like tree) and AIC_1 the value of the alternative model (*i.e.*, the non clock-like tree). If ΔAIC is 10 or higher, the null model is likely to lack substantial support and the global clock may be considered as rejected (Burnham and Anderson 2002). Finally, parametric bootstrap approaches as well as the Bayesian information criterion (BIC) have also been suggested to test the clock-like behavior of a data set (Bollback 2005).

Molecular clock estimations when the global clock is rejected

There are several approaches to enable molecular clock analyses even if the applicability of the global clock is rejected. We want to stress, however, that utilizing these approaches requires a detailed understanding of the procedure itself as well as the underlying assumptions. Therefore, we will introduce only a small selection of possible approaches and suggest studying the primary literature of these and other respective approaches if dating in the absence of a global clock is intended.

Two possible strategies are "tree shopping" and "gene shopping" (Takezaki *et al.* 1995, Hedges *et al.* 1996). In principle, they are special cases of the above-mentioned global clock approach. In the case of "tree shopping" one would prune the original tree by removing clades and/or lineages that appear to increase rate heterogeneity (*e.g.*, extraordinary long branches/clades) and which are of minor importance for the dating of the nodes in question. Subsequently, the global molecular clock may be accepted. "Gene shopping" refers to individual clock tests for every gene previously used in a combined analysis. The rejection of the global clock in the test for the combined gene tree might have been due to considerable rate heterogeneity in a single gene but not necessarily in all fragments used. It has to be noted, however, that the arbitrary selection of lineages and/or genes and the implicit manipulation of the data set may be problematic and is controversial (Cutler 2000). Problems may be particularly severe when using LRTs for testing the global clock. If the clock is rejected in a multi-locus data set, it may be accepted in a single-locus data set not because the selected gene works "better", but simply because the sequence lengths get shorter and therefore the absolute difference in log likelihoods between the clock and non clock-like trees is smaller. As a

consequence, the calculated ratio may less often exceed the critical value of the chi-squared distribution (see above). However, if critical lineages are removed by tree shopping, the degrees of freedom for the chi-squared approximation also become lower and therefore the probability that the clock is accepted in the reduced data set.

In cases where the global clock is still rejected, or where tree and gene shopping is not possible, other approaches have been suggested. One strategy is to refrain from the application of a global clock (one rate for the whole phylogeny) but to apply different rates to individual clades within the phylogeny (the local clock approach, see Fig. 2B). However, this might be difficult because of the potentially large number of possibilities to assign different substitution rates along a given tree (Sanderson 1998). A potential way to bypass this arbitrary application of individual rates (yet allowing substitution rates to differ along a phylogeny) was developed by Sanderson in two key papers (Sanderson 1997, 2002). Whereas the first paper introduced a non-parametric method, the non-parametric rate smoothing (NPRS), the second paper presented a semi-parametric penalized likelihood approach. In principle, this method allows rates to change from an ancestral to descendant lineages but penalizes strong deviations from the ancestral rate. A similar approach was developed by Thorne *et al.* (1998) using a Bayesian framework (see also Kishino *et al.* 2001). In order to overcome the above-mentioned problem associated with the local clock approach, Yang (2004) developed a hybrid algorithm that groups branches using a clustering algorithm (a further development of this approach was presented by Aris-Brosou 2007).

For further information on variable-rate, molecular-dating methods see the excellent reviews of Welch and Bromham (2005) and Rutschmann (2006).

Calibration of the clock

In order to calculate divergence times from node depths within a given phylogeny, either calibration point(s) within the phylogeny or an external molecular clock rate (*e.g.*, universal, taxon-specific, or trait-specific clock rates) are necessary (Table 1). It should be noted that the validity and accuracy of both calibration points and external molecular clock rates are often controversial, and that calibrating the clock might be the most sensitive part of estimating divergence time. Errors and variability of calibrations often account for large discrepancies in molecular clock estimations using the same data set (Bromham *et al.* 1999, Bromham and Penny 2003, Pulquerio and Nichols 2007) and may outscore many other problems in molecular clock analyses.

Whereas molecular clock analyses based on calibration points are often less critically discussed than approaches based on external (*e.g.*, "universal") molecular clock rates, the latter approach might be more widely used in the literature. Approaches based on external molecular clock rates are not *per se* inferior to approaches using calibration points. Both approaches are based on a set of assumptions, which are often violated, and both approaches have their own advantages and disadvantages, which need to be assessed carefully for each individual analysis (Table 1).

Calibration via calibration points or bounds from externally derived dates

As mentioned above, substitution rates can be estimated from ancestral DNA, fossils, or biogeographical events with these dates either being point estimates, or upper and lower bounds (Table 2, Fig. 1). Calibration points can be used for interpolation of divergence times (*i.e.*, the event to be estimated falls within the calibration points or within the calibration point and the tip of the branch), for extrapolation of divergence times (*i.e.*, the event to be estimated falls beyond the calibration point(s)), or a combination of both. Due to the fact that extrapolation has fewer constraints, it is inferior over interpolations and uncertainties will increase with the distance between the calibration point and the estimated event (Bromham and Penny 2003). In other words, distant calibration

Table 1. Pros and cons of estimating divergence times from calibration points and bounds from externally derived dates or *via* external molecular clock rates (*e.g.*, universal, taxon-specific, or trait-specific clock rates). See text for details.

	Calibration points or bounds from externally derived dates	External clock rate
Pros	- locus-independent approach - multi-locus analyses possible	- no calibration point(s) required
Cons	- reliable calibration points rarely available - accuracy and error of the calibration point(s) often difficult to assess - often only bounds but no points available for calibration	- only applicable to data sets with strict molecular clock behavior - external clock rates are typically locus-specific and thus only applicable to single gene - only relatively few external clock rates available
Examples	- closure of the Isthmus of Panama - Mediterranean salinity crisis	- avian clock (see Weir and Schluter 2008) - trait-specific Protostomia COI clock (this paper)

points (*e.g.*, in only distantly related lineages) may affect the accuracy of the analyses (Bromham *et al.* 1999) because of lineage-specific effects or saturation (see below). Moreover, from a statistical point of view, the accuracy of molecular clock estimations can be increased by using multiple calibration points.

Ideally, one would use ancestral DNA/RNA of known age for directly estimating substitution rates in a given phylogeny (Drummond *et al.* 2002, 2003, 2006). However, with the exception of some viral and bacterial sequences, this source of information usually is not available (Table 2). Moreover, ancestral DNA isolated from, for example, subfossil or museum specimens (Lambert *et al.* 2002, Paxinos *et al.* 2002), is often relatively young and may not be adequate for estimating older phylogenetic events (*sensu* Ho 2007, also see below for ancestral polymorphism and the power gap).

More widely used approaches for estimating substitution rates involve indirect methods, that is, fossil and biogeographical data. However, these approaches may also have serious faults (Table 2) affecting molecular clock analyses. Fossils suffer from the fact that they can provide only minimum age estimates as a fossil is necessarily younger than the phylogenetic event that led to its existence and as there is an inherent problem with missing taxa (Marshall 1990, Cutler 2000, Hedges and Kumar 2004, Benton and Donoghue 2007, Ho 2007). Thus, fossil data are best used as lower constraints in molecular clock analyses. Moreover, fossils are often notoriously difficult to identify and to classify within a modern DNA-based phylogenetic framework. Doyle and Donoghue (1993) stressed the fact that fossils can rarely be assigned to branches in a phylogeny and that fossils are often not ancestors of extant taxa, but rather extinct sister groups (also see Cutler 2000)—problems that may also apply to ancestral DNA (see above). These problems might be responsible for large discrepancies of divergence times estimated from fossil data and those estimated from biogeographical data with the former one being, in part, an order of magnitude higher than the latter one (Luttikhuizen *et al.* 2003, Govindarajan *et al.* 2005, Pulquerio and Nichols 2007).

Calibrations based on biogeographical data, however, suffer from the fact that phylogenetic events may or may not be associated with major biogeographical events. The separation of a pair of Pacific/Caribbean geminate species, for example, may be the result of the closure of the Isthmus of Panama some 3.0-2.5 million years ago (Mya). However, it also could have separated an unknown period of time prior to the closure of the isthmus. This is why in relevant phylogenetic studies workers suggest using the youngest phylogenetic event(s) from a set of events to calibrate the clock (*e.g.*, Knowlton and Weigt 1998). However, gene flow between trans-isthmian subpopulations may even have persisted until after the final closure of the isthmus due to passive dispersal, and thus the youngest phylogenetic event may not always reflect the time of closure of the isthmus either.

However, even if we assume that a phylogenetic event is directly linked to a geological event, several problems persist. One problem is the exact age of geological events to be used for calibration. Whereas some events like the beginning and end of the Mediterranean salinity crisis are dated with high accuracy (Krijgsman *et al.* 1999, also see "Examples of molecular clock estimations in the caenogastropod subfamily Pyrgulinae" below), the timings of other major events like the closure of the Isthmus of Panama are subject to ongoing controversy. Whereas Cronin and Dowsett (1996) suggested major hydrological impacts of the barrier starting some 3.5 Mya, with leakages between 3.1 and 2.8 Mya (also see the discussion in Knowlton and Weigt 1998), newer data indicate an initial decrease of circulation through the Panama Strait 4.5-4.0 Mya with temporary re-openings near 3.8 and 3.4-3.3 Mya, and a final closure 3.0-2.5 Mya (reviewed in Bartoli *et al.* 2005). Another problem is that during a certain geological event, species with different biology and ecology may have different divergence times. Taking the example of the closure of the Isthmus of Panama, benthic species adapted to specific coastal habitats may have diverged much earlier than, for example, small pelagic taxa (also see Schubart *et al.* 1998). Thus, timing of calibration points for molecular clocks ideally involves a specific assessment of a geological event in the context of the biology of the respective species.

Whereas dating uncertainties of vicariance events (such as the Isthmus) are often manageable, dispersal events linked to major geological changes are even more difficult to assess. The geological origin of newly evolved oceanic islands, for example, cannot typically be used to calibrate the clock for distinct phylogenetic groups from isolated islands. This is because the colonization of these islands occurred via dispersal an often unknown period of time after the geological origin of the islands and the geological age of an island may serve as lower bound for molecular clock calibrations, at best. In general, the utilization of dispersal events for molecular clock approaches is very controversial and vicariance events are more suitable.

The example above already indicates the complexity and difficulties associated with using calibration points and bounds (no matter whether ancestral DNA/RNA, fossil, or biogeographical data) for molecular clock analyses. Unfortunately, in the literature poor calibration "points" (which are, in fact, frequently only bounds or ranges at best; also see section on external clock rates below) are often subjected to sophisticated molecular clock algorithms. This striking imbalance already noted by Bandelt (2007) is also in our opinion one of the most critical points in molecular clock approaches. Typically, errors in calibration points

Table 2. Pros and cons of estimating divergence times from ancestral DNA/RNA, fossils, or biogeographical data (see text for details).

	Ancestral DNA/RNA	Fossils	Biogeographical data
Pros	- allows for direct calibration	- often readily available	- comparative analyses of different taxa possible
Cons	- rarely available - difficulties to place on phylogeny - ancestral DNA often cannot be assumed to originate from an ancestors	- difficulties to place on phylogeny - fossils cannot be assumed to be ancestors - fossils can provide only minimum age estimates for divergence events - sometimes uncertainties in dating fossils (both for absolute values and errors of date estimation) - often difficult to identify and to classify (problems with homoplasies) - often severe problems with missing taxa	- often uncertainties in dating biogeographical events - potential mis-linkage of phylogenetic and biogeographical events - typically only one or few calibration points in a given phylogeny - often taxon specific differences - biogeographical events can often provide only upper or lower bounds

out-compete errors of the actual molecular clock analyses (*e.g.*, Wilke 2004).

Calibration via external clock rates

Problems associated with estimating divergence times utilizing external clock rates are listed (Table 1) and discussed in more detail in the section "External molecular clock rates in invertebrates" below.

Molecular dating without calibration points or external clock rates

For many taxa and genes, neither calibration points nor bounds are available and no external clock rates might be applicable. This, however, may not prevent molecular clock analyses. Although absolute divergence times might not be assessable from phylogenies without calibration, the molecular clock approach also allows for estimation of relative divergence times. In other words, it is possible to compare different MRCA in a given phylogeny and to test whether their divergence times are significantly different. Such an approach, may, for example, be of interest in analyses of radiation patterns.

In a recent study, Geyer *et al.* (2006) used relative divergence time estimates for members of a solute carrier protein family in different taxa to infer the temporal evolution of its protein-subfamilies. In order to test whether two specific protein subfamilies represent the youngest members of this family, the variance of node depths of selected splits was calculated for the 100 best trees from a Bayesian search. Pairwise comparisons of relative divergence times of selected MRCA using paired Student's *t*-tests indicated that the depths of the nodes (and therefore their ages) differed significantly, which, in turn, allowed for the identification of the youngest MRCA in the given phylogeny (also see "Examples of molecular clock estimations in the caenogastropod subfamily Pyrgulinae" below).

Ancestral polymorphism

The amount of polymorphism present in an ancestral population prior to the separation of the descending species introduces a bias into molecular clock analyses if not corrected for. This is because calculating the depth of a node in a gene tree yields not the age of the divergence of the two descending species (t_{sps} in Fig. 3) but the age of the split of the analyzed genetic lineages (t_{MRCA} in Fig. 3). The latter event predates the former by an unknown amount of time (*ap* in Fig. 3), leading to a temporal overestimation of the age of the species divergence. Potential solutions for this problem are discussed in the literature (*e.g.*, Edwards and Beerli 2000, Arbogast *et al.* 2002).

In practice, ancestral polymorphism is often difficult to estimate because it typically requires detailed information of population sizes. In phylogenetic studies, however, the sampling design is usually optimized to reflect species level or higher-level relationships and not polymorphism at the population level. Thus, in many cases authors refrain from calculating and correcting for ancestral polymorphism. Instead, they consider the estimated uncorrected values to be maximum values, with the actual values being smaller by an unknown figure.

Ancestral polymorphism may vary among species. In molluscs, for example, we have found typical values for ancestral polymorphism corresponding to a time frame ranging from 0.1 to 0.4 million years (My) (Wilke *et al.*, unpubl. data, also see section "Examples of molecular clock estimations subfamily Pyrgulinae" below). Using these values simply to demonstrate the effect of ancestral polymorphism, an uncorrected phylogenetic event of, for example, 1.0 Mya, may actually be only 0.6-0.9 My old (the rate of overestimation thus is approx. 10-70%; also see Edwards and Beerli 2000). For older events of, for example, 5.0 Mya this bias would be much smaller, *i.e.*, 2-9%. Although this bias should not be

neglected, ancestral polymorphism for older phylogenetic events is often an order of magnitude smaller than the overall confidence limit of clock estimations.

Appropriate time frame

The accuracy of molecular clock analyses crucially depends on the performance of the gene(s) used, *i.e.*, how well the observed mutation rate corresponds to the actual mutation rate. Phylogenetic events in closely related taxa, corresponding to low divergence times, are characterized by a major problem, here called the "power gap". For these young phylogenetic events, the number of mutational differences is very low, causing stochastic effects to severely bias molecular clock analyses (also see Walsh *et al.* 1999).

Walsh *et al.* (1999) suggested a power test to determine whether a specific data set is sufficient for resolving possible polytomies among clades resulting from divergence events within a relatively short time interval. This test statistic might also serve as a basis for calculating the extant of the power gap in a given data set. Based on equation 3 in Braun and Kimball (2001) and equation 2 in Walsh and Friesen (2001), the length of an internode (t) in years that can be detected with a given data set becomes:

$$t = \frac{-\ln(\beta)}{L\lambda}$$

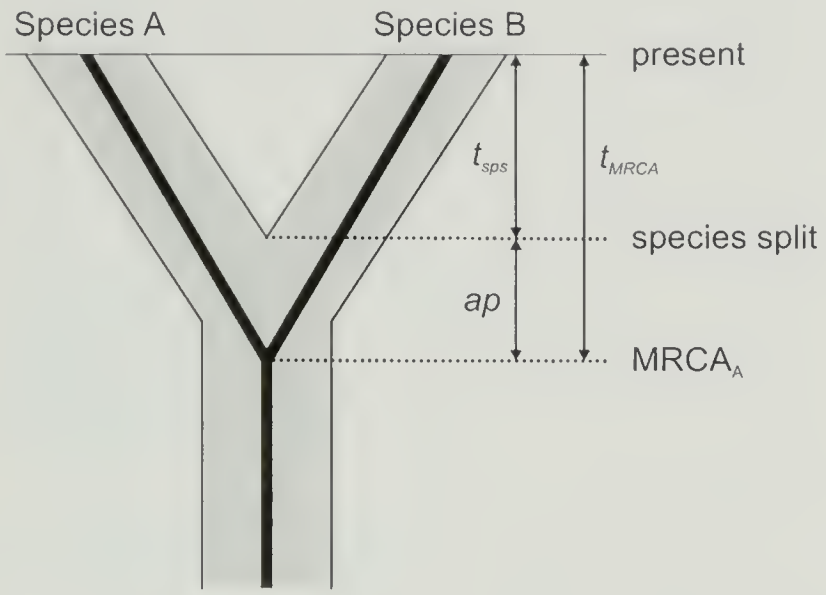

Figure 3. Species tree with an underlying gene tree showing the effect of ancestral polymorphism (*ap*) on the estimation of divergence times. Calculating the age of the most recent common ancestor (t_{MRCA}) of the genetic lineages (black lines) predates the age of the split of the species A and B (t_{sps}) by an unknown amount of time (*ap*). The fraction of this ancestral polymorphism of the total time estimate depends on the age of the species split: the older the split, the smaller the ancestral polymorphism bias (modified from Edwards and Beerli 2000).

where β is the type II error (*e.g.*, 0.05 is the 95% probability of observing one or more substitutions, and 0.20 is the 80% probability), L is the sequence length in bp, and λ is the substitution rate (substitutions per site and year).

We will provide an example of the power test utilizing the widely used COI fragment of approx. 658 bp length defined by the universal primers of Folmer *et al.* (1994). Based on a mean substitution rate of 1.3% My^{-1} (= 1.3 10^{-8} y) for the COI gene for selected invertebrate taxa (see Table 3) and a power of 80% ($\beta = 0.20$) set by convention (see Walsh and Friesen 2001), this fragment should be sufficient to resolve internodes with an age of about 200,000 years or more. A more conservative estimate of t based on a power of 95% ($\beta = 0.05$) yields an internode length of about 360,000 years. In other words, this fragment should not be used to date phylogenetic events with an age of <200,000 years because in such short time periods, the clock does not "tick" often enough in order to calculate reliable divergence times.

Distantly related taxa, however, are often characterized by saturational effects. Mutational saturation occurs when multiple mutations at a given site lead to a randomization of the phylogenetic signal with the number of observed differences being lower than the expected number of differences (Fig. 4). This, in turn, leads to an underestimation of observed divergence times, particularly for older phylogenetic events (also see Arbogast *et al.* 2002). The problem of saturation has been raised early on in molecular clock analyses. Brown *et al.* (1979: fig. 3) suggested in their landmark paper on the temporal evolution of mitochondrial genes saturational effects for divergence times >10 Mya. Problems with saturation may, to a certain extent, be mitigated through the application of sophisticated models of sequence evolution (see Kelchner and Thomas 2007). Moreover, relaxed molecular clocks, which incorporate rate variations across lineages (Fig. 2), may be less prone to saturational effects than strict molecular clock approaches. Nonetheless, tests for saturation, which are implemented in some phylogenetic software packages (*e.g.*, DAMBE, Xia and Xie 2001) should be performed for all molecular clock data sets under the respective model of sequence evolution, as data sets with significant levels of saturation are not suitable for molecular clock estimations. This is particularly important for molecular clock approaches utilizing external molecular clock rates as they are typically based on the strict clock model, thus not allowing for rate variation throughout time.

Arbogast *et al.* (2002) pointed out that estimating divergence times between both distantly and closely related taxa are challenging due to the problems discussed above. Whereas many workers are aware of problems in distantly related taxa (*i.e.*, saturation), problems with closely related taxa (*i.e.*, ancestral polymorphism, power gap), are more frequently neglected.

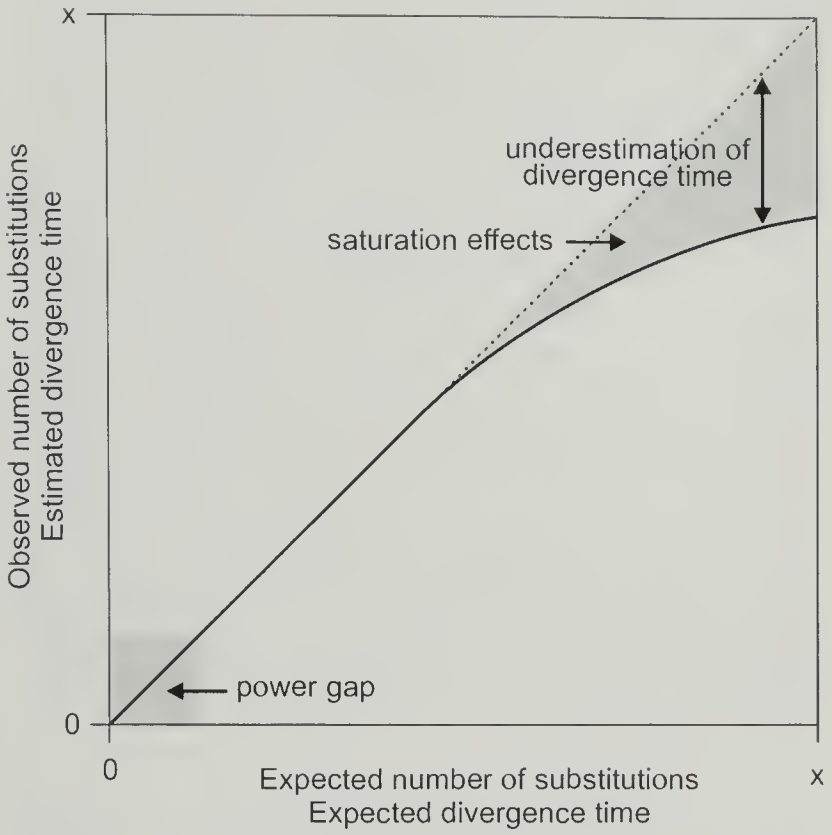

Figure 4. Effect of saturation on estimating divergence times. For genetically divergent taxa, saturational effects lead to a randomization of the phylogenetic signal with the number of observed mutations (solid line) being lower than the actual number of differences (dotted line). This causes an underestimation of divergence times.

Estimation of confidence limits of clock estimations

Calculation of confidence limits is a crucial aspect of clock estimations (Hillis *et al.* 1996, Wilke 2004). This is because confidence limits can be very large (Bromham *et al.* 1998, Bromham and Penny 2003), often making estimates without considering variability meaningless.

Confidence limits are largely affected by two major groups of errors: (a) molecular clock variations and (b) uncertainties of calibration points or external molecular clock rates.

Causes of molecular clock variations within and between lineages can have two major sources (reviewed in Bromham and Penny 2003). First, the molecular clock is probabilistic and ticks at irregular intervals. This behavior, commonly described by a Poisson process, potentially causes large confidence intervals. Second, there might be differences in substitution rates within and between lineages (for biological variables that account for deviations from rate constancy, see below).

Confidence limits of calibration points and external molecular clock rates include uncertainties in the timing of ancestral DNA, fossils, or geological events, time lags of biogeographical and phylogenetic events as well as the variation of external molecular clock rates. In early molecular clock studies, assessments of clock variations were largely neglected. This has led to numerous, partly conflicting molecular clock estimates, which in turn raised general criticism of the molecular clock approach.

Today, estimation of molecular clock confidence limits is standard procedure and many molecular clock software packages incorporate algorithms or approaches for quantification of uncertainties utilizing, for example, bootstrapping, Bayesian posterior distribution, or Poisson distribution (see reviews of Welch and Bromham 2005, Rutschmann 2006). Whereas most publicly available packages account for variation of the clock itself, not all consider uncertainties of calibration points or external molecular clock rates. In this case, the total error of the clock can be calculated using, for example, a propagation of uncertainty analysis (see "Examples of molecular clock estimations in the caenogastropod subfamily Pyrgulinae" below).

Sampling design and interpretation of data

Molecular clock approaches allow for a dating of the MRCA of extant lineages. In this regard, the clock approach is unambiguous; split I in Fig. 5, for example, shows the age of the MRCA of taxa 1+2 and taxon 3. The interpretation of the phylogenetic and taxonomic relevance of such events, however, might be affected by sampling design (*e.g.*, missing taxa) and may be subject to misinterpretation. Wilke (2004), for example, compared the results of two molecular clock analyses of similar sets of rissooidean snails that differed in their age estimates by more than an order of a magnitude. He showed that the difference was largely due to missing taxa together with misinterpretations of the results. Thus, these problems, though rarely discussed in the literature, may affect molecular clock analyses more severely than many other problems discussed in the present paper.

To demonstrate possible adverse effects of missing taxa, we provide a sample data set with ten species (Fig. 5) with the complete phylogeny (including extinct and unsampled) taxa to the left and a phylogeny with four missing taxa to the right. As mentioned above, molecular clock analyses estimate the age of MRCAs in a given phylogeny. In a complete phylogeny, that is, a phylogeny that contains all species of a given taxon, these age estimates of MRCAs can directly be used for phylogenetic interpretations. The age of node IV, for example, represents the age of genus A, and node II the onset of the intra-generic diversification within genus C. In an incomplete phylogeny such interpretations, however, may be erroneous. Node *IV* in the right tree is the MRCA of three taxa of genus A and three taxa of genus C. It does, however, not correspond to the age of genus C because that genus is much younger and represents the sister to the extinct genus B (see node III in the complete phylogeny). Also, node *II* in the sampled phylogeny does not represent the age of the onset of diversification in

genus C because taxon 5 (not sampled) is older than any of the sampled taxa (see node II in the complete phylogeny).

In most cases we do not know whether a phylogeny is complete or not. Thus, molecular clock estimates are best explained within the phylogenetic concept of the MRCA. If taxonomic interpretations were to be made, then this should be done within the context of minimum and maximum ages. In a given phylogeny, the observed **onset of** the **diversification** within a supra-specific taxon (genus and beyond) should be expressed as **minimum** age. Node *II* in the sampled phylogeny thus is the minimum age of diversification within genus C. Assuming a robust phylogeny, additional taxa previously not sampled cannot render this age younger; they only can render it older (see node II in the complete phylogeny). In contrast, the **age** of a given supra-specific taxon should be expressed as **maximum** age. Node *IV* in the sampled phylogeny thus is the maximum age of genus C. Additional taxa cannot render the age of genus C older; they only can render it younger (see node III in the complete phylogeny).

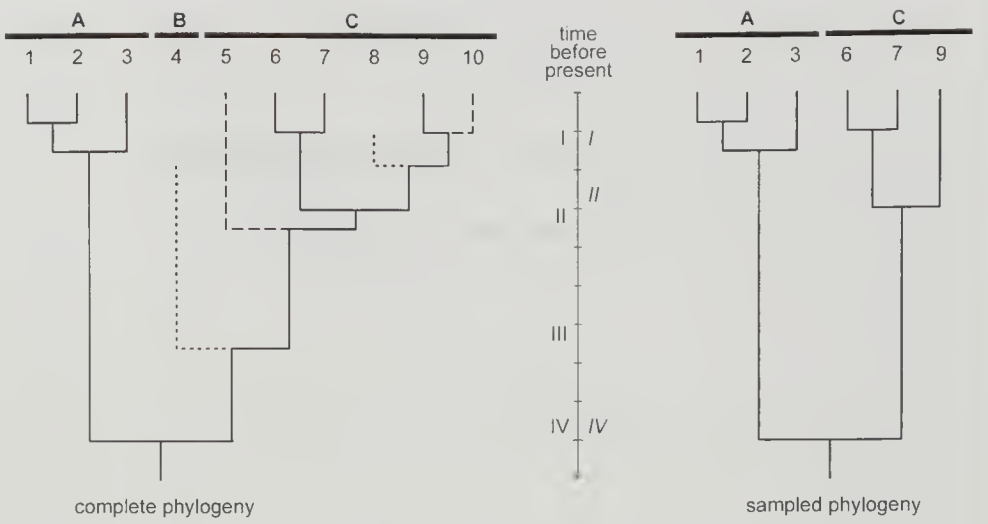

Figure 5. Effects of unsampled (dashed lines) and extinct (dotted lines) species on inferring the timing of phylogenetic events within three putative genera (A-C). Left, complete phylogeny; right, phylogeny with only six out of ten taxa sampled. Major nodes are marked with Roman numerals. See text for details.

EXTERNAL MOLECULAR CLOCK RATES IN INVERTEBRATES

Problems with external molecular clock rates

During our survey of molecular clock analyses reported in the literature, we noted an interesting bias. Whereas putative calibration points are often uncritically unitized for clock approaches (often simply as "pers. comm."), external molecular clock rates (particularly "universal" molecular clock rates) are typically dismissed outright as invalid. In fact, there are several theoretical and practical studies demonstrating that molecular clock rates can largely vary among genes and organisms (*e.g.*, Thomas *et al.* 2006). While we agree on these findings, we also would like to raise a cautionary note. An external molecular clock rate can only be as good as the individual rates upon which it is based. Given the uncertainties of many calibration points, conflicts between fossil and biogeography-based data as well as general clock problems such as ancestral polymorphism, missing taxa, and saturation (see above), external molecular clock rates (particularly those applicable to a larger taxon) are hard to establish. Thus, a universal, taxon-specific or trait-specific molecular clock rate may be rejected because there is no such rate, but they may also be rejected because of potential errors in individual rates on which they are based.

Nonetheless, there is a strong and compelling theoretical background suggesting that there is no clock universal for all genes and taxa, and that mutation rates *per se* vary among genes and broad taxonomic groups (*e.g.*, Ayala 1997, Drake *et al.* 1998, Bromham and Penny 2003, Takahata 2007).

If we ask whether the concept of a "universal" clock can possibly be saved, we have to understand which biological factors affect mutation rates. Ayala (1999) invoked five biological variables that account for deviations from rate constancy within and between lineages:

(1) generation time (shorter generation time "accelerates the clock" as it shortens the time for fixing new mutations, particularly if DNA replication-dependent errors are the major source of mutations; also see Takahata 2007),

(2) population size (larger population sizes will "slow the clock" because of increased times for fixing new mutations),

(3) species-specific differences in properties that affect DNA replication (different species may, for example, have different DNA polymerases with different error rates; Bromham and Penny 2003),

(4) changes in the function of a protein as evolutionary time proceeds, and

(5) stochasticity of natural selection.

Other factors might include:

(6) body size (smaller species tend to have faster rates of molecular evolution, Gillooly *et al.* 2005, Lanfear *et al.* 2007, but see Thomas *et al.* 2006),

(7) body temperature, including ectothermy *vs.* endothermy (body temperature affects metabolic rates, which in turn affects production of free radicals causing mutations, Gillooly *et al.* 2005), and

(8) life history, particularly reproductive traits (mutation rates are presumably higher in hermaphrodites compared with gonochorists, Davison 2006, also see Foltz *et al.* 2004).

According to Gillooly *et al.* (2005), many biological factors can be linked to two major hypotheses explaining rate heterogeneity: the metabolic rate hypothesis (higher metabolic rates are related to higher production of mutation-causing free radicals) and the generation time hypothesis (most mutations are caused by DNA replication errors during division in germ cell lines) (but see Lanfear *et al.* 2007).

It should be noted that most of these effects are largely hypothetical and have only rarely been tested in the context of the molecular clock. Moreover, the relationship between these variables and substitution rates might not be universal, but gene and taxon specific, and the underlying mechanisms often are still poorly understood (*e.g.*, Lanfear *et al.* 2007).

In fact, an extensive study conducted by Thomas *et al.* (2006) could not find a relationship between body size and mutation rates and most workers simply attribute lineage specific differences to the fickle process of natural selection (*e.g.*, Ayala 1999, Takahata 2007).

Gillooly *et al.* (2005), however, introduced a controversial model accounting for body size and temperature effects on metabolic rates, which supposedly could explain rate heterogeneity in different genes, taxa, and environments. Moreover, the authors argue that this model suggests a single molecular clock that ticks according to mass-specific metabolic energy.

Although this model was recently rejected by Lanfear *et al.* (2007), the effect of these and other biological factors could explain why the existence of an universal molecular clock had to be rejected for larger and biologically diverse groups, such as invertebrates (Thomas *et al.* 2006), but appears to be valid in some smaller groups with similar biology and life history like birds (Weir and Schluter 2008).

In fact, knowledge of the relevant factors affecting the clock could help reduce deviation from rate constancy within larger sets of taxa and lead to the establishment of a series of gene-specific molecular clock rates for groups of species that share similar biological and life history traits. An example for a potential "trait-specific" molecular clock rate in invertebrates is given in the following section.

A potential trait-specific molecular clock rate for the Protostomia

As outlined above, several biological and life history properties of animals might affect the tick rate of the clock. At the same time, clock rates can vary considerably among genes, and the performance of a given gene might be poor for relatively young (power gap) and relatively old (saturational effects) divergence events.

Acknowledging that invertebrates are a paraphyletic and highly diverse group, Wilke (2003) first attempted to establish a specific COI clock for the Protostomia, a clade of bilateral animals including the three major groups Ecdysozoa (*e.g.*, Arthropoda and Nematoda), Lophotrochozoa (*e.g.*, Mollusca and Annelida), and Platyzoa (*e.g.*, Platyhelminthes and Rotifera).

Based on published and his own estimates of molecular clock rates for the COI gene in taxa separated by less than 10 Mya (*i.e.*, the presumed time frame in which the COI gene is not saturated), Wilke found relatively coherent rates ranging from 0.7 to 1.2% My^{-1} (uncorrected substitution rates) or 1.4 to 2.4% My^{-1} (uncorrected divergence rates). These published individual rates were later reanalyzed and refined within a tree-base approach by Albrecht *et al.* (2006) utilizing Kimura's two-parameter model (K2P) model.

In this paper, we build upon the studies of Wilke (2003) and Albrecht *et al.* (2006) in order to establish a preliminary trait-specific molecular clock rate for the COI gene in the Protostomia.

The basic idea of this trait-specific clock rate is to find (within a larger taxon) groups of species:

(1) that share biological and life history characteristics supposedly affecting rate heterogeneity (*e.g.*, mode of reproduction, generation time, body size and temperature, population size),

(2) which individual clock rates can be calibrated with robust calibration points, and

(3) where molecular clock estimations are not affected by the power gap or significant degrees of saturation.

These individual rates can be assessed for dispersion and, if applicable, average trait-specific clock rates could be established together with their errors. The trait-specific clock rate for the COI gene in the Protostomia suggested here involves data from a total of 12 pairs of species from several higher taxonomic groups within the Protostomia with the following characteristics:

- they are aquatic,
- they are dioecious (see point 8 under variables that account for deviations from rate constancy between lineages above),
- they have a generation time of approximately one year (see point 1 above),
- they are ectothermic and live in tropical or subtropical waters (see point 7 above), and
- they are relatively small with body sizes differing by not more than an order of magnitude (see point 6 above).

In order to calculate an average trait-specific clock rate for these taxa, we obtained the COI sequences used in the original publications (see Table 3), tested the applicability of a strict molecular clock, and analyzed substitution rates under the assumption of the molecular clock and under different models of sequence evolution in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003).

Note that we were unable to correct for ancestral polymorphism in the clock data sets because of missing

Table 3. Molecular clock (substitution) rates for the COI gene under different models of sequence evolution based on a total of 12 pairs of sister taxa from 5 groups of Protostomia separated by biogeographical events. These taxa share major biological and life history traits. Mean trait-specific molecular clock rates (R_{MC}) together with their standard deviations are given for each model. All values are uncorrected for ancestral polymorphism (JC, Jukes-Cantor model; K2P, Kimura's two-parameter model; F81, Felsenstein 1981 model; HKY, Hasegawa-Kishino-Yano model; GTR, general time reversible model; I, invariable sites; and Γ, gamma distribution).

Taxon	Reference	# Taxon pairs	Mean body size in mm	Divergence time in My	Molecular clock rates (in % My^{-1})for selected models of sequence evolution						
					JC	K2P	F81	HKY	HKY+ I+Γ	GTR	GTR+ I+Γ
Salenthydrobia/Peringia (Gastropoda)	Wilke (2003)	1	3-4	5.64*	1.33	1.29	1.36	1.32	1.60	1.29	1.96
Chlorostoma (= *Tegula*) spp. (Gastropoda)	Hellberg and Vacquier (1999)	1	10-20	2.75**	1.37	1.40	1.37	1.37	1.48	1.37	2.06
Alpheus spp. (Decapoda)	Knowlton and Weigt (1998)	7***	20-50	2.75**	1.21	1.23	1.17	1.18	1.89	1.03	1.94
Sesarma spp. (Decapoda)	Schubart *et al.* (1998)	2	15-30	2.75**	1.01	1.03	1.03	1.10	1.61	1.02	1.59
Alvinella/Paralvinella (Annelida)	Chevaldonné *et al.* (2002)	1	2-3	2.75**	1.20	1.21	1.20	1.21	1.26	1.19	1.25
			Mean (n = 5)		**1.22**	**1.23**	**1.23**	**1.24**	**1.57**	**1.18**	**1.76**
			95% confidence interval (n = 5)		**0.27**	**0.26**	**0.28**	**0.22**	**0.45**	**0.31**	**0.66**

* Mediterranean Salinity Crisis, timing for its climax taken from Krijgsman *et al.* (1999).

** Closure of the Isthmus of Panama, timing based on average estimates suggested by Bartoli *et al.* (2005).

*** From 15 pairs of geminate sister species suggested by Knowlton and Weigt (1998), the seven most closely related pairs of species were used here (also see Albrecht *et al.* (2006)).

population-level data (see section on ancestral polymorphism). Thus, the trait-specific clock rate suggested here might be slightly overestimated, but because of the relatively old biogeographical events used for calibration (see Table 3), we would expect the bias to be <10%.

Nonetheless, the trait-specific clock rates suggested here for several major models of sequence evolution are surprisingly coherent. The 95% confidence intervals for individual models are typically around 20%. Even among models, the average clock rates are very similar, indicating that they are relatively robust against model misspecifications. The only exceptions are the models with gamma distribution and invariables sites (Γ+I), which show, as expected, an elevated clock rate and elevated confidence intervals.

Of course, the trait-specific COI clock suggested here would need further refinement involving more taxa and more independent calibration events in order to assess its validity for a large set of taxa. Nonetheless, this example already indicates that deviations from rate constancy can be mitigated with relatively simple means. Moreover, if the validity of this trait-specific clock would be confirmed, it may not only be applicable to many different species; it also could help to establish a general predictive model for substitution rates taking saturation as well as specific life history, biological, and biochemical characteristics into account.

A simple fool's guide to molecular clock approaches

The flowchart given (Fig. 6) intends to provide relatively simple and conservative, yet sound, guidance through crucial steps of molecular clock analyses. It is simple because it is based on a series of straightforward tests and tools readily available, and it is conservative because it does not attempt to deal with problems the solution of which would require expert knowledge (*e.g.*, estimation of divergence times from saturated data).

The guide is applicable to molecular dating of data sets for which (a) calibration points or bounds exist, (b) an external clock rate is available, or (c) for which no such information exists. In the latter case, however, only estimations of relative divergence times (see section "Molecular dating without calibration points or external clock rates" above) would be possible.

The initial information required is whether the data set in questions shows significant levels of saturation. The appropriate model of sequence evolution (typically the best

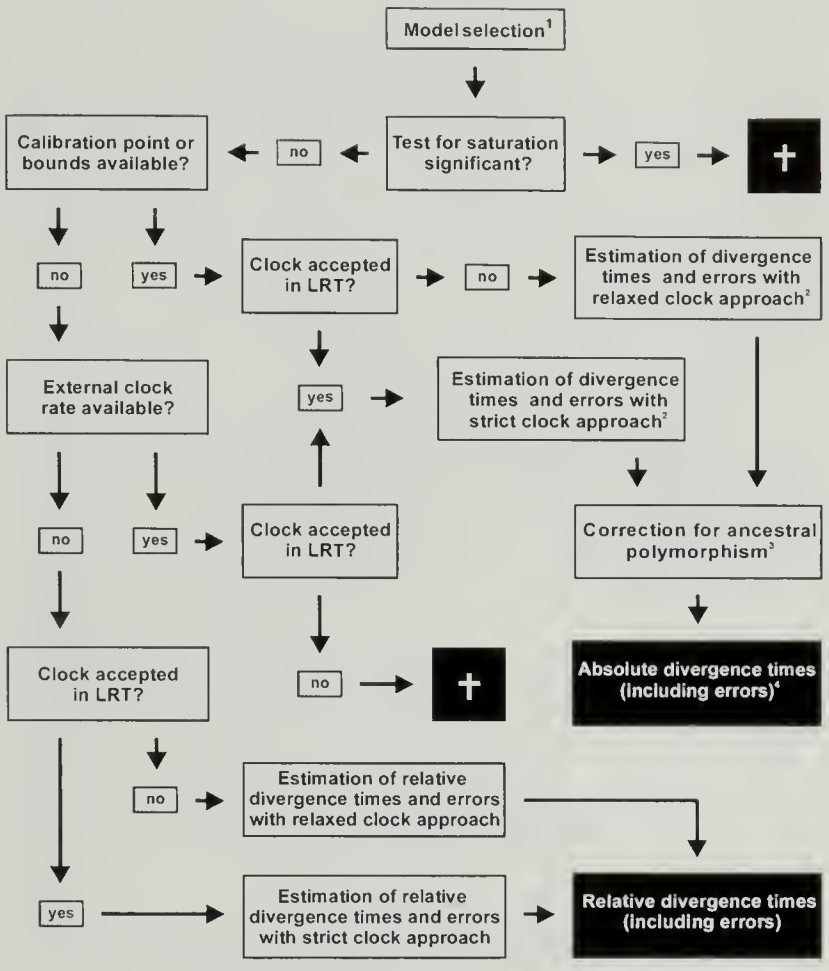

Figure 6. Simple fool's guide for molecular clock analyses. See text for details. [1]Typically, the best fit model of sequence evolution is used; approaches utilizing external clock rates may, however, require a pre-defined model. [2]Error estimations should include both errors of molecular clock variations as well as uncertainties of calibration points or external molecular clock rates. [3]If correction for ancestral polymorphism is not possible, divergence times should be treated as maximum divergence times. [4]For young phylogenetic events, the power gap should be tested.

fit model) has to be selected using, for example, the program Modeltest (Posada and Crandall 1998), MrModeltest (Nylander 2004), or similar software tools. Then the data set can be tested under the chosen model for substantial nucleotide saturation (*e.g.*, using the program DAMBE, Xia and Xie 2001). If the data set is saturated, molecular dating is not advisable.

If the test reveals no substantial saturation, then the question arises whether at least one calibration point or at least one lower and one upper bound for estimating substitution rates exist. If so, the tree can be calibrated with those points (see below). If not, the guide asks for the existence of an external molecular clock rate. If available, this external rate could be utilized to calibrate the clock. Otherwise, only estimations of relative divergence times might be possible.

In cases were both calibration point(s) and an external clock rate are available, we suggest using both approaches and comparing the results for consistence (*e.g.*, Wilke *et al.* 2007, also see section "Examples of molecular clock estimations in the caenogastropod subfamily Pyrgulinae" below).

Calibration point or bounds available

If at least one calibration point or two bounds are available, the clock has to be tested (*e.g.*, using LRT) in order to decide whether a strict molecular clock approach can be used for estimating divergence times or whether methods that account for rate heterogeneity ("relaxed clocks") have to be applied. If the clock is accepted, one global rate of substitution can be assumed and several packages are available to estimate divergence times and to provide error estimates (reviewed in Rutschmann 2006). Note that not all of these packages can deal with multiple calibration points.

If the clock is rejected, then methods should be applied that either correct for or incorporate rate heterogeneity (reviewed in Rutschmann 2006). However, as with strict clock approaches, not all of these methods can deal with multiple calibration points.

Whether strict or relaxed clock approaches are used, it should be checked if error estimations also account for uncertainties in the calibration point(s). If not, this error should be incorporated via, for example, propagation of uncertainty (see section "Examples of molecular clock estimations in the caenogastropod subfamily Pyrgulinae" below). Finally, divergence times should be corrected for ancestral polymorphism, if possible. If the data set does not allow for correction of ancestral polymorphism, divergence times should be treated as maximum times. Moreover, if divergence times are very low, it should be tested whether the data set is sufficient for resolving such young phylogenetic events (see section "Appropriate time frame" above).

External molecular clock rate available

If an external clock rate is available, the data set has to be tested for clock like behavior. If the clock is rejected, molecular dating is not advisable, as the applicability of an external rate typically requires one global rate of substitution (strict clock). If the clock is accepted, estimation of divergence times is as described above.

Some external clock rates are available for a specific model of sequence evolution only. In this case, the same model would need to be applied to the data set in question. Such model misspecifications are, however, controversially discussed (particularly for data sets with distantly related taxa) and should be used with caution.

Another consideration is that external clock rates may or may not be corrected for ancestral polymorphism. If uncorrected rates are used, it might be difficult to estimate the bias in the data set in question, and dates for young

divergence events should be treated with particular caution (see section "Examples of molecular clock estimations in the caenogastropod subfamily Pyrgulinae" below).

No calibration point or bounds and no external molecular clock rate available

If no information is available for calibrating the tree, relative divergence times can be estimated. Depending on whether the clock is accepted, strict or relaxed clock approaches should be used. Relative clock approaches are best implemented by setting the age of the root to 1 by default. Then, after testing for normality of node depth distribution, either paired Mann-Whitney *U*-tests or Student's *t*-tests can be used to study whether two specific MRCA significantly differ in their age utilizing replicates from the phylogenetic search. Although still rarely applied in phylogenetic studies, such relative clock approaches are powerful tools for testing hypotheses in evolutionary biology in the absence of specific divergence times.

EXAMPLES OF MOLECULAR CLOCK ESTIMATIONS IN THE CAENOGASTROPOD SUBFAMILY PYRGULINAE

To demonstrate different molecular clock approaches in our model taxon, the subfamily Pyrgulinae, we here use the two hydrobiid data sets of Wilke *et al.* (2007) for three largely independent clock analyses. The full data set A contains combined fragments of the mitochondrial COI gene, the mitochondrial LSU rRNA gene, and the nuclear SSU rRNA gene. The reduced data set B only contains COI sequences. The data set B is used for clock estimations utilizing the trait-specific COI Protostomia clock introduced above. Data set A serves as the basis for estimating divergence times based on an available calibration point as well as for relative clock estimations. The following descriptions are based on the flow chart (Fig. 6) presented above.

I. Time estimation with calibration point (data set A)

Model selection

The data set was analysed in MrModeltest 2.3. The models suggested were HKY+I+Γ (Hasegawa-Kishino-Yano model with invariable sites and Γ distribution), GTR+I+Γ (general time reversible model with invariable sites and Γ distribution), and TrNef+I (Tamura-Nei model with equal base frequencies and invariable sites) for the COI, LSU rRNA, and SSU rRNA fragments, respectively.

Test for saturation significant?

In order to test whether the individual partitions show significant levels of saturation, the test of Xia *et al.* (2003), as implemented in the software package DAMBE 4.2.13 (Xia and Xie 2001), was used with the proportion of invariable sites suggested by MrModeltest (*i.e.*, 0.6082 for COI, 0.5805 for LSU rRNA, and 0.9406 for SSU rRNA). The test did not reveal a significant degree of saturation even under the very conservative assumption of an extremely asymmetrical tree for any of the three data partitions. Therefore, the data set is considered to be suitable for further molecular clock analyses.

Calibration point or bounds available?

For the Pyrgulinae, the known phylogenetic age of the monotypic genus *Salentlydrobia* Wilke, 2003 (see Wilke 2003, 2004) could be used as the calibration point for estimating timing of evolutionary events (see node A in Fig. 7). Ecological and biogeographical data strongly indicate that *Salentlydrobia* originated during the Messinian salinity crisis (MSC), that is, between 5.96 and 5.33 Mya (see Krijgsman *et al.* 1999 for the dating of the MSC). As *Salentlydrobia* belongs to the potential sister subfamily of the Pyrgulinae (*i.e.*, the Hydrobiinae), and as their relationships do not show signs of saturation, it is here assumed that the substitution rates in these taxa are similar.

Clock accepted in LRT?

For the LRT, we first ran two analyses in MrBayes 3.1.2 (clock enforced and clock not enforced) with the partitioned model suggested by MrModeltest (see above) until the chains converged on similar results (*i.e.*, <0.01 after 1.000.000 generations). Then, the best tree from each analysis was used for the LRT. With $\log L_0 = -5493.43$, $\log L_1 = -5488.52$, $-2\log \Lambda = 9.82$, and $df = 19$, the clock hypothesis was not rejected ($P < 0.05$).

Estimation of divergence times and errors with strict clock approach

For calculation of the age of the split between the Black Sea/Asia Minor pyrgulinids and the Pyrgulinae from the Balkan (node B in Fig. 7) as well as the split between the Lake Ohrid pyrgulinids from their sister taxon (node C in Fig. 7), we are using all trees generated from the (clock-enforced) Bayesian search, except for those that fall within a predefined burn-in of 10%.

For each individual tree, we calculated the age of nodes B and C using the rule of three with the known variables being the age of node A (*i.e.*, the climax of the MSC with 5.64 Mya), the node depth of node A, and the depth of nodes B or C.

Separately for nodes B and C, we then averaged their ages over all trees and calculated the respective 95% confidence intervals (resulting in 7.72 ± 2.37 Mya for node B and 3.00 ± 1.04 Mya for node C). Finally, the error of the calibration point has to be added to this error of the clock.

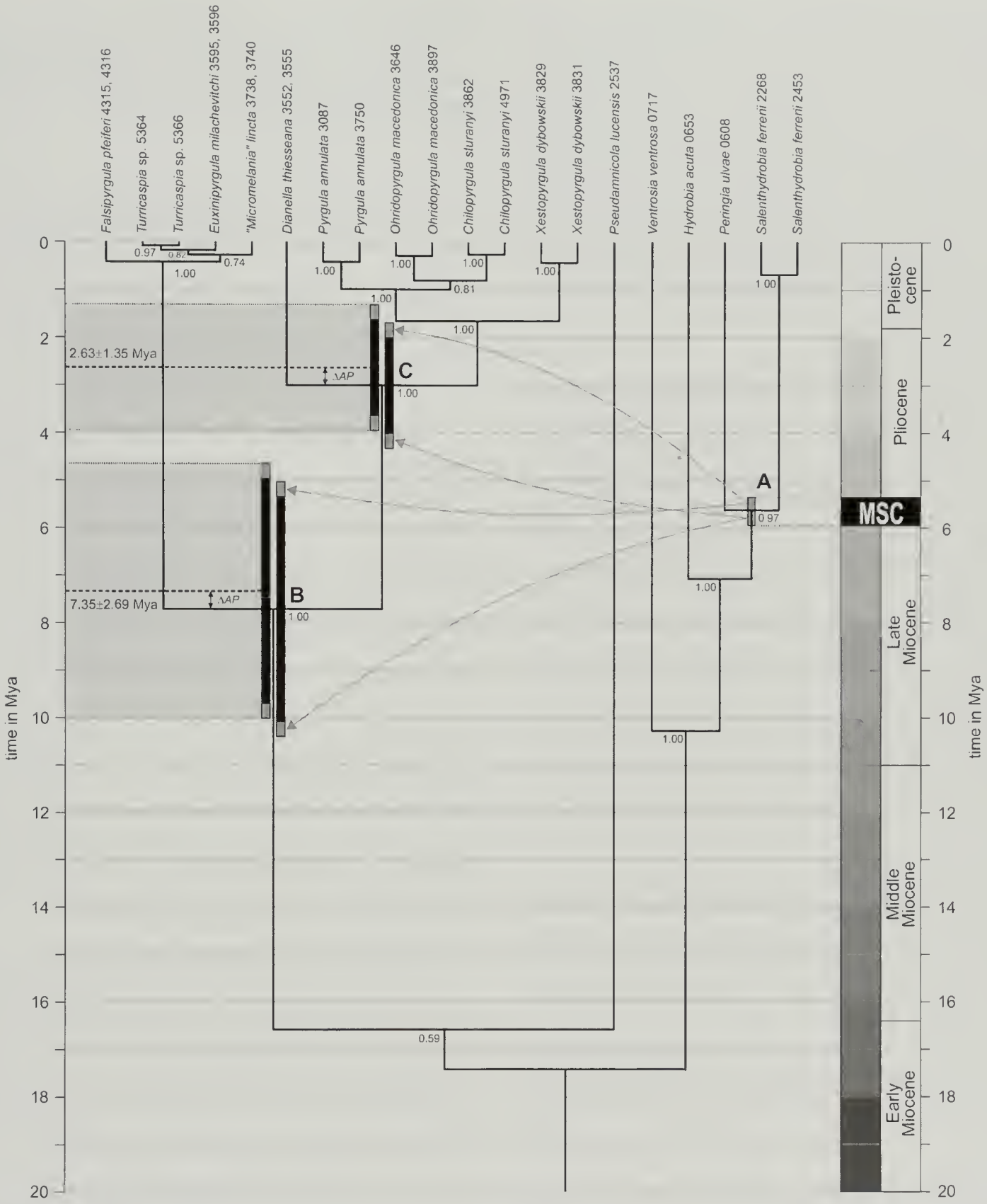

Figure 7. Estimation of divergence times utilizing a calibration point according to approach I. Shown is the best Bayesian tree under the clock criterion based on three gene fragments for representatives of the nominal subfamilies Pyrgulinae, Pseudamnicolinae, and Hydrobiinae (modified from Wilke *et al.* 2007). The outgroup taxon was removed *a posteriori*. The calibration point (*i.e.*, the origin of the genus *Salenthydrobia* that is associated with the Messinian salinity crisis [MSC; marked A]) and the time frame of major phylogenetic events (*i.e.*, the split of the Ponto-Caspian/Asia Minor taxa from the Balkan taxa [B], and the split of Lake Ohrid pyrgulinids from their sister taxon [C]) are shown on the tree. Branch length variations are plotted as black bars at nodes B and C. For estimating the total error of our clock calculations, we also incorporated the error of the calibration point (gray bars, see text for details). Bars right to the node are not corrected for ancestral polymorphism (ΔAP); bars to the left constitute corrected values. The timing of the events in question and their confidence intervals are shown as light gray bands. Posterior probabilities are given for all nodes.

Unfortunately, we do not have any information as to when the split between *Salenthydrobia* and its sister taxon occurred within the MSC. Thus, each point between the onset of the MSC (*i.e.*, 5.96 Mya) and its end (*i.e.*, 5.33 Mya) is equally likely. As we used the climax of the MSC for our clock estimation, we here suggest a pragmatic approach for calculating an approximate total error of the clock. We simply add the difference from the climax to both the beginning and end of the MSC (*i.e.*, ± 0.315 My) to the confidence interval of the clock estimations. The values for node B and C thus would be 7.72 ± 2.69 Mya and 3.00 ± 1.35 Mya, respectively (see Fig. 7)

Correction for ancestral polymorphism

To infer the amount of ancestral polymorphism (Fig. 3) within our data set in the absence of sufficient population genetics data, we here use an approach based on a suggestion of Edwards and Beerli (2000). We assume that the effective population sizes in extant species reflect the population sizes of the ancestral species. Hence, averaging the sequence diversity within the four descendant species of node C (Fig. 7) might provide a rough estimate of the ancestral polymorphism that was present when splits C and B occurred. The node depth under the chosen HKY+I+Γ model is 0.0039 for *Pyrgula annulata* (Linnaeus, 1767), 0.0029 for *Ohridopyrgula macedonica* (Brusina, 1896), 0.0028 for *Chilopyrgula sturanyi* Brusina, 1896, and 0.0042 for *Xestopyrgula dybowskii* Polinski, 1929 (mean node depth: 0.0034). Using node A as calibration point, the amount of ancestral polymorphism corresponds to approx. 0.37 My.

Estimation of absolute divergence times including errors

Deducing the ancestral polymorphism corresponding to 0.37 My from our uncorrected divergence time estimations results in corrected divergence times and confidence intervals of **7.35 ± 2.69 Mya** for node B and **2.63 ± 1.35 Mya** ago for node C (see Fig. 7).

II. Time estimation with external trait-specific COI clock (data set B)

Model selection

The COI data set was analysed in MrModeltest 2.3, which suggested the HKY+I+Γ model based on the Akaike information criterion.

Test for saturation significant?

Utilizing the test of Xia *et al.* (2003) and the proportion of invariable sites suggested by MrModeltest (*i.e.*, 0.6082), the test did not reveal a significant degree of saturation, even under the very conservative assumption of an extremely asymmetrical tree. The COI data set is therefore considered to be suitable for further molecular clock analyses.

Calibration point or bounds available?

We here ignore the *Salenthydrobia*-calibration point used above and continue with the flow chart assuming that there is no calibration point available.

Clock accepted in LRT?

The COI data set was used to run two analyses in MrBayes 3.1.2 (one with the clock enforced and one without enforced clock) under the HKY+I+Γ model suggested by MrModeltest (see above) until the chains converged on similar results (*i.e.*, <0.01 after 1.000.000 generations). Then, the best tree from each analysis was used for the LRT. The clock hypothesis was not rejected ($-2\log \Lambda = 10.66$, $df = 19$, $P < 0.05$).

Estimation of divergence times and errors with strict clock approach

For calculating the age of the split between the Black Sea/Asia Minor pyrgulinids and the Pyrgulinae from the Balkan (node B in Fig. 7) as well as the split between the Lake Ohrid pyrgulinids from its sister taxon (node C in Fig. 7), we use the external trait-specific COI clock rate suggested in the present paper. Based on a rate and 95% confidence interval of 1.57 ± 0.45% My^{-1} under the HKY+I+Γ model (see Table 3), we used all trees generated from the Bayesian search (under the clock criterion), except for those that fall within a predefined burn-in of 10%.

For each individual tree (in our case 90,000), we calculated the depths of nodes B and C and their respective standard deviations from the tree files with an R-routine (available upon request), resulting in mean node depths of 11.13 ± 1.90% and 4.46 ± 0.80% for nodes B and C, respectively. Alternatively this calculation can be carried out utilizing the program TreeAnnotator1.4.8 from the BEAST package (Drummond and Rambaut 2007).

Using the rule of three with the known variables being the depth of nodes B (11.13%) and C (4.46%) and the external clock rate of 1.57% My^{-1}, we calculated the mean age resulting in 7.11 Mya for node B and 2.85 Mya for node C.

Finally we calculated the total error of these estimates by combining the error of node depth (standard deviations of 1.90% and 0.80% for nodes B and C, respectively) with the error of the trait-specific clock (the standard deviation of 0.23% My^{-1} corresponds to a confidence interval of 0.45% My^{-1}) by utilizing the method of error propagation (note that this method is based on standard deviations rather than confidence intervals):

$$\Delta G = \sqrt{\left(\frac{1}{y}\Delta x\right)^2 + \left(\frac{\bar{x}}{y^2}\Delta y\right)^2}$$

with ΔG being the total error, Δx the error of the node depth, Δy the error of the external clock rate, $\bar{x}$ the mean node depth, and y the external clock rate.

Based on a mean node depth of $\bar{x} = 11.13\%$, a relative node depth error of $\Delta x = 1.90\%$, a relative error of the external clock rate of $\Delta y = 0.23\%$ My^{-1}, and an external clock rate of $y = 1.57\%$ My^{-1}, the total error (as standard deviation) for node B in My is:

$$\Delta G = \sqrt{\left(\frac{1}{1.57}1.9\right)^2 + \left(\frac{11.13}{(1.57)^2}0.23\right)^2} = 1.59$$

Multiplying this standard deviation of 1.59 My with 1.96 results in a 95% confidence interval of 3.10 My. Hence, the age and confidence intervals for node B would be 7.11 ± 3.10 Mya (the corresponding values for node C are 2.85 ± 1.29 Mya).

Correction for ancestral polymorphism

Correction for ancestral polymorphism in the present pyrgulinid data set is not possible. This is because the external clock rate used here is not corrected due to the lack of knowledge of intraspecific diversities within the taxa used for establishing this trait-specific clock rate. However, assuming that the extent of ancestral polymorphism in the latter data sets is similar to the one in our pyrgulinid data set, some approximate information on a potential bias can be given. If, for example, phylogenetic events to be estimated in the pyrgulinid data set have an age similar to the average age of those events used for establishing the trait specific-clock (here approx. 3 Mya, see Table 3), then the bias might be small. If the event to be estimated is older, then we likely will see an underestimation of time. However, if the events to be estimated are younger than those used to establish the trait-specific clock, then we will see an overestimation of divergence times.

Estimation of absolute divergence times including errors

Without correcting for ancestral polymorphism, the values presented above would have to serve as approximate final values. The time estimate of **2.85 ± 1.29 Mya** for node C might represent a relatively unaffected value. The estimate of **7.11 ± 3.10 Mya** for node B, however, is likely underestimated by an unknown value.

III. Relative time estimations (data set A)

Model selection

The best-fit models of sequence evolution suggested by MrModeltest 2.3 were HKY+I+Γ, GTR+I+Γ, and TrNef+I for the COI, LSU rRNA, and SSU rRNA fragments, respectively (see section "Time estimation with calibration point" above).

Test for saturation significant?

The test of Xia *et al.* (2003) did not reveal a significant degree of saturation for any of the three data partitions (see section "Time estimation with calibration point" above). Therefore, the data set is considered to be suitable for further molecular clock analyses.

Calibration point or bounds available?

We here assume that no calibration points and no external rates are available.

Clock accepted in LRT?

The LRT did not reject the clock hypothesis ($-2\log \Lambda = 9.82$, $df = 19$, $P < 0.05$, see section "Time estimation with calibration point" above).

Estimation of relative divergence times including errors

Calculation of relative divergence times of phylogenetic events can be done using all trees generated from the Bayesian search under the clock criterion, except for those that fall within the burn-in. For each individual tree, the relative age of a given node is estimated by either setting the node depth of the root to one or by simply using absolute node depth as relative divergence time. In most cases, relative divergence times, however, are meaningless. Instead there often is an interest in testing whether a specific split occurred simultaneously with another split in the phylogeny.

In Fig. 7, for example, node A (the split of *Salenthydrobia* from its sister taxon) appears to be younger than node B (the split of the BlackSea/Asia Minor pyrgulinids from the Balkan pyrgulinids). In order to test this assumption, the following statistics can be used. For each individual tree of the Bayesian search (with the trees from the burn-in ignored), the depth of node A is compared to the depth of node B either using a paired Student's *t*-test or a paired Mann-Whitney *U*-test, depending on whether the data are distributed normally.

As a Shapiro-Wilk test did not reject normal distribution of the data ($P > 0.05$), a paired Student's *t*-test was used to test whether depths of the nodes A and B (and therefore their ages) differ significantly. As the test was significant ($P < 0.01$), it can be assumed that node A is significantly younger than node B and that these phylogenetic events do not coincide in time.

CONCLUSIONS

Over the past few decades, molecular clock approaches have become increasingly popular, and it is now widely accepted that the molecular clock is an important source of information in evolutionary biology. Although not exact, it can provide useful information on divergence times, and a number of approaches are being developed to mitigate problems associated with the clock. This concerns both major clock strategies—the application of calibration points or

bounds and the application of external molecular clock rates.

For approaches based on calibration points and bounds, we show that they have the advantage of being locus- (gene-) independent, but that reliable calibration points are rarely available, that the accuracy and the error of calibration points often are difficult to assess, and that some suggested calibration points can only serve as upper or lower bounds, at best. We also show that data typically used for calibrating trees (*i.e.*, ancestral DNA/RNA, fossils, or biogeographical information) all suffer from specific problems that might severely bias molecular clock estimations.

Approaches based on external clock rates, however, have the advantage of not requiring such calibration points or bounds. They, however, typically call for a strict molecular clock behavior of the data set in question and are usually locus-specific. Moreover, relatively few external clock rates are available, and many of them are controversial. Whereas universal clocks are often dismissed outright, newer studies suggest that there might be a single substitution rate (including error) for a range of taxa that share biological and life history characteristics supposedly affecting rate heterogeneity, *i.e.*, a trait-specific molecular clock. One such trait-specific clock within the Protostomia is introduced in the present paper.

Common to all the approaches above is that they crucially depend on the performance of the gene(s) used, with the lower end of the performance (corresponding to low divergence times) being affected by the "power gap" and the upper end (corresponding to high divergence times) being affected by saturation. In addition, ancestral polymorphism may cause overestimation of divergence times, particularly affecting young phylogenetic events.

Another problem in molecular clock approaches is the estimation of confidence limits of the clock. Whereas many available software tools account for the stochastic nature of the clock, not all tools can account for the uncertainties of calibration points or external molecular clock rates.

Finally, we show that the arguably single most important source of errors in molecular clock estimates is not the underlying statistics, but misinterpretation of the results of clock analyses due to problems with sampling design (missing and extinct taxa).

Nonetheless, the examples presented here for two largely independent molecular clock strategies (calibration point vs. external trait-specific clock) yielded concurrent results, differing by less than 10% (*i.e.*, 7.35 ± 2.69 and 2.63 ± 1.35 Mya vs. 7.11 ± 3.10 and 2.85 ± 1.29 Mya). Although being an isolated case, it adds to the increasing evidence that many problems with molecular clocks and associated data are manageable and that the estimation of meaningful confidence intervals is crucial for a judicious interpretation of the results.

GLOSSARY

Akaike's information criterion (AIC): A method of model selection developed by Akaike (1974). The AIC suggests the best model out of a candidate set of models based on the differences between the individual AIC values of each model. These values are calculated using the likelihood estimator and a penalizing term that increases with the number of model parameters. By doing so, AIC provides a measure of uncertainty of each model rather than a significance value (for further information on model selection see Burnham and Anderson 2002).

Ancestral polymorphism: The amount of heterogeneity that is present in an ancestral population prior to the separation of the descending species. As a consequence, genetic divergence predates species divergence by a certain amount of time. This amount corresponds to the coalescent analogue of the polymorphism in the ancestral species. It averages $2N_e$ generations (with N_e being the effective population size) in a random mating population (see Arbogast *et al.* 2002, also see Fig. 3).

Coalescent theory: A population genetics approach that models the history of gene copies backwards in time. The theory provides a mathematical framework describing the characteristics of coalescent events, *i.e.*, lineages finding their most recent common ancestor (MRCA). The theory was developed by Kingman (1982).

Divergence rate: Substitution rate × 2.

Divergence time: Time since separation of descendent taxa from a most recent common ancestor (MRCA).

Effective population size: Wright (1938) defined the term as "the number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration". To date, there are several definitions for effective population size (see Ewens 2004).

Generation time effect: Assuming that DNA replication-dependent errors constitute a major fraction of the overall number of mutations, taxa with shorter generation times accumulate more mutations per unit time than taxa with longer generation times. Consequently, the substitution rate of the former would be higher (see Takahata 2007).

Global clock: A global clock assumes a single substitution rate along all branches of a given phylogeny. It is also termed as "strict clock". Note that some workers, however, use this term synonymously with "universal clock".

Likelihood ratio test (LRT): A model testing approach based on the difference in likelihood estimators of two nested models. The LRT approximately follows a chi-squared distribution with q degrees of freedom. If the likelihood ratio exceeds the critical value of the chi-squared distribution with

q degrees of freedom, the difference between both models is considered to be significant.

Local clock: A substitution rate for a specific clade within a given phylogeny. Following this concept, several different rates may be assigned to different clades of a given phylogeny. This approach requires *a priori* information justifying the subdivision in rate-specific clades. Note that some workers, however, use this term for a clock that is applicable to a set of closely related taxa, *i.e.*, a taxon-specific clock.

Molecular clock: A concept that correlates the number of substitutions to time, assuming that (a) the mutations are selectively neutral (or nearly neutral) and (b) the substitution rate is uniform. Consequently, the number of substitutions that separate two gene copies would be a function of the elapsed time since their most recent common ancestor (see Hillis *et al.* 1996).

Molecular clock rate: Number of substitutions per site and year and given as a fraction of one or in percent. Molecular clock rates are either based on substitution or divergence rates (divergence rate = substitution rate × 2). Also see the term "Substitution rate".

Nested models: A model is considered to be nested if it constitutes a special case of a more general, *i.e.*, a more parameter-rich model.

No-clock model: A term for the general model in molecular clock testing approaches against which the nested model (*e.g.*, a global clock) is tested.

Node depth: The distance between a specific node and the tip of an ultrametric phylogenetic tree in number of substitution per site. The value is either given as a fraction of one or as a percent.

Poisson process: Describes the accumulation of discrete, independent events (such as mutations) over time. The waiting times between the events are exponentially distributed.

Relative clock: A concept that correlates number of substitutions per site to relative time. This concept allows for comparing the relative age of MRCAs in a given phylogeny.

Relaxed clock: A dating approach that relaxes the assumption of a single substitution rate within a phylogeny and allows rates to vary. These approaches often assume that rate variation is comparatively small between an ancestral and a descending lineage, *i.e.*, that rates are auto-correlated (see Fig. 2, but see Drummond *et al.* 2006).

Saturation: Difference between the expected and observed number of mutations in a given gene due to multiple (hence "invisible") mutations at one or more sites (Fig. 4).

Strict clock: see "Global clock"

Substitution rate: Here used in terms of number of fixed mutations per site and time unit. In molecular clock approaches, the term "substitution rate" is often used synonymously with the term "molecular clock rate" and given in number of substitution per site and year. As the number of substitutions per site can either be given as a fraction of one or in percent, a substitution rate of, for example, 0.01 My^{-1} equals a rate of 1.0% My^{-1}.

Trait-specific clock: A single molecular clock rate (including error) of a specific gene that can be assigned to a range of taxa that share similar biological and life history characteristics supposedly affecting rate heterogeneity.

Universal clock: A single molecular clock rate (including error) of a specific gene (or group of closely related genes) for all taxa of a broader taxonomic group.

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Molluscan models in evolutionary biology: Apple snails (Gastropoda: Ampullariidae) as a system for addressing fundamental questions*

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Abstract: Molluscs constitute the second largest phylum in terms of the number of described species and possess a wide array of characteristics and adaptations for living in marine, terrestrial, and freshwater habitats. They are morphologically diverse and appear in the fossil record as far back as the early Cambrian (~560 mybp). Despite their high diversity and long evolutionary history, molluscs are often underused as models for the study of general aspects of evolutionary biology. Freshwater snails in the family Ampullariidae have a global tropical and subtropical distribution and high diversity with more than 150 species in nine currently recognized genera, making them an ideal group to address questions of historical biogeography and some of the underlying mechanisms of speciation. They exhibit a wide range of morphological, behavioral, and physiological adaptations that have probably played a role in the processes of diversification. Here we review some of the salient aspects of ampullariid evolution and present some early results from ongoing research in order to illustrate the excellent opportunity that this group provides as a system for addressing numerous questions in evolutionary biology, particularly with regard to the generation of biodiversity and its distribution around the globe. Specifically, we suggest that ampullariids have great potential to inform (1) biogeography, both on a global scale and a smaller intra-continental scale, (2) speciation and the generation of biodiversity, through analysis of trophic relations and habitat partitioning, and addressing issues such as Rapoport's Rule and the latitudinal biodiversity gradient, and (3) the evolution of physiological and behavioral adaptations. Also, a number of species in the family have become highly successful invasives, providing unintentional experiments that may offer insights into rapid evolutionary changes that often accompany introductions, as well as illuminating invasion biology in general.

Key words: biogeography, speciation, freshwater, *Pomacea*

Molluscs are second only to arthropods in number of described species, roughly estimated at about 100,000, with a further 100,000 or so as yet undescribed (Lindberg *et al.* 2004). Although 60-70% of molluscs are marine (van Bruggen 1995), they are also well represented in freshwater and terrestrial habitats. Their adaptations in these environments are displayed through a variety of trophic, ecological, and morphological characteristics (Lindberg *et al.* 2004). Yet despite their high biodiversity and multifaceted life histories and habits, molluscs remain underused in addressing general aspects of evolutionary biology. Several features of the group, including its long history, global distribution, ecological and morphological diversity, and high biodiversity, make it amenable to providing fundamental insights into many evolutionary issues, including patterns of historical biogeography, mechanisms generating biodiversity, and the underlying processes of adaptation and speciation. Freshwater snails offer many opportunities for such studies (*e.g.*, DeJong *et al.* 2001, Mavárez *et al.* 2002, Facon *et al.* 2003, Albrecht *et al.* 2007, Strong *et al.* 2008), and among them the operculate family Ampullariidae seems particularly valuable in this regard.

The Ampullariidae have a primarily circumtropical distribution, reaching their highest diversity in South America. There are records of ampullariids from the Lower Cretaceous, ~145 million years before present (mybp), and the Upper Jurassic, ~160 mybp, in Africa and Asia respectively (Wang 1984, Tracey *et al.* 1993, Van Damme and Pickford 1995), and their fossil record dates back at least 50 mybp in the Neotropics (Melchor *et al.* 2002). More than 150 nominal species are recognized in nine extant genera: *Afropomus* Pilsbry and

* From the symposium "Molluscs as models in evolutionary biology: from local speciation to global radiation" presented at the World Congress of Malacology, held from 15 to 20 July 2007 in Antwerp, Belgium.

Bequaert, 1927, *Saulea* Gray, 1867, and *Lanistes* Montfort, 1810 are African; *Pila* Röding, 1798 is African and Asian; *Asolene* d'Orbigny, 1838, *Felipponea* Dall, 1919, *Marisa* Gray, 1824, and *Pomella* Gray, 1847 are South American; *Pomacea* Perry, 1810 ranges from Argentina to the southeastern U.S.A. and the Caribbean (Berthold 1991, Cowie and Thiengo 2003).

While the overall family-level morphology of ampullariids is relatively constrained, many species exhibit wide ontogenetic and ecophenotypic conchological variation, making identification and delimiting of species based on conchology alone very difficult. Internal anatomy offers some resolution (Thiengo 1989, Thiengo *et al.* 1993, 2007), but molecular analyses have begun to make it possible to identify well-demarcated lineages (species) (Cowie *et al.* 2006), and provide a phylogenetic framework to resolve fundamental taxonomic and systematic problems and address major evolutionary questions. Their long evolutionary history, wide geographic distribution, and high biodiversity make them especially well suited for studying biogeography, biodiversification, and novel adaptations to provide further insights into evolutionary biology in general.

This paper makes no attempt to review ampullariid evolution, phylogenetics, or systematics comprehensively. Rather, we summarize the basic knowledge of the evolution and biogeography of the group and discuss some of the opportunities mentioned above, especially in the light of our ongoing research on ampullariids, to illustrate their potential as vehicles for addressing questions in evolutionary biology.

BIOGEOGRAPHY

The family Ampullariidae is thought to have originated in the part of Gondwana that is now Africa. The origin of the family more than 150 mybp was followed by spread and diversification across Africa, Asia, and the Neotropics. Its absence in Australia is thought to be a result of the early separation of that continent (>160 mybp) prior to ampullariids reaching it (Berthold 1991). Studies of the wide distributions of the diverse species in Africa and the Neotropics should allow insights into both the higher level (generic) origins and patterns of diversification within the family. This will, in turn, provide additional insight into the biological, phylogenetic, and evolutionary consequences of the break up of Gondwana, by corroborating or contradicting patterns revealed by other groups of plants and animals. Insights gained from those ampullariid taxa with narrow distributions (*Afropomus*, *Saulea*, *Felipponea*, and perhaps *Asolene*) may also provide more detailed information about the precise geographic relationships and pathways of dispersal between particular sub-regions of the main parts of Gondwana, in particular certain parts of eastern South America and western Africa.

Clarification of the exact order and timing of ampullariid diversification and associated biogeographic patterns will not only provide a much better understanding of evolution within the group, but also contribute significantly to our knowledge of the mode and tempo of evolution, adaptive radiation, and distribution of other freshwater fauna. Also, because the distributions of ampullariids and speciation within the family are probably influenced by both vicariant events like the splitting of Gondwana and passive, long distance dispersal with flow in major river systems, studying ampullarids may help clarify the relative roles of each, an ongoing debate in biogeography (Cowie and Holland 2006, Holland and Cowie 2006, Nelson 2006).

Global

The global, historical biogeography of the Ampullariidae is not fully understood. Resolving remaining questions about the origins and diversification of the genera will require additional fossil and molecular data and a more complete phylogenetic analysis. However, published hypotheses, based until now only on anatomical and morphological data (Berthold 1991, Simone 2004), have provided an important starting point in answering these questions, and new molecular information is also refining our understanding. According to the morphological analyses (Berthold 1991, Bieler 1993), the Ampullariidae originated in Gondwana and, 140 million years ago, were restricted to parts of Gondwana as follows: *Afropomus* and *Saulea* in southern Africa, *Lanistes* in southern Africa and Madagascar, and the most recent common ancestor (MRCA) of *Pila* and the Recent Neotropical genera in southern Africa, Madagascar, southwestern India, and eastern South America (Fig. 1A). The subsequent break up of Gondwana led to diversification of this MRCA on the different land masses. It gave rise in South America to five genera: *Pomella*, *Felipponea*, *Asolene*, *Marisa*, and *Pomacea* with the first three diversifying early and *Pomacea* and *Marisa* being more derived (Berthold 1991, Bieler 1993). In Africa it gave rise to *Pila*, which spread also into Asia. *Afropomus*, *Saulea*, and *Lanistes* remained on the African continent and, in the case of *Lanistes*, in Madagascar (Fig. 1A). However, recent studies using DNA sequence data challenge this scenario (Schultheiß *et al.* 2007, Jorgensen *et al.* 2008). In contrast to Berthold's (1991) phylogeny, *Pila* and *Lanistes* appear as sister taxa in most molecular analyses, suggesting a different Gondwanan distribution of the Ampullariidae from that Berthold had suggested. According to the molecular scenario, the ampullariid ancestor gave rise to two lineages: that giving rise to modern *Afropomus* and that giving rise to all other extant ampullariids. This second lineage then split into two lineages. One of these gave rise to the sister taxa *Lanistes* and *Pila* and diversified within Africa, colonizing Madagascar and, in the case of *Pila*, Asia; the other, the MRCA of *Saulea* and the New World taxa, colonized South America

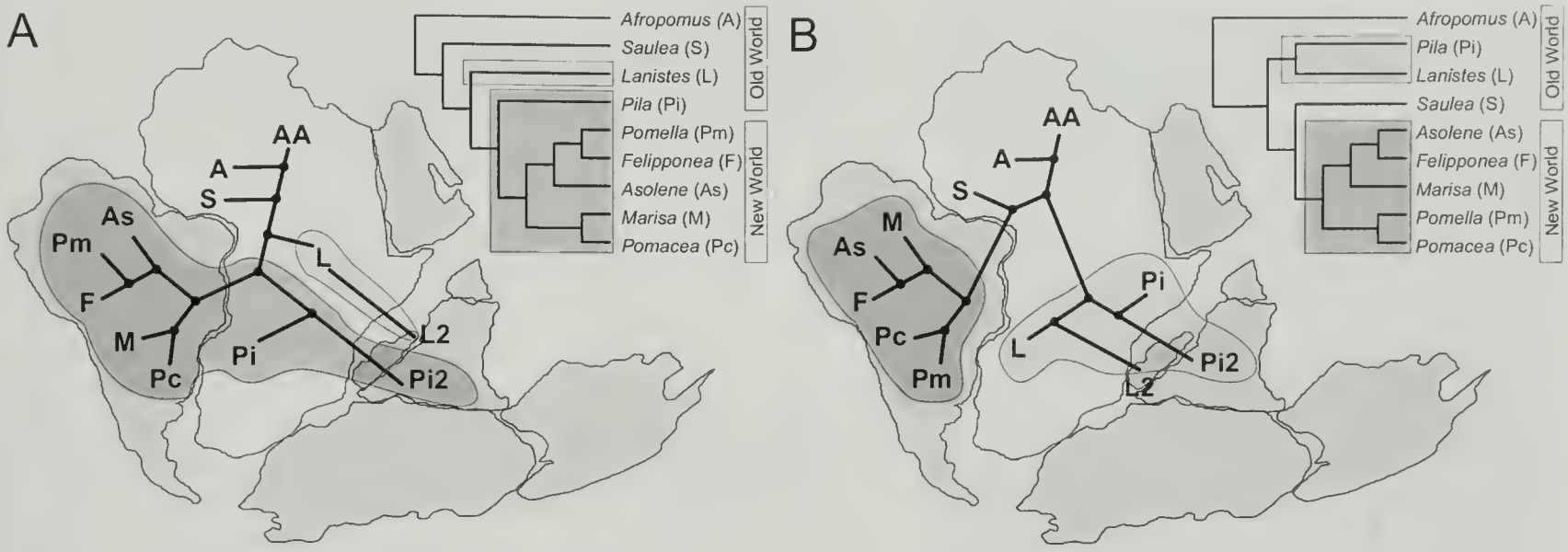

Figure 1. Two hypotheses of Ampullariidae biogeography and diversification. A, the morphological based hypothesis of Berthold (1991) assumes an ampullariid ancestor (AA) giving rise to *Afropomus*, *Saulea*, and *Lanistes* in southern Africa, with *Lanistes* spreading to Madagascar, and the MRCA of *Pila* and recent Neotropical genera splitting in Africa with *Pila* diversifying in southern Africa, Madagascar and Asia, and the five Neotropical genera diversifying throughout South and Central America. B, the DNA based scenario (Hayes 2007, Schultheiß *et al.* 2007, Jørgensen *et al.* 2008) showing the initial divergence of two main lineages in Africa, one giving rise to *Afropomus* and the other diversifying again and giving rise to *Pila* and *Lanistes*, which diversified within Africa but also colonized Madagascar with *Pila* spreading to Asia. The final lineage, probably sharing a MRCA with *Saulea*, colonized South America, diversifying into the five currently recognized New World genera. Shading highlights represent the major differences between the two hypotheses. Late Jurassic Gondwana maps (*ca.* 150 mybp) are redrawn from Scotese (2002).

and gave rise to the modern *Saulea* in Africa and the five New World genera (Fig. 1B). However, ongoing research (Hayes *et al.* 2009) shows that this relationship is sensitive to the inclusion of Cyclophoridae as an outgroup (cf. McArthur and Harasewych 2003), which results in *Saulea* being basal in a monophyletic African clade (*Saulea*, *Pila*, *Lanistes*, *Afropomus*).

Old World

Of the four African ampullariid genera, *Lanistes*, *Pila*, *Afropomus*, and *Saulea*, the latter two are monotypic and restricted to Liberia, Sierra Leone, and the Ivory Coast (Brown 1994). This area is known for its high proportion of endemic freshwater fauna and is considered a distinct freshwater bioregion, Upper Guinea (Thieme *et al.* 2005). The ampullariid fauna in this small region has been considered old and relictual (Van Damme 1984), and given the region's close geological ties with northeastern South America, makes it a likely candidate for the location of the sister taxa of the New World ampullariids. This contrasts somewhat with Berthold's (1991) scenario in which he placed the original distribution of ampullariids in South America along the southern coast of what is now Brazil. *Pila* and *Lanistes* are generally more widespread in Africa though both genera have widespread as well as locally restricted species. Fossils of both genera are known from the late Cenozoic of the Albertine Rift Valley (Van Damme and Pickford 1995).

The five African species of *Pila* currently recognized (Brown 1994) are distributed across Africa with no clearly discernable biogeographic pattern. *Pila ovata* (Olivier, 1804) is the only widespread species, although with four of its historically named forms being relatively distinct (Mandahl-Barth 1954). *Lanistes* is more speciose than African *Pila* with 19 currently recognized species, mostly with very limited (known) distributions (Brown 1994). *Lanistes* occurs both in Madagascar and Africa from the lower Nile south to KwaZulu-Natal and the Okavango Delta (Brown 1994). Presuming an age of *Lanistes* of tens of millions of years, and with the difficulty of delimiting species morphologically, intensive geographical sampling and molecular analysis, as in other ampullariid groups, will probably reveal additional cryptic species. For instance, *Lanistes ovum* Peters, 1845 is the only widespread species of *Lanistes*, and although many nominal taxa have been reduced to synonymy with it, some at least may represent cryptic species, with *L. ovum* in fact being a species complex rather than a single species. Molecular analyses of this widely distributed African taxon will be necessary to resolve this issue. The biogeography of *Lanistes* especially is a field wide open for investigation.

In Asia, *Pila* is the only native genus. Prashad (1925) recognized ten species of *Pila* in India, including two species of *Turbinicola* Annandale and Prashad, 1921, which is a junior synonym (Berthold 1991). Otherwise, Asian *Pila*, of which

there may be about 25 species based on Berthold's (1991) estimate of about 30 species in the genus as a whole, have not been revised comprehensively and their distributions, systematics, and biogeography are therefore also ripe for study.

Neotropics

In general, Neotropical freshwater biogeography may be better understood than that of Africa, especially with regard to ampullariids. Tectonic events and climatic fluctuations have probably influenced the diversity and distribution of New World ampullariids. The emergence of the Antillean archipelago (~49 mybp; Graham 2003) may have facilitated diversification by both vicariance and dispersal. Connection of the West Indies to northwestern South America, ending 32 mybp (Iturralde-Vinent and MacPhee 1999), may have been important. Other 'land-based' scenarios are also possible. There are two main groups of hypotheses to explain Neotropical freshwater biodiversity. Refuge hypotheses (Haffer 1982) posit that diversification resulted primarily from multiple habitat fragmentation and coalescence events driven primarily by Pleistocene climate changes (1.8 million - 11,000 ybp). Hydrogeological hypotheses (Lundberg 1998) suggest that current diversity was reached much earlier, resulting from the changing relationships among South American river systems and their drainages 90-10 mybp. Hydrogeological changes related to tectonic events drove diversification by fracturing and reuniting aquatic habitats multiple times, leading to allopatric speciation. These hypotheses place divergences among drainage biotas much earlier than refuge hypotheses and offer multiple time points that may be correlated to cladogenic events (Sivasundar *et al.* 2001, Montoya-Burgos 2003). Finally, the rise and completion of the Isthmus of Panama ~3 mybp (Coates and Obando 1996) provided non-marine connections between South and Central American drainages. Phylogeographic patterns in several freshwater fish genera suggest multiple waves of dispersal through Central America from South America (Bermingham and Martin 1998, Perdices *et al.* 2002).

A combination of hypotheses may thus explain New World ampullariid diversification. But historical biogeographic inferences rely on the fossil record and knowledge of phylogenetic relationships of extant taxa; in both regards New World ampullariids are poorly known. Limited fossil evidence places *Pomacea* in South America ~50 mybp but an earlier, possibly Gondwanan, origin has been suggested (Berthold 1991, Melchor *et al.* 2002), with origin and diversification of contemporary New World taxa occurring in South America soon after breakup of the supercontinent (~180 mybp). An ancient origin of South American ampullariids supports the hydrogeological hypothesis but remains conjecture without knowledge of the temporal pattern of diversification. Thus, New World ampullariids have the potential to illuminate and discriminate among these various general hypotheses of the diversification of the freshwater biota.

In South and Central America, based on molecular analysis (Fig. 1B), the lineage that probably gave rise to the genus *Saulea* in Africa diversified into five currently recognized genera, *Pomella*, *Asolene*, *Marisa*, *Felipponea*, and *Pomacea*. Reconstruction of the relationships within the family, based on morphology, placed *Asolene* as the most basal of the New World ampullariids with close ties to both *Felipponea* and *Pomella* (Berthold 1991, Bieler 1993). *Pomacea* and *Marisa* were placed in more derived positions and as sister taxa (Fig. 1A). *Pomella* has a rather disjunct distribution, with *Pomella americanista* (Ihering, 1919) and *Pomella megastoma* (Sowerby, 1825) (subgenus *Pomella sensu stricto*) occurring in the south (Argentina, Uruguay, Paraguay, southern Brazil) and *Pomella sinamarina* (Bruguière, 1792) (subgenus *Surinamia*) in the north of the continent (Guyana, Suriname, French Guiana). Similarly, the distribution of *Asolene* is non-contiguous, with some species occurring in the south and others restricted to the north. The three species recognized by Cowie and Thiengo (2003) in *Felipponea* are restricted to the south (Argentina, Uruguay, Paraguay, southern Brazil). Taxa in the more derived *Marisa-Pomacea* clade have much wider and somewhat more contiguous distributions. The two species of *Marisa* are distributed from southern Brazil through northern South America and Trinidad and Tobago. *Marisa planogyra* Pilsbry, 1933 occurs primarily in the south and *Marisa cornuarietis* (Linnaeus, 1758) in the north, with some possible overlap in northern Brazil. *Pomacea* is the largest and most diverse genus and has the widest distribution, occurring from Argentina through Central America, the Caribbean and into southeastern North America.

Multi-gene phylogenetic results from recent work on New World ampullariids are largely in agreement with previous hypotheses, placing *Pomacea* as the most derived group of New World ampullariids (Hayes 2007, Hayes *et al.* 2009). However, *Pomacea* as currently recognized (Cowie and Thiengo 2003) is not monophyletic, as also suggested by Simone (2004) in his morphological study. Similarly, Hayes (2007) found no support for monophyletic *Asolene*, *Felipponea*, or *Marisa*. Instead, both species of *Marisa*, two species of *Felipponea*, and several species of *Asolene* were recovered in a single well-supported basal clade, sister to a clade consisting of other species of *Asolene* and *Pomacea* (Fig. 2). These two clades were in turn sister to the larger well-supported group containing the remaining *Pomacea* species and the only *Pomella* species included in the analysis, *Pomella megastoma*. In the past, assignment of species to these genera, especially to *Asolene*, *Felipponea*, and *Pomella*, based on morphological criteria, has been inconsistent (Cowie and Thiengo 2003); these molecular results will help to circumscribe these poorly understood genera. Also, these preliminary data begin to clarify

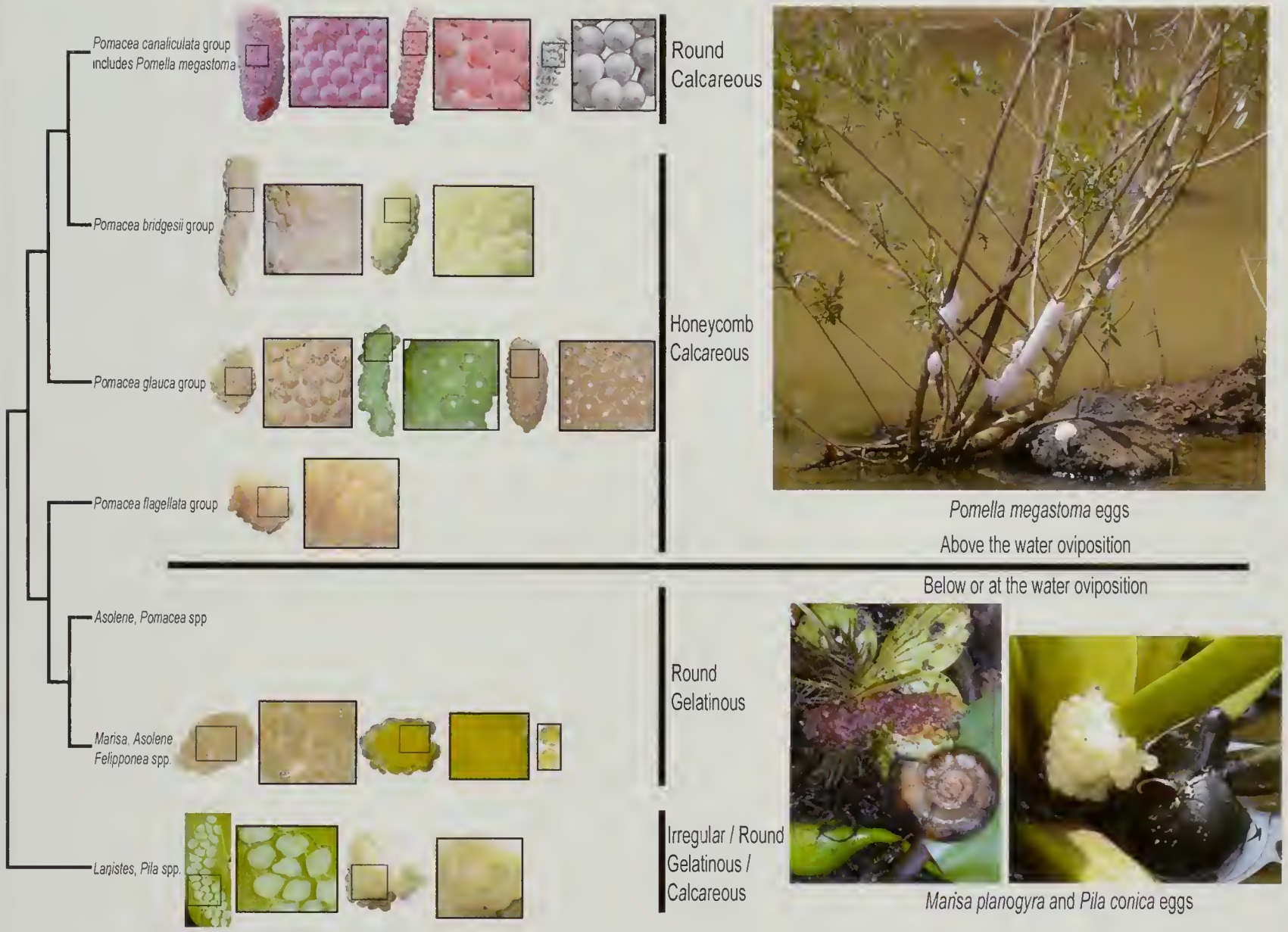

Figure 2. Ampullariidae phylogeny based on analysis of one mitochondrial and three nuclear genes showing the relationships among the major clades of the monophyletic New World genera (Hayes 2007). Egg morphology and oviposition location are mapped onto the major groups illustrating the evolutionary shift from laying gelatinous eggs below the water to laying calcareous eggs on emergent vegetation. Representative egg clutches from species within each of the major lineages illustrate a high level of morphological conservation within groups. From left to right egg clutches are: Top row, *Pomacea insularum* (d'Orbigny, 1835), *Pomacea canaliculata*, *Pomacea paludosa*; second row, *Pomacea diffusa* Blume, 1957, *Pomacea bridgesii* (Reeve, 1856); third row, *Pomacea* sp., *Pomacea glauca*, *Pomacea guyanensis* (Lamarck, 1822); fourth row, *Pomacea catemacensis* (Baker, 1922); fifth row, *Asolene spixii* (d'Orbigny, 1838), *Felipponea* sp.; bottom row, *Lanistes ovum*, *Pila conica* (Wood, 1828). Photo credits: J. F. R. Amato (*Felipponea* sp.), K. C. M. Heiler (*Lanistes ovum*), J.-P. Pointier (*Pomacea glauca*), R. C. Joshi (*Pila conica*), K. Gallagher (*Asolene spixii*), S. C. Thiengo (*Marisa planogyra*), and K. A. Hayes (all others).

the biogeographic patterns of diversification in New World ampullariids and reveal insights into factors like desiccation resistance, oviposition, and predation pressure that may have played a role in this diversification (Hayes 2009). These issues are discussed below.

BIODIVERSITY AND SPECIATION

The family Ampullariidae contains more than 150 species, and reaches its highest diversity in the Neotropics with the genus *Pomacea*, which contains 117 nominally valid species (Cowie and Thiengo 2003). The question of why there are so many Neotropical species has intrigued scientists since Alfred Russel Wallace (1852) first proposed that rivers act as barriers, driving allopatric speciation in Amazonian monkeys. Since then several explanations have been proposed, including Haffer's (1969) explanation of bird diversity in the context of the so-called "refuge hypothesis" and explanations of freshwater ichthyofaunal diversity based on the "hydrogeological hypothesis" (Lundberg *et al.* 1998, Montoya-Burgos 2003). Most explanations of high levels of Neotropical diversity have

been based on vertebrates, invoking vicariance as the primary isolating mechanism (Hall and Harvey 2002, Costa 2003, Ribas *et al.* 2005).

Conspicuously lacking are studies of the huge diversity of invertebrates, including Neotropical freshwater molluscs and studies looking specifically at the complex inter- and intraspecific interactions that drive speciation. Ampullariids have the potential to provide insight into these issues, as well as to reveal other less studied mechanisms generating diversity (Vermeij and Covich 1978, Endler 1982).

Since Darwin (1859), evolutionary biologists have suggested that ecology plays a vital part in the origin of species (Schluter 2001, Via 2002). The evolutionary ecology of apple snails has great potential for shedding light on the various processes involved in population divergence and ultimately speciation. Here, we address three of these, which may overlap; there are probably others equally amenable to investigation using these snails. First, the interaction between apple snails and their predators, their trophic relations, may influence natural selection regimes acting on both predator and prey. Studies focusing on these interactions should provide key insights into the evolution of these relationships and predator prey co-evolution in general, which in turn will provide information about how such processes help shape biodiversity. Second, habitat partitioning among ampullariids within an ecosystem may provide the necessary isolation for divergent selection to reinforce adaptations leading to reproductive isolation. These interactions may represent major drivers of evolutionary change. Third, because of their wide latitudinal distribution and high species level diversity, ampullariids offer an exciting opportunity to investigate large-scale patterns, or rules, of biodiversity, including Rapoport's Rule and the latitudinal diversity gradient.

Trophic relations

Throughout their range, ampullariids are major constituents of tropical/subtropical freshwater diversity and are key taxa in important aquatic ecosystems such as the Florida Everglades, the Llanos of Venezuela, and the Pantanal of central South America (Donnay and Beissinger 1993, Fellerhoff 2002, Brown *et al.* 2006). They may even serve as important indicators of ecosystem health (Ogden *et al.* 2005). In these systems, they are the main food of snail kites, which include the endangered Everglades Snail Kite, *Rostrhamus sociabilis plumbeus*, and closely related congeners (*i.e.*, *Rostrhamus hamatus*). Because of the abundance of apple snails in these ecosystems and their role in the diet of a variety of animals (birds, fishes, turtles, crocodilians), they could be considered keystone prey species and an important link between aquatic and terrestrial food chains (Donnay and Beissinger 1993, Ebenman and Jonsson 2005). Studies of trophic relationships indicate that these links may have a large influence on species diversity (Paine 1966, Kondoh 2003).

Populations of species coupled in predator-prey relationships may often be ecologically linked through both conspicuous predator-prey interactions (Connell 1961, Vermeij 1982) and more cryptic evolutionary dynamics (Yoshida *et al.* 2007). For instance, variation in the distribution and abundance of apple snail species has been shown to influence the distribution (Angehr 1999), abundance (Darby *et al.* 2006), and behavior (Tanaka *et al.* 2006) of snail kites, which have evolved both morphologically and behaviorally for extreme specialization on apple snails (Beissinger *et al.* 1994). At the same time, predation pressure, by kites and other predators, has probably shaped the morphological and behavioral adaptations of apple snails. For example, Reed and Janzen (1999) determined that the foraging behavior of limpkins (*Aramus guarauna*) resulted in disruptive selection on shell size in *Pomacea flagellata* (Say, 1829) in Costa Rica. They also observed directional selection against larger, light-colored snails by snail kites. Dieckmann and Doebeli (1999) modeled just such a predator-prey system and found that predator-prey interactions, when coupled with demographic stochasticity and the resulting genetic drift and assortative mating, often leads to evolutionary branching, which could result in sympatric speciation. Providing further evidence of possibly important evolutionary interactions, Snyder and Snyder (1971) found that *Pomacea paludosa* (Say, 1829), *Pomacea glauca* (Linnaeus, 1758), and *Pomacea dolioides* (Reeve, 1856) exhibit alarm responses to chemical cues from turtle predators, injured or dead conspecifics, and mechanical disturbance.

Similar processes may also have shaped the diversity of Old World ampullariids. Van Damme and Pickford (1995) suggested that rapid radiations of *Lanistes* spp. and changes in *Pila* spp. in the Rift Valley lakes of East Africa were probably the result of selection pressure from specialized predators, particularly fishes. Rapid and successive morphological changes in these two genera were inferred to have occurred *ca.* 8-2.5 mybp. A series of impressive *Lanistes* radiations involving rapid, major changes in shell morphology provides a good model for understanding speciation processes (Van Damme and Pickford 1995). Specifically, two successive radiations occurred, first in Paleolake Obweruka and later in Lake Malawi, both demonstrating convergence on anti-predatory behaviors and morphologies characteristic of a number of Rift Valley lake mollusc species. Some of the patterns seen, particularly thalassoidism (*i.e.*, shell form resembling marine gastropod species), were attributed to predator-prey interactions that may have triggered speciation. The repetitive ampullariid radiations were considered as conforming to a punctuated equilibrium model of evolutionary change (Van Damme and Pickford 1995) although such interpretations of African Great Lake fossil gastropod faunas

have long been criticized (*e.g.*, Jones 1981). The fossil radiations may be useful in understanding patterns of more recent radiations of African ampullariids, especially if change in shell morphology can be linked to genetic change.

Almost all theory on the tempo and mode of speciation in Lake Malawi, while providing major insight into evolutionary processes, rests on the study of cichlid fishes (Kocher 2004). The recent ampullariid radiation in Lake Malawi was studied morphologically by Berthold (1990), but a detailed molecular study of these snails would potentially shed further light on these questions using a non-fish model system. Recent studies of the Lake Malawi radiation (Jørgensen *et al.* 2008, Schultheiß, Van Bocxlaer, Albrecht, and Wilke, pers. comm.) revealed relatively low genetic variation within this clade. This might indicate a young evolutionary age of the radiation, a suggestion previously made by Berthold (1990). In combination with modern morphometric analyses, molecular methods will help to identify general patterns of diversification in ancient lakes, which will help clarify any differences in mode and tempo of speciation between vertebrate taxa like cichlid fish and invertebrates like the ampullariid genus *Lanistes*.

Habitat partitioning

In addition to predator-prey interactions, other aspects of ampullariid ecology have probably influenced their current diversity. African *Lanistes* have both lacustrine (*e.g.*, Lake Malawi, see above) and riverine (*e.g.*, Congo River basin) radiations. In Lake Malawi *Lanistes nyassanus* Dohrn, 1865 and *Lanistes solidus* Smith, 1877 differ in their use of microhabitats along a depth gradient, probably related to food availability and differential response to cichlid predators (Louda *et al.* 1984). Further, using the *Lanistes* spp. of Lake Malawi as an example, Berthold (1991) explored aspects of speciation and evolution of shell sculpture within the framework of a multidimensional niche concept. He proposed that speciation of Lake Malawi *Lanistes* was driven by differential adaptations to wave action, food resources, and predators (particularly habitat and behavioral shifts for predator avoidance). Such a scenario has the classic elements that would be anticipated in a case of ecological speciation, whereby reproductive isolation builds between two populations that accumulate adaptations to unique aspects of their environment (Schluter 2001).

The Congo River basin radiation of *Lanistes* consists of *Lanistes bicarinatus* Germain, 1907, *Lanistes congicus* Boettger, 1891, *Lanistes intortus* Martens, 1877, and *Lanistes usenduweensis* (Dupuis and Putzeys, 1901). The high levels of conchological variation among these species makes inferring their monophyly difficult based on morphological analysis. Nonetheless, this great variation suggests a role of ecology in species diversification. Investigation of this radiation should focus initially on ascertaining its age, documenting genetic variation, determining monophyly, and identifying common ancestors. However, while it is an example of neither true riverine nor true lacustrine speciation, it offers the possibility of investigating speciation in a habitat type (river basin) that is more permanent on both ecological and evolutionary timescales than most lake habitats (Giller and Malmquist 1998). If future molecular investigations reject the monophyly of the Congo species, then the basin might be interpreted as a refuge that has been stable over the long term rather than a place of species radiation.

Large-scale biodiversity rules

A number of patterns of species diversity have been documented across a wide range of taxonomic groups. Most notably, these patterns include Rapoport's Rule – a positive correlation between species ranges and latitude (Stevens 1989), Bergmann's rule – increasing body size with increasing latitude in mammals and birds (Bergmann 1847), and the latitudinal biodiversity gradient – decreasing species richness from tropical to polar latitudes (Dobzhansky 1950). The latitudinal biodiversity gradient is one of the longest recognized and most universally accepted patterns in nature (Darwin 1859, Wallace 1878, Hutchinson 1959, Wright *et al.* 2006), yet there remains little agreement regarding the underlying mechanisms responsible for it (Mittelbach *et al.* 2007).

Three broad categories of explanations have been proposed to explain the gradient, involving ecological, evolutionary, and historical hypotheses (Mittelbach *et al.* 2007). Ecological hypotheses focus on processes of species coexistence and the maintenance of species diversity through species interactions, and apple snails have been mentioned above as a system with which to investigate such processes. Evolutionary hypotheses focus on rates of diversification. And historical explanations are based on the persistence and extent of tropical environments. Understanding the relationship between latitude and speciation has been hindered by a lack of comparative analyses across a single clade that inhabits both tropical and temperate regions. Here again, apple snails may serve as a good system for investigating the underlying processes, and they can be used to test explicitly several of the proposed hypotheses.

The oft-cited "diversification rate hypothesis" suggests that high tropical diversity results from high rates of speciation (Fischer 1960) caused by one or more of the following: (1) greater opportunities for reproductive isolation because lower latitudes contain larger area (Terborgh 1973), (2) increased rates of molecular evolution due to higher metabolic rates in warmer regions (Rohde 1992, Wright *et al.* 2006), (3) enhanced biotic interactions because of increased specialization and reduced dispersal (Dobzhansky 1950, Janzen 1967), and (4) lower extinction rates due to increased climatic stability (Darwin 1859, Fischer 1960) or larger population sizes (Terborgh 1973).

Using ampullariids to investigate the various mechanisms responsible for higher levels of tropical than of temperate diversity will require data from paleontology, biogeography, ecology, and phylogenetics. However, using preliminary data (Hayes 2009) we can begin addressing at least one of these hypotheses. The explanation of Rohde (1992), supported by Wright *et al.* (2006), posits that higher tropical diversity results from an increased rate of molecular evolution in the tropics relative to higher latitudes. Species of *Pomacea* are an ideal group to test this hypothesis, as they range from temperate Argentina to the southeastern U.S.A. Using the approach of Wright *et al.* (2006), rate heterogeneity in molecular evolution can be tested using sister taxa, one of which occurs in the tropics and the other in a temperate region (*e.g.*, *Pomacea canaliculata* (Lamarck, 1822) and *Pomacea dolioides*). Hayes (2009) found that ampullariid diversity indeed decreases with increasing latitude. If future research finds a difference in rate of evolution, these taxa could be used to investigate further the possible mechanisms driving the differences.

The "historical time and area hypothesis" contends that areas with tropical climates are historically larger and older, which has allowed more opportunity for diversification (Fischer 1960, Wiens *et al.* 2006). If this were the primary driver of greater tropical versus temperate diversity, we should expect tropical species to be older and temperate species to be nested within clades of tropical taxa. Also we should expect diversity to be correlated with the age of geographical regions. Data emerging from ongoing work on ampullariids (Schultheiß *et al.* 2007, Jørgensen *et al.* 2008, Hayes *et al.* 2009) are beginning to provide the phylogenetic and biogeographic framework to address such hypotheses.

PHYSIOLOGY AND BEHAVIOR

In addition to addressing broad questions of biogeography and speciation, apple snails provide an excellent system for studying the evolution of physiological and behavioral adaptations, aspects of which may have profound implications for the generation of diversity, and for addressing important questions in behavioral ecology and evolution. Mapping apple snail oviposition location onto a preliminary phylogeny, Hayes (2007) found that laying eggs on emergent vegetation or other above-water hard surfaces is a synapomorphy that unites the most derived clade consisting predominantly of snails currently referred to *Pomacea*. Other ampullariids, including Old World and basal New World taxa, oviposit either on vegetation below or at the water line or in mud close to it (Cowie 2002) (Fig. 2). This observation, combined with the fact that this derived group is also the most speciose and covers the widest geographical range, leads to speculation that this shift to above water oviposition may have been a key innovation that accompanied the diversification and spread of the group. Other characteristics in the above-water egg-laying group seem to include longer siphons (for aerial respiration), increased lung size, and increased desiccation resistance (Cowie 2002). All these factors may be correlated with the success of the group.

Unique egg morphologies are associated with each of the clades in this above-water oviposition group, with the most derived group having spherical eggs that cluster relatively loosely in the egg mass. The more basal taxa in this group lay eggs that are honeycombed or polygonal in shape and abut tightly against one another within the egg mass (Fig. 2). It is possible that the derived condition of spherical, loosely clustered eggs may also have contributed to the success of these taxa through increased hatching rate resulting from more efficient respiration through the egg shell although respiration rates in clutches with different morphologies have yet to be measured.

Nuptial feeding is any form of nutrient transfer from the male to female during or directly after courtship or copulation. Burela and Martín (2007) reported nuptial feeding in *Pomacea canaliculata*, the first time it has been reported in a gastropod. Such behavior has implications for sexual selection and fitness. Burela and Martín (2007) discussed several possible advantages, including enhanced male fitness through benefits conferred to the offspring via additional nutrients, and mate attraction, mate acceptance, or increasing the length of copulation to maximize sperm transfer. Either way, this is a fascinating behavior that has interesting evolutionary implications for apple snails and mating behavior in general. Burela and Martín (2007) suggested that given the high level of similarity in the general body plan across the Ampullariidae, this behavior is probably not exclusive to this species, and may be found more widely.

BIOLOGICAL INVASIONS

Invasive species are now recognized throughout the world as a major economic and environmental threat (Pimentel *et al.* 2005, Puth and Post 2005). While these alien invasions cause tremendous agricultural, conservation, and human health problems, the rapid evolutionary changes that often accompany such unplanned invasion experiments may permit a greater understanding of the natural world, and at the same time provide insights into a variety of ecological and evolutionary processes (Sax *et al.* 2007). That rapid evolutionary changes occur after the introduction of alien species has become increasingly well documented (Cox 2004, Carroll *et al.* 2005, Huey *et al.* 2005), and more studies are taking advantage of these "accidental experiments" to investigate contemporary evolution. Such changes may often

take place in tens to hundreds of generations instead of the millions that most evolutionary processes are normally thought to occur over, and they take place in both the alien and the native species that interact during invasions (Sax *et al.* 2007). A number of ampullariid species have become invasive outside their native ranges, particularly species of *Pomacea* and *Marisa* (Joshi and Sebastian 2006, Rawlings *et al.* 2007, Hayes *et al.* 2008). It is possible that the adaptive genetic changes necessary to be successful invasives are occurring rapidly in these species, and these processes may be understood better by integrating ecological and evolutionary perspectives. Wada and Matsukura (2007) have shown that *Pomacea canaliculata* has adopted at least two strategies for dealing with overwintering in its introduced range in Japan: burial in mud or seeking refuge under rice straw before the onset of winter. They found seasonal differences in cold hardiness of snails, suggesting that cold winters may impose strong natural selection on such populations. It is still uncertain whether this is an adaptation acquired after introduction or one possessed by source populations in their native ranges. However, in either case it demonstrates that ampullariids may be an illuminating system for studying adaptive strategies of introduced species. Because multiple ampullariid species have been introduced, comparative studies among them may reveal key differences in such strategies. The results of such studies will not only strengthen our understanding of invasion biology but may also allow us to investigate the patterns and tempo of adaptive genetic changes along with the influence of founder events on these processes.

CONCLUSIONS

More than 150 years after Darwin's revolutionary idea of descent with modification, there remain a number of unanswered questions fundamental to our understanding of evolution and biodiversity. How many species are there? How are these species distributed? What are the processes that generate this biodiversity? Many of these questions remain unanswered simply because of the complexity of the evolutionary process. For example, the myriad mechanisms that might lead to the evolution of reproductive isolation (*i.e.*, speciation) are often difficult to disentangle. Yet addressing these issues in a range of groups, particularly those with the highest diversity, may reveal additional insights that will go a long way to answering these big evolutionary questions. Ampullariids offer an excellent system for addressing many of these questions, particularly regarding the generation of biodiversity and how it spread and diversified around the globe. Lessons learned from this group may be generalized not only to other freshwater taxa but also to more profound and over-arching themes in evolutionary biology.

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Land snail models in island biogeography: A tale of two snails*

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Abstract: Oceanic islands have long been important in evolutionary biology. Land snails are a major component of oceanic island biotas and have much to offer as systems for addressing major questions in evolution and biogeography. We review patterns of within-archipelago biogeography and diversification in two large Hawaiian land snail groups, the Succineidae and the Achatinellinae. Molecular studies suggest that long-distance oceanic dispersal and colonization of the Hawaiian Islands has been rare but between-island dispersal has been far more common. Long-distance oceanic dispersal is the most important driver for deep phylogenetic divergence. Dispersal is also important within the archipelago, while among-island vicariant processes result in only a portion of tip clade diversity. The Achatinellinae are monophyletic but there is evidence of a deep phylogenetic split between the two Hawaiian succineid clades, a result of two independent colonizations reflecting two oceanic dispersal events. Hawaiian succineids have also dispersed to Samoa and Tahiti. Dispersal is an important biogeographical phenomenon, and its role in shaping distributions of island lineages should not be underestimated. Because of their relatively sedentary nature, yet a proclivity for long-distance passive dispersal, island snails can facilitate insights into mechanisms of evolutionary diversification. Important phylogenetic lessons are emerging from studies of island snails and such studies will eventually allow estimation of ages of species groups, speciation rates, timing of the processes involved in community assembly, and other dynamics, all of which are important contributions to the overall understanding of evolution.

Key words: Succineidae, Achatinellinae, phylogeography, vicariance, Hawaii

Islands have long been considered important model systems for the study of evolution and biogeography. In the Pacific, the Hawaiian and Galapagos Islands in particular have provided numerous novel scientific insights (*e.g.,* Cain 1984, Grant 1986). Land snails are an important component of biodiversity in many oceanic island groups (*e.g.,* Cowie 2004, Cameron *et al.* 2007) and have great potential as informative models with which to address major evolutionary, especially biogeographic, questions.

Specific biological attributes of island snails that render them informative as models for biogeographic analyses include their high levels of species endemism and often broad distributions of families. This allows phylogenetic studies to use a hierarchical systematic approach to illuminate nested evolutionary patterns from the levels of populations to families. A number of snail families have remarkable passive dispersal and colonization abilities, given geological time, in seeming contrast with their low active mobility and often relatively small spatial ranges at the species level (*e.g.,* Barker and Mayhill 1999, Fontaine *et al.* 2007). Certain land snails are adept at dispersing across vast geographic distances, including ocean basins. This has been widely acknowledged and documented (*e.g.,* Darwin 1859, Anonymous 1936, Rees 1965, Dundee *et al.* 1967, Vagvolgyi 1975) and is evidenced by the worldwide distributions of a number of lineages. It is now supported by molecular evidence (Gittenberger *et al.* 2006).

In this paper, we review the phylogenetics, biogeography, and phylogeography of two major groups of land snails, the Succineidae and Achatinellinae, that have diversified across the Hawaiian archipelago in contrasting ways, illustrating the value of studies of island snails in evolution and biogeography. Within each group, we first address interspecific phylogenetic relationships and colonization patterns and then focus on intraspecific phylogeography. Since the Hawaiian land snail fauna is highly threatened (Solem 1990, Lydeard *et al.* 2004), understanding diversification patterns and evolutionary history can also provide valuable insights for conservation (*e.g.,* Holland and Hadfield 2002).

THE HAWAIIAN ISLANDS AND THEIR BIOTA

The Hawaiian archipelago consists of a sequence of oceanic islands formed as the Pacific plate moves northwestward over a stationary "hot spot" in the earth's mantle. The hot spot sends magma up through the plate, creating a chain

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of volcanoes, each sequentially younger than the one that preceded it, northwestward away from the hot spot. High islands eventually subside and erode to become low atolls, then submerged seamounts, and are ultimately subducted as the Pacific plate slides under the adjacent tectonic plate (Price and Clague 2002). The current islands are divided into the younger "high" islands and the older northwestern islands, which have become low atolls or small, eroded pinnacles or islets. Currently, the oldest, northwestern-most island is Kure Atoll (29 Ma) and the oldest high island is Kauai (5.1 Ma), with the youngest island, Hawaii itself, being less than 0.5 Ma and still forming.

The islands harbor unique forms of plants and animals that are the products of evolution in isolation over tens of millions of years, as older islands vanished and new islands formed. Most of the current diversity occurs on the high islands, from the island of Kauai in the northwest to the island of Hawaii in the southeast (Fig. 1). The islands' extreme isolation, diversity of microhabitats, and dynamic geology have led to spectacular biological endemism as well as prominence as a global biodiversity hotspot (Simon 1987, Ziegler 2002). The unique, terrestrial evolutionary radiations have attracted scientific attention for over a century (Gulick 1905, Carson 1987, Wagner and Funk 1995, Hormiga *et al.* 2003). In recent decades, however, the Hawaiian biota has become increasingly threatened. Anthropogenic habitat destruction and the devastating impacts of non-native species (Staples and Cowie 2001, Eldredge and Evenhuis 2003) have led to high levels of extinction (Vitousek 1988, Pimm *et al.* 1994, Wagner *et al.* 1999).

There are over 750 nomenclaturally valid, native, land snail species in 11 families in the Hawaiian Islands; over 99% of these species are endemic (Cowie 1995, Cowie *et al.* 1995). It has long been assumed that each endemic group (genus, subfamily, family) of species within this huge diversity has resulted from *in situ* speciation following a single colonization by a single ancestral lineage (Zimmerman 1948) although until recently this had not been rigorously tested. Ascertaining biogeographic origins of land snail radiations is important for understanding their natural history, and the fact that multiple origins have now been demonstrated in some Hawaiian terrestrial invertebrate groups (Gillespie *et al.* 1994, Robinson and Sattler 2001, Rundell *et al.* 2004) suggests that other taxa may have similarly complex evolutionary histories.

Hawaiian land snails have suffered higher levels of extinction than perhaps any other group of Hawaiian organisms, with estimates as high as 90% of species now extinct (Cowie 2001) and most of the remainder seriously threatened. As a consequence of their present conservation status, there is an urgent need for data regarding the systematics and evolutionary history of the fauna, especially as legislative decisions regarding conservation require an unambiguous understanding of taxonomic status (*e.g.*, Avise 2000, Holland and Hadfield 2004, 2007).

COMPARATIVE BIOGEOGRAPHY

Integration of molecular phylogenetics with geological history is a powerful approach that permits estimation of lineage ages and polarity. Knowing absolute island ages, questions regarding temporal aspects of radiations such as rates of evolutionary diversification can be addressed; DNA sequences can provide high resolution, quantitative data relevant, for instance, to colonization patterns of many taxa including endemic terrestrial snails (Chiba 1999, Goodacre 2002, Holland and Hadfield 2002, Rundell *et al.* 2004, Holland and Cowie 2007).

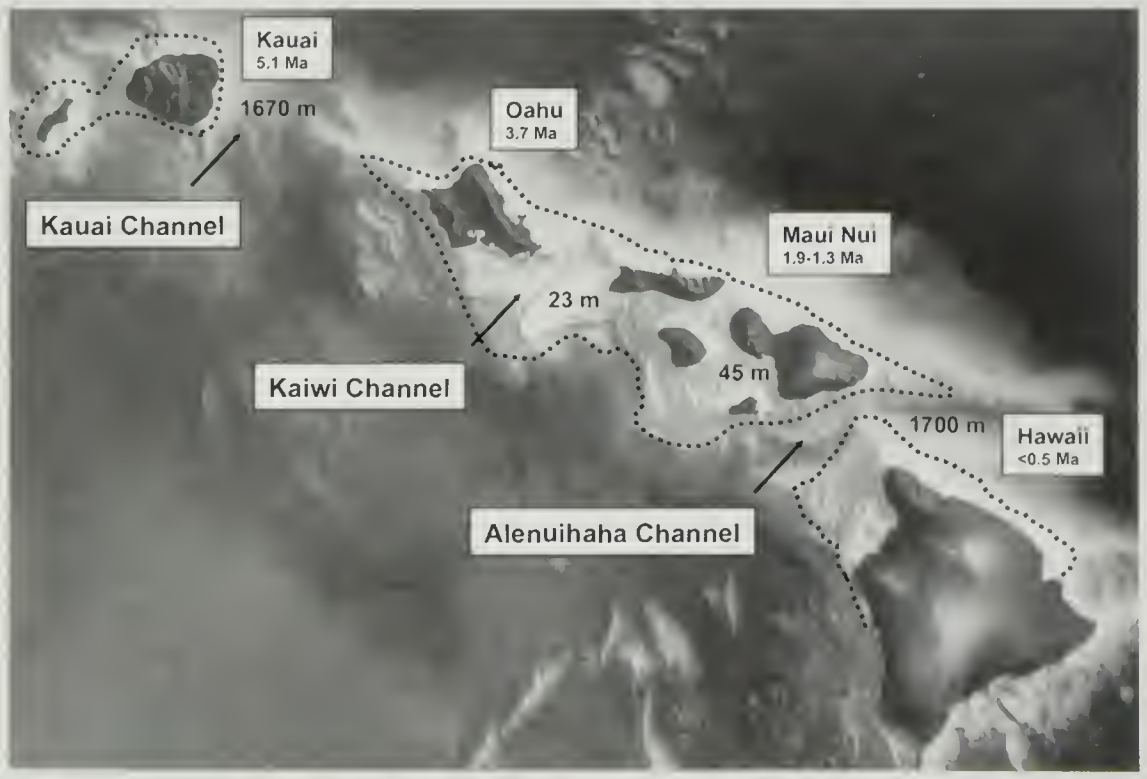

Figure 1. Map of the main Hawaiian Islands showing submarine topography and selected channel depths. Dotted lines show now submerged, maximum historical shorelines for each volcano as it formed. The islands of Maui, Lanai, Molokai, and Kahoolawe are known as Maui Nui. These islands and Oahu formed a single super island as recently as 18,000 years ago during the last glacial maximum.

Molecular phylogeography addresses spatial patterns of evolutionary diversity within species or species complexes and has recently been expanded to include comparison of phylogeographic patterns of multiple co-distributed lineages, comparative phylogeography (Arbogast and Kenagy 2001). Such studies have revealed pervasive and unanticipated biogeographic patterns and suggest that biotic assemblages contain much greater cryptic biological diversity than traditional systematics has recognized (*e.g.*, Bird *et al.* 2007, Sha *et al.* 2007, Victoriano *et al.* 2008). In a comparative framework, phylogeography can be used to evaluate both historical patterns and evolutionary processes, providing a basis for new avenues of research into the regional historical, ecological, and coevolutionary factors generating and maintaining biodiversity.

The well-documented geological history and known ages of the Hawaiian Islands allow examination of the timing of lineage splitting and species formation. The phylogenetic pattern characteristic of Hawaii's more diverse endemic clades, especially nonvolant groups with limited active dispersal ability, is for the older, basal members of a clade to occur on the oldest island and for successively more recently derived members of the clade to occur on successively younger islands (Wagner and Funk 1995, Fleischer *et al.* 1998, Roderick and Gillespie 1998, Price and Clague 2002, Cowie and Holland 2006), a pattern termed the progression rule of island biogeography (Wagner and Funk 1995). Since land snails are generally considered poor active long-distance dispersers; they are predicted to adhere to the progression rule pattern on oceanic archipelagos.

Native invertebrates, among them the endemic land snails, comprise the most species-rich faunal radiations in the Hawaiian Islands and offer valuable opportunities for the study of historical biogeography and evolutionary diversification of insular radiations in a comparative phylogenetic framework.

HAWAIIAN LAND SNAILS AS BIOGEOGRAPHIC MODELS

Hawaiian land snail phylogenies provide opportunities to investigate a number of general issues in evolutionary biology, including the relative roles of dispersal versus vicariance in the generation and maintenance of endemic lineages and the predictions of the "taxon cycle" (*sensu* Wilson 1961).

Since the early 1980s, a debate has periodically flared up in the primary biogeographic literature regarding the importance of vicariance versus dispersal in shaping distributions of plants and animals. Since most species have patchy distributions at some scale, and patchiness or allopatry has long been considered to play an important role in diversification (Mayr 1942), the mechanisms contributing to natural distribution patterns are fundamental to evolutionary biology and our understanding of biodiversity (see de Queiroz 2005, Cowie and Holland 2006).

According to the predictions of Wilson's (1961) taxon cycle, island lineages initially colonize marginal habitat, eventually dispersing and diversifying into higher elevation cloud forests, and ultimately go extinct as habitat changes and islands erode and subside. One of the key features of Wilson's taxon cycle is the notion that oceanic islands serve as evolutionary blind alleys (Whittaker 1998) and, therefore, do not give rise to further long-distance colonization.

Also, the diverse Hawaiian land snails provide natural systems with which to use molecular techniques to investigate many such patterns of island biogeography and lineage splitting (Holland and Hadfield 2002, 2004, Rundell *et al.* 2004, Cowie and Holland 2006, Holland and Cowie 2007) as well as to address more basic systematics issues (Holland and Hadfield 2007).

HAWAIIAN SUCCINEIDAE

Succineids occur worldwide (Pilsbry 1948, Patterson 1971), reaching their highest diversity in Pacific islands, India, and the Americas (Barker 2001). Though often associated with riparian areas (*e.g.*, Kerney and Cameron 1979), succineids also occur in a range of different habitats (Barker 2001, Rundell *et al.* 2004). There are 42 recognized Hawaiian succineid species, all endemic, with 35 single-island endemics (Cowie *et al.* 1995), suggesting that dispersal between islands occurs, but is sufficiently rare to allow speciation. The Hawaiian Succineidae have radiated into a diverse array of habitats, from montane rainforests to xeric coastal dunes (Cowie 1995, Cowie *et al.* 1995). Their shell types appear to reflect these ecological differences, making them candidates for studies of adaptive radiation, convergence, and morphological evolution.

We have been unable to find approximately half of the described species despite intensive field efforts focused on type localities and other suitable habitat, *e.g.*, *Catinella rubida* Pease, 1870 from Kauai, the type species of the genus *Catinella* Pease, 1870. These species are either extremely rare or possibly extinct. Rundell *et al.* (2004) estimated that as many as one third of the Hawaiian succineid species may be extinct, but this may well be an underestimate.

Phylogenetics and biogeography

Although the family in the Hawaiian Islands was historically assumed to be monophyletic and the result of a single colonization (Zimmerman 1948), Rundell *et al.* (2004) showed, based on mtDNA evidence, that this is not the case. Here we present a global, multi-locus data set that permits this surprising biogeographic result to be investigated further.

A combined multi-locus phylogeny of 40 ingroup species indicates that the Hawaiian succineids fall into two monophyletic groups (Fig. 2). Clade A (Fig. 2) includes succineids from Kauai, Oahu, Molokai, Maui, Lanai, and Hawaii, with a species from Samoa nested within this clade, sister to a species from Kauai. The general patterns of relationships within this clade provide support, though weakly, for the progression rule of successive colonization from older islands (Kauai, 5.1 Ma, Oahu, 3.7 Ma) to younger islands (West Maui, 1.3 Ma, Hawaii, <0.5 Ma), with species from Kauai and Oahu associated with the basal nodes of the clade. Basal to this clade is a species from the islands of the South Pacific (Samoan, Marquesas, Cook, and Austral Islands), indicating an ancient South Pacific origin. Clade B includes species from the island of Hawaii plus *Succinea caduca* Mighels, 1845, which is the one Hawaiian succineid that occurs on all six main islands (Holland and Cowie 2007), with a species from Tahiti nested within the clade, sister to a species from the island of Hawaii. Basal to Clade B is a species from Thailand (that is also found as a modern invasive species in Hawaii), suggesting a southeast Asian origin. Clade A is the older of the Hawaiian succineid lineages. The endemic Hawaiian succineids are not monophyletic and the two main Hawaiian lineages differ in their geographic origin and inferred relative time of colonization.

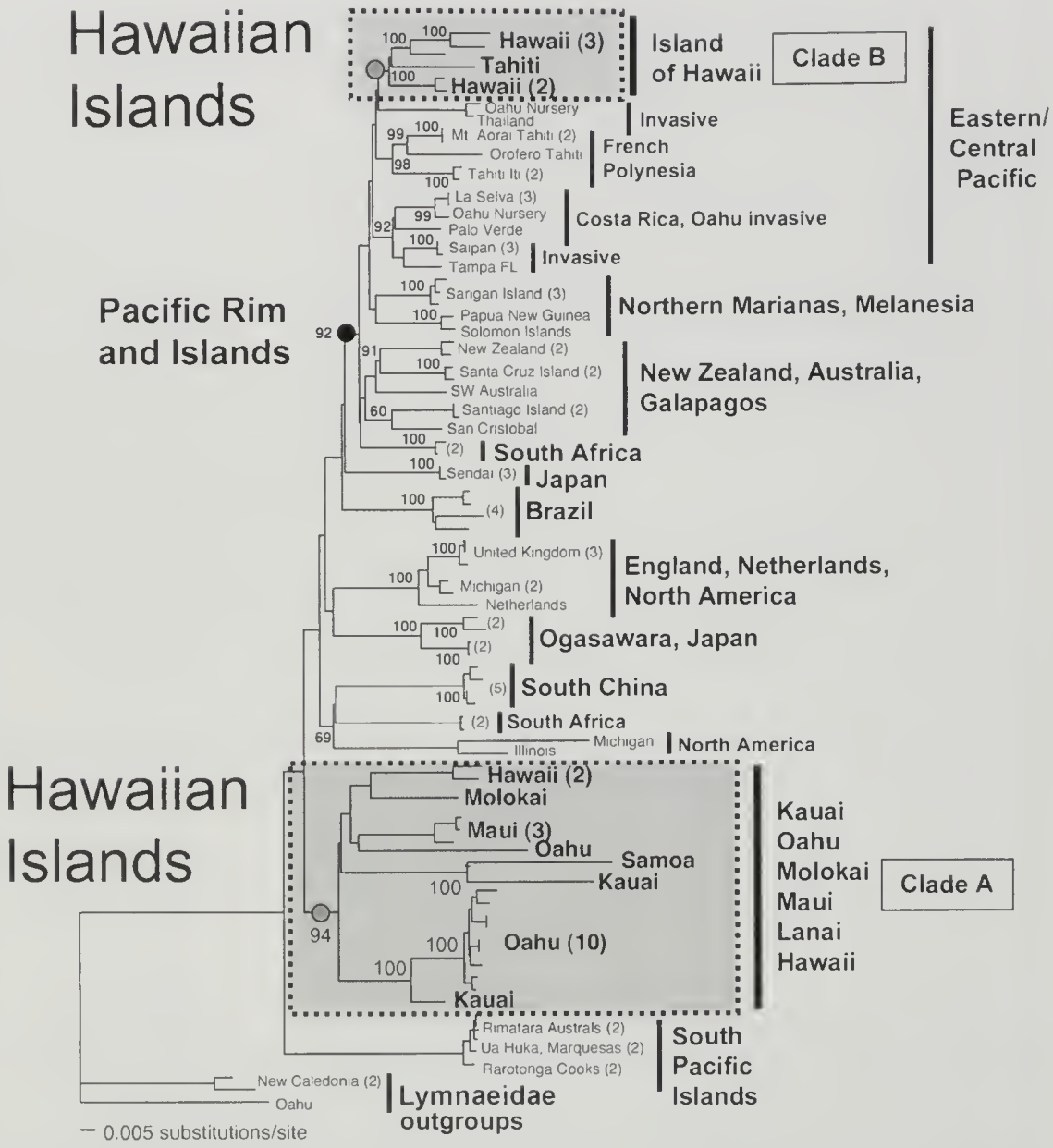

Figure 2. Phylogenetic tree for the Succineidae, based on three genes, two mitochondrial and one nuclear (COI, 16S, 18S; 1740 base pairs), for 40 ingroup species and two outgroups. This area phylogram was generated with PAUP (Swofford 2002) using a maximum-likelihood approach. Numbers near nodes represent bootstrap support based on 1000 replicates. Numbers in parentheses show numbers of specimens sequenced per lineage. The shaded boxes highlight the two Hawaiian lineages.

An Asian or Australasian origin has been thought likely for a number of other Pacific island land snail groups (Cowie 1996, Pokryszko 1997). The phylogenetic analyses presented here, with Thai species basal to Clade B (Fig. 2), are consistent with this suggestion. The positions of a Samoan species in Clade A and a Tahitian species in Clade B suggest that Samoa was colonized from Kauai and Tahiti was colonized from Hawaii. These may be the first recognized cases of natural colonizations of other locations by endemic Hawaiian animal species, contrasting, since they are both rainforest not marginal habitat species, with predictions of Wilson's (1961) "taxon cycle", which considers endemic island lineages to be essentially evolutionary blind alleys.

The absolute ages of the Hawaiian succineid lineages are uncertain but theoretically an ancestral succineid could have arrived in the Hawaiian archipelago as early as about 29 Ma ago. This is the age of Kure, the adjacent older islands in the chain having already vanished below the ocean surface, and islands have been consistently present above sea level since then (Carson and Clague 1995, Clague 1996). However, many groups of Hawaiian plants and animals exist exclusively at high elevation. Therefore, since there were probably periods of hundreds of thousands of years when, as older islands subsided and eroded and newer islands were as yet low, only low elevation habitats were available. Absence of high elevation habitat may thus preclude an ancient origin (*i.e.*, 29 Ma) (Price and Clague 2002). In particular, such a scenario may

have happened when Kauai, the oldest, high island, emerged because Nihoa (the adjacent older island) had already declined greatly in elevation (Price and Clague 2002). Nevertheless, some modern Hawaiian succineids inhabit coastal dune lands and low-elevation forests, indicating that their ancestors could also have thrived in similar environments; land of sufficient, if low, suitable elevation was indeed consistently available from 29 Ma ago to the present. Archaeological and paleontological evidence suggests that succineids, amastrids, and other endemic Hawaiian land snail species probably once had ranges that extended into low elevation dry forest and arid habitats (Christensen and Kirch 1986). Phylogenetic studies of other Hawaiian invertebrate radiations suggest a few origins far exceeding the age of Kauai (Russo *et al.* 1995, Jordan *et al.* 2003).

The island of Hawaii is less than 0.5 Ma old, and 22 (19 endemic) of the 42 recognized Hawaiian succineid species are from this island (Cowie *et al.* 1995). Additional work on the evolution of succineid species on the island of Hawaii, such as application of molecular clock theory to robust phylogenies consisting of additional nuclear genes and the possible inclusion of extinct species from museum collections, may provide more insights into the complexity and rate of evolutionary change.

Single species phylogeography: *Succinea caduca*

Although the Hawaiian succineids are primarily single-island endemics, *Succinea caduca* Mighels, 1845 occurs on all six major high islands (Holland and Cowie 2006). Holland and Cowie (2007) used mtDNA COI sequences to evaluate geographic patterns of variation in *S. caduca*, sampling 24 populations on the six islands. Six clades based on 276 COI sequences were revealed, indicating substantial geographic genetic structuring. Low nucleotide diversity and low pairwise molecular-divergence values within populations coupled with higher between-population values suggested multiple founder events. High overall haplotype diversity suggested diversification involving rare dispersal, fragmentation by historical lava flows, and variation in habitat structure. Within-island rather than between-island population comparisons accounted for the majority of molecular variance. Population partitioning patterns suggested that genetic fragmentation has been driven by punctuated, passive dispersal of groups of closely related haplotypes that subsequently expanded and persisted in isolation for long periods (average >2 Ma), and that inter-island dispersal has led to population fragmentation. However, Mantel tests for isolation by distance, statistical correlation of geographic and genetic distance, were not significant. Thus, rather than a widespread panmictic distribution that was subsequently divided into its present day distribution, we see evidence that dispersal has led to a chaotic pattern of genetic partitioning. Historical availability of mesic coastal habitat, together with effective dispersal, may explain the long-term persistence and unusual multi-island distribution of this species, which contrasts with the single island endemism of most Hawaiian succineids as well as much of the Hawaiian biota (Holland and Cowie 2007).

Haplotype networks from the northwestern portion of the archipelago, representing the older islands of Kauai and Oahu that are separated by the Kauai Channel (Fig. 1) and have never been connected, are broken into 5 clades (Holland and Cowie 2007). One of these clades contains haplotypes from Kauai and Oahu, demonstrating unambiguously that dispersal across the Kauai Channel has occurred. Other haplotypes from Oahu and all those from Maui Nui (Maui, Molokai, Lanai) group in a single clade and provide support for vicariant separations and evidence that Pleistocene island connections may have been important in enhancing gene flow. Vicariant separation in this clade has not led to deep divergence, a reflection of the geologically recent separation of the Maui Nui component islands (Price and Elliott-Fisk 2004), which conforms with the estimated most recent sea level minimum at the last glacial maximum 18,000 years ago, about 130 m below the current level. Haplotypes from the island of Hawaii cluster in a separate, single island clade. Thus, *Succinea caduca* provides evidence for both within (Kauai, Oahu) and between (Kauai–Oahu, Maui–Hawaii) island diversification, suggesting that a combination of vicariance and dispersal has led to the allopatric diversification of this species.

HAWAIIAN ACHATINELLINAE

With 99 recognized species, all single-island endemics, in four genera (Cowie *et al.* 1995, Holland and Hadfield 2004), the endemic Hawaiian tree snails (Achatinellinae) are a species-rich radiation. The richly colored, highly varied banding patterns of their shells captured the attention of early naturalists and shell collectors, many of whom collected thousands of snails during the late 1800s and early 1900s (Hadfield 1986). Historically, appreciation for Hawaiian tree snails helped to inspire and promote general awareness of the diverse and unique Hawaiian biota. In recent years, however, the Hawaiian tree snails have also gained scientific and regulatory attention because of their dire conservation status. Of the 41 species of *Achatinella* Swainson, 1828 recognized by the U.S. Fish and Wildlife Service, only 10 are now thought to survive and are listed as endangered, with range reductions approaching 90% (U.S. Fish and Wildlife Service 1993). All other achatinelline species are seriously threatened.

Phylogenetics and biogeography

The molecular phylogenetic pattern for the Hawaiian tree snails (Fig. 3; see also Thacker and Hadfield 2000, Holland and Hadfield 2004) contrasts markedly with the topology for Hawaiian succineids (Fig. 2). The extant species analyzed

form a monophyletic group of three main clades: the Oahu Clade (1) consisting only of five *Achatinella* species from Oahu; the Maui Nui Clade (2) with 13 species from three genera and four islands (Oahu, Molokai, Lanai, Maui); and the Mixed Clade (3) with five species from three genera and four islands (Oahu, Molokai, Maui, Hawaii). The outgroup (*Auriculella* sp., Achatinellidae: Auriculellinae) was selected based on Holland and Hadfield (2004).

Achatinella mustelina Mighels, 1845, endemic to the oldest mountain range on the island, was basal to the other four members of the clade. Therefore, in reconstructing the biogeographic pattern and colonization sequence for the tree snails (Fig. 4), we begin with *A. mustelina*. As few as two of the twelve colonization events depicted in Fig. 4 may have involved dispersal over an ocean channel; the remainder could have taken place via past island connections (forested land bridges) and relatively recent vicariant separation. However, this is the minimum possible number of ocean channel crossings based on this analysis.

The following genetic divergence values were generated using mtDNA COI sequence data, for which published divergence rates for invertebrates are typically in the range of 1.4 to 2.2% per million years (Knowlton and Weigt 1998). The mean divergence among the 12 species of *Partulina* Pfeiffer, 1854 (5.4%) was lower than that among the *Achatinella* species, all from a single island (6.1%), despite the *Partulina* species coming from what are presently four islands (Maui, Molokai, Lanai, Hawaii) (Fig. 1), suggesting that the *Partulina* and *Perdicella* species in the Maui Nui Clade are younger than *Achatinella* species. The mean genetic divergence among species of all three genera from the Maui Nui Clade was 5.4%, slightly lower than that for all members of the genus *Partulina* at 5.5%. Interestingly, the divergence between *Partulina physa* (Newcomb, 1854) from the island of Hawaii and the next closest relative, *P. tappaniana* (Adams, 1851), was low (3.3%) in spite of substantial differences in shell morphology and separation of the islands by the Alenuihaha Channel. This relatively close relationship, spanning a deep marine feature, reflects historical dispersal across a marine barrier.

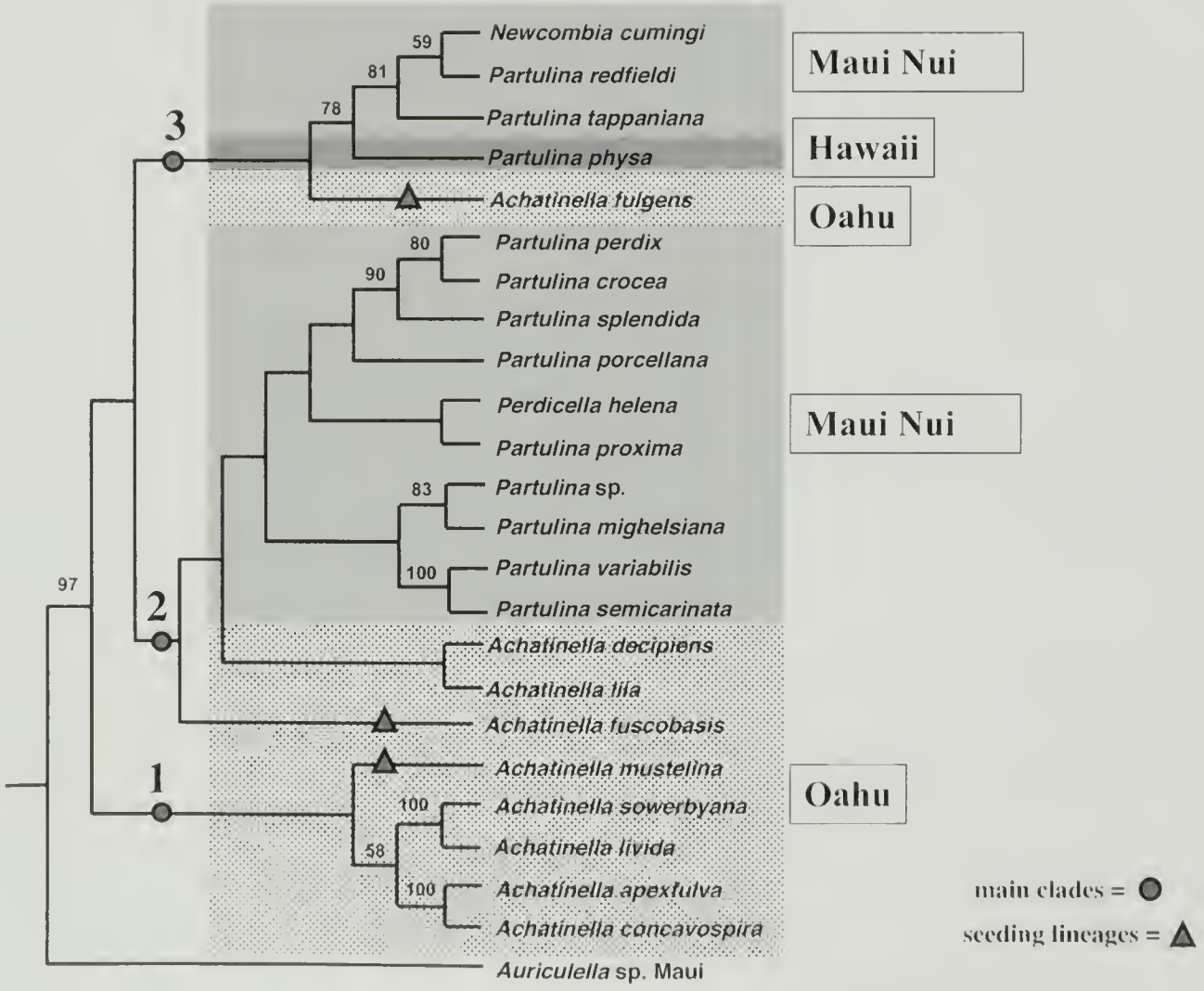

Figure 3. Maximum likelihood cladogram generated using PAUP (Swofford 2002) for the achatinelline tree snails reconstructed using COI gene sequences and 1000 bootstrap replicates. Some of the main features include monophyly of the subfamily and family (see Holland and Hadfield 2004), progression rule pattern of biogeography, and presence of seeding lineages from the oldest island in the group in each of the three main clades.

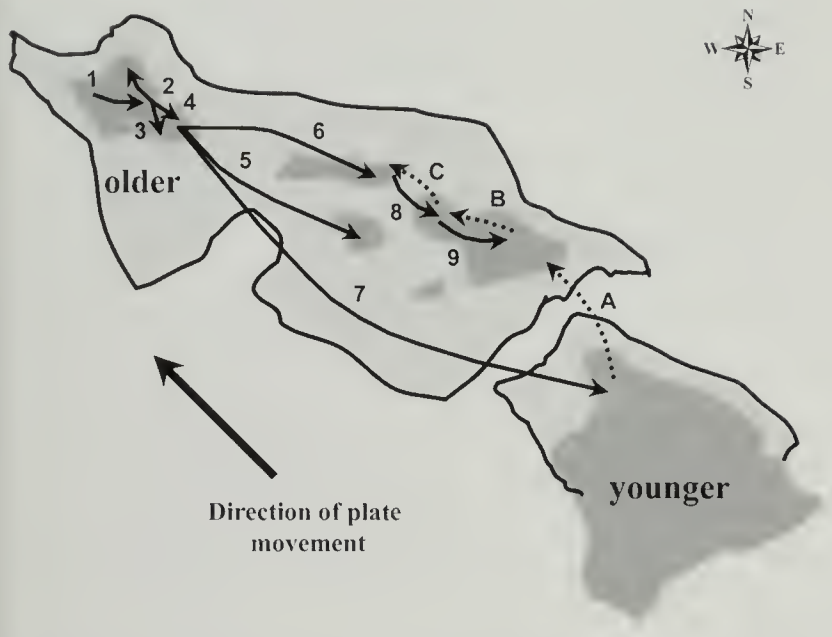

Figure 4. Island colonization sequence inferred from molecular evidence for achatinelline tree snails, based on the phylogeny of Holland and Hadfield (2004). Map includes submarine historical shorelines. Solid arrows follow the progression rule and represent forward colonization events from older to younger geological features, numbered according the order of the nodes in the tree (Fig. 3) and starting from *Achatinella mustelina*. Dotted arrows show back colonizations, from newer to older geological features or islands.

In a phylogenetic reconstruction that includes species representing all five achatinellid subfamilies, the Achatinellinae are monophyletic and well supported (R. H. Cowie *et al.*, unpubl. data). Achatinellid sequences formed a monophyletic group with high bootstrap support (91–100%).

Single species phylogeography: *Achatinella mustelina*

Holland and Hadfield (2002) used mitochondrial DNA (mtDNA) COI sequences to evaluate phylogeographic structure within and among 21 populations of *Achatinella mustelina* ($N = 78$). In contrast to the multi-island distribution of *Succinea caduca*, *A. mustelina* has a relatively small distribution spanning a single mountain range on Oahu. Pairwise intraspecific mtDNA sequence divergence between haplotypes ranged from 0 to 5.3%, and population genetic partitioning and mountain topography were strongly correlated. Maximum genetic distances were observed across deep, largely deforested valleys and sheer mountain ridges, independent of geographic distance. However, in certain areas where forest cover is presently fragmented, there was little sequence divergence despite large geographic distances. Genetic data were used to define five evolutionarily significant units (ESUs) that will guide conservation decisions regarding, for instance, placement of predator-exclusion fences, captive propagation, and eventual re-introduction and translocation.

To account for the observed phylogeographic pattern, an evolutionary scenario was proposed (Holland and Hadfield 2002) beginning with an initial panmictic phase, followed by long-term, large-scale habitat fragmentation, and finally recent fine-scale fragmentation resulting in the current patchy distribution. In the early geological history of the island, western Oahu consisted of a single massive shield volcano, similar in shape to the less than half-million year old volcanoes on the island of Hawaii. The modern topography of western Oahu is deeply eroded and rugged, with complex features resulting from action of wind and rain over its 3.7 Ma history. It is possible that large populations of tree snails gradually became separated from one another by vicariant forces resulting from the formation of valleys and ridges, and isolated by distance into the present pattern of "islands" of genetically cohesive populations. In support of this idea, a Mantel test (Holland and Hadfield 2002) demonstrated a correlation between genetic and geographic distance (in contrast to *Succinea caduca*). *Achatinella mustelina* has a far more restricted and genetically structured distribution than *S. caduca*, indicating relatively limited dispersal ability, possibly related to the former's much larger and heavier shell.

CONCLUSIONS

The molecular studies of Hawaiian land snails reviewed here strongly support the current shift in perception in historical biogeography from vicariance as the dominant process to more of a balance between dispersal and vicariance driving allopatric diversification.

In the Hawaiian Succineidae, molecular data reveal two independent colonization events from two different, distant geographic origins. Once the two lineages became established and began to radiate, each acted as a colonization source for long-distance dispersal to two different South Pacific island groups. Within one Hawaiian succineid lineage, dispersal across marine barriers occurred multiple times, resulting in one species (*Succinea caduca*) that occurs on all six major high islands. Closely related haplotypes that span a deep permanent feature such as the Kauai Channel attest to the dispersal ability of this species.

For the Hawaiian tree snails dispersal has also helped to shape their natural history in important ways. Sister species that span the Alenuihaha Channel between the relatively young islands of Maui and Hawaii attest to recent across-ocean dispersal. However, the fact that all 99 species are single-island endemics indicates that dispersal of these sedentary, heavy-shelled tree snails, while it certainly has occurred, is rare. Patterns of molecular diversification in the achatinellines indicate an Oahu origin, progression rule pattern, isolation by distance, and at least two channel-crossing dispersal events.

In contrast to the deep separation of two distinct lineages in the succineids, the achatinellines form a monophyletic clade (Holland and Hadfield 2004). However, preliminary molecular data indicate that the family Achatinellidae, which consists of five subfamilies (Cowie *et al.* 1995), two of which are endemic to Hawaii, colonized the Hawaiian Islands via four independent events (Holland and Cowie, unpubl. data).

Based on the well understood geological history of the Hawaiian Islands and the use of molecular phylogenetics, it has been possible to reconstruct the evolutionary and biogeographical history of two major groups of land snails, including the timing of lineage splitting and direction of colonization, while at the same time determining their geographic origins and whether presumptive radiations comprise monophyletic groups. Because of the relatively sedentary nature of land snails, yet the proclivity of some for long-distance passive dispersal, these groups have facilitated a major insight into the mechanisms via which evolutionary diversification takes place. Important phylogenetic lessons emerging from these studies of island snails include: (1) assumptions of monophyly are not always met; (2) in multi-island lineages composed of single island endemic species, monophyletic clades may include species from different islands; and (3) although some lineages conform to the isolation by distance and progression rule patterns of biogeography, others do not. Studies of island land snails will eventually lead to estimations of ages of species groups, speciation rates, timing of the processes involved in community assembly, and other dynamics, all of which are important contributions to the overall understanding of mechanisms of evolution.

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Molecular phylogeny, taxonomy, and evolution of the land snail genus *Pyrenaearia* (Gastropoda, Helicoidea)*

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Abstract: We reconstructed the molecular phylogeny of 78 specimens, including nearly all nominal species of the genus *Pyrenaearia*, and discussed the implications of the molecular phylogeny for species delimitation. The four basal clades obtained by mitochondrial COI and 16S sequences were highly congruent with nuclear ITS markers, shell morphology, and geographic distribution patterns, and were considered different species under the phylogenetic species concept: *Pyrenaearia carascalopsis* (Bourguignat in Fagot, 1884), *P. carascalensis* (Férussac, 1821), *P. parva* Ortiz de Zárate, 1956, and *P. cantabrica* (Hidalgo, 1873). Evidence of reproductive isolation between coexisting species of these basal clades has also been obtained. Because of genetic divergence, together with peculiarities of habitat use, distributional range, and shell morphology, *P. organiaca* (Fagot, 1905) and *P. navasi* (Fagot, 1907) were regarded as valid species. Further subdivisions within *Pyrenaearia* were also considered. Some incongruencies were found between shell morphology and DNA-based taxonomy, including the presence of hairs in adult shells and shell shape adaptations to different altitudes. The deepest split in *Pyrenaearia* involves four different lineages and represents an ancient event, occurring during the Pleistocene or even the Pliocene. Subsequent *Pyrenaearia* speciation within the four main groups has been a very recent process, and it is hypothesized to have occurred during Pleistocene cycles of climatic cooling and warming. Chronologically, a third speciation process is occurring from the Würm de-glaciation to the present time. All these processes included allopatric speciation of the high-altitude taxa during warmer interglacial periods, but parapatric or peripatric speciation events could also be involved. Based on the present day distribution of the species and their different altitudinal preferences, the whole genus could constitute a good model to investigate the evolutionary processes that created this diversity. It could also be an excellent group of closely related taxa for the study of the effect of Plio-Pleistocene climate changes on species distribution, population structure, speciation processes, secondary contacts, and passive dispersal.

Key words: DNA, systematics, distribution, land snails

The genus *Pyrenaearia* Hesse, 1921 is endemic in the north Iberian Peninsula and southern France, where it occurs in the Cantabrian Mountains and the Pyrenees (Fig. 1). Species of this genus generally live at high altitudes in open limestone habitats and occupy very restricted geographical areas (Altonaga *et al.* 1994, Puente 1994). They usually occur on rock walls (Ortiz de Zárate 1956), but above 2000 m they live predominantly under stones (Gómez-Moliner, pers. obs.). Based on distributional patterns, the 16 currently recognized nominal species in the genus can be divided in six species groups. (1) Six morphospecies are endemic in the Cantabrian Mountains, where *Pyrenaearia cantabrica* (Hidalgo, 1873) has a relatively wide distribution, while *P. covadongae* Ortiz de Zárate, 1956, *P. daanidentata* Raven, 1988, *P. oberthueri* (Ancey, 1884), *P. poncebensis* Ortiz de Zárate, 1956, and *P. schaufussi* (Kobelt in Rossmässler, 1876) are confined to the Picos de Europa Massif. (2) *Pyrenaearia velascoi* (Hidalgo, 1867) is restricted to the Basque Mountains. (3) Another five morphospecies are endemic in the central and western Pyrenees with *P. carascalensis* (Férussac, 1821) showing the widest distribution, whereas *P. carascalopsis* (Bourguignat in Fagot, 1884), *P. cotiellae* (Fagot, 1906), and *P. esserana* (Bourguignat in Fagot, 1888) are limited to small areas in the central Pyrenees. *Pyrenaearia transfuga* (Fagot 1885) is even restricted to a single locality (Escot) in the Aspe valley at the northern slope of the western Pyrenees. (4) Two species are confined to the eastern pre-Pyrenees: *P. organiaca* (Fagot, 1905) and *P. parva* Ortiz de Zárate, 1956. (5) *Pyrenaearia molae* Haas, 1924 lives in the mountains of Tarragona (Catalonia). (6) Finally, *P. navasi* (Fagot, 1907) is

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Figure 1. Map showing collection sites of the specimens used in this study. The small box at the lower left corner indicates the distribution of the genus *Pyrenaearia* in black.

the only species living south of the Ebro river valley, where it is confined to Mount Moncayo. Nevertheless, Prieto (1991) recognized two subspecies within *P. navasi*: *P. n. navasi* and *P. n. sylvatica*.

Most *Pyrenaearia* spp. are considered as sub-alpine organisms (Ortiz de Zárate 1956, Prieto 1986, Puente 1994), including *Pyrenaearia daanidentata*, *P. oberthueri*, *P. velascoi*, *P. carascalensis*, *P. carascalopsis*, *P. cotiellae*, *P. esserana*, *P. parva*, and *P. navasi navasi*. These cold-adapted species have allopatric distributions, with often strongly isolated populations inhabiting the highest mountains, while other taxa are confined to the valleys (*P. covadongae*, *P. poncebensis*, *P. schaufussi*, *P. transfuga*, *P. organiaca* and *P. navasi sylvatica*). Only *P. cantabrica* shows a wide altitudinal range from 200 m to 2600 m above sea level.

The taxonomic validity of the 16 nominal *Pyrenaearia* species and subspecies is not clear. Most species show no diagnostic differences in their reproductive apparatus although characters such as body color and relative sizes of structures in the reproductive system have been used to distinguish a few taxa (Ortiz de Zárate 1956, Puente 1994). Thus, taxon identification in *Pyrenaearia* is based mostly on shell characters, including shell size, shape and color, peristome morphology, and the presence of hairs on the shell surface (Ortiz de Zárate 1956, Puente 1994). However, shell morphology can be profoundly influenced by environmental cues, including altitude (Raven 1988), and may be subject to parallel or convergent evolution, resulting sometimes in arbitrary classifications and incorrect species determination (*e.g.*, Giusti and Manganelli 1992, Uit de Weerd *et al.* 2004).

Delimiting species and reconstructing their phylogenetic relationships are the two major goals of systematics (Wiens and Penkrot 2002). In this context, recent developments in DNA-based taxonomy aimed at defining species using the evolutionary and phylogenetic species concepts (Simpson 1961, Wiley 1978, Cracraft 1983, Coyne and Orr 2004) are extremely useful in validating morphospecies in several animal groups (Vogler and Monaghan 2007). Molecular genetic tools have also become very popular for reconstructing phylogenetic relationships in animal species (Avise 2000) and for identifying speciation processes, analyzing population structure, and inferring phylogeographic patterns.

The present work is the first molecular phylogenetic study of the genus *Pyrenaearia*. We used DNA sequence data to (1) assess the validity of the 16 nominal *Pyrenaearia* species under the morphological, phylogenetic, and biological species concepts, (2) reconstruct their phylogenetic relationships, and (3) evaluate the geographic limits of the different lineages and taxa. Based on this information we develop some tentative speciation scenarios in relation to the Plio-Pleistocene climatic changes.

MATERIALS AND METHODS

Seventy-eight specimens of *Pyrenaearia* were included in this study with *Hygromia limbata* (Draparnaud, 1805) and *Iberus gualtieranus* (Linnaeus, 1758) as outgroups. Locality and sample data are provided (Fig. 1, Appendix 1). Whenever possible, topotypes were included in the analysis. Freshly collected animals were kept frozen at -20 °C until analysis. Collection specimens were preserved in 70% ethanol.

Individual DNA was extracted from foot muscle (15 to 20 mg), using the DNAeasy Tissue kit (QIAGEN). In some cases tissue was removed, squashed, and homogenized in sucrose buffer in order to eliminate excess mucopolysaccharides (Moorsel *et al.* 2000).

Polymerase chain reaction (PCR) was used to amplify two mitochondrial gene fragments, cytochrome oxidase subunit I (COI) and 16S rRNA (16S), and one complete nuclear gene, the ribosomal internal transcribed spacer 1 (ITS-1).

The COI fragment was initially amplified using the primers described by Folmer *et al.* (1994) [LCO 1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′), HCO 2198 (5′-TAAACTTCAGGGTGACCAAAAATCA-3′)]. The amplicons were sequenced for various *Pyrenaearia* species and a

new specific forward primer was designed for this study to amplify a shorter fragment of about 500 base pair (bp) [COIP1F (5′-TAATGTTGTAGTTACTGC-3′)]. The COIP1F primer was used together with the HCO 2198 reverse primer of Folmer *et al.* (1994). The 16S fragment was amplified by the primers of Palumbi *et al.* (1991) [16SarL (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SbrH (5′-CCGGTCTGAACTCAGATCACGT-3′)]. The ITS-1 marker was amplified using the primers ITS1L (5′-TCCGTAGGTGAACCTGCGGAAGGAT-3′) and 58C (5′-TGCGTTCAAGATATCGATGTTCAA-3′) (Hillis and Dixon 1991). Amplicons were sequenced using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) run on an ABI PRISM Model 3100 Avant Genetic Analyzer. The sequences were deposited in GenBank (accession numbers in Appendix 1). All sequences were aligned with Clustal X version 1.8 (Thompson *et al.* 1997) with default settings and they were refined manually where necessary.

Phylogenetic analyses using PAUP* 4.0b 10(PPC) (Swofford 2002) were performed for the three DNA fragments independently as well as for the combined data set. Gaps were treated as missing data. The best model of sequence evolution was selected using the Akaike information criterion (AIC) implemented in Modeltest v3.06 (Posada and Crandall 1998).

A heuristic search was performed for the maximum parsimony (MP) analyses, with ten random-addition replicates, using the tree bisection reconnection (TBR) option generating multiples trees to determine the most parsimonious solution. Weights of transversions (Tv) and transitions (Ts) varied depending on the fragment and were estimated by maximum likelihood. The weighting scheme was 4:1 for COI and 1:1 for 16S and ITS-1.

Neighbor-joining (NJ) (Saitou and Nei 1987) trees were constructed using the GTR+I+G model (Rodríguez *et al.* 1990) for all data matrices, according to Modeltest results. Uncorrected pairwise "p" distances were also calculated for the four data sets (see Table 1). For both the MP and NJ analyses, bootstrap (BS) confidence estimates were based on 1000 replicates (Felsenstein 1985). Any BS values higher that 70% were considered as providing strong support (Hillis and Bull 1993).

Bayesian analyses (BA) were performed using the MrBayes v3.0 package (Huelsenbeck and Ronquist 2001). The GTR model was used for the four data matrices, and rate variation across sites was modeled using a gamma distribution for COI data set and equal distribution for 16S, ITS-1, and combined data sets, with a proportion of the sites being variants. The Markov Chain Monte Carlo (MCMC) search was run with four chains for 2 million generations, with trees being sampled every 100 generations (the first 2000 trees were discarded as "burn-in"). In the combined analyses, variation was partitioned among genes.

Table 1. Molecular diversity indices for the different clades of *Pyrenaearia* for COI (first line), 16S rRNA (second line), and ITS-1 (third line). Values of number of haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and mean number of nucleotide differences (k) are reported.

	CLADE 1	CLADE 2	CLADE 3	CLADE 4
H	4	3	20	27
	3	4	23	24
	3	1	8	5
h	0.9	0.833	0.965	0.986
	0.7	1.0	0.953	0.975
	0.7	0	0.713	0.321
π	0.021	0.004	0.03	0.027
	0.008	0.006	0.027	0.016
	0.005	0	0.002	0.002
k	9.4	2.0	13.361	12.068
	3.1	2.5	10.729	6.383
	3.4	0	1.112	1.444

RESULTS

Sequence characteristics and genetic divergences

The number of mtDNA haplotypes (H), haplotype diversity (h), nucleotide diversity (π), and number of nucleotide differences (k) were calculated for each gene fragment and for the combined data set (Table 1).

The aligned COI fragment comprised 443 base pairs (bp). Sequences were A:T rich (74.17%) with nucleotide composition of: T (43.72%), C (11.2%), A (30.45%), and G (14.63%). One hundred and thirteen (25.51%) positions were parsimony informative. Using the *Drosophila* mitochondrial genetic code, 100 (68.03%) of the 147 amino acids had synonymous nucleotide substitutions and 47 (31.97%) had no substitutions.

The aligned 16S fragment consisted of 408 bp. The sequences were A:T rich (69.84%), with nucleotide compositions of: T (33.78%), C (12.54%), A (36.06%), and G (17.62%). Eighty-nine (21.81%) positions were parsimony informative.

The aligned ITS-1 gene comprised 692 characters. The proportion of A:T (40.95%) was lower, with nucleotide compositions of: T (22.18%), C (27.12%), A (18.77%), and G (31.93%). Seventeen (2.46%) positions were parsimony informative.

The combined data set of the COI, 16S, and ITS-1 analyses consisted of 1543 bp, 1069 of which (66.69%) were invariable, while 284 (18.4%) were variable but parsimony uninformative and 230 (14.91%) were parsimony informative.

Phylogenetic analyses

The four NJ trees had nearly identical topologies (Figs. 2-4) and were also almost identical to the MP and BA

topologies (not shown). The p-distances based on COI were larger than those based on 16S. All haplotypes were grouped within four basal clades. Average p-distances between these four clades were higher than 8.5% and 4.5% for COI and 16S, respectively (Table 2). The mean values of the average pairwise p-distances between a basal clade and the other three (Table 2) were very similar for each mtDNA gene fragment (8.6-9.8% for COI; 5.2-6.9% for 16S).

ITS-1 was nearly 10× more conservative than COI and 16S (Table 2) and also recovered four major groups, the p-distances between which amounted to a maximum of 1.1%. The four ITS-1 groups corresponded to mtDNA clades, with clade 3a joined together with the other clade 3 subgroups in a common polytomy.

The combined data set of the three genes, COI, 16S, and ITS-1, yielded a well-supported topology (Fig. 4). Basal nodes were not resolved but the monophyly of each of the four basal clades was strongly supported by bootstrap values (>95%), except for clades 3 and 4 in the MP analysis. The four basal clades joined the same populations in the COI, ITS-1, and the concatenated phylogenetic analyses (Figs. 2-4). Clade 3a was recovered as an independent lineage in the 16S tree. These results suggest confidence in the phylogeny obtained for *Pyrenaearia*, since the separate and combined analyses of COI, 16S, and ITS-1 did not yield conflicting results. Therefore, we will use the combined phylogeny for further discussion.

Clade 1 comprised the haplotypes of *Pyrenaearia carascalopsis* and *P. esserana*, with the former constituting a paraphyletic group. Clade 2 comprised the haplotypes of *P. parva* from two localities, including topotypes. Clade 3 grouped *P. carascalensis* together with *P. cotiellae*, *P. molae*, *P. navasi*, *P. organiaca*, *P. transfuga* and *P. velascoi*. *Pyrenaearia organiaca* was the basal group of clade 3 and showed the greatest divergence within it (mean values of the average p-distances of 7.7% and 6.9% for COI and 16S, respectively) (Table 3). *Pyrenaearia navasi* was the sister group of clades 3c-f. The mean values of the average p-distances among clade 3 subgroups (3b-f) were below 5.0%, 3.6%, and 0.3% for COI, 16S and ITS-1, respectively (Table 3). The analysis fully resolved the phylogenetic position of *P. organiaca* and *P. navasi*. Nevertheless, the phylogenetic relationships of *P. carascalensis*, *P. cotiellae*, and *P. molae* could not be resolved.

The monophyly of *P. velascoi* was well supported (BS >85%) and this nominal species was grouped in subclade 3f with *P. transfuga* and with the populations of *P. carascalensis* from the West-Pyrenees. *Pyrenaearia transfuga* and *P. carascalensis* appeared in an unresolved polytomy. Excluding *P. velascoi*, all the haplotypes of the Cantabrian *Pyrenaearia* populations were grouped in clade 4, which thus comprised *P. cantabrica*, *P. oberthueri*, *P. daanidentata*, *P. poncebensis*, and *P. schaufussi*.

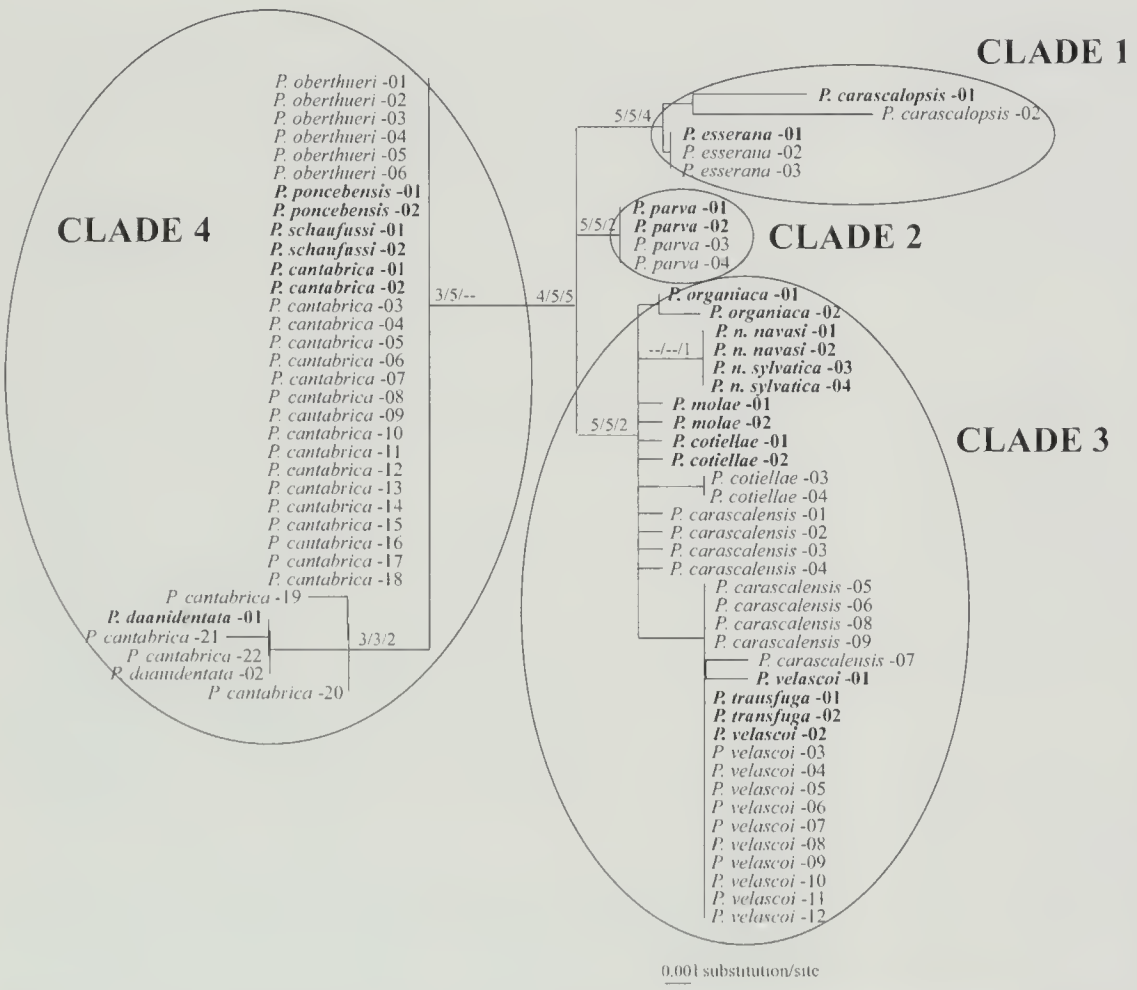

Figure 2. Unrooted NJ tree of nuclear ITS-1 from *Pyrenaearia* specimens. Bootstrap values under NJ, MP, and posterior probabilities under MB are shown at each node (NJ/MP/MB) when >70%. Number codes indicate bootstrap values and posterior probabilities: 1, 100%; 2, 99-95%; 3, 94-90%; 4, 89-80%; and 5, 79-65%.

DISCUSSION

Species delineation and taxonomy

The present study is the first attempt ever to determine species delineation of *Pyrenaearia* using DNA-based systematics. All haplotypes were unambiguously grouped within four main clades. The phylogeny of *Pyrenaearia* was highly consistent with the current geographical distribution of the clades. According to the explicit tree-based species delimitation protocol recommended by Wiens and Penkrot (2002) and the geographic distribution, we conclude that the four clades should

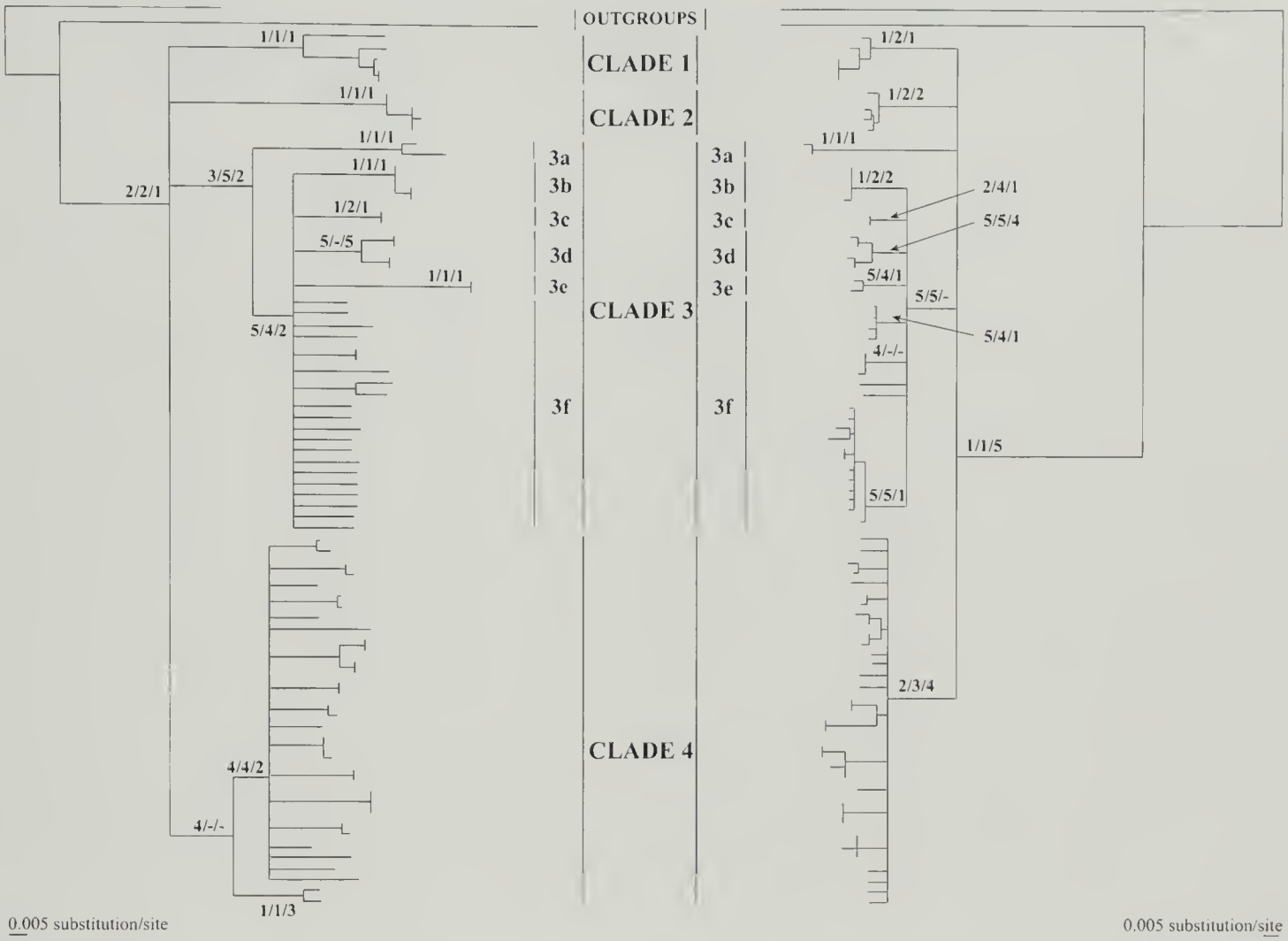

Figure 3. Molecular phylogenies of COI (left) and 16S rRNA (right) by NJ analyses. Bootstrap values under NJ, MP, and posterior probabilities under MB, are given at each node (NJ/MP/MB) when >70%. Bootstrap values are indicated by number codes (see legend of Fig. 2).

be considered different species under the phylogenetic species concept. With the exception of clades 3f and 4, all the clades are allopatric and, consequently, their reproductive isolation could not be tested. The species inhabiting the Pyrenees, the eastern pre-Pyrenees, the Tarragona, and the Moncayo mountains involved three basal clades.

Pyrenaearia carascalopsis and *P. esserana* formed a strongly supported phylogroup (clade 1). Both nominal species were not reciprocally monophyletic suggesting that they constitute a single taxon instead of two different species as proposed by Fagot (1906). In addition, some authors placed both nominal species in the synonym *P. carascalensis* (Germain 1930, Puente 1994). Yet our analyses showed that this opinion cannot be supported, and we instead agree with Prieto (1986) who joined *P. carascalopsis* and *P. esserana* in a single taxon because both taxa have neither well-defined morphological differences, nor particular geographical ranges.

Pyrenaearia parva (clade 2) is a valid morphospecies based on differences in shell morphology and body color (Ortiz de Zárate 1956, Prieto 1986, Puente 1994). It constitutes a well-defined clade that will not be discussed further. In this case, morphological and molecular evidence concerning the species status of *P. parva* were concordant.

Clade 3 joined seven nominal species. *Pyrenaearia organiaca* and *P. navasi* were recovered as two well-supported clades. Both morphospecies are very different from other *Pyrenaearia* taxa in terms of shell shape and geographic distribution. *Pyrenaearia organiaca* lives at low altitudes in the eastern pre-Pyrenees and its shell is very different in size, color, and sculpture (Ortíz de Zárate 1956, Bech 1990, Puente 1994). *Pyrenaearia navasi* is the only *Pyrenaearia* taxon occurring south of the Ebro valley and it also has a unique shell shape, shell color, and fragility (Prieto 1991). Therefore, we suggest that *P. organiaca* and *P. navasi* should be treated as valid species, based on phylogenetic and morphological criteria. The four *P. navasi* haplotypes form an unresolved intraspecific polytomy, thus eventual subspecific categories cannot be recognized. *Pyrenaearia carascalensis*, *P. molae*, *P. cotiellae*, *P. velascoi*, and *P. transfuga* formed a species complex (referred to as *P. carascalensis s.l.*) whose taxonomy can only be resolved after more intensive sampling and/or by screening more-sensitive markers. *Pyrenaearia cotiellae*,

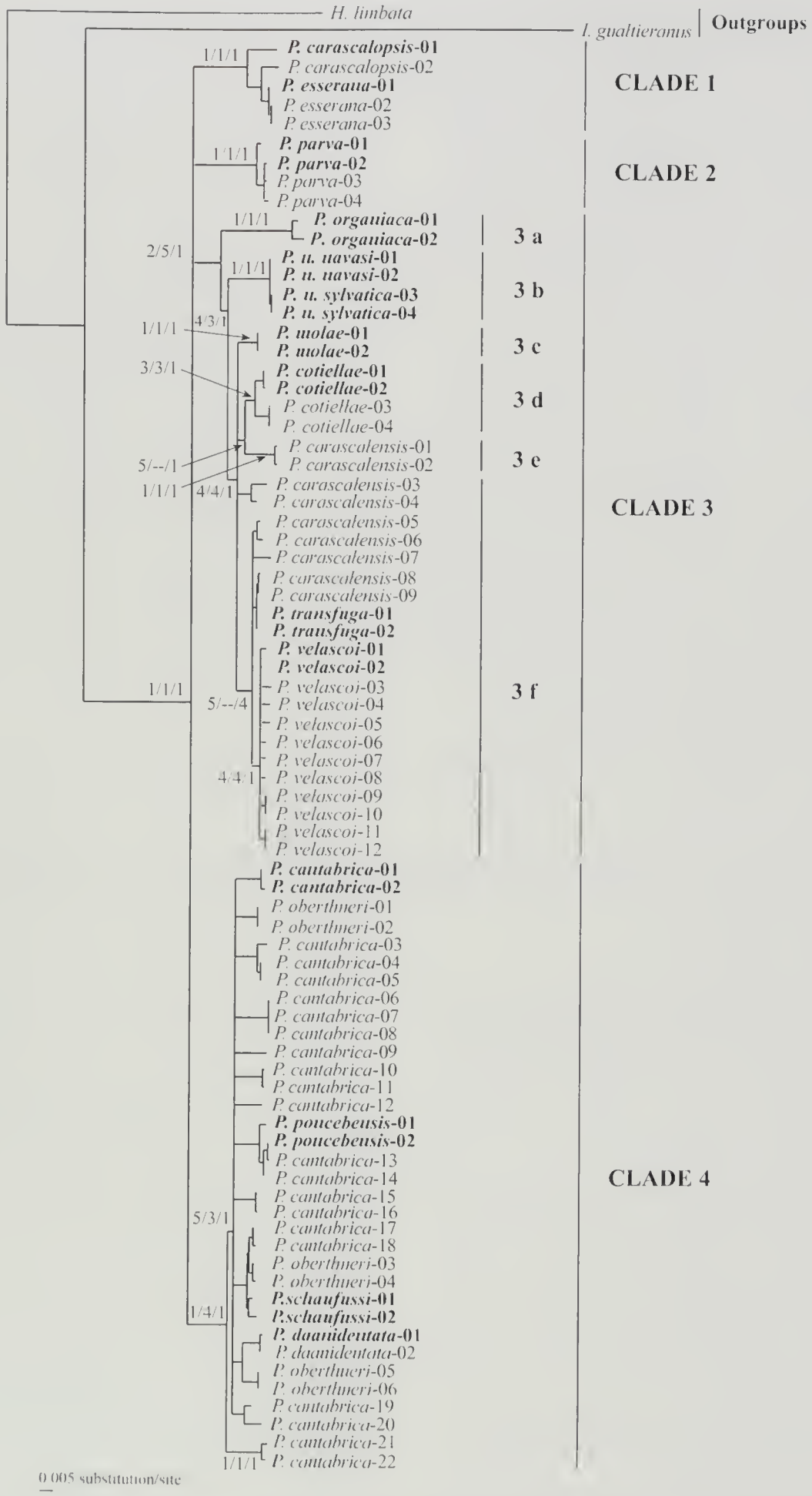

Figure 4. Molecular phylogeny of *Pyrenaearia* as revealed by (NJ) analysis for the combined data set of the COI, 16S rRNA, and ITS-1 genes. Bootstrap values under NJ, MP, and posterior probabilities under MB are shown at each node (NJ/MP/MB) when >70%. Topotype specimens are indicated in bold. Bootstrap values are indicated by number codes (see legend of Fig. 2).

Table 2. Uncorrected average (± *SD*) p-distances of COI (first line), 16S (second line), and ITS-1 (third line) within and among clades. Mean values of the average p-distances between a clade and the other three are also indicated.

	CLADE 1	CLADE 2	CLADE 3	CLADE 4
	2.12 %			
CLADE 1	0.77 %			
	0.52 %			
	11.36 % ± 0.0238	0.45 %		
CLADE 2	6.26 % ± 0.003	0.63 %		
	0.72 % ± 0.0035	0 %		
	10.48 % ± 0.0067	9.50 % ± 0.0066	3.01 %	
CLADE 3	7.23 % ± 0.0048	5.76 % ± 0.0041	2.82 %	
	1.07 % ± 0.0030	0.70 % ± 0.0008	0.16 %	
	8.94 % ± 0.0053	9.06 % ± 0.0053	8.50 % ± 0.0090	2.72 %
CLADE 4	6.72 % ± 0.0038	4.56 % ± 0.0044	6.05 % ± 0.0061	1.6 %
	1.24 % ± 0.0038	0.81 % ± 0.0016	1.08 % ± 0.0018	0.21 %
	9.81 % ± 0.0116	9.42 % ± 0.0095	8.79 % ± 0.011	8.56 % ± 0.0087
Mean values	6.94 % ± 0.0052	5.24 % ± 0.0077	6.17 % ± 0.007	5.99 % ± 0.0076
	1.13 % ± 0.0036	0.76 % ± 0.0016	1.05 % ± 0.0022	1.08 % ± 0.0023

Table 3. Uncorrected average (± *SD*) p-distances of COI (first line), 16S rRNA (second line), and ITS1 (third line) within and among clade 3 subgroups. Mean values of the average p-distances between clade 3 subgroups and the other five are also indicated.

	CLADE 3a	CLADE 3b	CLADE 3c	CLADE 3d	CLADE 3e	CLADE 3f
	1.58 %					
CLADE 3a	0.25 %					
	0.15 %					
	8.92 % ± 0.0040	0.3 %				
CLADE 3b	5.98 % ± 0.0017	0.13 %				
	0.37 % ± 0.0007	0 % ± 0.0000				
	7.44 % ± 0.0026	5.64 % ± 0.0024	0 %			
CLADE 3c	6.16 % ± 0.0014	2.83 % ± 0.0012	0 %			
	0.22 % ± 0.0008	0.15 % ± 0.000	0 %			
	7.90 % ± 0.0034	5.42 % ± 0.0033	3.5 % ± 0.0012	1.05 %		
CLADE 3d	6.66 % ± 0.0023	3.21 % ± 0.0031	2.39 % ± 0.003	0.92 %		
	0.29 % ± 0.0011	0.22 % ± 0.0008	0.07 % ± 0.0008	0.1 %		
	8.80 % ± 0.000	7.45 % ± 0.0024	5.19 % ± 0.000	4.4 % ± 0.0012	0 %	
CLADE 3e	6.18 % ± 0.0032	3.85 % ± 0.0012	1.76 % ± 0.000	1.95 % ± 0.0026	0.76 %	
	0.22 % ± 0.0009	0.15 % ± 0.000	0 % ± 0.000	0.07 % ± 0.0008	0 %	
	7.36 % ± 0.0050	4.51 % ± 0.0048	2.44 % ± 0.0045	1.97 % ± 0.0036	4.01 % ± 0.0047	0.88 %
CLADE 3f	7.31 % ± 0.0057	3.63 % ± 0.0032	2.63 % ± 0.0043	3.21 % ± 0.004	2.95 % ± 0.0033	1.24 %
	0.37 % ± 0.0010	0.3 % ± 0.0006	0.15 % ± 0.0006	0.22 % ± 0.0006	0.15 % ± 0.0006	0.06 %
	7.71 % ± 0.0073	4.92 % ± 0.009	3.17 % ± 0.0126	2.72 % ± 0.0132	4.58 % ± 0.0122	3.24 % ± 0.0121
Mean values	6.93 % ± 0.0071	3.53 % ± 0.0038	2.57 % ± 0.0043	3.06 % ± 0.0052	2.86 % ± 0.0063	3.21 % ± 0.0051
	0.34 % ± 0.0011	0.27 % ± 0.0008	0.13 % ± 0.0007	0.2 % ± 0.001	0.13 % ± 0.0007	0.22 % ± 0.0009

P. molae, and *P. velascoi* are monophyletic lineages and constitute three phylogeographic units in the sense of Avise (1989). Therefore, we suggest that they should maintain their nomenclatorial identity within the *P. carascalensis s.l.* group. Definition of other phylogeographic units within *P. carascalensis s.l.* would require a wider geographic sampling.

Finally, clade 4 comprised five nominal species from the Cantabrian mountains: *Pyrenaearia cantabrica*, *P.*

daanidentata, *P. oberthueri*, *P. poncebensis*, and *P. schaufussi*. Haplotypes of *P. poncebensis* and *P. schaufussi* topotypes were assigned to the *P. cantabrica* haplogroup. Thus, we include both names in the synonymy of *P. cantabrica*. Moreover, the morphological distinction between *P. schaufussi* and *P. cantabrica* has never been well documented (Azpeitia 1925, Ortiz de Zárate 1956, Prieto 1986, Puente 1994) and both taxa were usually defined by their geographical distribution (Altonaga *et al.* 1994), with *P. cantabrica* living in the West-Cantabrian mountains and *P. schaufussi* in the East. The morphospecies *P. poncebensis* was defined by the presence of periostracal hairs on adult shells (Ortiz de Zárate 1956). Haired *Pyrenaearia* specimens have been observed in several localities, interspersed between the localities of *P. cantabrica* (Puente 1994) and recently, we have observed haired adult shells even in the locus typicus of *P. cantabrica*. Clearly, our molecular results suggest that the presence of hairs in adult shells is not diagnostic in *Pyreanaearia* but rather represents a microhabitat adaptation that probably allows snails to retain water from fog in dry places. This hypothesis is based on the observation that haired *P. poncebensis* live under overhanging rocks in the locus typicus while smooth, hairless shells occur on neighboring vertical walls.

Pyrenaearia oberthueri did not represent a monophyletic group within clade 4. This taxon possesses an altitudinal cline in shell size and shape (Ortiz de Zárate 1956), with small, conical forms at higher altitudes (*oberthueri* morph) and larger, flattened shells in the valleys (*cantabrica* morph). Both morphs possess a bluish shell color with a brownish-red peristome (Ortiz de Zárate 1956). As such, *P. oberthueri* seems to be merely a high-altitude form of *P. cantabrica*, living above 1500 m in the Central Massif of Picos de Europa. The bluish color of the valley morph shells is probably a local variation of *P. cantabrica*. More studies are needed to survey and understand this clinal variation and to uncover additional geographical structure in populations (*e.g.*, haplotypes from Santa Ana mountains).

Pyrenaearia daanidentata is the only monophyletic morphospecies in clade 4. It is restricted to the highest altitudes (>2000 m) of the West Massif of Picos de Europa (Peña Santa) and its shell differs strongly from that of *P. cantabrica* by growing slower, being smaller and possessing two strong teeth in the peristome (Raven 1988). Based on the phylogenetic results, together with morphology and geographic distribution, *P. daanidentata* should retain its taxonomic identity. Whether this taxon has a specific or subspecific status requires further research.

Interestingly, *Pyrenaearia cantabrica* and *P. velascoi* (clades 4 and 3f, respectively) coexist above 1300 m in three mountains of the Basque country: Gorbea [Aldamin (30TWN1865), Peña Lekanda (30TWN1668)] and Beriain (30TWN8349) (Prieto 1986; present work). Yet, without exception, the haplotypes of *P. velascoi* and *P. cantabrica* from these areas were assigned to clades 3f and 4, respectively. These results, together with the absence of intermediate shell forms in these localities (Gómez-Moliner, pers. obs.), suggest that both taxa do not hybridize, so that *P. cantabrica* and *P. carascalensis s.l.* (including the nominal species *P. transfuga* and *P. velascoi*) should be considered different species under the morphological, biological, and phylogenetic species concepts.

Biogeographical considerations and divergence times

The phylogenetic relationships of the four basal clades remain unclear since they were joined in an unresolved polytomy. It is not excluded that this could be a hard polytomy (Walsh *et al.* 1999) if the radiation of the four clades happened in a short period of time. This seems to be supported by the very similar mean p-distances between them. However, from the beginning of the *Pyrenaearia* diversification to the present time, several lineages could have become extinct, although fossil evidence has not been reported. Missing evolutionary intermediates can indeed lead to phylogenetic artifacts, including unresolved nodes (Emerson 2002, Holland and Hadfield 2004).

A molecular clock is required for estimating divergence times. Unfortunately, the evolutionary rate of COI, 16S, or ITS-1 sequences of *Pyrenaearia* is unknown. Evolutionary rates of mtDNA are often estimated at 2% pairwise sequence divergence per million years (My) for invertebrates (DeSalle *et al.* 1987). Yet, faster clocks have been postulated in some terrestrial snails (Thomaz *et al.* 1996), so that mtDNA evolutionary rates of terrestrial gastropods tentatively vary between 1% and 10% per My (Thomaz *et al.* 1996, Douris *et al.* 1998, Chiba 1999, Hayashi and Chiba 2000). Sometimes, 2-5% sequence divergence per My has been tentatively used for several helicoids (Thomaz *et al.* 1996, Pfenninger *et al.* 2003). Hayasi and Chiba (2000) estimated a divergence rate of 10% for 16S in a bradybaenid using more reliable data than previous speculations. The mean values of the uncorrected average p-distances among basal clades were 8.5-9.8% for COI and 5.2-7.2% for 16S. Using a fast rate of 10% per My, the four clades would have separated during the Pleistocene (0.5-1.0 My). However, applying a more conventional divergence rate of 2% would imply that separation of the four clades could be dated between 2.6 and 4.9 My ago, in the Pliocene. Consequently, the split of the four major lineages in *Pyrenaearia* probably predated the Pleistocene glaciations as also postulated for other animal groups (Hewitt 1996, Taberlet *et al.* 1998). The genetic distances of mtDNA genes between clade 3a and the other subgroups of clade 3 (b-f) were similar to those indicated for the four basal clades (7.7%, 6.9% for COI, 16S) and probably predated the Pleistocene glaciations as well. The mean values of the average pairwise

genetic distances among the clades 3b-f ranged between 2.8% and 5.7% for COI and 2.2%-3.3% for 16S. Hence, the separation of clades 3b-f could be dated during Pleistocene glaciations [from Günz on, 600-500 thousand years (ky)], when considering the fast molecular rate of 10% per My.

Consequently, *Pyrenaearia* speciation within the four main groups is supposed to have occurred during cooling and warming cycles of the Pleistocene, which were particularly intense during the last 600 ky. During Pleistocene cooling periods, the high-altitude adapted populations had to migrate from their warm period refuges on the top of the mountains to lower altitudes in the valleys of the Cantabro-Pyrenaean region and the Ebro valley as a consequence of the permanent ice coverage of the mountains. In some cases, this downward migration was probably followed by expansions of the distributional ranges of cold-adapted species, resulting in possible secondary contacts. The presence of *Pyrenaearia velascoi* in the Cantabrian mountains seems to be the result of one of such expansion events that occurred probably during the last glaciation (Würm, 120-18 ky). This expansion of *P. carascalensis s.l.* to the west would have resulted in the secondary contact between the pyrenaean *P. carascalensis s.l.* and the cantabrian *P. cantabrica* lineages, but the previous allopatric speciation of these two lineages must have early enough to build up reproductive isolation.

The most recent speciation process began with the last deglaciation phase, starting ca. 14,000 ky BP, resulting in the shrinkage of the distribution range of cold-adapted species. This may have led to, for example, the isolation and subsequent genetic differentiation between *Pyrenaearia carascalensis s.s.* and *P. velascoi*. The small population of *P. carascalensis* living in Aspe valley (=*P. transfuga* morphospecies) could be a relict population surviving at low altitudes or may have been established by passive transport (heavy wind, rainstorms, birds) as was described in some other terrestrial molluscs (Gittenberger *et al.* 2006). The separation of two populations of *P. navasi*, one restricted to high altitudes (above 2000 m), the other sheltered within deciduous forests in the same mountainous system seems to also postdate the Last Glacial Maximum. The resolution of all these and some other questions needs more field and laboratory research.

Several workers have demonstrated that terrestrial molluscs are valuable organisms to investigate evolutionary processes in species with poor dispersal abilities (Davison and Clarke 2000, Hausdorf and Henning 2004, Schilthuizen *et al.* 2004, 2006, Uit de Weerd 2004, Ketmaier *et al.* 2006). The land snail genus *Pyrenaearia* is a polytypic group of closely related taxa that could constitute a good model organism for molecular phylogenetic studies. The existence of several taxa, which are confined to high-altitudes and, consequently, are intensely affected by insularity phenomena, makes these organisms suitable for the study of allopatric and/or peripatric speciation processes of isolated lineages. Moreover, the presence of some species living at high altitudes in close vicinity to other species restricted to the valleys within the same mountainous system also makes them a good model to study parapatric speciation. The effects of geo-historical climate changes on high-altitude species is poorly understood (Haubrich and Schmitt 2007). The genus *Pyrenaearia* could also represent an ideal group for the study of the effects of cyclic climate changes on geographic distribution of gene lineages and population structure in a group containing several taxa with subalpine affinities.

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Appendix 1. Species abbreviations, geographical coordinates (using Spanish grid references), and GenBank accession numbers for all the sequences of *Pyrenaearia*. Asterisks (*) and bold indicate topotypes.

Abbreviation	Localities-U.T.M.	GenBank access number of COI	GenBank access number of 16S rRNA	GenBank access number of ITS1
P. carascalopsis*-01	Port d´Salau (Lleida) 31TCH4733	EU310004	EU310083	EU310162
P. carascalopsis-02	Alto Aneu (Lleida) 31TCH4133	EU310005	EU310084	EU310163
P. esserana*-01	La Renclusa (Huesca) 31TCH0727	EU310006	EU310085	EU310164
P. esserana-02	Port de Viella (Lleida) 31TCH1624	EU310007	EU310086	EU310165
P. esserana-03	Port de Viella (Lleida) 31TCH1624	EU310008	EU310087	EU310166
P. parva*-01	Pedraforca (Barcelona) 31TCG9377	EU310009	EU310088	EU310167
P. parva*-02	Pedraforca (Barcelona) 31TCG9377	EU310010	EU310089	EU310168
P. parva-03	Sierra de Cadí (Barcelona) 31TCG9582	EU310011	EU310090	EU310169
P. parva-04	Sierra de Cadí (Barcelona) 31TCG9582	EU310012	EU310091	EU310170
P. organiaca*-01	Congost d´Organya (Lleida) 31TCG6377	EU310013	EU310092	EU310171
P. organiaca*-02	Congost d´Organya (Lleida) 31TCG6377	EU310014	EU310093	EU310172
P. navasi navasi*-01	Moncayo (Zaragoza) 30TWM9726	EU310015	EU310094	EU310173
P. navasi navasi*-02	Moncayo (Zaragoza) 30TWM9726	EU310016	EU310095	EU310174
P. navasi sylvatica*-03	Moncayo (Zaragoza) forest 30TWM9829	EU310017	EU310096	EU310175
P. navasi sylvatica*-04	Moncayo (Zaragoza) forest 30TWM9829	EU310018	EU310097	EU310176
P. molae*-01	Mola de Colldejou (Tarragona) 31TCF2153	EU310019	EU310098	EU310177
P. molae*-02	Mola de Colldejou (Tarragona) 31TCF2153	EU310020	EU310099	EU310178
P. cotiellae*-01	Circo de Armeña (Huesca) 31TBH8010	EU310021	EU310100	EU310179
P. cotiellae*-02	Circo de Armeña (Huesca) 31TBH8010	EU310022	EU310101	EU310180
P. cotiellae-03	Collado de Sahún (Huesca) 31TBH8616	EU310023	EU310102	EU310181
P. cotiellae-04	Collado de Sahún (Huesca) 31TBH8616	EU310024	EU310103	EU310182
P. carascalensis-01	Muntaya de Casamanya (Andorra) 31TCH8215	EU310025	EU310104	EU310183
P. carascalensis-02	Muntaya de Casamanya (Andorra) 31TCH8215	EU310026	EU310105	EU310184
P. carascalensis-03	Port Boucharo. Gavarnie (Francia) 30T YN4032	EU310027	EU310106	EU310185
P. carascalensis-04	Port Boucharo. Gavarnie (Francia) 30T YN4032	EU310028	EU310107	EU310186
P. carascalensis-05	Pico Anie (Navarra) 30TXN8555	EU310029	EU310108	EU310187
P. carascalensis-06	Pico Anie (Navarra) 30TXN8555	EU310030	EU310109	EU310188
P. carascalensis -07	Pico Anie (Navarra) 30TXN8555	EU310031	EU310110	EU310189
P. carascalensis-08	Portalet (Huesca) 30TYN1042	EU310032	EU310111	EU310190
P. carascalensis-09	Pico Anie (Navarra) 30TXN8555	EU310033	EU310112	EU310191
P. transfuga*-01	Pont d´Esquit (Francia) 30TXN9559	EU310034	EU310113	EU310192
P. transfuga*-02	Pont d´Esquit (Francia) 30TXN9559	EU310035	EU310114	EU310193
P. velascoi*-01	Aldamin (Bizkaia) 30TWN1865	EU310036	EU310115	EU310194
P. velascoi*-02	Aldamin (Bizkaia) 30TWN1865	EU310037	EU310116	EU310195
P. velascoi-03	Gorbea frente Aldamin (Bizkaia) 30TWN1865	EU310038	EU310117	EU310196
P. velascoi-04	Txindoki (Gipuzkoa) 30TWN7463	EU310039	EU310118	EU310197
P. velascoi-05	Txindoki (Gipuzkoa) 30TWN7463	EU310040	EU310119	EU310198
P. velascoi-06	Aitzgorri (Gipuzkoa) 30TWN5456	EU310041	EU310120	EU310199
P. velascoi -07	Monte Altxueta (Navarra) 30TWN8456	EU310042	EU310121	EU310200
P. velascoi-08	Aitzgorri (Gipuzkoa) 30TWN5456	EU310043	EU310122	EU310201
P. velascoi-09	Peña Lekanda (Bizkaia) 30TWN1668	EU310044	EU310123	EU310202
P. velascoi-10	Peña Lekanda (Bizkaia) 30TWN1668	EU310045	EU310124	EU310203
P. velascoi-11	Beriain (Navarra) 30TWN8349	EU310046	EU310125	EU310204
P. velascoi-12	Beriain (Navarra) 30TWN8349	EU310047	EU310126	EU310205
P. cantabrica*-01	Caldas de Oviedo (Asturias) 30TTP6300	EU310048	EU310127	EU310206
P. cantabrica*-02	Caldas de Oviedo (Asturias) 30TTP6300	EU310049	EU310128	EU310207
P. cantabrica-03	Peña Lekanda (Bizkaia) 30TWN1668	EU310050	EU310129	EU310208
P. cantabrica-04	Txarlazo (Bizkaia) 30TVN9659	EU310051	EU310130	EU310209
P. cantabrica-05	Txarlazo (Bizkaia) 30TVN9659	EU310052	EU310131	EU310210

Appendix 1. (continued)

Abbreviation	Localities-U.T.M.	GenBank access number of COI	GenBank access number of 16S rRNA	GenBank access number of ITS1
P. cantabrica-06	Beriain (Navarra) 30TWN8349	EU310053	EU310132	EU310211
P. cantabrica-07	Beriain (Navarra) 30TWN8349	EU310054	EU310133	EU310212
P. cantabrica-08	Beriain (Navarra) 30TWN8349	EU310055	EU310134	EU310213
P. cantabrica-09	Proaza (Asturias) 29TQH4192	EU310056	EU310135	EU310214
P. cantabrica-10	Langro-Pto Tarna (Asturias) 30TUN1281	EU310057	EU310136	EU310215
P. cantabrica-11	Langro-Pto Tarna (Asturias) 30TUN1281	EU310058	EU310137	EU310216
P. cantabrica-12	Aldamin (Bizkaia) 30TWN1865	EU310059	EU310138	EU310217
P. cantabrica-13	Poncebos (Asturias) 30TUN5191	EU310060	EU310139	EU310218
P. cantabrica-14	Poncebos (Asturias) 30TUN5191	EU310061	EU310140	EU310219
P. cantabrica-15	Caranga-Quirós (Asturias) 29TQH4089	EU310062	EU310141	EU310220
P. cantabrica-16	Caranga-Quirós (Asturias) 29TQH4089	EU310063	EU310142	EU310221
P. cantabrica-17	Cordiñanes-Caín (Asturias) 30TUN4582	EU310064	EU310143	EU310222
P. cantabrica-18	Cordiñanes-Caín (Asturias) 30TUN4582	EU310065	EU310144	EU310223
P. cantabrica-19	Desfiladero de los Beyos (Asturias) 30TUN3088	EU310066	EU310145	EU310224
P. cantabrica-20	Desfiladero de los Beyos (Asturias) 30TUN3088	EU310067	EU310146	EU310225
P. cantabrica-21	Anboto (Bizkaia) 30TWN3270	EU310068	EU310147	EU310226
P. cantabrica-22	Anboto (Bizkaia) 30TWN3270	EU310069	EU310148	EU310227
P. daanidentata*-01	Hoyo del Burro (León) 30TUN4082	EU310070	EU310149	EU310228
P. daanidentata-02	Canal del Perro (León) 30TUN4082	EU310071	EU310150	EU310229
P. oberthueri-01	Vega Urriello (Asturias) 30TUN5285	EU310072	EU310151	EU310230
P. oberthueri-02	Vega Urriello (Asturias) 30TUN5285	EU310073	EU310152	EU310231
P. oberthueri-03	Vega Urriello (Asturias) 30TUN5285	EU310074	EU310153	EU310232
P. oberthueri-04	Vega Urriello (Asturias) 30TUN5285	EU310075	EU310154	EU310233
P. oberthueri-05	Peña Santa Ana (León) 30TUN5282	EU310076	EU310155	EU310234
P. oberthueri-06	Peña Santa Ana (León) 30TUN5282	EU310077	EU310156	EU310235
P. poncebensis*-01	Poncebos (Asturias) 30TUN5191	EU310078	EU310157	EU310236
P. poncebensis*-02	Poncebos (Asturias) 30TUN5191	EU310079	EU310158	EU310237
P. schaufussi*-01	Urdón (Asturias) 30TUN6791	EU310080	EU310159	EU310238
P. schaufussi*-02	Urdón (Asturias) 30TUN6791	EU310081	EU310160	EU310239
Hygromia limbata	Pagasarri (Bizkaia) 30TWN0485	EU310082	EU310161	EU310240
Iberus gualtieranus	Huercal de Almería (Almería) 30SWF4880	AY928568	AY928596	EU446026

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Documenting molluscan evolution from ancient long-lived lakes: The case of *Toxosoma* Conrad, 1874 (Gastropoda, Cochliopidae) in Miocene Amazonian Lake Pebas*

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Abstract: Long-lived lakes with their often *in situ* evolved faunas form an excellent model system to study evolution. The paleontological record of long-lived lakes provides possibilities to investigate the tempo and mode of evolutionary change. However, in order to demonstrate such processes, a combined rigorous temporal and spatial framework is needed in order to make sure (1) alleged evolutionarily successive morphs are not misidentified immigrant species and (2) morphological variation is not due to ecophenotypy. Other factors that may cause the development of discrete morphs, such as sexual dimorphism, also must be considered. In this paper, evolution in the Miocene snail genus *Toxosoma* Conrad, 1874 from long-lived Lake Pebas in western Amazonia is studied within such a temporal and paleo-environmental framework.

Key words: anagenesis, cladogenesis, temporal/spatial framework, gastropod evolution

Long-lived lakes are considered as laboratories and archives of evolution (Martens 1997). Faunas of these lakes, such as the well-known African cichlid fish, have served in many groundbreaking studies on adaptation, niche partitioning, sexual selection, and other mechanisms underlying the process of speciation. Molluscs (and ostracods) are abundant in these lakes and contain hard shells that have a good fossilization potential. Their fossils have frequently been used in exploring these archives of evolution (*e.g.*, Williamson 1981a, 1981b, Mensink 1984, Geary 1990, Geary *et al.* 2002, Harzhauser and Mandic 2004, Wesselingh 2007). However, evolutionary insights based on the fossil record of long-lived lakes have been diverse and partially contradictory. A well-known example is the debate on alleged punctuated evolutionary change in Kenyan Lake Turkana mollusc faunas as proposed by Williamson (1981a, 1981b) and hotly contested afterwards (see references in Bocxlaer *et al.* 2007). In several studies, morphological change in successive stratigraphic intervals has been taken as evidence for evolution, underestimating the potential role of ecosystem variability and intrinsic biological processes involved in the generation of morphological variation in molluscs.

In this paper we aim to demonstrate that the fossil record of long-lived lakes is very well suited for the study of evolutionary patterns as long as adequate account is taken of the spatial and temporal context. The study is based on stratigraphic successive morphological change in snails of the genus *Toxosoma* Conrad, 1874 (Rissooidea: Cochliopidae), in Amazonian Lake Pebas during the Middle and early Late Miocene. The methodological context by which evolution might be demonstrable is outlined.

LONG-LIVED LAKES

Long-lived lakes are lakes that combine geological longevity and the development of endemic faunas. Geological longevity is not strictly delimited but usually taken as being longer than an interglacial time interval. The current Lake Victoria in East Africa, with its more than thousand endemic cichlid fish species, was completely desiccated only about 13 ka ago (Johnson *et al.* 1996). However, Quaternary Lake Ioanina in northern Greece existed for at least several hundreds of thousands of years, yet almost completely lacked endemic species (Frogley and Preece 2007). In order to develop endemic faunas, a lake must have been habitable for some time. Thirteen thousand years is the shortest known long-lived lake. Although lakes may persist over long time periods, it is the temporal continuity of ecological conditions under which lacustrine biota can live that is the most important feature of long-lived lakes (Wesselingh 2007). Typically, ecological continuity is on the order of 100 ka to several Ma. The faunas in long-lived lakes contain a significant proportion of endemic species. These lakes often show high

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within-habitat diversity. The endemic species can be anagenetic derivatives of more widespread sister species (references in Wesselingh 2007), but in many of the long-lived lakes *in situ* radiations occur (Michel 1994, Müller *et al.* 1999, Nevesskaja *et al.* 2001, Geary *et al.* 2002). Such radiations have led to species flocks, *i.e.*, monophyletic clades of mostly or entirely endemic species (West and Michel 2000). Long-lived lake faunas have a broad range of morphologies, often containing marine-like features such as unusually thick and strongly ornamented shells and apertural modifications including siphonal canals. A general trait shared by endemic taxa in long-lived lakes is the tendency to evolve K-reproductive strategies (Michel 1994). An exception has been documented from Late Miocene lake Pannon of central-eastern Europe, where secondary opportunist, endemic bivalve species evolved as r-strategists (Harzhauser and Mandic 2004).

Long-lived lakes can be found in rift valleys (such as Lake Baikal in Russia and Lake Tanganyika in East Africa), in karst depressions (Lake Pozo in Indonesia and Lake Ohrid on the Balkans), in tectonic depressions (Lake Titicaca in South America and Lake Biwa in Japan), and even in meteorite craters (Miocene Lake Steinheim in southern Germany). They may be located at or below sea-level and contain brackish water such as the Caspian Sea and Neogene Paratethyan lakes of southeastern Europe. The oldest, long-lived lacustrine mollusc fauna known is that of the Permian South American Parana Lake (Wesselingh 2007). Lake Pebas, occupying western Amazonia during the Early to early Late Miocene (ca. 23-11 Ma), was located in the Andean foreland basins and adjacent pericratonic basins. At its maximum, the lake measured over 1 million km^2 in area (Wesselingh *et al.* 2002).

EVOLUTION AND THE FOSSIL RECORD

When working with fossils, species concepts are almost entirely morphologically based. Species can be defined by certain, unique combinations of ranges of morphological characters. However, (sexual) dimorphism is an inherent biological process known to produce distinct morphs within species. The possibility that different morphs may represent males and females must therefore be considered in evolutionary studies as well. Freshwater gastropod species are also well known to show ecophenotypic variation, *i.e.*, morphological variation related to habitat differences lacking a hereditary basis. Several mollusc groups exist whose species, as identified by DNA or ecological preferences, cannot be

Toxosoma contortum Wesselingh, 2006

Intermediate sized elongate to ovate-conical shell
Aperture flaring
Plane of aperture tilted backwards
Aperture contorted by well developed columellar plicae
Well developed teeth behind aperture within outerlip

Toxosoma grande Wesselingh, 2006

Large bulbous, comparatively thin shell
Convex whorl profile
Lacking teeth/spiral ridges inside outerlip
Columellar plicae absent or very low
Large and wide aperture

Toxosoma denticulatum Wesselingh, 2006

Intermediate-large ovate to ovate-conical shell
Thick shell
Suture very shallow
Whorl profile comparatively flat
Apertural margins reinforced
Columellar plicae very robuste
Well developed teeth behind aperture within outerlip
High aperture

Toxosoma eboreum Conrad, 1874

Intermediate sized ovate-conical shell
Thick shell
Somewhat deepened suture
Semiconvex whorl profile
Base bodywhorl slightly bulbous
Columellar plicae small but well developed
Teeth behind aperture within outerlip of variable strength

Figure 1. *Toxosoma* species from the Middle to early Late Miocene Pebas Formation of western Amazonia. Specimen and locality information in Wesselingh (2006). Scale bar = 1 mm.

distinguished on shell morphology, either by the lack of sufficient characters or by an abundance of apparently widely variable and overlapping characters. Within long-lived lakes taxa that are extremely difficult to separate based on shell characters are present, among others, Lake Tanganika snail species of the genus *Lavigeria* Bourguignat, 1888 (Michels 2004). However, detailed study of distribution patterns, ecology, and DNA may lead to grouping of specimens that turn out to have more or less subtle but distinctive morphological traits after all (Papadopoulos *et al.* 2004). Nevertheless, it can be expected that shell morphology cannot always be informative as to species discrimination. This may especially be the case in evolutionary transitional forms. The importance of sound systematics is not a trivial issue. Bocxlaer *et al.* (2007) showed that the erroneous generic identification of gastropod taxa in Lake Turkana has severely corrupted the evolutionary claims of Williamson (1981a, 1981b).

Evolution in fossil biotas can be defined as morphological change in lineages (anagenesis) and the origination of novel morphologies within clades (cladogenesis) through time in as far these cannot be ascribed to ecophenotypy or polymorphism. In order to demonstrate evolution, both control over stratigraphic time (temporal control) as well as the architecture of depositional environments is needed to address ecophenotypy (spatial control).

MATERIALS AND METHODS

Toxosoma eboreum Conrad, 1874 is the single *Toxosoma* species (Gastropoda, Cochliopidae) known from Middle Miocene intervals of the Pebas Formation (Wesselingh 2006). In successive late Middle to early Late Miocene intervals, three species are found (*Toxosoma denticulatum* Wesselingh, 2006; *Toxosoma contortum* Wesselingh, 2006; *Toxosoma grande* Wesselingh, 2006). The species have a range of shell characters that allow for their discrimination (Fig. 1).

Since *Toxosoma* is endemic to the Pebas system, it is possible that the latter three species originated from a single Middle Miocene ancestor (see Discussion section below). Together with a further three species found in earlier stratigraphic intervals in the Pebas Formation, *Toxosoma* may constitute a classical species flock. In the Pebas system, several putative species flocks occur, including a flock of the cochliopid genus *Sioliella* Haas, 1949. In order to assess whether the three species are not mere ecophenotypes or sexual dimorphs we have measured specimens from different stratigraphic and depositional environments. A total of 278 specimens belonging to the four species were retrieved from 17 Miocene Pebas Formation localities in Colombian and Peruvian Amazonia (Table 1, Fig. 2, Appendix 1). A single unidentified specimen was also included in the analyses.

Table 1. Localities; detailed information (including coordinates, collection dates, and maps) can be found in Wesselingh (2006) and Wesselingh *et al.* (2002, 2006b). MZ, mollusc zonation from Wesselingh *et al.* (2006b). ENZ, inferred environment from facies analyses (Räsänen *et al.* 1998, Wesselingh *et al.* 2006a) and from multivariate analysis of mollusc samples (Wesselingh *et al.* 2002). F, fluvial; ML, marginal lacustrine (prodelta, interdistributary bay, shoreface); L, lake shelf and bottom.

Sample	Locality	MZ	ENV
F4	Mocagua (Amazonas, Colombia)	10	ML
F6	Mocagua (Amazonas, Colombia)	10	ML
F16	Los Chorros I (Amazonas, Colombia)	10	F
F21	Los Chorros III (Amazonas, Colombia)	10	ML
F22	Los Chorros III (Amazonas, Colombia)	10	L
F29	Puerto Nariño I (Amazonas, Colombia)	11	ML-F
F32	Macedonia (Amazonas, Colombia)	11	ML
F34	Zaragoza (Amazonas, Colombia)	11	ML
F363	Iquitos Puerto GansoAzul (Loreto, Peru)	6	ML-L
F367a	Nuevo Horizonte III (Loreto, Peru)	9	L
F417	Pebas XIII (Loreto, Peru)	7	L
F489	Santa Elena I (Loreto, Peru)	8	ML
F498	Santa Elena II (Loreto, Peru)	8	L
F535	Santa Rosa de Pichana (Loreto, Peru)	7	L
F685	Tamshiyacu (Loreto, Peru)	7	ML
F702	Porvenir II (Loreto, Peru)	9	F
F707	Porvenir IV (Loreto, Peru)	9	L
F830	Momon III (Loreto, Peru)	7	L-ML
F836	Nuevo Horizonte II (Loreto, Peru)	9	L

The specimens were first identified using apertural outline and inclination, whorl profile, nature and number of columellar plicae, and grooves/denticles within the outer lip (Fig. 1). Three characters [height (H), height of aperture (Hap), and width of body whorl half a whorl before termination (WBW)] were measured with a resolution of 20 μm (Fig. 3). In *Toxosoma* specimens, the last quarter of the body whorl has a tendency to flare, which can be considered an adult apertural modification (Papadopoulos *et al.* 2004). The extent of flaring can vary considerably within and between populations. The maximum shell width (W) that is linked to the extent of flaring is too variable a measure and has been excluded from study. In many of the specimens, the aperture is damaged, precluding width measurements. In this study, the width of the shell half a whorl before the aperture is used in order to circumvent the above-mentioned problems. In three of the four species, the aperture tends to become tilted, making the height of the aperture (Hap) another potentially problematic character for study. However, we observed a remarkable correlation between W and

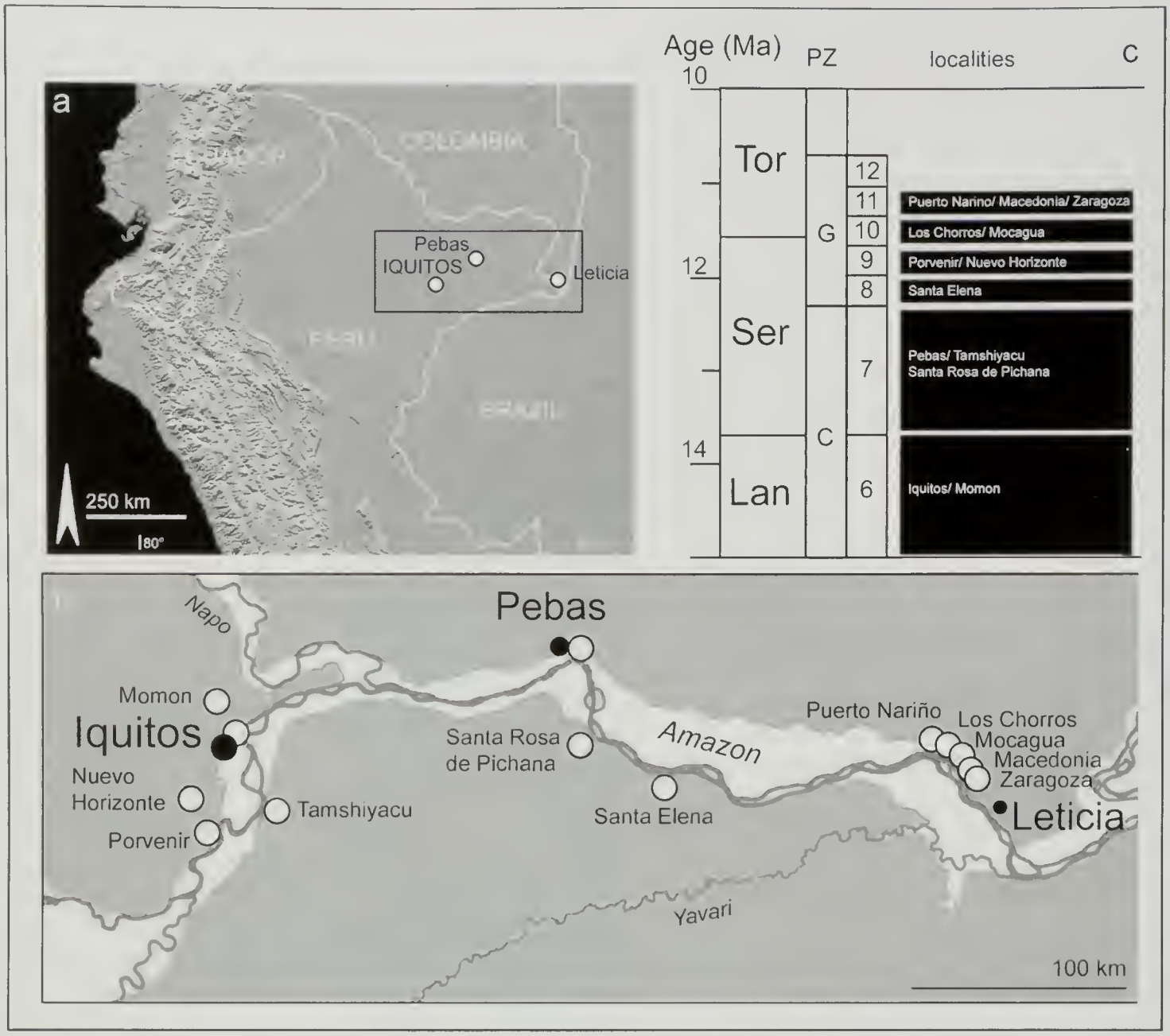

Figure 2. Localities and stratigraphic context of studied material. A, fieldwork area. B, localities: white areas denote alluvial plains, grey areas indicate uplands (so called "terra firme") that are underlain mostly by Neogene deposits. C, stratigraphic framework for the studied samples. Tor, Tortonian; Ser, Serravallian; Lan, Langhian; PZ, pollen zones (Hoorn 1993); G, *Grimsdalea* zone; C, *Crassoretitriletes* zone; numbers refer to mollusc zones from Wesselingh *et al.* (2006b).

Hap, strongly suggesting that the Hap is a usable character in spite of variations in the tilt of the apertural plane. Finally, the number of whorls was counted (Fig. 3).

A Principal Components Analysis (PCA) was performed on the variables H, Hap, WBW, and Wh (N = 276) with Primer 6.1 (Clarke and Warwick 2001). Width (W) was excluded because of the large number of missing measurements. The first two principal components were plotted in morphological space for each stratigraphical interval. The depositional environments of the *Toxosoma* species are inferred from sedimentary facies analyses (Räsänen *et al.* 1998, Wesselingh *et al.* 2006a) and from multivariate analysis of mollusc samples (Wesselingh *et al.* 2002). A variety of habitats, ranging from fluvial to lake bottoms, have been discerned.

RESULTS

In stratigraphic zones MZ6-MZ8, only the species *Toxosoma eboreum* is present. In samples from zones MZ9-MZ11, *T. contortum*, *T. grande*, and *T. denticulatum* co-occur. Within species, size differences were observed in different samples. All identifications apart from one (see Discussion below) were unambiguous.

Shell measurements are presented (Appendix 1). The first two principal components (PC1 and PC2) plotted here account for 97% of the variance in the data (per-axis inertia: PC1 = 0.85; PC2 = 0.12). PC1 represents mostly size parameters (H, Hap, WBW-1). The second PCA axis is strongly determined by the number of whorls. PC1 and PC2

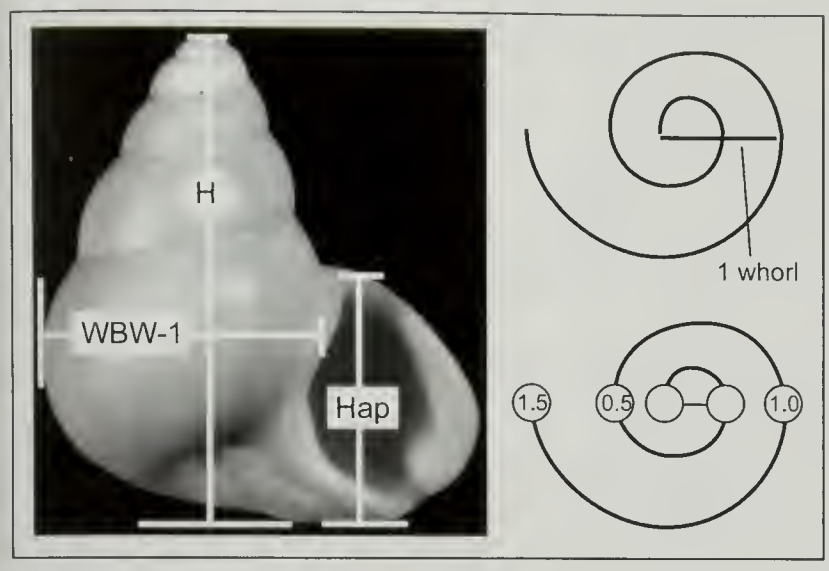

Figure 3. Characters measured. H, height; WBW-1, width of body whorl between half a whorl and one whorl before the termination of the shell; Hap, height of aperture. Whorls are counted as in upper right. The width per whorl is counted as in lower right.

scores are provided for the different species per stratigraphic interval (Fig. 4).

Toxosoma eboreum occurs in the lower three stratigraphic zones (MZ6-MZ8). The populations in the MZ6 samples have low PC1 scores and variability. PC1 scores and variability are larger in the overlying two stratigraphic zones. The populations in all three zones have intermediate PC2 scores.

In stratigraphic zones MZ9-MZ11, *Toxosoma contortum* is characterized by low PC1 scores and intermediate PC2 scores. In MZ9, PC1 scores are most variable and partially overlap those of *T. eboreum* in the underlying MZ8. In the overlying MZ10 and MZ11, the average PC1 score slightly increases.

In MZ9, *Toxosoma grande* and *T. denticulatum* have very similar PC1 scores, but are well distinguished by PC2 scores (low for *T. denticulatum* and high for *T. grande*). In the overlying MZ10 and MZ11, the PC2 values of both species largely overlap. A notable increase in PC1 values and variability is found for *T. grande* in successive stratigraphic zones.

The unidentified *Toxosoma* species from interval MZ10 scores between PC values of *T. contortum* and *T. grande* in that interval. In three of the four species (*Toxosoma eboreum*, *T. contorum*, *T. grande*) an increase in PC1 values in successive time intervals is observed and corresponds to an anagenetic increase in size.

DISCUSSION

The data indicate anagenetic change in *Toxosoma eboreum* in stratigraphic zones MZ6-MZ8. The apparent existence of two morphs within MZ7 is discussed below. It is possible that *T. eboreum* evolved from *T. ovatum* Wesselingh, 2006 that is common in MZ5.

The apparent (because of low number of samples) sudden occurrence in MZ9 of *Toxosoma contortum*, *T. denticulatum*, and *T. grande* is interpreted to result from cladogenesis (see below). More detailed sampling is required to establish the exact order of branching. The three species are well delimited through morphological characters as well as PC scores in MZ9 samples. However, the PC scores of the latter two species largely overlap in the upper two stratigraphic zones (MZ10 and MZ11). The PC1 values of both *T. contortum* and *T. grande* increase in successive intervals, again indicating anagenetic change towards larger sizes. On the scale of the mollusc biozones, both apparent "punctuated" (the occurrence of three species in MZ9) as well as gradual change (*e.g.*, directional change within *T. contortum*, *T. eboreum*, and *T. denticulatum* lineages) occurs. The exact time associated with the biozones is unknown but presumably on the order of several hundred thousand years per zone (Wesselingh *et al.* 2006a). The current sampling density and time control does not allow assessing whether the cladogenesis between MZ8 and MZ9 was sudden.

The unidentified specimen in MZ10 may be an aberrant specimen of one of the three species occurring in that zone. It also may represent a fifth species. However, we consider the last option unlikely as no similar specimens have been found in the large collection of Pebas material available. Finally, the unidentified morph may also represent a hybrid between *Toxosoma contortum* and *T. grande* or *T. denticulatum*. The limits imposed by the fossil record do not allow for an interpretation of the unidentified species.

Toxosoma eboreum lived in lacustrine and marginal lacustrine habitats (see Table 1; Wesselingh *et al.* 2002). *Toxosoma grande* is a species from marginal lake habitats (interdistributary bay, delta, and pro-delta) and fluvial habitats (see Table 1; Wesselingh *et al.* 2002). The lack of morphological intermediates with the other two co-occurring *Toxosoma* species in marginal lacustrine habitats indicates that they are not ecophenotypic morphs.

Toxosoma denticulatum inhabited lake margins and lake shelves although low numbers occur in samples from lake bottom settings that were not sampled in this study. *Toxosoma contortum* has a similar distribution although more common in lake bottom samples. Since both species occur in the same habitats (Table 1) and lack morphological intermediates, they too are unlikely to be ecophenotypic variants.

The three *Toxosoma* species occurring in MZ9-MZ11 have partially overlapping environmental optima. *Toxosoma grande* has its optimum in marginal lacustrine habitats, *T. denticulatum* on the lake shelf, and *T. contortum* on the lake shelf and lake bottom. A number of large samples containing

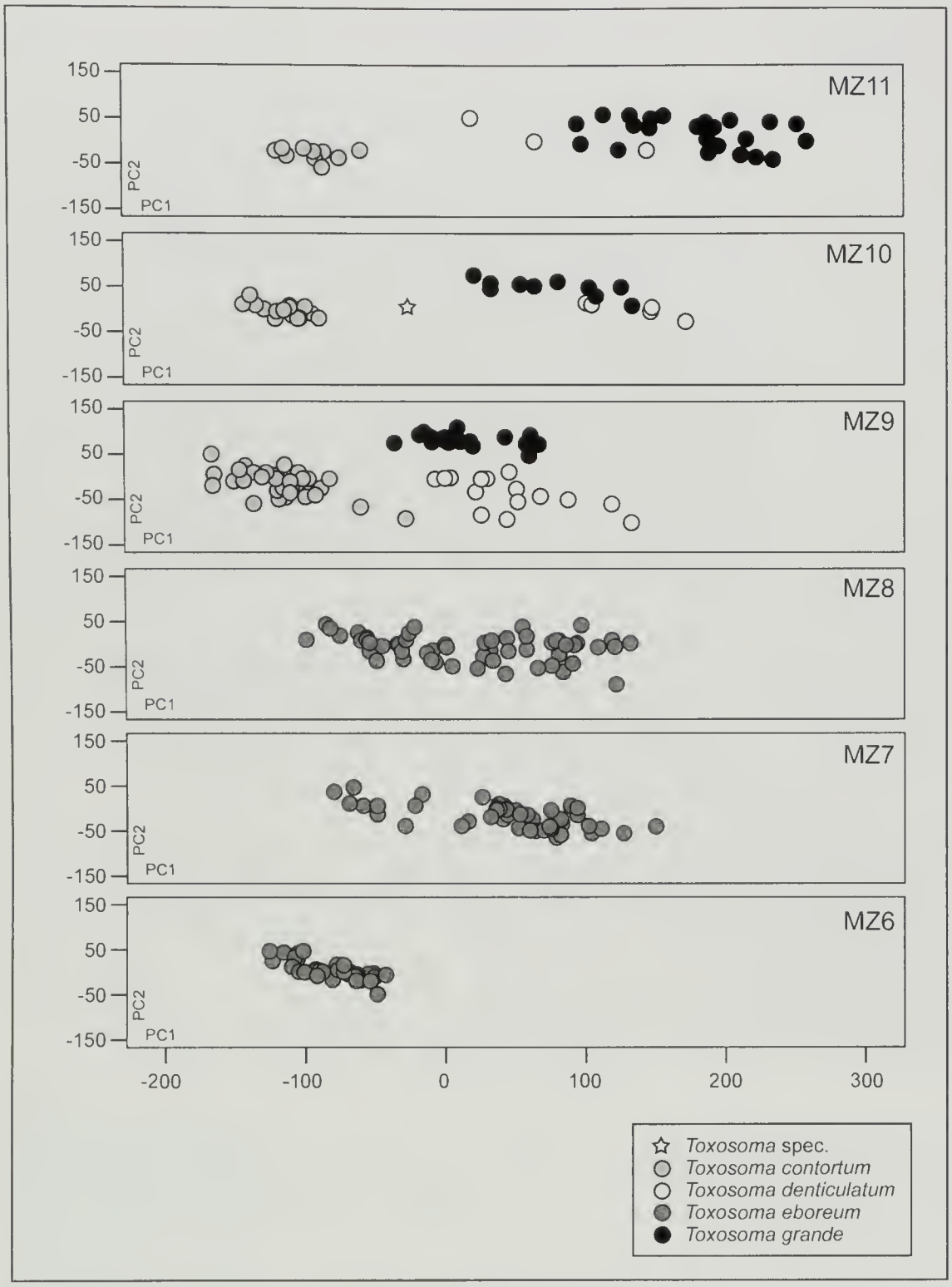

Figure 4. Character development in Pebasian *Toxosoma* species. MZ numbers refer to mollusc zones. PC1 and PC2 refer to the PCA axes.

solely *T. grande* suggest that the form is not simply a male or female sexual dimorph, as at least some of the specimens corresponding to the other sex would be expected. Various samples containing only *T. contortum* were found in stratigraphic zones and depositional environments where *T. denticulatum* also may have lived. This also indicates that it is unlikely that both forms are male and female morphs of a single species. Furthermore, in such a case a similar development in early ontogeny would be expected with divergence in adult stages (Shaver 1953). In some samples of *T. eboreum* and *T. denticulatum*, apparent slender and broad morphs co-occurred. Pronounced sexual dimorphism has been demonstrated to occur in some cochliopid gastropod species (Taylor 1987). Width/whorl measurements on both afore-mentioned *Toxosoma* species refute the presence of two well-delimited forms that might have represented sexual dimorphs (Fig. 5).

Documenting morphological change through time does not equate with documenting evolution. Other possible causes for morphological variability, such as ecophenotypy and sexual dimorphism, need to be accounted for. Furthermore, one needs to ascertain that the successive morphs are members of a single lineage. Endemic lineages in fossil long-lived lakes, such as the gastropod genus *Toxosoma* in Miocene Lake Pebas, comply with the latter assumption. The inferred cladogenetic event giving rise to three *Toxosoma* species in interval MZ9 may also have resulted from the invasion of species that developed elsewhere in the large (>1 million km^2) Pebas system. However, the morphological variability of closely related species is very low within stratigraphic intervals, even in samples from locations far apart (Wesselingh *et al.* 2006b). The geological record also provides opportunities to consider the potential role of environmental variation in generating morphological variation, and the shells themselves may provide clues about the possibility of dimorphism, making them a very suitable model to study speciation.

The three species whose distributional optima are in lacustrine habitats (*Toxosoma eboreum, T. denticulatum*, and *T. contortum*) developed thick shells with pronounced denticulation. The single marginal lacustrine-to-fluvial species (*T. grande*) has a comparatively thin shell, large aperture, and lacks well-developed denticles. The thick shells of the lacustrine species are possibly related to very abundant shell-cracking fish populations in the lake habitats. Teeth of sciaenid fish, a shell-cracking group, are often found in lacustrine samples from the Pebas Formation. A causal relationship is speculative at the moment. Scars of failed predatory attacks are common

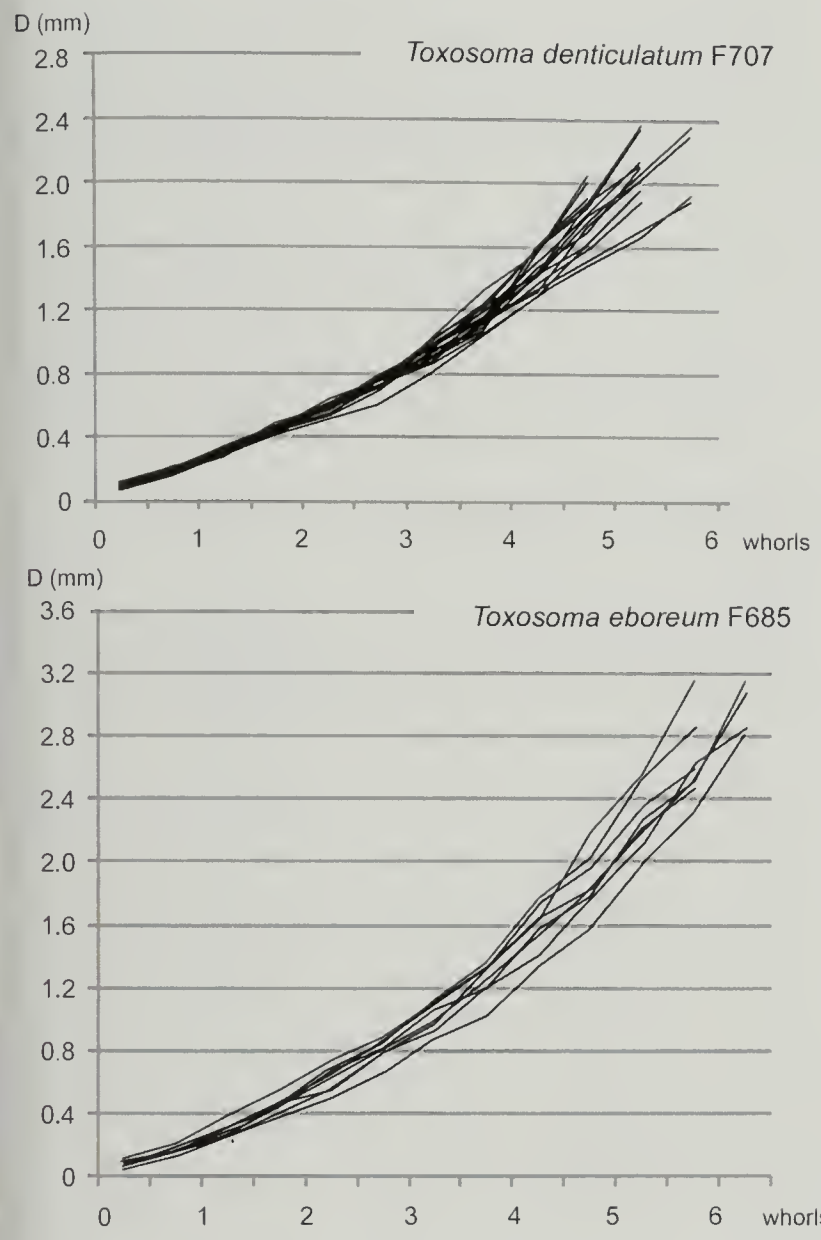

Figure 5. Width per half revolution (see Fig. 3 for explanation). *Toxosoma eboreum* and *T. denticulatum* have almost identical growth curves, the latter grows only to larger sizes and appears to develop two morphs that may represent sexual dimorphs in later stages of ontogeny. D, diameter. F707 and F685 refer to sample numbers (see Table 1).

on *Toxosoma* shells, however. In the lacustrine species such scars appear to match attacks from crushing predators (exemplified by the damage illustrated in Wesselingh 2007 on Pebasian *Pachydon obliquus* Gabb, 1869). The outer lip of *T. grande* is often damaged in a way that suggests the action of predatory crabs.

Geary (1990) studied morphological change within species of *Melanopsis* Ferussac, 1807 in the Late Miocene long-lived Lake Pannon in Austria and Hungary and found anagenetic change followed by a gradual cladogenetic split. The split probably developed over a time interval of approx. one million years and was accompanied by the shift of the two daughter species into different depositional environments. Geary's (1990) study is one of the very few documentations of gradual evolutionary change in long-lived lakes and shows that fossilized, long-lived lake shells provide great potential for such studies, as is corroborated with the *Toxosoma* study presented here.

CONCLUSIONS

In the case of the gastropod genus *Toxosoma,* an apparent cladogenetic event is documented in the Middle to early Late Miocene intervals of the Pebas Formation. The morphs correspond to species, and sexual dimorphism is found implausible as a possible cause for the observed morphologies.

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Appendix 1. Measurements of *Toxosoma*. H, height; Hap, height of aperture; WH, number of whorls *100; WBW, width of shell half a whorl before termination (see Fig. 3).

Sample	Species	H	Hap	WH	WBW
F685	*T. eboreum*	510	260	610	285
F685	*T. eboreum*	460	220	580	265
F685	*T. eboreum*	465	260	550	275
F685	*T. eboreum*	470	240	590	275
F685	*T. eboreum*	440	220	550	255
F685	*T. eboreum*	445	210	580	255
F685	*T. eboreum*	465	230	605	265
F685	*T. eboreum*	485	230	570	285
F685	*T. eboreum*	480	230	605	260
F685	*T. eboreum*	450	215	560	260
F685	*T. eboreum*	495	240	610	280
F685	*T. eboreum*	445	220	570	250
F685	*T. eboreum*	525	275	595	295
F685	*T. eboreum*	475	240	555	290
F685	*T. eboreum*	440	215	545	250
F685	*T. eboreum*	475	230	580	275
F685	*T. eboreum*	430	210	530	245
F685	*T. eboreum*	465	240	560	265
F685	*T. eboreum*	480	220	620	270
F685	*T. eboreum*	475	230	615	275
F685	*T. eboreum*	450	225	600	255
F685	*T. eboreum*	435	230	560	255
F685	*T. eboreum*	500	250	600	275
F685	*T. eboreum*	455	230	605	260
F685	*T. eboreum*	475	220	600	270
F685	*T. eboreum*	485	250	595	280
F685	*T. eboreum*	470	230	595	265
F707	*T. contortum*	285	135	505	140
F707	*T. contortum*	335	150	600	160
F707	*T. contortum*	285	140	550	140
F707	*T. contortum*	340	135	600	155
F707	*T. contortum*	310	175	530	170

Appendix 1. (Continued)

Sample	Species	H	Hap	WH	WBW
F707	*T. contortum*	290	130	575	140
F707	*T. contortum*	355	150	580	180
F707	*T. contortum*	325	150	605	165
F707	*T. contortum*	380	165	620	185
F707	*T. contortum*	300	130	565	155
F707	*T. contortum*	305	140	550	155
F707	*T. contortum*	345	160	590	170
F707	*T. contortum*	320	155	560	165
F707	*T. contortum*	305	140	530	155
F707	*T. contortum*	315	145	550	160
F029	*T. grande*	555	295	520	290
F029	*T. grande*	535	335	515	295
F029	*T. grande*	520	265	495	275
F029	*T. grande*	595	295	510	340
F029	*T. grande*	610	310	515	340
F029	*T. grande*	575	310	550	320
F029	*T. grande*	550	320	510	325
F029	*T. grande*	515	275	500	325
F029	*T. grande*	505	275	520	295
F029	*T. grande*	515	290	505	290
F032	*T. grande*	485	250	515	265
F032	*T. grande*	580	335	590	300
F032	*T. grande*	560	290	580	305
F032	*T. grande*	500	290	575	265
F032	*T. grande*	590	325	595	320
F032	*T. grande*	560	315	565	295
F032	*T. grande*	550	310	525	295
F032	*T. grande*	520	295	525	275
F032	*T. grande*	550	310	550	300
F032	*T. grande*	575	320	585	300
F032	*T. grande*	600	350	555	330
F032	*T. grande*	495	265	495	275
F032	*T. grande*	550	310	525	310
F032	*T. grande*	495	245	560	260
F032	*T. contortum*	375	170	575	190
F032	*T. contortum*	350	160	580	185
F032	*T. contortum*	370	125	590	175
F032	*T. contortum*	380	140	590	180
F032	*T. contortum*	380	120	610	175
F032	*T. contortum*	350	125	585	165
F032	*T. contortum*	340	115	575	175
F032	*T. contortum*	375	115	575	175
F032	*T. contortum*	350	140	570	175
F032	*T. contortum*	340	135	570	165
F417	*T. eboreum*	455	230	570	255
F417	*T. eboreum*	435	215	555	255
F535	*T. eboreum*	425	240	560	250
F535	*T. eboreum*	360	185	550	200
F535	*T. eboreum*	420	215	585	235
F535	*T. eboreum*	390	195	525	230
F535	*T. eboreum*	355	175	545	195

Appendix 1. (Continued)

Sample	Species	H	Hap	WH	WBW
F535	*T. eboreum*	370	185	570	205
F535	*T. eboreum*	445	240	570	250
F535	*T. eboreum*	420	205	595	235
F535	*T. eboreum*	370	180	550	210
F535	*T. eboreum*	340	175	520	195
F535	*T. eboreum*	435	230	560	240
F535	*T. eboreum*	435	215	575	245
F535	*T. eboreum*	390	190	550	225
F535	*T. eboreum*	350	185	510	200
F535	*T. eboreum*	390	180	595	220
F836	*T. denticulatum*	520	290	655	250
F836	*T. denticulatum*	400	240	560	215
F836	*T. denticulatum*	445	245	650	225
F836	*T. denticulatum*	435	245	545	245
F836	*T. denticulatum*	410	210	560	205
F836	*T. denticulatum*	430	230	560	235
F836	*T. denticulatum*	400	235	560	210
F836	*T. denticulatum*	430	235	640	220
F836	*T. denticulatum*	470	205	580	240
F367	*T. denticulatum*	485	260	605	240
F367	*T. denticulatum*	505	280	615	255
F367	*T. denticulatum*	455	250	610	220
F367	*T. denticulatum*	415	255	590	215
F367	*T. denticulatum*	455	255	600	250
F367	*T. denticulatum*	430	240	560	215
F367	*T. contortum*	330	170	545	165
F367	*T. contortum*	340	160	560	175
F367	*T. contortum*	340	160	600	170
F367	*T. contortum*	310	150	545	150
F367	*T. contortum*	325	145	550	160
F367	*T. contortum*	330	140	585	165
F367	*T. contortum*	410	180	645	190
F367	*T. contortum*	325	160	580	160
F367	*T. contortum*	340	145	580	165
F367	*T. contortum*	310	145	615	155
F367	*T. contortum*	300	150	565	150
F367	*T. contortum*	325	150	560	160
F367	*T. contortum*	350	175	560	175
F367	*T. contortum*	315	145	545	165
F367	*T. contortum*	335	160	560	175
F367	*T. contortum*	335	150	565	165
F367	*T. contortum*	350	155	595	175
F367	*T. contortum*	335	155	590	160
F367	*T. contortum*	315	145	555	160
F367	*T. contortum*	300	140	540	155
F034	*T. denticulatum*	460	255	555	235
F034	*T. denticulatum*	520	295	575	270
F034	*T. denticulatum*	415	220	505	245
F498	*T. eboreum*	415	205	605	230
F498	*T. eboreum*	405	200	570	220
F498	*T. eboreum*	355	180	530	205

Appendix 1. (Continued)

Sample	Species	H	Hap	WH	WBW
F498	*T. eboreum*	390	185	555	205
F498	*T. eboreum*	405	195	575	215
F498	*T. eboreum*	385	170	570	185
F498	*T. eboreum*	410	200	595	215
F498	*T. eboreum*	435	220	570	240
F498	*T. eboreum*	460	200	620	245
F498	*T. eboreum*	395	170	590	180
F498	*T. eboreum*	350	165	510	180
F498	*T. eboreum*	400	180	555	195
F498	*T. eboreum*	420	230	585	245
F498	*T. eboreum*	430	220	610	230
F498	*T. eboreum*	370	175	540	200
F498	*T. eboreum*	380	195	590	220
F498	*T. eboreum*	380	160	545	190
F498	*T. eboreum*	370	170	545	205
F498	*T. eboreum*	375	190	560	200
F498	*T. eboreum*	405	200	560	240
F498	*T. eboreum*	410	195	590	215
F498	*T. eboreum*	340	150	545	180
F498	*T. eboreum*	400	190	570	190
F498	*T. eboreum*	360	170	535	180
F498	*T. eboreum*	385	165	550	190
F498	*T. eboreum*	350	170	520	180
F489	*T. eboreum*	490	235	615	250
F489	*T. eboreum*	395	185	545	210
F489	*T. eboreum*	455	200	540	255
F489	*T. eboreum*	445	210	550	225
F489	*T. eboreum*	470	210	565	250
F489	*T. eboreum*	420	195	555	220
F489	*T. eboreum*	495	215	575	255
F489	*T. eboreum*	500	220	550	270
F489	*T. eboreum*	460	215	515	255
F489	*T. eboreum*	500	220	510	275
F489	*T. eboreum*	470	205	535	255
F489	*T. eboreum*	535	230	640	265
F489	*T. eboreum*	475	230	550	260
F489	*T. eboreum*	505	245	545	290
F489	*T. eboreum*	485	225	545	260
F489	*T. eboreum*	495	210	600	250
F489	*T. eboreum*	400	180	530	210
F489	*T. eboreum*	500	220	555	265
F489	*T. eboreum*	480	220	545	270
F489	*T. eboreum*	495	210	555	270
F489	*T. eboreum*	525	240	550	290
F489	*T. eboreum*	405	190	515	200
F489	*T. eboreum*	435	180	560	215
F489	*T. eboreum*	460	190	590	235
F489	*T. eboreum*	510	250	560	280
F489	*T. eboreum*	505	220	595	255
F489	*T. eboreum*	450	205	545	235
F489	*T. eboreum*	510	225	560	280

Appendix 1. (Continued)

Sample	Species	H	Hap	WH	WBW
F489	*T. eboreum*	460	200	570	250
F489	*T. eboreum*	485	205	605	250
F022	*T. contortum*	345	140	545	160
F022	*T. contortum*	350	150	565	175
F022	*T. contortum*	335	155	570	165
F022	*T. contortum*	340	150	550	180
F022	*T. contortum*	345	150	575	165
F022	*T. contortum*	350	150	575	155
F022	*T. contortum*	350	165	575	170
F022	*T. contortum*	335	135	575	160
F022	*T. contortum*	335	150	550	165
F022	*T. contortum*	320	135	555	165
F022	*T. contortum*	330	135	560	170
F022	*T. contortum*	315	145	545	150
F022	*T. contortum*	300	145	545	155
F022	*T. contortum*	300	155	525	155
F022	*T. contortum*	330	155	555	160
F004	*T. grande*	460	255	495	270
F004	*T. grande*	515	280	545	270
F004	*T. grande*	420	245	500	240
F004	*T. grande*	450	250	505	255
F004	*T. grande*	445	240	500	250
F702	*T. grande*	420	210	465	220
F702	*T. grande*	475	220	480	250
F702	*T. grande*	430	180	465	220
F702	*T. grande*	435	195	475	235
F702	*T. grande*	460	240	480	235
F702	*T. grande*	400	195	460	215
F702	*T. grande*	415	180	465	220
F702	*T. grande*	405	190	455	215
F702	*T. grande*	425	205	475	225
F702	*T. grande*	415	200	475	225
F702	*T. grande*	380	185	480	215
F702	*T. grande*	450	225	465	235
F702	*T. grande*	435	205	485	230
F702	*T. grande*	405	185	460	215
F702	*T. grande*	405	220	480	225
F702	*T. grande*	470	215	505	250
F702	*T. grande*	420	185	475	210
F702	*T. grande*	470	220	460	245
F702	*T. grande*	420	195	445	240
F363	*T. eboreum*	360	180	560	220
F363	*T. eboreum*	370	160	560	215
F363	*T. eboreum*	335	175	550	210
F363	*T. eboreum*	340	170	540	205
F363	*T. eboreum*	360	180	575	200
F363	*T. eboreum*	330	165	550	195
F363	*T. eboreum*	325	150	525	190
F363	*T. eboreum*	315	170	515	185
F363	*T. eboreum*	360	180	560	180
F363	*T. eboreum*	355	165	560	175

Appendix 1. (Continued)

Sample	Species	H	Hap	WH	WBW
F363	*T. eboreum*	385	190	565	170
F363	*T. eboreum*	320	145	530	165
F363	*T. eboreum*	370	160	570	160
F363	*T. eboreum*	330	155	510	155
F363	*T. eboreum*	340	160	520	150
F006	*T. denticulatum*	550	290	580	285
F006	*T. denticulatum*	520	280	560	290
F006	*T. denticulatum*	490	260	540	260
F006	*T. denticulatum*	525	280	550	285
F006	*T. denticulatum*	485	270	545	265
F016	*T. grande*	430	220	510	250
F016	*T. grande*	495	245	505	270
F016	*T.* spec.	390	205	550	200
F016	*T. grande*	490	265	525	270
F016	*T. grande*	505	260	505	290
F021	*T. grande*	420	220	480	240
F830	*T. eboreum*	345	160	550	180
F830	*T. eboreum*	355	160	555	205
F830	*T. eboreum*	335	160	555	190
F830	*T. eboreum*	345	160	560	180
F830	*T. eboreum*	320	165	510	190
F830	*T. eboreum*	320	160	545	180
F830	*T. eboreum*	360	165	555	200
F830	*T. eboreum*	365	170	560	195
F830	*T. eboreum*	340	160	555	190
F830	*T. eboreum*	350	155	550	180
F830	*T. eboreum*	340	160	555	195
F830	*T. eboreum*	355	160	550	195
F830	*T. eboreum*	365	160	565	205
F830	*T. eboreum*	390	170	560	200
F830	*T. eboreum*	350	170	555	200
F830	*T. eboreum*	330	155	555	180
F830	*T. eboreum*	335	155	555	180
F830	*T. eboreum*	365	170	575	195
F830	*T. eboreum*	375	175	575	195
F830	*T. eboreum*	380	170	605	205
F830	*T. eboreum*	340	160	565	185
F830	*T. eboreum*	305	145	510	185
F830	*T. eboreum*	350	175	540	195

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Morphological cladistic analysis as a model for character evaluation in primitive living chitons (Polyplacophora, Lepidopleurina)*

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Abstract: Chitons are often referred to as "living fossils" in part because they are proposed as one of the earliest-diverging groups of living molluscs, but also because the gross morphology of the polyplacophoran shell has been conserved for hundreds of millions of years. As such, the analysis of evolution and radiation within polyplacophorans is of considerable interest not only for resolving the shape of pan-molluscan phylogeny but also as model organisms for the study of character evolution. This study presents a new, rigorous cladistic analysis of the morphological characters used in taxonomic descriptions for chitons in the living suborder Lepidopleurina Thiele, 1910 (the earliest-derived living group of chitons). Shell-based characters alone entirely fail to recover any recognized subdivisions within the group, which may raise serious questions about the application of fossil data (from isolated shell valves). New analysis including characters from girdle armature and gill arrangements recovers some genera within the group but also points to the lack of monophyly within the main genus *Leptochiton* Gray, 1847. Additional characters from molecular data and soft anatomy, used in combination, are clearly needed to resolve questions of chiton relationships. However, the data sets currently available already provide interesting insights into the analytical power of traditional morphology as well as some knowledge about the early evolution and radiation of this group.

Key words: morphology, molluscan evolution, cladistics, *Leptochiton*

Phylogenetic studies have shown that chitons (Polyplacophora) retain many features that are plesiomorphic within molluscs and indeed appear to have close morphological similarity with the hypothesized common ancestor of the Mollusca (*e.g.*, Haszprunar 1996, Sigwart and Sutton 2007; Fig. 1A). Nevertheless, given the radical disparity encompassed by molluscan morphology, understanding the patterns of evolution within the Polyplacophora is of particular importance to undertand how a *bauplan* that has remained so conserved over hundreds of millions of years in the direct living descendants of the ancestral chiton may have also given rise to forms as different as bivalves and cephalopods. The living chitons are contained in the subclass Neoloricata, which has a fossil record extending from the Carboniferous (ca. 350 Mya) to Recent. However, in spite of this deep fossil record, the majority of fossil species are known only from isolated, disarticulated plates, which makes it very difficult to infer the morphology of the whole animal (Cherns 2004, Vendrasco *et al.* 2004, Sigwart and Sutton 2007). The three major clades within the Neoloricata (Lepidopleurida, Chitonina, and Acanthochitonina) are separated stratigraphically in the fossil record as well as morphologically, which has resulted in a broad acceptance of their monophyly and phylogenetic separation at the ordinal level (Buckland-Nicks 1995, Okusu *et al.* 2003). The suborder Lepidopleurina is uncontroversially accepted to be the earliest-derived group of living chitons (Fig. 1B). In spite of this broadly accepted separation, to date there is limited understanding of the relationships between taxa within the major clades of chitons and not a robust testing of the monophyly of these groups across the broad range of taxa they each cover. Living members of the order Lepidopleurida (suborder Lepidopleurina) include nine genera comprising five families (*sensu* Sirenko 2006); but the genus *Leptochiton* Gray, 1847 contains about 80 of the 120 valid extant species in the order. Complex shell and girdle features that are used to define non-lepidopleuran chitons are often lacking in these species, which are small, plain, and superficially homogenous in their external morphology. However, species are described using the same conventional features across all chitons—shell sculpture, girdle armature microstructure, gills, and gross radular morphology. In addition, several morphological features, particularly from soft anatomy, have been proposed to assess the intra-clade relationships of chitons, particularly sperm acrosome and egg hull structures (Hodgson *et al.* 1988, Sirenko 1993, Buckland-Nicks 1995, 2006), relative positions of gonopores

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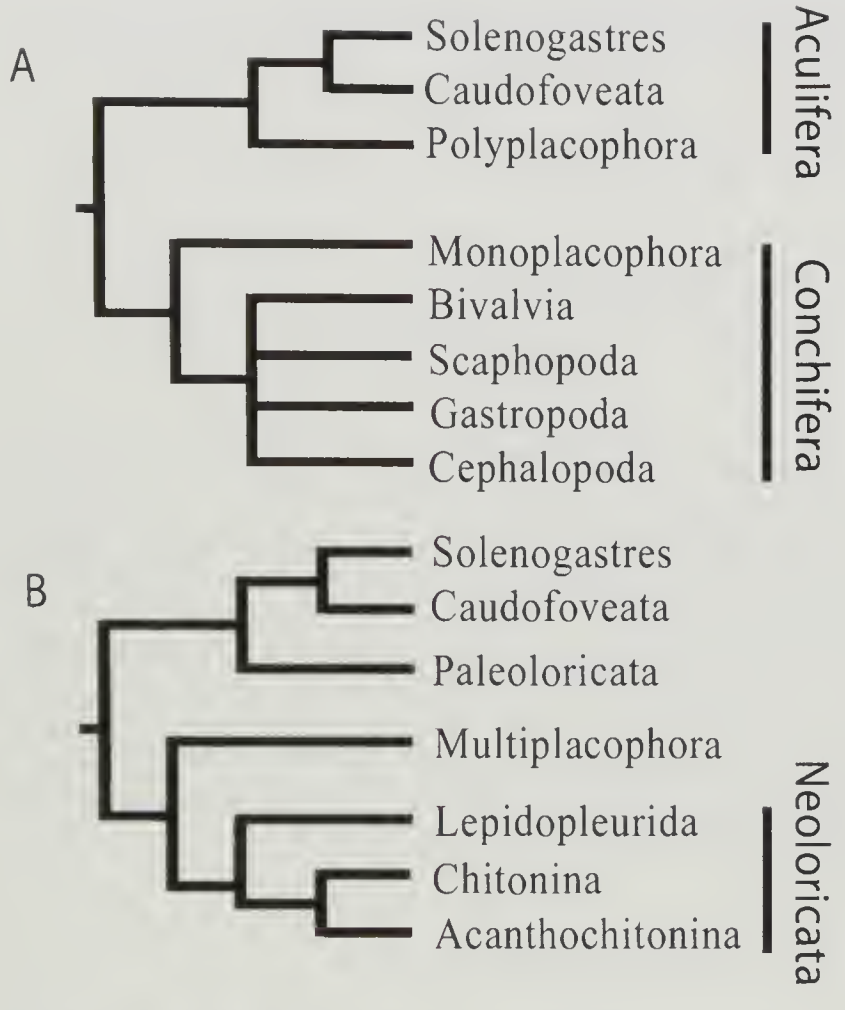

Figure 1. A, generalized topology of the aculiferan model of molluscan evolution, showing relationships between living classes of molluscs. B, generalized topology of major clades within the Aculifera (redrawn from Sigwart and Sutton 2007). All living chitons are classified in the subclass Neoloricata, in the two orders Chitonida (containing two suborders, Chitonina and Acanthochitonina) and Lepidopleurida (the focus of the present study).

and nephridiopores within the gill row (Sirenko 1993, Sigwart 2008b), and patterns of aesthete canals within the valves (Fernandez *et al.* 2007, Vendrasco *et al.* 2008).

The aim of this paper is to empirically assess the usefulness of the morphological features that are currently used to define species, by testing their ability to build phylogenetic hypotheses within and between the taxa that comprise this clade. This is accomplished here by using a subset of exemplar lepidopleuran taxa. The hypothesis tested is that if the characters used to diagnose and to describe species were defined expressly to separate species, genera, and other taxonomic subsets, then the expected results of a quantitative phylogenetic analysis of those characters would be a tree replicating the established Linnaean hierarchy. In fact, many non-diagnostic characters are regularly described for chitons, and the accepted diagnoses of genera in particular are fraught with inconsistency. Consequently, this study also examines the information content of morphological characters.

MATERIALS AND METHODS

Characters and taxa

This study draws on morphological examination by the author of preserved specimen material, particularly from the collections of the Museum national d'Histoire naturelle (MNHN, Paris); Zoologische Staatssammlung München (ZSM, Münich), Naturhistorisches Museum in Wien (NHMW, Vienna); and the National Museum of Ireland, Natural History Division (NMINH, Dublin). Sixty-nine morphological characters were described based on features used in every modern species description, derived from a standard implicitly set by the work of Kaas and Van Belle (1985). Character states are defined based on features identified in the literature and from the author's personal observation of specimen morphology. Codings for the present morphological matrix are based on original anatomical observation by the author. In principle, these character states are defined based on variability that is morphologically constant for all individuals within a species but may vary, encompassing the different states defined, in different species. The characters include 32 shell features, 27 girdle features, two aspects of gross body shape, two radula characters, and six characters of gill arrangement (Table 1). Part of this data set was based on the matrix of shell features published by Sigwart *et al.* (2007), incorporating new refinements to the characters used in that analysis. Characters were described with two (binary), three, or four potential character states. All characters were considered to be unordered (that is, evolutionary change could hypothetically transform freely between any of the described states) and without polarity (no ancestral state is assumed). All characters were equally weighted for the analysis. The complete character matrix is presented in Table 2. The characters were coded for 39 ingroup taxa selected to cover the morphological diversity of the living Lepidopleurina. Two species of *Callochiton* and the monotypic *Choriplax grayi* (H. Adams and Angas, 1864) were coded as outgroup taxa. The genus *Callochiton* resolved as sister to the family Leptochitonidae in a molecular phylogenetic analysis (Okusu *et al.* 2003). This is a controversial result which has not been supported by other studies (Buckland-Nicks 2006, Sirenko 2006, B. Lieb, pers. comm.). However, as there is so little information about the internal topology of any of the major polyplacophoran clades, this is the best evidence for choosing a most proximal outgroup from living taxa. *Choriplax* has traditionally been classified within the Lepidopleurina but has recently been considered by Sirenko (2006) to be an advanced form with secondarily derived features in common with lepidopleurans.

Phylogenetic analysis, consensus, and support

The complete matrix was initially subjected to a permutation tail probability test (PTP) to assess data quality, using

Table 1. Characters formulated from external morphology and used to code chiton taxa in the phylogenetic analysis. CI (confidence index) from main analysis of complete matrix.

	Character description	CI
1	Ratio: apophyses outside diameter / valve width: ≤0.8 (0); >0.8 (1).	0.125
2	Ratio: combined diameter of apophyses / valve width: ≤0.4 (0); >0.4 (1).	0.125
3	Thickened on terminal margins: no (0); yes (1).	0.250
4	General character of arch (intermediate plates): straight sides (0); rounded (1); concave (2).	0.333
5	Dorsal elevation (height / width) of intermediate plates: ≤0.4 (0); >0.4 (1).	0.200
6	Valves beaked: no (0); yes (1).	0.111
7	Lateral area elevated on intermediate plates: no (0); yes (1).	0.091
8	Intermediate plates with distinct diagonal separating lateral areas: no (0); yes (1).	0.077
9	Apophyses' jugal margin: straight (0); concave (1).	0.091
10	Head valve shape: semicircular (0); shape < semicircle (1); shape > semicircle (2).	0.222
11	Tail valve shape: semicircular (0); shape < semicircle (1); shape > semicircle (2).	0.286
12	Mucro prominent: no (0); yes (1).	0.100
13	Mucro position: posterior (0); median (1); anterior (2).	0.095
14	Post-mucronal slope: straight (0); concave (1); convex (2).	0.125
15	Articulamentum character: weak, transparent (0); moderate, opaque at least in major central areas (1); strong, forming thickened calluses and/or extending into insertions (2).	0.182
16	Insertion plates present: no (0); yes (1).	0.333
17	Tail valve apophyses shape same as on intermediate valves: yes (0); no, difference(s) (1).	0.077
18	Intermediate valve shape: trapezoidal (0); rectangular (1); ovate or circular (2).	0.400
19	Intermediate valves: anterior margin: straight (0); concave (1); convex (2).	0.167
20	Intermediate valves: posterior margin: straight (0); convex (1); concave around apex (2).	0.182
21	Tegmentum—general sculpture: smooth (0); granulose (1); pustulose or joined (2).	0.250
22	Tegmentum—gradation of sculpture: regular (0); larger toward margin (1); faded posteriorly (2).	0.333
23	Tegmentum—dominant granule shape: no granules (0); roundish (1); square or irregular (2).	0.250
24	Central areas of intermediate valves—distinct jugal sculpture: not distinct from pleural area (0); longitudinally granulate in jugal area; pleural areas coarser (1); jugal sculpture in quincunx, grading to radiating longitudinal or diagonal series (2).	0.167
25	Intermediate valves—sculpture with longitudinal rows: no pattern (0); longitudinal rows (1); quincunx or diagonal series (2); irregular, wavy, or zigzag (3).	0.214
26	Intermediate valves—sculpture interstices: close / narrow or sandy (0); evenly distributed, series of granules may be coalescing / beading (1); widespread or punctured (2).	0.118
27	Areas of intermediate plates with corresponding sculpture as terminal plates: no (0); yes (1).	0.200
28	Ratio: apophyses inside separation / valve width: ≤0.4 (0); >0.4 (1).	0.100
29	Thick periostracum forming pustules: absent (0); present (1).	1.000
30	Valve carinated or keeled: no (0); semi-carinated or keeled at posterior (1); yes (2).	0.133
31	Lateral area sculpture in radiating rows: no pattern or quincunx (0); strong radiating rows (1); central sculpture continuing in longitudinal rows (2); wavy or zigzag (3).	0.300
32	Tail valve more than twice the length of intermediate valves: no (0); yes (1).	0.500
33	Dorsal girdle scales—length / width ratio of typical scale (total length / width at base): absent (0); square (1); length ≥1.5 × width (2); width ≥1.5 × length (3).	0.200
34	Dorsal girdle scales: absent (0); straight (1); distally curved (2).	0.333
35	Dorsal girdle scales or spicules—generalized density: "dense" (1); unremarkable (2); "sparse" (3).	0.133
36	Dorsal girdle scales—distribution: absent (0); regular (1); irregular (2).	0.200
37	Dorsal girdle scales or spicules—lateral size gradient: no, equal at all points (0); smaller toward margin (1); larger toward margin (2).	0.167
38	Dorsal girdle scales—shape: absent (0); square top (1); round top (2); pointed (3).	0.250
39	Dorsal girdle scales—texture: absent (0); smooth to weakly ribbed or striated (1); distinct ribs, more than 10 (2).	0.273

Table 1. (Continued)

	Character description	CI
40	Ventral girdle scales—length relative to typical dorsal scale: absent (0); equal length (1); shorter, half or less length (2); longer, 1.5 times length or more (3).	0.250
41	Ventral girdle scales—width relative to typical dorsal scale: absent (0); equal width (1); narrower half or less length (2); wider 1.5 times length or more (3).	0.250
42	Ventral girdle scales—texture: absent (0); smooth to weakly ribbed or striated (1); distinct ribs, 10 or less (1); distinct ribs, more than 10 (2).	0.286
43	Ventral girdle scales or spicules: absent (0); straight (1); distally curved (2).	0.667
44	Ventral girdle scales or spicules—shape: absent (0); elongate oval, round top or pointed scales (1); rectangular scales (2); spiculose (3).	0.375
45	Ventral girdle scales or spicules—generalized density: "dense" (1); unremarkable (2); "sparse" (3).	0.167
46	Ventral girdle scales or spicules—distribution: absent (0); regular (1); irregular (2).	0.286
47	Ventral girdle scales or spicules—lateral size gradient: no, equal at all points (0); smaller toward foot (1); larger toward foot (2).	0.500
48	Dorsal spicules, including intersegmental armature—length relative to major dorsal scales: absent (0); spicules form major dorsal coverage, scales absent (1); dorsal or intersegmental spicules not substantially longer than major dorsal scales (2); dorsal or intersegmental spicules substantially longer, three times length of dorsal scales or longer (3).	0.429
49	Dorsal spicules, including intersegmental armature—shape: absent (0); straight (1); distally curved (2).	0.500
50	Dorsal spicules, including intersegmental armature—terminal shape: absent (0); sharp, pointed (1); blunt (2).	0.182
51	Intersegmental spicules—complex base: absent or simple (0); spicules in chitinous cupules (1); spicules in complex *rigschaftnadel* (2).	0.167
52	Dorsal spicules, including intersegmental armature—texture: absent (0); smooth, strongly tapered (1); striated or grooved (2); smooth, cylindrical (3).	0.154
53	Marginal scales / spicules—length relative to typical dorsal armature: absent or not differentiated (0); same length (1); shorter (2); substantially longer, three times length or longer (3).	0.200
54	Marginal scales / spicules—width relative to typical dorsal armature: absent or not differentiated (0); same width (1); narrower (2).	0.182
55	Marginal scales / spicules—texture: absent (0); smooth, strongly tapered (1); striated or grooved (2); smooth, cylindrical (3).	0.176
56	Marginal spicules—shape: absent (0); straight (1); distally curved (2).	0.222
57	Marginal spicules—terminal shape: absent (0); pointed, sharp (1); blunt (2).	0.154
58	Dorsal sutural spines differentiated from major armature: no (0); yes (1).	0.077
59	Marginal spicules—complex base: absent or simple (0); spicules in chitinous cupules (1); spicules in complex *rigschaftnadel* (2).	0.182
60	Ratio: animal body length / width: ≤2 (0); >2 (1).	0.063
61	Maximum adult body length (mm): <10 mm (0); ≥10 mm (1).	0.125
62	Radula—number of cusps on major lateral teeth: monocuspidate (0); bicuspidate (1); tricuspidate (2).	0.154
63	Radula—smallest cusp on major lateral teeth: monocuspidate or equal (0); anterior / interior (1); posterior / exterior (2); central, tricuspid with two outer denticles equal (3).	0.214
64	Gills—number per side in adult animal: ≤4 (0); 5-10 (1); 11-16 (2); >16 (3).	0.167
65	Gills—size distribution: largest at posterior, or all equal (0); smallest anterior and posterior (1).	0.100
66	Gills interrupted by anal interspace: yes (0); no, gills on anal stem (1).	0.125
67	Gills "merobranchial", posterior: yes (0); no, other arrangement (1).	1.000
68	Gill coverage, as percentage of foot length: <40% (0); ≥40% (1).	0.200
69	Gills abanal (single gill posterior of nephridiopore in adult animal): no (0); yes (1)	0.500

Table 2. Character-taxon data set utilized for the phylogenetic analysis. Asterisks (*) denote taxa designated as outgroups. Ingroup taxa are listed in alphabetical order by species epithet. For some characters, transitional states are coded r = {0,1}; t = {1,2}.

	1 2 3 4 5 6 123456789012345678901234567890123456789012345678901234567890123456789
**Callochiton bouveti* Thiele, 1906	11000111101020110120101010100200001010033111 2??11101111220101223001 11
**Callochiton septemvalvis* (Montagu, 1803)	11000111100000110122101020100200001010022111 2?2111013212101012230011 1
**Choriplax grayi* (H. Adams and Angas, 1864)	11110001022022211121202011100110213 2?31??????????????????????1120?????1
Parachiton acuminiatus (Theile, 1909)	00010011001001000100102011110?r12122?32111112??2110231121001120???0?0
Leptochiton aequispinus (Bergenhayn, 1933)	01020000100020200110101020110200 21???2211211???000001131200010021 10?0
Leptochiton algesirensis (Capellini, 1859)	11010001010020?0110010?01?110?r01222112221 1t222121111212000110201010
Leptochiton alveolus (Sars MS, Lovén, 1846)	10011000000011?01?1010100??10100212 11?2112112?12110 2????1011002?10?0
Leptochiton americanus Kaas and Van Belle, 1985	11000001?10001201200101 11?10011021 1??3111111?1?21101?????0?0110110?0
Nierstraszella andamanica (Smith, 1906)	1110010010000r20110200002210120000120001111312?11202000010111021 1010
Leptochiton asellus (Gmelin, 1791)	110?0101101101?0??2220101?0101r02212122221122 1?????12111000101 1?1000
Leptochiton binghami (Boone, 1928)	???0?110???121????02101111?0210211?13131113 2?311010000000?01?0???0?0
Leptochiton bouchieti Sirenko, 2001	00010000000021001110101022110000001210031112122112223131112012 1000000
Lepidopleurus cajetanus (Poli, 1791) .	0001?01110?122?0??0100101211002011120?232111?122111t122110011202?1000
Leptochiton cancellatus (Jeffreys, 1839)	11010011111121001100101010110?10122211212112212211 1?????10100?111000
Parachiton communis Saito, 1996	01010011002000101100102010100001212 1?33111121?????0000000112331 1000
Deshayesiella curvata (Capenter MS, Pilsbry, 1892)	01010111020120200?021??11000002021 2?13221111 21?2110112111 0?1110111000
Leptochiton deforgesi Sirenko, 2001	10010000000111001100102022110000112 1122312112122122111312121021000000
Leptochiton denhartogi Strack, 2003	11010011111121001100101011110010122 1112111111212211011121100000011 1000
Leptochiton foresti (Leloup, 1981)	11100100100010200102101012100210001 2000111 13?1?11202112210101201 1?010
Leptochiton hirasei (Taki and Taki, 1929)	00010001 10?12100020010 1?1?0000r0211??111211 21?2110111211011020 11?0?0
Leptochiton inquinatus (Reeve, 1847)	01010011100000001100 10?01?1000r01211123121 112?2212121121t1?11001?1000
Leptochiton intermedius (Salvini-Plawen, 1968)	10010001000001100100101012110000?????????????????????????????1?????0?0
Leptochiton japonicus (Theile, 1909)	01020000100020200110101020110200 21???2211211???000001131200010021 1000
Leptochiton juvenis (Leloup, 1981)	00000101000020101100111210110200211111212111212212001231111012321 1000
Leptochiton kerguelensis Haddon, 1886	00011011000011001?0000112?10000021 1?1211111112211 12?????10001011?0?0
Nierstraszella lineata (Nierstrasz, 1905)	110r00010100012011110?203110113000120001111312?11202000010111021 1010

Table 2. (Continued)

	1	2	3	4	5	6
	123456789012345678901234567890123456789012345678901234567890123456789					
Leptochiton medinae (Plate, 1899)	000100010?0100001100101110100010112?13211211112222021121100011011100					
Hanleya nagelfar (Lovén, 1846)	110010010000100000200000?0000000200001211 32??11101000000112031101 0					
Leptochiton n. sp. 4 Sigwart, 2008a	000100010001200011201011111000002121122331112123122100000100021000000					
Leptochiton n. sp. 5 Sigwart, 2008a	000000000000210011001112221101002111221111111231221311121201210000 00					
Oldroydia percrassa (Dall, 1894)	110001001100012012 22?0111?10011000 10?00131112121110 1????00111021100?					
Leptochiton pergranatus Dall, 1889	??00000110?021100?02101011 1?02r0212?132331131223110100000 0?01?02??000					
Ferreiraella plana (Nierstrasz, 1905)	110201001021012012111010 20?10100001200000000200112021121 20?01203??0?0					
Parachiton politus Saito, 1996	010100100020001001001020101000012121?22211112 1?21202000000112331 1000					
Leptochiton rugatus (Capenter MS, Pilsbry, 1892)	??010011?1011?00110020?01?1?00r012??1t311211?1?22202112221??1001?1000					
Leptochiton saitoi Sirenko, 2001	001000010000201011001011111002000022000000032221211111111110023111000					
Leptochiton scabridus (Jeffreys, 1880)	000100011 00?1100012011101?000110112?113122112?221111????1?00201?1000					
Leptochiton thandari Sirenko, 2001	100000001000000011001011110101002121121111112122121111111120021000000					
Leptochiton vanbellei Sirenko, 2001	100110000001100001001010221102000022000111212221122111311121121000000					
Leptochiton vaubani Kaas, 1991	110011000000110011101012211002202122031111112222110100000100111300010					
Leptochiton vietnamensis Sirenko, 1998	010001010000201001001010101000000022100000032221111111111110011211000					
Ferreiraella xylophaga Gowlett-Holmes and Jones, 1992	100000000200102112110000021102000020000000002001110111111111123301010					

50 permutation replicates (Faith and Cranston 1991). Having determined that the data differ significantly from random, they were then analyzed in the standard software package PAUP* version 4.0b10 (Swofford 2002), using a heuristic search algorithm. To improve the efficiency of analysis, an initial heuristic search was performed with ten random addition sequence replicates to determine the size (*n* steps) of the shortest common most parsimonious trees (MPTs). The analysis was then repeated with 100 random addition sequence replicates, limiting the search to trees of length *n* or less. All trees of minimum length were retained as the primary set of MPTs to a maximum of 3000 MPTs. To assess the relative degree of clade support, bootstrap values were calculated from the analysis, using 50 bootstrap replicates of 10 random addition sequence replicates each.

Strict consensus cladograms can show dramatic reduction of resolution when the positions of a few ingroups are highly mobile (*e.g.*, Wilkinson 1999). Reduced consensus methods can recover additional ingroup relationships by removing these unresolved taxa. It is important to note that taxa are pruned only after the analysis is performed using the complete data matrix so no data are selectively "ignored". This study employed the "Strict" program in the software package RedCon version 3.0 (Wilkinson 2001) to produce a set of strict reduced consensus trees.

Two methods were employed to examine the influence of the two major data partitions (shell characters and girdle characters). First, consensus index (CI) values for the two data sets were compared from within the main analysis (the value for each character of the minimum number of potential state changes, divided by the minimum number actually observed on the tree). Two secondary analyses were run on restricted sections of the matrix (one with shell characters only, one with girdle characters alone) with the same parameters and protocol as the main analysis (initial PTP test and heuristic search protocol in PAUP* outlined above).

RESULTS

The permutation tail probability calculated for the matrix was 0.02, which is significant ($P < 0.05$), indicating that the matrix should contain a resolvable phylogenetic signal. The phylogenetic analysis resulted in 68 MPTs, each of length 517

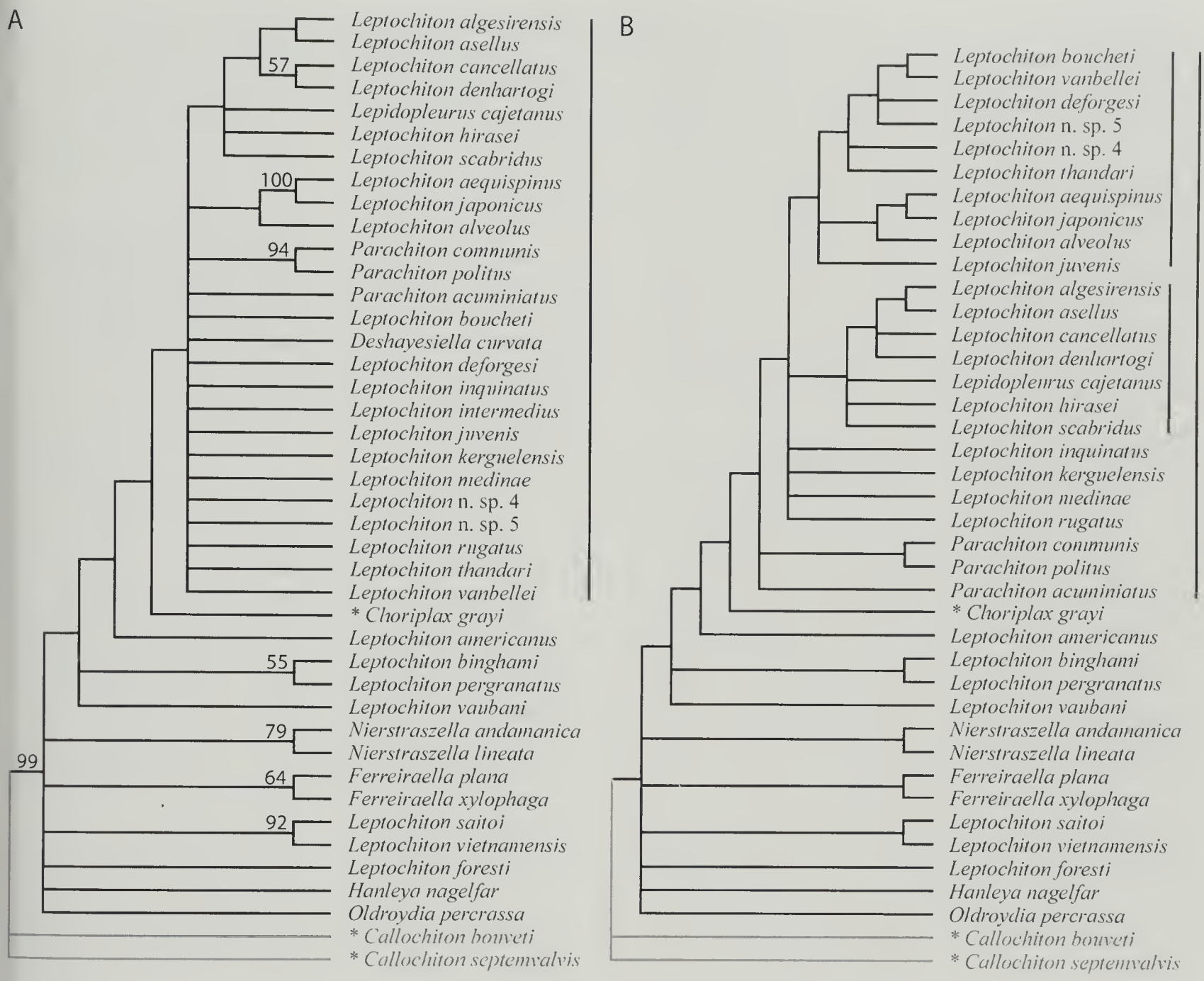

Figure 2. Phylogenetic trees resulting from analysis of morphological characters for Lepidopleurida. Asterisks (*) denote taxa designated as outgroups. A, strict consensus of 68 MPTs; bootstrap values are marked on internal nodes (where the bootstrap value is over 50). B, preferred tree, strict reduced consensus tree with *Leptochiton intermedius* and *Deshayesiella curvata* pruned from trees contributing to Fig. 1A. Major internal clades discussed in the text are marked with vertical lines to the right: predominantly Pacific *Leptochiton* spp. (top, *Leptochiton boucheti–Leptochiton juvenis*); predominantly Atlantic *Leptochiton* spp. (lower, *Leptochiton algesirensis–Leptochiton scabridus*); major *Leptochiton* clade including *Parachiton* (*Leptochiton boucheti–Parachiton acuminatus*).

steps (Fig. 2A). The strict consensus of these trees recovers a monophyletic Lepidopleurida including *Choriplax*, relative to the *Callochiton* outgroup (Fig. 2A). Whether *Choriplax* is designated an outgroup taxon or an ingroup taxon does not change the topology of the resulting trees. Un-rooted trees also resolve the same topology in all cases. Within the ingroup, a single major clade containing most *Leptochiton* species (21 of 28 taxa included in the analysis), including the type species *Leptochiton asellus* (Gmelin, 1791) as well as *Lepidopleurus* Risso, 1826, *Parachiton* Thiele, 1909, and *Deshayesiella* Carpenter in Dall, 1879. The remaining lepidopleurans are arranged as a grade sister to this main clade. Strong sister-taxa relationships resolve *Nierstraszella* Sirenko, 1992 and *Ferreiraella* Sirenko, 1988 as monophyletic (each genus represents its own family). The monophyly of Lepidopleurina is supported by three synapomorphies: merobranchial gill arrangement (unique to Lepidopleurina), adanal gill placement (although this is reversed in *Choriplax*), and lack of shell insertion plates (although this is reversed in *Ferreiraella* as well as in *Choriplax*).

Reduced consensus analysis resulted in a set of seven additional strict reduced consensus trees; four excluded a single taxon from the trees, two trees excluded two taxa, and one excluded nine taxa. Pruning *Leptochiton intermedius* (Salvini-Plawen, 1968) produced better resolution within the main *Leptochiton* clade. However, other permutations recovered in these seven SRC trees did not change the topology of the cladogram. The two SRC trees that pruned two taxa both excluded *L. intermedius* and one of *Parachiton acuminatus* (Theile, 1909) or *Deshayesiella curvata* (Capenter MS, Pilsbry, 1892). *Parachiton acuminatus* is the type species of the genus *Parachiton* so its exclusion is not preferred. The preferred final tree representing the phylogeny, based on this character matrix, is the SRC tree excluding *L. intermedius* and *D. curvata* (Fig. 2B).

Bootstrap values for the nodes recovered were generally low, with support (>50) for local sister-group relationships but low support (<50) for the major clade of *Leptochiton* and allies (Fig. 2A). Within this major *Leptochiton* clade, there is one consistently resolved group of primarily Atlantic species although one Japanese species, *Leptochiton hirasei* (Taki and Taki, 1929), is included. This is the only cluster of species that resolves consistently in all MPTs although bootstrap support for this clade is low. Reduced consensus methods further resolve a separate group of Pacific species of *Leptochiton* although one Atlantic species, *Leptochiton alveolus* (Sars MS, Lovén, 1846), is included (Fig. 2B).

Consistency index (CI) values for most characters were quite low (Table 1). The average CI values for the shell characters (0.22) were lower but not substantially different from the average CI for girdle characters (0.26). The analysis of 32 shell characters resulted in 348 MPTs of length 206 steps; analysis of 27 girdle characters resulted in a large number of MPTs (>3000), length 180 steps. Consensus trees from each of these analyses resulted in a totally unresolved polytomy that failed to separate the outgroup and ingroup taxa.

DISCUSSION

Lepidopleuran chitons are recognized by three major synapomorphies: the absence (or very minimal development) of shell insertion plates; latero-ventral shell eaves that anchor the valves in the fleshy girdle; and the posterior (*i.e.*, "merobranchial") and adanal gill arrangement. The adanal condition is identified by the placement of multiple gills posterior to the nephridiopore; gills are added to both the anterior and posterior ends of the gill row during ontogeny. These features (absent insertion plates and gill arrangement) are traditionally considered plesiomorphic within chitons. Fossil polyplacophoran valves with morphology very similar to those in living lepidopleurans are known in many taxa from the Lower Carboniferous (ca. 350 Mya), whereas shell insertion plates first appear in the fossil record in the Permian (ca. 290-250 Mya) and become more common in Cretaceous taxa and later. Merobranchial adanal gills, although not preserved in the fossil record, are a feature unique to lepidopleurans, and this is assumed to be the ancestral condition on that basis.

Sirenko (2006) revised the classification of Lepidopleurida and assigned the genera *Choriplax*, *Hemiarthrum*, and *Weedingia* to the family Hemiarthridae in the Chitonida. These three genera had previously been classified in the Lepidopleurida because, although they have shell insertion plates, the insertion plates are weak and unslitted. Their removal from the Lepidopleurida was based on their abanal gill arrangement (with only one gill located posterior to the nephridiopore in adult animals). Sirenko (2006) interpreted the gill arrangement in these three genera to be indicative that they are more derived and that the insertion plates in their shells are secondarily simplified. In the present analyis, *Choriplax* resolves within Lepidopleurina.

The nature of nephridiopore arrangement in lepidopleurans and all chitons clearly requires further study. Other genera that are uncontroversially considered to be within Chitonida may have adanal (plesiomorphic) gill arrangements; *Chaetopleura*, *Stenoplax*, *Callistochiton*, and *Onithochiton* species all have three or more gills posterior to the nephridiopore in adult animals. This classification of gill arrangements may not provide the straightforward synapomorphy that has been proposed.

In the present analysis, the large and dominant genus *Leptochiton* is clearly non-monophyletic. The major clade (Fig. 2B) could be considered to represent the family Leptochitonidae including *Parachiton*. Although the shell morphology that defines *Parachiton* (having a substantially enlarged tail valve) is distinctive, it may be interesting to consider in future studies whether that feature has evolved more than once. Outside of the major "Leptochitonidae" clade, the two other lepidopleuran families represented by more than one species are resolved in this tree: Nierstraszellidae (*Nierstraszella* spp.) and Ferreiraellidae (*Ferreiraella* spp.) as part of a basal polytomy including *Hanleya* (Hanleyidae) and *Oldroydia* (Protochitonidae). The consistent recovery of Mediterranean and North Atlantic taxa in close topological proximity (although not supported by bootstrap values) indicates that there may be a single regional radiation that links these taxa, which is worthy of further study. The two genera included, *Lepidopleurus* and *Leptochiton*, have been the subject of taxonomic controversy; the type species of both genera are included in this putative North Atlantic clade.

The first cladistic analysis published to assess the relationships within a clade of chitons was presented by Sigwart *et al.* (2007); as that analysis was intended to assess the phylogenetic

position of a new fossil species, it used only shell morphology. The resulting phylogeny did not recover any of the family or genus-level groups within Lepidopleurina although there were some relationships between individual sister taxa that were well supported (bootstrap >50), as was also found in this expanded study.

Understanding the potential usefulness of the major data sets available is an important step in building our understanding of the evolution of this group. The analysis of the two largest data partitions in this study, shell morphology characters and girdle morphology characters, unambiguously demonstrates that employing more characters produces more resolution. Analysis of either data set alone results in large numbers of trees with effectively random distribution of taxa. This was also demonstrated by the large number of initial MPTs recovered in the study of Sigwart *et al.* (2007) using shell character data.

In studies of molecular phylogenetic methods, it is well known that resolution and support recovered by phylogenetic analyses improves with increased character sampling (*i.e.*, number of genes and/or length of sequences). This argument suggests that adding more data of any type produces better resolved and presumably more accurate trees (*e.g.*, Poe and Swofford 1999, Simmons and Miya 2004). Questions remain unresolved about the relative benefits of adding more characters or more taxa in phylogenetic reconstruction, particularly using morphological data (Graybeal 1998, Cobbett *et al.* 2007). Some workers have even argued that large-scale cladistic data sets of morphological data are less informative than those concentrating on few well-described characters (Scotland *et al.* 2003). The basis for this position is that morphological data alone cannot provide a sufficient character base to derive robust phylogenetic hypotheses, so it may be more accurate to rely on molecular data and use a few broadly defined morphological features as a reassuring appendum. This has been informally called the "Christmas tree" approach to the use of morphological attributes: using morphological ornaments to make an adequate tree more aesthetic. In fact, one argument for the dominance of molecular phylogenetic methods to the exclusion of morphological data is that "much of the useful morphological diversity has already been scrutinized" (Scotland *et al.* 2003: 545). This statement is at odds with the state of anatomical knowledge for many biological groups, of which chitons are a quite typical example.

Other studies have concluded that "much more [morphological] variation could be included in phylogenetic analyses than is used presently" (Poe and Wiens 2000: 33-34). This is true not only of computational phylogenetic analyses but of the descriptive taxonomic studies that form a basis for phylogenetic investigation. The sources of data to describe species of chitons rely almost exclusively on external anatomy (gills, girdle, and shell morphology), with use of radular morphology (Saito 2004) and aesthete arrangement (Sirenko 2001, Fernandez *et al.* 2007) gaining popularity and regular usage. Further sources of data, such as gamete morphology (Buckland-Nicks 2006) and nephridiopore arrangement (Sirenko 1993, Sigwart 2008b) and others will become standard sources of morphological character data as anatomical descriptions are published for larger numbers of closely related taxa.

The present study, based on "classical" morphological characters used in traditional species descriptions of chitons, functions as a model to demonstrate that cladistic analyses of morphological data sets can successfully generate phylogenetic hypotheses. Whether or not the hypotheses proposed by these cladograms are "true" will be determined by the progressive accumulation of a body of work examining the internal relationships of taxa within well-established clades, based on multiple data sets including genetic and novel morphological information.

Morphological data that are available from currently routine description and analysis of specimens can be used to produce phylogenetic hypotheses and provide new insights into the relationships within known clades. The anecdotal superficial homogeneity of chiton morphology is disputed by this finding. Maximizing the number of morphological characters in analysis improves the quality of phylogenetic signal recovered (making the transition from a complete polytomy to resolved clades). Application of morphological data is critical not only because it is sensible to use all available data for living taxa, but also because it creates a link to fossil taxa. Encompassing all forms of data for fossil and living species is critical to understanding the ongoing evolution of chitons and other groups of living fossils.

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The use of developmental sequences for assessing evolutionary change in gastropods*

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Abstract: First introduced by Ernst Haeckel in the nineteenth century, the use of developmental sequences has recently seen a renaissance as part of the study of the evolutionary biology of embryos; here we review briefly the literature describing gastropod developmental sequences, appraising the extent to which it has contributed to this renaissance. Gastropods have figured extensively in studies of early development with cell lineage analysis available for numerous taxa. Phylogenetic comparisons of these data reveal strong evolutionary signals, particularly in relation to early cell divisions. In contrast, although the description of post cell division developmental stages, including functional elements of development, in gastropods is extensive, interspecific comparisons are rare and tend to focus instead on developmental mode. However, a recent comparison of the sequence of functional and morphological events in a clade of basommatophoran snails demonstrated several alterations in the timing of developmental events (*i.e.*, heterochronies) across the phylogeny. Many gastropod groups may offer the potential to carry out similar investigations of the evolutionary importance of sequence heterochrony and to try to unravel the mechanistic basis for such patterns in developmental sequences.

Key words: Pulmonata, Basommatophora, embryo, development, heterochrony

"*....Haeckel can be seen as the father of a sequence-based phylogenetic embryology*" (Richardson and Keuck 2002: 495).

DEVELOPMENTAL SEQUENCES IN BIOLOGY

Haeckel is perhaps best known for his Biogenetic Law, which, in its simplest interpretation, proposed that ontogeny is a brief and rapid re-run of phylogeny, evolution occurring by terminal addition (Haeckel 1866). Much has been made of the rescinding of this law, and Haeckel's reputation and work has to some extent been damaged by these discussions (Garstang 1922, deBeer 1958, Gould 1977, Richardson 1995, 1998, Richardson and Jeffery 2002, Richardson and Keuck 2002). However, it is frequently overlooked that, in the process of formulating his now discredited law, Haeckel made other valuable contributions to the field of evolutionary biology (Richardson and Keuck 2002). First, he flagged the importance of variation in embryonic development between species, the same variation that underpins much of the current emphasis in "Evo-Devo" research (Raff 2000, Arthur 2002). Second, he was first to champion the use of developmental sequences in cross-species comparisons (Richardson and Keuck 2002): his approach of using letters of the alphabet to represent the succession of developmental stages that could be lost or replaced through evolutionary time provided a significant advance on other approaches that focused on the relative rates of development of different traits (*e.g.*, as emphasized by workers such as Wilhelm His) and is very similar to approaches currently being advocated for analyzing changes in developmental sequence between descendent and ancestral species (*i.e.*, heterochronies—see below).

Related to the use of developmental sequences, another of Haeckel's major contributions to biology was the coining of the term *heterochrony*, which he used to describe "anomalies" to his biogenetic law (Richardson and Keuck 2002). Such anomalies were alterations to developmental sequences across species, whereby developmental events appeared either later or earlier in ontogeny (Haeckel also flagged heterotopes, which were alterations in the position of developmental events within the embryo). While Haeckel viewed heterochronies as exceptions to his biogenic law, they appear to have assumed major importance in the investigation of evolution and have even been suggested by some to be one of the key drivers of evolutionary change (*e.g.*, Gould 1977, but see also Raff 1996). Much of this emphasis of heterochrony as a major evolutionary shaping force has centered around shifts in the developmental timing of morphological traits and, in particular, body size (as a proxy for developmental time) in relation to reproductive maturation. This so-called global heterochrony shifted the emphasis from sequences to one concerned mostly with size and shape (*i.e.*, towards an allometric approach).

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It is only comparatively recently that those interested in heterochrony have re-established the Haeckelian focus on using developmental sequences and the potential importance of comparing these sequences across taxa in order to elucidate patterns in the evolution of development (*e.g.*, Smith 2001, 2003, Bininda-Emonds *et al.* 2002). This "new" emphasis has seen the development of sophisticated analytical procedures that allow the formal comparisons of changes in developmental sequence across species within an explicit phylogenetic context (sequence heterochronies) (Mabee and Trendler 1996, Smith 1996, 1997, 2001, Richardson *et al.* 2001, Bininda-Emonds *et al.* 2002, Jeffery *et al.* 2002a, 2002b, 2005, Schulmeister and Wheeler 2004). Given the great wealth of information that exists on gastropod development, stretching back over one hundred years, coupled with a voluminous recent literature marrying molecular techniques with classical embryology, the question can be posed as to what extent has the study of gastropod development figured in this renaissance? Here we address this question by reviewing briefly work that has aimed to compare developmental sequences in gastropods. We demonstrate that work on gastropods has been at the forefront of investigations assessing the evolutionary importance of very early developmental stages but that, paradoxically, despite a voluminous literature documenting patterns for individual species, cross-species comparisons of events later in the developmental sequence appear to be rare. Finally, we present the findings of a recent study that begins, in a small way, to redress this imbalance.

Developmental sequences in gastropods

Cell lineage analysis

There is a long and distinguished history of studying cell lineages in gastropods, initiated by workers such as Blochmann (1882), Conklin (1897), and Delsman (1914) [see Raven (1958) and Fretter and Graham (1962) for summaries of this early work and Lindberg and Guralnick (2003) for a list of papers]. However, it was not until Hyman (1951) that the information from these pioneering studies alongside that on cell lineages for other spiralian taxa were used to propose a link between development and evolution; this reluctance to link ontogeny with phylogeny was almost certainly a reaction to the controversy surrounding recapitulation (Lindberg and Guralnick 2003).

In the past couple of decades, with the advent of sophisticated approaches for exploring phylogenetic relationships, gastropods have again been at the center of research linking early cell-cleavage patterns with evolution although it should be noted that many of these analyses draw on data from the studies at the turn of the nineteenth century (Freeman and Lundelius 1992, van den Biggelar 1993, van den Biggelar and Haszprunar 1996, Ponder and Lindberg 1997). In particular, there has been a focus on the timing of the formation of the 4d mesentoblast (the precursor of the mesoderm), with the relation of this timing to the cleavage of other cells being linked to the evolution of the major gastropod groups (van den Biggelar and Haszpruner 1996, Lindberg and Guralnick 2003). The main observation is that the onset of the 3d macromere division, which leads to the formation of the 4d mesentoblast, is accelerated through evolutionary time. Hence, in more derived gastropod groups such as the caenogastropods and heterobranchs, it occurs at the 24-cell stage compared with at the 63-cell stage in the stem gastropod taxa (*e.g.*, Patellogastropoda and Vestigastropoda). In effect, these studies demonstrate a heterochrony in the sequence of very early development, with a shift in the timing of developmental events between ancestral and descendent taxa.

A more recent analysis on more extensive cell-lineage data for more taxa (Lindberg and Guralinick 2003) confirmed that there was congruence between phylogenetic trees derived using cell lineages and those using morphological and molecular approaches. This study also identified a long branch within the cell-lineage phylogeny, indicating a large number of developmental event changes, between the Patellogastropoda/Vestigastropoda and Neritopsina/Apogastropoda clades. This evolutionary change again indicated an acceleration in development, a shortening of the trochophore stage, and an accompanying lengthening of the veliger stage. It was proposed that this shift towards the earlier development of a longer planktotrophic stage may have been a response to increased levels of primary production in the oceans during the Silurian period. In effect, this is a heterochronous change in the timing of developmental stage, and it is clear that the investigation of cell lineages in relation to gastropod evolution has led us full circle in terms of the important link between ontogeny and phylogeny.

Sequences in later developmental events

The evidence for an evolutionary role for development from extensive phylogenetic analysis of very early gastropod developmental events has not been extended to any great degree to the later stages of development. This is somewhat surprising, given the extent to which the developmental events of many gastropod species have been described in detail [for example see Raven (1958) and Fretter and Graham (1962)]. Indeed, the only substantial phylogenetic analysis on gastropod development has focused on an assessment of the evolution of developmental mode rather than developmental sequences *per se*. Collin (2004) mapped the developmental mode (of 72 calyptraeid gastropods) onto a phylogeny and found that there was no evidence that phylogenetic effects had constrained the evolution of this trait; species with planktotrophic, lecithotrophic, or direct development with

nurse eggs all had the potential to evolve a different developmental mode.

Other studies that focus more on developmental sequences are either qualitative or restricted in their comparative element. Page (1994), for example, provided an interesting qualitative comparison of the occurrence of eight developmental structures in opisthobranchs and prosobranchs. She concluded that young planktonic opisthobranch larvae represented a good approximation of an ancestral gastropod larva by not expressing many of the structures of the definitive body found early in prosobranch development (*i.e.*, at the veliger stage) until late in the larval phase. Gibson (2003), in contrast, found that one group of opisthobranchs, the Notospidea, possessed adult characters (notum differentiation, adult shell growth, lack of operculum) during the early larval stage. Collin and Wise (1997) included observations of early cell development in their investigation of development in the pyramidellid *Odostomia columbiana* Dall and Bartsch, 1907 and concluded that larvae with unequal cleavage and early development of eyes and tentacles might represent the common ancestors of pyramellids and opisthobranchs. At the same time, gastropods have been used by workers taking a functional approach to embryonic development, including studies of embryonic ionic balance (*e.g.*, Taylor 1977), calcification (*e.g.*, Bielefeld and Becker 1991), respiration (*e.g.*, Baldwin 1935), and muscle and nerve development (*e.g.*, Croll and Voronezhskaya 1996, Page 1998, Yamanaka *et al.* 2000).

Clearly, there is a lot of information on the later developmental stages of gastropod species both in terms of morphological and functional traits, including some evidence for differences in developmental sequences of these traits between species. More extensive, formal analysis of these data could shed much light on the role that developmental sequences have played in gastropod evolution; indeed, gastropods might provide particularly good models for integrated approaches that focus on functional as well as morphological aspects of development. In the next section, we describe a study that takes such a formal and integrative approach.

Case study: A quantitative comparison of developmental sequences in basommatophoran gastropods

So far, we have seen that there is an imbalance between cell lineage and later developmental characters when it comes to comparative approaches with developmental sequences in gastropods, with a lack of studies focusing on later development. In fact, the bulk of studies explicitly testing for differences in developmental sequences across phylogenies have been for mammals. In these examples, there is clear evidence for heterochrony in terms of altered *sequences* of developmental events between ancestors and descendents. There are, for example, differences in the timing of the central nervous system and the craniofacial apparatus between eutherians and marsupials (Smith 1997, Nunn and Smith 1998). Jeffery *et al.* (2002a) also demonstrated that, within the amniotes, mammals were characterized by delayed development of the eyes and that there were several heterochronies involving shifts in cardiac events relative to non-cardiac events. However, a more recent study suggested that the role of sequence heterochrony in mammals may not be that extensive; Bininda-Emonds *et al.* (2004) hypothesized that a lack of heterochronies in mammals might be expected due to the time during which the organo-genetic period occurs being spent in the protective environment of the amniotic egg. In many invertebrate groups, however, including many gastropods, embryonic development is external and, potentially, more open to evolutionary processes. Hence, we might predict that alterations to the developmental sequence might be more pronounced in such instances.

A recent study by Smirthwaite *et al.* (2007) took the first step towards exploring heterochrony in invertebrates, by comparing developmental sequences in three clades of basommatophoran snails. This work involved observing the precise timings of "traditional" morphological stages and functional/physiological traits in 12 species, mapping these events onto developmental time lines and then making a formal comparison of the relative timing of these events among species.

The morphological stages used were those that have been described previously by workers such as Raven (1958) and Cummin (1972) and included the trochophore, veliger, and hippo stages (Figs. 1A-C). It is important to note that it was the *onset* of these stages that were used as events. The physiological and functional events typically used occurred after these morphological stages (but see below) and included the formation of eyespots, heart, and radula, the onset of body flexing and contraction of mantle muscle, the time when the animal migrated from the egg capsule and, finally, the egg mass (Figs. 1-2).

Once event timings had been determined, timelines were constructed for each species, with that for *Lymnaea stagnalis* (Linnaeus, 1758) used as the "standard" (Fig. 2) and event-pair cracking (PARSIMOV approach—Jeffery *et al.* 2005) was used to test formally for heterochronies. In essence, this technique involves comparing the timing of each pair of events by classifying each event depending on whether it occurred earlier, simultaneously, or later than each other event in all other species. These scores are then mapped onto the phylogeny for the group.

This analysis provided formal support for several heterochronies, some of which are illustrated in Fig. 2. The lines on the first panel of this figure (*i.e.*, Fig. 2A) illustrate three traits (hippo, eyespot formation, and mantle muscle control) that do not change their timing relative to one another

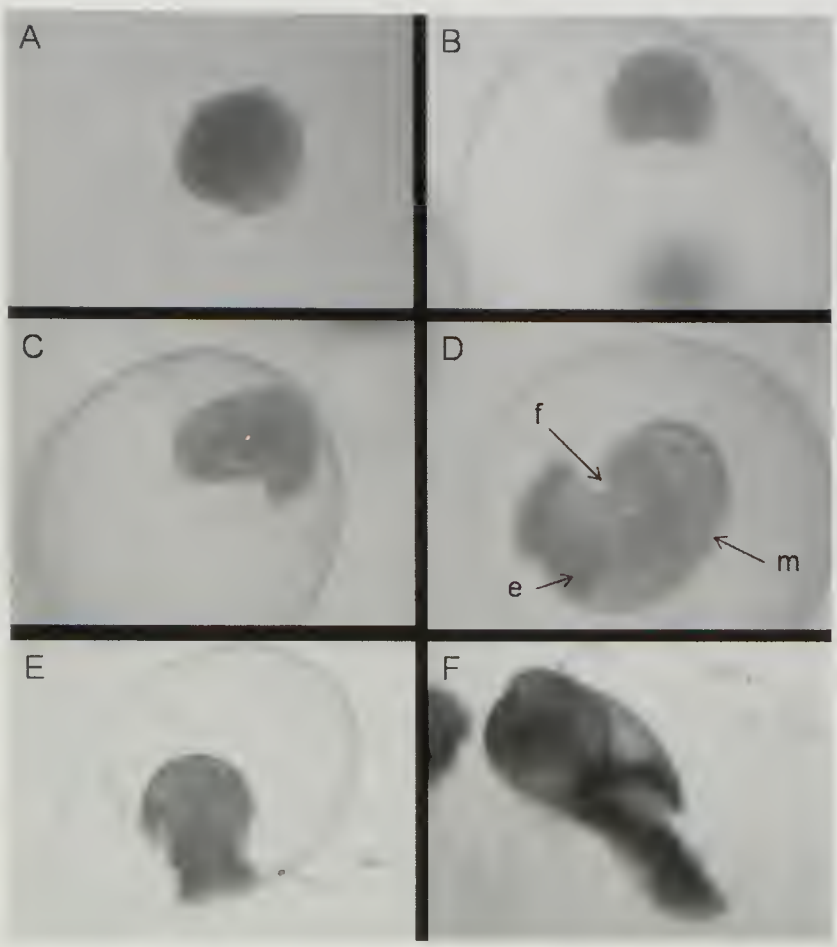

Figure 1. Developmental stages of *Lymnaea stagnalis* embryos: A, trochophore; B, veliger; C, early hippo; D, free-swimming hippo; E, crawling hippo; F, juvenile snail migrating from egg mass. Arrows and letter for free-swimming hippo indicate the location of the eye (e), body flexing (f), and mantle muscle contraction (m).

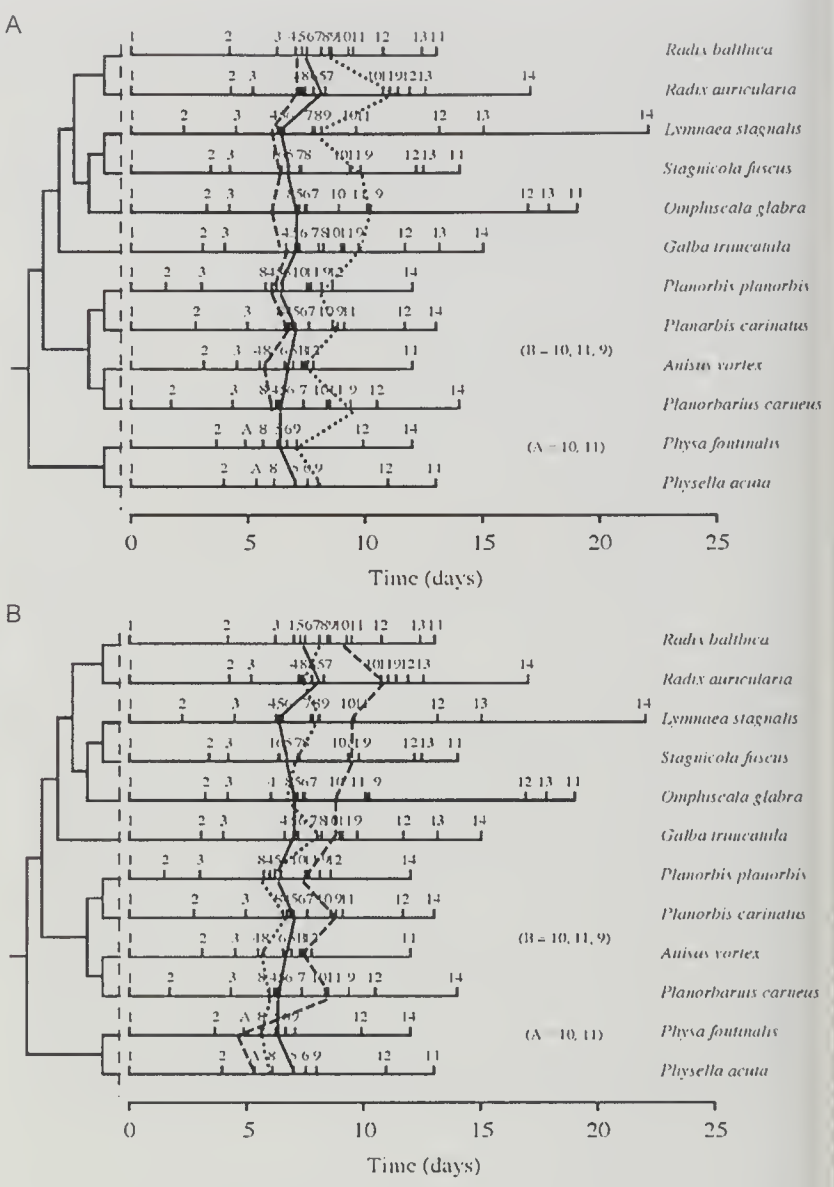

Figure 2. Sequences of events mapped on a time line for twelve species of basommatophoran snail; the three main clades represent (from top to bottom) the Lymnaeidae, Planorbidae, and Physidae. Event labeling (note: event timing was measured as the starting point for that event): 1, laying; 2, trochophore; 3, veliger; 4, hippo; 5, eyespot formed; 6, heart beat; 7, free swimming; 8, body flexing; 9, mantle muscle contraction; 10, attachment to egg; 11, crawling; 12, radula; 13, emergence from egg capsule; 14, migration from the egg mass. A, illustrates three non-heterochronous events, *i.e.*, which do not change their relative sequence across species: hippo, dashed line; eyespot formation, solid line; mantle muscle control, dotted line. B, illustrates sequence heterochronies among three events: in the two *Physa* species, attachment to the egg capsule (solid line) occurs earlier in development than body flexing (dotted line) and eyespot formation (dashed line) compared with in other species; body flexing (dotted line) occurs earlier in the Physidae, Planorbidae, and two species of Lymnaeidae (*Radix balthica* Linnaeus, 1758 and *Omphiscola glabra*) than in the other species within Lymnaeidae.

across species; hence, these traits are non-heterochronous. In contrast, the second panel (Fig. 2B) illustrates three traits that are heterochronous. One of these heterochronies involves the early occurrence of the embryo attaching to the egg capsule wall in relation to eyespot formation and body flexing in the two species of *Physa* Draparnaud, 1801 compared with all other species. Eyespot formation and body flexing were also heterochronous; within the Planorbidae and the Physidae, body flexing occurred before the eye was formed, whereas only two [*Radix auricularia* (Linnaeus, 1758) and *Omphiscola glabra* (O. F. Müller, 1774)] of the six species in the family Lymnaeidae showed this timing pattern. Hence, it appears that, within this clade of gastropods, heterochronies are associated with speciation events and larger taxonomic divergences at the family level. The number of events changing their position was also similar in relative terms to those identified for mammals (Bininda-Emonds *et al.* 2004).

Where can the investigation of developmental sequences in gastropods take us?

The study by Smirthwaite *et al.* (2007) provides evidence that sequence heterochrony occurs in a clade of gastropods, which suggests that, potentially, heterochrony make have played a part in the evolution of this group. However, we must be wary of assuming that, because we have demonstrated

heterochrony as essentially a *pattern* of evolutionary change, that the evolutionary mechanism must also involve heterochrony; there is no necessity for pattern and mechanism of evolutionary change both to be heterochronous (Spicer and Rundle 2007). Indeed, we would suggest that one of the big challenges for research on developmental sequences will be to establish whether there is a link between heterochronic process and heterochrony as a pattern (Spicer and Rundle 2007). Clearly, this approach will need to be guided by comparative studies such as that by Smirthwaite *et al.* (2007) that flag functional heterochronies between extant species, allowing investigations such as whether alterations to developmental sequences between species can be replicated within species. Such intraspecific changes in developmental sequence have been termed heterokairies (Spicer and Burggren 2003, Spicer and Rundle 2006, 2007) and their investigation should promote research on developmental sequences.

So might gastropods be a good model for future studies on the role of developmental sequences in evolution? We feel that the answer to this question is yes, for several reasons. First, the embryonic development of many gastropods is external and visible, which means that observations of early development and manipulations of the developmental environment are tractable. Second, phylogenies have been constructed for several groups and the relatedness of the major gastropod clades has also been formulated, allowing the explicit test of patterns in sequences within an evolutionary framework. Finally, the extensive information on cell lineages within the gastropods has meant that they have been one of the groups at the forefront of the development of techniques for generating cell-fate maps such as fluorescent stains used with confocal microscopy. Such techniques have already allowed some workers to trace developmental pathways from cell cleavage through to structures such as nerves, muscles, the mantle, and cilia within gastropod species (Render 1997, Hejnol *et al.* 2007, Wanninger *et al.* 2008). The emphasis so far has been in elucidating how major taxonomic divergences may be linked with different developmental pathways, yet it might be possible that species-level divergence might also be driven by small-scale variation in induction patterns during early cell divisions. Such variation might also be linked to later differences in the developmental sequence. Clearly, this is a highly speculative hypothesis but perhaps one that deserves consideration. At the same time, the ability to measure gene expression and up-regulation in gastropod embryos (Lartillot *et al.* 2002, Hinman *et al.* 2003) will also enhance our understanding of the genetic basis of developmental sequence change. Coupled with studies that assess fitness implications, there is real potential to take a truly integrated approach to the study of developmental sequences using gastropods (Spicer and Rundle 2006).

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Alien non-marine snails and slugs of priority quarantine importance in the United States: A preliminary risk assessment

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Abstract: In 2002, the U.S. Department of Agriculture requested assistance from the American Malacological Society in the development of a list of non-native snails and slugs of top national quarantine significance. From a review of the major pest snail and slug literature, together with our own experience, we developed a preliminary list of gastropod species displaying significant potential to damage natural ecosystems or agriculture, or human health or commerce, and either entirely absent from the United States to our knowledge or restricted to narrow areas of introduction. Comments on the list from the worldwide malacological community were then solicited and led us to modify the original list. We then evaluated the taxa on this list by ranking them according to 12 attributes—seven biological variables and five aspects of human interaction—based on thorough review of the detailed literature. The ranked list that emerged from this risk assessment process included 46 taxa (species or species-groups) in 18 families. The highest ranked taxa were in the Ampullariidae, Hygromiidae, Cochlicellidae, Helicidae, Veronicellidae, Succineidae, Achatinidae, and Planorbidae. We validated the risk assessment model by scoring a suite of non-native snail and slug species already present in the United States. The list is not definitive but rather is offered as a framework for additional research. There remain important gaps in biological knowledge of many of the taxa evaluated, and rigorous reporting of economic impacts is extremely limited. We expect the prioritizing and listing of taxa to be dynamic, not only as these knowledge gaps are filled but also as environmental, agricultural, international trade, and societal factors change.

Key words: Gastropoda, invasive species, life-history, natural ecosystems, pests

Alien species are being moved around the world at unprecedented rates as a result of the globalization of trade and the increased ability of people to travel widely. These alien species have serious impacts on agriculture, the natural environment, commerce, and human health and well-being (Bright 1998, Cox 1999, Mack *et al.* 2000, Staples and Cowie 2001), and these effects may be complex (Didham *et al.* 2007). In the United States, annual costs associated with damage to the environment and to agriculture caused by alien species have been most recently estimated as US$120 billion (Pimentel *et al.* 2005). Combined costs for the United States (Pimentel *et al.* 2000), the United Kingdom, Australia, South Africa, India, and Brazil have been estimated as US$314 billion per year (Pimentel *et al.* 2001). Although the level of uncertainty is high, these estimates indicate that the problem is severe.

While much attention is paid to invasive plants (*e.g.*, Gordon *et al.* 2008), insects (Simberloff 1986), and pathogens (Palm 2001), with some notable exceptions (*e.g.*, zebra mussels (*Dreissena polymorpha* (Pallas, 1771)): Britton and McMahon 2005; apple snails (*Pomacea* spp.): Hayes *et al.* 2008; New Zealand mud snails (*Potamopyrgus antipodarum* (Gray, 1853)): Kerans *et al.* 2005, Hall *et al.* 2006), molluscs receive relatively little attention (Keller *et al.* 2007). Nonetheless, invasive molluscs can have important impacts on agriculture (Godan 1983, Henderson 1989, 1996, Barker 2002a), biodiversity (Coote and Loève 2003, Lydeard *et al.* 2004), and human health (Madsen and Frandsen 1989, Pointier *et al.* 2005, Hollingsworth and Cowie 2006, Boaventura *et al.* 2007, Hollingsworth *et al.* 2007) and can become major public nuisances (Civeyrel and Simberloff 1996).

Quarantine measures to limit the spread of invasive species include pre-introduction screening of species to assess their potential for invasiveness (Ruesink *et al.* 1995). Formal systems of weed risk assessment have been put into regulatory use widely for plants (Gordon *et al.* 2008), driven in part by the continuing demands of the global horticulture trade to move many species to new localities, with the horticultural industry playing probably by far the most important role in the introduction of invasive plants (Dehnen-Schmutz *et al.* 2007). Similar science-based risk assessment protocols based on the guidelines of the International Plant Protection Convention (IPPC) have been developed by Australia, New Zealand, and

other countries for other major groups of organisms. There have been many assessments of individual species of concern (*e.g.*, Ruesink *et al.* 1995) and many jurisdictions have lists of prohibited species, but for the most part these have not been developed by applying objective, science-based, standardized protocols. Some countries have nascent protocols but have yet to implement them widely (*e.g.*, Mito and Uesugi 2004, Gederaas *et al.* 2007).

Many studies of various animal and plant groups, reviewed by Kolar and Lodge (2001) and Hayes and Barry (2008), have attempted to develop formal screening protocols by assessing potential risk based on suites of characters thought *a priori* to correlate with invasiveness, *e.g.*, in fish (Kolar and Lodge 2002), birds (Veltman *et al.* 1996, Duncan *et al.* 2001), and reptiles and amphibians (Bomford *et al.* 2008). The goals of such screening systems are primarily to provide an objective means of analyzing the legal, deliberate import of alien species. But they could also be used to allocate special attention to the interception of species transported inadvertently that are potentially invasive. However, increasingly it is being suggested that any species-level characteristics that might identify successful invaders are both taxon and location specific (Sakai *et al.* 2001, Hayes and Barry 2008), and general approaches to risk analysis of potential invasive species remain challenging (Stohlgren and Schnase 2006).

With some notable exceptions, most alien snails and slugs are transported inadvertently (Cowie and Robinson 2003). Quarantine agencies around the world routinely intercept numerous species of snails and slugs. Robinson (1999) listed those that were intercepted by U. S. quarantine officials between 1993 and 1998. The purpose of the present study was, on behalf of the American Malacological Society (AMS) and at the request of the U. S. Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine (USDA-APHIS-PPQ), to develop a much shorter list of the snail and slug species considered as top priority for prevention of their introduction and establishment in the United States. This list would then be used by USDA-APHIS-PPQ officials as a list of species of quarantine importance to the United States and upon which to focus their attention. A preliminary version of the list (Cowie 2002a) was submitted to the USDA; the present paper is a revised version based on further analysis and more extensive review of the literature.

MATERIALS AND METHODS

Scope

Species to be considered were species not present in the United States or, if present, only distributed highly locally and with the possibility of eradication or at least containment. A number of species found only in Hawaii, although widespread there, were considered containable with respect to invasion of the remainder of the United States and were therefore included. Only species falling under the jurisdiction of USDA-APHIS-PPQ were included, that is, pest species with the potential to cause damage to either agriculture or natural ecosystems. Marine species (the responsibility of the National Marine Fisheries Service) were excluded, as were species only affecting endangered species (the responsibility of the U. S. Fish and Wildlife Service). However, we treated these constraints fairly broadly because they are often inter-related and considered pest problems in four areas: agriculture (including livestock health), environment, human health, and commerce.

Initially, the charge from the USDA was to generate a list of 15 species, selected and prioritized using an explicit protocol. It soon became clear that a simple list of 15 species would not serve the interests of USDA-APHIS-PPQ adequately, for the following reasons. (1) Most snail and slug species are generalist herbivores. They do not in general exhibit the kind of precise host-specificity exhibited, for instance, by many of the insect pests upon which PPQ focuses greater attention. Congeners (and even less closely related species) are therefore likely to have similar feeding habits, and listing just one species would exclude other, related species that may not differ markedly in pest potential. (2) Detailed information regarding species-level differences in feeding preferences among related species is available for few taxa. Therefore, listing one and not others of a number of species in a group (*e.g.*, a genus) might again divert attention away from potential pests. (3) Distinguishing closely related species is difficult even for experts in the group and would be impossible for PPQ field personnel without extensive training, except in certain clear cases. (4) Limiting the list to just 15 species could result in a focus on only a few taxonomic groups that include multiple species considered potential pests while omitting species in other groups that might be equally problematic but for which information was limited. Conversely, selecting 15 well-known species from a range of larger groups might also have meant omitting other species in those groups that were potential pests. For these reasons, we decided to create a prioritized list of larger taxonomic groups (families) with a number of known or potential pests considered within each.

Development of an initial unranked list

Focusing primarily on species intercepted by USDA-APHIS-PPQ (Robinson 1999; D. G. Robinson, unpubl. data), we developed a preliminary list by scanning the literature on mollusc pests worldwide, including primarily Godan (1983), Henderson (1989, 1996), Barker (2002a), augmented by our own knowledge. Some well-known pests were immediately excluded from the list because they were already widely distributed in the United States, *e.g.*, *Deroceras reticulatum*

(Müller, 1774) (Barker 2002a), *Cornu aspersum* (Müller, 1774) (Dundee 1974, Roth and Sadeghian 2003). Other less well-known taxa were evaluated provisionally but omitted from the list, including, notably, the following.

Bradybaena similaris (Rang, 1831) (Bradybaenidae). This species is probably already too widespread in the United States, occurring in much of the southeast (Dundee 1974).

Otala lactea (Müller, 1774) (Helicidae). This species is a minor plant pest but is probably already too widespread in the United States, as it is known from southeastern states, Arizona, and a number of counties in California (Roth and Sadeghian 2003).

Theba Risso, 1826 (Helicidae). *Theba pisana* (Müller, 1774) is a serious pest (Baker 1989, 1991, 2002, Coupland 1996), currently confined to a small number of localities in southern California (Roth and Sadeghian 2003), and is included in the list. However, no other species in the genus appears to have pest potential as none is referred to in the pest snail literature.

Trochulus Chemnitz, 1786 (Hygromiidae). There is no clear evidence that these species have pest potential (D. G. Robinson, unpubl. data) and they are not mentioned widely in the pest snail literature.

Xerotricha Monterosato, 1892 (Hygromiidae). *Xerotricha conspurcata* (Draparnaud, 1801) is established in four or five counties in the San Francisco Bay area, and although USDA-APHIS-PPQ still takes action on it when intercepted, the agency decided some years ago not to address these infestations. We therefore excluded it and other *Xerotricha* spp. from our analyses.

Milax gagates (Draparnaud, 1801) (Milacidae). This species is a major pest in Europe and elsewhere (Barker 2002a) but is already probably too widespread in the United States, occurring in much of eastern North America, the Pacific Northwest, and California (Pilsbry 1948, Roth and Sadeghian 2003).

Gonaxis Taylor, 1877 (Streptaxidae). At least two species of *Gonaxis* have been introduced to Hawaii as putative biocontrol agents for *Achatina fulica* Bowdich, 1822 (Cowie 1997). However, although they have been implicated in the decline of native snail species, there is no evidence that they are a serious problem on the scale of that caused by the better known predator *Englandina rosea* (Férussac, 1821) (Cowie 2001a). They are not listed by Robinson (1999) as having been intercepted and there is no intention of introducing them deliberately to the mainland United States.

Subulinidae. Too little is known of the pest potential of subulinids; they are rarely mentioned in the pest literature; and a number of species are already widespread in the United States (Robinson and Slapcinsky 2005).

Belocaulus angustipes (Heynemann, 1885) (Veronicellidae). This slug may not be important as a major plant pest but is known as a disease vector (Rueda *et al.* 2002), although it is probably already too widespread in the United States (D. G. Robinson, unpubl. data).

Aegopinella nitidula (Draparnaud, 1805) (Zonitidae). This small European land snail has been reported in British Columbia, with the suggestion that it could affect the native land snail fauna through predation (Forsyth *et al.* 2001). However, there is no evidence of this and it is not listed by Robinson (1999) as having been intercepted.

Pomacea diffusa Blume, 1957 (Ampullariidae). We include all other species of *Pomacea* Perry, 1810, but this species, which is often referred to incorrectly as *Pomacea bridgesii* (Reeve, 1856) (Rawlings *et al.* 2007), has been considered a microherbivore (feeding on algae) (Howells 2002) and therefore not a potential pest, although its food preferences may be wider (Aditya and Raut 2001). It is also widely used as a domestic aquarium snail. Regulatory changes have banned live *Pomacea* spp., with the exception of *P. bridgesii* (*i.e.*, *P. diffusa*), from any United States trade.

Potamopyrgus antipodarum (Gray, 1853) (Hydrobiidae). This freshwater species may outcompete native species and change stream ecology but is probably already too widespread in the United States to be eradicated or contained, having been found in ten western states, as well as in the Great Lakes (Kerans *et al.* 2005, Hall *et al.* 2006, Bersine *et al.* 2008).

Thiaridae. Within this freshwater family, the two most invasive species, *Melanoides tuberculata* (Müller, 1774) and *Tarebia granifera* (Lamarck, 1816), are already too widespread in the United States, the former having been reported from at least 15 states, the latter from seven (Dundee and Paine 1977, Burch and Tottenham 1980, Mitchell *et al.* 2007, NatureServe 2008).

Triculinae (Pomatiopsidae). Some of these freshwater taxa transmit *Schistosoma* and most triculines can transmit *Paragonimus*, helminth parasites infecting people (Davis *et al.* 1999). However, none of them is a threat, as their ecological requirements probably cannot be met in the United States (G. M. Davis, pers. comm.).

Consultation with the malacological community

Having developed a preliminary version of this list we disseminated it widely over the Internet, primarily through the MOLLUSCA listserver, with an explanation of the purpose of the project and a request for comments and suggestions of additional or alternative species to include on it. The MOLLUSCA listserver has approximately 1,000 members throughout the world. The message was also sent to the AMS membership of about 340 malacologists although many of these are also subscribers to MOLLUSCA. Responses were received from over 20 people. The first author also presented a talk at the 2002 annual meeting of the AMS, outlining the progress of the project and again requesting

input. A number of helpful comments were made by various conference attendees. All these comments were considered when developing the final prioritized list.

Scoring taxa and prioritizing the list

Following this consultation phase we evaluated each of the species or species-groups in the list according to 12 non-exclusive attributes that are generally thought to correlate with a species' invasiveness and that seemed particularly pertinent to non-marine molluscs (*e.g.*, Veltman *et al.* 1996, Goodwin *et al.* 1999, Lockwood 1999, Duncan *et al.* 2001, Kolar and Lodge 2001, Sakai *et al.* 2001, Daehler *et al.* 2004, Leung *et al.* 2004, Marchetti *et al.* 2004, Theoharides and Dukes 2007, Alonso and Castro-Díez 2008, Bomford *et al.* 2008, Hayes and Barry 2008). Our evaluations were based on information obtained via a thorough search of the literature.

Species and species groups were scored by giving them a '1' if the data suggested that an attribute would enhance their pest potential and a '0' if the data suggested it would not do so. If an attribute was mixed or would enhance pest potential only somewhat, we scored it as '0.5', and if the data were insufficient, we did not assign a score. We were conservative in using 0.5 or not assigning a score if there was any question about giving 1 or 0.

For each species or group we summed the scores to obtain S, a simple measure of the pest potential of each species or group. This measure, however, downplays a species' pest potential when fewer attributes can be scored (*i.e.*, when we had less knowledge). We therefore also divided each value of S by the total number of attributes scored, to obtain P, a proportional measure of pest potential not influenced by the number of scores, and ranging from 0 to 1, least to greatest concern. The species/groups were then ranked from highest to lowest based on the values of S and P.

The attributes scored included both biological attributes of the species and attributes related to their interaction with people. The biological attributes evaluated were as follows.

Range. If a species has a wide natural climatic range, it could invade a larger area within the United States. For example, among the Ampullariidae, one or more species of *Pomacea* occur from temperate Argentina to the Amazon basin and have the potential to spread widely in the United States (scored as 1), contrasting with the two species of *Marisa* (scored as 0), which are more restricted in South America and thus probably less likely to become widespread in the United States (Rawlings *et al.* 2007, Hayes *et al.* 2008). Similarly, among Helicidae, *Otala punctata* is confined to the western Mediterranean, primarily close to the coast, a limited climatic range (scored as 0), whereas *Theba pisana* occurs from the southwest of the British Isles to the eastern Mediterranean, a much wider geographic span, but nonetheless almost exclusively close to the coast (Cowie 1990), and therefore *T. pisana* was scored as 0.5 rather than 1. The extent of the natural ranges of some species has been confounded by human-mediated spread, *e.g. Archachatina marginata* and *Achatina fulica* (Raut and Barker 2002), or by misidentification, *e.g.*, *Achatina achatina* (Bequaert 1950), and are probably smaller than sometimes supposed. Nevertheless, *A. fulica* may have the potential to spread widely within the United States (Smith 2005). Ranges were determined by scanning the literature, web sites, and from our personal knowledge. Detailed data for many species are unavailable, and while those with very wide or very narrow ranges are easy to assess, others are more difficult. Our scoring of range size was thus in some cases somewhat subjective.

Phylogenetic relationships. If a species is closely related to known pests (pest status assessed below), the likelihood of it becoming a pest is greater (Hayes *et al.* 2008, examples in Barker 2002a). We scored taxa as 1 if in the same or a very closely related genus as a known serious pest, 0.5 if in a less closely related genus or in the same or a very closely related genus as a less serious pest, and 0 if more distantly related to any known pest. Species known themselves to be serious pests were scored as 1.

Adult size. Larger species are favored for deliberate introductions (Mead 1979, Smith 2005, Thiengo *et al.* 2007) but for inadvertent introductions smaller species have a greater chance of evading quarantine (Cowie and Robinson 2003). For species we knew to be introduced predominantly deliberately, we scored large size (maximum shell dimension of snails and maximum extended length of slugs roughly >2 cm) as increasing invasive potential (1), whereas for species introduced primarily accidentally we scored small size (roughly <1 cm) as increasing invasive potential. Deliberately introduced taxa <1 cm and accidentally introduced taxa >2 cm were scored as 0. Intermediate-sized snails (1-2 cm), regardless of mode of introduction, were scored as 0.5. Assessments were based on information from basic field guides and the taxonomic literature, augmented by our knowledge of probable modes of introduction (*e.g.*, Cowie 1998a, Cowie and Robinson 2003).

Egg/juvenile size. Production of smaller and therefore more readily dispersed offspring could lead to a species' more rapid and wider dispersal once introduced (cf. Vagvolgyi 1975, Paulay and Meyer 2002). Egg size is reflected by hatchling size and is broadly correlated with adult size (Heller 2001). Heller (2001) tabulated known egg sizes for terrestrial species and we augmented those data with information for additional species from other published sources: Barrientos (1998) (*Ovachlamys fulgens*); Staikou and Lazaridou-Dimitriadou (1991) (*Xeropicta*); Thompson (1957) (*Euglandina*); Turner and McCabe (1990) and Barnes *et al.* (2008) (*Pomacea*); Liang (1974), Liang and van der Schalie (1975), O'Keeffe (1985), Parashar *et al.* (1986), Raut *et al.* (1992), and Saha (1993)

(Planorbidae); Chi and Wagner (1962) (*Oncomelania*). For a few taxa we relied on our personal experience (*Otala punctata*, *Cochlicella* spp., *Succinea tenella*), and for one, *Limicolaria aurora*, on data for a congeneric (Ergonmwan 2007). We could not find information for other species. We scored eggs <3 mm in diameter as small (1), those >7 mm as large (0), and those between these sizes as intermediate (0.5). Heller (2001) gave ranges of sizes for some species and we have combined some species into groups (*e.g.*, *Pomacea*, *Helix*) for our analyses. Thus, for the few taxa in which egg size data straddled these categories, we were conservative and scored them as 0.5.

Reproductive potential. In general, larger snails produce more eggs over their lifetime (Heller 2001) although there is great variation in both longevity and productivity among species. However, if a species produces large numbers of young in a short period of time, *e.g.*, an annual reproductive season, the chances of it being more invasive may be greater (Keller *et al.* 2007). Annual productivity data were obtained from: Hodasi (1979) (*Achatina achatina*); Raut and Barker (2002) (*Achatina fulica*); Plummer (1975) (*Archachatina marginata*); Barrientos (1998) (*Ovachlamys fulgens*); Cowie (1984) and Baker (1991) (*Theba pisana*, *Cernuella virgata* (da Costa, 1778)); Baur and Raboud (1988) (*Arianta arbustorum*); Lazaridou and Chatziioannou (2005) (*Xerolenta obvia*); Baker and Hawke (1991) (*Cochlicella acuta*); Rueda *et al.* (2002) (*Sarasinula plebeia*, *Leidyula moreleti*); Cowie (2002b) (*Pomacea*); Keller *et al.* (2007) (*Marisa cornuarietis*, *Biomphalaria glabrata*); Dillon (2000; annualized from data in his table 4.1) (*Biomphalaria*, *Bulinus*). For *Limicolaria aurora* we used data from a congeneric (Ergonmwan 2007). We scored mean per snail annual production of >1,000 eggs as 1, of 500-1,000 eggs as 0.5, and of <500 eggs as 0. In some cases productivity appears highly variable among regions, straddling categories (*e.g.*, *Achatina fulica*; Raut and Barker 2002); we scored these as 0.5.

Semelparous or iteroparous. Semelparous species put all their reproductive effort into a single reproductive event (or season), a life-history trade-off that results in a shortened life-cycle. Semelparity is probably correlated with high reproductive potential so semelparous species may be more invasive than iteroparous species (Dillon 2000, Heller 2001, Barker 2002b). We treated species with an annual (or shorter) life-cycle as semelparous, scoring them as 1. Other species were scored as semelparous if they breed only during one season before dying, regardless of their overall life-cycle, which may be biennial or longer (Heller 2001). Iteroparous species, including some that reproduce more or less continuously over multiple years (Dillon 2000), were scored as 0. We based our scores on the following: Raut and Barker (2002) (Achatinidae); Txurruka *et al.* (1996) (*Arion ater*); South (1992) (Arionidae, *Tandonia budapestensis*); Barrientos (1998) (*Ovachlamys fulgens*); Baur and Raboud (1988) (*Arianta arbustorum*); Cowie (1984), Baker (1989, 1991, 2002), and Baker *et al.* (1991) (*Theba pisana*, *Cernuella virgata*, *Cochlicella* spp.); Heller (2001), Staikou *et al.* (1988), and Staikou and Lazaridou-Dimitriadou (1991) (*Helix*, *Xeropicta*); Lazaridou and Chatziioannou (2005) (*Xerolenta obvia*); Barker (2002b) (*Tandonia sowerbii*); Cowie (2002b) (Ampullariidae); Dazo *et al.* (1966), Sturrock (1973), and Loreau and Baluku (1987) (*Biomphalaria*, *Bulinus*); Yapi *et al.* (1994) (*Indoplanorbis exustus*); Remais *et al.* (2007) (*Oncomelania*). *Eobania vermiculata* is "marginally iteroparous", with most individuals reproducing only once but a significant number reproducing for at least one additional season (Lazaridou-Dimitriadou and Kattoulas 1991); we scored it as 0.5. In some cases we generalized from information for one or a few species, *e.g.*, *Xeropicta* in our list: information for *Xeropicta vestalis* (Pfeiffer, 1841) (Heller 2001) and *Xeropicta derbentina* (Krynicki, 1836) (Staikou and Lazaridou-Dimitriadou 1991, Kiss *et al.* 2005).

Breeding system. Selfing or parthenogenetic rather than outcrossing species may be better invaders (Foltz *et al.* 1984, Baur and Bengtsson 1987, Dybdahl and Kane 2005). All ampullariids and pomatiopsids were scored as outcrossing (0) as they have separate sexes and no records of parthenogenesis (Dillon 2000, Cowie 2002b). All other species on the list are hermaphrodites. None exhibits parthenogenesis (Jordaens *et al.* 2007), but selfing may occur to a greater or lesser degree in most species, along something of a continuum of strategies. Many normally outcrossing species may self under rare circumstances, especially if kept in isolation (Duncan 1975), though usually producing eggs/young at a very much reduced rate. For example, achatinids, helicids, and hygromiids are generally considered obligate outcrossers (*e.g.*, Duncan 1975, Barker 1999, Raut and Barker 2002) although limited selfing may be possible (*e.g.*, *Arianta arbustorum*; Heller 2001); all were scored as outcrossing. *Arion lusitanicus* is predominantly, if not exclusively, outcrossing (Foltz *et al.* 1982). Some species adopt either strategy although in some cases selfing only in isolation, *e.g.*, *Arion ater* (Foltz *et al.* 1982), *Sarasinula plebeia* (Rueda *et al.* 2002) and *Laevicaulis alte* (Duncan 1975); they were scored as 0.5. Most planorbids are capable of outcrossing and selfing although preference for one mode or the other differs among species (Jarne *et al.* 1993, Dillon 2000, Jordaens *et al.* 2007). Even in a preferential outcrosser, *Biomphalaria glabrata* (Say 1818), there is little loss in productivity when forced to self (Paraense 1959). However, planorbids were scored as 0.5, since although the potential in some species to self without loss of fecundity is equivalent, from the current perspective, to being selfers, it is not known how widely this applies in the taxa considered. The capacity to self is widespread in Succineidae, but whether important in natural situations and in our listed taxa is not known (Barker 2001); they were not assigned a score. *Ovachlamys fulgens* selfs readily with no loss of fecundity and this may be the predominant mode (Barrientos 1998), as it is

in *Tandonia budapestensis* and *Tandonia sowerbii* (Foltz *et al.* 1984); these were scored as 1.

The human-interaction attributes evaluated were as follows.

Introduction pressure. Frequent interception implies higher introduction pressure and hence greater likelihood of establishment (Cowie and Robinson 2003). Species listed by Robinson (1999: table 3) were the species most commonly intercepted by USDA-APHIS-PPQ during 1993-1998; those on our list we scored as 1. Robinson (1999) also mentioned *Helix pomatia*, *Cantareus apertus*, *Achatina* spp., and *Archachatina marginata* as being frequently intercepted; they also were scored as 1. Others scored as 1 include *Xeropicta* spp., based on Kiss *et al.* (2005, reporting on *Xeropicta derbentina*), *Succinea tenella*, based on Cowie *et al.* (2008), *Pomacea* spp. and *Marisa* spp. because of their worldwide popularity in the aquarium trade (Rawlings *et al.* 2007, Hayes *et al.* 2008), and a number of taxa based on data (D. G. Robinson, unpubl. data) accumulated since 1998 (Robinson 1999). We scored other species as 0.5 if they were listed by Godan (1983) or Robinson (1999) as having been intercepted entering the United States or Canada. Others were scored as 0, and no score was assigned if we were unsure of their introduction pressure.

Invasion history. Invasiveness elsewhere in the world suggests a greater likelihood of becoming invasive in the United States. Species known to be invasive (as opposed to simply recorded as present, *e.g.*, *Macrochlamys indica*: Robinson 1999; Barker and Efford 2004) elsewhere in the world (including Hawaii, as being distinct from the continental United States) were scored as 1 based on the literature, including the following: Mead (1979), Raut and Barker (2002), Smith (2005), and Thiengo *et al.* (2007) (*Achatina fulica*); Grimm (2001) and Shoaib and Cagáň (2004) (*Arion lusitanicus*, *Xerolenta obvia*); Hollingsworth *et al.* (2007) (*Parmarion martensi*); Robinson and Fields (2004) (*Zachrysia provisoria*); Robinson (1999) and Cowie *et al.* (2008) (*Ovachlamys fulgens*); Baker (1989, 2002, 2008) (*Theba pisana*, *Cernuella virgata*, *Cochlicella* spp.); Kiss *et al.* (2005) (*Xeropicta*); Barker (1999, 2002a) (*Tandonia budapestensis*, *T. sowerbii*); Cowie *et al.* (2008) (*Succinea tenella*); Cowie (1998b) and Cowie *et al.* (2008) (*Laevicaulis alte*, *Sarasinula plebeia*, *Veronicella cubensis*); Cowie (2002b), Rawlings *et al.* (2007), and Hayes *et al.* (2008) (*Pomacea* spp.); Coelho da Silva *et al.* (1997), Pointier *et al.* (2005), Majoros *et al.* (2008) (*Indoplanorbis exustus*, *Biomphalaria* spp., with *Bulinus* spp. explicitly not considered invasive). *Pila* was scored as 0.5 on the basis of its localized but serious invasive status on one of the Hawaiian Islands (Tran *et al.* 2008), as was *Limicolaria aurora* because of its invasive status in Martinique (Raut and Barker 2002). *Cantareus apertus*, a Mediterranean species, is invasive in southern Germany (Godan 1983); *Eobania vermiculata*, another Mediterranean species, is locally established in California (Roth and Sadeghian 2003) and Japan (Ueshima *et al.* 2004), and may be invasive; *Tandonia rustica*, a central European species is arguably invasive in Western Europe, where it is widespread (*e.g.*, Philp 1987); all were scored as 0.5. Species that appeared not to have become invasive anywhere or that were explicitly stated to be only minimally invasive, were scored as 0. Species for which we were unsure were not scored.

Major pest elsewhere. If a species is a major pest elsewhere of a crop grown in the United States, or causes other major problems elsewhere (*e.g.*, environmental damage, human disease), there is a greater likelihood that it will cause serious problems in the United States. Species scored as having a history of invasion (above) are often considered invasive on the basis of being major pests where introduced. The two attributes are closely linked. Some species, however, lack an extensive history of invasion but are pests (perhaps relatively minor pests) within their native ranges (*e.g.*, *Arion ater*, *Mariaella dussumieri*). Many, if not most, snails and slugs can act as intermediate hosts of human and livestock parasites (Godan 1983, Grewal *et al.* 2003). Assessment of whether a species causes sufficient problems to be categorized as a major pest is somewhat subjective. We have been conservative in scoring as such only those taxa that are explicitly referred to in the literature as causing substantial problems. Many species have been reported as pests although many of them may cause little loss. Numerous crops have been listed as susceptible to damage by certain species but with no indication of the severity of the problem (*e.g.*, Raut and Barker 2002: table 3.1). And some species have been reported as pests but only on the basis of occasionally being found in association with a particular crop, as we suspect is the case for many of the instances listed by Godan (1983). Our assessments were based primarily on the following: Raut and Barker (2002) (Achatinidae); Frank (1996) and Grimm (2001) (*Arion lusitanicus*); Godan (1983), South (1992), and Barker (2002b) (Arionidae, Milacidae); Kumar and Ahmed (2000) (*Macrochlamys indica*); Godan (1983) (*Mariaella dussumieri*, *Parmarion martensi*); Hollingsworth *et al.* (2007) (*Parmarion martensi*); Robinson and Fields (2004) (*Zachrysia* spp.); Sanderson and Sirgel (2002) (*Theba pisana*); Godan (1983) (*Helix*, *Arianta* [as '*Helicigona*'] *arbustorum*, *Cantareus apertus*, *Eobania vermiculata*, *Otala punctata*, *Xerolenta obvia*); Baker (1989, 2002, 2008) and Coupland (1996) (*Theba pisana*, *Cernuella virgata*, *Cochlicella* spp.); Kiss *et al.* (2005) (*Xeropicta*); Cowie *et al.* (2008) (*Succinea tenella*); de Jager and Daneel (2002) (*Elisolimax flavescens*); Godan (1983), Raut (1996), Hata *et al.* (1997), Rueda *et al.* (2002), Fields and Robinson (2004), USDA-APHIS-PPQ (2006), Hollingsworth *et al.* (2007), Naranjo-García *et al.* (2007), and Cowie *et al.* (2008) (Veronicellidae); Stange (2006) (*Zachrysia provisoria*, *Ovachlamys fulgens*, *Veronicella sloanii*); Cowie (2002b), Joshi and Sebastian (2006), and

Rawlings *et al.* (2007) (Ampullariidae); Stevens (2002) and Pointier *et al.* (2005) (Planorbidae); Davis *et al.* (1999) (*Oncomelania*).

A "multi-pest". The severity of the problems caused has been scored above, according to whether a species is a major pest. Here we score species as 1 if they cause problems in more than one of agriculture (including livestock health), environment, human health, and commerce, regardless of degree. Thus, for example, *Achatina fulica* is not only a serious plant pest but also an important vector of parasitic diseases, as well as a major public nuisance (Mead 1979, Civeyrel and Simberloff 1996, Raut and Barker 2002, Smith 2005, Thiengo *et al.* 2007); *Veronicella cubensis* is an important parasitic disease vector (Hollingsworth *et al.* 2007) as well as an agricultural and garden pest; *Pomacea* spp. are major crop pests (Cowie 2002b, Joshi and Sebastian 2006) and important parasite vectors (Hollingsworth and Cowie 2006). Other species may cause serious problems in one area but only minor problems in another. For instance, *Parmarion martensi* is a plant pest in Malaysia (Godan 1983) and a possibly serious human disease vector in Hawaii (Hollingsworth *et al.* 2007). Other taxa cause problems in more than one area but they are not severe in either. For example, *Arion ater* is a minor crop pest and also causes environmental damage by feeding on young tree seedlings (South 1992); *Pila* spp. are local or minor crop pests (Cowie 2002b, Levin *et al.* 2006) and recognized parasite vectors (Hollingsworth and Cowie 2006), as are *Indoplanorbis exustus* (Stevens 2002), *Thelidomus aspera* (Lindo *et al.* 2002), and *Diplosolenodes occidentalis* (Rueda *et al.* 2002); *Laevicaulis alte* is a disease vector, although not as important as *Veronicella cubensis* or *Parmarion martensi* (Hollingsworth *et al.* (2007), and a relatively minor plant pest (Raut 1996). All were scored as 1.

Economic potential. We evaluated whether the problems a species could cause would be likely to result in major economic loss in the United States, including costs of control or eradication. This attribute overlaps with the attribute of being a major pest elsewhere, but is explicitly focused on economic cost. Our evaluation was based on the likelihood of the taxon becoming widespread in the United States and on either quantified assessments of costs in other regions, *e.g.*, Baker (1989) (*Cernuella virgata*, *Cochlicella* spp., *Theba pisana*), Andrews (1989) (*Sarasinula plebeia*), Cheng (1989), Naylor (1996), and Levin *et al.* (2006) (*Pomacea*), or unquantified statements of the pest's economic importance, *e.g.*, Mead (1979) and Raut and Barker (2002) (*Achatina fulica*), Frank (1996) and Grimm (2001) (*Arion lusitanicus*), South (1992) (*Tandonia budapestensis*), de Jager and Daneel (2002) (*Elisolimax flavescens*). If we found no report in the literature explicitly indicating major economic costs or only highly localized costs, or found explicit statements that a species/group was not a major economic problem we scored it as 0, *e.g.*, *Archachatina marginata* and *Limicolaria aurora* (Raut and Barker 2002). Others, for which the economic literature was limited or equivocal, or for which we considered the potential economic costs unlikely to be widespread were scored as 0.5, *e.g.*, *Achatina achatina* (Raut and Barker 2002), *Zachrysia provisoria* (Robinson and Fields 2004), *Ovachlamys fulgens* (Stange 2006, Cowie *et al.* 2008), *Tandonia* spp. (South 1992), *Veronicella cubensis* (USDA-APHIS-PPQ 2006), *Veronicella sloanii* (Stange 2006), *Pila* spp. (Cowie 2002b, Levin *et al.* 2006).

Validating the model

We assessed the appropriateness of the model by scoring the following representative suite of species that have already been introduced to the United States and determining whether it would accurately predict their invasion status. We excluded information from the United States when scoring the species' attributes, to avoid circularity. We selected a non-random sample of taxa that (1) have been subject to relatively substantial amounts of research in the United States, so that there is an appropriate level of knowledge of their distributions and impacts, (2) are already widespread in the United States, and (3) represent a range of impacts. Scores of attributes were obtained as follows.

Deroceras reticulatum (Müller, 1774) (terrestrial slug, Agriolimacidae): native range (Kerney and Cameron 1979, Barker 1999), adult size (Kerney and Cameron 1979), egg size, reproductive potential, and semelparity/iteroparity (Heller 2001), breeding system (Foltz *et al.* 1984), introduction pressure (Robinson 1999), invasion history (Barker 1999), pest status, and economic damage (Barker 2002a).

Cepaea nemoralis (Linnaeus, 1758) (terrestrial snail, Helicidae): native range, adult size, and invasion history (Kerney and Cameron 1979), phylogenetic relationships (scored as 0.5 since *Cepaea* is somewhat closely related to *Cornu*), egg size (Heller 2001), reproductive potential and semelparity/iteroparity (Cowie 1984), breeding system (helicids in general are outcrossers: Duncan 1975), introduction pressure (Robinson 1999), pest status, and economic damage (Godan 1983, Henderson 1989, 1996, Barker 2002a).

Cornu aspersum (Müller, 1774) (terrestrial snail, Helicidae): native range and adult size (scored as 0.5 because it is probably introduced both deliberately and accidentally: Barker 1999) (Kerney and Cameron 1979), egg size (Heller 2001), reproductive potential (Desbuquois *et al.* 2000), semelparity/iteroparity (R. H. Cowie, pers. obs.), breeding system (Selander and Hudson 1976), introduction pressure (Robinson 1999), invasion history (Barker 1999), pest status, and economic damage (Godan 1983, Sanderson and Sirgel 2002).

Potamopyrgus antipodarum (Gray, 1853) (freshwater snail, Hydrobiidae): native range, adult size, reproductive potential, breeding system, invasion history (Alonso and Castro-Díez 2008, Radea *et al.* 2008), juvenile size (Radea *et al.*

2008), semelparity/iteroparity (Winterbourn 1970), invasion pressure (Robinson 1999, Alonso and Castro-Díez 2008), and pest status (Alonso and Castro-Díez 2008, Holomuzi and Biggs 1999).

Milax gagates (Draparnaud, 1801) (terrestrial slug, Milacidae): native range and adult size (Kerney and Cameron 1979), egg size (Heller 2001), semelparity/iteroparity (South 1992), breeding system (Foltz *et al.* 1984), introduction pressure (Robinson 1999), invasion history (Barker 1999), pest status, and economic damage (Godan 1983, Henderson 1989, 1996, South 1992, Barker 2002a).

Rumina decollata (Linnaeus, 1758) (terrestrial snail, Subulinidae): native range (Batts 1957), phylogenetic relationships (scored as 0 as it is not known as a pest nor closely related to a known pest), adult size (scored as 0.5 because it is probably introduced both deliberately and accidentally: Cowie 2001a), invasion history (De Francesco and Lagiglia 2007), egg size (Heller 2001), reproductive potential (extrapolated from Batts 1957, Selander and Hudson 1976, Fisher and Orth 1985), semelparity/iteroparity (Dundee 1986), breeding system (Batts 1957, Selander and Hudson 1976, Fisher and Orth 1985), pest status, status as a "multi-pest", and economic damage (Cowie 2001a).

Melanoides tuberculata (Müller, 1774) (freshwater snail, Thiaridae): native range (not assigned a score because it is now so widespread that its true region of origin and its extent is not known), phylogenetic relationships (scored as 1 as it is itself a minor pest), adult size (Dudgeon 1986, Pointier *et al.* 1994) (scored as 0.5 because it is probably introduced both deliberately and accidentally: Cowie and Robinson 2003), juvenile size (Dudgeon 1986, Pointier *et al.* 1992), reproductive potential (Berry and Kadri 1974), semelparity/iteroparity (Berry and Kadri 1974, Dudgeon 1986, Pointier *et al.* 1992), breeding system (Berry and Kadri 1974, Dudgeon 1986, Ben-Ami and Heller 2007), introduction pressure (Robinson 1999), invasion history (Berry and Kadri 1974, Dudgeon 1986, Pointier *et al.* 1994, Pointier 1999, Cowie 2001b), pest potential, status as a 'multi-pest', and economic damage (Berry and Kadri 1974, Dudgeon 1986, Pointier 1999, Ben-Ami and Heller 2001).

RESULTS AND DISCUSSION

The prioritized list

We created a ranked list of 46 species or groups of species representing 18 families (Table 1). Ranks based on simple (S) and proportional (P) values for each taxon were generally similar. However, some species exhibited relatively large disparities between the two scores although none reflected grossly different placement of these species in the overall rankings, for instance from the top to the bottom third. Nevertheless, we argue that the rank based on P values probably captures the true pest potential better, as it is less biased by the number of attributes it was possible to score for a particular species. The S rank will inevitably increase as more attributes are scored (unless they are all scored as 0), which is not the case for the P rank. The data for the individual attributes on which these scores and ranks are based are provided (Appendix 1). The evaluated species/groups belong to 18 families (Table 2). The top-ranked 12 species or groups fell in eight families, and these eight families included 28 of the 46 taxa evaluated (Table 2).

The top-ranked potential pest groups were the Ampullariidae and Hygromiidae (Table 2). The former ranked highly because of *Pomacea* spp. These freshwater snails have become major pests of rice and other crops in southeast Asia and Hawaii (Joshi and Sebastian 2006). Four species of *Pomacea* have been introduced to the continental United States, where they threaten rice crops and natural ecosystems (Rawlings *et al.* 2007). Cowie and Thiengo (2003) recognized 117 nomenclaturally valid species, many of which may have a similar pest potential to those already introduced. Hygromiids ranked highly because of *Cernuella* spp. and *Xeropicta* spp. *Cernuella virgata* has become a major cereal and pasture pest in Australia (Baker 2002). These and many other hygromiids are especially prone to being introduced in association with domestic tiles imported to the United States from southern Europe (Robinson 1999). Some of them also occur in temperate localities in their native Europe (Kerney and Cameron 1979) and collectively they thus have the potential to invade large parts of the United States.

Helicidae and the closely related Cochlicellidae ranked immediately below the ampullariids and hygromiids (Table 2). Helicids ranked highly essentially because of the value for *Theba pisana* (Table 1, Appendix 1). The value for cochlicellids (Appendix 1) was based on information for *Cochlicella acuta* (Müller, 1774) and *C. barbara* (Linnaeus, 1758). Both *T. pisana* and these cochlicellids have become pests in various parts of the world where they have been introduced, notably in Australia where they are major cereal and pasture pests (Baker 2002). *Theba pisana* is also a pest of grape vines in South Africa (Sanderson and Sirgel 2002) and was formerly an important citrus pest in California but was thought to have been eradicated (Armitage 1949). It has now reappeared but is not widespread (Roth and Sagedhian 2003).

Veronicellid slugs ranked next highest (Table 2). Veronicellids are large slugs. *Sarasinula plebeia* and *Veronicella cubensis* especially can become extremely abundant and are important pests in numerous crops, horticultural facilities, and gardens, and can become a public nuisance in urban/suburban areas (Rueda *et al.* 2002, Naranjo-García *et al.* 2007, R. H. Cowie, pers. obs.). *Laevicaulis alte* is less well recognized

Table 1. List of mollusc species and species-groups of potential major pest significance to the United States, ranked according to their pest potential from greatest (1) to least (46). S and P denote Simple and Proportional values and the ranks based on them (see methods).

Species/species-group	Family[a]	S score	P score	S rank	P rank
Cernuella Schlüter, 1838	Hygromiidae	9.5	0.79	1	1
Pomacea Perry, 1810[b]	Ampullariidae	9.5	0.79	1	1
Cochlicella Férussac, 1821	Cochlicellidae	9.0	0.75	3	3
Theba pisana (Müller, 1774)	Helicidae	9.0	0.75	3	3
Sarasinula plebeia (Fischer, 1868)	Veronicellidae	6.5	0.72	9	5
Xeropicta Monterosato, 1892	Hygromiidae	6.5	0.72	9	5
Laevicaulis alte (Férussac, 1822)	Veronicellidae	5.5	0.69	12	7
Succinea tenella Morelet, 1865[c]	Succineidae	5.5	0.69	12	7
Veronicella cubensis (Pfeiffer, 1840)	Veronicellidae	5.5	0.69	12	7
Achatina fulica Bowdich, 1822	Achatinidae	7.5	0.68	5	10
Indoplanorbis exustus (Deshayes, 1834)	Planorbidae	7.5	0.68	5	10
Biomphalaria Preston, 1910[d]	Planorbidae	7.0	0.64	7	12
Bulinus Müller, 1781	Planorbidae	6.5	0.59	9	13
Ovachlamys fulgens (Gude, 1900)	Chronidae	7.0	0.58	7	14
Zachrysia provisoria (Pfeiffer, 1858)	Pleurodontidae	4.5	0.56	22	15
Tandonia budapestensis (Hazay, 1881)	Milacidae	5.5	0.55	12	16
Xerolenta obvia (Menke, 1828)	Hygromiidae	5.5	0.55	12	16
Arion lusitanicus Auct., *non* Mabille, 1868[e]	Arionidae	5.5	0.50	12	18
Elisolimax flavescens (Keferstein, 1866)	Urocyclidae	4.0	0.50	24	18
Marisa Gray, 1824	Ampullariidae	5.0	0.50	18	18
Parmarion martensi Simroth, 1893	Ariophantidae	4.0	0.50	24	18
Pila Röding, 1798	Ampullariidae	5.0	0.50	18	18
Tandonia sowerbii (Férussac, 1823)	Milacidae	5.0	0.50	18	18
Cantareus apertus (Born, 1778)	Helicidae	4.5	0.45	22	24
Eobania vermiculata (Müller, 1774)	Helicidae	5.0	0.45	18	24
Veronicella sloanei (Cuvier, 1817)	Veronicellidae	3.0	0.43	30	26
Diplosolenodes occidentalis (Guilding, 1825)	Veronicellidae	2.5	0.42	35	27
Macrochlamys indica Godwin-Austen, 1888	Ariophantidae	2.5	0.42	35	27
Succinea s.g. *Calcisuccinea* Pilsbry, 1948[f]	Succineidae	2.5	0.42	35	27
Arion ater (Linnaeus, 1758)	Arionidae	4.0	0.40	24	30
Oncomelania Gredler, 1881	Pomatiopsidae	4.0	0.40	24	30
Enidae Woodward, 1903	Enidae	3.5	0.39	29	32
Achatina achatina (Linnaeus, 1758)	Achatinidae	4.0	0.33	24	33
Thelidomus aspera (Férussac, 1821)	Pleurodontidae	2.5	0.31	35	34
Zachrysia auricoma (Férussac, 1821)	Pleurodontidae	2.5	0.31	35	34
Euglandina Crosse and Fischer, 1870[g]	Spiraxidae	2.5	0.28	35	36
Tandonia rustica (Millet, 1843)	Milacidae	2.5	0.28	35	36
Helix Linnaeus, 1758	Helicidae	3.0	0.27	30	38
Limicolaria aurora (Jay, 1839)	Achatinidae	3.0	0.27	30	38
Otala punctata (Müller, 1774)	Helicidae	3.0	0.27	30	38
Archachatina marginata (Swainson, 1821)	Achatinidae	3.0	0.25	30	41
Mariaella dussumieri Gray, 1855	Ariophantidae	2.0	0.25	43	41
Arianta arbustorum (Linnaeus, 1758)	Helicidae	2.5	0.21	35	43
Acusta tourannensis (Souleyet, 1842)	Bradybaenidae	1.5	0.19	44	44

(continued)

Table 1. (continued)

Species/species-group	Family[a]	S score	P score	S rank	P rank
Leidyula moreleti (Crosse and Fischer, 1872)	Veronicellidae	1.5	0.19	44	44
Zachrysia trinitaria (Pfeiffer, 1858)	Pleurodontidae	1.0	0.13	46	46

[a] All assignments to family from Robinson (1999), except for Wilke *et al.* (2001) for Pomatiopsidae and Vaught (1989) for Ariophantidae, while accepting that some are in flux (*e.g.*, Wade *et al.* 2007).

[b] All species of *Pomacea* except *P. diffusa* Blume, 1957, which is often referred to, incorrectly (Rawlings *et al.* 2007, Hayes *et al.* 2008), as *P. bridgesii* (Reeve, 1856), and the native *P. paludosa* (Say, 1829).

[c] May also include the similar *Succinea horticola* Reinhardt, 1877.

[d] All species of *Biomphalaria* except the native *B. obstructa* (Morelet, 1849).

[e] *Arion lusitanicus* Auct., *non* Mabille is now referred to as *Arion vulgaris* Moquin-Tandon, 1855 by many workers. *Arion lusitanicus* Mabille, 1868 is increasingly acknowledged as a species of *Mesarion* Hesse, 1926, restricted to Spain and Portugal. The issue is not satisfactorily resolved.

[f] Only species of *Succinea* (*Calcisuccinea*) not native to the United States.

[g] Only species of *Euglandina* not native to the United States.

as a major pest, but some of its other attributes resulted in a high ranking (Table 1, Appendix 1). This may be an instance in which differential knowledge of the attributes scored among these veronicellids resulted in a higher ranking of a species (*L. alte*) than its potential may warrant, relative to other species (*S. plebeia* and *V. cubensis*), and reflects the need for caution when interpreting the results of analyses of this kind when based on limited knowledge.

The succineids, achatinids, and planorbids included the remaining taxa in the top ranked 12 (Table 1). In general, succineids have not been considered significant pests until recently as a number of species, notably *Succinea tenella*, are increasingly transported around the world in the horticultural trade (Cowie *et al.* 2008). What their impacts will be is not entirely clear. *Achatina fulica* has often been thought of as one of the world's worst land snail pests (Mead 1979, Raut and Barker 2002) and was the driver of the high ranking of the achatinids (Table 2). Like most snails and slugs, it can act as a vector of human and animal diseases and, with its large size and potential for explosive population growth following introduction, can become a major public nuisance (Poucher 1975, Civeyrel and Simberloff 1996). Other achatinids ranked much lower. However, complacency about them would be misplaced, as little is known about the biology of most of them and many are difficult for untrained specialists to distinguish. Quarantine officials should be vigilant of any achatinids. The planorbids' biological attributes make them potentially highly invasive (Appendix 1). The planorbids role as potential pests is primarily in the arena of human disease, as they are major parasite vectors. However, in this regard their potential is more difficult to evaluate than the more straightforward agricultural potential of most of the other taxa evaluated and it may be that sanitary conditions and people's behavior may minimize the chance of the parasites cycling in the United States (D. S. Woodruff, pers. comm.). The potential of planorbids may be overestimated by our model.

Table 2. Families ranked according to the highest rank achieved by a species or group of species in each family, with the number of species or groups ranked in the top 12 (based on P rank) for each family, and the total number of species or groups that we assessed in each family.

Family	Highest species or group rank (P/S)	Number of species or groups in top 12	Total number of species or groups assessed
Ampullariidae	1/1	1	3
Hygromiidae	1/1	2	3
Helicidae	3/3	1	6
Cochlicellidae	3/3	1	1
Veronicellidae	5/9	3	6
Succineidae	7/12	1	2
Achatinidae	10/5	1	4
Planorbidae	10/5	2	3
Chronidae	14/7	0	1
Pleurodontidae	15/22	0	4
Milacidae	16/12	0	3
Arionidae	18/12	0	2
Ariophantidae	18/24	0	3
Urocyclidae	18/24	0	1
Pomatiopsidae	29/24	0	1
Enidae	31/29	0	1
Spiraxidae	36/35	0	1
Bradybaenidae	44/43	0	1

Validation of the model

To test the validity of our model, we scored a number of additional species and compared the outcome with their known status in the United States. Their attribute scores are available (Appendix 2).

Deroceras reticulatum (Agriolimacidae) scored 7.5 (S value) and 0.68 (P value), ranking it 5 and 10, respectively, among the more serious 'potential' invaders, and appropriately predicting its wide distribution and major pest status in the United States (chapters in Barker 2002a).

Cepaea nemoralis (Helicidae) scored 3.5 (S) and 0.29 (P), ranking it 29 and 36, respectively, toward the bottom of the list. While it is widespread in the eastern United States (Brussard 1975, Whitson 2005), it appears not to be a pest and although a role as a competitor of native snail species has been suggested (Whitson 2005), it has not been demonstrated. The prediction of the model, especially the P rank, which we deem more appropriate, concurs with the essentially non-pest status of this species in the United States.

Cornu aspersum (Helicidae) scored 7.0 (S) and 0.58 (P), ranking it 7 and 14, respectively, among the top-ranked one third. While it is widely distributed in the United States (Roth and Sadeghian 2003), it is only a major agricultural pest, notably of citrus, in California (*e.g.*, Sakovich 2002). Elsewhere it may be more of a garden nuisance. Nevertheless, its status as invasive in the United States is unquestionable and its ranking may reflect the relatively lesser relevance of its biological attributes (which included relatively few 1 scores) as opposed to its human interaction attributes (see discussion below). Thus, the model, at least regarding the P rank, may have underestimated its potential.

Potamopyrgus antipodarum (Hydrobiidae) scored 7.5 (S) and 0.63 (P), ranking it 5 and 13, respectively, among the top third. This ranking is reflected appropriately in its increasing spread through much of the western United States and increasing but as yet somewhat limited documentation of its ecological impacts (Kerans *et al.* 2005, Hall *et al.* 2006).

Milax gagates (Milacidae) scored 7.0 (S) and 0.58 (P), ranking it 7 and 14, respectively, also in the top third. Although widely distributed in the United States (Pilsbry 1948, Roth and Sadeghian 2003), the relative lack of literature (*e.g.*, Godan (1983) reports it as damaging Brussels sprouts; it is not mentioned in the chapters of Barker (2002a) dealing with the United States) suggests that it has not yet become a major widespread pest. In this case the model (at least the S value) may have overestimated this species' potential, although given its pest status in Europe, it would be unwise to assume this. However, simply changing the multi-pest score from 0 to 1 on the basis of its damaging endemic plants in Hawaii (Cowie 1997), changes its scores to 8.0 (S) and 0.73 (P), thereby ranking it 5 (both S and P ranks) and illustrating both the sensitivity of the ranking system to minor changes in the scores and perhaps the serious potential of this species as both an agricultural and environmental pest.

Rumina decollata (Subulinidae) scored 5.0 (S) and 0.42 (P), ranking it 18 and 27, respectively. Having been initially introduced accidentally, it has now been spread deliberately as a putative control agent for *Cornu aspersum*, and is now found widely in southern states from the east coast to California (Cowie 2001a). It has not been considered a serious agricultural pest although it may occasionally become sufficiently abundant in domestic gardens to be considered a nuisance (Fisher and Orth 1985, Cowie 2001a). As a facultative snail predator, it has been suggested that it could affect native, including endangered, snail species, but any such impacts have not been documented (Cowie 2001a). Thus, its wide distribution but low, though not negligible, effects are reflected appropriately in its ranking in the middle third.

Melanoides tuberculata (Thiaridae) scored 6.5 (S) and 0.59 (P), ranking it 9 (S) and 13 (P), among the top third. This ranking of its invasive potential is reflected in its presence in 15 states (Mitchell *et al.* 2007). Almost no studies have attempted to demonstrate any negative effects. However, it can reach high densities and acts as a vector of various trematode parasites. Thus it may have serious ecological impacts as a result of both competition with other freshwater organisms (including native snails and mussels) and transmission of parasites to fish (including endangered species) and indirectly to birds; it potentially may also have a human health impact as a result of the indirect transmission of trematodes to people (Mitchell *et al.* 2007).

Broadly, the model appropriately predicted the invasive pest status of this range of species, suggesting that it works at a gross level. Nevertheless, it is clearly sensitive to minor scoring changes and to the scoring algorithm used, and because some of the scores, especially the human attribute ones, are somewhat subjective, the model can only provide a rather general categorization.

Alternative models

In addition to the uncertainty in an analysis of this kind resulting from a lack of adequate basic knowledge of the attributes scored, subjectivity in scoring some of them, and choice of ranking algorithm, one could arguably include other attributes or weight the attributes differentially, as certain ones may be more important than others in determining potential invasiveness. Notably, climate/habitat match, introduction pressure, and being invasive elsewhere seem to be especially important (*e.g.*, Kolar and Lodge 2001, Theoharides and Dukes 2007, Bomford *et al.* 2008, Hayes and Barry 2008). However, weighting some attributes more than others would involve even more subjectivity than is already inherent in our model

and we preferred to take the more objective approach of not weighting. Nevertheless, some of the categories are strongly related to each other (*e.g.*, invasion history, major pest elsewhere, economic potential) and by including scores for each of them we are in a sense positively weighting the more fundamental underlying attribute. Also, many of the biological attributes scored do indeed seem to be generally correlated with the human interaction attributes. Furthermore, by scanning Appendix 1, it is possible to identify those species that, for instance, are frequently intercepted, that are invasive/pests elsewhere, and so on, and to emphasize certain attributes in order to fine-tune or re-evaluate the ranking of a particular species or group of species. By doing so, it may be possible to tailor quarantine interventions to the threats from individual species or groups. In simplest terms, however, if a species is an invasive pest elsewhere and occurs in habitats/climates represented in the United States, in the absence of any more sophisticated risk assessment, the simplest approach is to assume that it also has that pest potential in the United States.

CONCLUSIONS

Our extensive review of the pest snail and slug literature and consultation with the malacological community, combined with our testing of the model against known alien pests in the United States, makes us confident that our prioritized list does indeed include those taxa most likely to become pests in the United States if they breach quarantine and/or if they cannot be contained locally. The ampullariid genus *Pomacea*, hygromiids, *Cochlicella* spp., helicids (notably *Theba pisana*), veronicellids, succineids, achatinids (primarily *Achatina fulica*), and planorbids topped the list. However, while the ranks, particularly the P ranks, assigned to these species/groups may be reasonable approximations of the relative seriousness of their threats, they should not be adhered to rigidly. Similarly, paying strict attention to the relative rankings of the other taxa that constitute the remainder of the list is also probably not warranted, especially as these species rank as potential pests for a variety of reasons in addition to their potential specifically as agricultural pests.

Other snail and slug species not listed may well have pest potential of which we are currently unaware or may develop pest potential as a result of future environmental changes, changes in agricultural practice, and changes in commercial activities including import/export routes and societal preferences. Notable among these are the numerous hygromiid species from around the Mediterranean, where the group exhibits immense diversity, exemplified by the long list of hygromiids given by Robinson (1999: 438) and of '*Helicella*' species given by Godan (1983: 272).

A key need, however, is better knowledge of the basic biology of many of these potential pests, and rigorous documentation of the levels of damage they cause (including economic data) rather than statements such as 'is a pest of legumes' or 'causes damage to fruit trees', which do not permit assessment of the severity of damage caused. Also, the relative lack of study of their environmental as opposed to agricultural impacts means that the potential of some species to cause serious environmental harm may be underestimated in studies such as this, since with little knowledge, it may not be possible to assign a score for their environmental pest status and potential economic impact on the environment.

Nevertheless, we consider this prioritized list of potential pest snails and slugs of quarantine importance to the United States to be a good approximation that we hope will be used as a basis for further development and more detailed evaluation of the pest potential of the taxa included.

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Appendix 1. Scores of each of the 46 taxa evaluated against the 12 attributes related to potential invasiveness (see text for explanation).

Taxon	Present in USA[a]	Native range	Phylogenetic relationships	Adult size	Egg/ juvenile size	Reproductive potential	Semelparous/ iteroparous	Breeding system	Introduction pressure	Invasion history	Major pest	Multi-pest	Economic damage
Land snails and slugs													
Achatinidae													
Achatina achatina	N	0	1	1	0	0	0	0	1	0	0.5	0	0.5
Achatina fulica	R	-	1	1	0	0.5	0	0	1	1	1	1	1
Archachatina marginata	N	0	1	1	0	0	0	0	1	0	0	0	0
Limicolaria aurora	N	0	0	1	0.5	0	-	0	0.5	0.5	0.5	0	0
Arionidae													
Arion ater	N	-	1	0	0.5	-	1	0.5	0.5	0	0	0.5	0
Arion lusitanicus	N	0	1	0	0.5	-	1	0	0	1	1	0	1
Ariophantidae													
Macrochlamys indica	N	-	1	0.5	-	-	-	-	0	0.5	0.5	0	-
Mariaella dussumieri	N	0	1	0.5	-	-	-	-	0	0	0.5	0	0
Parmarion martensi	R	0	1	0.5	-	-	-	-	0	1	0.5	1	0
Bradybaenidae													
Acusta tourannensis	N	0	0.5	0.5	-	-	-	-	0.5	0	0	0	0
Cochlicellidae													
Cochlicella	N	1	1	1	1	0	1	0	1	1	1	0	1
Chronidae													
Ovachlamys fulgens	R	0	1	0	1	0	1	1	1	1	0.5	0	0.5
Enidae													
Enidae	N	0.5	1	0.5	1	-	-	-	0.5	0	0	0	0
Helicidae													
Arianta arbustorum	N	0.5	0	0.5	0.5	0	0	0	0.5	0	0.5	0	0
Cantareus apertus	R	0	1	1	-	-	0	0	1	1	0.5	0	0
Eobania vermiculata	R	0.5	1	1	0.5	-	0.5	0	1	0.5	0	0	0
Helix	R	0	0.5	1	0.5	-	0	0	1	0	0	0	0
Otala punctata	R	0	1	1	0.5	-	0	0	0.5	0	0	0	0
Theba pisana	R	0.5	1	0.5	1	1	1	0	1	1	1	0	1
Hygromiidae													
Cernuella	R	1	1	0.5	1	1	1	0	1	1	1	0	1
Xerolenta obvia	R	1	-	0.5	1	0	1	0	0.5	1	0.5	0	-
Xeropicta	N	1	-	0.5	1	-	1	0	1	1	1	0	-
Milacidae													
Tandonia budapestensis	R	0	1	0.5	-	-	1	0	0.5	1	1	0	0.5
Tandonia rustica	N	0	1	0.5	0.5	-	-	-	0	0.5	0	0	0
Tandonia sowerbii	N	0	1	0.5	0.5	-	1	0	-	1	0.5	0	0.5
Pleurodontidae													
Thelidomus aspera	N	0	1	0	-	-	-	-	0.5	0	0	1	0
Zachrysia auricoma	N	0	1	0	-	-	-	-	0.5	0.5	0.5	0	0
Zachrysia provisoria	R	0	1	0	-	-	-	-	0.5	1	1	0	1
Zachrysia trinitaria	R	0	1	0	-	-	-	-	0	0	0	0	0
Spiraxidae													
Euglandina[b]	N	0	1	0	1	-	-	-	0.5	0	0	0	0
Succineidae													
Succinea (Calcisuccinea)[c]	N	0	1	0.5	-	-	-	-	1	0	-	0	-
Succinea tenella[d]	R	0	1	1	1	-	-	-	1	1	0.5	0	-

Appendix 1. (continued)

Taxon	Present in USA[a]	Native range	Phylogenetic relationships	Adult size	Egg/ juvenile size	Repro-ductive potential	Semelparous/ iteroparous	Breeding system	Intro-duction pressure	Inva-sion history	Major pest	Multi-pest	Economic damage
Urocyclidae													
Elisolimax flavescens	N	0	1	0.5	-	-	-	-	0.5	0	1	0	1
Veronicellidae													
Diplosolenodes occidentalis	N	-	1	0	-	-	-	-	0.5	-	0	1	0
Laevicaulis alte	R	1	1	0	-	-	-	0.5	0.5	1	0.5	1	-
Leidyula moreleti	N	0	1	0	-	-	-	-	0.5	0	0	0	0
Sarasinula plebeia	R	-	1	0	-	0	-	0.5	1	1	1	1	1
Veronicella cubensis	R	0	1	0	-	-	-	-	1	1	1	1	0.5
Veronicella sloanii	N	0	1	0	-	-	-	-	0.5	-	1	0	0.5
Freshwater snails													
Ampullariidae													
Marisa	R	0	0.5	1	-	1	0	0	1	0.5	1	0	-
Pila	R	1	0.5	1	-	-	0	0	0.5	0.5	0.5	1	0
Pomacea[e]	R	1	1	1	0.5	1	0	0	1	1	1	1	1
Planorbidae													
Biomphalaria[f]	N	1	1	1	1	0.5	0	0.5	0	1	1	0	-
Bulinus	N	1	1	1	1	1	0	0.5	0	0	1	0	-
Indoplanorbis exustus	R	0	1	0.5	1	1	0	0.5	0.5	1	1	1	-
Pomatiopsidae													
Oncomelania	N	0	1	1	1	-	0	0	0	0	1	0	-

[a] Not present (N) or locally restricted (R).
[b] Only species of *Euglandina* not native to the United States.
[c] Only species of *Succinea* (*Calcisuccinea*) not native to the United States.
[d] May also include the similar *Succinea horticola*.
[e] All species of *Pomacea* except *P. diffusa* (often referred to, incorrectly, as *P. bridgesii*) and the native *P. paludosa*.
[f] All species of *Biomphalaria* except the native *B. obstructa*.

Appendix 2. Scores of each of the seven species already present in the United States that were used to validate the model for assessing invasive potential.

Taxon	Native range	Phylogenetic relationships	Adult size	Egg/ juvenile size	Repro-ductive potential	Semelparous/ iteroparous	Breeding system	Introduction pressure	Invasion history	Major pest	Multi-pest	Economic damage
Agriolimacidae												
Deroceras reticulatum	-	1	0	1	0.5	1	0	1	1	1	0	1
Helicidae												
Cepaea nemoralis	1	0.5	0.5	0.5	0	0	0	0.5	0.5	0	0	0
Cornu aspersum	1	1	0.5	0.5	0	0	0	1	1	1	0	1
Hydrobiidae												
Potamopyrgus antipodarum	0	1	1	1	0	1	1	0.5	1	1	0	0
Milacidae												
Milax gagates	0.5	1	0	1	-	1	0	0.5	1	1	0	1
Subulinidae												
Rumina decollata	0.5	0	0.5	1	0.5	1	0.5	0	1	0	0	0
Thiaridae												
Melanoides tuberculata	-	1	0.5	1	0	0	1	0.5	1	0.5	1	0

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New small deep-sea species of Gastropoda from the Campos Basin off Brazil

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Abstract: During environmental characterization of the Campos Basin, Rio de Janeiro State (22°S), about 120 samples from depths between 400 and 2000 m were dredged with a 0.25-m^2 box-core, and a high biodiversity of micro-molluscs was found. Although only dead animals were collected, the shells were often in a good state of preservation. Six new species, belonging to gastropods families Aclididae (*Aclis kanela* n. sp.), Trochidae (*Mirachelus urueuauau* n. sp. and *Calliotropis pataxo* n. sp.), Skeneidae (*Palazzia pankakare* n. sp. and *Adeuomphalus xerente* n. sp.), and Tornidae (*Ponderinella xacriaba* n. sp.) are described.

Key words: biodiversity, deep-water, continental slope, new species

This paper consists of observations on several groups of southern Atlantic gastropods, accumulated during the past 10 years. It is based on material from the continental slope of Brazil obtained during environmental characterization of the Campos Basin, the main Brazilian oil-production area, sponsored by the Brazilian petroleum company Petrobras. Finds of very small and well-preserved shells are still rare worldwide, and there are no published records from the Brazilian coast. This is the most extensive collection of deep-water molluscs from South America.

Of the 1575 species of marine molluscs reported for Brazil by Rios (1994), 1112 belong to the Gastropoda. Only 126 (11.33%) of these are reported to occur on the continental slope (below 200 m depth); many also occur on the continental shelf. These numbers are not indicative of an impoverished malacological fauna but rather reflect quite limited collecting effort at such depths.

The first gastropod species from Brazilian deep water was reported by Watson (1879), who described *Margarites dnopherus* (Watson, 1879). Many of the 126 above-mentioned deep-water species were also described or reported by him. After the great 19th century exploratory expeditions (the "Challenger," "Albatross," and others), no additional reports on Brazilian deep-water molluscs appeared. This situation began to change after 1980, when Leal and co-workers described some new macro-mollusc species (Leal and Bouchet 1989, 1991, Leal and Rios 1990, Leal and Simone 1998, 2000). Other reports appeared by Harasewych (1983) and more recently by Absalão *et al.* (2001), Absalão and Pimenta (2003, 2005), Absalão and Santos (2004), Zelaya *et al.* (2006), Simone (1999, 2002, 2003, 2005), Simone and Birman (2006), Simone and Cunha (2006), Barros *et al.* (2007), Lima and Barros (2007), and Lima *et al.* (2007). Despite the efforts of these investigators, our knowledge about molluscs and especially about deep-water micro-molluscs is incomplete.

Our limited knowledge about biodiversity of these deep waters is even worse when we consider our lack of knowledge about the anatomy of the soft parts and the radulae of most deep-water species, making it difficult to determine their generic and/or sub-generic placement. Therefore, despite taxonomic difficulties, the goal of this study was to describe several newly discovered deep-water micro-gastropod species.

MATERIALS AND METHODS

About 120 samples were dredged with a 0.25-m^2 box-core from the continental slope off Rio de Janeiro state, at depths ranging from 700 to 1950 m. Dredging was performed by the R/V *Astro-Garoupa* between 2001 and 2003 as part of the program "Environmental Characterization of Campos Basin, RJ, Brazil" under the auspices of PETROBRAS S.A.

Each sample was washed through a 0.5-mm mesh and preserved in 70% ethanol. In the laboratory, these residual materials were sorted under magnification and the molluscs picked out. Although no live specimens were collected, the shells were often in a good state of preservation.

Shells were mounted on specimen stubs and photographed under a Scanning Electron Microscope (ZEISS EVO 40) at "Gerência de Bioestatigrafia e Paleoecologia Aplicada (BPA)" belonging to the Centro de Pesquisas da Petrobrás (CENPES).

Most of the material is deposited in the mollusc collection of Departamento de Zoologia, Instituto de Biologia da Universidade Federal do Rio de Janeiro (IBUFRJ) unless otherwise stated. Other abbreviations and terms used in this paper are: MNRJ (Museu Nacional do Rio de Janeiro), MNHN

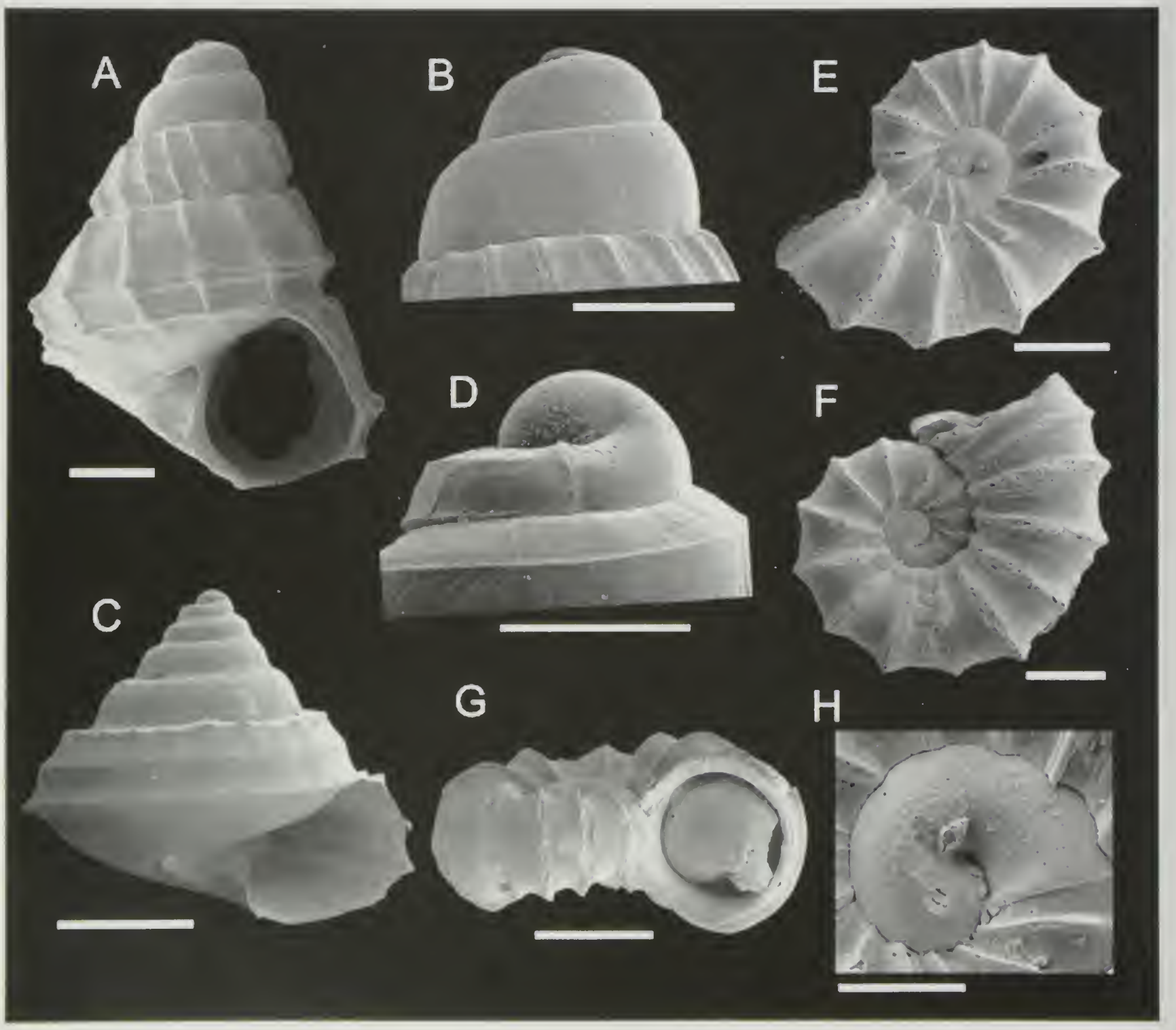
(Muséum national d'Histoire naturelle Paris), BC (Bacia de Campos = Campos Basin), PETROBRAS (Brazilian Petroleum Company), n. sp. (new species), Norte (north), and Sul (south).

RESULTS

Family Trochidae Rafinesque, 1915
Genus *Mirachelus* Woodring, 1928
Mirachelus urueuauau new species (Figs. 1A-B)

Description

Shell small, conical, and solid, of about 4 whorls. Protoconch relatively large and smooth, with about 2½ whorls. Teleoconch with 12 well-separated, thin axial cordlets. There is a spiral cord on the lower ⅓ of the whorl, forming nodules where it crosses the axial cordlets. No microsculpture. Shell profile pagoda-like, with a deep suture. Base almost straight, with a smooth cord bordering the umbilicus. Aperture subcircular-polyhedric because the outer lip is



Figures 1. A-B, *Mirachelus urueuauau* n. sp. Holotype IBUFRJ 18031. A, whole shell, frontal view; B, protoconch. C-D, *Calliotropis pataxo* n. sp. Holotype IBUFRJ 18035. C, whole shell, frontal view; D, protoconch. E-H, *Palazzia pankakare* n. sp. Holotype IBUFRJ 18041. E, dorsal view; F, ventral view; G, frontal view; H, protoconch. Scale bar for A-C: 500 µm; D: 250 µm; E-G: 400 µm; H: 200 µm.

marked to both the spiral cord and basal cord. Columella short, slightly arched. Radula and operculum unknown.

Etymology

This species is named in honor of the Uru-eu-au-au Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Holotype

IBUFRJ 18031; 2.65 mm length, 2.26 mm width, Campos Basin, BC Sul I #75 (22°31′28″S, 40°03′50″W), 19.XI.2002, 1050 m.

Paratypes

MNRJ 12847, BC Sul II #80 (22°24′30″S, 39°57′28″W), 20.VI.2003, 1044 m; IBUFRJ 18032, BC Sul II #86 (22°31′37″S, 39°55′14″W), 16.VI.2003, 1630 m; MNHN, BC Sul II #81 (22°26′28″S, 39°54′08″W), 21.VI.2003, 1345 m; IBUFRJ 18033 BC Norte I #61 (21°52′51″S, 39°48′11″W), 12.XII.2002, 1350 m.

Additional material

IBUFRJ 18034 BC Norte II #61 (21°52′51″S, 39°48′12″W), 26.VI.2003, 1372 m.

Remarks

The only species of *Mirachelus* previously reported for Brazil is *Mirachelus clinocnemus* Quinn, 1979 (Quinn 1979: 18-19, figs. 33-34). Rios (1994, fig. 83) illustrated the species with an SEM photograph. *Mirachelus urueuauau* n. sp. can be distinguished from *M. clinocnemus* by its more delicate axial riblets, compared to the thicker axial ribs in *M. clinocnemus*. The spaces between the axial riblets in *M. urueuauau* n. sp. is about 4 times the riblet width whereas the spaces are almost equal to the width of the ribs in *M. clinocnemus*. Finally, the base is almost straight and smooth in *M. urueuauau* n. sp., while in *M. clinocnemus* it is convex and has three strong spiral cords. The umbilicus is wider in *M. urueuauau* n. sp. than in *M. clinocnemus*. Although the holotype of *Mirachelus urueuauau* n. sp. is only a fourth whorl shell, these differences cannot be attributed to size differences. *Mirachelus corbis* (Dall, 1889) (Quinn 1979: 18, figs. 35-36) is a very distinctive species, with a more turreted shell profile, stronger and numerous axial ribs, an additional spiral cord on the whorls, an excavated suture, and an ornamented base rib. Despite its small size, *Mirachelus urueuauau* n. sp. cannot be confused with any other species of the genus.

Genus *Calliotropis* Seguenza, 1903

Calliotropis pataxo new species (Figs. 1C-D)

Description

Shell small, broadly conical, of about 6 whorls, iridescent, carinate, and with broad umbilicus. Shell profile straight or slightly concave, strongly stepped. Protoconch small, smooth, with slightly more than one whorl, ornamented with very small irregular stellar nodules connected by protuberances concentrated on the initial part. Proto-teleoconch boundary well marked. First and second whorls of teleoconch with a narrow keel on the shoulder, then becoming obsolete and arising again on the body whorl as a more nodulose keel. Body whorl with three keels: one nodulose near the suture; the second in the middle of the whorl, less evident than the others; and the third with very light nodules, delimiting the body whorl from the base. Opisthocline axial growth scars present on the entire shell, but more visible on the first two teleoconch whorls. There are about 22 low but somewhat pointed nodules on the spiral keel on the body whorl, suggesting the presence of very retractive axial ribs. Base convex, smooth or with hardly discernible axial cords. Umbilicus broad and deep, bordered by somewhat evanescent cord; two additional umbilical cords may also be present inside. Aperture subquadrate, narrow; inner lip slightly reflected. Columella slightly oblique and arched.

Etymology

This species is named in honor of the Pataxó Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Holotype

IBUFRJ 18035, 1.22 mm length, 1.40 mm width, Campos Basin, BC Sul I #73 (22°41′35″S, 40°00′45″W), 22.XI.2002, 1950 m.

Paratypes

MNRJ 12848, BC Sul II #77 (22°36′12″S, 39°58′22″W), 13.VI.2003, 1670 m; IBUFRJ 18036, BC Sul II #83 (22°30′34″S, 39°51′44″W), 16.VI.2003, 1970 m; MNHN, BC Norte I #46 (22°10′55″S, 39°49′00″W), 10.XII.2002, 1350 m; IBUFRJ 18037, BC Norte I #62 (21°52′41″S, 39°46′17″W), 11.XII.2002, 1650 m.

Additional material

IBUFRJ 18038, BC Norte I #63 (21°52′44″S, 39°40′45″W), 11.XII.2002, 1950 m; IBUFRJ 18039, BC Norte II #57 (21°57′15″S, 39°47′41″W), 28.VI.2003, 1587 m.

Remarks

There are only three species previously reported for Brazil: *Calliotropis actinophora* (Dall, 1890), *Calliotropis aeglees* (Watson, 1879), and *Calliotropis calatha* (Dall, 1927) (Rios 1994). The first two are deep-water species reported from off

northern and northeastern Brazil; the third is reported from the edge of the continental shelf off southern Brazil. *Calliotropis pataxo* n. sp. can be distinguished from *C. actinophora* (type illustrated by Quinn 1979, figs. 21-22) by its smaller spire angle, less-rounded profile of the body whorl, absence of thin radial riblets, absence of nodulous middle spiral keel, and absence of two strong spiral cords on the base. It can be distinguished from *C. aeglees* (type illustrated by Quinn 1979, figs. 11-12) by its stepped shell; the less-nodulose spiral cords, especially the supra-sutural cord; absence of axial riblets on the first whorl; absence of a third keel forming the whorl periphery; and absence of three spiral cords on the base. It can be distinguished from *C. calathia* (type illustrated by Quinn 1979, figs. 15-16) by the less-expanded body whorl, absence of axial riblets, sutures not being channeled, less-prominent middle keel, and absence of four spiral cords on the base. *Calliotropis rhina* (Watson, 1886) (type illustrated by Quinn 1979, figs. 27-28) shows a much higher spire than *Calliotropis pataxo* n. sp., in addition to more nodulous spiral cords and spiral cords on the base. *Calliotropis lissocona* (Dall, 1881) (type illustrated by Quinn, 1979, figs. 13-14) has a smaller spire angle than *C. pataxo* n. sp., shows no trace of a middle keel, has two very strong spiral cords on the base, and a nodulose spiral cord bordering the umbilicus; none of these cords is present in *C. pataxo* n. sp. In summary, the stepped shell, the slight nodulose ornamentation, and smooth base preclude any chance of confusing *Calliotropis pataxo* n. sp. with other species of the Atlantic basin.

Family Skeneidae Thiele, 1929
Genus *Palazzia* Warén, 1991
Palazzia pankakare new species (Figs. 1E-H)

Description

Shell small, diameter about 1.5 mm, white, solid, planispiral; spire not projecting beyond the last whorl profile. Protoconch with about 1.5 whorls, with clear cut between proto-teleoconch boundary. It is entirely covered by irregular corrugations. Teleoconch with 15 annular ribs (triangular in section) and broad smooth interspaces between them, these spaces about 3-4 times the width of the annular ribs. No spiral sculpture nor pits present. Lips strong but not thickened. Aperture holostomate, circular. Operculum unknown.

Etymology

This species is named in honor of the Pankakaré Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Holotype

IBUFRJ 18041, shell diameter 1.40 mm, height 0.68 mm, Campos Basin, BC Sul I #77 (22°36′03″S, 39°57′54″W), 16.XI.2002, 1650 m.

Paratypes

MNRJ 12849, BC Sul I #85 (22°29′33″S, 39°56′17″W), 19.XI.2002, 1350 m; IBUFRJ 18042, BC Sul II #85 (22°30′21″S, 39°56′53″W), 21.VI.2003, 1353 m; MNHN, BC Sul II #86 (22°31′37″S, 39°55′14″W), 16.VI.2003, 1630 m; IBUFRJ 18043, BC Norte I #61 (21°52′51″S, 39°48′11″W), 12.XII.2002, 1350 m.

Additional material

IBUFRJ 18044, BC Norte II #61 (21°52′51″S, 39°48′12″W), 26.VI.2003, 1372 m.

Remarks

There is no record of this genus in the South Atlantic, and *Palazzia planorbis* (Dall, 1927) and *Palazzia ansonia* (Palazzi, 1988) are the only known species reported for the North Atlantic (Warén 1991a: 77, figs. 16A-D; 78-79, figs. 17A-G, 18A-C). *Palazzia pankakare* n. sp. can be distinguished from the other congeners by its incomplete and much more numerous axial rings. The micro-ornamentation of the protoconch of *P. pankakare* n. sp. is identical those showed by *P. planorbis* and *P. ansonia* (see Warén 1991a: 79, figs. 18A-C). The genus *Ammonicera* Vayssière, 1893 has conchological similarities with *Palazzia pankakare* n. sp. but is distinguished from *Palazzia* by its spirally ornamented protoconch (Rolán 1992). Despite the good fit of *P. pankakare* n. sp. to genus *Palazzia*, Warén (1991a: 75) states that "the teleoconch surface is finely and irregularly pitted" and we were unable to find any sign of pits on the teleoconch. So, attribution to the generic level should be viewed with caution.

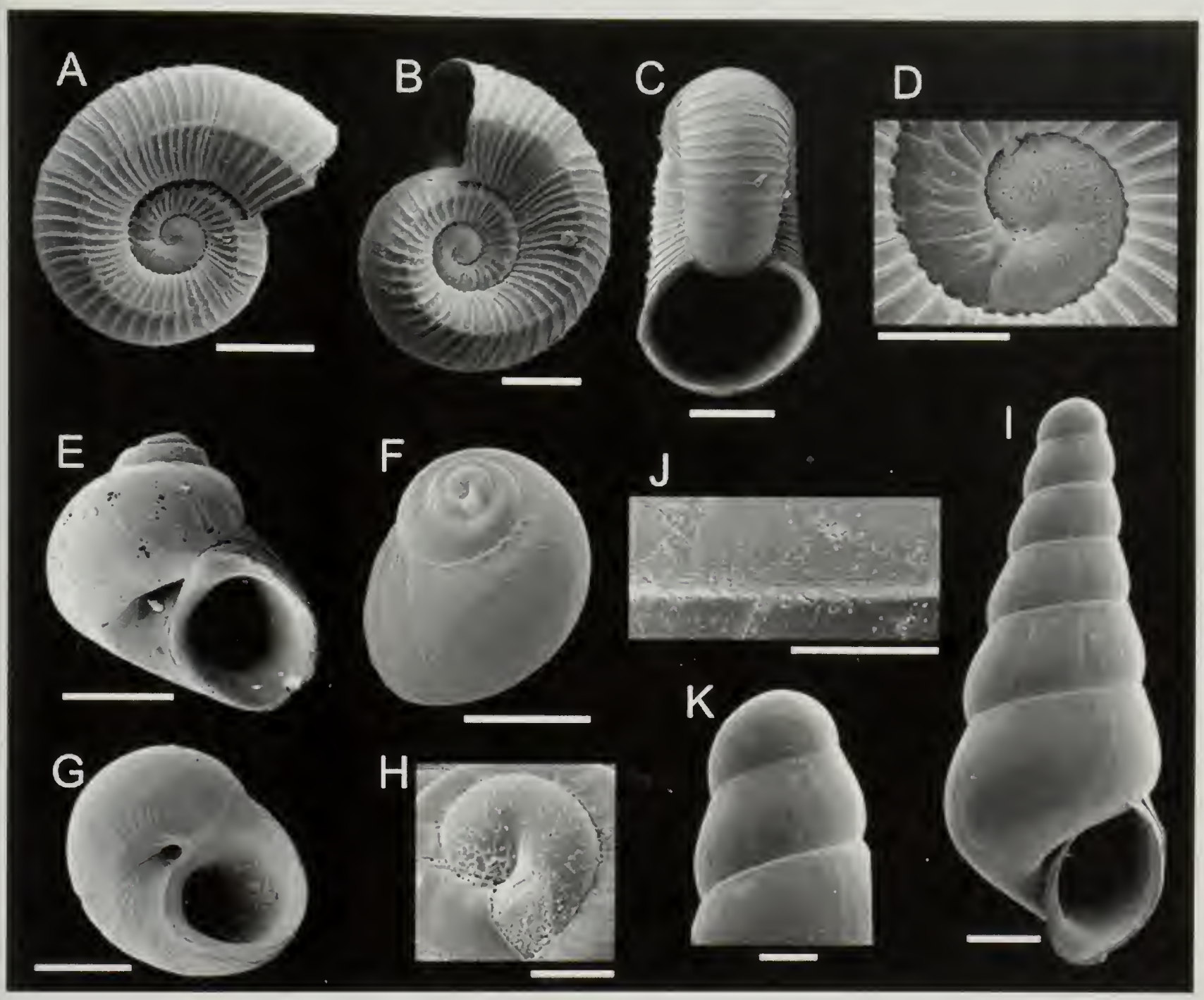
Genus *Adeuomphalus* Seguenza, 1876
Adeuomphalus xerente new species (Figs. 2A-D)

Description

Shell planospiral, small, white, with axial rings and spiral cords. Protoconch size about 1.75 whorls, with well-marked boundary with teleoconch. Protoconch with irregular stellar nodules connected by protuberances that spread over ¾ of the protoconch length. Teleoconch with about 60 narrow, equally spaced rings. Width of interspaces about 2-4 times the width of ring. Peripherally there is a spiral cord on both the dorsal and ventral side of the shell, and sometimes it is crossed by rings forming low and rounded nodules. Lips not thickened, aperture irregularly ovoid with anterior whorl projecting slightly over it. Operculum and radula unknown.

Etymology

This species is named in honor of the Xerente Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.



Figures 2. A-D, *Adeuomphalus xerente* n. sp. Holotype IBUFRJ 18045. A, dorsal view; B, ventral view; C, frontal view; D, protoconch. E-H, *Poderinella xacriaba* n. sp. Holotype IBUFRJ 18048. E, whole shell, frontal view; F, dorsal view; G, base; H, protoconch. I-K, *Aclis kanela* n. sp. Holotype IBUFRJ 18056. I, whole shell, frontal view; J, protoconch and K, detail of suture. Scale bar for A-C, I: 250 μm; D, H, J-K: 100 μm; E, G: 500 μm

Holotype

IBUFRJ 18045, shell diameter 0.74 mm, height 0.55 mm, Campos Basin, BC Sul II #85 (22°30′21″S, 39°56′53″W), 21.VI.2003, 1353 m.

Paratypes

MNRJ 12850, BC Sul II #86 (22°31′37″S, 39°55′14″W), 16.VI.2003, 1630 m; IBUFRJ18046, BC Norte I #46 (22°10′55″S, 39°49′00″W), 10.XII.2002, 1350 m; MNHN, BC Norte I #61 (21°52′51″S, 39°48′11″W), 12.XII.2002, 1350 m; IBUFRJ 18047, BC Norte II #45 (22°10′53″S, 39°52′18″W), 01.VII.2003, 1039 m.

Remarks

There is no record of this genus in the South Atlantic, and *Adeuomphalus ammoniformis* Seguenza, 1876 is the only known species reported for the North Atlantic (Warén 1991a: 74-76, figs. 14F, 15A-B). It can be distinguished from *Adeuomphalus xerente* n. sp. by its much more numerous axial rings (about 85); *A. xerente* n. sp. has only about 60. These rings in *A. ammoniformis*

also disappear abruptly towards the periphery of the shell, and frequently have a tubercle ⅔ from the periphery; in *Adeuomphalus xerente* n. sp. the axial rings are constant over all the shell, not disappearing in any part. The tubercles are more discrete than those in *A. ammoniformis*. The protoconch ornamentation of *Adeuomphalus xerente* n. sp. is identical to that described for *A. ammoniformis*. But despite that, the two species can be clearly distinguished.

Family Tornidae Sacco, 1896
Genus *Ponderinella* Marshall, 1988
Ponderinella xacriaba new species (Figs. 2E-H)

Description

Shell ovoid-conical, almost globular, small, smooth, solid, and white. Resembing a small naticid. Protoconch with about 1.5 whorls, ornamented with delicate irregular lines that ramify and anastomose (very small irregular stellar nodules connected by protuberances), and a well-marked boundary with the teleoconch. Teleoconch with convex whorls, smooth except for irregular growth scars. Suture well marked, bordered at body whorl by a slight depression. Base smooth, umbilicus moderately narrow. Lips strong, aperture rounded, prosocline, slightly pointed posteriorly. Operculum and radula unknown.

Etymology

This species is named in honor of the Xacriaba Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Holotype

IBUFRJ 18048, 1.23 mm length, 1.23 mm width, Campos Basin, BC Sul I, #73 (22°41′35″S, 40°00′45″W), 22.XI.2002, 1950 m.

Paratypes

MNRJ 12851, BC Sul I #77 (22°36′03″S, 39°57′54″W), 16.XI.2002, 1650 m; IBUFRJ 18049, BC Sul I #82 (22°28′49″S, 39°53′24″W), 17.XI.2002, 1650 m; MNHN, BC Sul I #85 (22°29′33″S, 39°56′17″W), 19.XI.2002, 1350 m; IBUFRJ 18050, BC Sul II #81 (22°26′28″S, 39°54′08″W), 21.VI.2003, 1345 m.

Additional material

IBUFRJ 18051, BC Sul II #85 (22°30′21″S, 39°56′53″W), 21.VI.2003, 1353 m; IBUFRJ 18052, BC Norte I #46 (22°10′55″S, 39°49′00″W), 10.XII.2002, 1350 m; IBUFRJ 18053, BC Norte I #61 (21°52′51″S, 39°48′11″W), 12.XII.2002, 1350 m; IBUFRJ 18054, BC Norte II #50 (22°04′33″S, 39°52′05″W), 30.VI.2003, 1030 m; IBUFRJ 18055, BC Norte II #61 (21°52′51″S, 39°48′12″W), 26.VI.2003, 1372 m.

Remarks

At first glance, *Ponderinella xacriaba* n. sp. resembles *Cirsonella* in the general shell shape, but the protoconch ornamentation and aperture are very distinctive. While *Cirsonella* has a protoconch with very fine irregular spiral lines (Warén, 1991b: 210, fig. E), *Ponderinella xacriaba* n. sp. has tiny irregular stellar nodules connected by protuberances (Fig. 2H). The aperture of *Cirsonella* is regularly rounded (Warén, 1991b: 212, figs. 11A-D), whereas in *P. xacriaba* n. sp. it is posteriorly constricted (Fig. 2E). These differences preclude assignment of this new taxon to the genus *Cirsonella*. The genus *Ponderinella*, on other hand, was originally created to accommodate a characteristic group from the southeastern Pacific. Recently, Rolán and Rubio (2002) assigned some eastern Atlantic species to this genus, and it seems to be an appropriate systematic placement for *Ponderinella xacriaba* n. sp. as well.

The genus *Ponderinella* was, until now, restricted to the eastern Atlantic. The record of *Ponderinella xacriaba* n. sp. expands the genus distribution to the southwestern Atlantic. Among Atlantic *Ponderinella*, *P. xacriaba* n. sp. is distinguished from *Ponderinella tornatica* (Moolenbeek and Hoenselaar, 1995) (Rolán and Rubio 2002: 39, figs. 101-106) which has a strong peripheral keel on the body whorl and another keel bordering the umbilicus, whereas *P. xacriaba* n. sp. is devoid of such characters. *Ponderinella minutissima* Rolán and Rubio, 2002 (pp. 42-43, figs. 118-126) has a thickened umbilical cord, which is absent in *P. xacriaba* n. sp. *Ponderinella skeneoides* Rolán and Rubio, 2002 (pp. 40-41, figs. 107-117) is the most similar species to *P. xacriaba* n. sp. Although *P. skeneoides* has a very variable shell profile, some of them (Rolán and Rubio2002: 41, fig. 115) resemble those present in *P. xacriaba* n. sp., and in addition both species share the same kind of protoconch ornamentation (Rolán and Rubio 2002: 41, fig. 116, and Figs. 2E-F herein). Despite these similarities, the two species can be distinguished because *P. skeneoides* usually has a much broader umbilicus (Rolán and Rubio 2002: 41, figs. 107-110), an umbilical cord (sometimes nodulose) (Rolán and Rubio 2002: 41, figs. 108, 113-114), and a less-rounded aperture with the ventral part somewhat retracted (same illustrations mentioned above); *P. xacriaba* n. sp. has a narrower umbilicus, no umbilical cord, and a more-rounded aperture with no retraction on the ventral side (Figs. 2E, 2G).

Family Aclididae Sars, 1878
Genus *Aclis* Lovén, 1846
Aclis kanela new species (Figs. 2I-K)

Description

Shell small, white, thin, conical-elongated. Protoconch globose, smooth, with about 1½ whorls. Proto-teleoconch

transition not discernible. Teleoconch with convex profile; in the third and fourth whorls, the main whorl diameter is in the anterior part of the whorl. Whorls increasing moderately in diameter. Smooth, except for irregular axial growing scars. Suture well impressed, with posterior border clearly extending over the anterior one, forming a projecting border (Fig. 2K). Supra-sutural microscopic spiral striation, visible only under very strong magnification, disappears towards the middle of the whorl. Base conical, convex. Aperture ovoid, peristome reflected on anterior side, with no thickening. Umbilicus narrow, partially covered by parietal wall (fig. 2I).

Etymology

This species is named in honor of the Kanela Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Holotype

IBUFRJ 18056, 1.90 mm length, 0.75 mm width, Campos Basin, BC Sul I #75 (22°31′28″S, 40°03′50″W), 19.XI.2002, 1050 m.

Paratypes

MNRJ 12852, BC Sul I #80 (22°24′31″S, 39°57′28″W), 20.XI.2002, 1050 m; IBUFRJ 18057, BC Sul II #75 (22°31′28″S, 40°03′49″W), 18.VI.2003, 1043 m; MNHN, BC Sul II #84 (22°26′28″S, 39°58′53.3″W), 20.VI.2003, 1046 m; IBUFRJ 18058, BC Norte I #45 (22°10′54″S, 39°52′19″W), 10.XII.2002, 1050 m.

Additional material

IBUFRJ 18059, BC Norte I #60 (21°52′50″S, 39°51′04″W), 12.XII.2002, 1050 m; IBUFRJ 18060, BC Norte II #63 (21°52′43″S, 39°40′41″W), 26.VI.2003, 1941 m.

Remarks

Five species of *Aclis* have been previously reported for Brazil (Rios 1994). Two of them are shallow-water species (*Aclis bermudensis* Dall and Bartsch, 1911 and *Aclis underwoodae* (Bartsch, 1947)) and show distinctive shell profiles. *Aclis hyalina* Watson, 1881 was reallocated to the genus *Costaclis* Bartsch, 1947 by Bouchet and Warén (1986). The other two, *Aclis sarissa* Watson, 1881 and *Aclis macrostoma* Barros, Lima, and Francisco, 2007 are deep-water species from northern Brazil and will be discussed below. *Aclis sarissa* shows the typical elongated-turreted *Aclis* shell profile, with the first whorls markedly smaller than the last ones; whereas in *Aclis kanela* n. sp. the increase in shell width is more regular. The apex is also blunter than in the two former species. Finally, *A. sarissa* shows very fine axial lines, which are lacking in *A. kanela* n. sp. *Aclis macrostoma* and *A. kanela* n. sp. share the same type of supra-sutural microscopic spiral striae. *Aclis macrostoma* has a more pointed protoconch than *A. kanela* n. sp., a much more obtuse spire angle, and an opisthocline aperture with reflected lips posteriorly and anteriorly; *A. kanela* n. sp. has the aperture almost orthocline and reflected lips restricted on the anterior side. A similar protoconch is present in *Aclis attenuans* Jeffreys, 1883 (Bouchet and Warén 1986: 305, fig. 730), but the whorls are regularly convex, whereas in *Aclis kanela* n. sp. the greatest whorl diameter is on the anterior part of the whorl; moreover, the lip in *A. attenuans* is more extensively reflected, whereas this reflection is more restricted in *A. kanela* n. sp. Finally, *A. attenuans* is a Mediterranean species, while *A. kanela* n. sp. is a South American one.

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The genera *Myonera*, *Octoporia*, and *Protocuspidaria* (Pelecypoda, Cuspidariidae) from deep waters of Campos Basin, Rio de Janeiro, Brazil with descriptions of two new species

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Abstract: Eight species of Cuspidariidae belonging to the genera *Myonera* Dall, 1886, *Octoporia* Scarlato and Starobogatov, 1983, and *Protocuspidaria* Allen and Morgan, 1981 were obtained in samples from the continental slope (700-2000 m depth) at Campos Basin, Rio de Janeiro state (22°S), Brazil. *Myonera paucistriata* Dall, 1886 and *Protocuspidaria verityi* Allen and Morgan, 1981 are now documented from the Campos Basin. Though previously reported for Brazil, the known range of *Octoporia octaporosa* (Allen and Morgan, 1981) is enlarged southward. *Myonera limatula* (Dall, 1881) and *Protocuspidaria atlantica* Allen and Morgan, 1981 are reported from the South Atlantic Ocean for the first time. *Myonera kaiwa* sp. nov. and *Protocuspidaria jarauara* sp. nov. are described herein. *Myonera* sp., an eighth taxon, is present as one unique specimen. The presence of micro-pits on the shell surface is reported for the first time in the Septibranchia.

Key words: Mollusca, Anomalodesmata, biodiversity, continental slope, micro-pits

The septibranchs comprise some of the most intriguing pelecypods, with anatomical adaptations for a carnivorous diet. Reflecting the importance of the group in deep waters, in the last 30 years the number of described species has increased considerably, with many faunal surveys and taxonomic reviews (Kuroda 1952, Bernard 1974, Knudsen 1982, Poutiers 1984, Poutiers and Bernard 1995). In spite of this activity, there are still only a few studies regarding exclusively the septibranchs in deep waters off Brazil (*e.g.*, Marini 1974).

The classification of septibranchs has a long taxonomic history (Pelseneer 1911, Thiele 1935, Newell 1969, Allen and Morgan 1981, Morton 1981, Scarlato and Starobogatov 1983, Poutiers and Bernard 1995, Harper *et al.* 2006). Scarlato and Starobogatov (1983) proposed a new classification based on the branchial apparatus-septum structure that increased the number of higher-rank taxa, many of them new. Poutiers and Bernard (1995) completed a revision of species from the Pacific Ocean, and their classification disagreed with that of Scarlato and Starobogatov (1983). Harper *et al.* (2000) and Morton (2003: 378, table 3), both based on morphological data, did not recognize the higher-rank taxa classification proposed by Scarlato and Starobogatov (1983). Additionally, Dreyer *et al.* (2003) and Harper *et al.* (2006) addressed the same issue, but worked with all anomalodesmatans, and with molecular tools as well, also did not support Scarlato and Starobogatov (1983).

In our opinion, there is insufficient justification to adopt the higher-rank taxa proposed by Scarlato and Starobogatov (1983) and we follow the classification of Poutiers and Bernard (1995), regarding *Myonera* Dall, 1886, *Octoporia* Scarlato and Starobogatov, 1983, and *Protocuspidaria* Allen and Morgan, 1981 as full genera of Cuspidariidae.

MATERIALS AND METHODS

All material was dredged with a 0.25-m^2 box-corer, from the continental slope off Rio de Janeiro state, at depths ranging from 700 to 1950 m. The dredging was carried out by the R/V *Astro-Garoupa* between 2001 and 2003 as part of the program "Environmental Characterization of Campos Basin, RJ, Brazil" under the auspices of PETROBRAS S.A.

Each sample was washed through a mesh of 0.05 mm and preserved in 70% ethanol. In the laboratory, the residues were examined under magnification and the molluscs picked out. Although no live specimens were collected, many of the shells were in a good state of preservation.

Shells were mounted on specimen stubs and photographed under a Scanning Electron Microscope (ZEISS EVO 40), at the Gerência de Bioestatigrafia e Paleoecologia Aplicada (BPA), belonging to the Petrobras Research Center (Centro de Pesquisas da Petrobras–CENPES). The diameters of the micro-pits were measured on the SEM photos.

Though other authors have given the distribution of species studied herein, only those who expanded the known geographic range of these species were included in the tables of distribution.

The material is deposited in the Mollusca collection of the following institutions: Departamento de Zoologia, Instituto

de Biologia, Universidade Federal do Rio de Janeiro (IBUFRJ); Museu de Zoologia, Universidade de São Paulo (MZUSP); Museu Nacional, Universidade Federal do Rio de Janeiro (MNRJ); and Muséum national d'Histoire naturelle, Paris (MNHN).

RESULTS

Cuspidariidae Dall, 1886
Genus *Myonera* Dall, 1886
Type species: *Myonera paucistriata* Dall, 1886 by original designation in Dall, 1886b

Genus characterization

Shell small, outline variable, inequilateral, rostrate, usually inflated. Externally with concentric and/or radial ornamentation. Hinge edentulous. Ligament internal, deflected and posteriorly pointed. Gills with four or five pairs of pores. (Adapted from Allen and Morgan 1981, Poutiers and Bernard 1995).

Myonera paucistriata Dall, 1886 (Figs. 1A-D)
Neaera paucistriata Bush, 1885: 473 [*nomen nudum*]
Myonera paucistriata Dall, 1886: 302; Abbott 1974: 568; Allen and Morgan 1981: 473; Rios 1994: 303
Cuspidaria paucistriata: Pelseneer, 1911: 80

Characterization

Shell white, small (max. length 8 mm), elongated, inequilateral, rostrate, inflated. Umbo large, centralized, rostrum short, ventrally pointed, two keels between umbo and postero-ventral margin, anterior margin rounded. Anteriorly with an undulating appearance. External surface smooth. Micro-pits absent. Hinge edentulous. Resilifer posteriorly pointed.

Distribution of *Myonera paucistriata* Dall, 1886

References	Locality	Depth (m)
Dall (1886b)	Type locality: U. S. Coast Survey Steamer 'Blake' sta. 226 and 230 [St. Vicent]; sta. 43 [near Tortugas, Florida].	620-849
Abbott (1974)	North Carolina to West Indies.	353-1609
Allen and Morgan (1981)	Pacific Ocean: Hawaiian Islands. Atlantic Ocean: northwest Atlantic, west coast of Malabar, Bay of Biscay.	678-3806
Rios (1994)	North Carolina to West Indies. Brazil.	600-760
Rosenberg (2005)	USA: Florida: Florida Keys. 35°N to 34.42°S; 80°W to 4.25°W.	166-1609
Present study	Campos Basin, Rio de Janeiro, Brazil.	1050-1930

Discussion

The name *Myonera* was introduced as a subgenus of *Neaera* by Dall (1886a). Later, Dall (1886b) established *Myonera* as a genus and included *Neaera sulcifera* Jeffreys, 1881, *Neaera angularis* Jeffreys, 1876, *Neaera lamellifera* Dall, 1881, *Neaera limatula* Dall, 1881 [plus its synonym *Neaera contracta* Jeffreys, 1881], *Myonera laticella* Dall, 1886, *Neaera undata* Verrill, 1884, and *Neaera fragilissima* Smith, 1885, and designated *Myonera paucistriata* as the type species. Currently, some of these species are allocated to other genera (*e.g.*, *Cuspidaria sulcifera*, *Cuspidaria contracta*, *Cuspidaria undata*, *Cardiomya fragilissima*).

Some classifications still rank *Myonera* as a subgenus of *Cuspidaria* (Thiele 1935, Allen and Morgan 1981), as a section of the genus *Cuspidaria* (Fischer 1887) or, most frequently, as a genus of the family Cuspidariidae (Grassé 1960, Vokes 1967, Bernard 1974, Rios 1994, Poutiers and Bernard 1995, Absalão *et al.* 2003).

Scarlato and Starobogatov (1983) proposed the family Myoneridae, comprising 11 genera, a classification not followed by many authors (Rios 1994, Poutiers and Bernard 1995, Harper *et al.* 2000, 2006, Morton 2003, Dreyer *et al.* 2003). We agree with Poutiers and Bernard (1995: 139) that "although the usage of several ordinal taxa [...] may prove to be useful, many of them seem presently unwarranted."

Material examined

IBUFRJ 17855 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.01, [1 valve], IBUFRJ 17860 (22°07′17″S, 39°50′02″W, 1230 m), 13.V.02, [2 valves], IBUFRJ 17865 (22°04′52″S, 39°49′04″W, 1330 m), 09.V.02, [2 valves], IBUFRJ 17866 (22°02′36″S, 39°49′36″W, 1330 m), 08.V.02, [1 valve], IBUFRJ 17867 (22°03′27″S, 39°45′07″W, 1730 m), 08.V.02, [2 valves], IBUFRJ 17871 (22°05′45″S, 39°45′55″W, 1730 m), 09.V.02, [1 valve], IBUFRJ 17880 (22°09′10″S, 39°44′50″W, 1930 m), 08.V.02, [1 valve], IBUFRJ 17911 (22°04′44″S, 39°46′31″W, 1650 m), 24.XI.02, [2 valves], IBUFRJ 17912 (21°57′15″S, 39°47′43″W, 1650 m), 14.XII.02, [1 valve], IBUFRJ 17924 (21°52′41″S, 39°46′17″W, 1650 m), 11.XII.02, [1 valve], IBUFRJ 17925 (22°41′10″S, 40°02′20″W, 1650 m), 13.VI.03, [3 valves], IBUFRJ 17935 (21°52′41″S, 39°46′17″W, 1650 m), 26.VI.03, [1 valve], IBUFRJ 17936 (21°57′15″S, 39°47′41″W, 1650 m), 28.VI.03, [3 valves], IBUFRJ 17954 (22°04′45″S, 39°46′31″W, 1650 m), 27.VI.03, [4 valves], IBUFRJ 17979 (22°11′04″S, 39°47′04″W, 1650 m), 22.VI.03, [7 valves], IBUFRJ 18025 (21°57′15″S, 39°49′37″W, 1350 m), 25.VI.03, [6 valves], IBUFRJ 18026 (22°26′27″S, 39°58′51″W, 1050 m), 20.XI.02, [1 valve], IBUFRJ 18027 (21°57′15″S, 39°49′37″W, 1350 m), 14.XII.02, [2 valves], IBUFRJ 18030 (21°52′41″S, 39°46′17″W, 1650 m), 11.XII.02, [6 valves].

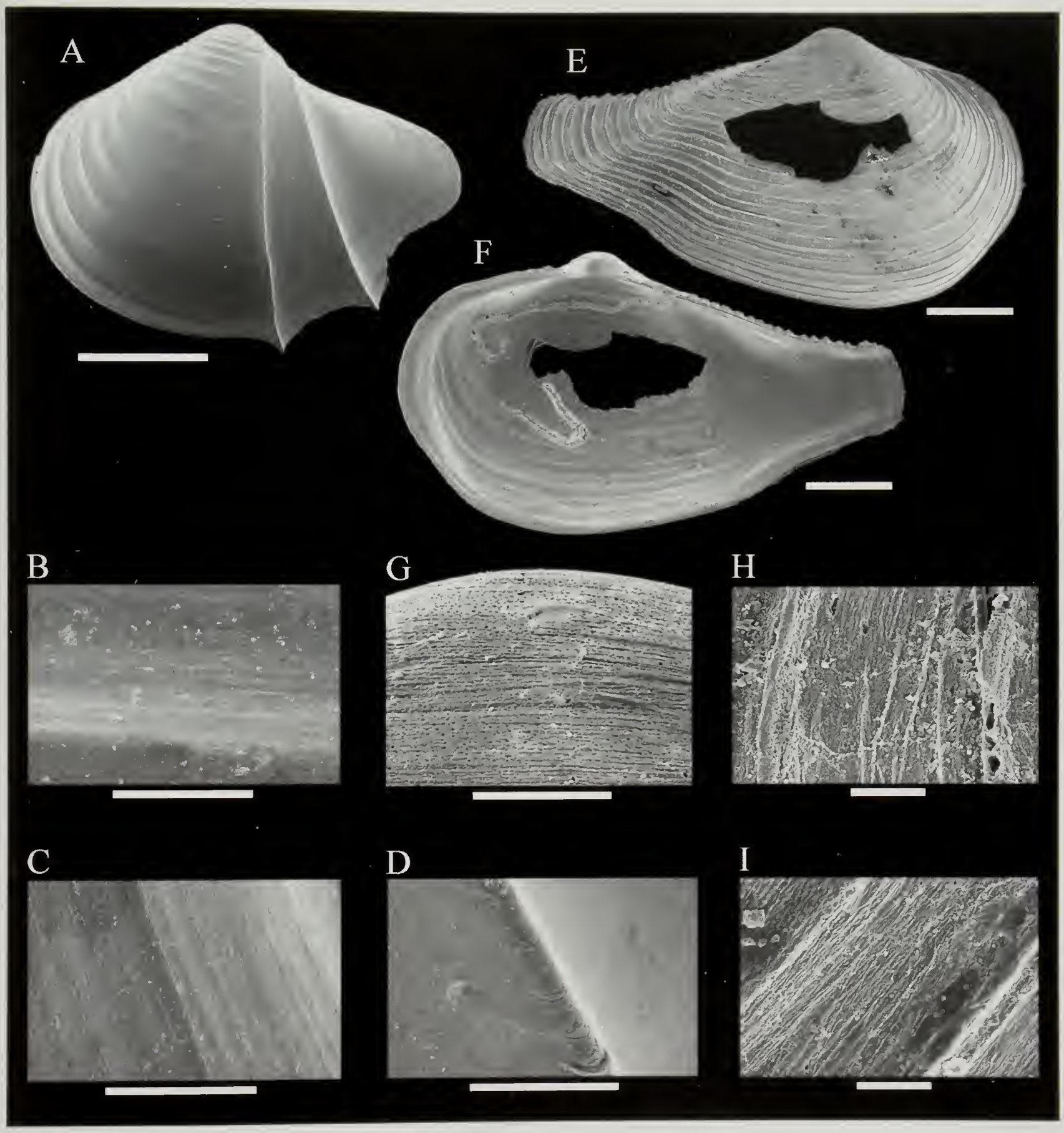

Figure 1. A-D, *Myonera paucistriata* Dall, 1886. IBUFRJ 17924. A, external view; B, umbo detail; C, anterior margin detail; D, rostrum detail. E-I, *Myonera limatula* (Dall, 1881). IBUFRJ 14798. E, external view; F, internal view; G, umbo detail; H, rostrum detail; I, anterior margin detail. Scale bar A: 2 mm; E-F: 1 mm; B-D, G-I: 100 μm.

Discussion

The name *Neaera paucistriata* was first cited in Bush (1885), but in a manner that does not comply with articles 12 and 16 of the Code (ICZN 1999), characterizing it as a *nomen nudum*. A proper description of this species was made by Dall (1886b) as *Myonera paucistriata*.

This is probably the most common of all septibranchs (Allen and Morgan 1981). In Campos Basin, it was present in 18 of 117 stations where pelecypods occurred.

Regarding micro-pits, this species is different from all other *Myonera* in this study, because, for *Myonera paucistriata*, no micro-pits were observed on the shell surface (Figs. 1B-D).

Myonera limatula (Dall, 1881) (Figs. 1E-I)
Neaera limatula Dall, 1881: 112
Neaera contracta: Jeffreys, 1881: 941, pl. LXXI, fig. 4
Myonera limatula: Dall, 1886: 304, pl. III, fig. 5

Characterization

Shell white, small (max. length 6 mm), elongate, inequilateral, rostrate, somewhat inflated. Umbo large, closer to anterior end, rostrum oblique, pointing downwards, postero-ventral sinuation slightly pronounced, anterior margin rounded. Externally with about 20 equidistant concentric lamellae with fine concentric growth lines between them. Micro-pits present on umbo (size: $\bar{x} = 3$ µm ± 0.9 *SD*) and restricted to distal surface of concentric lamellae on the rostrum (size: $\bar{x} = 2$ µm ± 0.5 *SD*). These pits are unevenly distributed over the shell, being concentrated on the posterior part. Hinge edentulous. Resilifer somewhat oblique.

Distribution of *Myonera limatula* (Dall, 1881)

References	Locality	Depth (m)
Dall (1881)	Type locality: U. S. Coast Survey Steamer 'Blake' sta. 44 [near Tortugas, Florida].	985
Abbott (1974)	off Nantucket, Massachusetts, Florida Strait	985-1000
Poutiers and Bernard (1995)	Northwest and West Central Atlantic.	230-1000
Rosenberg (2005)	USA: Florida: West Florida. 42°N to 25°N.	985-1000
Present study	Campos Basin, Rio de Janeiro, Brazil.	1700

Material examined

IBUFRJ 14798 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [1 valve].

Discussion

The hinge plate of *Myonera limatula* has a lamellar process on the postero-dorsal margin of the right valve (Fig. 1F). This was not mentioned in the original description (Dall 1881: 112-113) but was noted for *Neaera contracta* (Jeffreys, 1881), a junior synonym of *M. limatula*. Jeffreys (1881: 941) noted one laminar tooth on the posterior side of the right valve, extending parallel to the hinge plate. Dall (1886b: 304) argued that the posterior margin of the right valve is beveled off and is not a tooth, and we agree with this interpretation.

Unlike *Myonera paucistriata*, micro-pits are present in *Myonera limatula*. These micro-pits are restricted to the umbo region (Fig. 1G) and on concentric lamellae, which shows a gradient of distribution of the micro-pits, being very abundant on the rostrum region (Fig. 1H) and vanishing towards the anterior and ventral margins (Fig. 1I). In Campos Basin it was present at 1 of 117 stations where pelecypods occurred.

Myonera sp. (Figs. 2A-E)

Description

Shell small (max. length 3 mm), fragile, inequilateral. Umbo slender, small, closer to anterior end, dorsal margin straight, rostrum long, postero-ventral sinuation absent, anterior margin rounded. Ornamented from anterior to posterior ends with seven equally spaced concentric continuous ridges, which are present from the middle to the ventral margin of the shell. Hardly visible concentric growth striae. Micro-pits (size: $\bar{x} = 3$ µm ± 0.5 *SD*) are abundant at umbo and less numerous at rostrum, but vanish toward antero-ventral margin and are absent from the ridges of shell. Hinge edentulous. Resilifer elongated, posteriorly pointed.

Distribution

Restricted to Campos Basin, Rio de Janeiro state, Brazil.

Material examined

IBUFRJ 14795 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [1 spec.].

Discussion

A very rare taxon, only one specimen was found. Although we strongly suspect that it is new to science, a formal epithet will be delayed until additional material is collected.

Myonera sp. can be distinguished from all other Atlantic species by a unique set of characters: the straight dorsal margin and the narrow length of the dorso-ventral axis, which is the smallest among the known Atlantic species. The most similar species in the South Atlantic is *Myonera alleni* Poutiers, 1995 (figured at Allen and Morgan 1981: 472, fig. 35 as *Myonera atlantica*). *Myonera* sp. can be distinguished from *M. alleni* by, in the former, the absence of postero-ventral sinuation and rostral ridge, antero-dorsal margin straight (Fig. 2A). Often *M. alleni* shows incomplete concentric ridges, whereas in *Myonera* sp. these are always complete from the

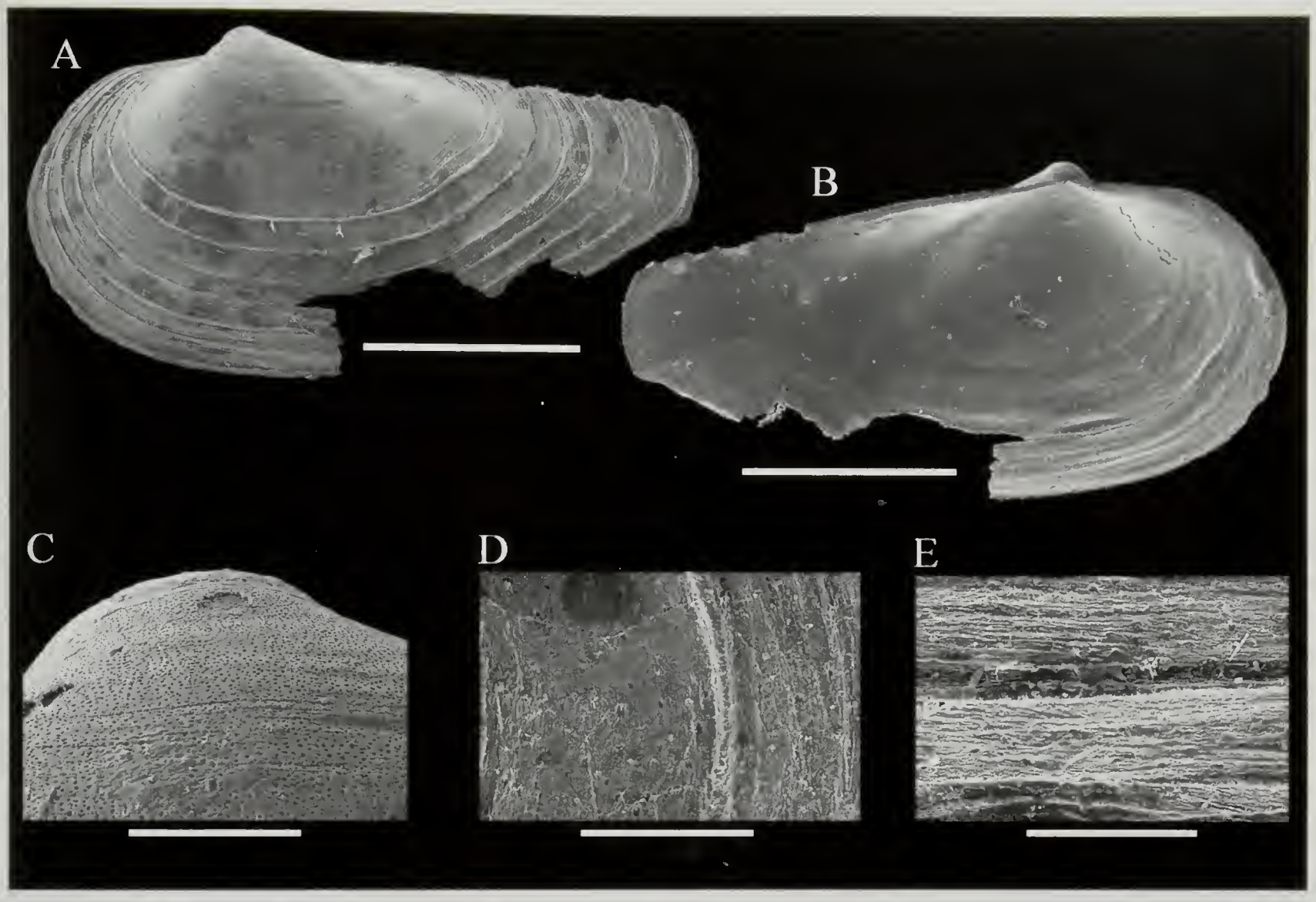

Figure 2. A-E, *Myonera* sp. IBUFRJ 14795. A, external view; B, internal view; C, umbo detail; D, rostrum detail; E, anterior margin detail. Scale bar A-B: 1 mm; C-E: 100 μm.

anterior to the posterior end, including the rostral region (Fig. 2A).

The micro-pits of *Myonera limatula* show an opposite distribution to those of *Myonera* sp. In the former species the micro-pits are present on the umbo but are restricted to the distal margins of the concentric lamellae on the remainder of the shell. In *Myonera* sp. they occur, except on the concentric lamellae, over the umbo region (Fig. 2C) and over the rostrum region (Fig. 2D) vanishing towards the antero-ventral margin (Fig. 2E). Since *Myonera* sp. is a bit smaller than *M. limatula*, such differences in pit distribution might be explained by the intrinsic differences among growth stages, but when one compares the same shell regions of both species it is clear that such differences are not related to growth. In addition, the material of *M. limatula* and *Myonera* sp. is not worn, so such differences cannot be attributed to preservation stage of the shell either.

Myonera kaiwa sp. nov. (Figs. 3A-I)

Description

Shell white, small (max. length 5 mm), elongate, inequilateral, rostrate, inflated. Umbo large, closer to anterior end, rostrum slender, elongate, gently curved dorsally, postero-ventral margin slightly sinuate, anterior margin well rounded. Ornamented with about five concentric foliaceous lamellae, and countless growth lines between them. Rostral ridge present, with a secondary ridge parallel to the dorsal margin usually visible, with growth scars and about seven faint longitudinal striae formed by the periostracum, more conspicuous at the rostrum end. Micro-pits absent on lamellae, but present over entire shell. The micro-pits are more abundant and larger (size: $\bar{x}$ = 10 μm ± 2.8 *SD*) on the rostrum, decreasing in number and in size (size: $\bar{x}$ = 5 μm ± 0.6 *SD*) towards the anterior margin. Hinge edentulous. Resilifer elongate, deflected, posteriorly pointed.

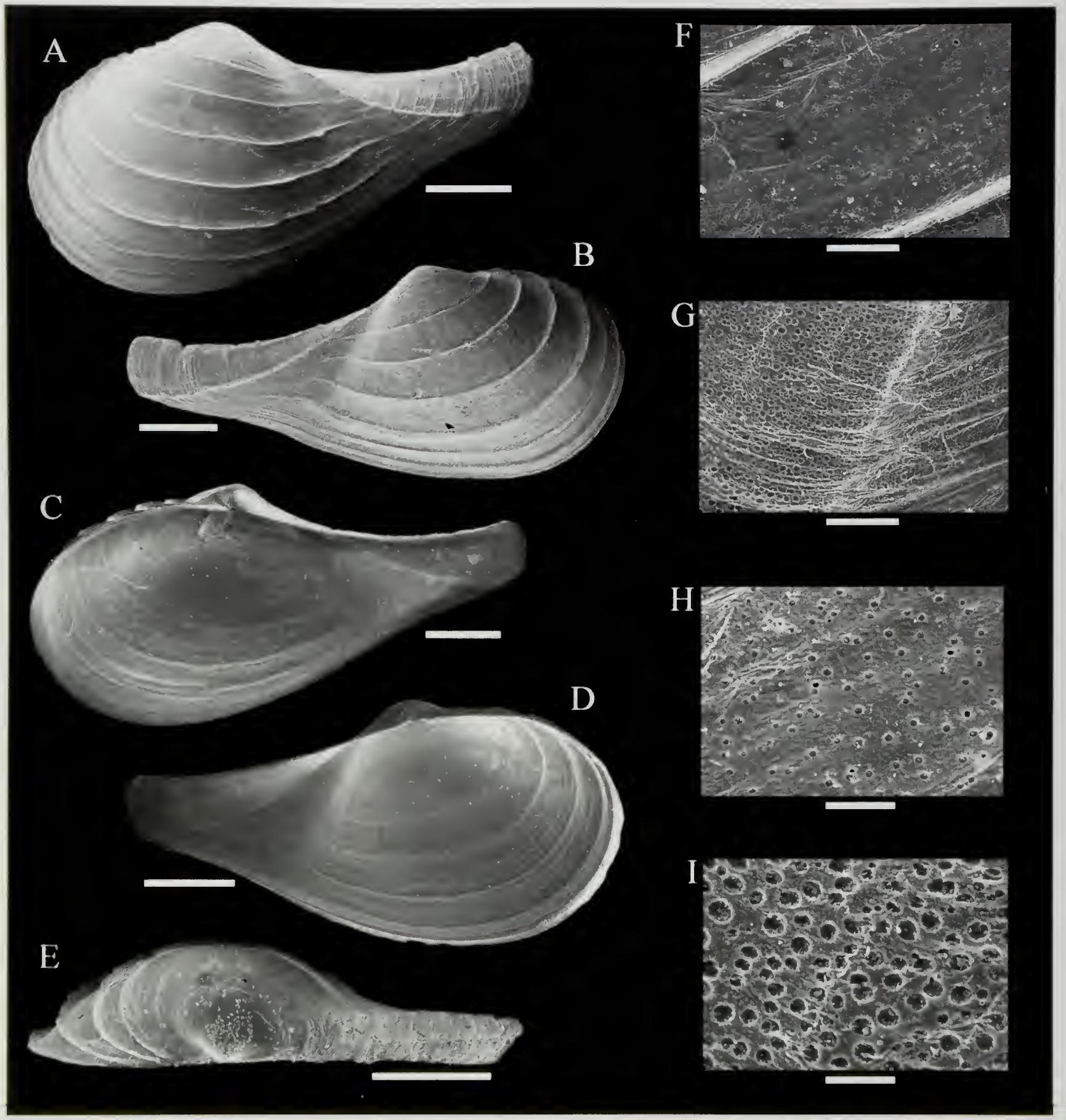

Figure 3. A-I, *Myonera kaiwa* sp. nov. A, external view, left valve. Holotype IBUFRJ 17923; B, external view, right valve. Paratype IBUFRJ 17934; C, internal view, right valve. Paratype IBUFRJ 17933; D, internal view, left valve. Paratype IBUFRJ 17932; E, dorsal view. Paratype IBUFRJ 17886. F-I, Paratype IBUFRJ 14799. F, anterior margin; G, rostrum detail; H, anterior margin detail; I, rostrum detail. Scale bar A-E: 1 mm; F-G: 100 μm; H-I: 40 μm.

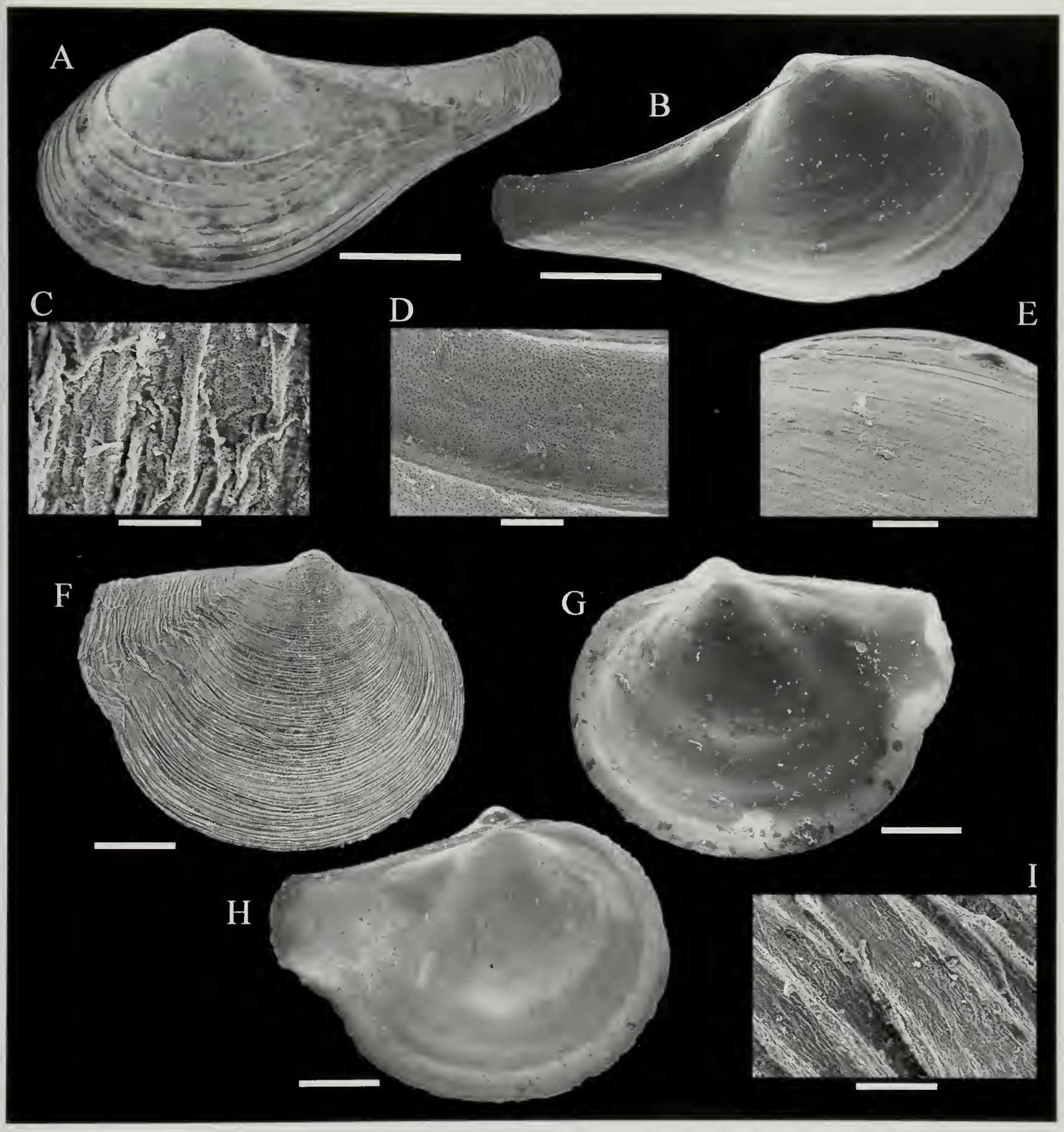

Figure 4. A-E, *Octoporia octaporosa* (Allen and Morgan, 1981). A-B, IBUFRJ 14805. A, external view; B, internal view; C, rostrum detail. IBUFRJ 14802; D, anterior margin detail. IBUFRJ 14800; E, umbo detail. IBUFRJ 14804. F-I, *Protocuspidaria verityi* Allen and Morgan, 1981. F-G, I, IBUFRJ 14998. F, external view; G, internal view, right valve; H, internal view, left valve. IBUFRJ 17896; I, anterior margin detail. Scale bar A-B: 1 mm; C, E: 25 μm; D, I: 50 μm; F-H: 500 μm.

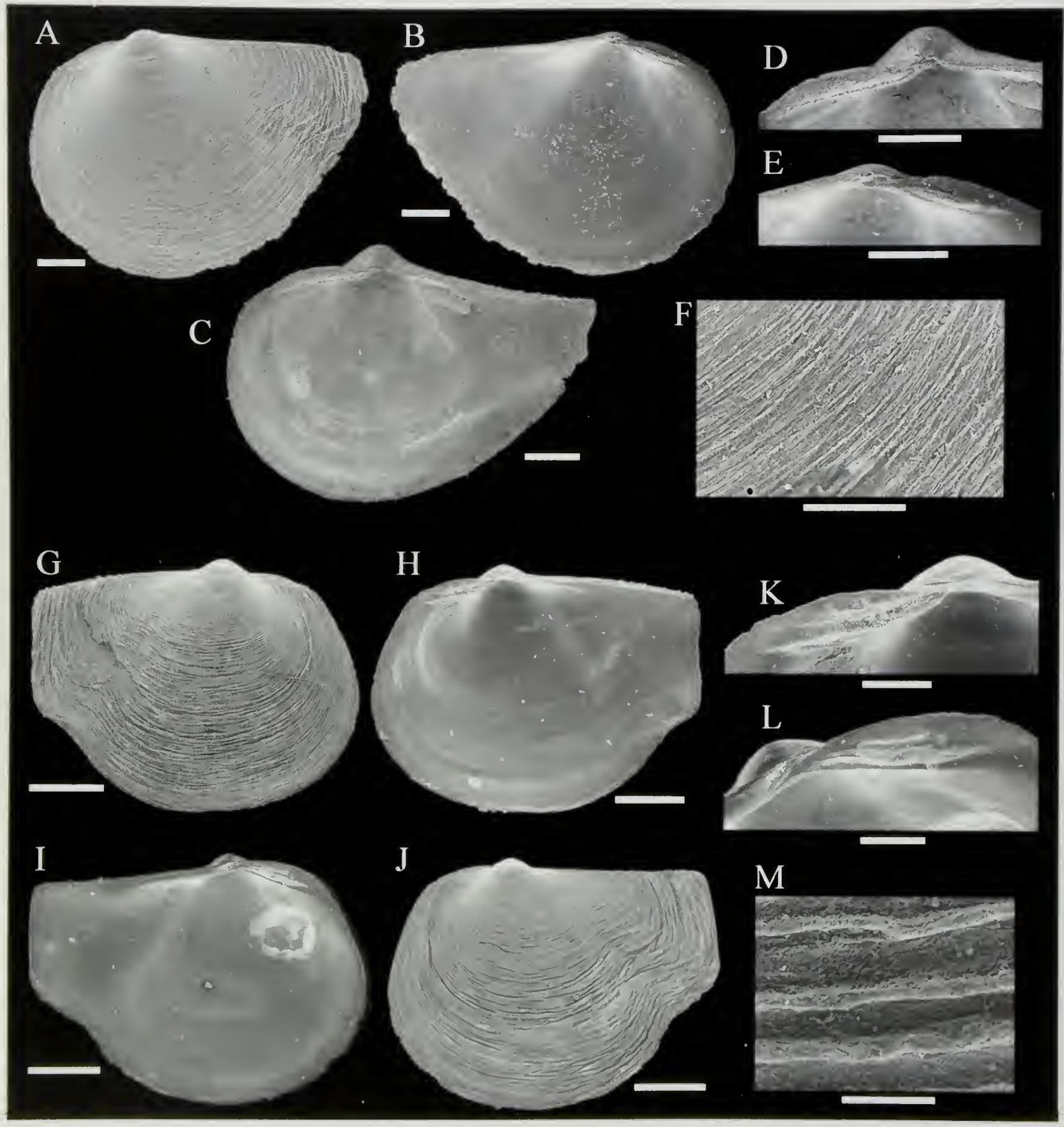

Figure 5. A-F, *Protocuspidaria atlantica* Allen and Morgan, 1981. A-B, E-F, IBUFRJ 14997. C-D, IBUFRJ 18006. A, external view; B-C, internal view from left and right valves, respectively; D-E, hinge detail; F, anterior margin detail. G-M, *Protocuspidaria jaranara* sp. nov. Holotype IBUFRJ 14996. G, J, external view from right and left valves, respectively; H-I, internal view from right and left valves, respectively; K-L, hinge detail; M, anterior margin detail. Scale bar C-D, G-J: 500 μm; A-B, E, K-L: 200 μm; F, M: 50 μm.

Etymology

This species is named in honor of the Kaiwa Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Distribution

Restricted to Campos Basin, Rio de Janeiro state, Brazil.

Holotype

IBUFRJ 17923 (21°52′41″S, 39°46′17″W, 1650 m), 26.VI.03, [left valve].

Paratypes

IBUFRJ 17934 (21°52′41″S, 39°46′17″W, 1650 m), 26. VI.03, [1 valve], IBUFRJ 17933 (21°52′41″S 39°46′17″W, 1650 m), 26.VI.03, [1 valve], IBUFRJ 17932 (21°52′41″S, 39°46′17″W, 1650 m), 26.VI.03, [1 valve], IBUFRJ 17886 (22°05′11″S, 39°42′40″W, 1930 m), 08.V.02, [1 valve], IBUFRJ 14799 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [1 valve], MNRJ 12859 (22°36′03″S, 39°57′54″W, 1650 m), 16.XI.02, [2 valves], MZUSP 40595 (22°04′45″S, 39°46′31″W, 1650 m), 27.VI.03, [2 valves], MNHN (22°37′02″S, 39°56′20″W, 1950 m), 13.VI.03, [2 valves], MNHN (22°03′27″S, 39°45′07″W, 1730 m), 08.V.02, [3 valves].

Other material examined

IBUFRJ 17879 · (22°09′10″S, 39°44′50″W, 1930 m), 08.V.02, [15 valves], IBUFRJ 17885 (22°06′52″S, 39°44′13″W, 1930 m), 08.V.02, [6 valves], IBUFRJ 17887 (22°05′11″S, 39°42′40″W, 1930 m), 08.V.02, [7 valves], IBUFRJ 17904 (22°01′16″S, 39°43′44″W, 1950 m), 25.XI.02, [4 valves], IBUFRJ 17909 (22°04′44″S, 39°46′31″W, 1650 m), 24.XI.02, [5 valves], IBUFRJ 17916 (21°57′26″S, 39°40′33″W, 1950 m), 11.XII.02, [6 valves], IBUFRJ 17917 (21°52′44″S, 39°40′45″W, 1950 m), 11.XII.02, [4 valves], IBUFRJ 17940 (21°52′43″S, 39°40′41″W, 1950 m), 26.VI.03, [1 valve], IBUFRJ 17950 (22°41′35″S, 40°00′45″W, 1950 m), 22.XI.02, [5 valves], IBUFRJ 17988 (21°57′26″S, 39°40′34″W, 1950 m), 27.VI.03, [5 valves], IBUFRJ 18000 (22°33′08″S, 39°54′21″W, 1950 m), 15.VI.03, [3 valves], IBUFRJ 18011 (22°41′31″S, 40°00′47″W, 1950 m), 06.XII.03, [7 valves], IBUFRJ 18014 (22°30′34″S, 9°51′44″W, 1950 m), 16.VI.03, [7 valves].

Discussion

The most similar species in the South Atlantic is *Myonera alleni*. *Myonera kaiwa* sp. nov. can be distinguished from *M. alleni*, and also from *Myonera* sp. (this study) by less-marked sinuation on the postero-ventral margin, a more concave postero-dorsal margin, a longer and more slender rostrum, and the concentric foliaceous lamellae extending to the umbo and complete to the rostral ridge (Figs. 3A-B).

Regarding the micro-pits, *Myonera kaiwa* sp. nov. is the only taxon that shows this set of features: pits restricted to the shell (not occurring on the concentric ridges) (Figs. 3F-G), the largest pits and a size gradient (Figs. 3H-I). In the Campos Basin, it was present at 20 of 117 stations where pelecypods occurred.

Genus *Octoporia* Scarlato and Starobogatov, 1983

Type species: *Cuspidaria* (*Myonera*) *octaporosa* Allen and Morgan, 1981 original designation by Scarlato and Starobogatov (1983)

Genus characterization

Shell small, elongate, inequilateral, rostrum long. Sculptured with growth lines and concentric ribs, these more conspicuous on anterior part. Hinge edentulous. Septum with 8-20 pairs of pores. (Adapted from Allen and Morgan 1981, Scarlato and Starobogatov 1983, Poutiers and Bernard 1995).

Discussion

The genus *Octoporia* includes species that resemble the shells of *Myonera* but shows anatomical similarities with *Halonympha* Dall, 1886 (Poutiers and Bernard 1995). The name *Octoporia* was introduced by Scarlato and Starobogatov (1983) as a genus with only one known species, *Cuspidaria* (*Myonera*) *octaporosa* (Allen and Morgan, 1981), in their new family Halonymphidae, which accommodates species with 8-20 pairs of septal pores. Subsequently, Krylova (1994) revised *Octoporia*, describing new species.

Currently, the family Hanonymphidae is not generally recognized [except by Scarlato and Starobogatov (1983) and Krylova (1994)] but the name *Octoporia* was accepted as a genus by Poutiers and Bernard (1995) and as a subgenus of *Halonympha* by Harper *et al.* (2006), in both cases, as a taxonomic category in Cuspidariidae.

Octoporia octaporosa (Allen and Morgan, 1981) (Figs. 4A-E)

Cuspidaria (*Myonera*) *octaporosa* Allen and Morgan, 1981: 476-479, figs. 40-41

Octoporia octaporosa: Scarlato and Starobogatov 1983 translated *in* Poutiers and Bernard 1995: 176; Krylova 1994: 40

Characterization

Shell white, small (max. length 5 mm), elongate, inequilateral, rostrate. Umbo small, triangular, blunt, centralized, postero-dorsal margin concave, rostrum slender, faintly postero-ventral sinuation, anterior margin rounded. Ornamentation varying from almost smooth to covered by five or more slender concentric ribs, usually present from ventral margin to middle of shell. Concentric growth lines may be present over entire shell but are much more conspicuous on rostrum. Rostral ridge slight. Additional irregular radial lines barely

visible on rostrum. Micro-pits equally distributed over entire shell and lamellae, but smaller (size: $\bar{x}$ = 0.5 µm ± 0.1 *SD*) on the umbo and on rostrum and larger on the anterior margin (size: $\bar{x}$ = 1 µm ± 0.6 *SD*). Hinge edentulous. Resilifer not visible in present material.

Distribution of *Octoporia octaporosa* (Allen and Morgan, 1981)

References	Locality	Depth (m)
Allen and Morgan (1981)	Type locality: Atlantis II, sta. 92, 36°20.0′N, 67°56.0′W.	4800
	Other material: 37°24.0′N to 0.0°46.0′S; 69°26.2′W to 29°28.0′W.	3459-5000
Present study	Campos Basin, Rio de Janeiro, Brazil.	900-1950

Material examined

IBUFRJ 17857 (22°03′03″S, 39°50′32″W, 1230 m), 13.V.02, [5 valves], IBUFRJ 17863 (22°06′58″S, 39°48′41″W, 1330 m), 09.V.02, [5 valves], IBUFRJ 17869 (22°03′27″S, 39°45′07″W, 1730 m), 08.V.02, [19 valves], IBUFRJ 17870 (22°05′45″S, 39°45′55″W, 1730 m), 09.V.02, [12 valves], IBUFRJ 17875 (22°08′23″S, 39°46′23″W, 1730 m), 09.V.02, [12 valves], IBUFRJ 17881 (22°09′10″S, 39°44′50″W, 1930 m), 08.V.02, [37 valves], IBUFRJ 17882 (22°06′52″S, 39°44′13″W, 1930 m), 08.V.02, [15 valves], IBUFRJ 17889 (22°05′11″S, 39°42′40″W, 1930 m), 08.V.02, [29 valves], IBUFRJ 17890 (22°38′01″S, 40°17′26″W, 900 m), 18.V.02, [1 valve], IBUFRJ 17895 (22°41′18″S, 40°14′05″W, 1100 m), 15.V.02, [4 valves], IBUFRJ 17900 (22°11′04″S, 39°47′04″W, 1650 m), 25.XI.02, [11 valves], IBUFRJ 17901 (22°11′16″S, 39°43′44″W, 1950 m), 25.XI.02, [14 valves], IBUFRJ 17910 (22°04′44″S, 39°46′31″W, 1650 m), 24.XI.02, [10 valves], IBUFRJ 17907 (22°04′46″S, 39°43′02″W, 1950 m), 24.XI.02, [8 valves], IBUFRJ 17915 (21°57′26″S, 39°40′33″W, 1950 m), 11.XII.02, [13 valves], IBUFRJ 17922 (21°52′41″S, 39°46′17″W, 1650 m), 11.XII.02, [34 valves], IBUFRJ17958 (22°46′59″S, 40°07′49″W, 1650 m), 22.XI.02, [9 valves], IBUFRJ 17981 (22°38′53″S, 40°04′14″W, 1350 m), 23.XI.02, [1 valve], IBUFRJ 18003 (22°41′03″S, 40°02′29″W, 1650 m), 23.XI.02, [23 valves], IBUFRJ 17947 (22°41′35″S, 40°00′45″W, 1950 m), 22.XI.02, [75 valves], IBUFRJ 17985 (22°31′28″S, 40°03′50″W, 1050 m), 19.XI.02, [16 valves], IBUFRJ 17966 (22°36′03″S, 39°57′54″W, 1650 m), 16.XI.02, [7 valves], IBUFRJ 17964 (22°37′02″S, 39°56′20″W, 1950 m), 23.XI.02, [22 valves], IBUFRJ 18021 (22°24′31″S, 39°57′28″W, 1050 m), 20.XI.02, [6 valves], IBUFRJ 17995 (22°27′18″S, 39°54′50″W, 1350 m), 17.XI.02, [2 valves], IBUFRJ 17930 (22°30′35″S, 39°51′45″W, 1950 m), 23.XI.02, [32 valves], IBUFRJ 14800 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [1 valve], IBUFRJ 14801 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [4 valves], IBUFRJ 14802 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [4 valves], IBUFRJ 14803 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [11 valves], IBUFRJ 14804 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [5 valves], IBUFRJ 14805 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [4 valves], IBUFRJ 14806 (21°57′05″S, 39°49′58″W, 1200 m), 24.IX.2001 [1 valve], IBUFRJ 18022 (22°33′10″S, 39°54′22″W, 1950 m), 23.XI.02, [72 valves], IBUFRJ 17959 (22°10′53″S, 39°52′18″W, 1050 m), 01.VII.03, [4 valves], IBUFRJ 17978 (22°11′04″S, 39°47′04″W, 1650), 22.VI.03, [10 valves], IBUFRJ 17990 (22°11′16″S, 39°43′44″W, 1950 m), 22.VI.03, [18 valves], IBUFRJ 18024 (22°04′33″S, 39°52′05″W, 1050 m), 30.VI.03, [2 valves], IBUFRJ 18023 (22°04′43″S, 39°49′09″W, 1350 m), 25.VI.03, [1 valve], IBUFRJ 17953 (22°04′45″S, 39°46′31″W, 1650 m), 27.VI.03, [2 valves], IBUFRJ 17996 (22°04′45″S, 39°41′58″W, 1950 m), 27.VI.03, [15 valves], IBUFRJ 17938 (21°57′15″S, 39°47′41″W, 1650 m), 28.VI.03, [7 valves], IBUFRJ 17989 (21°57′26″S, 39°40′34″W, 1950 m), 27.VI.03, [29 valves], IBUFRJ 18012 (21°52′51″S, 39°48′12″W, 1350 m), 26.VI.03, [2 valves], IBUFRJ 17931 (21°52′41″S, 39°46′17″W, 1650 m), 26.VI.03, [27 valves], IBUFRJ 17939 (21°52′43″S, 39°40′41″W, 1950 m), 26.VI.03, [56 valves], IBUFRJ 17974 (22°48′05″S, 40°06′38″W, 1950 m), 06.XII.03, [25 valves], IBUFRJ 17926 (22°41′10″S, 40°02′20″W, 1650 m), 13.VI.03, [6 valves], IBUFRJ 18004 (22°34′05″S, 40°00′12″W, 1350 m), 15.VI.03, [2 valves], IBUFRJ 17946 (22°36′12″S, 39°58′22″W, 1650 m), 13.VI.03, [16 valves], IBUFRJ 18007 (22°28′46″S, 39°53′27″W, 1650 m), 17.VI.03, [8 valves], IBUFRJ 17977 (22°31′37″S, 39°55′14″W, 1650 m), 16.VI.03, [2 valves], MNHN (22°31′28″S, 40°03′49″W, 1050 m), 18. VI.03, [17 valves].

Discussion

This species may show variation in rostrum shape and features of ornamentation. The elongated rostrum is normally concave, pointing dorsally (Figs. 4A-B) but some specimens show the rostrum almost straight. The shell ornamentation grades from almost smooth to about 9 concentric ribs. We suspect that the shell illustrated by Poutiers (1984: 291, fig. 4) as *Cuspidaria* sp. is in fact *Octoporia octaporosa*, but only direct examination of this shell could resolve this matter.

This species exhibits a simple pattern of distribution of micro-pits. Though smaller on rostrum (Fig. 4C) and on the lamellae, specifically on the distal ends (Fig. 4D), the micro-pits are dispersed over the entire shell (Figs. 4C-E). Since there is no information about micro-pits on *Octoporia* spp. and because *Octoporia octaporosa* is the only species of the genus *Octoporia* studied herein, it is not possible to compare this pattern of distribution of micro-pits with other congeneric species. This species was quite abundant

in our samples (743 valves), and in the Campos Basin it was present at 49 of 117 stations where pelecypods occurred.

Genus *Protocuspidaria* Allen and Morgan, 1981
Type species: *Protocuspidaria verityi* Allen and Morgan, 1981 by original designation in Allen and Morgan (1981)

Genus characterization

Shell small, rounded, equivalve, inequilateral, rostrate, laterally compressed. Umbo small, hemispherical, closer to anterior end. Rostrum very short, with variable width. Postero-ventral sinuation with individual variation, anterior margin rounded, and postero-dorsal margin nearly straight. Shell surface covered by countless striae, more conspicuous toward ventral margin and rostrum. Hinge edentulous, or with anterior lateral tooth on one or both valves. Resilifer small, central. Septum thin, membranous, with no muscle attachments to the shell (Adapted from Allen and Morgan 1981, Krylova 1995, Poutiers and Bernard 1995).

Discussion

This genus poses a great challenge, in part because, as pointed out by Allen and Morgan (1981: 495), "All species have a similar external appearance, in that they are small, rounded, laterally flattened with a very short rostrum", and still according to Allen and Morgan (1981: 500), "there is sufficient variation between individuals to make identification other than by reference to dentition extremely difficult."

The genus *Protocuspidaria* was established by Allen and Morgan (1981), with species showing quite variable outlines. Three subgenera are characterized by the presence or absence of hinge teeth, or by which valve bears the teeth. Accordingly, the subgenus *Protocuspidaria* is characterized by an anterior tooth only on the right valve, the subgenus *Edentaria* Allen and Morgan, 1981 is characterized by a hinge devoid of teeth on both valves, and the subgenus *Bidentaria* Allen and Morgan, 1981 is characterized by an anterior tooth on both valves (Allen and Morgan 1981: 495, 497, and 499, respectively). Poutiers and Bernard (1995) stated that this subgeneric division does not represent this group in a realistic way. According to them, this scheme of separation based on the hinge structure fails to distinguish each group, but in spite of that they keep the subgeneric division proposed by Allen and Morgan (1981).

Scarlato and Starobogatov (1983) proposed the superfamily Protocuspidarioidea and the family Protocuspidariidae, with each subgenus of Allen and Morgan (1981) raised to genus level. Subsequently, Poutiers (1984: 295, fig. 6a-b) described *Protocuspidaria* (*Edentaria*) *thomassini* and Krylova (1995) added 10 new species but recognized the family Protocuspidariidae with only two genera: *Protocuspidaria* (with three subgenera: *Protocuspidaria*, *Bidentaria*, and *Edentaria*) diagnosed by the presence of 7 tentacles on the siphon border and *Multitentaculata* Krylova, 1995 that shows between 7 and 33 tentacles on the siphon border. Besides that, Krylova (1995) subdivided *Multitentaculata* according to the hinge. The subgenus *Multitentaculata s.s.* lacks teeth, whereas the subgenus *Dentaria* would be characterized by the presence of an anterior lateral tooth on the right valve. According to Krylova (1995: 34) "the tentacles number at siphon border is, at time, the unique distinguishing morphological character between the subgenus *Protocuspidaria* and *Multitentaculata*." At the same time, Poutiers and Bernard (1995) and Morton (2003: 378, table 3) did not recognizes the family Protocuspidariidae.

Because there are intense discussions about the phylogeny of Septibranchia (Dreyer *et al.* 2003, Harper *et al.* 2006) and because of the lack of taxonomic evidence to maintain the higher-rank level of *Protocuspidaria*, we prefer to follow the authors that retain *Protocuspidaria* at the genus level (*e.g.*, Allen and Morgan 1981, Poutiers and Bernard 1995) although some divisions may prove to be useful in the future. Micro-pits were absent in all species examined herein.

Protocuspidaria (*Protocuspidaria*) *verityi* Allen and Morgan, 1981 (Figs. 4F-I)
Protocuspidaria (*P.*) *verityi* Allen and Morgan, 1981: 496-497, figs. 61-62; Krylova, 1995: 31.

Characterization

Shell white, small (max. recorded length 5 mm), rounded, inequilateral, rostrate. Umbo small, closer to anterior end. Anterior margin rounded, usually giving rise to a plateau immediately next to the umbo. Rostrum truncate, usually short, variable in height. Postero-ventral sinuation with individual variation. Dorsal margin straight. Shell surface covered by countless striae. Micro-pits absent. Hinge with anterior lateral tooth only on right valve. Resilifer small, central.

Distribution of *Protocuspidaria (Protocuspidaria) verityi* Allen and Morgan, 1981

References	Locality	Depth (m)
Allen and Morgan (1981)	Type locality: Atlantis II, sta. 167, 7°58.0′S, 34°17.0′W to 7°50.0′S, 34°17.0′W.	943-1007
	Other material: 47°35.5′N to 36°05.2′S; 11°35.0′E to 68° 31.0′W.	943-4706
Present study	Campos Basin, Rio de Janeiro, Brazil.	750-1950

Material examined

IBUFRJ 17854 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.01, [1 valve], IBUFRJ 17859 (22°07′17″S, 39°50′02″W, 1230 m), 13.V.02, [5 valves], IBUFRJ 17861 (22°06′58″S, 39°48′41″W, 1330 m), 09.V.02, [3 valves], IBUFRJ 17873 (22°05′45″S, 39°45′55″W, 1730 m), 09.V.02, [4 valves], IBUFRJ 17878 (22°09′10″S, 39°44′50″W, 1930 m), 08.V.02, [2 valves], IBUFRJ 17893 (22°33′31″S, 40°12′05″W, 900 m), 18.V.02, [2 valves], IBUFRJ 17896 (22°39′34″S, 40°08′22″W, 1200 m), 15.V.02, [1 valve], IBUFRJ 17897 (22°10′54″S, 39°52′19″W, 1050 m), 10.XII.02, [7 valves], IBUFRJ 17902 (22°11′16″S, 39°43′44″W, 1950 m), 25.XI.02, [3 valves], IBUFRJ 17905 (22°04′43″S, 39°49′08″W, 1350 m), 24.XI.02, [1 valve], IBUFRJ 17906 (22°04′46″S, 39°43′02″W, 1950 m), 24.XI.02, [2 valves], IBUFRJ 17918 (21°52′44″S, 39°40′45″W, 1950 m), 11.XII.02, [3 valves], IBUFRJ 17941 (21°52′43″S, 39°40′41″W, 1950 m), 26.VI.03, [2 valves], IBUFRJ 18019 (22°27′31″S, 40°09′23″W, 750 m), 18.VI.03, [3 valves], IBUFRJ 17944 (22°36′12″S, 39°58′22″W, 1650 m), 13.VI.03, [1 valve], IBUFRJ 17956 (22°46′59″S, 40°07′49″W, 1650 m), 22.XI.02, [4 valves], IBUFRJ 17962 (22°10′53″S, 39°52′18″W, 1050 m), 01.VII.03, [1 valve], IBUFRJ 17980 (22°11′04″S, 39°47′04″W, 1650 m), 22.VI.03, [1 valve], IBUFRJ 17963 (22°37′02″S, 39°56′20″W, 1950 m), 23.XI.02, [2 valves], IBUFRJ 17969 (22°28′49″S, 39°53′24″W, 1650 m), 17.XI.02, [1 valve], IBUFRJ 17968 (22°36′03″S, 39°57′54″W, 1650 m), 16.XI.02, [1 valve], IBUFRJ 17982 (22°38′53″S, 40°04′14″W, 1350 m), 23.XI.02, [3 valves], IBUFRJ 17991 (22°11′16″S, 39°43′44″W, 1950 m), 22.VI.03, [1 valve], IBUFRJ 17992 (22°29′33″S, 39°56′17″W, 1350 m), 19.XI.02, [1 valve], IBUFRJ 17997 (22°04′45″S, 39°41′58″W, 1950 m), 27.VI.03, [2 valves], IBUFRJ 17998 (22°37′02″S, 39°56′20″W, 1950 m), 13.VI.03, [2 valves], IBUFRJ 18001 (22°33′08″S, 39°54′21″W, 1950 m), 15.VI.03, [2 valves], IBUFRJ 18016 (22°26′28″S, 39°54′08″W, 1350 m), 21.VI.03, [1 valve], IBUFRJ 18017 (22°24′30″S, 39°57′28″W, 1050 m), 20.VI.03, [2 valves], IBUFRJ 18018 (22°35′04″S, 40°08′53″W, 1050 m), 21.XI.02, [2 valves], IBUFRJ 18020 (21°52′59″S, 39°55′32″W, 750 m), 29.VI.03, [1 valve], IBUFRJ 17949 (22°41′35″S, 40°00′45″W, 1950 m), 22.XI.02, [2 valves], IBUFRJ 17874 (22°08′23″S, 39°46′23″W, 1730 m), 09.V.02, [1 valve], IBUFRJ 17884 (22°06′52″S, 39°44′13″W, 1930 m), 08.V.02, [3 valves], IBUFRJ 17986 (22°31′28″S, 40°03′50″W, 1050 m), 19.XI.02, [4 valves], MNHN (22°03′03″S, 39°50′32″W, 1230 m), 13.V.02, [2 valves].

Discussion

Despite the many individuals examined, no specimen showed micro-pits on any part of the shell (Fig. 4I). Since we cannot find micro-pits at any other *Protocuspidaria* species studied herein, we suppose that the absence of micro-pits might be a character of the generic level. In the Campos Basin it was present at 36 of 117 stations where pelecypods occurred.

Protocuspidaria (Bidentaria) atlantica Allen and Morgan, 1981 (Figs. 5A-F)

Protocuspidaria (*B.*) *atlantica* Allen and Morgan, 1981: 499, figs. 64-67; Krylova, 1995: 33

Characterization

Shell white, small (max. recorded length 5 mm), rounded, inequilateral, rostrate. Umbo small, hemispherical, closer to anterior end. Anterior margin rounded, usually giving rise to a plateau immediately next to the umbo. Rostrum truncate, variable in height. Postero-ventral sinuation with individual variation, from almost inconspicuous to quite accentuated. Dorsal margin straight. Shell surface covered by countless striae. Micro-pits absent. Hinge with anterior lateral tooth on both valves. Resilifer small, central.

Distribution of *Protocuspidaria (Bidentaria) atlantica* Allen and Morgan, 1981

References	Locality	Depth (m)
Allen and Morgan (1981)	Type locality: Discovery, sta. 6696, 28°6.0′N, 13°28.0′W.	1780
	Other material: 46°31.2′N to 28°06.0′N; 66°47.0′W to 10°19.5′W.	1150-4706
Present study	Campos Basin, Rio de Janeiro, Brazil.	900-1950

Material examined

IBUFRJ 14997 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [5 valves], IBUFRJ 14998 (21°57′05″S, 39°49′58″W, 1200 m), 24.IX.2001 [4 valves], IBUFRJ 17858 (22°05′04″S, 39°50′01″W, 1230 m), 09.V.02, [4 valves], IBUFRJ 17864 (22°04′52″S, 39°49′04″W, 1330 m), 09.V.02, [2 valves], IBUFRJ 17891 (22°38′01″S, 40°17′26″W, 900 m), 18.V.02, [2 valves], IBUFRJ 17894 (22°37′54″S, 40°13′36″W, 1000 m), 19.V.02, [3 valves], IBUFRJ 17921 (21°52′41″S, 39°46′17″W, 1650 m), 11.XII.02, [3 valves], IBUFRJ 17928 (22°41′10″S, 40°02′20″W, 1650 m), 13.VI.03, [2 valves], IBUFRJ 17929 (22°30′35″S, 39°51′45″W, 1950 m), 23.XI.02, [1 valve], IBUFRJ 17937 (21°57′15″S, 39°47′41″W, 1650 m), 28.VI.03, [2 valves], IBUFRJ 17945 (22°36′12″S, 39°58′22″W, 1650 m), 13.VI.03, [1 valve], IBUFRJ 17961 (22°10′53″S, 39°52′18″W, 1050 m), 01.VII.03, [7 valves], IBUFRJ 17965 (22°37′02″S, 39°56′20″W, 1950 m), 23.XI.02, [1 valve], IBUFRJ 17970 (22°28′49″S, 39°53′24″W, 1650 m), 17.XI.02, [1 valve], IBUFRJ 17973 (22°48′05″S, 40°06′38″W, 1950 m), 06.XII.03, [3 valves], IBUFRJ 17975 (22°31′37″S, 39°55′14″W, 1650 m), 16.VI.03, [3 valves], IBUFRJ 17983 (22°38′53″S, 40°04′14″W, 1350 m),

23.XI.02, [3 valves], IBUFRJ 17984 (22°31′28″S, 40°03′50″W, 1050 m), 19.XI.02, [2 valves], IBUFRJ 17987 (21°57′26″S, 39°40′34″W, 1950 m), 27.VI.03, [2 valves], IBUFRJ 17993 (22°29′33″S, 39°56′17″W, 1350 m), 19.XI.02, [3 valves], IBUFRJ 17994 (22°27′18″S, 39°54′50″W, 1350 m), 17.XI.02, [3 valves], IBUFRJ 18002 (22°41′03″S, 40°02′29″W, 1650 m), 23.XI.02, [3 valves], IBUFRJ 18005 (22°34′05″S, 40°00′12″W, 1350 m), 15.VI.03, [1 valve], IBUFRJ 18006 (22°28′46″S, 39°53′27″W, 1650 m), 17.VI.03, [1 valve], IBUFRJ 18009 (22°31′28″S, 40°03′49″W, 1050 m), 18.VI.03, [8 valves], IBUFRJ 18013 (21°52′51″S, 39°48′12″W, 1350 m), 26.VI.03, [4 valves], IBUFRJ 18015 (21°52′51″S, 39°48′11″W, 1350 m), 12.XII.02, [5 valves], MNHN (22°41′31″S, 40°00′47″W, 1950 m), 06.XII.03, [2 valves].

Discussion

This species shows the most variable outline and teeth variation in shape, but the presence of the anterior lateral teeth in both valves is diagnostic (Figs. 5D-E). These teeth can vary in their degree of development according to the size of the specimen (*e.g.*, Allen and Morgan 1981, Poutiers and Bernard 1995), but this kind of variation, or expression, lacks taxonomic significance. Like all other species of this genus, no micro-pits were observed, even at high magnification (Fig. 5F). In the Campos Basin, it was present at 28 of 117 stations where pelecypods occurred.

Protocuspidaria (*Bidentaria*) *jarauara* sp. nov. (Figs. 5G-M)
Myonera aff. *ruginosa auct. non* Jeffreys, 1881: Absalão *et al.*, 2003: 327, figs. 10-11

Description

Shell white, small (max. recorded length 5 mm), rounded, inequilateral, rostrate, laterally compressed. Umbo small, closer to anterior end. Anterior margin rounded, an anterior plateau present immediately next to the umbo. Rostrum large, truncate. Ventral and dorsal margins of the rostrum subparallel. Shell surface covered by countless striae. Micro-pits absent. Hinge with bifid anterior lateral tooth on both valves. Resilifer small, central.

Etymology

This species is named in honor of the Jarauara Indians, one of the indigenous peoples of Brazil. The name is employed as a noun in apposition.

Distribution

Restricted to Campos Basin, Rio de Janeiro state, Brazil.

Holotype

IBUFRJ 14996 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [1 spec.].

Paratype

IBUFRJ 17888 (22°05′11″S, 39°42′40″W, 1930 m), 08.V.02, [2 valves], MNRJ 12860 (22°10′54″S, 39°48′59″W, 1350 m), 25.VI.03, [2 valves], MZUSP 40957 (22°04′45″S, 39°46′31″W, 1650 m), 27.VI.03, [2 valves], MNHN (22°10′55″S, 39°49′00″W, 1350 m), 10.XII.02, [2 valves], MNHN (21°52′44″S, 39°40′45″W, 1950 m), 11.XII.02, [2 valves].

Other material examined

IBUFRJ 17862 (22°06′58″S, 39°48′41″W, 1330 m), 09.V.02, [1 valve], IBUFRJ 17872 (22°05′45″S, 39°45′55″W, 1730 m), 09.V.02, [4 valves], IBUFRJ 17876 (22°08′23″S, 39°46′23″W, 1730 m), 09.V.02, [1 valve], IBUFRJ 17877 (22°09′10″S, 39°44′50″W, 1930 m), 08.V.02, [1 valve], IBUFRJ 17883 (22°06′52″S, 39°44′13″W, 1930 m), 08.V.02, [5 valves], IBUFRJ 17892 (22°38′01″S, 40°17′26″W, 900 m), 18.V.02, [1 valve], IBUFRJ 17898 (22°10′55″S, 39°49′00″W, 1350 m), 10.XII.02, [1 valve], IBUFRJ 17903 (22°11′16″S, 39°43′44″W, 1950 m), 25.XI.02, [1 valve], IBUFRJ 17908 (22°04′46″S, 39°43′02″W, 1950 m), 24.XI.02, [1 valve], IBUFRJ 17913 (21°57′15″S, 39°47′43″W, 1650 m), 14.XII.02, [1 valve], IBUFRJ 17914 (21°57′26″S, 39°40′33″W, 1950 m), 11.XII.02, [1 valve], IBUFRJ 17920 (21°52′41″S, 39°46′17″W, 1650 m), 11.XII.02, [1 valve], IBUFRJ 17927 (22°41′10″S, 40°02′20″W, 1650 m), 13.VI.03, [1 valve], IBUFRJ 17942 (21°52′43″S, 39°40′41″W, 1950 m), 26.VI.03, [2 valves], IBUFRJ 17943 (22°36′12″S, 39°58′22″W, 1650 m), 13.VI.03, [1 valve], IBUFRJ 17948 (22°41′35″S, 40°00′45″W, 1950 m), 22.XI.02, [1 valve], IBUFRJ 17955 (22°46′59″S, 40°07′49″W, 1650 m), 22.XI.02, [1 valve], IBUFRJ 17957 (22°46′59″S, 40°07′49″W, 1650 m), 22.XI.02, [3 valves], IBUFRJ 17960 (22°10′53″S, 39°52′18″W, 1050 m), 01.VII.03, [2 valves], IBUFRJ 17971 (22°28′49″S, 39°53′24″W, 1650 m), 17.XI.02, [3 valves], IBUFRJ 17972 (22°48′05″S, 40°06′38″W, 1950 m), 06.XII.03, [1 valve], IBUFRJ 17976 (22°31′37″S, 39°55′14″W, 1650 m), 16.VI.03, [4 valves], IBUFRJ 18028 (22°31′36″S, 39°55′15″W, 1650 m), 16.XI.02, [1 valve], IBUFRJ 18061 (21°58′36″S, 39°46′30″W, 1700 m), 08.X.2001 [1 valve].

Discussion

The diagnostic character of this species is a bifid anterior lateral tooth on both valves (Figs. 5K-L), since this bifid tooth is absent in all other species previously reported in the genus. The presence of this tooth could suggest that a fourth subgenus is present—and still unnamed—if one used exclusively hinge characters to determine the subgenera of *Protocuspidaria*. But, because we do not have any other information about soft parts or any other kind of data beyond the conchological one, we prefer to keep the new species in *Bidentaria*.

Exteriorly, *Protocuspidaria* (*Bidentaria*) *jarauara* sp. nov. could be initially confused with *Protocuspidaria* (*Bidentaria*)

atlantica and *Protocuspidaria* (*Protocuspidaria*) *verityi*, but the hinge differences clearly distinguish the three species.

Absalão *et al.* (2003: 327) has previously assigned *Protocuspidaria* (*Bidentaria*) *jaranara* sp. nov. to the Brazilian coast under the name *Myonera* aff. *ruginosa* Jeffreys, 1881. In fact, the description of the genus *Protocuspidaria* is very similar to the description given for *Myonera ruginosa* Jeffreys (1881: 942, pl. LXXI, fig. 7), which distinguishes *M. ruginosa* by the external surface of the shell covered by narrow concentric striae, a short and truncated rostrum, anterior border rounded, a small prominent umbo, and an anterior tooth on the left valve. So, the identification of *M. ruginosa* to Brazil must be disregarded. The transfer of *M. ruginosa* to the genus *Protocuspidaria* was first suggested by Allen and Morgan (1981: 995) and followed by Krylova (1995: 29).

No micro-pits were observed, even at high magnification (Fig. 5M). In the Campos Basin it was present at 29 of 117 stations where pelecypods occurred.

GENERAL DISCUSSION

Reflecting the difficulties involved in collecting material from deep waters, and despite the efforts of several investigators over the past 30 years (Allen and Turner 1974, Allen and Morgan 1981, Leal and Simone 2000, Absalão *et al.* 2001, 2003, 2005, Simone 2002, 2003, Absalão and Pimenta 2003, 2005, Absalão and Santos 2004, Caetano *et al.* 2006, Simone and Cunha 2006, Zelaya *et al.* 2006, Barros *et al.* 2007, Lima and Barros 2007), the Brazilian deep-water species are essentially unknown. Most (five of eight) of the species reported here were not previously recorded in Brazilian waters. Two species are new to science (*Myonera kaiwa* sp. nov. and *Protocuspidaria jaranara* sp. nov.) and for one taxon, *Myonera* sp., a formal epithet will be delayed until additional material is available. Except for *Myonera paucistriata*, which is probably the most common of all septibranchs at Campos Basin, all others species studied herein have their known range expanded geographically and/or bathymetrically. *Protocuspidaria verityi* though well represented at North Atlantic Ocean, has been scarcely represented at South Atlantic Ocean, with an occurrence gap between the latitudinal coordinates 09° and 36°S. *Myonera limatula* and *Protocuspidaria atlantica* are for the first time recorded in the South Atlantic Ocean and Brazil. *Octoporia octaporosa* had been previously recorded for the South Atlantic Ocean, but we have enlarged its range to the south. Bathymetrically, *O. octaporosa* (900 m), *P. verityi* (750 m), and *P. atlantica* (900 m) show their shallowest record, while *M. limatula* (1700 m) shows its deepest record. These new data show that our understanding of the taxonomic composition and distribution of deep-water pelecypod species inhabiting Brazilian coast is still unsatisfactory.

Under high resolution of a Scanning Electron Microscope (SEM), a character not yet reported for septibranchs was observed: the presence of micro-pits on the shell surface. The distribution of micro-pits found on the species studied here is not random and seems to be a taxonomically useful pattern.

Only the type species of *Octoporia*, *O. octaporosa*, is represented in our samples. This is numerically the most abundant species sampled and, in spite of the variation in the shell ornamentation, which grades from almost smooth to concentrically ribbed, all specimens show the same pattern of distribution of the micro-pits. On *Protocuspidaria*, despite the many individuals and the three species examined in this paper, no micro-pits were observed on any part of the shell. These findings suggest the importance of the micro-pits for taxonomic proposals. For the genus *Myonera*, only the genus type species, *M. paucistriata*, has no micro-pits. The other species studied herein (four taxa) exhibit, each one, a different pattern of distribution of the micro-pits on the shell surface and additional research is needed to establish the potential use of such micro-pits in this taxonomic category.

The micro-pits resembles the "pores" described for polyplacophorans that house the aesthetes and have been observed in other molluscs [*e.g.*, according to Reindl and Haszprunar (1996), in Polyplacophora, *Leptochiton cancellatus* (Sowerby, 1839); Gastropoda, *Diodora graeca* (Linnaeus, 1758); and Pelecypoda, *Arca noae* Linnaeus, 1758)]. The homology of these pits is not established and their probable function is a matter for speculation, with widely differing interpretations. Some authors have suggested that their function is sensory (Baxter *et al.* 1990), or for excretion (Waller 1980), or for maintenance of the periostracum (Baxter *et al.* 1987). The function of such micro-pits for septibranchs is thus still unclear and open to future research.

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X-ray quantitative texture analysis on *Helix aspersa aspera* (Pulmonata) shells selected or not for increased weight

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Abstract: X-ray Quantitative Texture Analysis (QTA) results are examined for the outer aragonitic shell layers of *Helix aspersa aspersa* (Müller, 1774) to probe the relevance of the approach to non-flat surfaces. Two sets of *H. aspersa aspersa* were studied, for a total of 29 samples. Quantitative texture analysis showed that although the nature of the texture present was roughly constant, the textural strength varied significantly among specimens because of biologically inherited surface irregularities. A statistical analysis showed that textural strength exhibited larger standard deviations for snails selected for greater shell weight than for control snails. The *H. aspersa aspersa* aragonite texture is the same as observed in previous studies, with <110> shell growth directions. This texture causes elastic behavior of the mineral part of the shell, which accommodates moderate shear and compression. We furthermore determine that the colored bands at the shell surface were aligned with the <020> crystal directions.

Key words: *Helix* texture, aragonite, shell growth

Quantitative Texture Analysis (QTA) is frequently used to characterize the macroscopic organization of layered crystals in mollusc shells. A high degree of order (or textural strength) has been reported (Hedegaard and Wenk 1998, Chateigner *et al.* 1999), which varies among taxa, with qualitatively identical textures in closely related species (Chateigner *et al.* 2000). In a single specimen, textures can vary with location in the shell, either between different layers as in *Cypraea testudinaria* (Linnaeus, 1758) (Chateigner *et al.* 1996) or in the same layer, *e.g.*, in Pterioida (Zolotoyabko and Quintana 2002, Checa and Rodriguez-Navarro 2005). The organic matrices control the inorganic crystal orientation (Falini *et al.* 1996) and the crystal shapes themselves (Aizenberg *et al.* 1996), but these two traits can be seen as non-redundant characters in terms of phylogeny. For instance, it has been demonstrated that scanning electronic microscopy images can be misleadingly interpreted in terms of orientation (Chateigner *et al.* 2000). QTA has also been proposed to link living species to extinct fossils in the Bivalvia (Chateigner *et al.* 2002).

Two types of QTA have been applied to the Mollusca in the literature, which differ in the radiation used, thereby probing different material scales. While X-ray diffraction was formerly used (Chateigner *et al.* 1996, 1999, 2000, 2002, Hedegaard and Wenk 1998), Electron Backscatter Diffraction (EBSD) has more recently provided a way for local characterization of texture variation in molluscs (Checa *et al.* 2005, Rousseau *et al.* 2005). Dealing with X-ray analysis using whole X-ray diffraction profiles accounts for all the crystallites, even the smallest ones. However, the X-ray beam extends on the specimens' surface for several mm^2 during the measurements. This simple fact alters the results because of the irregular surface of specimens and prevents quantitative results. Only once in the literature have QTA measurements been made on two specimens of the same species, *Helix aspersa*, and these indicated variability in the quantification of the texture although qualitatively, orientations were the same (Chateigner *et al.* 2000).

Quantitative variation in the results (*e.g.*, variation in textural strength) could also come from real variation in specimen textures, for instance due to a growth anomaly, rather than from an artifact from the irregular irradiation of the surface. These two latter effects could also be explained by the natural texture variation inherent to a species reared in different conditions. In Europe, snail production has increased considerably in the last two decades. Snail farming could give rise to modifications of shell growth that could be undesirable from an economical point of view. In particular, rearing larger snails for an increased tissue weight could modify the shell texture because of the faster growth (Dupont-Nivet *et al.* 2000b). In turn, the texture modifications could affect other shell characteristics (mechanical properties, colors, etc.).

Here we statistically analyzed X-ray QTA results of outer aragonitic layers of *Helix aspersa aspersa* shells, obtained under controlled conditions to optimize growth. Our first aim was to examine a potential effect of the selection method used to increase weight on the degree of preferred orientation.

We then determined the distribution of QTA results on equally shaped samples to show how this distribution changed in two lines of animals, one selected for growth and the other not. Both lines were reared in the same environmental conditions. Our second aim was to exemplify how QTA results can vary between individuals when using X-ray investigations of texture, using the usual measurement characteristics. The certification of the methodology allowed further identification of alignment of given crystal axes with specific shell directions, such as in between shell layers and with colored bands.

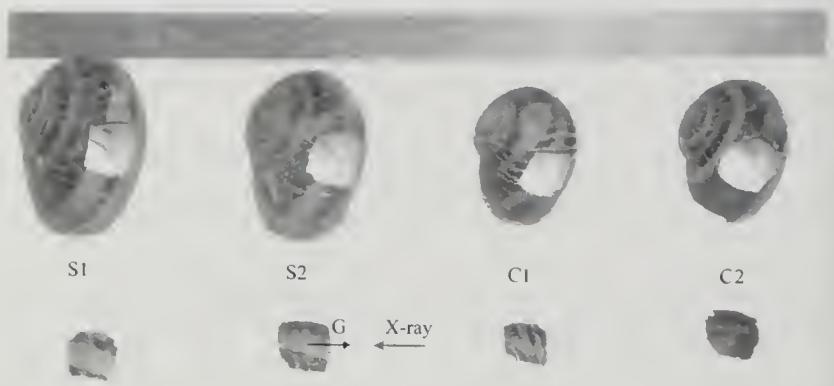

Figure 1. Shell samples used for X-ray measurements. S-specimens come from a line selected for increased weight while C-specimens were control samples. The arrows indicate the growth direction **G** that guides sample positioning with respect to the X-ray beam.

MATERIALS AND METHODS

The specimens of *Helix aspersa aspersa* used in these experiments were from two lines: one selected for increased weight (S) and a control line (C). In the S line, at each generation, the largest snails were chosen for reproduction while in the C line, breeders were chosen at random. The selection procedure is further described in Dupont-Nivet *et al.* (2000b). Animals were all reared in the same room with environmental conditions optimal for growth (density, food, temperature, relative humidity, and photoperiod) as detailed elsewhere (Dupont-Nivet *et al.* 1998, 2000a). Selected snails used in this experiment were from the 7th generation. Their mean weight was 16.98 g versus 10.61 g for the control snails. We collected 'adult' snails, *i.e.*, snails for which the peristome was reflected and, thus, shell growth was completed. Fourteen and fifteen samples were analyzed for the C and S lines, respectively. They were chosen from the whole population available, with weight and age close to the population means, *i.e.*, at a similar growth stage. Shell specimens were all prepared the same day according to the following procedure. Animals were frozen at –18 °C, thawed after one day, and the body manually separated from the shell. Shells were washed with water and air-dried.

For the X-ray QTA experiments, a sample of approx. 1 × 1 cm^2 was cut out the mollusc shells about 0.5 cm from an omnipresent growth irregularity near the macroscopic margin of the shell (Fig. 1). The position of the sample on the diffractometer was as in Chateigner *et al.* (1999). The pole figure plotting was with the projection normal as the **N** direction of the shell, with the **G** and **M** directions respectively vertical and horizontal in the pole figure projection planes (Fig. 2).

The *Helix aspersa aspersa* shell is composed typically of 95% crystallized biogenic aragonite, and of approx. 5% in volume of residual materials, mainly intercrystalline and intracrystalline biomolecules. However, these two latter components are not visible and do not perturb the X-ray diffraction diagrams because of their weak presence and scattering factors and their poor crystallization. Aragonite is one of the three $CaCO_3$ polymorphs and crystallizes in the orthorhombic Pmcn space group with the following cell parameters: a = 4.9611 Å, b = 7.9672 Å, and c = 5.7407 Å for the reference non-biogenic mineral (Pilarti *et al.* 1998).

The X-ray QTA measurements were carried out using a 4-circle diffractometer and a monochromatized Cu-Kα averaged radiation (1.5418 Å) in point focused tube mode (Ricote and Chateigner 2004), with a beam cross-section of 1 × 1 mm^2. The sample was mounted in the center of an Eulerian Cradle (Huber) and rotated in all necessary space directions (at a fixed X-ray incident angle $\omega = 16.64°$, scanning for $0 < \chi < 60°$ and $0 < \phi < 355°$ with 5° steps). Each diagram was acquired for 60 seconds, using the Curved Position Sensitive detector (CPS 120, INEL) which spans all the Bragg diffracted intensities in a 120° 2θ-range at once for all given sample orientations (Fig. 3).

After acquisition, data treatment and QTA involved the so-called "combined analysis" (Chateigner 2004) which used as a first step Rietveld's (1969) refinement of all the 936 resulting diagrams. After this step, integral intensities were

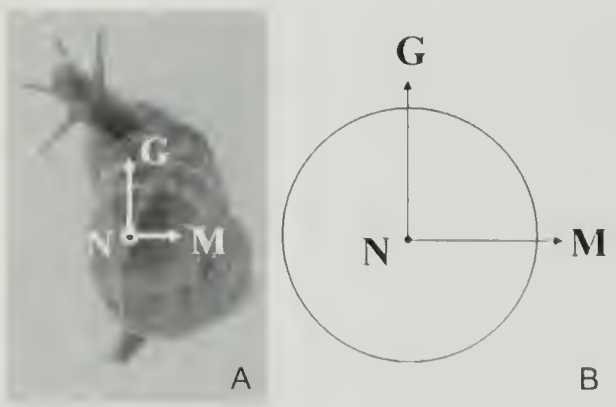

Figure 2. A, Sample reference frame with margin (**M**), growth (**G**), and normal (**N**) directions; B, corresponding pole figure frame.

extracted by the Le Bail extraction procedure (Le Bail *et al.* 1988) and used for quantitative texture analysis with the E-WIMV algorithm (Lutterotti *et al.* 2004). During this latter step, the Orientation Distribution Function (ODF) (Matthies *et al.* 1987) was refined. These steps were iterated 4 times to find the best solution at the convergence of the program, after which pole figures were reconstructed. Instrumental aberrations were calibrated on a standard LaB_6 powder from NIST (SRM660b) and de-convoluted for all the acquired data. Defocusing aberrations with the tilt angle were refined on each sample since they depend on the sample curvature, using a polynomial approach (Chateigner 2004). Pole figures were normalized into orientation density values, expressed in "multiples of random distribution" units (or m.r.d.). In these units, samples without any texture (powders) exhibit homogeneous pole figures at the 1 m.r.d. level, while textured samples show maxima and minima in the pole figures, respectively above and below 1 m.r.d., the former corresponding to the texture components. The E-WIMV approach provided the maximum and minimum values of the ODF, which were quantitative appreciations of the texture strength for specific points of the orientation space. An overall texture strength value was the texture index F^2 (Bunge 1982). During the Rietveld and E-WIMV cycles, the phase cell parameters were also refined, together with other effects that could be detected (crystallite sizes, d-spacing microstrains, stresses, etc). Quality of the results were assessed by the reliability factors for the Rietveld (R_B, R_w, R_{exp}) and ODF (R_{wT}, R_{BT}) refinements, respectively, as defined for this combined analysis (Chateigner 2004) and implemented in the MAUD package (Lutterotti *et al.* 1999).

Scanning Electron Microscope images were obtained from secondary electrons using a Philips XL 30 FEG instrument at an operating voltage of 20 kV. Energy Dispersive Spectrometry could reveal only a single composition with respect to the $CaCO_3$ stoichiometry. Four to five locations were measured for each specimen.

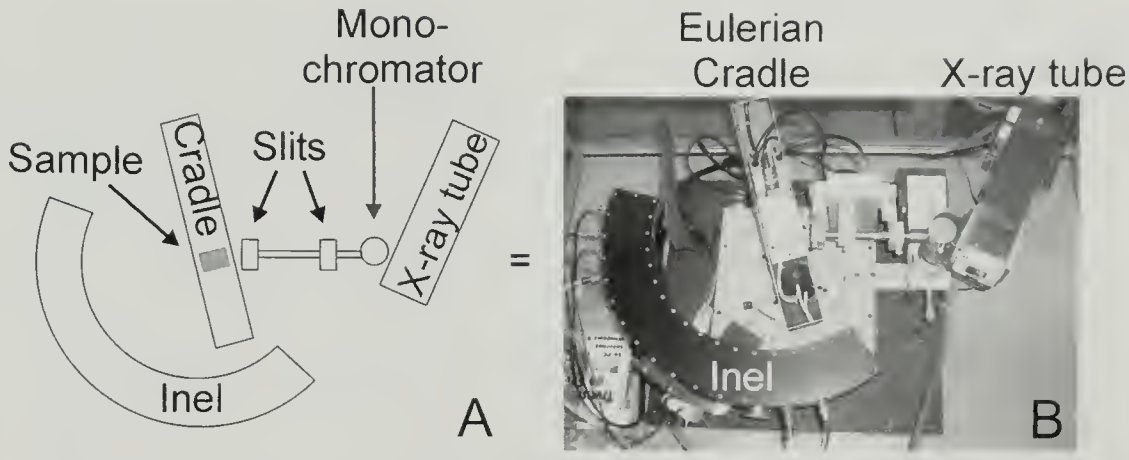

Figure 3. A, Schematic and, B, picture of the X-ray instrumental set-up. Scale: 1: 20.

RESULTS AND DISCUSSION

Individual X-ray diagrams, measured on the same sample for different orientations, clearly showed the unique presence of textured aragonite (Fig. 4). Diagrams measured for different orientations of the sample exhibited different peak intensity ratios, indicating the texture was probably strong. None of these diagrams corresponded to randomly oriented powder. Peak positions corresponded only to the aragonite unit-cell. For this phase and our X-ray energy, the linear absorption coefficient was $\mu = 208\ cm^{-1}$, which corresponded to 99% of the diffracted intensity coming from typically the first 46 μm of the shell, *i.e.*, approx. two thirds of the total shell thickness, then probing the outer, crossed-lamellar layers. For this probe depth the diffraction peaks were very narrow, a signature of well-developed crystallites, to sizes larger than our instrumental limit of typically 1 μm for their mean smallest dimension. No micro-distortion of the d-spacings could be detected, indicating that crystallization occurred in a smooth manner in these layers.

The refinements of both textures (E-WIMV) and structures (Rietveld) were in good agreement with the experimental values as indicated by the reliability factors

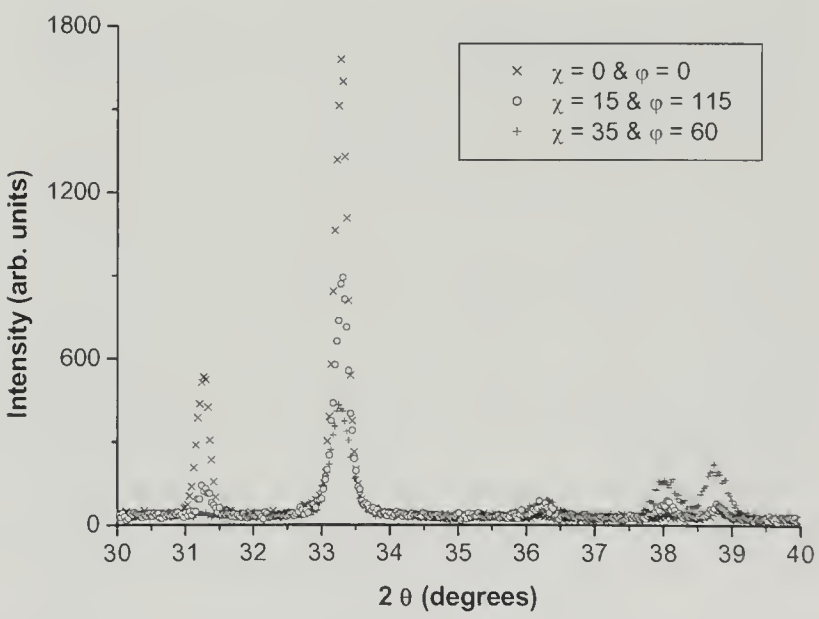

Figure 4. Plot of three spectra measured for three different couples of tilt and azimuth angles, respectively χ and ϕ. The intensity ratio changes as a function of these two rotations, indicating preferred orientation.

(Table 1). On the 29 measured individuals, the Rietveld reliability factors ranged from roughly 14 to 30%, which for 936 diagrams (3×10^6 measured points per sample) was considered as very good, and was also indicated by the low standard deviations ranging between 1 and 2 r.m.s. For the texture refinement (taking out extremes S1-13 and S2-7), reliability factors ranged from approx. 12% to 32% (with 5 to 6 r.m.s. standard deviation) which again were satisfactory for the level of textures shown (Chateigner 2005). Refined cell parameters corresponded to the values of synthetic non-biogenic aragonite, and no difference was observed between the two sample sets within the standard deviations. We conclude selection does not affect aragonite structure. Orientation distribution function minima were all close to 0 m.r.d., indicating that 100% of the total shell volume was textured. Orientation distribution function maxima were very large and fluctuated strongly, with a tendency for larger ODF max in the control samples, even if both sets overlapped within their standard deviations. Variation of the ODF max values reached 35% of the mean value in C samples versus 50% in S. The same maximum variations were visible for the texture index values. Interestingly, standard deviations for texture strengths (ODF max and F^2) were lower for C samples than for S samples. This is particularly significant if we bear in

Table 1. Refinement results from the MAUD package. Grayish specimens represent extreme results (larger texture reliability factors then worst refinements) and have been removed for mean and standard deviation calculations. The standard deviation resulting from the refinements on the parameters is typically 2 units on the last shown digit.

Specimen	R_B (%)	R_w (%)	R_{exp} (%)	R_{BT} (%)	R_{wT} (%)	a (Å)	b (Å)	c (Å)	ODF min (m.r.d.)	ODF max (m.r.d.)	F^2 (m.r.d.)^2
S1-1	21.79	27.70	19.18	18.94	17.33	4.9503	7.9540	5.7410	0.0005	142.00	12.60
S1-3	19.95	25.46	17.39	15.13	13.74	4.9604	7.9760	5.7493	0.0027	160.33	14.45
S1-5	19.88	25.46	17.88	13.39	12.89	4.9680	7.9803	5.7500	0.0006	223.39	20.4
S1-8	20.09	25.97	17.16	15.21	15.35	4.9579	7.9658	5.7438	0.0001	278.05	33.98
S1-9	22.41	28.73	18.45	16.61	15.89	4.9764	7.9956	5.7520	0.0001	252.27	21.97
S1-10	19.52	24.85	19.52	13.79	13.92	4.9672	7.9809	5.7528	0.0344	94.99	7.60
S1-13	17.01	21.44	15.34	13.23	11.74	4.9615	7.9676	5.7408	0.0057	183.23	18.12
S1-14	23.27	30.09	20.90	15.31	13.88	4.9687	7.9813	5.7444	0.0001	191.49	19.62
S1-21	19.83	25.51	17.64	12.77	11.85	4.9579	7.9662	5.7408	0.0002	177.16	24.13
S2-2	20.2	25.77	17.82	13.82	13.26	4.9674	7.9852	5.7453	0.0026	180.38	17.48
S2-3	19.62	25.21	17.60	13.08	12.96	4.9580	7.9740	5.7426	0.0003	206.53	19.58
S2-5	22.72	29.16	17.59	20.62	20.15	4.9559	7.9830	5.7465	0.0154	97.94	9.42
S2-7	20.07	25.12	14.24	36.67	35.8	4.9636	7.9888	5.7409	0.0390	85.40	5.30
S2-11	20.07	28.16	16.79	14.40	14.53	4.9545	7.9627	5.7448	0.0004	142.40	18.61
S211b	22.56	28.85	17.85	19.00	18.35	4.9539	7.9589	5.7433	0.0014	161.01	16.85
Mean	**20.60**	**26.50**	**17.69**	**16.80**	**16.11**	**4.9614**	**7.9747**	**5.7452**	**0.0069**	**171.77**	**17.34**
σ(r.m.s.)	***1.64***	***2.24***	***1.58***	***6.00***	***5.94***	***0.0070***	***0.0118***	***0.0041***	***0.0128***	***47.04***	***7.07***
C1-2	20.73	26.65	17.87	15.08	13.65	4.9561	7.9568	5.7377	0.0081	190.80	15.18
C1-4	19.97	25.53	15.74	22.24	19.32	4.9546	7.9681	5.7420	0.0130	182.90	20.00
C1-6	21.01	26.90	18.17	14.26	13.73	4.9715	7.9833	5.7390	0.0023	173.77	19.32
C1-7	19.59	25.27	17.69	12.63	11.25	4.9553	7.9613	5.7405	0.0016	202.65	28.24
C1-9	22.46	28.72	18.23	16.09	14.73	4.9649	7.9762	5.7395	0.0029	193.19	24.06
C1-11	23.42	29.51	16.46	31.44	28.00	4.9756	7.9829	5.7434	0.0006	166.57	20.27
C1-12	22.83	29.66	18.23	14.84	13.14	4.9664	7.9795	5.7417	0.0008	189.94	25.92
C1-17	19.31	24.8	16.74	15.96	14.52	4.9614	7.9741	5.7397	0.0053	150.38	16.3
C1-23	19.43	24.68	15.82	19.40	22.15	4.9691	7.9867	5.7542	0.0082	266.34	24.23
C2-5	22.23	28.48	17.30	18.67	16.91	4.9579	7.9576	5.7400	0.0003	217.91	27.95
C2-6	20.21	26.05	17.75	13.52	13.68	4.9524	7.9619	5.7382	0.0003	272.31	24.79
C2-7	20.09	25.81	17.67	14.29	12.57	4.9547	7.9518	5.7364	0.0034	163.58	15.07
C2-14	21.57	27.37	16.93	20.25	17.17	4.9560	7.9677	5.7436	0.0009	184.00	22.74
C2-19	20.66	26.48	15.91	18.02	17.88	4.9547	7.9685	5.7406	0.0019	168.25	18.72
Mean	**20.97**	**26.85**	**17.18**	**17.62**	**16.54**	**4.9608**	**7.9697**	**5.7412**	**0.0035**	**194.47**	**21.65**
σ(r.m.s.)	***1.33***	***1.67***	***0.91***	***4.86***	***4.58***	***0.0074***	***0.0110***	***0.0043***	***0.0038***	***36.07***	***4.45***

mind that S samples were flatter than C's, and consequently should have provided less fluctuating X-ray results. Histograms of the F^2 fluctuations are presented for S (Fig. 5A) and C (Fig. 5B) samples, respectively. The error bars on these diagrams are standard deviations. One can clearly notice the reduced variability of F^2 on C samples.

The significantly stronger textures observed for control shells underlined the higher degree of orientation in control samples. Smaller standard deviation of F^2 and ODF max in C compared to S indicated a larger textural resemblance among the control specimens than in the ones selected for increased weight. This could be attributed to the larger shells obtained by selection, which implies a faster, less acute, crystallographic growth.

The overlap of the results (including standard deviations) indicated that the overall preferred crystallite orientation was only mildly dependent on the selection carried out although

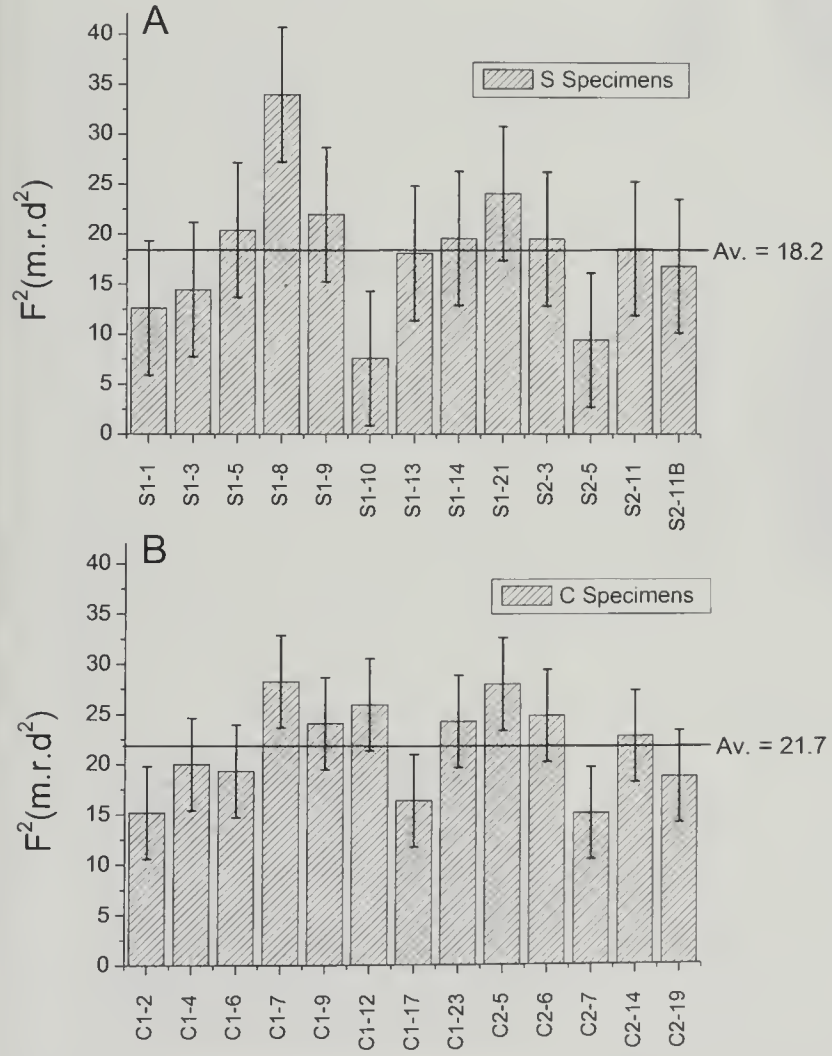

Figure 5. A, F^2 histogram for S (S2-7 omitted) specimens with an average (Av.) of 18.2 m.r.d.^{2}, including an error bar indicating the standard deviation for the entire class: 6.7 r.m.s. and B, F^2 histogram for C (C1-11 omitted) specimens with an average of 21.7 m.r.d.^{2} including an error bar indicating the standard deviation for the entire class: 4.5 r.m.s.

the degree of this orientation was slightly influenced. To better visualize the influence of selection, one can calculate (Fig. 6) the {020} and {002} pole figures for the extremes as well as for an average sample (C2-14, the closest to the F^2 average value) including their minimum and maximum pole distribution values.

This figure shows that the same mean preferred-orientation components were present in the samples, including the extreme samples: QTA provided reliable results although the maximum densities were subjected to deviations due to surface effects like roughness, flatness, and growth anomalies. The textures stabilized corresponded to crystallite **c**-axes (revealed by the {002} pole figures) aligned with the macroscopic normal of the shell, while the **b**-axes (revealed by the {020} pole figures) of aragonite were mainly aligned at approx. 10° from **G**. Slight variations between samples included the error created by positioning the samples on the diffractometer. However, the **b**-axes were always found at this 10° angle from **G**, certifying our positioning. The full width at half maximum of the **c**-axes distribution was around 20° in the direction of the **b**-axes and around 10° perpendicularly.

The fact that all samples exhibited pole figures which had the same shape but different maximum ODF values (and F^2 values) indicated that the direction of the preferred orientation of the crystallites did not change with selection, but the number of crystallites that oriented within a given width of distribution did. Indeed, the non-oriented part of the irradiated samples was in practice zero for all specimens in both C and S sets (Table 1), but the maxima for S were more variable than for control specimens, indicating that selection for larger size induced order-disorder fluctuations of the aragonite layers throughout the population. Adult age, *i.e.*, time to complete growth (until the peristome was reflected), was not significantly different between lines (Dupont-Nivet *et al.* 2000b). This means that larger size in S snails was achieved only through faster growth, with 60% more weight in S animals in the same growing time. The fact that the preferred orientation of crystallites changed only slightly with selection showed that this was a phenomena which was determined more by the species than by growth conditions. However, the faster growth seemed to have potentially unfavorable effects through order-disorder fluctuations of the aragonite layers. Other experiments showed that neither mortality nor shell shape or shell proportion (ratio between shell and animal weight) differed significantly between both lines (Dupont-Nivet, pers. comm.).

Preferred orientations also condition mechanical properties of aggregates, in particular when the constituting crystals possess strong anisotropy of their elastic stiffness constants, as for aragonite. The elastic mechanical behavior of the mineral aragonite fraction can be simulated from the ODF-weighted average of the single crystal stiffness tensor,

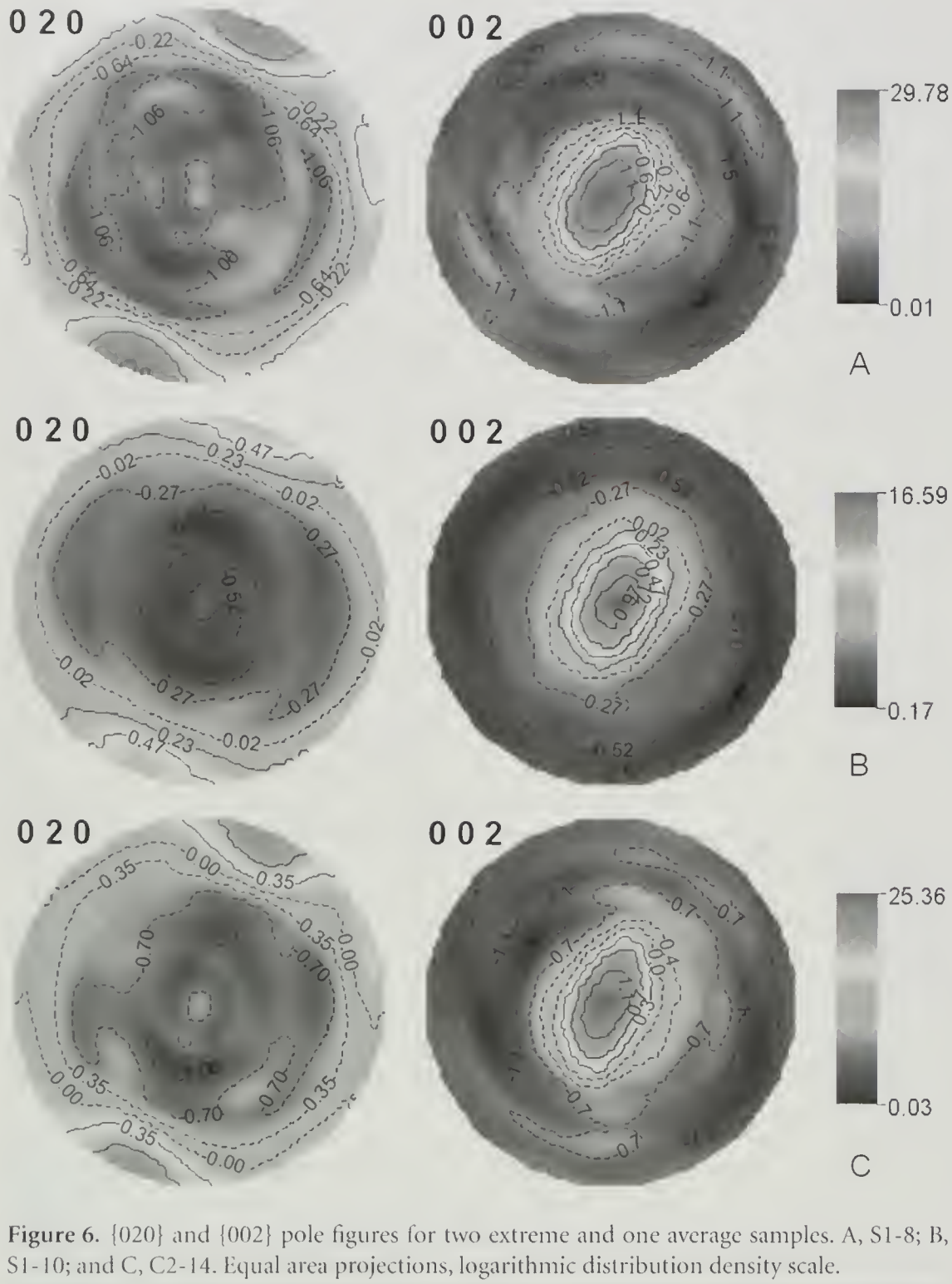

Figure 6. {020} and {002} pole figures for two extreme and one average samples. A, S1-8; B, S1-10; and C, C2-14. Equal area projections, logarithmic distribution density scale.

under the hypothesis of regular grain boundary behaviors, using the geometric mean approach (Ouhenia *et al.* 2008). In such an approach, we ignore the biocomposite nature of the shell and organic-inorganic interactions, and illustrate how the mineral part of the shell affects elastic behavior of the *Helix aspersa aspersa* shell. For an aragonite single crystal there are 9 independent values for the c_{ij} stiffness values (in GPa): c_{11} = 159.58, c_{22} = 86.97, c_{33} = 85.03, c_{44} = 41.32, c_{55} = 25.64, c_{66} = 42.74, c_{12} 36.63, c_{13} = 1.97, and c_{23} = 15.91. In the frame of this calculation, axes 1, 2, and 3 for i and j indices are the **M**, **G**, and **N** directions, respectively. Using the ODF geometric mean, we obtained mean macroscopic stiffness values of (in GPa): c^{M}_{11} = 117, c^{M}_{22} = 107, c^{M}_{33} = 86, c^{M}_{44} = 36, c^{M}_{55} = 34, c^{M}_{66} = 41, c^{M}_{12} = 31, c^{M}_{13} = 14, and c^{M}_{23} = 15, within 6% of the standard deviation, and no significant difference occurred between mean values for the S and C sample sets. Several orientation effects on the macroscopic constants of the shell, compared to the single crystal values, kept the c_{33} constant practically unchanged around 85 GPa. This is because of the strong c-axis orientation with **N**. However, the texture imposes an average value for c^{M}_{11} and c^{M}_{22}, intermediate values compared to the single crystal. This causes a homogeneous mechanical response of the mineral part for compression in the (**G**, **M**) plane of the shell. All the off-diagonal c_{ij} coefficients are homogenized, being much less anisotropic than in the single crystal, giving rise to moderate anisotropic transverse strains in the shell. Because of this, c^{M}_{13} is 7 times larger than in a single crystal, at only a small expense of c^{M}_{12}. The shear coefficients c^{M}_{44}, c^{M}_{55}, and c^{M}_{66} were balanced compared to the single crystal values, and in particular c_{44} and c_{55}. This allows the shell to accommodate relatively large shear coefficients along all directions. Hence, from an elastic anisotropic theory point of view, the strong texture exhibited by the *H. aspersa aspersa* shell behaves, at least for the mineral part, in an optimal manner relative to moderate compressive and shear forces. This is caused by balanced weak and strong elastic coefficients that are not optimal in all macroscopic directions of the shell as observed for instance in the marine gastropod *Charonia lampas lampas* (Linnaeus, 1758) (Ouhenia *et al.* 2008).

One of our objectives was to determine if growth selection had important detrimental effects on shell characteristics. Indeed, even if it is not a selected trait, shell strength is a key point in snail farming. Animals are often manipulated (structure changes, sorting, collecting) which creates multiple chances for shell fracture. These broken shells are problematic for snail survival (dehydration) and growth, and also for the commercial value of animals. The results of this study clearly show that selection did not compromise shell structure, at least in our experimental conditions. However, any side effects

of faster growth should be checked on a regular basis. Moreover, we should check changes in shell thickness and measured shell strength and correlate them with crystallographic results to test if order-disorder fluctuations of the aragonite layers have unfavorable effects.

Looking at a local scale, the crystal organizations on SEM images, the different orientations of crystallites can be made visible. From the shell top ((**G**, **M**) plane) *Helix aspersa aspersa* has successively alternating lamellae (Fig. 7). The crystals are elongated alternatively along a direction at ~ −62° (arrow) from the vertical direction (**G**) and a direction of about 53° from it. This results in a counterclockwise angle between the respective elongations of ~115° in the two succeeding elongation directions.

The two main visible directions of crystal elongation showed a striking angular correspondence between the (110) and (-110) crystallographic planes within aragonite (or between the [110]* and [-110]* reciprocal crystallographic directions, respectively normal to the (110) and (-110) planes). Calculating the angles between (100) and (-110) planes using the cell-parameters (a = 4.96 Å and b = 7.97 Å), one finds an angle of ~116°. When looking at the {110} pole figure (Fig. 8A), the two directions of crystal elongation in the two *Helix aspersa aspersa* layers clearly could be identified to the [110]* and [-110]* crystalline directions (Fig. 8B). However, this does not mean that crystallographic alignment occurred with two components of orientation. Indeed, looking at Fig. 6C, only one, previously described orientation component was present throughout the shell, which corresponded to the {110} four-fold multiplicity of Fig. 8A.

We conclude that elongation of the crystals is operated along the {110} plane of stacking directions. From one layer to the next, this biologically driven growth of crystals occurred without loss of crystallographic orientation, but with change in for individuals selected for growth from one of the {110} planes (for instance (110)) to the other (*e.g.*, (-110)). This is a different growth scheme than observed in the gastropod *Cypraea testudinaria* (Linnaeus, 1758), in which the crossed lamellae of either the radial or co-marginal layers were obtained by a twinning relationship (Chateigner *et al.* 1996). In *Helix aspersa aspersa*, we always found the same single-components of orientation whatever the size of the shell, *i.e.*, whatever the thickness probed, indicating all the layers do keep the crystal orientation. This appears a common pattern in land snails, the same textures having been observed in *Helix pomatia* (Linnaeus, 1758), *Helmintoglypta* (Binney, 1897), and *Englandina* (Férussac, 1818) (Chateigner *et al.* 2000) while all the marine gastropods analyzed to date showed orientation modifications from layer to layer (Chateigner *et al.* 1996, 2000, Checa and Rodriguez-Navarro 2005).

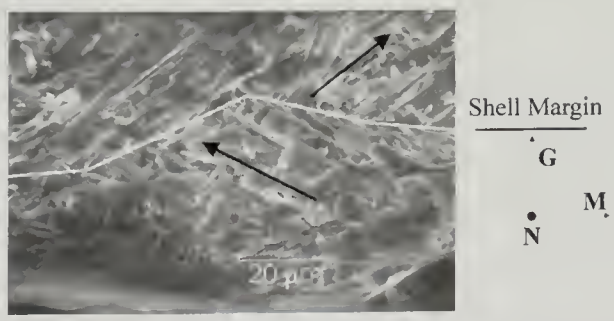

Figure 7. SEM photograph of a fractured *Helix aspersa aspersa* shell parallel to the (**G**, **M**) plane. Magnification is 742 ×. The bottom of the image corresponds to the outermost CCL (Comarginal Crossed-Lamellar) layer while the top of the image is the next inner RCL (Radial Crossed-Lamellar) layer. Arrows illustrate the main crystal directions in the two layers. The shell frame is indicated on the right.

Finally, looking at brownish bands on the surface of *Helix aspersa aspersa* shells (Figs. 1-2), these typically made an angle around 10° with the growth direction **G**. Such angular values were retrieved between the <020> crystallographic directions and **G** ({020} pole figure (Fig. 6C and Fig. 8B)). A close directional relationship may exist between the alignment of the colored bands at the shell surface and the <020> crystallographic directions, using for instance pending bonds from the carbonate groups as they coincide with the observed orientations (Fig. 8C). Such bands were recently associated in *Helix aspersa aspersa* to the presence of un-substituted, methyl terminated chains of 8-13 conjugated double-bond polyenes, either isolated or bound to other molecules, the density of which rendered the color intensity (Hedegaard *et al.* 2006). Since crystallographic texture in molluscs is associated with inter-crystalline macromolecule interaction, the fact that the colored bands of *H. aspersa aspersa* were linked to the specific [020] direction indicates bound polyenes or pigments in this species.

In conclusion, comparison of QTA results of 29 specimens of *Helix aspersa aspersa*, using a statistical analysis, indicated quantitative agreement within standard deviations of ~5 m.r.d.^{2} for the texture index and 40-50 m.r.d. for the maximum value of the orientation distribution function, suggesting such standard deviations vary from about 20 to 35%. We observed a difference between control specimens and the ones selected for larger size: growth stimulation affects the preferred orientations. The standard samples have a higher average texture index with less variability (lower standard deviation). These results indicated the degree to which one can see texture variation between individuals, at least for *H. aspersa aspersa* having a quite regular but curved shell shape.

A clear identification of the elongation direction of the individual crystals in the radial and co-marginal crossed lamellar layers indicated <110> crystallographic directions whatever the layer, while the crystals in different layers all had the same orientation. The colored bands at the surface of the shells were linked to the **b**-axis of the aragonite structure. The elastic behavior of the mineral part of the shell is averaged by

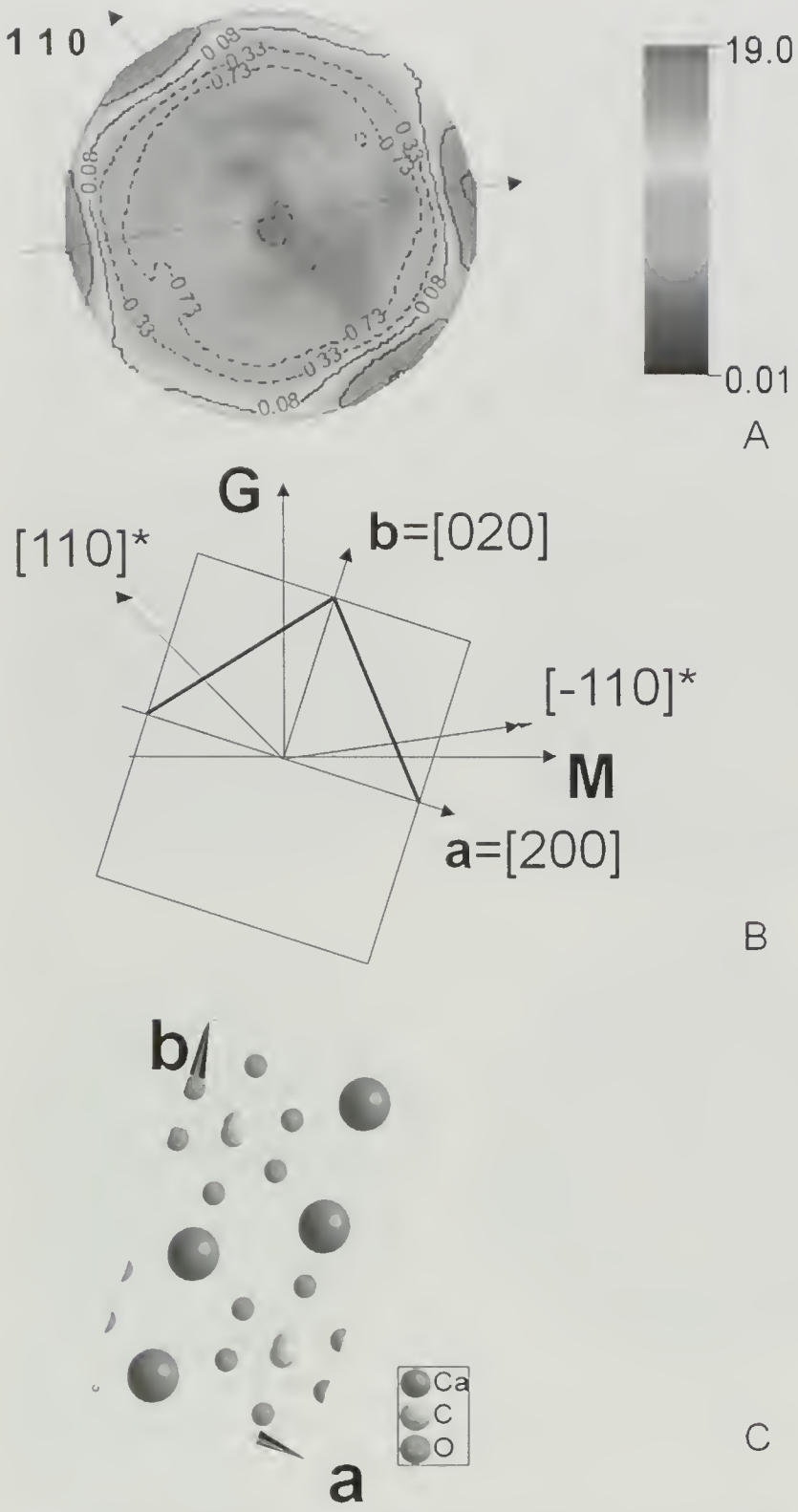

Figure 8. A, {110} pole figure of *Hexlix aspersa aspersa* C2-14; B, schematic of the corresponding main crystalline directions; and C, one theoretical aragonite unit-cell in the (**G**, **N**, **M**) shell reference frame.

the crystallite dispersions to accommodate moderate shear and compression stresses.

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Mollusc survey of the lower Bruneau River, Owyhee County, Idaho, U.S.A.

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Abstract: The Bruneau River in southwestern Idaho is a largely free-flowing desert stream characterized by a snowmelt-driven hydrograph, flash floods, and geothermal inputs. We surveyed the lower Bruneau River from its confluence with the Snake River upstream to Hot Creek, a distance of approx. 41 river kilometers, between 1997 and 2008. We supplemented our work with a review of collections held at the Orma J. Smith Museum of Natural History, College of Idaho and with collections available online from national and international museums. The known mollusc fauna consists of 18 species (11 gastropods and 7 bivalves) from eight families. Molluscan richness is greatest in free-flowing stream reaches and is dominated by hydrobiid and unionid taxa.

Key words: gastropods, bivalves, invasive species

The freshwater molluscan fauna of Idaho has interested malacologists for many years (Hendersen 1924) yet its diverse taxa remain poorly understood (Frest and Johannes 2000, Frest *et al.* 2001). Further, the taxonomic status of some species in Idaho is under revision (Taylor 2003, Hershler and Liu 2004, Rogers and Wethington 2007, Wethington and Lydeard 2007) with considerable work yet to be completed. Of the 117 putative species in Idaho (Frest and Johannes 2000), few published accounts exist on their occurrence in specific stream segments. Similarly, the ecology of most gastropods, including species in Idaho, has received very little attention (Brown *et al.* 2008) resulting in a vague understanding of interactions between species and their environment. Conservation of freshwater molluscs requires: (1) an understanding of the relationships between species in a geographic area (*i.e.*, stabilized taxonomy), (2) an understanding of the distribution of species (*i.e.*, species inventory), and (3) an understanding of the ecology of species within the context of long-term species persistence (Brown and Johnson 2004, Stewart and Dillon 2004, Lysne *et al.* 2008). In reality, natural resource managers must frequently prioritize conservation goals with incomplete biological knowledge (Regan *et al.* 2003, 2005). Within this context, we join other efforts (Dillon 2008) to document molluscan biodiversity and to provide information for natural resource managers to set conservation priorities.

MATERIALS AND METHODS

Study area

The Bruneau River, in southwestern Idaho, is a largely free-flowing, high-desert stream originating in the mountains of northern Nevada and stretching approx. 185 km north to join the Snake River (Fig. 1). Only two known structures divert Bruneau River water between the Bruneau Arm and its headwaters: Buckaroo Diversion at Bruneau River kilometer (RKM) 34 and Harris Diversion at RKM 34.4. Mean instantaneous flow of the Bruneau River, measured at RKM 35.4 between 1987 and 2007 was 8.47 cubic meters second^{-1} (cms) and ranged from 2.86 cms in December to 30.58 cms in May (USGS 2008). Mean monthly water temperatures ranged from 14 °C in May to 21 °C in September, with a high of 26 °C in August (USGS 2008). The Bruneau River has few perennial tributaries but has numerous ephemeral creeks that can significantly increase flow during intense summer storms. A defining characteristic of the Bruneau River system is the geothermal aquifer that underlies much of southern Idaho (Berenbrock 1993) and contributes a base flow to the river year round. Water temperature of geothermal spring flows range between 11 °C and 40 °C (Myler *et al.* 2007), and an abrupt thermocline has been observed in the main channel as a result of geothermal, hyporheic additions to the river.

Field sampling

We surveyed sections of the Bruneau River from the Bruneau Arm of C. J. Strike Reservoir (*i.e.*, the confluence of the Bruneau and Snake Rivers; 42.92375°N, 115.90353°W) to Hot Creek (42.76226°N, 115.73084°W), a distance of approx. 41 km (Fig. 1). We used a Venturi dredge apparatus and SCUBA (Stephenson *et al.* 2004) to collect 432 samples from the Bruneau Arm of C. J. Strike Reservoir. A 0.25-m^2 quadrat was placed on the reservoir bottom and the substrate suctioned to a depth of approx. 5 cm. Samples were sorted by hand to identify, and return to the river, endangered gastropod species, if present. Remaining sample material was

preserved in 95% ETOH and shipped to EcoAnalysts Inc., Moscow, Idaho, for identification to the lowest possible taxonomic level. We estimated densities of taxa for species importance curves based on all collections between 1997 and 2008 where density information was available.

In the free-flowing reaches of the Bruneau River, we conducted visual inspections (via a benthic viewer) of all available habitat types (*e.g.*, springs, river, pools, riffles, submerged and emergent aquatic vegetation, fines, cobbles, and bedrock) below Hot Creek. We spent more than 100 person-hours surveying the stream and associated habitats for molluscs. We used hand collections only in the river below Hot Creek and made no attempt to quantify densities or total numbers of individuals of any taxa. Relative abundance of taxa for species importance curves was estimated qualitatively by professional judgment. We collected and preserved individuals in 95% ETOH only for purposes of voucher specimens. These individuals were identified to the lowest possible taxonomic level. Collections in the Bruneau Arm occurred throughout the year but our observations in the free-flowing Bruneau River were conducted, for safety reasons, between July and March when flows were <5.7 cms. Available information including approximate sample locations, physico-chemical data, and lists of non-molluscan taxa are available from the authors.

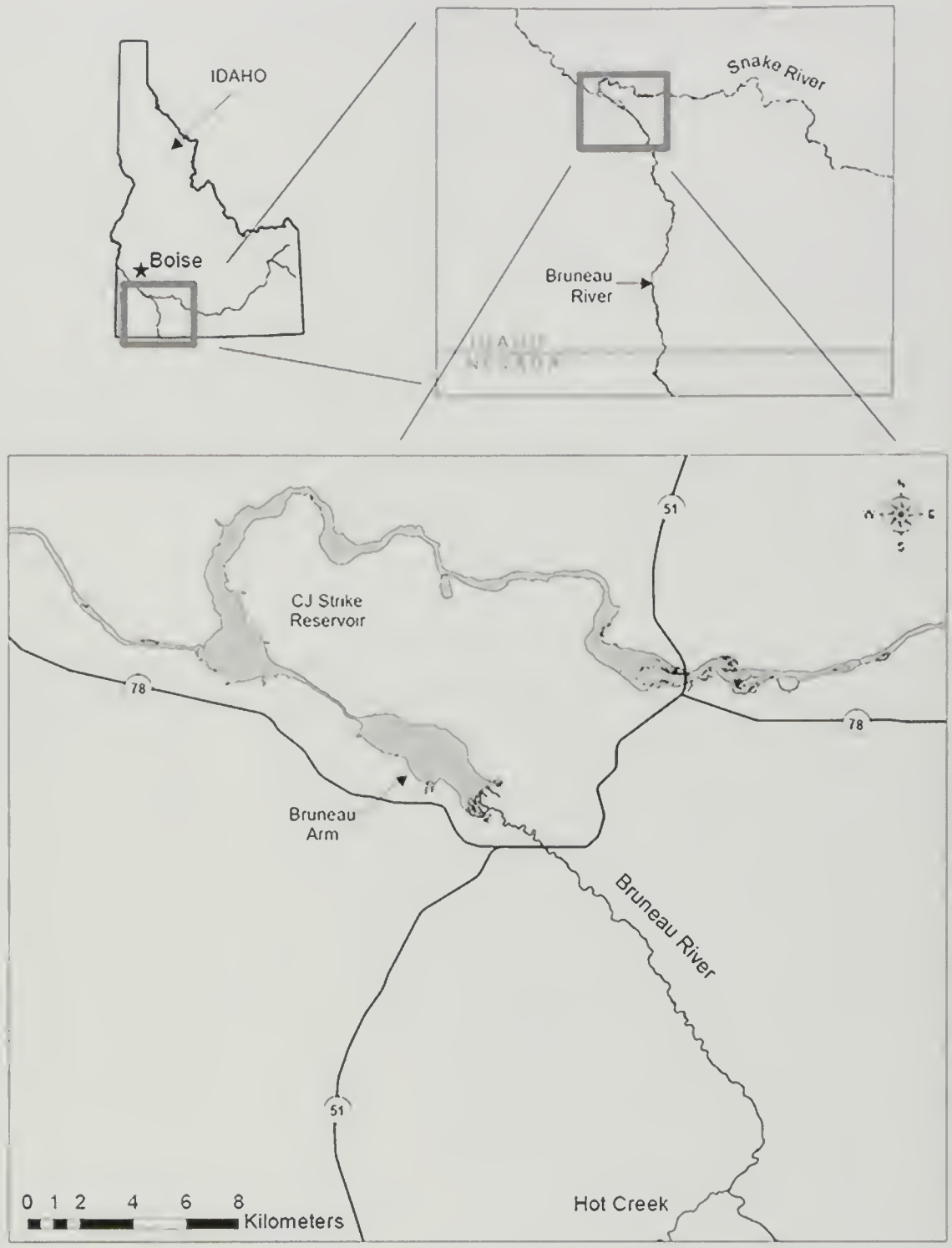

Figure 1. Figure of the study area in southwestern Idaho. Samples were observed or collected in the Bruneau Arm as well as the Bruneau River between Hot Creek and Highway 51.

Museum collections

We supplemented our work with a review of collections held at the Orma J. Smith Museum of Natural History, The College of Idaho (http://www.collegeofidaho.edu) between August 2007 and September 2008. The Smith Museum houses the largest collection of freshwater molluscs in Idaho and has staff dedicated to the curation and study of freshwater molluscs. We conducted a query of the Smith Museum's curation database resulting in approx. 1,100 lots from the Bruneau River. We also inspected approx. 150 un-cataloged lots of freshwater molluscs, looking for collections from the Bruneau River. In addition, we surveyed online databases and/or requested searches from The University of Michigan's Museum of Zoology, the California Academy of Sciences, and The Canadian Museum of Nature. From these searches, we obtained 418 lots containing molluscs from the Bruneau River. Detailed information on all collections is available from the authors. Voucher specimens from our work have been deposited at the Orma J. Smith Museum of Natural History. Identifications followed Burch (1989); nomenclature follows Turgeon *et al.* (1998) for most taxa and Mackie (2007) for the corbiculid and sphaeriid clams.

RESULTS

We documented eleven species of gastropods from five families, including the endangered, thermophillic springsnail *Pyrgulopsis bruneauensis* (Hershler, 1990) (Myler *et al.* 2007) and two non-native gastropods: *Potamopyrgus antipodarum* (Gray, 1853) and *Radix auricularia* (Linnaeus, 1758). We also documented seven species of bivalve molluscs from three families, including the invasive *Corbicula fluminea* (Müller, 1774). Table 1 lists species collected or observed, the location of collection, and information on the conservation status of each taxon globally and in Idaho (NatureServe 2008). Thirteen species of molluscs were collected or observed in the free-flowing reaches of the Bruneau River below Hot Creek. Of these molluscs, one hydrobiid gastropod, *Fluminicola fuscus* (Haldeman, 1847), and one unionid bivalve, *Gonidea angulata* (Lea, 1838), dominated numerically. Nine species of molluscs were collected from the impounded Bruneau Arm of C. J. Strike Reservoir. Of these, *Gyraulus parvus* (Say, 1817) and *Vorticifex effusa* (Lea, 1856) dominated numerically. Four species were found in both the free-flowing sections of the river and the reservoir and all are considered habitat generalists. The three non-native species (*C. fluminea*, *P. antipodarum*, and *R. auricularia*) occurred in the river and reservoir reaches.

We observed a change in species importance from reservoir to river habitats (Figs. 2A-B). Based on our collections, the Bruneau Arm molluscan community is dominated by *Vorticifex effusa* and *Gyraulus parvus*. Estimated densities of both species exceeded 1,500 individuals/m^2 whereas estimated densities for all other taxa fell below 400 individuals/m^2. By contrast, in the river below Hot Creek the molluscan community is dominated by *Fluminicola fuscus* and *Gonidea angulata*. Three additional species, *Gyraulus parvus*, *Physa gyrina*, and *Pyrgulopsis bruneauensis*, represented an intermediate relative abundance with the remaining taxa best described as uncommon.

DISCUSSION

The molluscan fauna of the Bruneau River below Hot Creek is rich (containing about 15% of the known Idaho fauna) relative to other tributaries of the Snake River in southern Idaho. The reasons for this diversity are not entirely known, but the relatively long length of the stream at the landscape scale, the diversity of habitats at multiple spatial scales, water chemistry, geothermal influences, and the relatively unimpaired conditions of the Bruneau River corridor are probably important influences.

Table 1. Molluscs present in the lower Bruneau River (below Hot Creek), Idaho. Data show presence related to general habitat as well as conservation status. Voucher specimens of each taxon are located in the Orma J. Smith Museum of Natural History, The College of Idaho. Habitat descriptors: RIV, river; RES, reservoir. Rankings are from NatureServe (2008): G1/S1, critically imperiled; G2/S2, imperiled; G3/S3, vulnerable; G4/S4, apparently secure; G5/S5, secure; SNR, state not ranked; EXO, exotic/introduced; NA, no ranking available.

Family	Genus	Species	Authority	Habitat	G Rank	S Rank
Gastropods						
Ancylidae	*Ferrissia*	*rivularis*	Say, 1817	RIV, RES	G5	SNR
Hydrobiidae	*Fluminicola*	*fuscus*	Haldeman, 1847	RIV	G2	S1
Hydrobiidae	*Potamopyrgus*	*antipodarum*	Gray, 1853	RIV, RES	G5	EXO
Hydrobiidae	*Pyrgulopsis*	*bruneauensis*	Hershler, 1990	RIV	G1	S1
Hydrobiidae	*Pyrgulopsis*	*robusta*	Walker, 1908	RES	G4	S1
Lymnaeidae	*Fossaria*	sp.	Westerlund, 1885	RES	NA	NA
Lymnaeidae	*Radix*	*auricularia*	Linnaeus, 1758	RIV	G5	EXO
Physidae	*Physa*	*gyrina*	Say, 1821	RIV, RES	G5	SNR
Planorbidae	*Gyraulus*	*parvus*	Say, 1817	RIV, RES	G5	SNR
Planorbidae	*Planorbella*	*subcrenata*	Carpenter, 1857	RIV	G5	SNR
Planorbidae	*Vorticifex*	*effusa*	Lea, 1856	RES	G3	SNR
Bivalves						
Corbiculidae	*Corbicula*	*fluminea*	Müller, 1774	RES	G5	EXO
Sphaeriidae	*Pisidium*	*casertanum*	Poli, 1791	RIV	G5	SNR
Sphaeriidae	*Pisidium*	*variabile*	Prime, 1852	RIV	NA	SNR
Sphaeriidae	*Pisidium*	*compressum*	Prime, 1852	RES	G5	SNR
Sphaeriidae	*Sphaerium*	*simile*	Say, 1817	RIV	G5	SNR
Unionidae	*Anodonta*	*californiensis*	Lea, 1852	RIV	G5	SNR
Unionidae	*Gonidea*	*angulata*	Lea, 1838	RIV	G3	SNR

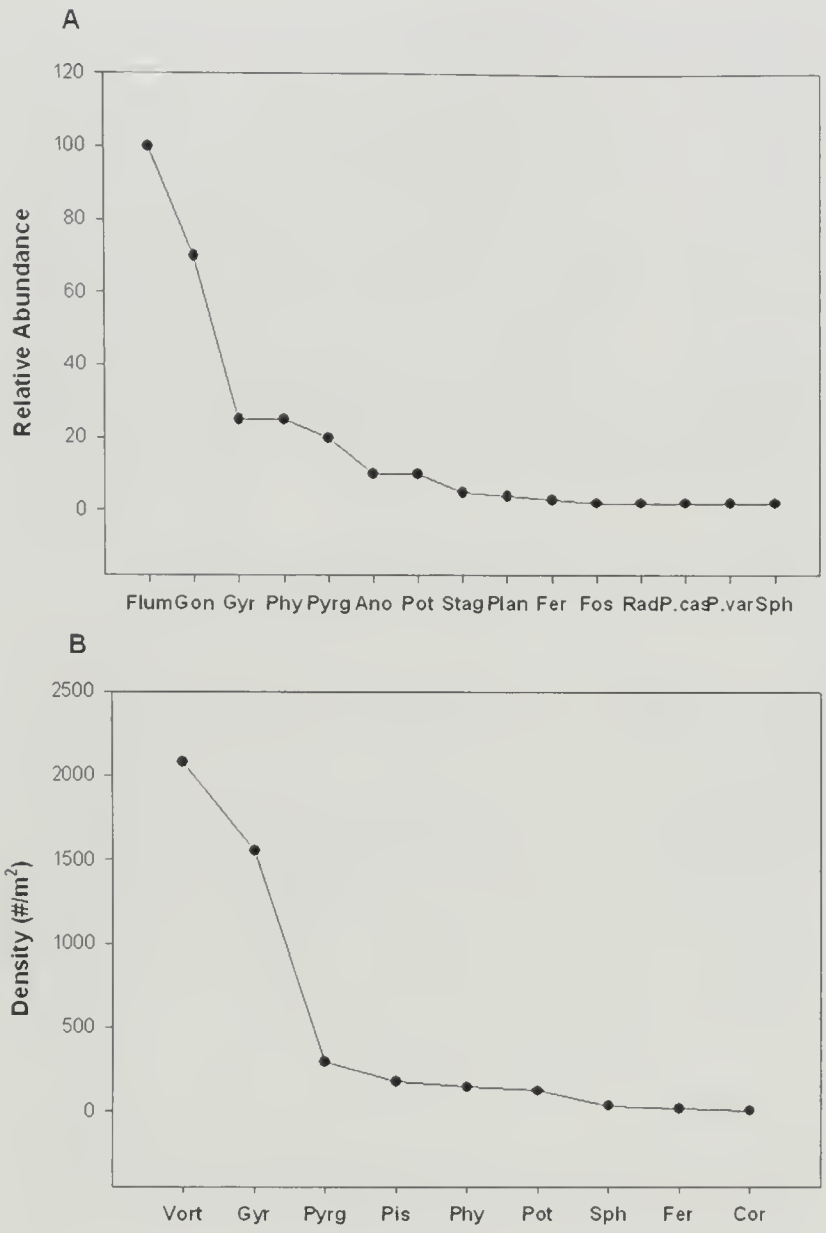

Figure 2. Importance curves for molluscs in the Bruneau River from Hot Creek to the Bruneau Arm of C. J. Strike Reservoir (A), and in the Bruneau Arm of C. J. Strike Reservoir (B). Abbreviations are: **Flum***inicola fuscus*, **Gon***idea angulata*, **Gyr***aulus parvus*, **Phy***sa gyrina*, **Pyrg***ulopsis bruneauensis* (A), **Ano***donta californiensis*, **Pot***amopyrgus antipodarum*, **Stag***nicola hinkleyi*, **Plan***orbella subcrenatum*, **Fer***rissia rivularis*, **Fos***saria* sp., **Rad***ix auricularia*, **P***isidium* **cas***ertanum*, **P***isidium* **var***iabile*, **Sph***aerium simile* (A), **Vor***ticifex effusa*, **Pyrg***ulopsis robusta* (B), **Pis***idium compressum*, **Sph***aerium* sp. (B), and **Cor***icula fluminea*.

The Bruneau River is a long (ca. 185 km) desert stream. It is generally larger, in terms of volume, than most tributaries in southern Idaho but is considerably smaller than the Boise, Payette, or Salmon Rivers further to the north. The underlying basalt geology (Ross and Savage 1967) permits the Bruneau to run relatively clean without the excessive sedimentation that is observed in many desert streams. Further, much of the Bruneau River runs through remote canyon-lands with steeply incised walls that limit grazing and other activities that tend to result in increased sedimentation (Allan 1995, Belsky *et al.* 1999). Similarly, the Bruneau River above our study area lacks irrigation diversions, intensive agriculture, or timber harvest practices that are typical of many landscapes surrounding Idaho streams to the east and north. The result is a desert stream system unique in Idaho with a natural flow regime and a relatively undisturbed stream corridor.

The Bruneau River has a steep gradient as it leaves the Owyhee Uplands which begin to attenuate below Hot Creek. As the river approaches the town of Bruneau, Idaho, the topography flattens out and the river begins to meander, which reduces stream velocity and sediment load and alters benthic habitat. As the river continues toward the Bruneau Arm of C. J. Strike Reservoir, it bisects a large wetland created by the impoundment of the Snake and Bruneau Rivers. The Bruneau Arm is essentially lentic and different ecologically from the river upstream with a very different species assemblage. These varied landscape scale habitat characteristics may explain the high molluscan diversity in the Bruneau River (Newton *et al.* 2008).

Perhaps more importantly, the Bruneau River below Hot Creek has increased habitat heterogeneity owing to the geothermal aquifer that underlies much of the Owyhee Uplands in southern Idaho. Water temperature is perhaps one of the most important determinants of where species are presently found (Allan 1995), it has important implications with regard to a species biology (Brown *et al.* 1998, Lysne and Koetsier 2006a), and can be used as a predictor of species presence (Malcom and Radke 2005). Hundreds of geothermal springs enter the Bruneau along its length (Myler *et al.* 2007), both above and below the water's surface, creating a patchwork of different habitats, thermal characteristics, and floral and faunal assemblages. The geothermal aquifer also moderates cold winter temperatures and provides thermal refugia for aquatic invertebrates eliminating the need for hibernation-like, overwintering behaviors observed in other Idaho molluscs (Lysne and Koetsier 2006b). For example, water temperature in the Bruneau River measured at 2/3 depth in February of 2008 was 4.8 °C, below the known thermal range of *Pyrgulopsis bruneauensis* (Mladenka and Minshall 2001). However, at the boundary layer, water temperature was 12.7 °C, within the thermal tolerance of the species, and the river supports *P. bruneauensis* activity year-round given the appropriate substrates (Myler *et al.* 2007). Other species may respond similarly to the unusual thermal regime: Richards (2004) found populations of *Taylorconcha serpenticola* to fluctuate seasonally but reproduction occurred in winter with the thermally constant water temperatures. Similarly, Mladenka and Minshall (2001) found that *Pyrgulopsis bruneauensis* reproduced year-round in super-heated springs. Thus, the varied geothermal habitat characteristics, at a smaller spatial scale than the landscape, may also contribute to the high molluscan diversity of the Bruneau River.

Downstream of Hot Creek the malacofauna is dominated numerically by two species: *Fluminicola fuscus* and *Gonidea angulata*. Conversely, the Bruneau Arm is dominated by *Gyraulus parvus* and *Vorticifex effusa* and no unionid bivalve molluscs were present (Stephenson *et al.* 2004). The non-native *Radix auricularia* was found only in the free-flowing river and the non-native *Potamopyrgus antipodarum* was found in both lotic and lentic habitats. The relative importance of molluscs in the river versus the reservoir follows habitat requirements of unionid mussels (Vaughn and Taylor 1999, Dillon 2000) and the comparative ecology of pulmonate and "prosobranch" snails (Brown *et al.* 1998, Brown and Johnson 2004). Pulmonate snails such as *Vorticifex effusa* and *Gyraulus parvus* are considered more tolerant of lentic systems with relatively eutrophic water quality whereas "prosobranchs" such as *Fluminicola fuscus* and unionids such as *Gonidea angulata* are more characteristic of lotic systems and relatively mesotrophic water quality.

Of note in the Bruneau River below Hot Creek are three species of intermediate relative abundance: *Gyraulus parvus*, *Physa gyrina*, and *Pyrgulopsis bruneauensis*. *Physa gyrina* occupies various habitat types and and is common throughout the area we studied. By contrast, based on our direct observations, both *G. parvus* and *Pyrgulopsis bruneauensis* are patchily distributed depending on the presence of suitable habitats.

The presence of *Potamopyrgus antipodarum* is of concern. This species is now the most abundant mollusc in parts of the middle Snake River (Richards *et al.* 2001) and can reach extremely high densities in some locations (Richards *et al.* 2004). The invasion of Bruneau River may be recent. Clark (1979) sampled two sites within the present study area in 1975, approx. 10 years prior to the invasion of the Snake River basin by this species, and failed to detect *P. antipodarum*. Similarly, surveys between Hot Creek and the Bruneau Arm completed between 1990 and 2005 also failed to detect and/or report the presence of *P. antipodarum* (Myler *et al.* 2007). Today the species is abundant in C. J. Strike Reservoir and appears to be moving upstream. The relatively recent establishment of *P. antipodarum* thus provides an opportunity to monitor its invasion and potential community level effects.

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The shell features of *Cornu aspersum* (synonym *Helix aspersa*) and *Helix pomatia*: Characteristics and comparison

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Abstract: We examine the morphometric, chemical, and physical properties of adult shells from breeding populations of *Cornu aspersum maxima* (Taylor, 1883), *Cornu aspersum aspersum* (Müller, 1774), and *Helix pomatia* (Linnaeus, 1758). The higher thermal requirements of the African subspecies *C. aspersum maxima* were confirmed by the fact that normal shell maturation, indicated by a decreasing calcium content as the snail ages, was related to an increased mean air temperature of over 22.9 °C during the breeding season. In contrast, normal shell maturation of the European subspecies *C. aspersum aspersum* occurred with a temperature in the range of 20.6-23.6 °C. Based on the results of texturometric analysis, shell puncture force increased with an increase in temperature during breeding. In contrast, shell puncture force decreased and collapsing force increased with increasing relative humidity. The mechanical strength of *C. aspersum* and *H. pomatia* shells was related to their chemical composition and the level of their structural maturity. Shells containing a higher percentage of calcium were characterized by lower mechanical strength than those containing a lower amount.

Key words: shell features, measurement methods, shell maturation

The physical, chemical, and morphometric features of snail shells are closely related to their internal structure. The internal structure of the shell and the mechanisms of its shaping differ in various mollusc species (Saleuddin and Hare 1970, Weiner and Traub 1980, Wilbur and Saleuddin 1983, Bowen and Hieng Tang 1996, Chateigner *et al.* 1996, 2000, Hedegaard and Wenk 1998, Kaplan 1998, Dauphin and Denis 2000). Shell formation relies on the production of an organic "matrix" on the outer surface of the mantle. The matrix is composed of specific glycoproteins and amino acids, creating an orderly environment for the crystallization of calcium carbonate, which is the main structural material of a shell. Calcium carbonate is synthesized in the form of a biomineral characteristic of molluscs, aragonite, with a higher specific gravity and different crystalline structure compared to calcite. In a mature shell, properly organized crystals of aragonite form several layers of microstructure specific to individual species of molluscs.

There are insufficient data on the shaping of selected physical, chemical, and morphometric features of shell structure which are of primary importance from the point of view of breeding the edible species of Helicidae. Information exists on the effect of microclimate and genotype on color diversification and the pattern of the shell in Helicidae (Albuquerque de Matos 1984, Lecompte *et al.* 1998). Moreover, methods for measuring the mechanical strength of the shell have been described for species with shell symmetry and a habitat different from those for Helicidae (Kent 1981, Garden 1998).

This study provides data regarding the shaping of selected physical, chemical, and morphometric features of shells in bred populations of *Cornu aspersum* and *Helix pomatia*. Biometric and physical-chemical analyses of mature shells from *C. aspersum maxima* and *C. aspersum aspersum* bred in variable microclimates were carried out. Shells from mature *H. pomatia* were used as a reference.

MATERIALS AND METHODS

Breeding populations of Helicidae

The following populations in an experimental snail farm in the National Research Institute of Animal Production – NRI, near Kraków (Poland) were included in this study: (1) "Balice" and "Albino" populations of *Cornu aspersum maxima*; (2) "Balice" and "French" populations of *Cornu aspersum aspersum*; and (3) the natural "Balice" population of Roman snail (*Helix pomatia*) occupying a park surrounding the Radziwiłł palace in Balice, near Kraków. Shells of mature individuals were collected. *Cornu aspersum* specimens were aged 0+ (first season) and 1+ (second season) years and *H. pomatia* specimens were aged 2+ to 3+ years. Snails were collected over two consecutive seasons in 2002 and 2003 from microclimatically variable breeding sites, such as field

enclosures, greenhouse enclosures, plastic basins, and a natural park.

Snail breeding for research

Cornu aspersum and *Helix pomatia* were bred in non-heated ground enclosures located either in a greenhouse or in a field from May to the end of September. Agricultural lime was applied annually at a rate of 0.5 kg/m^2 and the field sown with a mixture of pasture plants. The spring hatching density was 300 individuals per 1 m^2. The animals were provided with wooden pallets onto which feed was sprinkled. Animals could find shelter under the feeders. Breeding enclosures were equipped with a water spray. Throughout the entire study, snails were fed the same standard dry feed. In autumn, 7-month old *C. aspersum* specimens were somatically and commercially mature, in contrast to Roman snails that maturated 1-2 years later.

***Cornu aspersum* and *Helix pomatia* shell sampling**

Sixty mature snails were randomly selected from each population. Samples of mature *Cornu aspersum* (age 0+) were collected in autumn when the snails reached physiological and commercial maturity, on the basis of visual inspection of the shell. Shells were prepared and dried at room temperature for morphometric measurements and further physical and chemical analyses. Mechanically damaged shells and shells of irregular structure were discarded prior to the tests. Samples from reproductive adults (age 1+) of *Cornu aspersum* were collected in February, after the first hibernation, and in May, after reproduction in breeding enclosures. Damaged or irregular shells were discarded. *Cornu aspersum* aged 0+ and *Helix pomatia* from the natural populations were collected within a 750-m^2 zone, at a distance of 100 m from the breeding farm.

Microclimatic and breeding condition

Relative air humidity (%) was measured twice daily in June, July, and August in all the breeding areas of the snail farm at the NRI in Balice and in the park surrounding the Radziwiłł palace. Measurements were made in the morning and afternoon, using a portable electronic thermo-hygrometer, and a mean value for the whole season was calculated. The air temperature was also recorded.

Methods of measuring shell features used in the study

The following parameters were examined: seven morphometric parameters and indices; two physical parameters connected with shell mechanical strength; and five chemical parameters and indices related to the calcium and phosphorus content in the shell. The hibernating snail was boiled for 30 seconds in hot water, the carcass was removed from the shell, and the shell was dried at room temperature. Morphometric parameters were measured with an electronic scale and electronic micrometer screw according to the method adopted by the Nature Protection Laboratory of the Polish Academy of Science (Figs. 1A-C). The method to measure shell thickness involved crushing it and taking measurements of the thickness of ten fragments chosen at random to calculate average thickness. The shape index, or quotient of shell width and height, was used to classify *Cornu aspersum* (Chevallier 1977). The solidity index allows comparison of the solidity of shells of various sizes and weights (Cooke 1973, Ireland 1991). Another index designated "the weight ratio of calcium in the solidity index" allows comparison of the mineral fractions ratio in shell on solidity of shells of various sizes and weights too.

Puncture strength is related to the hardness of the most resistant internal layer of a shell, while collapse strength is related to external damage to a shell. A computerized TA.XT2

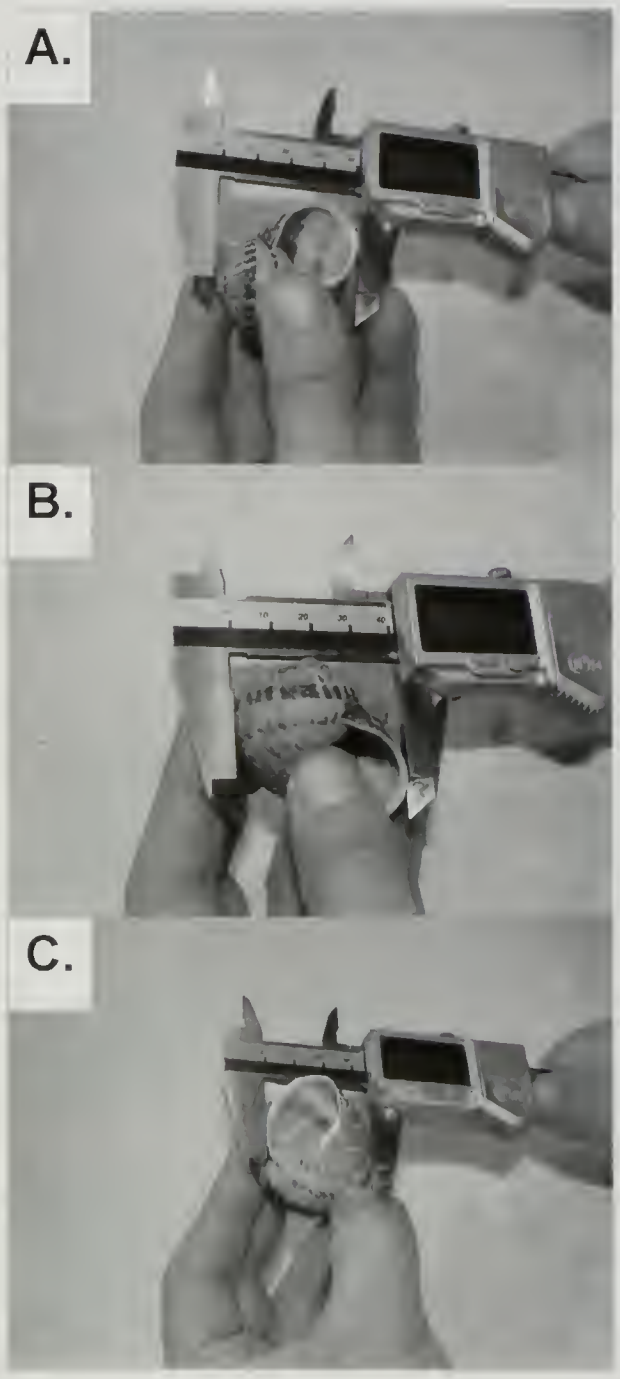

Figure 1. Method of measurement of (A) height, (B) width, and (C) diameter of shell.

Texture Analyser with a 25-kg load cell was used for measuring these parameters. The XT.RA Dimension (ver. 3.7) computer program by Stable Micro Systems, Haselemere, Surrey, UK was used for data collection. The shells were punctured at a rate of 0.1 mm s^{-1} by a needle plunger (SMS-P/2N) from the inside at the point where the widest coil was at its most convex (Fig. 2). The puncture force was defined as the force corresponding to the highest, usually first maximal peak on the force-deformation curve. In measuring collapse strength, pressure was exerted with a flat probe (SMS-P/4) 4 mm in diameter moving at a rate of 2 mm s^{-1} (Fig. 3). Shells were crushed from the outside at the middle part of the last coil. Because of the damage occurring, it was possible to make only one measurement of each shell, and it was impossible to use the same shell for both puncture and collapse strength. The values for these parameters were measured separately in two sub-samples, and the relations between the two forces were calculated using intra-group analysis of a single-factor regression. A list of measured features, corresponding methods, and terms of measurement is given (Table 1).

Statistical analysis of the results

Statistical relationships between pairs of measured shell features were calculated according to a single-factor regression analysis. The same method was used to analyze relationships between the features of the shells, relative humidity, and air temperature. A single-factor analysis of variance based on the least significant differences (LSD) test was performed in order to compare shell feature values between individual breeding populations in two subspecies of *Cornu aspersum*.

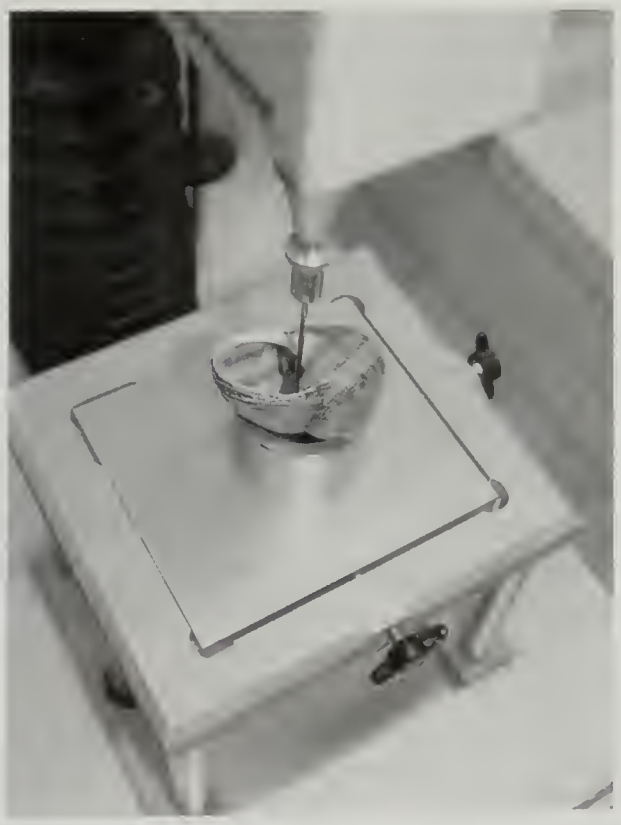

Figure 2. Method of measurement of puncture force of shell.

Figure 3. Method of measurement of collapse force of shell.

RESULTS

Comparison of *Cornu aspersum* and *Helix pomatia*

No statistically significant differences in shell weight, thickness, and diameter were found for age groups 0+ and 1+ of the two breeding populations of *Cornu aspersum maxima* and the two populations of *Cornu aspersum aspersum* (Table 2), indicating the uniformity of breeding conditions for all experimental groups. Statistically significant differences ($P < 0.05$ or $P < 0.01$) between populations of both subspecies were found for shell shape, measured using shape index value; mechanical strength, measured using collapsing force; and, in snails aged 1+, the calcium content in the shell solidity index. For *C. aspersum maxima,* collapsing force increased with age, whereas for *C. aspersum aspersum* it decreased. *Cornu aspersum maxima* shells, compared to *C. aspersum aspersum* shells, were always characterized by a higher weight, solidity index, diameter, thickness, and mechanical strength for puncture and collapse. Despite the close relationship between both subspecies and identical experimental conditions, their shells differed significantly in chemical composition. *Cornu aspersum maxima* shells, for corresponding age groups, contained less calcium and phosphorus and more raw ash than *C. aspersum aspersum* shells. The shells of *Helix pomatia*,

Table 1. List of measured features, corresponding methods, and terms of measurement.

Parameter	Unit	Measurement method
Shell weight	(g)	Measurement made after drying the shell at room temperature
Shell diameter	(mm)	Measurement method (Nature Protection Laboratory of the NAS)
Shell height	(mm)	Measurement method according to Chevallier (1977)
Shell width	(mm)	Measurement method according to Chevallier (1977)
Shell thickness	(mm)	Mean thickness of 10 measurement points
Shell volume	(ml)	Volume of water contained in a shell
Shape coefficient	-	Quotient of shell width and height, Chevallier (1977)
Solidity index	(g cm^{-2}) 100	S.i = [shell weight (height width)$^{-1}$]100 Interpretation: the lower the coefficient value, the less solid the shell Measurement method according to Cooke (1973)
Weight ratio of calcium in solidity index	(g cm^{-2}) 100	S.i. Ca = [Ca weight in shell (height width)$^{-1}$] 100 Interpretation: the lower the coefficient value, the less effect of the mineral fractions ratio in shell on the shell solidity.
Shell puncture force	(N)	TA.XT2 texture analyzer, SMS-P/2N needle 0.1 mm s^{-1}
Shell collapse force	(N)	TA.XT analyser, SMS-P/4 plumper, 2 mm s^{-1}
Calcium	(g, %)	Complexometric titration with sodium versenate (Hermanowicz *et al.* 1999)
Phosphorus	(mg, %)	Colorimetric method with ammonium molybdate and menthol as a reducer in a mineralized sample (Hermanowicz *et al.* 1999).
Raw ash	(g, %)	Ash determined at 550 °C (Hermanowicz *et al.* 1999)

maturating at the age of 2+-3+, were different from the shells of both subspecies of *C. aspersum*, containing more calcium in the solidity index, higher mechanical strength for collapse, and a lower value for shape index.

Effect of microclimatic conditions

On average, relative humidity in 2002 was higher for the whole breeding season (May-September) compared to 2003. The differences ranged between 7.2% (field enclosure) and 8.9% (park), and extreme season-related differences between the various locations ranged from 0.4% to 23.7% (Fig. 4). The mean air temperature in the greenhouse in 2002 was exactly the same as in 2003, and for the other breeding areas the differences ranged from 0.2 to 0.3 °C. However, the differences in the same season between locations were much more pronounced, ranging from 0.9 °C to 2.8 °C (Fig. 5).

For *Cornu aspersum aspersum* and *Cornu aspersum maxima*, in a 6-month breeding cycle, puncture force for mature snails decreased and the collapse force increased with increasing mean seasonal relative air humidity in the breeding areas used for the study. Correspondingly, the calcium content increased in *C. aspersum aspersum* shells, and the calcium and phosphorus content decreased in *C. aspersum maxima* shells (Table 3).

Similarly, the thickness of the shells of both *Cornu aspersum* subspecies increased with increasing mean seasonal temperature. In contrast to the effect of relative air humidity, the shell puncture force increased for the shells of both *C. aspersum* subspecies. The calcium content in the shells of both subspecies decreased with increasing temperature.

Interrelation between shell features

The regression analyses between the pairs of shell features indicated that the highest number of statistically significant ($P < 0.05$) and highly significant ($P < 0.01$) relationships were found in *Cornu aspersum maxima* shells, followed by *Cornu aspersum aspersum*, and *Helix pomatia* (Table 4).

Mechanical strength of Cornu aspersum maxima *shells*

The puncture force for *Helix aspersa maxima* shells increased ($P < 0.05$ or $P < 0.01$) with the increased weight, height, and thickness of the shell as well as with an increasing solidity index and shell collapsing force. Puncture force also increased with decreasing calcium content. Because mean calcium content in shells decreased and shell puncture force increased with snail age, declining calcium content should be considered an index of shell structural maturation, which is accompanied by increasing mechanical strength. No relationship was found between collapse force and the width, volume, and shape of a shell or its phosphorus content (Table 4).

However, relationships between the collapsing force and other shell features differed from the relationships described for puncture force. (1) Shell collapsing force decreased ($P < 0.05$) with increasing diameter, width, and volume of a shell whereas puncture force was not related to these features. (2) Shell collapsing force increased ($P < 0.01$) with decreasing phosphorus content. Just as calcium content was a measure of shell maturation in relation to puncture force, phosphorous played a similar role in relation to collapsing force, constituting an index of the organic structure of a shell.

Table 2. Mean values of features of mature shells of *Cornu aspersum* and *Helix pomatia* from all samples collected in 2002-2003. *, differences of statistical significance ($P < 0.05$); **, differences of high statistical significance ($P < 0.01$).

	Cornu aspersum maxima populations				*Cornu aspersum aspersum* populations				*Helix pomatia*
	"Balice"	"Albino"	"Balice"	"Albino"	"Balice	"French"	"Balice	"French"	
Shell parameters	age 0+		age 1+		age 0+		age 1+		age 2+ - 3+
Weight (g)	3.5	3.6	4.4	4.5	1.7	1.8	2.1	2.0	3.9
Solidity index [(g cm^{-2}) 100]	21.2	21.0	28.3*	26.2*	16.9*	17.8*	20.6	21.3	26.4
Diameter	33.8	35.1	33.0	32.8	27.1	27.2	27.0	27.2	32.4
Shell thickness (mm)	0.33	0.33	0.44	0.42	0.25	0.27	0.31	0.31	0.37
Shape index	1.05**	1.09**	1.05**	1.09**	1.08*	1.07*	1.08**	1.05**	0.97
Calcium content (g, %)	34.8	34.6	32.4	32.3	41.5	41.5	38.1	39.8	36.8
Calcium content in 150 shells (g)	179	182	212*	188*	107	113	117	117	212
Calcium content in solidity index (g cm^{-2})	7.2	7.1	9.1**	7.3**	7.1	7.4	7.7**	8.3**	9.7
Phosphorus content (mg, %)	0.0024	0.0026	0.0011*	0.0016*	0.0087**	0.0054**	0.0044**	0.0055**	0.0015
Raw ash content (g, %)	81.9	79.3	83.2	84.5	62.9	70.2	65.3	64.7	66.6
Shell puncture force (N)	20.0	20.0	26.7	26.0	9.5*	10.7*	14.6**	17.0**	23.2
Shell collapse force (N)	77.6**	62.0**	105.3**	79.8**	51.7**	64.9**	39.7**	50.0**	122.6

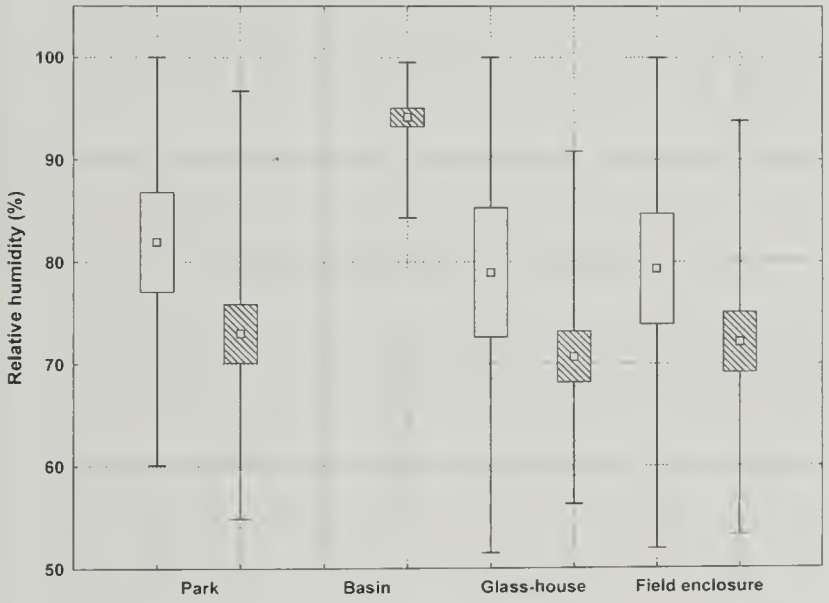

Figure 4. Mean relative humidity in 2002 (open boxes) and 2003 (hatched boxes). Box represents ± *SE*; whiskers indicate maximum and minimum values.

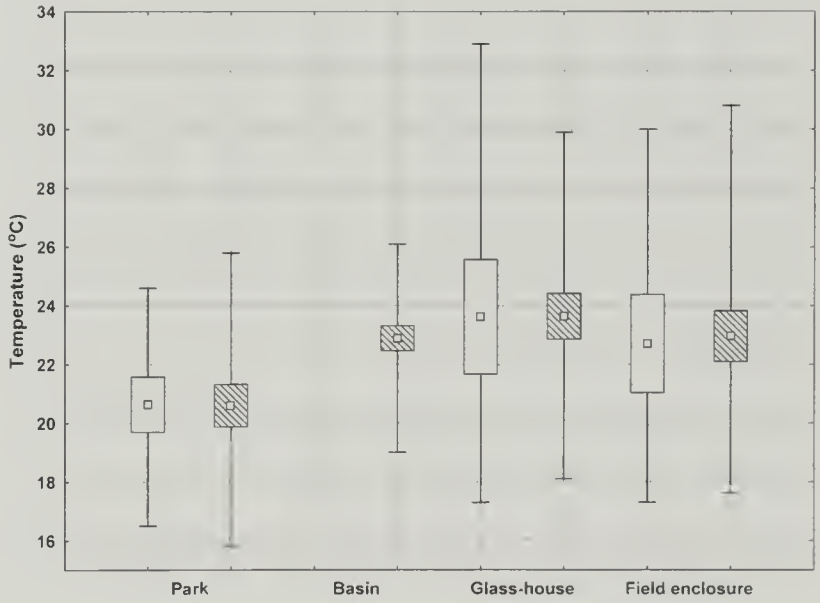

Figure 5. Mean temperature in 2002 (open boxes) and 2003 (hatched boxes). Box represents ± *SE*; whiskers indicate maximum and minimum values.

Mechanical strength of Cornu aspersum aspersum *shells*

The shell puncture force increased ($P < 0.05$ or $P < 0.01$) with increasing weight and thickness of a shell, and with an increase in the shell solidity index. As in the case of *Cornu aspersum maxima*, the puncture force increased ($P < 0.01$) with decreasing calcium content, providing further proof that decreasing calcium content constitutes an index of shell structural maturation. No relation was found between puncture force and the diameter, width, and volume of a shell. The increase in force with decreasing raw ash content proved to be statistically not significant. No correlations were found between the shell collapsing force and other shell features or even with the puncture force, this being an important difference between the shells of this subspecies and those of *Cornu aspersum maxima*.

Mechanical strength of Helix pomatia *shells*

In this study there was no statistically significant relationship between the mechanical strength of *Helix pomatia*

Table 3. Correlation coefficients (r) for statistically significant ($P < 0.05$ or 0.01) relationships between mean relative humidity or temperature in the snail breeding period and mean values of selected features of mature *Cornu aspersum* shells.

Climatic factor	*Cornu aspersum* subspecies	Weight	Diameter	Thickness	Calcium content (%)	Phosphorus content (%)	Raw ash content (%)	Puncture force	Collapse force
Relative humidity	*Cornu aspersum aspersum*	-0.79	-0.93		0.85[1]			-0.84	0.52[2]
	Cornu aspersum maxima		-0.75		-0.95[1]	-0.71	-0.74	-0.93	0.49
Temperature	*Cornu aspersum aspersum*			0.58	-0.74			0.88	
	Cornu aspersum maxima	0.73		0.53	-0.62[3]			0.98	

[1] for humidity over 80%, relationship between the features disappeared
[2] for humidity over 72%, relationship between the features disappeared
[3] relationship found for temperatures over 22.9 °C

shells and other shell features. Particulary, relatively high correlation coefficients between the shell puncture force and its shape and phosphorus content were not statistically significant. The same, statistically not significant were high correlation coefficients between the shell collapsing force and the shape index, calcium content, and phosphorus content.

Differences between *Helix pomatia* and *Cornu aspersum* regarding the relationships between the mechanical strength of a mature shell and its chemical composition were the result of differences in the ages of snails in separate age groups and differences related to sampling sites. Samples of the Roman snail originated mainly from the natural environment, with different microclimatic conditions than in the breeding enclosures of *C. aspersum.*

Relationship between Cornu aspersum *and* Helix pomatia *shell solidity and calcium content*

No relationships were found between the shell solidity index and the total content by weight of accumulated calcium, nor between the calcium content in the solidity index and the percentage of calcium content in shells. However, for *Cornu aspersum maxima*, *Cornu aspersum aspersum*, and *Helix pomatia,* significant relationships were found between the shell solidity index and the percentage of calcium content in the shell (Table 5) and calcium content in the solidity index.

DISCUSSION

Shell calcium, phosphorus content, and shell mechanical strength

The results of regression analysis indicated that increasing puncture and collapsing force in the case of *Cornu aspersum maxima* shells and increasing puncture force in *Cornu aspersum aspersum* were accompanied by a decrease in calcium content. Calcium content decreased with the age of the snail whereas shell mechanical strength increased with age, which confirms the relationship between the observed quantitative chemical changes and the structural maturation of a shell. Shell mechanical strength was also largely dependent on phosphorus content, despite the fact that this element constituted only a small fraction of the weight of the shell. Phosphorus was, however, an indicator of the organic matter contained in a mature shell, or was a residue after transformations important for shell maturation. In *C. aspersum maxima,* collapsing force decreased with an increase in phosphorus content, and in *Helix pomatia,* the force increased. The puncture force for *C. aspersum aspersum* and *H. pomatia* shells decreased with an increase in the phosphorus content.

The role of calcium and phosphorus as indicators of shell structure differentiation is confirmed by the fact that the percentage content of phosphorus was more closely correlated to shell collapsing force than to shell puncture force. However, puncture force, which increased with the increasing unitary weight of a shell and its solidity index, was negatively correlated with the percentage of calcium content for all the studied species and subspecies of snails.

It was assumed that both puncture and collapsing forces for both species of snails would increase with increasing shell weight, solidity index, and thickness. However, in both subspecies of *Cornu aspersum* and in the Roman snail (*Helix pomatia*) this assumption was true only for shell puncture force. In *Cornu aspersum aspersum,* no relationship was found for the collapsing force, and in the Roman snail, the shell collapsing force actually decreased with increases in weight and shell solidity index. The puncture force value does indeed depend on the above-mentioned physical features, but collapsing force was more dependent on the variable microstructural features of a shell in individual species and populations of snails.

Table 4. Correlation coefficients (r) for relationship between mean values of features of mature *Cornu aspersum* and *Helix pomatia* shells. *, statistically significant result ($P < 0.05$); **, highly statistically significant result ($P < 0.01$).

Parameter	Species	Height	Width	Diameter	Thickness	Volume	Shape index	Solidity index	Puncture force	Collapse force	Calcium	Phosphorus
Weight	*C. aspersum maxima*				0.86**			0.89*	0.98**	0.82*	-0.70**	
	C. aspersum aspersum	0.71**	0.62**	0.68**	0.78**	0.64**		0.77**	0.96**		-0.77**	
	Helix pomatia	0.89**				0.93*	0.92*					
Diameter	*C. aspersum maxima*	0.89**	0.98**		-0.52*	0.97**		-0.55*		-0.82*		0.74**
	C. aspersum aspersum	0.96**	0.98**			0.98**	0.65**					
	Helix pomatia		1.00**		1.00**							
Height	*C. aspersum maxima*		0.85**	0.89**	-0.51*	0.89**		-0.62*	0.75*	-0.77*		0.69**
	C. aspersum aspersum		0.96**	0.96**		0.96**					-0.55*	-0.54*
	Helix pomatia		0.98**			0.89*						
Width	*C. aspersum maxima*	0.85**		0.98**	-0.56*	0.98**		-0.61*		-0.80*		0.70**
	C. aspersum aspersum	0.96**		0.98**		0.98**		0.70**				
	Helix pomatia	0.98**		1.00**		0.96*						
Thickness	*C. aspersum maxima*	-0.51*	-0.56*	-0.52*		-0.64**		0.95**	0.99**	0.93**	-0.52*	-0.60*
	C. aspersum aspersum							0.94**	0.97**		-0.68**	
	Helix pomatia			1.00**								
Volume	*C. aspersum maxima*			0.97**	-0.64**			-0.69**		-0.80*		0.78**
	C. aspersum aspersum			0.98**				0.94**				
	Helix pomatia	0.89*	0.96*									
Solidity index	*C. aspersum maxima*			-0.55**	0.95**	-0.69**			0.98**	0.89**	-0.70**	-0.58*
	C. aspersum aspersum				0.94**	0.94**			0.98**		-0.62**	
	Helix pomatia											
Puncture force	*C. aspersum maxima*	0.75*			0.99**			0.98**		0.97**	-0.98**	
	C. aspersum aspersum				0.97**			0.98**			-0.94**	
	Helix pomatia											
Collapse force	*C. aspersum maxima*	-0.77*	-0.80*	-0.82**	0.93**	-0.80*		0.89**	0.97**			-0.85**
	C. aspersum aspersum											
	Helix pomatia											
Calcium	*C. aspersum maxima*				-0.52**			-0.68*	-0.98**			
	C. aspersum aspersum	0.55*			-0.68**			-0.67**	-0.94**			0.62*
	Helix pomatia							-0.73*				

Nutritional experiments with the edible land snail *Achatina fulica* Bowdich, 1822 indicate that the calcium content in mature shells was higher when snails were fed with a feed of relatively low calcium content, and that increased calcium content in the feed caused a significant decrease in shell thickness and inhibition of growth (Ireland 1991). The author suggests that toxic metals, which are present as a contaminant of calcium carbonate in the feed, have an adverse effect on the snails (Russel *et al.* 1981, Ireland 1986). Foreign admixtures, especially metallic ones, may weaken and disturb the aragonite crystalline structure, displacing calcium from carbonate chemical bonds. However, the possible negative effect of foreign admixtures should not be overestimated, as a decrease in the percentage of calcium content in *Cornu aspersum* and *Helix pomatia* shells was accompanied by increased thickness and mechanical strength.

Table 5. Correlation coefficients and linear regression equations between calcium content (x) in shell and solidity index (Y).

Feature (independent variable x)	Snail species	Regression equation	Coefficient of correlation	Significance of regression equation
Weight ratio of calcium in solidity index	*Cornu aspersum maxima*	Y = 2.0 x + 7.2	0.57	$P < 0.05$
	Cornu aspersum aspersum	Y = 2.6 x + 1.5	0.81	$P < 0.01$
	Helix pomatia	Y = 2.7 x – 1.6	0.81	$P < 0.05$
Percent calcium content in shell	*Cornu aspersum maxima*	Y = - 0.55 x + 41.7	-0.68	$P < 0.05$
	Cornu aspersum aspersum	Y = - 0.51 x + 39.1	-0.67	$P < 0.01$
	Helix pomatia	Y = - 0.74 x + 52.7	-0.73	$P < 0.05$

Microclimate and shell mechanical strength

Goodfriend (1986) analyzed the influence of microclimatic factors and calcium content in soil on shells of several natural populations of helicid species. The results were inconclusive since the research was carried out on individuals of unknown age, using various sources of food and drinking water.

In the present study, however, the increase in shell collapsing force with increased air humidity is explained by the effect of humidity on the calcium and phosphorus content in shells: as air humidity increased, the content of those two elements in *Cornu aspersum maxima* shells decreased, marking a faster and more complete structural maturation. The fact that in *C. aspersum maxima* shells the calcium and phosphorus content decreased with increasing air humidity by up to 79.0%, while in *Cornu aspersum aspersum* shells the situation was the opposite, with the content of both elements increasing, indicates the existence of physiological differences in shell maturation between the two subspecies. The natural populations of the former subspecies live in a Mediterranean climate on the north coast of Africa, which is much drier than the climate of the Atlantic coast of Europe from where the latter subspecies originates. Living in a dry climate, the former subspecies reacted to short, high increases of humidity up to a level of approx. 80% with normal shell maturation. The European subspecies reacted in the same way to decreasing humidity since its natural habitat has a very high humidity. For this reason, the collapsing force of the latter subspecies ceased to increase at an air humidity of over 72.1%, within an observed mean humidity range of 70.7%-94.4%.

The effect of air temperature on shell quality during the breeding season was clear. Results of intra-group regression analysis confirm a strong correlation between an increase in mean air temperature during the breeding season and increased shell puncture force, while at the same time the collapsing force for both subspecies of *Cornu aspersum* decreased. However, the region of origin had a discernible effect on the reaction rate of both subspecies since the correlation coefficients between a rise in the air temperature and shell puncture force were higher for thermophilic *C. aspersum maxima* than for *C. aspersum aspersum* originating from European Atlantic coast populations. The high thermal requirements necessary for the regular development of *C. aspersum maxima* shells are indicated by the fact that the correlation coefficient between a decrease in the shell calcium content, denoting a higher maturity of a shell, and a rise in air temperature was significant only for average temperatures of over 22.9 °C. In the case of *C. aspersum aspersum*, the coefficient was significant, and even higher than in the case of the first subspecies within the entire range of average temperatures used for the study, that is, from 20.6 °C to 23.6 °C.

Geographic and genetic origin and shell features

Regression indicated a statistically highly significant discrepancy between increasing calcium content in shell samples and decreasing solidity. For *Cornu aspersum* and *Helix pomatia*, these equations are similar, and for both subspecies of *C. aspersum* the values of regression coefficients are almost identical. This is physiological confirmation of a very close genetic relationship between those two subspecies.

As indicated by regression equations, the solidity index also increases with the weight of calcium in the index. The slope of the regression line is almost identical for *Cornu*

aspersum aspersum and *Helix pomatia*, suggesting a genetically fixed effect of similar environmental and climatic factors on both European taxa of snails in contrast to the North African *Cornu aspersum maxima*.

It is interesting that in studies regarding both subspecies of *Cornu aspersum*, there was a significant relationship between increasing shell thickness and decreasing calcium content in shells. This was probably related to changes in the content of organic structural components of the shell, and to changes in the crystalline structures of the aragonite layers of which a mature shell is composed (Weiner and Traub 1980, Weiner *et al.* 1983).

The thick, solid, and more mechanically resistant African *Cornu aspersum maxima* shell provides effective protection against water loss from the snail's body, whereas the European, less mechanically resistant *Cornu aspersum aspersum* shell from the wet Atlantic zone is evidently required to perform less of a protective function.

Conclusion

The greatest number of correlations between the features of *Cornu aspersum* or *Helix pomatia* shells indicated that the highest number of statistically significant ($P < 0.05$) and highly significant ($P < 0.01$) relationships was found in *Cornu aspersum maxima* shells, followed by *Cornu aspersum aspersum* and *Helix pomatia*. In particular, the increasing puncture and collapsing force in the case of *C. aspersum maxima* shells and increasing puncture force in *C. aspersum aspersum* were accompanied by a decrease in calcium content. Calcium content decreased in older snails.

Microclimatic factors have opposing effects on the shaping of individual parameters of mechanical strength. It was found that with the increase of average temperature during the snail breeding season, shell puncture force increased. Conversely, as average humidity increased, the puncture force decreased and the collapsing force increased. Therefore, achieving maximum mechanical strength requires the optimal combination of these two microclimatic factors.

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Rediscovery of the sacoglossan opisthobranch *Hermaea wrangeliae* (Ichikawa, 1993) in Okinawa, Japan

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Abstract: In December 2008, eight sacoglossan specimens of *Hermaea* Lovén, 1844 were collected from uniseriate red algae (cf. *Wrangelia tanegana*) at two sites on the west coast of Okinawa, Japan. These specimens are compared to the described *Aplysiopsis wrangeliae* Ichikawa, 1993 from Okinawa which we and other authors have previously suggested belongs to the genus *Hermaea*; we suggest that our specimens are attributable to *Hermaea wrangeliae* (Ichikawa, 1993).

Key words: Sacoglossa, Ascoglossa, *Aplysiopsis*, Rhodophyta, *Wrangelia*

Japan and Australia are two regions of high diversity for sacoglossan opisthobranchs, essentially "hot spots" compared to other geographic regions (Jensen 2007). Our work on Japanese shores (2002-2009) has supported Jensen's biogeographic analysis. For example, our comprehensive synthesis of the literature (the indexed as well as the less accessible, non-indexed sacoglossan records) indicates that there are at least 100-150 species of sacoglossans on Japanese shores (Trowbridge *et al.* 2007 and unpubl. data).

Many western Pacific and Indo-Pacific species of sacoglossans are insufficiently described. This problem is acute for the cerata-bearing species (Limapontioidea), particularly those species associated with red algae. Trowbridge *et al.* (2009a) recently reviewed the approx. 20 species in three sacoglossan families that feed on red algae in the order Ceramiales; we concluded that the Okinawan species described as *Aplysiopsis wrangeliae* Ichikawa, 1993 undoubtedly belonged to the genus *Hermaea* Lovén, 1844. Because specimens were neither archived at Seto Marine Laboratory (contrary to Ichikawa 1993) nor retained by the author (M. Ichikawa, pers. comm.) and because no internal morphological features were described, it was not possible to confirm the correct genus. This was unfortunate because sabot-shaped radular teeth would largely support placement in the genus *Aplysiopsis* Deshayes, 1835. [The term sabot-shaped refers to that of a Dutch wooden shoe (Jensen 2001); within the family Hermaeidae, sabot-shaped teeth occur in *Aplysiopsis* but not in *Hermaea* (Jensen 1996)].

Based on the algal host association described for *Aplysiopsis wrangeliae* (namely the red alga *Wrangelia tayloriana*) and the fact that no *Aplysiopsis* species has been shown to feed preferentially on red algae pointed to Ichikawa's species belonging to *Hermaea*. Our interpretation (Trowbridge *et al.* 2009a) was implicitly supported by Jensen (2007) who also listed the Okinawan species as *Hermaea wrangeliae*.

After recently collecting eight sacoglossan specimens that appear to fit the description of *Aplysiopsis wrangeliae*, we investigated this issue more explicitly, considering nine characters that differ between the sacoglossan genera *Aplysiopsis* and *Hermaea* (Jensen 1996 and pers. comm.).

COLLECTIONS

On 11 December 2008, we collected five specimens of *Hermaea* from about 0.5 L of delicately branching red algae from the shallow subtidal region (<0.5 m deep) at Zanpamisaki (26°26′15″N, 127°42′48″E) on the west coast of the main island of Okinawa, Japan. The algae grew profusely in dense turfs in sandy areas and as epiphytes on a variety of other macroalgae. The alga was uniseriate throughout (single row of cells attached end-to-end), diminutive (<5 cm tall), and extensively branched (Figs. 1H-M). N. Miyamoto, a phycologist resident in Okinawa, kindly confirmed the alga belonged to the order Ceramiales. The vegetative morphology was consistent with drawings by Okamura (1934) for *Wrangelia argus* (now considered to be *Wrangelia tanegana*). Non-reproductive algae, however, cannot be identified definitely to genus or species as most of the diagnostic traits relate to the temporally transient reproductive structures. Thus, we refer to the alga as cf. *Wrangelia*. Voucher specimens were made of this alga in case molecular identification might be possible.

The second collection was on 16 December 2008 at Sobe (26°23′12″N, 127°43′24″E) in Yomitan on the west coast

of the main island of Okinawa. We collected approx. 1.1 kg (wet weight) of the shallow subtidal green alga *Codium geppiorum* which often is covered in red algal epiphytes belonging to the Ceramiales. Although *C. geppiorum* has not previously been reported from Okinawa, Chang *et al.* (2002) recently redescribed the species complex and kindly identified our specimens in 2006 (J.-S. Chang, pers. comm.). While sorting through the *Codium*, we found three additional specimens of *Hermaea*. Because of logistic constraints (namely imminent departure from Okinawa), we did not have the opportunity to identify or characterize the red algal epiphytes.

DESCRIPTION AND DISCUSSION

External morphology

Type description

The holotype specimen—from Kuro Island, Okinawa—was 10 mm long (Ichikawa 1993); the length of the other five specimens was not provided. Key characters were auriculate rhinophores with a short ear-like flap and indented cerata containing digestive diverticulae. Other features included a transparent body, transverse stripes on rhinophores, white spots on the head, a large dark spot on the middle of the neck, and a white dotted line along the ventral border of the foot. There was a relatively schematic line drawing (Ichikawa 1993: fig. 3) and an out-of-focus photograph (plate II, fig. 11) to complete the half-page type description.

The assignment of Ichikawa's specimens to the genus *Aplysiopsis* was seemingly based on two attributes: auriculate rhinophores with a small flap and the presence of pale brown longitudinal lines on the sole of the foot of some (but not all) specimens. Based on Jensen (1996), such rhinophores are found in 2-3 genera: *Aplysiopsis, Hermaea,* and *Hermaeopsis* A. Costa, 1869 (the latter of which may be synonymous with *Hermaea*). They are also found in at least one *Costasiella* species, namely *Costasiella illa* (Marcus, 1965) (Jensen, pers. comm.). The second character is also problematic as it was found in only some individuals. No data were reported to evaluate the two other external characters that differ between the genera: the shape of the pericardium and the presence or absence of dorsal vessels (Table 1).

Our observations

Our Okinawan specimens ranged from 1.5 to 4.5 mm ($N = 8$). The auriculate rhinophores were distinctly grooved with a small flap; this character was readily observed at all angles (Figs. 1A-G). The specimens observed in detail had multiple transverse dark bands encircling the rhinophores. The intensity of the transverse markings varied considerably among specimens (Figs. 1A-G); the distal two bands were more pronounced than the proximal band. Three transverse bands were distinct in our larger specimens. The regions between the bands were heavily encrusted with white pigment granules (Fig. 1C); thus, the dark bands appear dark in part because of the absence of white dots and in part because of the presence of dark pigmentation. Under low magnification, the dark color appeared black, but under high magnification, the spots appeared to be dark wine-colored to red in color on well-fed specimens (Figs. 1C-D). Dark pigment spots were not restricted to the rhinophore bands: they were also distributed sparsely between the longitudinal rows of cerata, the dorsal surface of the head, the lateral sides of the body, and the proximal surfaces of the cerata.

There were 8-12 large cerata per sacoglossan as well as 7-13 diminutive ones generally alternating with larger ones (Figs. 1A-B, Table 2). Cerata were covered with white spots, particularly on the distal ends, and contained digestive diverticulae approx. 100-150 μm in diameter. There was a central core with lateral primary branches that extended at an approx. 80-90° angle from the central core. The laterals extended out in typically 3-4 directions. In one specimen, the laterals also branched; in the other specimens, they did not.

The overall shape of the cerata within individuals was temporally changeable from mildly lumpy to extremely spiky. The ceratal surface could be contracted down to the diverticulae, at which point the widest part of each ceras was near the distal-most branches, rendering the cerata very knob-like. At other times (in the same individual slugs), the ceratal surface could be inflated or raised away from the relatively inflexible diverticulae; the difference in appearance between the contracted and expanded cerata was strikingly apparent. Ceratal shape, therefore, is not a strong diagnostic feature for species description.

The white spots described by Ichikawa (1993) were concentrated on the head, the tips of the cerata, rhinophores, and lateral margin of the foot. The spots are presumed to be pigment granules, not epidermal glands, as they did not release white fluid when the specimens were prodded with a blunt probe or gently squeezed with forceps. When we turned individual specimens over, the line of white dots demarcating the edge of the foot was distinctive. Most specimens lacked white dots on the ventral surface of the foot but all had the dots along the edge; one specimen had sparsely distributed dots across the sole that were not at all obvious.

In all specimens we observed (of this species and other *Hermaea* spp.), the anterior end of the digestive tract occurred posterior to the eyes as the esophagus extends dorsally and then veers to the left side of the animal. This section of the digestive system contains a large number of fresh, red algal chloroplasts; however, in well-fed specimens, the internal structure (esophageal pouch) clearly extends to the animal's left (Figs. 1A-G). We observed the entry of chloroplasts into

Table 1. Comparison of two sacoglossan genera based on 6 differences (from Jensen 1996) and 3 supplementary attributes.

Evidence	Characters (#[1])	*Aplysiopsis*	*Hermaea*	*Aplysiopsis wrangeliae*[2]	*Hermaea* sp. (this study)
Primary attributes					
	pericardium (20)	elongate	oval	not shown in drawing or photograph; not described in text	oval to round
	dorsal vessels (21)	present	absent	not shown in drawing or photograph; not described in text	none seen
	shape of radular teeth (32)	sabot-shaped	blade-shaped	not described	blade-shaped
	lateral flanges on teeth (35)	absent	present or variable[3]	not described	unclear with light microscopy
	number of visceral ganglia (40)	3	2[4]	not described	2
	seminal receptacle (49)	absent	variable	not described	absent
Supplementary attributes					
	coloration	typically heavily pigmented	typically translucent	transparent[5]	translucent
	sole of foot	pair of stripes present in many species	no stripes	pair of stripes in some specimens	no stripes
	algal diet	septate green algae (*e.g.*, *Cladophora* or *Chaetomorpha*)	septate red algae (or diatoms in *Hermaea vancouverensis* O'Donoghue, 1924)	septate red alga *Wrangelia*	septate red algae

[1] character numbers based on Jensen (1996, table 3)
[2] based on species description of Ichikawa (1993)
[3] depends on whether or not *Hermaea* and *Hermaeopsis* are considered synonymous
[4] this character is supported for our present species but not for two other Japanese *Hermaea* species we have dissected (Hirano *et al.*, unpubl. obs.)
[5] translucent is undoubtedly more appropriate

the esophageal pouch during feeding and the movement of chloroplasts and fluid into the digestive diverticulae.

Potential discrepancies

There were three apparent discrepancies between Ichikawa's description and our specimens. First, Ichikawa (1993) reported three green transverse bands on the rhinophores whereas we observed red ones. If the dark spots were colored by ingested algal chloroplasts (as is true with many sacoglossans), then the red vs. green color discrepancy presumably reflects variation in feeding status of the specimens: red algal chloroplasts turn green as they age and disintegrate.

Second, the dark spot on the middle of the neck illustrated by Ichikawa (1993: 126 in fig. 3) was posterior to the eyes and symmetrical. Our observations differed slightly from this description: the structure we saw (esophageal pouch) extends to the animal's left (Figs. 1A-G). Ichikawa's photograph (plate II, fig. 11) appears consistent with ours (Fig. 1) despite the former being small and unfocused.

Third, Ichikawa (1993: 126) stated "Some of the paratype specimens have two light brown, longitudinal stripes on the sole of the foot, as in other species of *Aplysiopsis*". Such stripes occur in many Japanese species including *Aplysiopsis toyamana* (Baba, 1959), *Aplysiopsis minor* (Baba, 1959), and *Aplysiopsis orientalis* (Baba, 1949) but not *Aplysiopsis nigra* (Baba, 1949). We consider it rather inadequate to identify all specimens as *Aplysiopsis* based on a character found in only some individuals: genus determination should be based on characters in all conspecific specimens. None of our *Hermaea* specimens had such stripes. If Ichikawa's specimens were *Hermaea*, then how could we account for her observations? One possibility is that not all specimens have this trait, only some did. Another possibility is that she misinterpreted darker internal structures as superficial stripes. For example, the longitudinal trunks of the digestive

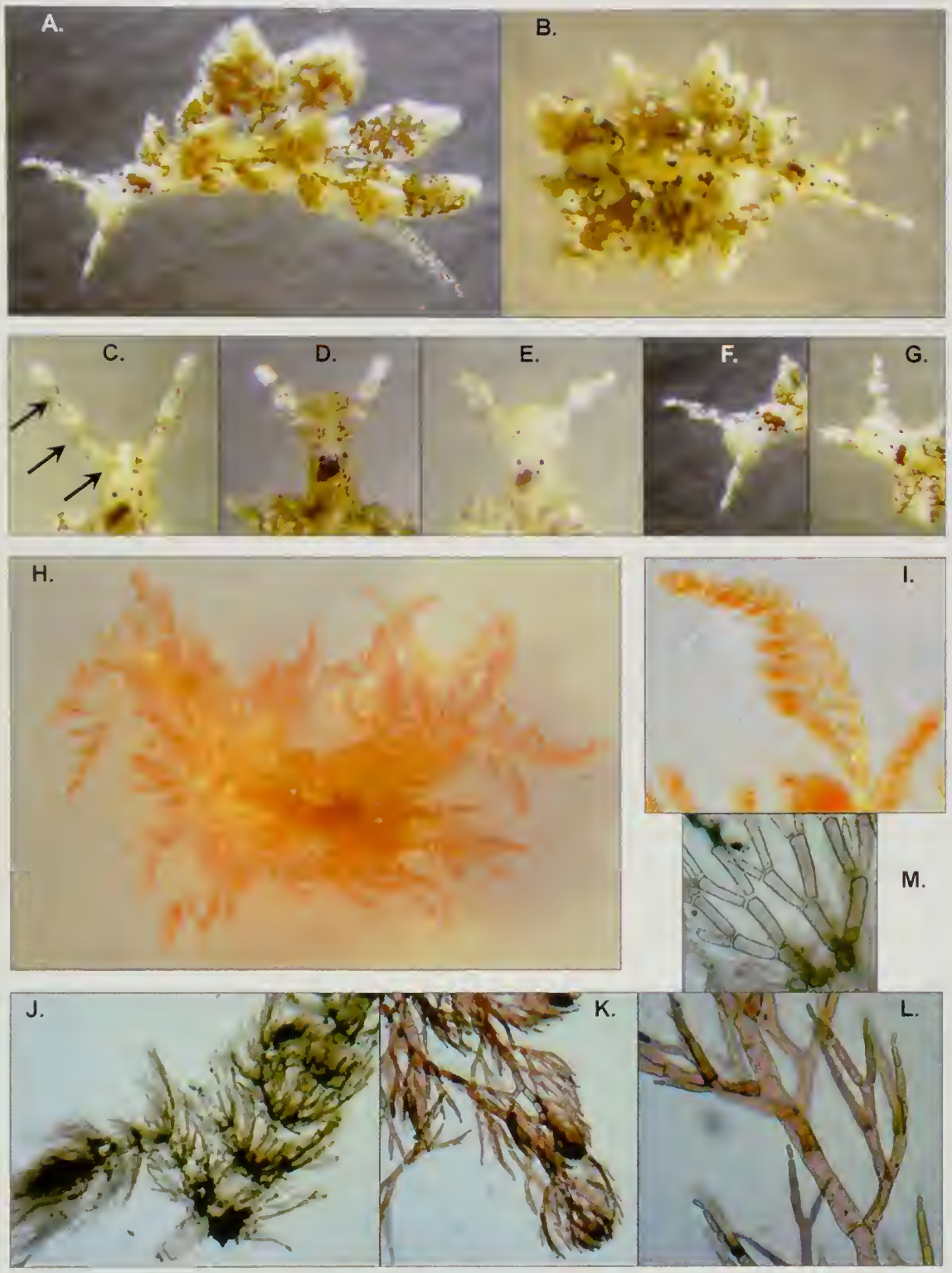

Figure 1. Specimens of *Hermaea wrangeliae* (Ichikawa, 1993) collected in December 2008 from two locations on the west coast of Okinawa, Japan. A-B, Oblique view of sacoglossan's side to illustrate the rhinophore shape, cerata attributes, presence of distinct esophageal pouch (pigmented by ingested red algal chloroplasts), and white pigment granules on rhinophores, cerata, tail, and elsewhere. C-G, Dorsal views of head with medial-lateral esophageal pouch (reddish brown structure). Transverse bands indicated by arrows in C; bands were less obvious in smaller specimens. Epidermal reddish-brown markings best seen in D. Specimens ranged in size from 1.5 to 4.5 mm long. H-L, Red algal host collected on 11 December 2008 from Zanpa-misaki, Okinawa. H, Entire thallus a few centimeters long; I, close-up of a single primary branch with lateral secondary branches. J, Close-up view of secondary branch, illustrating main axis with clusters of uniseriate branchlets arranged in whorls around the axis. K-M, Microscopic views of branchlets (ca. 100 µm long and 15-20 µm wide) with new branchlets arising at terminal ends of each cell. The sacoglossans puncture the cells of these branchlets.

diverticula (which contain chloroplasts) may be seen through the translucent sole of the foot. Variation in feeding (amount ingested and/or time since last meal) could cause variation in slug appearance.

Given the nature of Ichikawa's observations and descriptions of other sacoglossan species in the 1993 paper, we presume that our species is the one she described, but that the type description was superficial and rather stylized. A less parsimonious explanation (but not mutually exclusive) is that two or more sympatric *Hermaea* spp. feed on red algae in Okinawa.

Feeding and reproduction

Type description

Ichikawa (1993) reported *Aplysiopsis wrangeliae* from the red alga *Wrangelia tayloriana* (now considered synonymous with *Wrangelia tanegana* (see Guiry and Guiry 2009)). She stated that the sacoglossan species was "limited to" this alga, a rather strong statement given that she collected only six specimens and did not confirm actual feeding by the sacoglossan on the alga. She also stated that the body shape and color "perfectly match this host" (Ichikawa 1993: 126); the statement is potentially misleading as all or most *Hermaea* match their hosts, regardless of whether they are stenophagous or comparatively polyphagous on red algal hosts; the retention of ingested algal chloroplasts is the basis of this nutritional homochrony. Finally, no reproductive details were provided for this species.

Our observations

The specimens from Zanpa-misaki were collected from a Ceramialean red alga structurally similar to *Wrangelia tayloriana* (Figs. 1H-L). The uniseriate alga was a few centimeters long with delicately branching laterals that arose from the main axes in whorls at each node. The terminal branchlets (Figs.

Table 2. Details about 5 specimens of *Hermaea* collected from Zanpa-misaki, western Okinawa on 11 December 2008.

Body length (mm)	# of cerata on left side	# of cerata on right side	Total # of cerata	Notes
3.5	4 large + 5 small	4 large + 2 small	15	• 2-3 tiers of primary branches on digestive diverticulae in cerata • no secondary branches
4.0	3 large + 8 small	6 large + 5 small	22	• 3-4 tiers of primary branches on digestive diverticulae in cerata • no secondary branches
4.0	5 large + 6 small	5 large + 6 small	22	• 3-4 tiers of primary branches on digestive diverticulae in cerata • only 1 secondary branch
3.0	6 large + 4 small	6 large + 5 small	21	• 3-4 tiers of primary branches on digestive diverticulae in cerata • no secondary branches
1.5	6 large + 4 small	4 large + 4 small	18	• not examined

1K-M) were approx. 100 µm long and 15-20 µm wide; these were the cells punctured and drained by our specimens. We confirmed the actual feeding of all our *Hermaea* specimens on the red alga (Figs. 1H-M). The slugs were strongly attracted to the algal thalli and punctured and emptied the uniseriate cells.

On 11 December 2008, we observed two of the specimens initiate direct frontal contact involving rubbing the frontal part of their heads together. This behavior was similar to that observed in *Stiliger berghi* Baba, 1937 directly before copulation and spawning. Although we did not observe the actual copulation, an egg mass was deposited within the next few hours and then another one on a subsequent day. There was one embryo per capsule and the masses lacked extracapsular yolk. The timing of egg mass deposition did not enable us to measure uncleaved ova; we measured the capsules and embryos at the gastrula stage. Capsules averaged 106.3 × 121.0 µm (N = 15) and embryos averaged 60.7 × 67.9 µm (N = 15). These values are comparable to those of at least three other red algal feeders (*Hermaea bifida* (Montagu, 1815), *Stiliger fuscovittatus* Lance, 1962, and *Stiliger berghi*) (see Trowbridge *et al.* 2009a).

Internal anatomy

Type description

Ichikawa (1993) provided no details on internal anatomy of this species or of the other 11 new species described in the same publication. Thus, the four internal characters that could distinguish between *Aplysiopsis* and *Hermaea* (Table 1) could not be evaluated for the type specimen.

Our observations

We examined the radular morphology of two specimens to distinguish between *Aplysiopsis* (with sabot-shaped teeth) and *Hermaea* (with blade-shaped teeth) (see Jensen 1996: 109). The buccal masses were removed from formalin-preserved specimens (fixed in 5% formalin seawater), placed in dilute bleach (10% Clorox) to remove organic tissue, and then examined and photographed under a compound microscope. The radular teeth were fairly thick (laterally) and blade-shaped, thereby refuting the *Aplysiopsis* placement.

The radula of the 3.5 mm specimen had 8 teeth plus 1 "ghost" tooth (forming) in the ascending series of unused teeth; the slug had 17 teeth plus 2-3 pre-radular elements (basal rod-like elements) and 3 transitional ones (with short, incompletely formed blades) in the descending series of used teeth. The leading tooth was 32.8 µm long and 12.5 µm wide at the base. The 4 mm specimen had 9 teeth plus 1-2 ghost teeth in the ascending series and 17 teeth plus 2-3 preradular elements and 3 transitional teeth in the descending series (Fig. 2). The size of the leading tooth was 34.3 µm long and 14.0 µm wide at the base. The cutting edge of each radular tooth appeared smooth under the light microscope. There was a lateral ridge on each tooth but whether this constituted "lateral flanges" (see Table 1) was unclear with light microscopy. In the two specimens examined, the descending series of teeth terminated in a loose coil of used teeth (see Fig. 2), not in tight ball nor in a heap of disarticulated teeth.

The pharynx was rather small. The pharyngeal lips were moderately thick, and the dorsal septate muscle was rather low and thin. The ventral ascus muscle was also thin and steeply inclined to the longitudinal axis of the pharynx. The esophagus was very short and had a rather large, elongate esophageal pouch immediately behind the nerve ring. In living specimens, the pouch is externally visible (Fig. 1) due to pigmented algal chloroplasts. The digestive tract then reached a small stomach from which a very short intestine ran to the

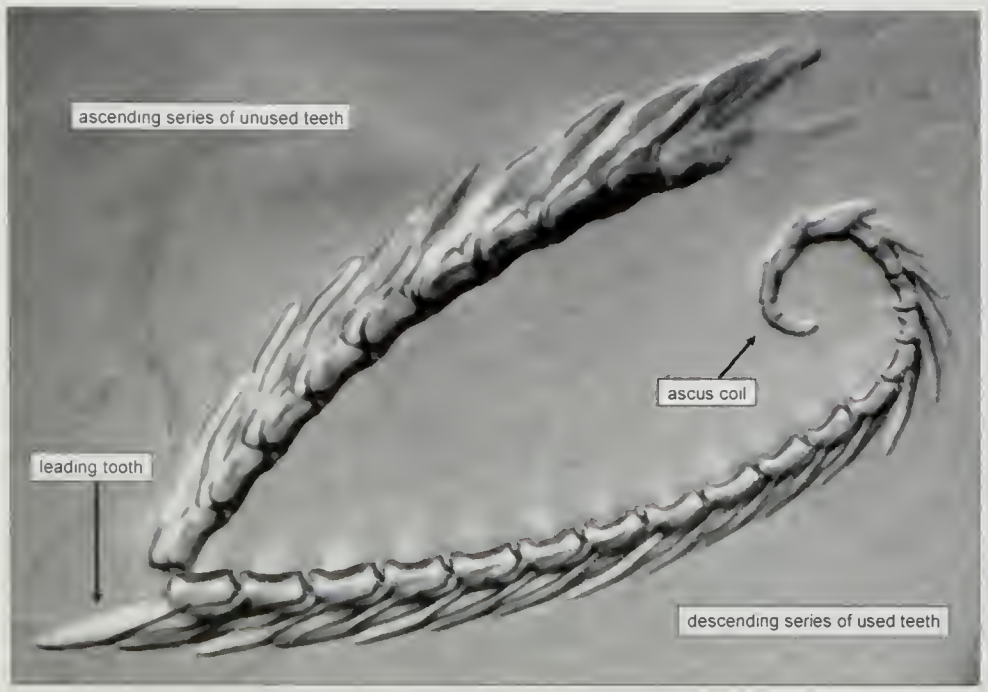

Figure 2. Radular ribbon of 4 mm specimen of *Hermaea wrangeliae* collected on 11 December 2008 from Zanpa-misaki, Okinawa. Leading tooth was 34.3 µm long and 14.0 µm wide at base. Photographed under a compound light microscope.

anus which opened mid-dorsally and a little posterior to the esophageal pouch. Two main digestive gland tubules ran along the dorso-lateral surface of the body (Figs. 1A-B). The digestive diverticulae extended from these large tubules into the cerata (Fig. 1A).

Another important character determining whether or not a sacoglossan belongs to the genus *Hermaea* is a penis with no stylet (*e.g.*, the superficially similar *Placida* Trinchese, 1876 has a stylet). Our dissection confirmed that the Okinawan specimens lack the stylet (*i.e.*, unarmed penis) and had a diaulic reproductive system (two openings rather than three). In addition, two visceral nerve ganglia were seen (Table 1), indicating our specimens belong to *Hermaea*. (However, we have observed three visceral ganglia—rather than two—in two other Japanese *Hermaea* spp. so this might be a variable character at the genus level or earlier studies may have been less detailed).

The taxonomy of the cerata-bearing sacoglossans such as *Hermaea* has long been unstable and, in many respects, not well defined. Many species originally described as *Aplysiopsis* or *Hermaeopsis* were subsequently moved into the genus *Hermaea*, particularly due to blade-shape of radular teeth and red algal diet (see Table 1). Thus, the fact that our recorded Okinawan species, originally described as *Aplysiopsis wrangeliae*, actually belongs to *Hermaea* is not surprising. The proper name of this species consequently should be *Hermaea wrangeliae* (Ichikawa, 1993). Important external features include the transverse markings on the rhinophores, the sparse, club-shaped cerata, and the branched digestive diverticulae within the cerata.

A far more difficult question—and one largely beyond the scope of this paper—is the relationship of this described species and many other ones (described and undescribed species) around the world listed by Burn (2006), Gosliner *et al.* (2008), and Trowbridge *et al.* (2009a). The European *Hermaea bifida* and the Australian *Hermaea evelinemarcusae* Jensen, 1993 have been well described and the Japanese species is definitely not synonymous with either species; the distinct transverse markings on the rhinophores are lacking in these species. However, most of the congeners have not been well described. For example, the tooth shape is generally similar among many *Hermaea* species. The knob-like cerata with branching digestive diverticulae have been illustrated for many *Hermaea* spp. (*e.g.*, Vogel 1971, Marcus 1972, Cervera *et al.* 1991). Yet, the branching patterns of digestive diverticulae are often highly stylized (as in Ichikawa 1993) so matching actual specimens to idealized illustrations is problematic; this issue is not limited to *Hermaea* but plagues the descriptions of the majority of cerata-bearing sacoglossans. Comparisons of reproductive features of congeners are also problematic as the size of uncleaved ova, capsules, and larval shells have been reported in only a few cases such as the European *Hermaea bifida*, and reproductive anatomy has rarely been reported.

Basic natural history details, morphological information, and algal host associations are sorely needed for sacoglossan species, particularly the red algal feeders, in all geographic regions other than the North Atlantic Ocean. This report on an Okinawan *Hermaea* and related reports on other Japanese sacoglossans (Hirano *et al.* 2007a, 2007b, 2007c, 2008a, 2008b, Trowbridge *et al.* 2008a, 2008b, 2009a, 2009b) attempt to identify and characterize the sacoglossan fauna in a highly diverse but understudied geographic region with an amazing flora of potential algal hosts.

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