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CONTENTS

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TAXONOMIC REVISION OF *POTAMOLITHUS AGAPETUS* PILSBRY, 1911, AND *POTAMOLITHUS BUSCHII* (FRAUENFELD, 1865) (GASTROPODA: HYDROBIIDAE)

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ABSTRACT

Potamolithus agapetus Pilsbry, 1911, and *P. buschii* (Frauenfeld, 1865) are related species that live sympatrically in Río de la Plata.

Studies carried out on populations of both species from Río de la Plata show that *P. agapetus* presents a marked secondary sexual dimorphism on shell shape and size.

The female shell is bigger than male shell, and its body whorl shape is subglobose, with a rounded angle at the basal periphery and another angle a short distance below the suture. The male body whorl shape is usually rounded without keels and seldom with a round angle at the basal periphery.

Females of *P. agapetus* are very similar to the shell of *P. buschii,* which lacks secondary sexual dimorphism. For that reason, *P. agapetus* females were excluded from the original description by Pilsbry (1911), and seemingly included in subsequent enlarged descriptions of *P. buschii.*

Both species share the same body whorl shape, but both present different degrees between angulose to globular shapes. They can be distinguished by shell color pattern, columella width, body whorl sculpture, head pigment pattern, eyebrow position, nuchal node size in females, gill filament number and range, and in the shape and number of cusps on the central and lateral radular teeth.

Key words: *Potamolithus,* Hydrobiidae, taxonomy, sexual dimorphism.

INTRODUCTION

The genus *Potamolithus* comprises small (up to 7 mm long), thick-shelled gastropods that inhabit rivers and streams (Pilsbry, 1911; López Armengol, 1985).

This genus, exclusively South American and endemic in Ribeira, Itajaí-açú and Jacuhy rivers in southern Brazil and Uruguay River, part of the Paraná and Río de la Plata drainage systems (López Armengol, 1985).

Controversial aspects of authorship and type species (ICZN Case 2801; López Armengol & Manceñido, 1992; Kabat, 1993; Kabat & Hershler, 1993; Manceñido & López Armengol, 1993) have been cleared up by ICZN action (ICZN Opinion 1779, 1994) fixing Pilsbry & Rush as the authors of this genus and *Potamolithus lapidum* (d'Orbigny, 1835) as its type species.

In 1911, Pilsbry presented a key to species and subspecies and a description of the known species which were arranged in four groups. Parodiz (1965) gave a description of *Potamolithus* species and added new characters and geographical data. Davis & Pons da Silva (1984) described the anatomy of *P.*

ribeirensis from Feitoría River, Brazil, and discussed phylogenetic relationships and convergence with other hydrobiid and pomatiopsid genera.

The descriptions of *Potamolithus* species were based on shell features, and only a few specimens were studied in some cases.

Potamolithus agapetus Pilsbry, 1911, and *Potamolithus buschii* (Frauenfeld, 1865) are sympatric in Río de la Plata (Pilsbry, 1911; López Armengol, 1985). According to Pilsbry (1911) both species belong to the "group of *P. buschii*" because they share the same general shell shape: both equally wide and high with a normal length spire, a simple lip, and a flattened columella. Juveniles of *P. buschii* are not always readily distinguishable from immature *P. agapetus. Potamolithus agapetus* was originally described as the smallest *Potamolithus* known and has a globular-conic shell. On the other hand, *P. buschii* was originally described as having a wide and carinate shell.

Studies carried out on populations of both species from Río de la Plata show that specimens with a shell morphology agreeing with the original description of *P. agapetus* are all males. On the other hand, a great variability
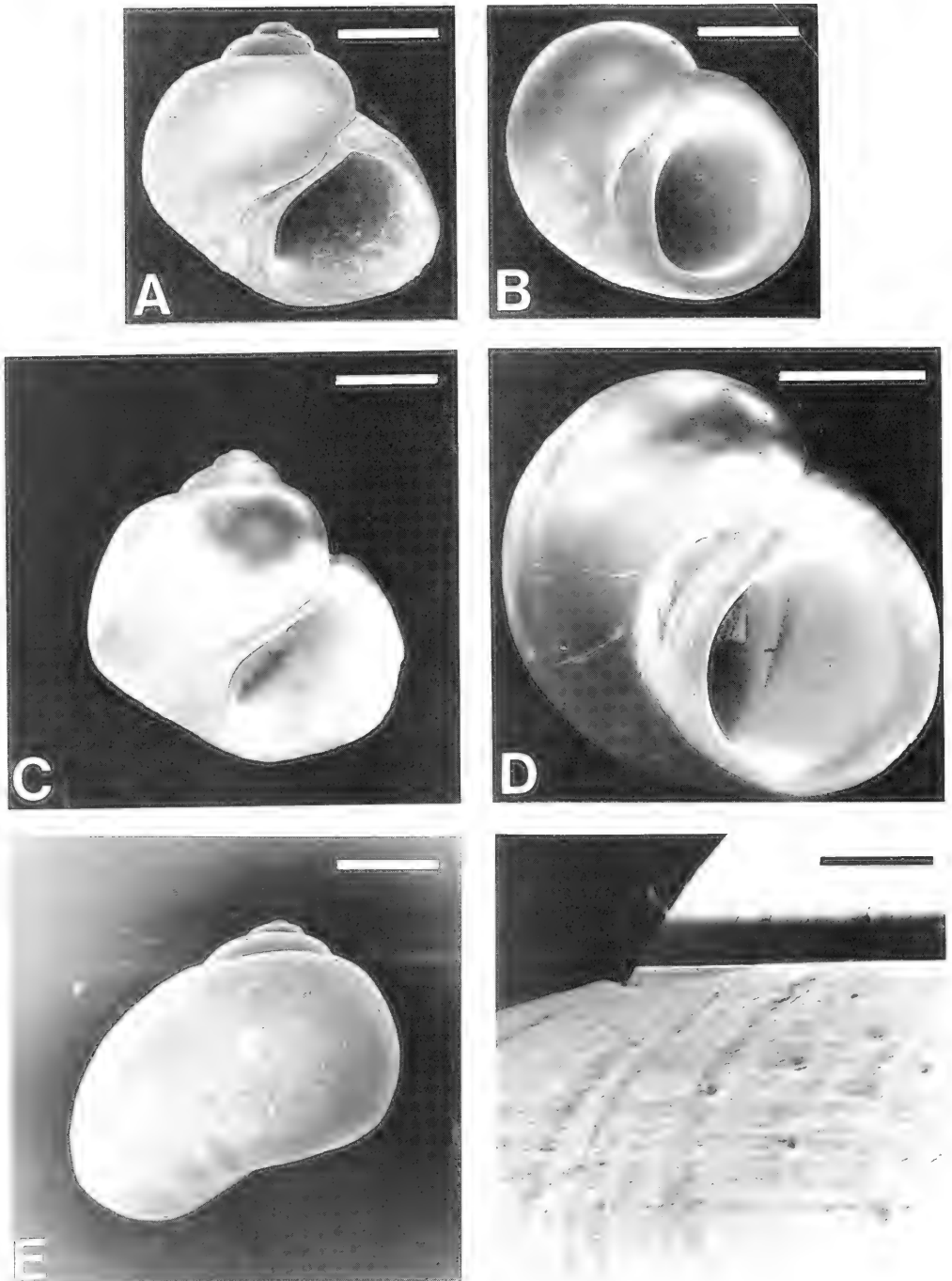
FIG. 1. Scanning electron micrographs of the shell of *P. agapetus.* A, B, males; C, D, females. Body whorl periphery: A, globose, and C, angular. F, lateral view: note the concave body whorl base. G, enlargement of the shell showing the surface faintly marked with growth-lines and some pits. Scale bar A–E = 1 mm; F = 50 μm.

A

B
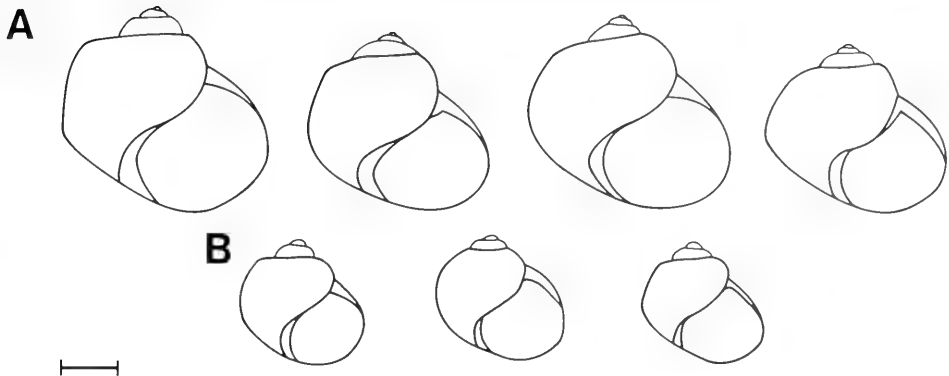
FIG. 2. Variation in the shape of the shell in *P. agapetus*. A, females. B, males. Scale bar = 1 mm.

TABLE 1. Whorl number of *P. agapetus*. Frequency of males and females at each whorl stage present at the localities studied. % = percentage of population.

Whorls	Anchorena beach			Río San Juan			Isla San Gabriel's		
	♂	♀	%	♂	♀	%	♂	♀	%
eroded	7	15	3.72	2	12	18.92	2	2	4.12
2.50	—	—	—	—	—	—	4	1	5.15
3.00	12	1	2.19	5	—	6.76	18	7	25.77
3.25	104	17	20.44	7	—	9.46	19	1	20.62
3.50	42	68	18.58	1	1	2.70	7	6	13.40
3.75	7	221	38.51	2	11	17.57	2	20	22.68
4.00	—	86	14.53	2	29	41.89	—	8	8.25
4.25	—	12	2.03	—	2	2.70	—	—	—
N =	172	420		19	55		52	45	

was observed in *P. buschii* in such characters as radula, head pigmentation, number of gill filaments, and the size of the female nuchal node.

The aim of this work is to redescribe both *P. agapetus* and *P. buschii*.

MATERIALS AND METHODS

Localities studied were: Río de la Plata, Anchorena beach, Argentina (34°29′S, 58°28′W), col.: López Armengol, 30-IV-1984, Colección Malacológica del Museo de La Plata, MLP 4652; mouth of Río San Juan where it empties into Río de la Plata, Uruguay (33°17′S, 57°58′W), col.: Pérez Duhalde, 15-VII-1989, MLP 4986; Río de la Plata, Isla San Gabriel, Uruguay (34°29′S, 57°52′W),col.: Ló-

pez Armengol-Casciotta, 17-III-1985, MLP 4655.

The samples were taken randomly and include all individuals of all size classes at a single site in the river. The sample for the number of individuals and their sex for each whorl number consisted of 592 specimens of *P. agapetus* and 289 specimens of *P. buschii.* These samples were drawn from an initial population of 3,404 individuals (MLP 4652).

Specimens were measured by ocular micrometer in a Wild M-5 stereoscopic microscope. All specimens studied were unparasitized. Measurements are those of Hershler & Landye (1988). The following ratios were formed using some of this data: shell length/body whorl length; body whorl length/shell width; shell length/shell width and aperture length/shell length.

TABLE 2. Shell measurements (mm) and ratios for 29 males and 44 females of 3.50 to 3.75 whorls of *P. agapetus* (MLP 4652). X̄ ± standard deviation (range). * = significant difference between sexes, P≤ .001.

Characters	males	females	SD P ≤ .001
Shell length	2.32 ± 0.25	2.88 ± 0.26	*
	(1.95 − 3.09)	(2.27 − 3.24)	
Body whorl length	2.08 ± 0.24	2.56 ± 0.24	*
	(1.76 − 2.77)	(1.95 − 2.96)	
Spire length	0.25 ± 0.04	0.32 ± 0.05	*
	(0.15 − 0.32)	(0.19 − 0.44)	
Shell width	2.28 ± 0.26	2.81 ± 0.27	*
	(1.95 − 3.09)	(2.20 − 3.21)	
Aperture length	1.74 ± 0.22	2.17 ± 0.20	*
	(1.45 − 2.39)	(1.76 − 2.52)	
Aperture width	1.22 ± 0.16	1.51 ± 0.15	*
	(1.01 − 1.76)	(1.20 − 1.83)	
Columella width	0.24 ± 0.04	0.30 ± 0.06	*
	(0.16 − 0.32)	(0.19 − 0.44)	
Umbilical area width	0.18 ± 0.07	0.22 ± 0.08	
	(0.06 − 0.38)	(0.06 − 0.38)	
Shell length/body whorl length	1.12 ± 0.02	1.13 ± 0.02	
	(1.08 − 1.18)	(1.08 − 1.17)	
Body whorl length/shell width	0.91 ± 0.03	0.91 ± 0.04	
	(0.84 − 0.99)	(0.83 − 0.98)	
Shell length/shell width	1.02 ± 0.02	1.02 ± 0.04	
	(0.92 − 1.09)	(0.96 − 1.07)	
Aperture length/shell length	0.73 ± 0.04	0.75 ± 0.04	
	(0.63 − 0.80)	(0.63 − 0.83)	

Number of whorls was counted according to Emberton (1985), but 0.25, 0.5 and 0.75 were the fractions considered. Body whorl and penultimate whorl convexity were calculated following Hershler & Landye (1988). Whorl convexity value is directly proportional to whorl convexity.

Shells and radulae were studied and photographed using scanning electron microscope (Jeol JSM-T 100). Heads were dried by the critical point method.

The position and distance between the base of penis or nuchal node with respect to the lobes of the eyes and the angle of the base of penis or nuchal node with respect to the mid-line of the neck were calculated on fixed material, following Davis et al. (1986).

Statistical analyses were limited to calculating the means, standard deviations, and standard 't' test for sexual dimorphism in shell measurements and ratios and gill filament number. The significance level accepted was P ≤ .001. Xi^2 was performed to evaluate sex ratio = 1:1.

RESULTS

Potamolithus agapetus Pilsbry 1911

Potamolithus agapetus Pilsbry 1911: 578, pl. 40, fig. 10, 10a.
Potamolithus agapetus Parodiz 1965: 9

Type material: Academy of Natural Sciences of Philadelphia 69,683.

Type locality: Río de la Plata, at Isla San Gabriel, near Colonia, Colonia Department, Uruguay.

Description

The shell is globose-conic to subglobose (Figs. 1, 2) and solid but not thick. The color is uniform light brown. The surface is rather smooth, faintly marked with growth lines (Fig. 1F). The spire is 11% of the shell length. The number of whorls is most frequently between 3.00 and 4.00 (Table 1), slightly convex (penultimate whorl convexity = 0.20 and body whorl convexity = 0.14) in outline. The

FIG. 3. Scatter-diagram for the number of whorls and shell length. A, *P. agapetus* (82 males and 61 females). B, *P. buschii* (128 males and 108 females). Note the sexual dimorphism in shell size in *P. agapetus*. Males (black circles), females (black triangles). One symbol may represents more than one specimen.

FIG. 4. Pigment patterns of *P. agapetus*. A, head-neck, dorsal view. B, penis, right side. Scale bar = 1 mm.

body whorl base is concave in dorsal view (Fig. 1E). The aperture is oblique, inclined about 35° to 42° ($\bar{X}$ = 39°) towards the coiling axis, rounded-ovate, and angular at the top. The columella is wide and flattened (Fig. 1B, D).

Shells with discontinous peristome have a thin outer lip, and the umbilical area can be present or absent. When it present, is narrow and bounded by an angle. In shells with continous peristome (Fig. 1B, D), the inner lip is heavily calloused and the outer lip is simple and thin. There is a rather conspicuous umbilical area bounded by an angle or an acute ridge. Some specimens have an umbilical opening.

There is sexual dimorphism in shape and shell size. The shape of the body whorl in males is usually globose (Figs. 1A, 2B). However, some males have a shell with a rounded angle below the suture or with two angles, one below the suture and the other at the basal periphery (Fig. 2B). Males have a rounded outer lip (Fig. 1B). The female body whorl shape is usually subglobose, with two rounded angles, one below the suture and the other at the basal periphery (Figs. 1C, 2A); the outer lip is sharp (Fig. 1D).

The females are larger than males with the same number of whorls (Fig. 3A). No sexual dimorphism in umbilical area width and calculated ratios were observed. Statistics on shell dimensions for males and females of

3.50 to 3.75 number of whorls are given in Table 2.

There was no significant difference in number of males and females at Anchorena Beach (0.76:1).

The head can be unpigmented or with a band of melanin in the snout, or with two V-shaped bands orientated with the vertex pointing from the snout to the neck. Another band runs dorsally in the middle of each tentacle. There is a concentration of white spheric granules above and around the eyes ("eyebrows") (Fig. 4A), and eye lobes are slight swellings at the base of each tentacle.

The neck of females bears a protuberance called nuchal node (Davis & Pons da Silva, 1984). The position of the nuchal node base is mainly to the right of the mid-line of the head. The nuchal node is $\bar{X}$ = 0.25 mm ± 0.02 (0.24–0.30) high (Fig. 5A, B). The distance between the base of the nuchal node and the eyes is $\bar{X}$ = 0.31 mm ± 0.09 (0.15–0.4). The angle of the base of the nuchal node (with respect to the mid-line of the neck) is $\bar{X}$ = 52° (34°–72°).

The penis is simple, without appendages; with a black spot at the distal end (Figs. 4B, 5C). The distance between the base of the penis and the lobes of the eyes is $\bar{X}$ = 0.14 mm ± 0.01 (0.12–0.15). The angle of the base of the penis (with respect to the mid-line of the neck) is $\bar{X}$ = 23° (14°–30°).

There are 19 to 28 gill filaments (Fig. 6),
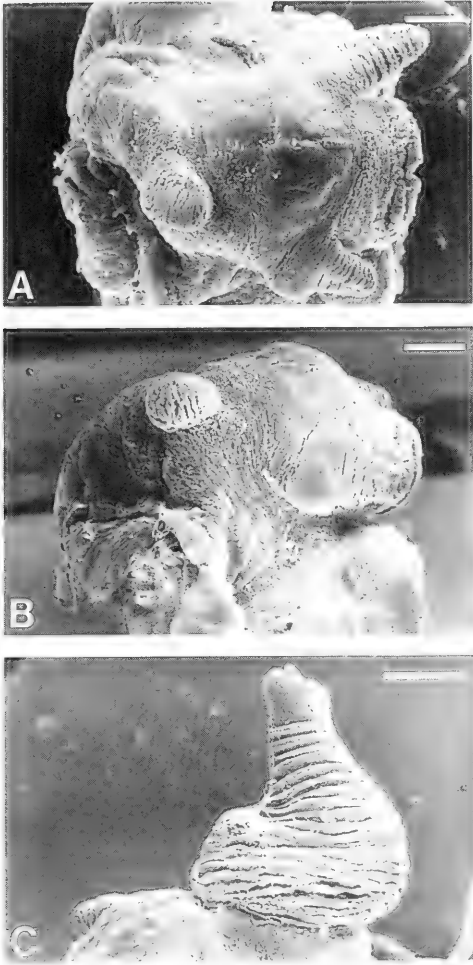
FIG. 5. Scanning electron micrographs of the head-neck of *P. agapetus*. A, dorsal view of female head-neck, showing the nuchal node. B, right side of the head-neck of a female. C, left side of the head-neck and fully erect penis of a male. Scale bar = 200 μm.

with no indication of sexual dimorphism in their number ($\bar{X}$ = 23.00 ± 2.30 and 23.87 ± 2.64 for males and females respectively).

Radula typically taenioglossate (Fig. 7), the statistics and cusp formulae given in Table 3. Distinctive features are: the concave hollow in the middle of the anterior cusp of the central teeth (Fig. 7C); the external edge of lateral angle of the central teeth is sometimes curved; the innermost pair of basal cusps

arise from the face of the tooth; there is a concave hollow between the basal cusps and basal process, and the basal process is prominent.

There were no differences among the blades of the lateral tooth (Fig. 7A, B, D, E). There is a pronounced posterior projection on the face of the lateral tooth (Fig. 7D).

Potamolithus buschii (Frauenfeld, 1865)

Lithoglyphus Buschii Frauenfeld, 1865, *ex* Dunker, *in litt.:* 530, pl. 11
Potamolithus buschii, Pilsbry & Rush 1896: 80
Potamolithus buschii, Pilsbry, 1896: 88
Potamolithus buschii, Pilsbry, 1911: 580, pl. 40, figs. 11–14, pl. 41b, fig. 2
Potamolithus buschii, Parodiz, 1965: 28, figs. 63–72

Type material: Naturhistorisches Museum, Vienna, Austria.

Type locality: ''Erst kürzlich von Buenos-Ayres [*sic.* Colonia Department, Uruguay] erhalten. Wird gefunden an der Mündung des St. Juan in den La Plata.''

Description

The shell is imperforate, solid, subglobose to globose in shape (Figs. 8–10). The shell is green, with irregular buff zigzag streaks (Fig. 11); some specimens (27% at Anchorena beach) have a dusky-brown band located sutural and peripheral on the body whorl (Fig. 11). The surface is smooth, although marked with growth-lines (Fig. 8F). The spire length is variable, between 9.60% and 15% of the shell length. The number of whorls is most frequently between 3.75 and 4.00 (Table 4), convex (penultimate whorl conv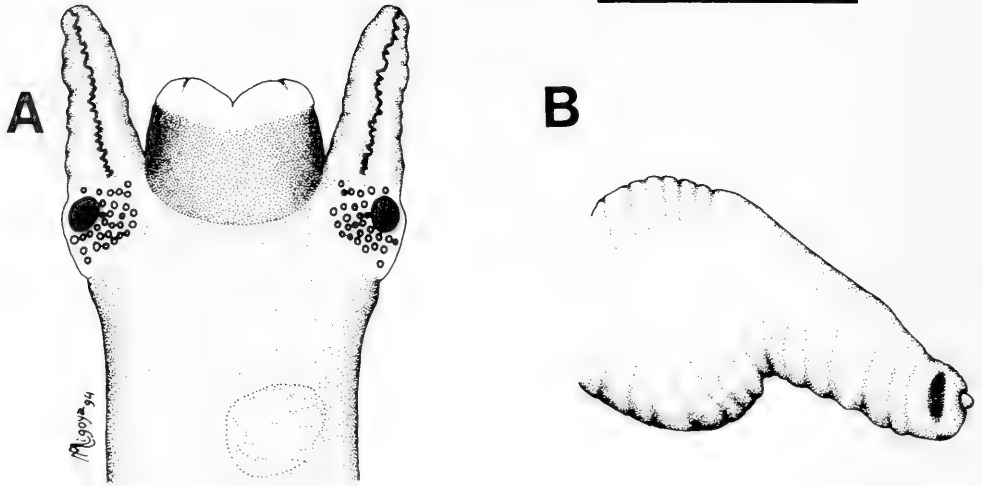exity = 0.17 and body whorl convexity = 0.21) inoutline. The body whorl can be carinate,strongly angular, with a rounded angle, or globose at the basal periphery (Figs. 8A–D, 10). The body whorl is convex above the basal periphery, usually having a low keel or rounded angle at the back and a short distance below the suture (Fig. 9A–C). There is also, sometimes, a second spiral ridge below the upper one and a concavity between both called sulcus (Fig. 9D). The base is flattened in dorsal view (Fig. 9A–C). The aperture is oblique, inclined about
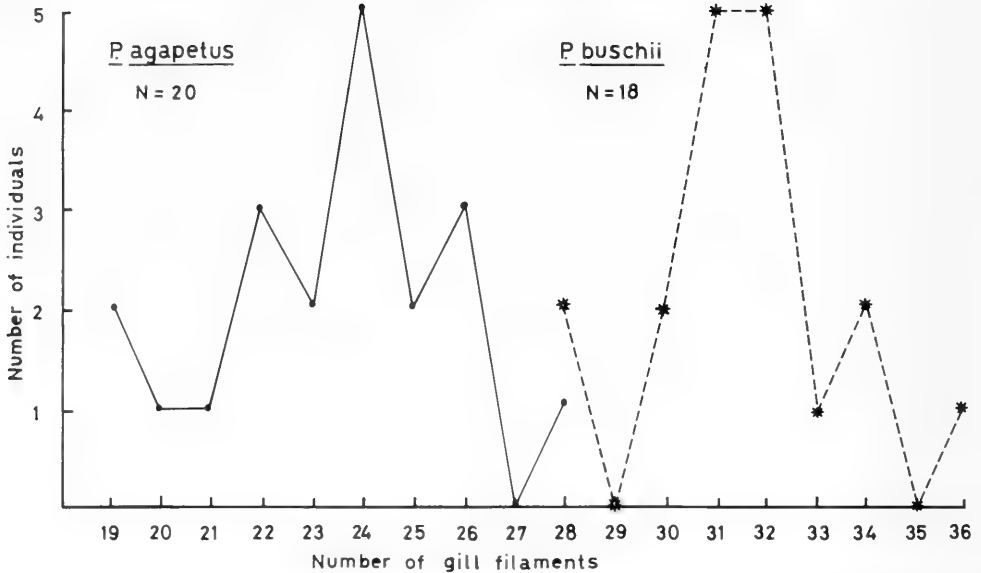
FIG. 6. Gill filament number in *P. agapetus* and *P. buschii*. Scatter-diagram between number of gill filaments and number of individuals.

40° to 54° (X̄ = 46°53′) towards the axis of coiling; basally rounded and angular at the top. Columella narrow and flattened or convex (Fig. 8E).

In shells with a discontinous peristome, the outer lip is thin and may or may not have an umbilical area. When the umbilical area is present, it is narrow and bounded by an angle. In shells with a continous peristome, the inner lip is heavily calloused and the outer lip is thick (Fig. 8E). Sometimes the peristome is edged with a black line. There is a well-developed concave umbilical area bounded by an angle or an acute ridge. Some specimens have an umbilical opening.

Statistics on shell dimensions for males and females of 3.50 to 3.75 whorls are given in Table 5. No sexual dimorphism in shell size was evident; females and males at the same number of whorls have the same size (Fig. 3B).

There was no significant difference in the number of males and females at Anchorena Beach (0.90:1).

The entire head is black (melanin), and there is a black band in the middle of each tentacle. Next to the eyes there is a hyaline band with white spheric granules on it (Fig. 12A).

The nuchal node is located to the right of the mid-line and is 0.06 mm high (Fig. 13A, B). The distance between the base of nuchal node and the lobes of the eyes is X̄ = 1.01 mm ± 0.24 (0.63–1.26). The angle of the base of nuchal node (with respect to the mid-line of the neck) is x̄ = 48° (27°–69°).

The penis is simple, without appendages (Fig. 13C). The penis bears two parallel bands of melanin running on both sides, one dorsal along the distal part and the other ventral (Fig. 12B). The distance between the base of penis and the lobes of the eyes is X̄ = 0.59 mm ±0.08 (0.45–0.75). The angle of the base of the penis (with respect to the mid-line of the neck) is X̄ = 32° (20°–45°).

There are 28 to 36 gill filaments (Fig. 6), with no indication of sexual dimorphism in their number (X̄ = 30.14 ± 1.57 and 32.45 ± 1.69 for males and females respectively).

Radula tipically taenioglossate (Fig. 14). The statistics and cusps formulae given in Table 3. Distinctive features are: that the middle of the anterior cusps of the central tooth is flat; the external edge of lateral angle is
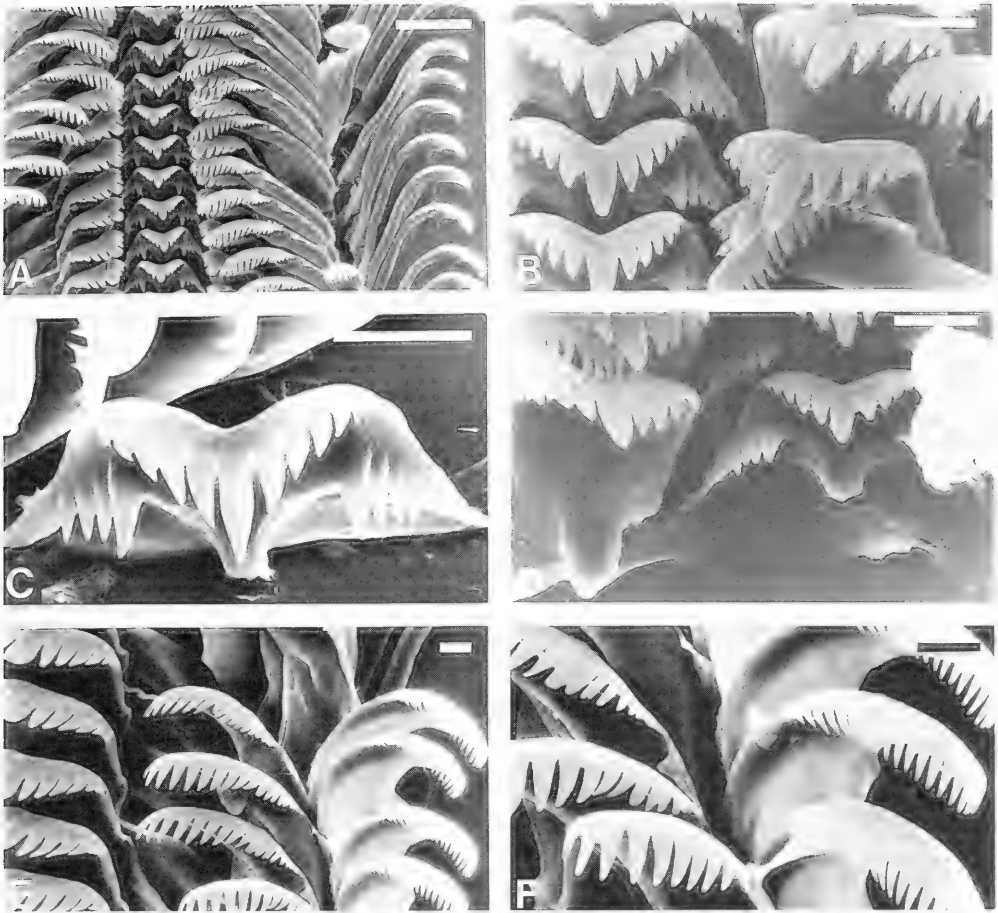
FIG. 7. Radula of *P. agapetus*. A, Section of radular ribbon excluding left outer marginals. B, enlargement of central and right lateral teeth. C, central tooth. D, central and left lateral teeth. E, left lateral and marginal teeth. F, right inner and outer marginal teeth. Scale bar A = 50 μm; B-F = 10 μm.

TABLE 3. Formulae for the most common cusps arrangements for the four radular teeth of *P. agapetus* and *P. buschii*.

Tooth	N	Formula (%)		
P. agapetus (4 radulae)				
Central	29	$\frac{6-1-6}{3-3}$ (79.30),	$\frac{6-1-5}{3-3}$ (17.24),	$\frac{5-1-6}{3-3}$ (3.45)
Lateral	38	4−1−5 (44.74); 5−1−4 (31.58); 5−1−5 (23.68)		
Inner marginal	35	15−22		
Outer marginal	21	17−23		
P. buschii (2 radulae)				
Central	51	$\frac{4-1-4}{2-2}$ (33.30);	$\frac{4-1-4}{2-3}$ (33.30);	$\frac{4-1-5}{2-2}$ (33.30)
Lateral	46	3−1−3 (80.43); 4−1−3 (10.87); 2−1−2 (8.70)		
Inner marginal	31	9−11		
Outer marginal	24	12−15		
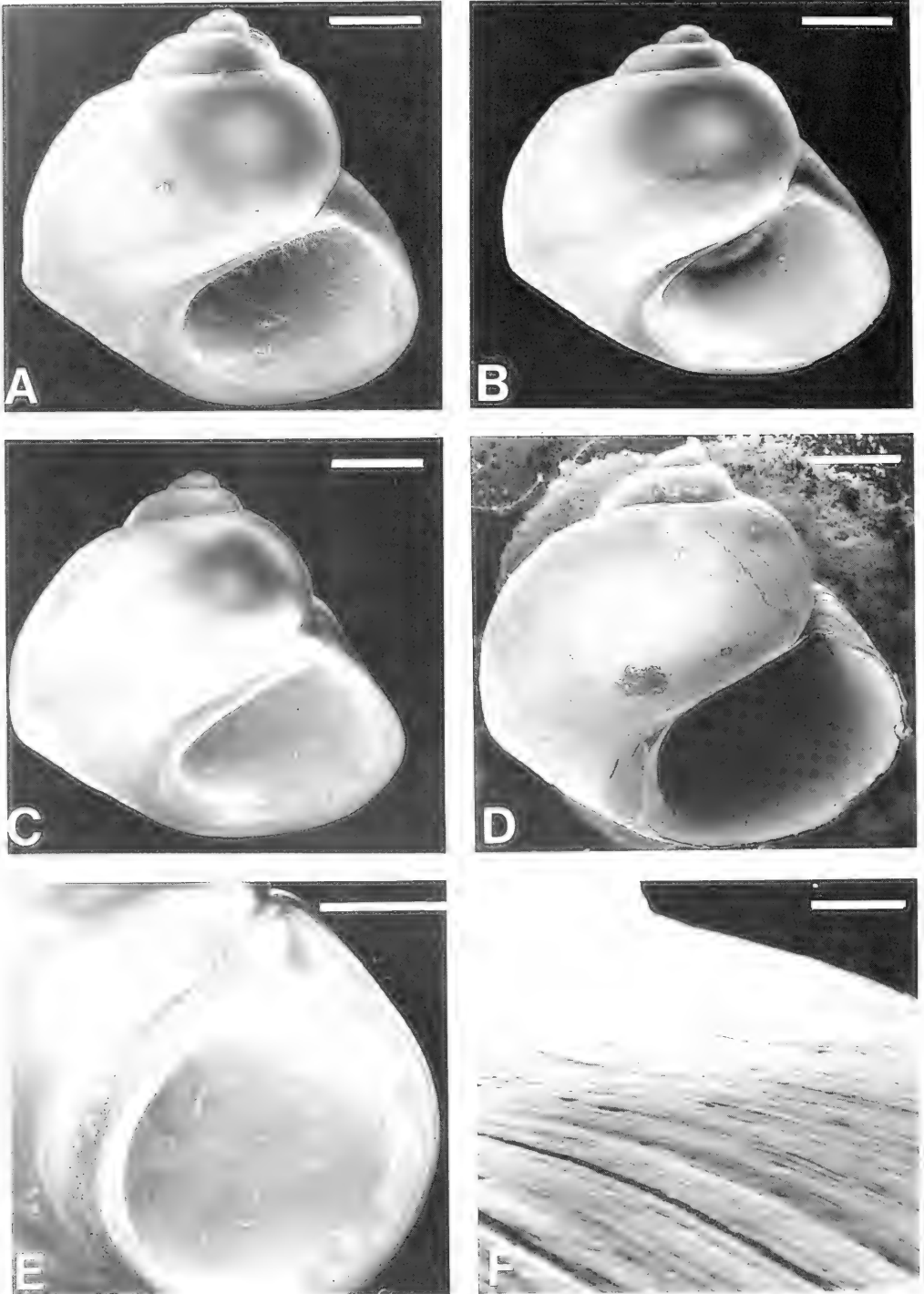
FIG. 8. Scanning electron micrographs of the shell of *P. buschii*. A–D, frontal view. Body whorl: A, carinated; B, strongly angular; C, rounded angle; D, globose. E, umbilical view. F, enlargement of the shell showing the surface marked with growth-lines and some pits. Scale bar A–E = 1 mm; F = 50 μm.
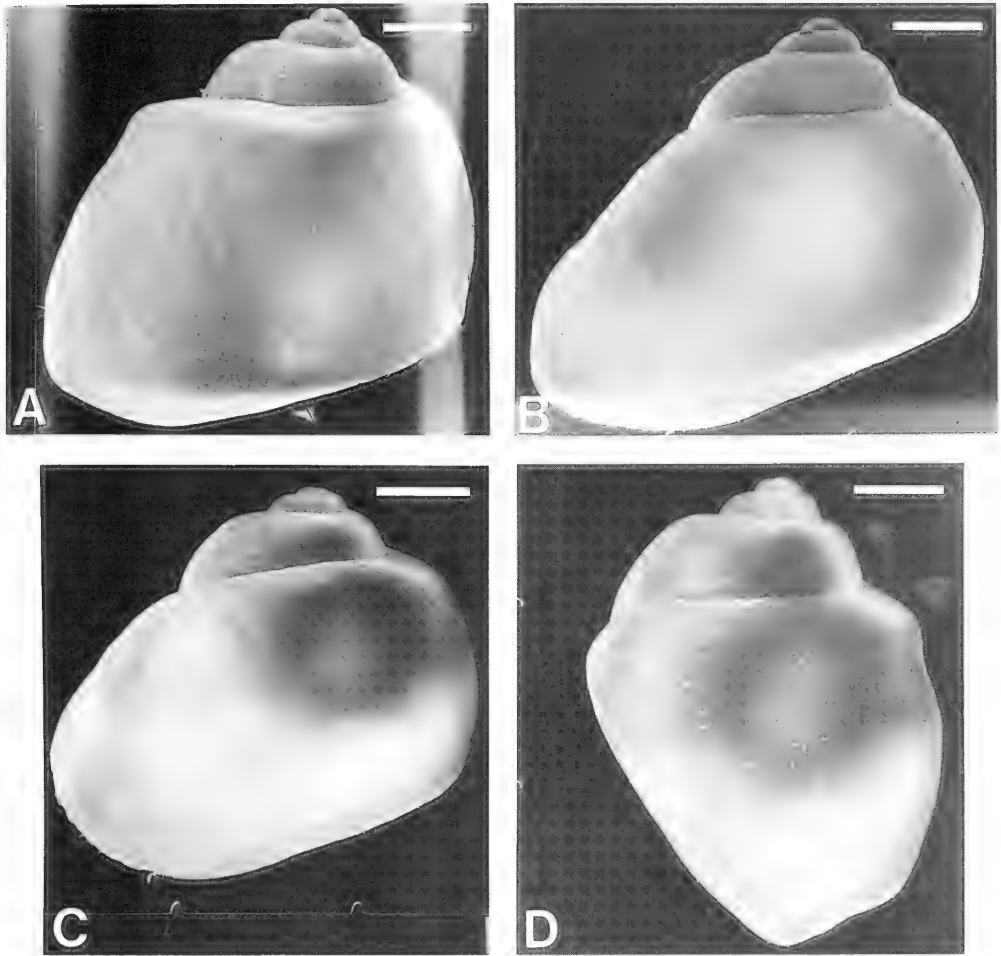
FIG. 9. Scanning electron micrographs of the shell of *P. buschii*. A–C, dorsal view. Note the flat body whorl base and the different degrees of subsutural carination: A, carinated; B, angular; C, globose. D, lateral view: note the sulcus between two ridges. Scale bar = 1 mm.

straight, and the innermost pair of basal cusps arise from the face of the tooth (Fig. 14C). The ventral part of basal cusps is a little concave and the basal process is not prominent. The central blade of lateral teeth widened with respect to the other cusps (Fig. 14A, B, D).

DISCUSSION AND CONCLUSIONS

Potamolithus agapetus shows marked secondary sexual dimorphism in shell shape and size. The shape of the body whorl in males is usually rounded, whereas the female is subglobose, with a rounded angle at short distance below the suture and another angle at the basal periphery. Like other gastropods showing sexual dimorphism, the female shell is larger than the male shell.

Potamolithus agapetus was described by Pilsbry (1911) as the smallest *Potamolithus* known, with body whorl evenly rounded, without keels or angles but his description did not include subglobose shells. Two
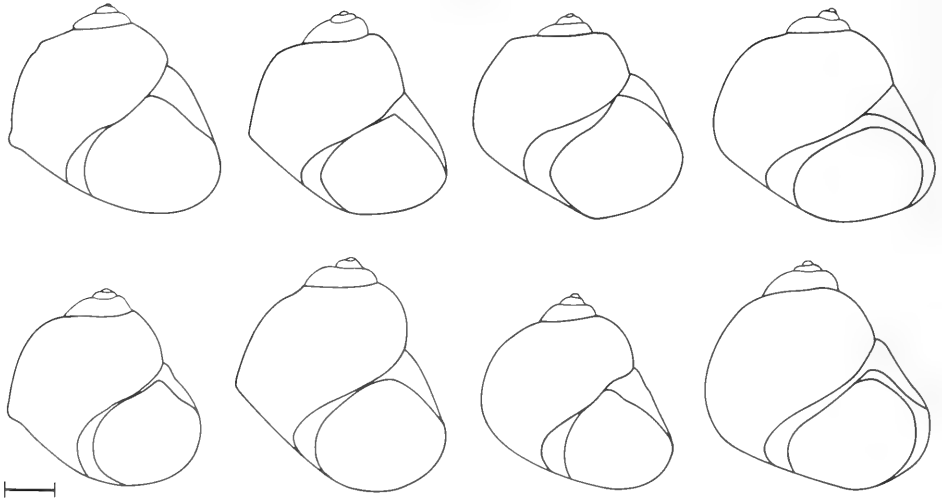
FIG. 10. Variation in the shape of the shell in *P. buschii*. Scale bar = 1 mm.

rounded angles are usually present in females.

Potamolithus buschii was described by Frauenfeld (1865) as having a wide, carinate shell, but in subsequent descriptions by Pilsbry (1911) and Parodiz (1965) the concept of this species changed. Pilsbry (1911) included the least angular forms of *P. buschii* from Isla San Gabriel (type locality of *P. agapetus*) and Parodiz (1965) stated that carinated shells were not the common form of the species.

Because *P. agapetus* and *P. buschii* are related species and sympatric in Río de la Plata, it is probable that *P. agapetus* females have been included in the descriptions of *P. buschii* by Pilsbry (1911) and Parodiz (1965). For example, Pilsbry (1911) showed in his Plate 40, fig. 14, the least angular form of *P. buschii,* which is very similar to the female form of *P. agapetus*. This is became both species share the body whorl shape ranging from angulose to globular, and broad umbilical area circled by an angular or acute ridge. The features that reliably to distinguish both species, as redefined herein are listed in Table 6.

1 mm

FIG. 11. Shell of *P. buschii,* showing the peripheral band and irregular buff zigzag streaks.

TABLE 4. Whorl number of *P. buschii*. Frequency of males and females at each whorl stage present at the localities studied. % = percentage of population.

Whorls	Anchorena Beach			Río San Juan			Isla San Gabriel		
	♂	♀	%	♂	♀	%	♂	♀	%
eroded	25	19	15.23	—	2	4.55	20	19	14.45
2.25	—	—	—	—	—	—	—	3	1.11
3.00	—	—	—	—	1	2.27	3	8	4.07
3.25	1	3	1.38	1	1	4.55	15	24	14.45
3.50	14	4	6.23	—	3	6.82	11	19	11.11
3.75	64	56	41.52	9	5	31.82	32	53	31.48
4.00	29	68	33.56	1	7	18.18	21	37	21.48
4.25	2	4	2.08	5	9	31.81	1	4	1.85
N =	135	154		16	28		103	167	

TABLE 5. Shell measurements (mm) and ratios for 78 males and 53 females of 3.50 to 3.75 whorls of *P. buschii*. X̄ ± standard deviation (range). There is no significant difference between sexes, $P \leq .001$.

Characters	males	females
Shell length	3.90 ± 0.39	3.77 ± 0.45
	$(2.84 - 4.5)$	$(2.34 - 4.68)$
Body whorl length	3.49 ± 0.35	3.39 ± 0.41
	$(2.52 - 4.14)$	$(2.16 - 4.32)$
Spire length	0.41 ± 0.09	0.39 ± 0.08
	$(0.18 - 0.63)$	$(0.18 - 0.63)$
Shell width	3.92 ± 0.39	3.87 ± 0.48
	$(2.52 - 4.68)$	$(2.61 - 4.86)$
Aperture length	3.03 ± 0.29	2.99 ± 0.31
	$(2.08 - 3.51)$	$(2.16 - 3.69)$
Aperture width	2.22 ± 0.23	2.17 ± 0.26
	$(1.39 - 2.61)$	$(1.35 - 2.70)$
Columella width	0.32 ± 0.08	0.34 ± 0.08
	$(0.09 - 0.45)$	$(0.18 - 0.54)$
Umbilical area width	0.24 ± 0.12	0.25 ± 0.13
	$(0.04 - 0.54)$	$(0.09 - 0.54)$
Shell length/body whorl length	1.11 ± 0.02	1.11 ± 0.02
	$(1.06 - 1.20)$	$(1.06 - 1.16)$
Body whorl length/shell width	0.89 ± 0.04	0.88 ± 0.03
	$(0.79 - 1.03)$	$(0.82 - 0.97)$
Shell length/shell width	1.01 ± 0.05	1.02 ± 0.04
	$(0.89 - 1.13)$	$(0.94 - 1.11)$
Aperture length/shell length	0.78 ± 0.05	0.79 ± 0.04
	$(0.67 - 0.92)$	$(0.69 - 0.92)$

LÓPEZ ARMENGOL
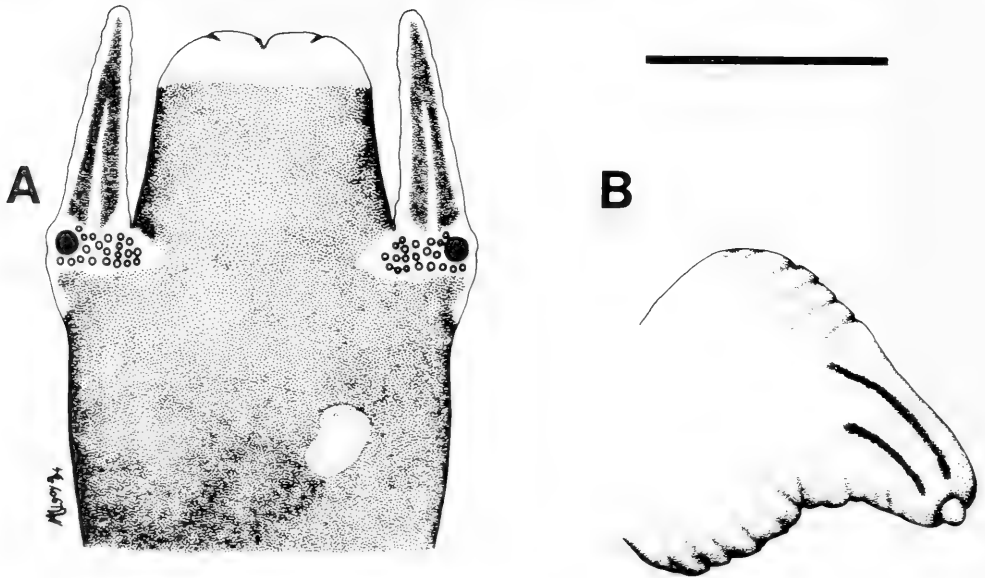
FIG. 12. Pigment patterns of *P. buschii.* A, head-neck, dorsal view. B, penis, right side. Scale bar = 1 mm.

TABLE 6. Characters distinguishing *P. agapetus* and *P. buschii.*

Characters	*P. agapetus*	*P. buschii*
Shell		
Irregular buff zigzag streaks	no	yes
Growth-lines	faintly marked	marked
Body whorl sculpture	rounded basal angle in females	rounded angle, strongly angular, or carena subsutural and basal in both sexes
Sulcus in dorsal view	no	no/yes
Body whorl base in dorsal view	concave	flat
Relationship between shell length and shell width	longer than wider	wider than longer
Aperture inclination	35° to 42°	40° to 54°
Columella	wide	narrow
Peristome	simple and thin	thicker, sometimes dark-edged
External Features		
Head pigment pattern	unpigmented	entirely black
Eyebrows position	above and around the eyes	in hyaline bands
Nuchal node size	0.25 mm	0.06 mm
Penis pigment pattern	black spot in distal end	two parallel bands
Gill Filaments		
Gill filaments number range	19–28	28–36
Radula		
Central teeth		
Middle of the anterior cusps	concave hollow	flat
External edge of lateral angle	sometimes curved	straight
Ventral part of basal cusps	concave	less concave
Basal process prominent	yes	no
Lateral teeth		
Central blade more developed	no	yes
Sexual Dimorphism in Shell	yes	no
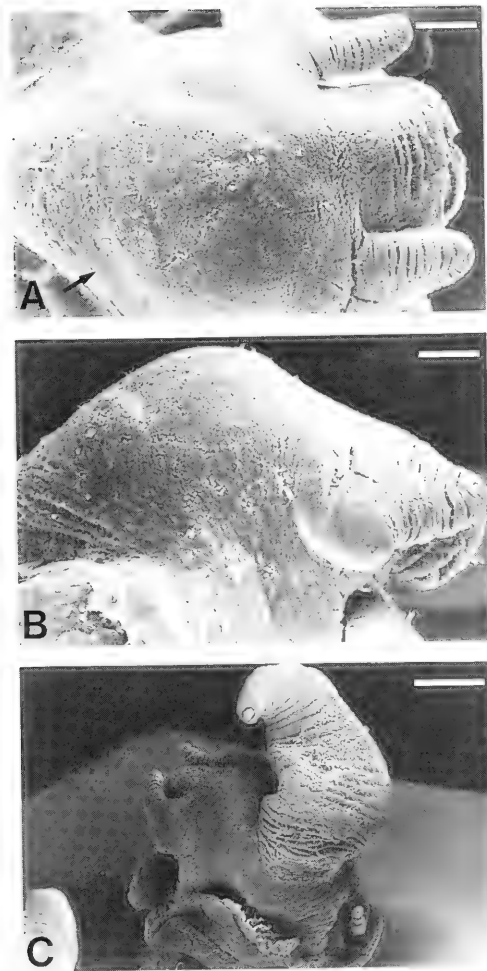
FIG. 13. Scanning electron micrographs of the head-neck of *P. buschii*. A, dorsal view of female head-neck, showing the nuchal node (arrow). B, right side of the female head-neck. C, left side of the head-neck and fully erect penis of a male. Scale bar A, B = 200 μm; C = 500 μm.
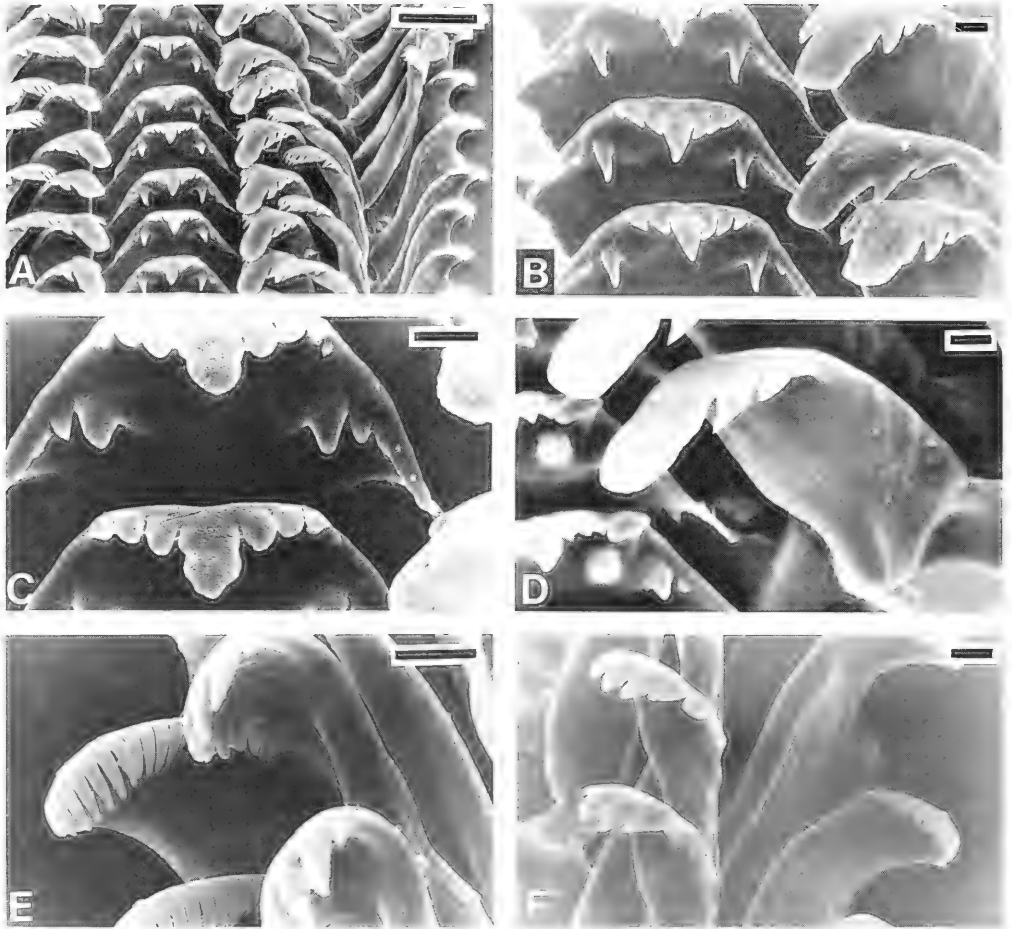
FIG. 14. Radula of *P. buschii.* A, section of radular ribbon excluding left outer marginals. B, enlargement of central and lateral teeth. C, central teeth. D, lateral teeth. E, left inner and outer marginal teeth. F, right inner and outer marginal teeth. Scale bar A = 50 μm; B-F = 10 μm.

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RECRUITMENT OF *DREISSENA POLYMORPHA:* DOES THE PRESENCE AND DENSITY OF CONSPECIFICS DETERMINE THE RECRUITMENT DENSITY AND PATTERN IN A POPULATION?

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ABSTRACT

Results of a field experiment conducted to examine the density and spatial pattern of recruitment in a population of *Dreissena polymorpha* in Lake St. Clair were consistent with the hypothesis that recruitment is in response to a chemical cue released by conspecific adults. The number of recruits were significantly higher in treatments in which conspecific adults were present. Analysis of the distribution of adults and recruits in low and high density treatments showed a strong spatial correlation between adults and recruits. However, the distribution of recruits in the low density treatment was more aggregated in comparison to the high density treatment. Comparison of density and size distribution of recruits between low and high density treatments and the adjacent natural population revealed recruits in the natural population were smaller and less dense than recruits in the experimental treatments. This results suggests that although recruitment is in response to conspecific adults, recruitment into a population with lower adult densities, as represented by the experimental treatments, may result in enhanced growth of the new recruits.

Key words: *Dreissena polymorpha,* recruitment, spatial, density, conspecific.

INTRODUCTION

Larval settlement and juvenile recruitment are the initial processes determining the structure of populations of many sessile, aquatic species (Rodriguez et al., 1993). Successful recruitment of a sessile organism depends on the behavioral adaptation of early life stages to meet or avoid biological and physical hazards (Schubart et al., 1995). The location of settlement and potential recruitment can affect the performance and ultimate survival of a sessile organism. Stimuli necessary for settlement involve a combination of factors, including speed of fluids and contours of the substratum (e.g., Sebens, 1983; Wethey, 1986; Butman, 1989; Pawlik & Hadfield, 1990; Pawlik et al., 1991; Johnson, 1994), luminosity (e.g., Crisp & Ritz, 1973; Young & Chia, 1982) and chemical cues (e.g., Morse & Morse, 1984, Pawlik, 1986; Raimondi, 1988). Perhaps the most widely examined of all settlement stimuli are the existence of chemical inducers associated with conspecific adults. Such cues are of great ecological importance, because the induction of settlement by conspecifics can account for the aggregated distribution of many benthic marine invertebrates (Rodriguez et al., 1993).

Aggregated distributions may increase the probability of fertilization in individuals that either release their gametes into the water column (e.g., Pearse & Arch, 1969; Russo, 1979; Pawlik, 1986) or have internal fertilization (e.g., Raimondi, 1991). Aggregation also acts as an effective defense mechanism (e.g., Garnick, 1978; Bernstein et al., 1981; Pawlik, 1986; Hoffman, 1989), increases filter-feeding efficiency (e.g., Barnes & Powell, 1950) and results in decreased juvenile mortality (e.g., Highsmith, 1982). Settlement induced by conspecific adults has been described in several benthic invertebrates, including polychaetes (Jensen & Morse, 1984; Pawlik, 1986), barnacles (Knight-Jones, 1953; Rittschof et al., 1984; Raimondi, 1988; Johnson & Strathmann, 1989; Crisp, 1990; Raimondi, 1991), echinoids (Highsmith, 1982; Burke, 1984) and molluscs (Seki & Kan-no, 1981).

The majority of studies of settlement and/or recruitment, however, have been confined to marine invertebrates. This is a reflection of the common planktonic larval stage characteristic of many benthic marine invertebrates. The recent invader *Dreissena polymorpha* (Pallas), the zebra mussel, is one of the few North American freshwater benthic invertebrates with a planktonic larval stage. Other

freshwater bivalves that possess a plank-tonic larval or juvenile stage include such ex-otic species as the quagga mussel, *Dreissena bugensis,* and the asian clam *Corbicula fluminea.* Most species of North American freshwater bivalves reproduce either via a specialized parasitic larval stage called glochidia (e.g., Unionidae) or through incubation of a small number of embryos that simply crawl away once ready for juvenile existence (e.g., Sphaeriidae) (Mackie, 1991). *Dreissena polymorpha* larvae may remain in the water column for 5 days to 5 weeks (Sprung, 1993) before settling onto hard substrata, undergoing metamorphosis and becoming juveniles. The incorporation of a new cohort or age class into the population is the stage of the larval life cycle referred to as recruitment (Connell, 1985).

Since its introduction into North America, researchers have devoted considerable energy to studying the ecology and the control of *D. polymorpha.* Extensive research has been conducted to determine the distribution (e.g., Hebert et al., 1991; Schaner et al., 1991; Dermott & Munawar, 1993), predict the spread (e.g., Strayer, 1991; Neary & Leach, 1992: Ramcharan et al., 1992) and ultimate impact (e.g., MacIsaac et al., 1992; Bunt et al., 1993) of *D. polymorpha* on lake ecosystems. However, in order to predict the spread or impact of *D. polymorpha,* we must first understand what factors regulate populations. For a sessile organism, population distribution is determined by dispersal ability and the extent of passive transport at a large spatial scale and suitable settlement sites at a small spatial scale (Minchinton & Scheibing, 1991). As a result, a more appropriate predictor of population structure and community patterns may be sought through the study of recruitment.

The results of a 1989 survey of *D. polymorpha* in Lake St. Clair (Hebert et al., 1991) revealed a marked heterogeneity in size and cohort structure among sites, depending on the density of *D. polymorpha.* Hebert et al. (1991) suggested that veliger settlement may be cued by a chemical released by conspecific individuals that is an attractant at low concentrations and a repellent at high concentrations. Wainman et al. (1995) also suggested that shell induced factor was the explanation for the difference in recruitment between experimental substrata with and without mussels present. Their experimental design, however, consisted of racks sus-pended below the water surface in the metal forbay of a thermal generating station, and therefore their results may not be representative of recruitment in the natural population.

The objectives of this study were twofold. Firstly, we determined whether or not the presence of conspecifics may be a cue to induce recruitment into a population of *D. polymorpha* at Lake St. Clair. Secondly, we determined whether or not the spatial arrangement of recruits was influenced by the presence and density of conspecifics. Results of recruitment in the experimental study were then compared to recruitment in the natural population in Lake St. Clair.

METHODS

Study Site

The study site was approximately 1 km from shore at 42°19′57.0″N, 82°33′19.5″W at 2.5 m depth near Stoney Point, Ontario, on the southeastern shore of Lake St. Clair (Fig. 1). Lake St. Clair is the smallest of the Great Lakes, with a total area of 1,114 km^2 (Bolsenga & Herdendorf, 1993). The mean depth is only 3 m, with maximum natural depth of 6.4 m and maximum depth along a dredged shipping channel of 8 m (Bolsenga & Herdendorf, 1993). Average annual temperature is 11.9°C. Temperatures range from near freezing for most of the winter to their summer average peak of 24°C in July and August. Substratum at the site was predominately silt and clay, with some fine sand and approximately 40% hard substrata, consisting of mainly rocks and Unionidae shells. *Dreissena polymorpha* were found on most submerged hard substrata. Densities of *D. polymorpha* at this site (± SE) were 15,735 (± 316)/m^2 (1992), 15,545 (± 310)/m^2 (1993) and 10,264 (± 109)/m^2 (1994) (Chase, unpublished data). Recruitment occurred in August and October in 1992, August in 1993, and in October in 1994. Population data from previous years showed good recruitment at this site (1992: 5,469 individuals/m^2; 1993: 10,393 individuals/m^2 (Chase, unpublished data).

Experimental Design

The experimental substrata consisted of plexiglass plates (18 × 18 cm). Each plate was attached to a cement block with a stain-
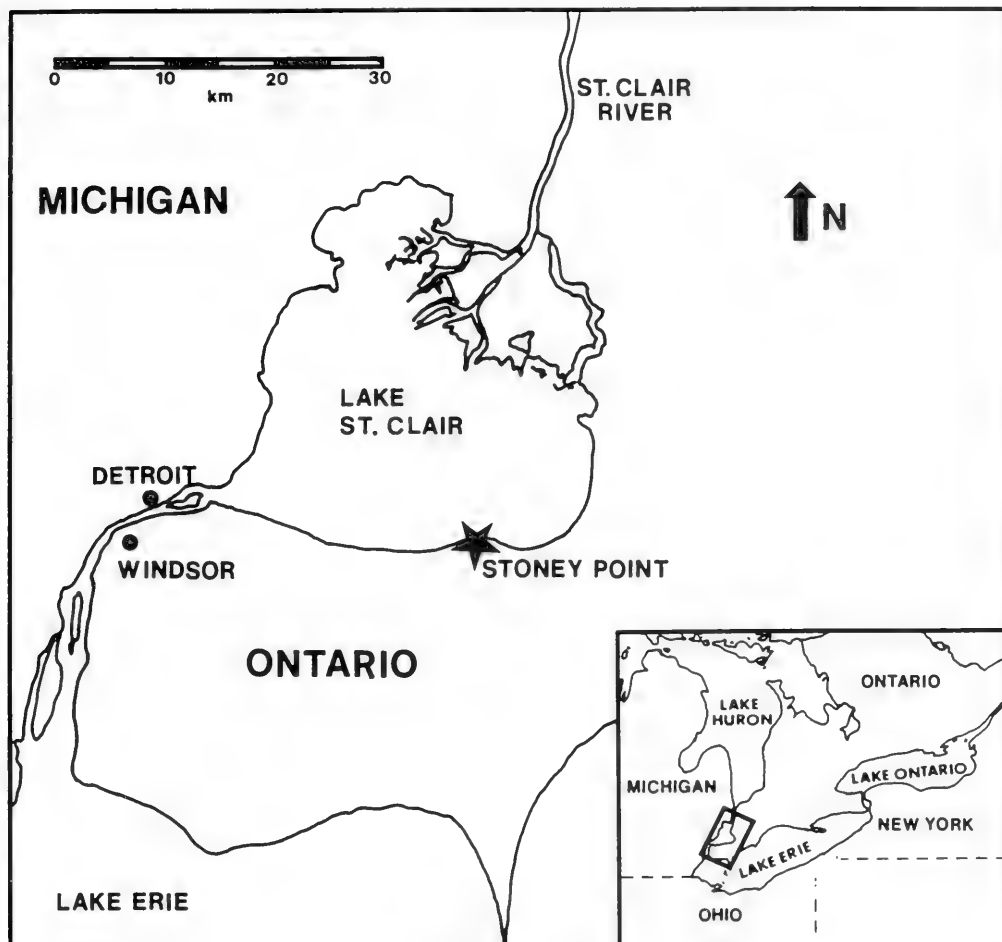
FIG. 1. Location of study site at Stoney Point, Ontario, Canada.

less steel bolt (Fig. 2b). The plates were immersed in lake water 2–3 days before use to remove any manufacturing chemicals that might have been present and which could prevent initial attachment by adult mussels.

On May 15, 1994, adult mussels (6–21 mm in length) were collected from Lake St. Clair using SCUBA, and subsequently returned to the laboratory. Mussels were randomly chosen and placed on the plexiglass plates in an aquaria to allow byssal thread attachment. Three experimental treatments were established:

(1) No adult mussels,
(2) Low adult density (average 167 ± 30 individuals/m²), and

(3) High adult density (average 4583 ± 419 individuals/m²).

Mussels on the plates remained in the laboratory for 48 hours, during which time they were fed dried *Chlorella* sp. (Beta Green, Natrol) ad lib. It was found that moderate temperature (approximately 15°C) and food addition enhanced byssal thread attachment of the adults (M. Chase, personal observation).

Data Collection

On May 17, 1994, the plates were randomly arranged in Lake St. Clair using SCUBA in a 6 × 3 configuration, with a 1-m perpendicular distance between blocks (Fig.
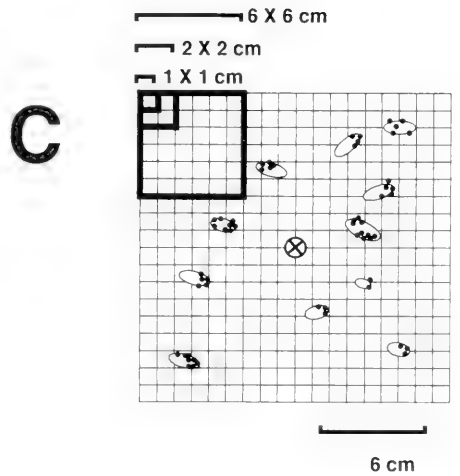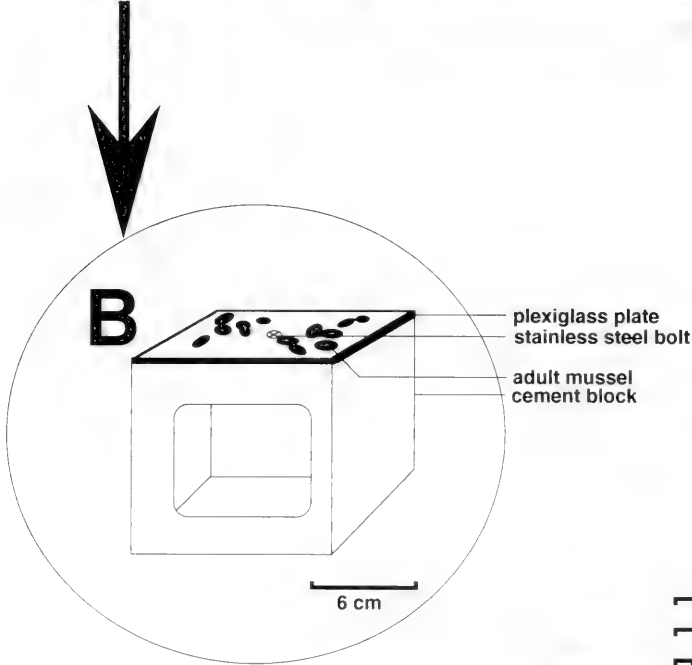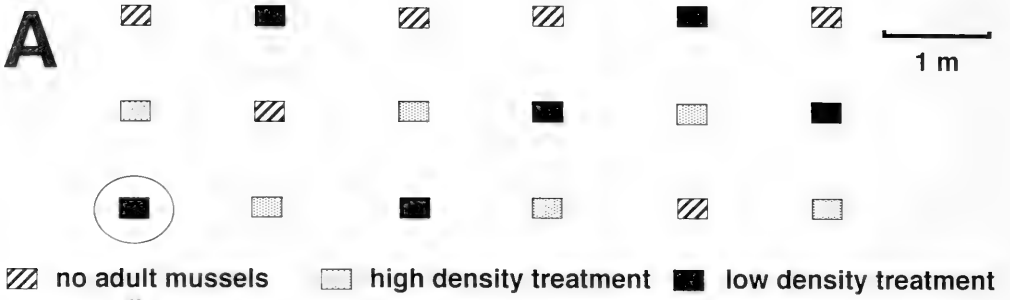
FIG. 2a. Schematic diagram of experimental design, which consisted of three treatments of adult mussel density—no adults, low density and high density—in Lake St. Clair. FIG. 2b. Diagram of one cement block as placed in the field with a plexiglass plate (18 × 18 cm) attached with a stainless steel bolt. FIG. 2c. Diagram of a plexiglass plate from the low density treatment showing division into 324 (1 × 1 cm) squares and subsequent analysis at three spatial scales; 1 × 1 cm, 2 × 2 cm and 6 × 6 cm. Open symbols represent adult mussels, closed symbols represent recruits.

2a). The plates were monitored visually twice monthly for recruitment.

The plates were removed on November 16, 1994, 182 days after deployment, and returned to the laboratory for examination. Of the 18 plates deployed, 16 were recovered, including 6 plates from the high density treatment, 5 plates of the low density treatment, and 5 plates of the no mussel treatment. In addition to retrieving the plates, 10 rocks were randomly collected from the surrounding area so that the density and size distribution of recruits from the experimental treatments could be compared to the natural population.

In the laboratory, each plate was divided into 324 (1 × 1 cm) squares (Fig. 2c). The number of adults and recruits in each square was recorded under 10× magnification using a Wild-Heerbrug microscope. Recruitment was defined as individuals between 0.8 and 4 mm in shell length. Adults and recruits were then removed from the plates.

Mussels from the natural population were removed from each of the rocks collected and preserved in ethanol. Densities of adults and recruits in the natural population were determined using the method of Bailey et al. (1995). The shell length of the recruits from both the plates and the natural population were measured at 6.4× magnification using a digitizing tablet interfaced with an IBM personal computer (Roff & Hopcroft, 1986). Length was measured as the longest distance between the umbo and the ventral margin.

Data Analysis

Counts of the number of recruits per plate in each treatment were Log_{10} (x + 1) transformed, and a one-way ANOVA was performed followed by a Tukey-Kramer test to make a posteriori comparisons of means.

Lengths of recruits on the plates were Log_{10} transformed and then compared within and among treatments by use of one-way ANOVA. Lengths of recruits in each treatment were then compared to Log_{10} transformed lengths of recruits in the natural population by one-way ANOVA.

To determine the effect of adult density on the spatial arrangement of recruits, the distribution of adults and recruits was examined at three spatial scales; 1 × 1 cm, 2 × 2 cm and 6 × 6 cm (Fig. 2c). A nested analysis of covariance was applied to determine how adults and recruits covaried at these scales.
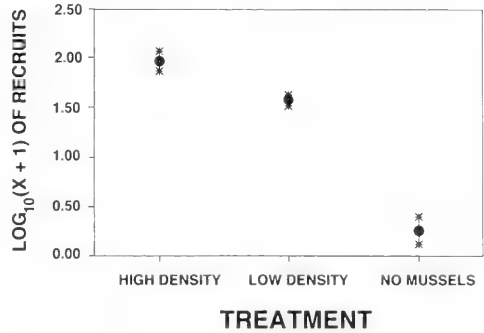
FIG. 3. Number of recruits (Log_{10} (X + 1) transformed) (± SE) in each of the experimental treatments; no adult mussels (n = 5 plates), low density (n = 5 plates) and high density (n = 6 plates).

This analysis quantified the strength and nature of the covariation of adults and recruits on the plates in the low and high density treatments.

RESULTS

Recruit Density

One-way ANOVA on log_{10} (x + 1) transformed number of recruits showed significant (F = 83.82, DF = 2,13, p < 0.001) differences among the treatments (Fig. 3). Pairwise comparisons of mean number of recruits in each treatment using the Tukey-Kramer test showed that the number of recruits in the no adult mussel treatment was significantly lower than the low density treatment (p < 0.001) and the number of recruits in the low density treatment was significantly lower than the high density treatment (p = 0.036) (Fig. 3). Examination of the relationship between recruit density and adult density within treatments revealed a positive linear relationship within the low density treatment (Fig. 4a) but a negative linear relationship within the high density treatment. Neither regression was significant (Low density: DF = 1,3, F = 2.84, r^2 = 0.49; high density: DF = 1,4, F = 7.314, r^2 = 0.65).

Size Distribution

One-way ANOVA of length of recruits between the five plates in the low density treatment was not significant (F = 1.26, DF = 4,203, p = 0.286); therefore, lengths of recruits in the low density treatment were pooled. Lengths of recruits from the six
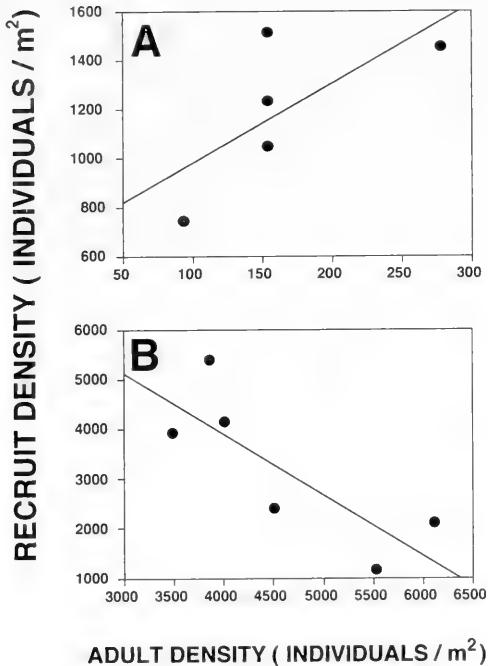
FIG. 4. Regression of recruit density versus adult density for the low density (A) and high density (B) treatments.

plates in the high density treatments were also pooled, because the one-way ANOVA of length of recruits between the high density treatments was not significant (F = 2.12, DF = 5,622, p = 0.062).

One-way ANOVA of length of recruits in the low and high density treatments was significant (F = 4.89, DF = 1,834, p = 0.027). The mean length of recruits in the low density treatment (1.89 ± 0.03 mm) was significantly larger than the mean length of recruits in the high density treatment (1.81 ± 0.02 mm), despite the small difference in means.

Because the lengths of recruits in the high and low density treatments differed, separate ANOVA's were performed to compare them to the natural population. One-way ANOVA revealed the length of the recruits in both the low (F = 175.04, DF = 1,353, p < 0.001) and the high (F = 197.81, DF = 1,773, p < 0.001) density treatments (Fig. 5) were significantly larger than the mean lengths in the natural population. The mean length of recruits in the natural population was 1.33 ± 0.03 mm (Fig. 5), about 500 μm less than in both experimental treatments.

Spatial Arrangement

Across both low and high adult density treatments, and all plates (Fig. 6), correlation between adults and recruits was high (r = 0.90 ± 0.07). There was no significant difference between the correlation values at either the 1 × 1, 2 × 2 or 6 × 6 cm spatial scales in the low adult density treatment, although the mean correlation at the 2 × 2 cm spatial scale was always the highest (2 × 2: r = 1.14 ± 0.08; 6 × 6: r = 0.87 ± 0.26). Although r > 1 is mathematically impossible in simple correlation analysis, such estimates are possible in nested covariance analysis. They should just be interpreted as high correlations at this scale. One-way ANOVA of correlation at the different spatial scales in the high adult density treatment revealed no significant difference between the 2 × 2 and the 6 × 6 cm spatial scales (F = 2.04, DF = 1, 10, p = 0.183) although the mean correlation at the 6 × 6 cm scale (r = 0.88 ± 0.07) was always higher than the mean correlation in the 2 × 2 cm scale (r = 0.74 ± 0.08). Correlation at the 1 × 1 cm scale was significantly lower than either the 2 × 2 or the 6 × 6 cm scales in the high density treatment (F = 17.01, DF = 2, 15, p < 0.001). Correlation at the 1 × 1 cm scale was also low in the low density treatments (mean r = 0.64 ± 0.5). Low correlation between adults and recruits at the 1 × 1 cm scale reflects the average length of adult mussels on the plates, which was 1.03 cm.

DISCUSSION

Recruitment Density

Results of the experimental study showed that the density of recruitment increased with the density of adults. Little recruitment was observed on plates with no adult mussels present. Several explanations may account for the pattern of recruitment observed in this study, including differential deposition and attachment, post depositional movement, and differential mortality after settlement (Johnson, 1994). Because this study examined only recruitment, it is difficult to determine which of the possible mechanisms may be underlying the settlement of D. polymorpha at Lake St. Clair. Recruitment is defined as the arrival of the first cohort or age class into the population (Connell, 1985), so it includes any post-settlement movement or
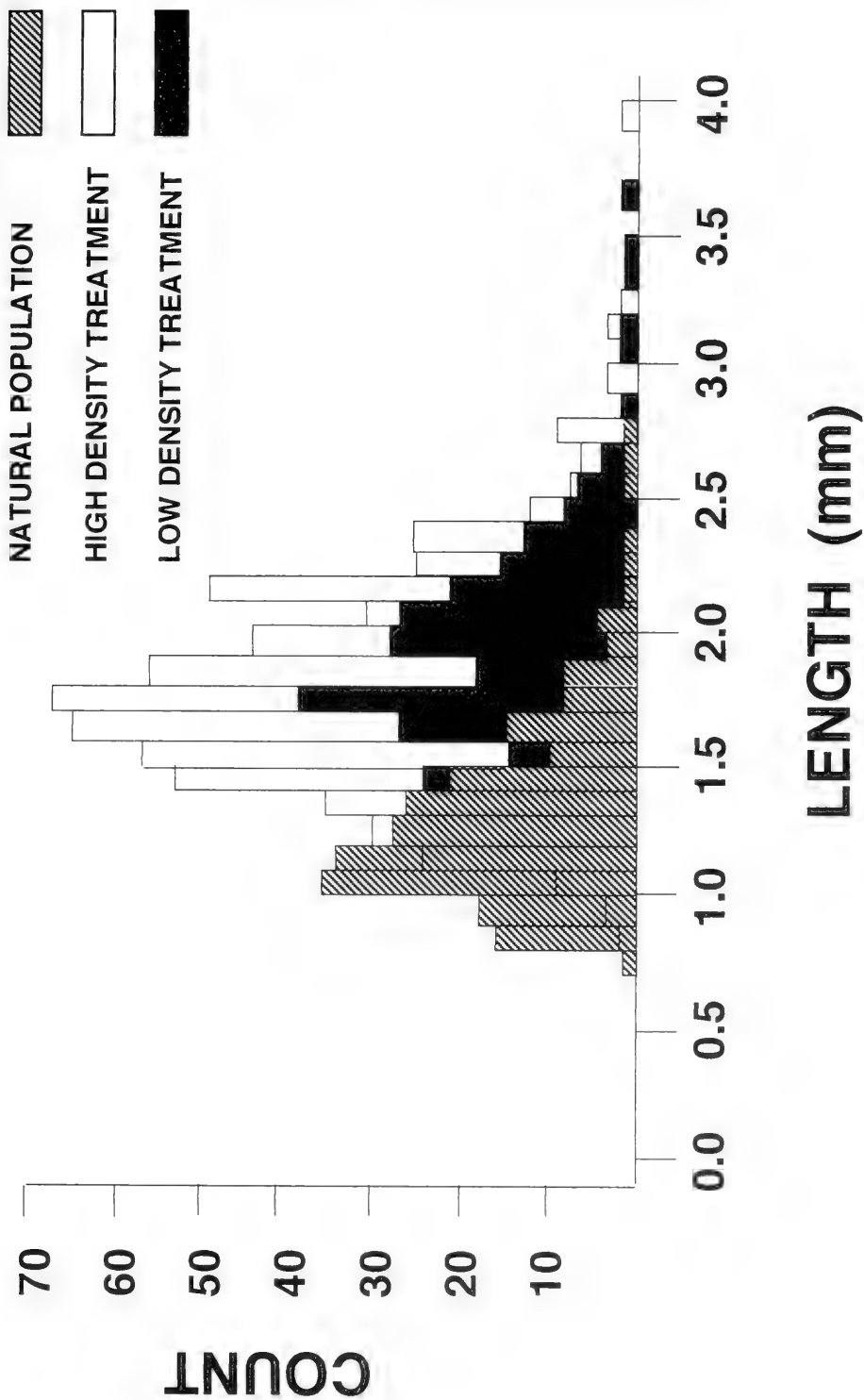
FIG. 5. Length-frequency histograms of recruit length in the high (n = 631) and low (n = 209) density experimental treatments and the adjacent natural population (n = 148).
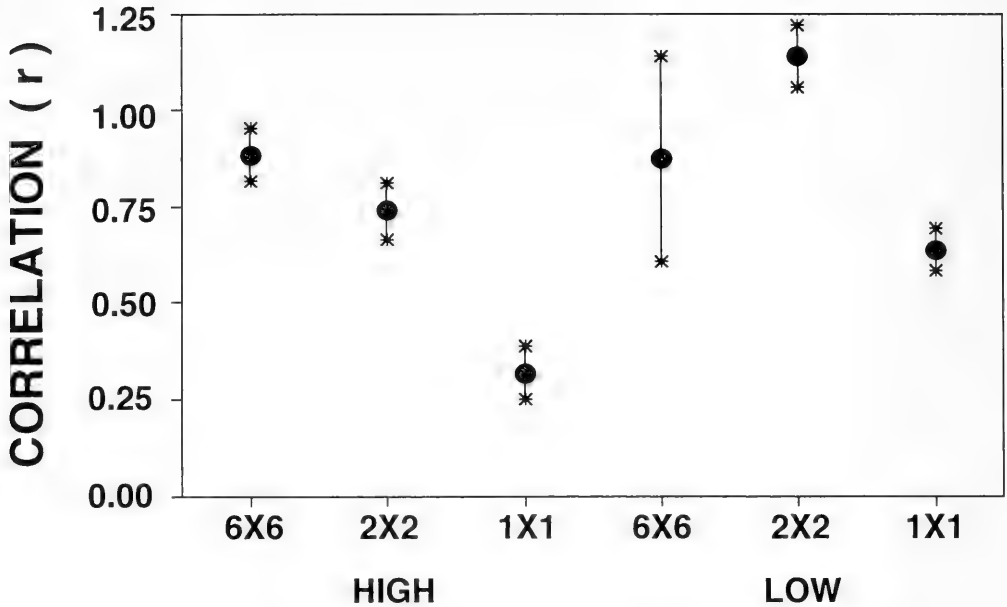
FIG. 6. Correlation (± SE) between adults and recruits in the high and low density treatments at each of the three spatial scales; 1 × 1 cm, 2 × 2 cm and 6 × 6 cm.

mortality that may have occurred. It is possible that settlement did occur on the clean plates but the mussels did not survive to the time of census. Alternatively, another factor may have been acting as a deterrent to prevent settlement to the clean plates. Even on plates with adults, recruitment occurred only on or very close to the adults. It was observed on several occasions that herbivorous snails were present on the clean plates. While the mussels are at no risk of predation from the snails, their presence may still act as a deterrent to recruitment. Barnacle recruitment can be reduced in the presence of limpets (Denley & Underwood, 1979; Miller, 1986) because of the biological disturbance (i.e., bulldozing) by the limpets. Johnson & Strathmann (1989) demonstrated reduced settlement of barnacle larvae as a result of prior occupation of the substratum. Their results indicated that mucus secretions may have been responsible for the reduction in settlement, because they may have affected the adhesion of the larvae or caused an alteration in the existing cues present on the substratum (Johnson & Strathmann, 1989). The most likely explanation of the recruitment pattern observed in this study, however, is that of differential deposition and attachment, that is, recruitment is in response to a cue released by conspecific adults. Wainman et al. (1995) also observed no settlement on treatments without mussels. In addition to treatments with and without adult mussels, Wainman et al. (1995) included treatments with mussel-sized stones. These treatments served as a control to ascertain whether recruitment was in response to a chemical cue released by the adults or simply in response to the heterogeneity of the substrata. Wainman et al. (1995) found settlement and recruitment was significantly lower on mussel-sized stones than on live mussels. This pattern was maintained even after 10–12 days despite reduction in the numbers of recruits. This pattern suggests that although there is post settlement mortality, the pattern of settlement with conspecifics was maintained. A laboratory study on settlement and metamorphosis of larval zebra and quagga mussels (Baldwin, 1995) provides further evidence for the presence of a chemical cue associated with conspecific adults. Baldwin (1995) found that in the laboratory *D. polymorpha* settled and metamorphosed more readily on natural substrata (adult shells) and in water from adult rearing tanks as opposed to water not exposed to adults. On the basis

of our study and the research described above, it appears that *D. polymorpha* responds to some chemical cue released by adult conspecifics that enhances settlement and recruitment. The exact nature of the cue, however, was beyond the scope of this study.

Our study also examined the effect of increased density of adult mussels on the density and spatial pattern of recruitment. Proximity to adults allows for synchronization of spawning and increased fertilization of spawned gametes as well as local introduction of food as a result of adult filtering activity. Large aggregations have a better chance of surviving physical disturbance and consequently gain a longer adult life span and overall increased fecundity (Pawlik, 1986). However, at high population densities, organisms may experience intense intraspecific competition for space (Wu, 1980; Hui & Moyse, 1987) and resources (Russo, 1979) and increased rates of both predation (Fairweather, 1988) and parasitization (Blower & Roughgarden, 1989). Therefore, at high population densities there may be selective pressure for individuals to avoid conspecifics at settlement (Satchell & Farrell, 1993).

Within this study, recruitment density was highest in the high density treatment. However, variation in the adult densities within each of the high and low density treatments enabled the examination of the relationship between recruit density and adult density within each treatment. This variation is the result of differential attachment of adult mussels within the laboratory and subsequent loss of adults during transportation and placement in the field site. Although the data are limited, it was observed that within the high density treatment (n = 6 plates) there is a negative relationship between adult mussel density and the density of recruits, whereas in the low density treatment (n = 5 plates), there is a positive relationship between adult mussel density and recruit density. It is possible that there is reduced recruitment with higher adult densities, but the adult densities employed in the high density treatments were not large enough to elicit such a response. When comparison was made between the recruitment densities from the experimental treatments and the natural population, it was observed that the recruitment density in the natural population was $1,257 \pm 178$ individuals per m^2 [which is comparable to the recruitment density in the low density treatment ($1,198 \pm 141$ individuals per m^2)] but much lower than the recruitment density in the high density treatment ($3,189 \pm 637$ individuals/m^2). Adult densities in the natural population were $8,029 \pm 506$ individuals per m^2 versus only $4,583 \pm 419$ individuals per m^2 for the high density treatments. This suggests that there may be some avoidance of the high adult density in the natural population.

Spatial Arrangement

Examination of the spatial arrangement by nested analysis of covariance revealed a strong correlation between adults and recruits, confirming the observation that at all scales we tended to find recruits when adults were present. When the spatial arrangement was examined on three spatial scales the low density treatment had the highest correlation at the 2×2 cm scale, whereas the high density treatment the highest correlation was at the 6×6 cm scale. However, correlation in the 2×2 and the 6×6 cm scales were not significantly different within treatments. This pattern suggests that while in the low density treatment the recruits are found closer to the adults than in the high density treatment, both treatments show the same conclusion that the recruitment occurs in response to the presence of adult conspecifics.

In a patchy environment (represented by the low density treatment), the recruits must be close to the adults to obtain whatever benefit — protection, enhanced feeding — that such an association would elicit. This is indicative of the higher correlation at the 2×2 cm scale. Hoffman (1989) suggested that gregarious settlement reduces stress on the vulnerable meta individual. Clumps of barnacles may also influence water flow in a way that enhances feeding (Barnes & Powell, 1950). However, in a more homogenous environment (represented by the high density treatment) such a close association may be detrimental because of competition for space and resources and the increased risks of predation of parasitism. In the high density treatment, the adults and recruits covaried on a larger scale (6×6 cm) than in the low density treatment, indicating a more uniform distribution. In addition, the ratio of recruits to adults was much lower in the high density treatment (0.8 ± 0.2) than the low density treatment (7.5 ± 0.8). Hebert et al. (1991) observed a marked heterogeneity in size and cohort structure in

D. polymorpha at different sites in Lake St. Clair in 1989. Members of the 1988 cohort had the smallest shell sizes at sites with the highest density, suggesting that their growth rates were slowed by intraspecific competition.

Natural Population

Comparison of the mean length of recruits revealed that recruits in the natural population were significantly smaller than recruits in either the low or the high density treatments. The largest mean length of recruits was in the low density treatment (1.89 mm), which may suggest that increased competition for food in the high density environment resulted in reduced growth of recruits. Such a scenario will confer an advantage of recruiting into a low density habitat with either more space to grow or decreased competition for food with larger mussels. This observation may also explain the reduction in growth of mussels at Lake St. Clair since their introduction. Population densities near Stoney Point were only 0.5 and 4,500 individuals per m^2 in 1988 and 1989 respectively (Hebert et al., 1991). At that time, Mackie (1991) reported that an overwintering young adult between 1–4 mm in shell length will attain a shell length of 15 to 20 mm by the end of the year. Our data have shown that overwintering young adults of similar size (1–4 mm) had shell lengths of only 9 mm (Chase, unpublished data) by the end of the next year. Population densities at Stoney Point now exceed 10,000 individuals per m^2 (Chase, unpublished data). Similar restriction in growth rates and survival of recent recruits of the barnacle *Pollicipes polymerus* were determined to be the result of competition between the established adults and the recruits for food resources (Page, 1986). When large adults were experimentally removed from an aggregate, the smaller barnacles were able to increase rapidly in size (Page, 1986). Larger barnacles may also have interfered with the water flow that brings food to the smaller barnacles (Page, 1986). In the mussel *Mytilus edulis,* Kautsky (1982) also reported that growth was suppressed in small mussels by increased density of large mussels. However, differences in size distribution and abundance may also be the result of differential recruitment between the experimental and natural population. This observation may also be the result of the raised level of the bricks in the water column, which may enhance growth of recruits. Pontius & Culver (1995) found that *D. polymorpha* higher in the water column had larger biomass, which may indicate they were better able to obtain food. However, the significant difference between the low and high density treatments suggests an explanation other than height in the water column.

Conclusion

It appears that for *D. polymorpha* at Lake St. Clair, the presence and density of conspecifics are important determinants of the recruitment density and pattern in the population. The presence of adult conspecifics may offer some chemical cue that induces recruitment into the population. However, recruitment into a low density habitat may be advantageous because it may enhance the growth of young recruits. Therefore, in *D. polymorpha* there appears to be a tradeoff between adult densities that are high enough to provide an attachment site and protection but low enough to enhance growth and possibly survival. Larger mussels may have a better chance at surviving the winter, and because fecundity is related to size in most benthic invertebrates (Hughes, 1971; Spight & Emlen, 1976; Brousseau, 1978; Sprung, 1987; Chase & Thomas, 1995), recruitment into a low density habitat may also enhance reproductive output, assuming this size differential is maintained.

The objective of this study was to examine recruitment. As such, the extent of post settlement mortality is unknown. It is possible the post settlement mortality was higher in the low density treatments than in either the high density treatment or the natural population. Thus, although our study suggests that recruitment into low density habitats may be advantageous because of enhanced growth and survival, it may have also suffered from a greater initial mortality. However, in terms of the ultimate survival and population structure of *D. polymorpha* at Lake St. Clair our results are valid.

offering constructive criticism. This research was funded through Natural Science and Engineering Research Council of Canada, Ontario Ministry of Natural Resources, and the Great Lakes University Research Fund (Lake Erie Trophic Transfer) grants to R. C. Bailey.

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ADDITIONAL NOTES ON NOMINA FIRST INTRODUCED BY TETSUAKI KIRA IN "COLOURED ILLUSTRATIONS OF THE SHELLS OF JAPAN"

Rüdiger Bieler[1] & Richard E. Petit[2]

The taxa, both available and unavailable, first proposed by Tetsuaki Kira in the numerous printings of his "Coloured Illustrations of the Shells of Japan" and the English edition, "Shells of the Western Pacific in Color, Vol. I" were recently listed by us (Bieler & Petit, 1990). At the time, we discussed 54 species-group and one genus-group name. Forty names were found to be available from this work, although only five of these had been formally designated as new taxa. The difficulty in recognizing some of these unannounced introductions is demonstrated by our having to add two new taxa that we overlooked despite extensive searching and comparing the various printings and editions in which some nude names have been introduced. There probably remain still others that have eluded us. Also, we add additional data on the previously listed genus-group name.

Laevistrombus Abbott, 1960

Laevistrombus Kira, 1955: 31 (*nomen nudum*).
Laevistrombus Kira. Abbott, 1960: 47–48 (type species designated: *Strombus canarium* Linné, 1758).

This name first appeared in the 3rd printing of the 1st edition of "Coloured Illustrations of the Shells of Japan" as a subgenus for two nominal species of *Strombus*: *S.* (*L.*) *canarium* Linné, 1758, and *S.* (*L.*) *isabella* Lamarck, 1822. No description or statement of differentiation was given, as required by ICZN *Code* Article 13a, nor was a type species designated. Subsequent printings remained unchanged at least through the 6th printing of the 2nd edition (1963). In the 9th printing of the 2nd edition (1964), *Laevistrombus* is elevated to genus-level and *L. isabella* emended to *L. canarium* "forma" *isabella*. The two intermediate printings have not been seen, but have no effect on this discussion.

When Abbott (1960: 47–48) treated *Laevistrombus* as a subgenus in his monograph of *Strombus,* he gave a brief description of *Laevistrombus* and designated *S. canarium* Linné as its type species. Although Abbott cited Kira as the author of *Laevistrombus,* the name had not previously been available and must take date and authorship from Abbott, 1960 (ICZN *Code* Article 50a).

Simplicifusus Kuroda & Habe, 1971

Simplicifusus Kira, 1962: 85 (*nomen nudum*).
Simplicifusus Kira, 1964: 77 (*nomen nudum*).
Simplicifusus Kira. Kuroda & Habe, 1971: 282, 184 (type species designated: *Fusinus simplex* Smith [sic; = *Fusus simplex* E. A. Smith, 1879]).

Simplicifusus first appeared in Kira's "Shells of the Western Pacific in Color" (1962: 85) as a subgenus of *Fusinus* for two species: *F.* (*S.*) *hyphalus* M. Smith and *F.* (*S.*) *simplex* (Smith) [= *Fusinus hyphalus* Maxwell Smith, 1940, and *Fusus simplex* E. A. Smith, 1879]. We cannot determine exactly when this name first appeared in the Japanese version of this work, "Coloured Illustrations of the Shells of Japan, Vol. I." It was not in the 6th printing (1963) but was in the 9th printing (1964). We have not seen the two intermediate printings. However, Kira (1962, 1964) gave no description or statement of differentiating characters as required by ICZN *Code* Article 13a.

Kuroda & Habe (1971) cited *Simplicifus* Kira as a genus (Japanese text, p. 282) and as a subgenus of *Fusinus* (English text, p. 184). A description of the genus is given (Japanese text, p. 282), and *Fusinus simplex* (Smith) is designated as type species (Japanese p. 282; English p. 184). Because *Simplicifusus* was not previously an available name, it must take date and authorship from Kuroda & Habe, 1971 (ICZN *Code* Article 50a).

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Pictodentalium Habe, 1963

Pictodentalium Kira, 1959: 105 (and subsequent years; *nomen nudum*).

Pictodentalium Habe, 1963: 255 (genus described; type species designated: *Dentalium (Pictodentalium) formosum hirasei* Kira, 1959).

In our previous paper (1990: 141), we showed that this genus-group name was a *nomen nudum* in all editions of Kira's works. It was treated in a systematic manner by Habe in 1963 (p. 255), who gave a description of it as a subgenus. He attributed the name to Kira and gave the type-species as *Dentalium (Pictodentalium) formosum hirasei* Kira, stating that designation was by monotypy. He then placed *D. (P.) formosum hirasei* Kira in the synonymy of *D. (P.) formosum* (A. Adams & Reeve, 1850). Because *Pictodentalium* had not previously been made available, it must take date and authorship from Habe, 1963 (ICZN *Code* Article 50a).

We thank Dr. Alan R. Kabat for bringing to our attention the omission of *Simplicifusus* in our earlier paper, and an anonymous reviewer for additional data on the availability of *Pictodentalium*.

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ON THE NEW NAMES INTRODUCED IN THE VARIOUS PRINTINGS OF "SHELLS OF THE WORLD IN COLOUR" [VOL. I BY TADASHIGE HABE AND KIYOSHI ITO; VOL. II BY TADASHIGE HABE AND SADAO KOSUGE]

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ABSTRACT

The two volumes of "Shells of the World in Colour" (Vol. I, "The Northern Pacific" by Habe & Ito; Vol. II, "The Tropical Pacific" by Habe & Kosuge) contain many gastropod and bivalve names denoted as new therein. Some of these are *nomina nuda* made available only in later publications. However, the volumes also contain new taxa that are made available but not indicated as such. The problem is compounded by the existence of multiple printings of both volumes in which unexplained nomenclatural changes have been made. Forty-four species-group names and two genus-group names date from these works. Twelve genus-group names indicated as new were not made available until later. All pertinent treatments of these taxa are listed.

INTRODUCTION

In our continuing efforts to determine the status and correct dates of publication of various taxa proposed by Japanese authors, this paper discusses names introduced in the two volumes of "Shells of the World in Colour" (Vol. I, "The Northern Pacific" by Habe & Ito; Vol. II, "The Tropical Pacific" by Habe & Kosuge). Both of these volumes went through numerous printings, with changes being made that are not indicated as such.

Neither book is easy to locate, and few workers have access to more than one printing (we have failed to locate any copies of some printings). This paper lists the changes between printings that affect zoological nomenclature. At least 14 genus-group and 44 species-group names are involved, spanning many marine gastropod and bivalve families.

Of particular importance is the determination of when a particular taxon was made available for taxonomic purposes. The descriptions of the species and subspecies in the two works under consideration are in Japanese and usually very brief. These species-group taxa are, however, accepted as being validly proposed. The genus-group taxa present more serious problems because 12 of the new names were introduced without fulfilling ICZN *Code* Article 13 requirements of providing a fixation of type species, and a differentiating description or indication

to such. They are here regarded as *nomina nuda* and became available only in later works. Two names, *Harpofusus* and *Megacrenella,* appear to fulfill the minimal requirements set by the *Code* and are here accepted as dating from their first appearance.

It is hoped that the following notes will be of value to systematists who must refer to these taxa. We have maintained original orthography when possible, and have not indicated some typographical errors and incorrect usages in order to avoid using "[sic]" as much as possible. Readers should be aware that in addition to these "new" names there are numerous changes between the editions involving generic or (for subspecies) specific allocations, re-identifications, and adjustments in spelling and latinization. The works apparently were newly typeset, at least in part, between printings, sometimes resulting in a compounding of problems. An example of the combination of intended and accidental changes is Habe & Ito's reference to a species of *Neptunea* (p. 66, pl. 33, fig. 8); this was initially identified as *Neptunea minor* and later (1977) corrected to "*Neptunea Rurosio,*" a lapsus for *N. kuroshio* Oyama, 1958.

An example of the taxonomic confusion in these works is the nominal subspecies *shirogai,* first introduced as "*Collisella pelta shirogai* Habe et Ito (nov.)" in 1965. The 1977 printing of the work, referring to the same illustration, not only still indicated it as being

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new, but changed the name of the species: "*Collisella cassis shirogai* Habe et Ito (nov.)." An additional layer of difficulty was introduced by printer's errors. For instance, "*Buccinum chishimananux* Habe et Ito (nov.)" of 1965a was meant to introduce a new subspecies, *nux,* for the species *chishimanum.*

Another example that has perplexed authors is *Harpa kawamurai* Habe, first introduced in the 3rd printing of Habe & Kosuge (1972) with no indication that it was new. *Harpa kajiyamai* Habe, which appeared at the same time, has never before been correctly cited in the literature.

Systematists are urged to cite these works *by printing.* The date of a particular printing can be easily determined from the colophon (inscription at end of each copy). For details on date determination, see Bieler & Petit (1990: 132).

LISTING OF NEW NAMES

(A) Habe & Ito, 1965 (in sequence of occurrence in volume; the work in which each taxon is considered to have been made available is shown by the usage of 1965a, 1965b, or a later date)

(1) *Collisella pelta shirogai,* 1965a
(2) *Omphalomargarites,* 1965b
(3) *Cirsotrema kagayai,* 1965a
(4) *Bulbus flavus elongatus,* 1965a
(5) *Trophonopsis scitula emphaticus,* 1965a
(6) *Boreotrophon paucicostatus,* 1965a
(7) *Nodulotrophon,* 1965b
(8) *Mohnia multicostata,* 1965a
(9) *Ancistrolepis trochoidea ovoidea,* 1965a
(10) *Fusipagoda,* 1965b
(11) *Buccinum chishimanum nux,* 1965a
(12) *Buccinum hosoyai,* 1965a
(13) *Buccinum opisthoplectum microconcha,* 1965a
(14) *Buccinum felis shikamai,* 1965a
(15) *Buccinum kawamurai,* 1965a
(16) *Clinopegma buccinoides,* 1965a
(17) *Neoberingus,* 1965b
(18) *Beringion,* 1965b
(19) *Harpofusus,* 1965a
(20) *Volutopsion,* 1965b
(21) *Buccinum subreticulatum,* 1965a
(22) *Buccinum ferrugineum,* 1965a
(23) *Buccinum kinukatsugi* Habe & Ito, 1968
(24) *Buccinum midori,* 1965a
(25) *Boreomelon stearnsii ryosukei,* 1965a

(26) *Fulgoraria (Musashia) kaneko hayashii,* 1965a
(27) *Decollidrillia,* 1965b
(28) *Decollidrillia nigra,* 1965a
(29) *Megacrenella,* 1965a
(30) *Adula californiensis chosenica,* 1965a
(31) *Megacardita ferruginosa koreana,* 1965a

(B) Habe & Kosuge, 1966 (in sequence of occurrence in volume; the work in which each taxon is considered to have been made available is shown by the usage of 1966a, 1966b, or a later date)

(32) *Patelloida (Collisellina) saccharinoides,* 1966a
(33) *Astralium yamamurae,* 1966a
(34) *Granulittorina,* 1966b
(35) *Granulittorina philippiana,* 1966a
(36) *Clypeomorus batillariaeformis,* 1966a
(37) *Ficadusta,* 1966b
(38) *Reticutriton,* 1966b
(39) *Spinidrupa,* 1966b
(40) *Pyrene testudinaria nigropardalis,* 1966a
(41) *Pyrene lacteoides,* 1966a
(42) *Plicarcularia gibbosuloidea,* 1966a
(43) *Hemifusus carinifer,* 1966a
(44) *Latirus stenomphalus,* 1966a
(45) *Vexillum rubrocostatum,* 1966a
(46) *Nebularia yaekoae,* 1966a
(47) *Harpa kawamurai* Habe, in Habe & Kosuge, 1972
(48) *Harpa kajiyamai* Habe, in Habe & Kosuge, 1972
(49) *Volutoconus grossi mcmichaeli,* 1966a
(50) *Brachytoma kurodai,* 1966a
(51) *Brachytoma kawamurai,* 1966a
(52) *Brachytoma vexillium,* 1966a
(53) *Eglisia brunnea,* 1966a
(54) *Mantellum perfragile,* 1966a
(55) *Anomiostrea,* 1966b
(56) *Laevicardium rubropictum,* 1966a
(57) *Vasticardium nigropunctatum,* 1966a
(58) *Macrotoma yamamurae,* 1966a

DISCUSSION BY VOLUME

"Shells of the World in Colour, Vol. I. The Northern Pacific." Tadashige Habe and Kiyoshi Ito

The first printing of "Shells of the World in Colour, Vol. I" is dated June 1, 1965 (Habe & Ito, 1965a). A paper by Habe & Ito published in *Venus (The Japanese Journal of Malacology)* on July 31, 1965 (1965b) also contains

descriptions of taxa, indicated as new therein, which had been shown as new in the book. In the next few years there were several printings of the book; Dr. Kosuge (personal comm., March 15, 1995) advises that the 11th printing appeared in March 1991.

Printings that we have seen:

Printing 1   June 1, 1965 (1965a)
          2   September 1, 1970
          4   August 1, 1972
          5   January 20, 1974
          8   October 1, 1977

The following new species appear (using the original arrangement of families). Important changes between printings and references from other sources are also listed.

GASTROPODA

Acmaeidae

(1) *Collisella cassis shirogai* Habe & Ito, 1965a

Collisella pelta shirogai Habe et Ito (nov.). Habe & Ito, 1965a: 11, pl. 4, fig, 18; 1970, 1972, 1974: ibid.
Collisella pelta shirogai subsp. nov. Habe & Ito, 1965b: 16, 29, pl. 4, fig. 8.
Collisella pelta shirogai Habe et Ito. Habe, 1977: 111 (cited as of 1965a).
Collisella cassis shirogai Habe et Ito (nov.). Habe & Ito, 1977: 11, pl. 4, fig. 18.

Trochidae

(2) *Omphalomargarites* Habe & Ito, 1965b

Omphalomargarites (nov) *vorticifera* (Dall, 1873). Habe & Ito, 1965a: 17, pl. 6, figs. 6, 7; 1970, 1972, 1974, 1977: ibid. (genus-group name = *nomen nudum*).
Omphalomargarites subgen. nov. Habe & Ito, 1965b: 17 (type species *Margarites vorticifera* (Dall, 1873), with no indication of genus in which it was to be placed).
Omphalomargarites gen. nov. Habe & Ito, 1965b: 30 (type species, *Margarites vorticifera* (Dall, 1873).
Omphalomargarites (gen. nov.) *vorticifera* (Dall). Habe & Ito, 1965b: 45 (plate caption for pl. 2).
Omphalomargarites Habe & Ito. Kuroda & Habe, 1971: 31(21) (with Habe & Ito, 1965b, given precedence over 1965a,

and with type species stated to be by original designation)
Omphalomargarites Habe et Ito. Habe, 1977: 90 (cited as of 1965a, with type as *Margarites vorticifera* (Dall, 1873) by monotypy; 1965b mentioned only as a *"cf."* reference).

Epitoniidae

(3) *Cirsotrema kagayai* Habe & Ito, 1965a

Cirsotrema kagayai Habe et Ito (nov.). Habe & Ito, 1965a: 29, pl. 7, fig. 25; 1970, 1972, 1974, 1977: ibid.
Cirsotrema kagayai sp. nov. Habe & Ito, 1965b: 17, 30, pl. 2, fig. 9.
Cirsotrema kagayai Habe et Ito. Habe, 1977: 56 (cited as of 1965a).

Naticidae

(4) *Bulbus flavus elongatus* Habe & Ito, 1965a

Bulbus flavus elongatus Habe et Ito (nov). Habe & Ito, 1965a: 31, pl. 8, fig. 8; 1970, 1972, 1974, 1977: ibid.
Bulbus flavus elongatus subsp. nov. Habe & Ito, 1965b: 17, 31, pl. 3, fig. 2.
Bulbus flavus elongatus Habe et Ito. Habe, 1977: 38 (cited as of 1965a).

Muricidae

(5) *Trophonopsis scitula emphaticus* Habe & Ito, 1965a

Trophonopsis scitula emphaticus Habe et Ito (nov.). Habe & Ito, 1965a: 36, pl. 10, fig. 10; 1970, 1972, 1974, 1977: ibid.
Trophonopsis scitula emphaticus subsp. nov. Habe & Ito, 1965b: 18, 31, pl. 2, fig. 1.
Trophonopsis scitulus emphatica Habe et Ito. Habe, 1977: 38 (cited as of 1965a).

(6) *Boreotrophon paucicostatus* Habe & Ito, 1965a

Boreotrophon paucicostatus Habe et Ito (nov: [sic]). Habe & Ito, 1965a: 37, pl. 10, fig. 13; 1970, 1972, 1974, 1977: ibid.
Boreotrophon paucicostatus sp. nov. Habe & Ito, 1965b: 18, 32, pl. 2, fig. 10.
Boreotrophon paucicostatus Habe et Ito. Habe, 1977: 95 (cited as of 1965a).

(7) *Nodulotrophon* Habe & Ito, 1965b

Nodulotrophon (nov.) *dalli* (Kobelt, 1878). Habe & Ito, 1965a: 37, pl. 10, fig. 14;

1970, 1972, 1974, 1977: ibid. (genus-group name = *nomen nudum*).

Nodulotrophon gen. nov. Habe & Ito, 1965b: 19, 32 (with type species as *Trophon dalli* Kobelt, 1878).

Nodulotrophon Habe et Ito. Habe, 1977: 87 (cited as of 1965a, with type, by monotypy, *Trophon dalli* Kobelt, 1878; 1965b not mentioned).

Taxonomic note: This genus-group name must date from 1965b because there was no description in 1965a.

Buccinidae

(8) *Mohnia multicostata* Habe & Ito, 1965a

Mohnia multicostata Habe et Ito (nov.). Habe & Ito, 1965a: 45, pl. 13, fig. 12; 1970, 1972, 1974, 1977: ibid.

Mohnia multicostata sp. nov. Habe & Ito, 1965b: 19, 33, pl. 2, fig. 2.

Mohnia multicostata Habe et Ito. Habe, 1977: 80 (cited as of 1965a).

(9) *Ancistrolepis trochoidea ovoidea* Habe & Ito, 1965a

Ancistrolepis trochoideus ovoideus Habe et Ito (nov.). Habe & Ito, 1965a: 46, pl. 13, fig. 18; 1970, 1972, 1974, 1977: ibid.

Ancistrolepis trochoideus ovoideus subsp. nov. Habe & Ito, 1965b: 20, 33, pl. 2, fig. 13.

Ancistrolepis trochoidea [Bathyancistrolepis] ovoidea Habe et Ito. Habe, 1977: 92 (cited as of 1965a).

(10) *Fusipagoda* Habe & Ito, 1965b

Fusipagoda (nov.) *exquisita* Dall, 1913. Habe & Ito, 1965a: 48; Habe & Ito, 1970, 1972, 1974, 1977: ibid. (genus-group name = *nomen nudum*).

Fusipagoda gen. nov., Habe & Ito, 1965b: 21 (with type species as *Mohnia exquisita* Dall).

Fusipagoda Habe et Ito. Habe, 1977: 43 (cited as of 1965b with type species as cited, by original designation; 1965a cited as "name only").

(11) *Buccinum chishimanum nux* Habe & Ito, 1965a

Buccinum chishimananux [sic] Habe et Ito (nov.). Habe & Ito, 1965a: 49, pl. 14, fig. 2.

Buccinum chishimanum nux subsp. nov. Habe & Ito, 1965b: 22, 36, pl. 2, fig. 7.

Buccimum [sic] *chishimana nux* Habe et Ito (nov.). Habe & Ito, 1970: 49, pl. 14, fig. 1; 1972, 1974, 1977: ibid.

Buccinum chishimanum nux Habe et Ito. Habe, 1977: 88 (cited as of 1965a).

(12) *Buccinum hosoyai* Habe & Ito, 1965a

Buccinum hosoyai Habe et Ito (nov.). Habe & Ito, 1965a: 49, pl. 14, fig. 2; Habe & Ito, 1970, 1972, 1974, 1977: ibid.

Buccinum hosoyai sp. nov. Habe & Ito, 1965b: 23, 36, pl. 2, fig. 8.

Buccinum hosoyai Habe et Ito. Habe, 1977: 49 (cited as of 1965a).

(13) *Buccinum opisthoplectum microconcha* Habe & Ito, 1965a

Buccinum opisthoplectum microconcha Habe et Ito (nov.). Habe & Ito, 1965a: 50, pl. 14, fig. 7; 1970, 1972, 1974, 1977: ibid.

Buccinum opisthoplectum microconcha subsp. nov. Habe & Ito, 1965b: 23, 37, pl. 2, fig. 6.

Buccinum opisthoplectum microconcha Habe et Ito. Habe, 1977: 75 (cited as of 1965a; stated to be a synonym of *Buccinum japonicum* A. Adams, 1861).

(14) *Buccinum felis shikamai* Habe & Ito, 1965a

Buccinum felis shikamai Habe et Ito (nov.). Habe & Ito, 1965a: 50; 1970, 1972, 1974, 1977: ibid.

Buccinum felis shikamai subsp. nov. Habe & Ito, 1965b: 23, 37, pl. 2, fig. 5.

Buccinum felis shikamai Habe et Ito. Habe, 1977: 110 (cited as of 1965b).

(15) *Buccinum kawamurai* Habe & Ito, 1965a

Buccinum kawamurai Habe et Ito (nov.). Habe & Ito, 1965a: 52, pl. 15, fig. 1; 1970, 1972, 1974, 1977: ibid.

Buccinum kawamurai sp. nov. Habe & Ito, 1965b: 26, 38, pl. 2, fig. 11.

Buccinum kawamurai Habe et Ito. Habe, 1977: 58 (cited as of 1965a).

(16) *Clinopegma buccinoides* Habe & Ito, 1965a

Clinopegma buccinoides Habe et Ito (nov.). Habe & Ito, 1965a: 55, pl. 16, fig. 1; 1970, 1972, 1974, 1977: ibid.

Clinopegma buccinoides sp. nov., Habe & Ito, 1965b: 27, 41, pl. 2, fig. 12.

Clinopegma buccinoides Habe et Ito. Habe, 1977: 28 (cited as of 1965a).

(17) *Neoberingius* Habe & Ito, 1965b

Neoberingius (nov.) *frielei* Dall, (1895) [sic]. Habe & Ito, 1965a: 57, pl. 17, fig. 3. (genus-group name = *nomen nudum*).
Neoberingius gen. nov. Habe & Ito, 1965b: 21, 35, pl. 3, fig. 7. (type species, *Beringius frielei* Dall, 1894 [sic]).
Neoberingius (nov.) *frielei* (Dall, 1895). Habe & Ito, 1970: 57, pl. 17, fig. 3; 1972, 1974, 1977: ibid.
Neoberingius Habe et Ito. Habe, 1977: 83 (cited as of 1965b, with type species as cited, by original designation; 1965a cited as "name only").

(18) *Beringion* Habe & Ito, 1965b

Beringion (nov.) *marshalli* (Dall, 1919). Habe & Ito, 1965a: 58, pl. 17, fig. 4; 1970, 1972, 1974, 1977: ibid. (genus-group name = *nomen nudum*).
Beringion gen. nov. Habe & Ito, 1965b: 21, 35, pl. 3, fig. 6 (with type species as *Beringius marshalli* Dall, 1919).
Beringion Habe et Ito. Habe, 1977: 27 (cited as of 1965b, with type species as cited, by original designation; 1965a referred to with comments: "f. 4 as *Beringion* (nov.) *marshalli;* f. 5, *B. beringii,* with a notice of 'the type-species of *Beringion*', name only").
Taxonomic note: Habe's statement (1977: 83) is ambiguous as the mention of "type species" in the Japanese text is in the context of "*Beringion* type species is figured," which appears in discussion of *B. beringii* (Middendorff). He cited the new genus as of 1965b, which we consider to be correct.

(19) *Harpofusus* Habe & Ito, 1965a

Harpofusus (nov.) *melonis* (Dall, 1891). Habe & Ito, 1965a: 59, pl. 18, fig. 1; 1970, 1972, 1974: ibid. (with type species, by monotypy, *Harpofusus melonis* (Dall, 1891)).
Harpofusus gen. nov. Habe & Ito, 1965b: 20, 34 (with type species as *Pyrulofusus melonis* Dall, 1891 [as *Pyrofusus* on p. 20]).
Pyrulofusus (*Harpofusus*) *melonis* (Dall, 1891). Habe & Ito, 1977: 59, pl. 18, fig. 1.
Harpofusus Habe et Ito. Habe, 1977: 46. (listed as a genus of Buccinidae, cited as

of 1965a, with type species, by monotypy, *Pyrulofusus melonis* (Dall, 1891) [= *Strombella melonis* Dall, 1891]).
Taxonomic note: Habe & Ito (1965a: 59) move this species from its previous placement (*Volutopsis,* name given in Japanese only) into a new genus, based on the yellowish-orange aperture coloration and the vertical shell folds and spiral ribs. Similarity to *Pyrulofusus* is also mentioned. This fulfills the ICZN *Code* requirements, and we date this taxon as of 1965a.

(20) *Volutopsion* Habe & Ito, 1965b

Volutopsion (nov.) *castaneus* (Mörch, 1858). Habe & Ito, 1965a: 62, pl. 20, fig. 6; 1970, 1972, 1974, 1977: ibid. (genus-group name = *nomen nudum*).
Volutopsion gen. nov. Habe & Ito, 1965b: 21, 35, pl. 2, fig. 15 (with type species as *Volutopsius castaneus* Dall [sic]).
Volutopsion Habe et Ito. Habe, 1977: 131 (cited as of 1965a with type species, by monotypy, *Volutopsion castaneus [-um]* (Mörch, 1858); 1965b is mentioned only as a "*cf.*" reference, with same type species indicated, but by original designation. We consider this genus name to be available from 1965b, with type species, by original designation, *Volutopsion castaneum* (Mörch, 1858) [= *Neptunea castanea* Mörch, 1858].

(21) *Buccinum subreticulatum* Habe & Ito, 1965a

Buccinum Subreticulatum [sic] Habe et Ito (nov.). Habe & Ito, 1965a: 73, pl. 27, fig. 4.
Buccinum subreticulatum sp. nov. Habe & Ito, 1965b: 24, 39, pl. 2, fig. 14.
Buccinum subreticulatum Habe et Ito (nov.). Habe & Ito, 1970: 73, pl. 27, fig. 4; 1972, 1974, 1977: ibid.
Buccinum subreticulatum Habe et Ito. Habe, 1977: 118 (cited as of 1965a).

(22) *Buccinum ferrugineum* Habe & Ito, 1965a

(23) *Buccinum kinukatsugi* Habe & Ito, 1968

Buccinum ferrugineum Habe et Ito (nov.). Habe & Ito, 1965a: 76, pl. 28, fig. 8.
Buccinum ferrugineum sp. nov. Habe & Ito, 1965b: 25, 40, pl. 3, fig. 3.
Buccinum kinukatsugi nom. nov. Habe & Ito,

1968: 2, 5, pl. 1, fig. 4 (new name for *Buccinum ferrugineum* Habe & Ito, 1965, *non* Born, 1780 [sic; = 1778]).

Buccinum kinukatsugi Habe et Ito (nov.). Habe & Ito, 1970: 76, pl. 28, fig. 8; 1972, 1974, 1977: ibid.

Buccinum kinukatsugi Habe et Ito. Habe, 1977: 63 (cited as of 1968).

(24) *Buccinum midori* Habe & Ito, 1965a

Buccinum midori Habe et Ito (nov.). Habe & Ito, 1965a: 76, pl. 28, fig. 9; 1970, 1972, 1974, 1977: ibid.

Buccinum midori sp. nov. Habe & Ito, 1965b: 25, 40, pl. 2, fig. 16.

Buccinum midori Habe et Ito. Habe, 1977: 75 (cited as of 1965a).

Volutidae

(25) *Boreomelon stearnsii ryosukei* Habe & Ito, 1965a

Boreomelon stearnsii ryosukei Habe et Ito (nov.). Habe & Ito, 1965a: 77, pl. 29, fig. 2; 1970, 1972, 1974: ibid.

Boreomelon stearnsii ryosukei subsp. nov. Habe & Ito, 1965b: 26, 42, pl. 2, fig. 17.

Boromelon [sic] *stearnsii ryosukei* Habe et Ito (nov.). Habe & Ito, 1977: 77, pl. 29, fig. 2.

Boreomelon stearnsii ryosukei Habe et Ito. Habe, 1977: 103 (cited as of 1965a).

(26) *Fulgoraria (Musashia) kaneko hayashii* Habe & Ito, 1965a

Fulgoraria (Musashia) kaneko hayashii Habe et Ito (nov.). Habe & Ito, 1965a: 77, pl. 29, fig. 4; 1970, 1972, 1974, 1977: ibid.

Fulgoraria (Musashia) kaneko hayashii subsp. nov. Habe & Ito, 1965b: 26, 42, pl. 3, fig. 5.

Fulgoraria (Musashia) kaneko hayashii Habe et Ito. Habe, 1977: 47 (cited as of 1965a).

Turridae

(27) *Decollidrillia* Habe & Ito, 1965b

(28) *Decollidrillia nigra* Habe & Ito, 1965a

Decollidrillia nigra Hade [sic] et Ito (nov.). Habe & Ito, 1965a: 80, pl. 30, fig. 6. (genus-group name = *nomen nudum*).

Decollidrillia nigra gen. et sp. nov. Habe & Ito, 1965b: 27, 43, pl. 4, fig. 6.

Decollidrillia nigra Habe et Ito (nov.). Habe & Ito, 1970: 80, pl. 30, fig. 6; 1972, 1974, 1977: ibid.

Decollidrillia Habe et Ito. Habe, 1977: 35 (cited as of 1965b, with type, by original designation, *D. nigra;* 1965a cited as "name only").

Decollidrillia nigra Hade [-be] et Ito. Habe, 1977: 83 (species name cited as of 1965a).

Taxonomic note: We agree that this new genus dates from 1965b, but type designation is by monotypy (Articles 13c, 68d).

Bivalvia

Mytilidae

(29) *Megacrenella* Habe & Ito, 1965a

Crenella (Megacrenella nov.) *columbiana* Dall, 1897. Habe & Ito, 1965a: 109, pl. 35, fig. 11; 1970, 1972, 1974, 1977: ibid. (with type species, by monotypy, *Crenella (Megacrenella) columbiana* (Dall, 1897)).

Megacrenella gen. nov. Habe & Ito, 1965b: 28, 44, pl. 3, fig. 4 (with type species as *Crenella columbiana* Dall, 1897; 1965a listed as a "*cf.*" reference).

Megacrenella Habe et Ito. Habe, 1977: 74 (cited as of 1965a, with type species, by original designation, *Crenella columbiana* Dall, 1897)

Taxonomic note: We consider the type indication as by monotypy. The two other nominal taxa mentioned in the Japanese text are clearly stated to be synonyms of *Crenella columbiana.* Habe & Ito (1965a: 100, in Japanese) refer to something that translates to "type species group," which we cannot accept as original designation. The authors discuss the position of the group, based on morphological characters, as standing between *Solamen* and *Crenella* (the latter name mentioned only in Japanese characters) and also indicate its relationship to *Arvella.* This appears to fulfill the ICZN *Code* requirements, and we date this taxon as of 1965a.

(30) *Adula californiensis chosenica* Habe & Ito, 1965a

Adula californiensis chosenica (Kuroda MS.) Habe et Ito (nov.). Habe & Ito, 1965a: 114, pl. 37, fig. 4; 1970, 1972, 1974, 1977: ibid.

Adula californiensis chosenica subsp. nov. Habe & Ito, 1965b: 28, 43, pl. 3, fig. 1.

Adula californiensis chosenica Habe et Ito. Habe, 1977: 31 (cited as of 1965a and stated to be a synonym of *A. schmidti* (Schrenck, 1867)).

Carditidae

(31) *Megacardita ferruginosa koreana* Habe & Ito, 1965a

Megacardita ferruginosa koreana Habe et Ito (nov.). Habe & Ito, 1965a: 128, pl. 43, fig. 8; 1970, 1972, 1974, 1977: ibid.

Megacardita ferruginea [sic] *koreana* subsp. nov. Habe & Ito, 1965b: 28, 45 (plate caption), pl. 3, fig. 8.

Megacardita ferruginea [sic] *koreanica* [sic] subsp. nov. Habe & Ito, 1965b: 44.

Megacardita ferruginosa koreana Habe et Ito. Habe, 1977: 65 (cited as of 1965a).

"Shells of the World in Colour, Vol. II. The Tropical Pacific." Tadashige Habe and Sadao Kosuge

First published January 15, 1966 (1966a), this work preceded an article in *Venus* by the same authors (1966b) in which new taxa, first appearing in Volume II, are proposed. There is no indication in Volume II that these taxa are newly introduced therein. The authors stated (1966b) that these "genera and species were figured and briefly described" in 1966a and that "they are redescribed in detail herewith in the nomenclatural value." Dr. Kosuge (personal comm., March 15, 1995) has confirmed that the genera all must date from the *Venus* article.

Dr. Kosuge also advises that there are at least ten printings of this work, the 10th appearing in March, 1991.

Printings that we have seen:

Printing		
	1	January 15, 1966 (1966a)
	2	November 1, 1966 (1966c)
	3	February 1, 1972
	5	November 11, 1974
	6	September 1, 1976

The following new species appear (using the original arrangement of families). Important changes between printings and references from other sources are also listed.

Gastropoda

Acmaeidae

(32) *Patelloida (Collisellina) saccharinoides* Habe & Kosuge, 1966a

Patelloida (Collisellina) saccharinoides Habe et Kosuge. Habe & Kosuge, 1966a: 6, pl. 2, fig. 10; 1966c, 1972, 1974, 1976: ibid.

Patelloida (Collisellina) saccharinoides Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 312.

Patelloida (Collisellina) saccharioides [sic] Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 326, pl. 29, fig. 6 (this spelling also on plate caption on same page).

Patelloida (Collisellina) saccharinoides Habe et Kosuge. Habe, 1977: 103 (cited as of 1966a).

Turbinidae

(33) *Astralium yamamurae* Habe & Kosuge, 1966a

Astralium yamamurai [sic] Habe et Kosuge. Habe & Kosuge, 1966a: 11; 1966c: ibid. (error in spelling corrected on page 121 and in all later usages)

Astralium (Distellifer [sic]*) yamamurae* Habe et Kosuge. Habe & Kosuge, 1966a: 121, pl. 45, fig. 11; 1966c, 1972, 1974, 1976: ibid.

Astralium yamamurae Habe et Kosuge. Habe & Kosuge, 1972: 11; 1974, 1976: ibid.

Astralium (Destellifer) yamamurae Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 313, 327 (with reference to 1966a, pl. 45, fig. 4 [sic; error for fig. 11]).

Astralium (Destellifer) yamamurae Habe et Kosuge. Habe, 1977: 133 (cited as of 1966a; 1966b cited as "name only").

Littorinidae

(34) *Granulilittorina* Habe & Kosuge, 1966b

(35) *Granulilittorina philippiana* Habe & Kosuge, 1966a

Granulilittorina philippiana Habe et Kosuge. Habe & Kosuge, 1966a: 20, pl. 6, fig. 13; 1966c; ibid. (genus-group name = *nomen nudum*).

Granulilittorina philippiana Habe et Kosuge (gen. et sp. nov.). Habe & Kosuge, 1966b: 313, 328 (with reference to 1966a, pl. 6, fig. 13).

Granulilittorina millegrana (Philippi) Habe et Kosuge. Habe & Kosuge, 1972, 1974, 1976: 20, pl. 6, fig. 13.

Granulilittorina Habe et Kosuge. Habe, 1977: 45. (cited as of 1966b, with type, by monotypy, *G. philippiana* Habe & Kosuge; 1966a not mentioned).

Granulilittorina philippiana Habe et Kosuge. Habe, 1977: 96 (cited as of 1966a; stated to be a synonym of *G. millegrana* (Philippi, 1848)).

Taxonomic note: In 1966a no indication was given that this was a newly introduced genus-group name. Rosewater (1970: 491–493) used *Granulilittorina* as a valid subgenus of *Nodilittorina*. In treating the genus-group name he cited both 1966a and 1966b. However, under the species name (in the synonymy of *N. (G.) millegrana*) he listed as of 1966b with "[figured in] Habe and Kosuge" 1966a (square brackets in quote are of Rosewater).

Cerithiidae

(36) *Clypeomorus batillariaeformis* Habe & Kosuge, 1966a

Clypeomrus [sic] *batillariaeformis* Habe et Kosuge. Habe & Kosuge, 1966a: 23, pl. 7, fig. 14; 1966c: ibid.

Clypeomorus batillariaeformis Habe et Kosuge (sp. nov.) Habe & Kosuge, 1966b: 314, 328, pl. 29, fig. 13 (with reference to 1966a, pl. 7, fig. 14).

Clypeomorus batillariaeformis Habe et Kosuge. Habe & Kosuge, 1972, 1974, 1976: 23, pl. 7, fig. 14.

Clypeomorus batillariaeformis Habe et Kosuge. Habe, 1977: 26 (cited as of 1966a; original misspelling of genus shown and corrected).

Taxonomic note: Houbrick (1985: 51) treated this species in detail and attributed it to Habe & Kosuge, 1966b, without any mention of 1966a.

Cypraeidae

(37) *Ficadusta* Habe & Kosuge, 1966b

Ficadusta pulchella (Swainson, 1823). Habe & Kosuge, 1966a: 40, pl. 14, figs. 15, 16; 1966c: ibid. (genus-group name = *nomen nudum*).

Ficadusta Habe et Kosuge (gen. nov.). Habe & Kosuge, 1966b: 314, 329 (with refer-

ence to 1966a; type species: *Cypraea pulchella* Swainson).

Ficadusta pulchella (Swainson). Habe & Kosuge, 1966b: 326 (plate expl.), pl. 29, figs. 11, 12.

Ficadusta pulchella (Swainson, 1923 [sic]). Habe & Kosuge, 1972: 40, pl. 14, figs. 15, 16; 1974, 1976: ibid. (type reset in 1972 to correct English common name).

Ficadusta Habe et Kosuge, 1966. Habe, 1977: 40 (cited as of 1966b, but with type by "monotypy," whereas in 1966b it was designated; 1966a listed as "name only").

Cymatiidae

(38) *Reticutriton* Habe & Kosuge, 1966b

Reticutriton pfeifferianum (Reeve, 1844). Habe & Kosuge, 1966a: 43, pl. 15, fig. 14; 1966c: ibid. (genus-group name = *nomen nudum*).

Reticutriton Habe et Kosuge (gen. nov.). Habe & Kosuge, 1966b: 315, 330 (with reference to 1966a; type species: *Triton pfeifferianus* Reeve).

Reticutriton pfeifferianus (Reeve, 1844). Habe & Kosuge, 1972: 43, pl. 15, fig. 14; 1974, 1976: ibid.

Reticutriton Habe et Kosuge. Habe, 1977: 102 (cited as of 1966b; 1966a not mentioned).

Muricidae

(39) *Spinidrupa* Habe & Kosuge, 1966b

Spinidrupa eurantha [sic] (A. Adams). Habe & Kosuge, 1966a: 54, pl. 20, fig. 4; 1966c, 1972, 1974, 1976: ibid. (genus-group name = *nomen nudum*).

Spinidrupa Habe et Kosuge (gen. nov.). Habe & Kosuge, 1966b: 315, 330 (with reference to 1966a; type species: *Murex eurantha* [sic] A. Adams [p. 315; as *euracantha* on p. 330; = *Murex euracanthus* A. Adams, 1851].

Spinidrupa Habe et Kosuge. Habe, 1977: 115 (cited as of 1966b; 1966a listed as "name only").

Pyrenidae (Columbellidae)

(40) *Pyrene testudinaria nigropardalis* Habe & Kosuge, 1966a

Pyrene testudinalia [sic] *nigropardalis* Habe et

Kosuge. Habe & Kosuge, 1966a: 57, pl. 21, fig. 3; 1966c: ibid.

Pyrene testudinaria nigropardalis Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 316, 331, pl. 29, fig. 7 (with reference to 1966a).

Pyrene testudinaria nigropardalis Habe et Kosuge. Habe & Kosuge, 1972, 1974, 1976: 57, pl. 21, fig. 3.

Pyrene testudinalia [sic] *nigropardalis* Habe et Kosuge. Habe, 1977: 83 (cited as of 1966a).

(41) *Pyrene lacteoides* Habe & Kosuge, 1966a

Pyrene lacteoides Habe et Kosuge. Habe & Kosuge, 1966a: 57, pl. 21, fig. 8; 1966c, 1972, 1974, 1976: ibid.

Pyrene lacteoides Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 316, 330, pl. 29, fig. 8 (with reference to 1966a).

Pyrene lacteoides Habe et Kosuge. Habe, 1977: 68 (cited as of 1966a).

Nassariidae

(42) *Plicarcularia gibbosuloidea* Habe & Kosuge, 1966a

Pliarcularia [sic] *gibbosuloidea* Habe et Kosuge. Habe & Kosuge, 1966a: 60, pl. 22, figs. 5, 6; 1966c, 1972, 1974, 1976: ibid.

Pliarcularia [sic] *gibbosuloidea* Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 317.

Plicarcularia gibbosuloidea Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 326 [pl. explanation], 331, pl. 29, figs. 2, 3 (with reference to 1966a).

Plicarcularia gibbosuloidea Habe & Kosuge. Habe, 1977: 44 (cited as of 1966a; original misspelling of genus shown and corrected).

Galeoidae (Galeolidae in 1966a: 64 and 1966c: 64; correct on p. 65 and in later printings)

(43) *Hemifusus carinifer* Habe & Kosuge, 1966a

Hemifusus carinifera [sic] Habe et Kosuge. Habe & Kosuge, 1966a: 64, pl. 23, fig. 2; 1966c, 1972, 1974, 1976: ibid.

Hemifusus cariniferus Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 317, 332, pl. 29, fig. 17 (with reference to 1966a).

Hemifusus cariniferus Habe et Kosuge. Habe, 1977: 29 (cited as of 1966a).

Note: Originally introduced as an adjective in the female form, the ending has to be adjusted to the masculine *-fer* (*-fer, -fera, -ferum,* meaning "bearing"; as opposed to *-ferus-a-um,* meaning "wild").

Fasciolariidae

(44) *Latirus stenomphalus* Habe & Kosuge, 1966a

Latirus stenomphalus Habe et Kosuge. Habe & Kosuge, 1966a: 68, 122, pl. 45, fig. 16 (with reference to Kira, [1954]: pl. 30, fig. 16, which is the species Kira figured as *Latirus recurvirostrum* Schubert & Wagner); 1966a, 1972, 1974, 1976: ibid.

Latirus stenomphalus Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 318, 334, (with reference to *Latirus recurvirostrum* Kira, 1954: pl. 30, fig. 16 [on p. 318] and to 1966a [p. 334]; misspelled *sttnomphalus* on p. 318). This reference to Kira is to the species he figured as *Latirus recurvirostrum* Schubert & Wagner.

Latirus stenomphalus Habe et Kosuge. Habe, 1977: 116 (cited as of 1966a).

Mitridae

(45) *Vexillum rubrocostatum* Habe & Kosuge, 1966a

Vexillum rubrocostatum Habe et Kosuge. Habe & Kosuge, 1966a: 73, pl. 28, fig. 9; 1966c, 1972, 1974, 1976: ibid.

Vexillum rubrocostatum Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 319, 333, pl. 29, fig. 4 (with reference to 1966a).

Vexillum rubrocostatum Habe et Kosuge. Habe, 1977: 102 (cited as of 1966a).

(46) *Nebularia yaekoae* Habe & Kosuge, 1966a

Nebularia yaekoae Habe et Kosuge. Habe & Kosuge, 1966a: 76, pl. 28, fig. 34; 1966c, 1972, 1974, 1976: ibid.

Nebularia yaekoae Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 319, 333, pl. 29, fig. 10 (with reference to 1966a).

Nebularia yaekoae Habe et Kosuge. Habe, 1977: 131 (cited as of 1966a).

Harpidae

(47) *Harpa kawamurai* Habe, in Habe & Kosuge, 1972

Harpa striata (Lamarck, 1816). Habe & Ko-

suge, 1966a: 79, pl. 30, fig. 2; supple-
mental pl. 1, fig. 2; 1966c: ibid.

Harpa kawamurai Habe. Habe, in Habe & Ko-
suge, 1972: 79, pl. 30, fig. 2; supplemen-
tal pl. 1, fig. 2; 1974, 1976 (no indication
that name is new).

Harpa kawamurai Habe & Kosuge, 1973 [sic].
Habe, 1975b: 10 (listed as "invalid" and
as "= *Harpa major* Röding, 1798").

Harpa kawamurai Habe & Kosuge, 1973 [sic].
Matsukuma & Okutani, 1986: 6.

Taxonomic note: The 3rd printing of Habe &
Kosuge, where this species first ap-
pears, is rare, and we have located only
one copy. Not listed by Habe (1977). The
Japanese text of Habe (1975b: 10)
states that, according to personal com-
munication with Dr. Rehder, this nominal
species is a form of *Harpa major* Röding,
1798.

(48) *Harpa kajiyamai* Habe, in Habe & Ko-
suge, 1972

Harpa cancellata (Röding, 1798). Habe &
Kuroda, 1966a: 79, pl. 30, fig. 3; supple-
mental pl. 1, fig. 3; 1966c: ibid.

Harpa kajiyamai Habe. Habe & Kosuge, 1972:
79, pl. 30, fig. 3; supplemental pl. 1, fig.
3.

Harpa kajiyamai Rehder, 1973: 244, pl. 188,
figs. 3, 4 (described from specimens re-
ceived from Habe, who was stated to
have recognized the species as new and
given it a provisional name, and re-
quested that it be named for the collec-
tor).

Harpa kajiyamai Rehder. Habe & Kosuge,
1974: 79, pl. 30, fig. 3; supplemental pl.
1, fig. 3; 1976: ibid.

Taxonomic note: Walls (1980: 191) in his list
of *Harpa* species includes both *H. kajiy-
amai* Habe, 1970 [sic], and *H. kajiyamai*
Rehder, 1973, indicating that both are "=
[*Harpa*] *harpa*," a synonymy we do not
endorse. This species name must be at-
tributed to Habe (1972). Not listed by
Habe (1977).

Volutidae

(49) *Volutoconus grossi mcmichaeli* Habe &
Kosuge, 1966a

Volutoconus grossi mcmichaeli Habe & Ko-
suge. Habe & Kosuge, 1966a: 86, pl. 33,
fig. 1; 1966c, 1972, 1974, 1976: ibid.

Volutoconus grossi mcmichaeli Habe et Ko-

suge (sp. nov.). Habe & Kosuge, 1966b:
320, 335, pl. 29, fig. 19 (with reference to
1966a).

Volutoconus grossi mcmichaeli Habe et Ko-
suge. Habe, 1977: 74 (cited as of 1966a).

Turridae

(50) *Brachytoma kurodai* Habe & Kosuge,
1966a

Brachytoma kurodai Habe et Kosuge. Habe &
Kosuge, 1966a: 96, pl. 38, fig. 13; 1966c,
1972, 1974, 1976: ibid.

Brachytoma kurodai Habe et Kosuge (sp.
nov.). Habe & Kosuge, 1966b: 320, 335,
pl. 29, fig. 14 (with reference to 1966a)

Brachytoma kurodai Habe et Kosuge. Habe,
1977: 66 (cited as of 1966a).

(51) *Brachytoma kawamurai* Habe & Kosuge,
1966a

Brachytoma kawamurai Habe et Kosuge.
Habe & Kosuge, 1966a: 96, pl. 38, fig.
14; 1966c, 1972, 1974, 1976: ibid.

Brachytoma kawamurai Habe et Kosuge (sp.
nov.). Habe & Kosuge, 1966b: 321, 336,
pl. 29, fig. 9 (with reference to 1966a)

Brachytoma kawamurai Habe et Kosuge.
Habe, 1977: 58 (cited as of 1966a).

(52) *Brachytoma vexillium* Habe & Kosuge,
1966a

Brachytoma vexillium Habe et Kosuge. Habe
& Kosuge, 1966a: 96, pl. 38, fig. 15;
1966c, 1972, 1974, 1976: ibid.

Brachytoma vexillum Habe et Kosuge (sp.
nov.). Habe & Kosuge, 1966b: 321, 336,
pl. 29, fig. 5 (with reference to 1966a).

Brachytoma vexillum Habe et Kosuge. Habe,
1977: 130 (cited as of 1966a; original
spelling not mentioned).

Taxonomic note: The original spelling of the
specific name, although obviously a mis-
spelling or typographical error, must be
retained in accordance with ICZN *Code*
Article 32.

Epitoniidae

(53) *Eglisia brunnea* Habe & Kosuge, 1966a

Eglisia brunnea Habe et Kosuge. Habe & Ko-
suge, 1966a: 103, pl. 40, fig. 16; 1966c:
ibid.

Eglisia brunnea Habe et Kosuge (sp. nov.).
Habe & Kosuge, 1966b: 322, 337, pl. 29,
fig. 18 (with reference to 1966a).

Eglisia lanceolata brunnea Habe et Kosuge. Habe & Kosuge, 1972: 103, pl. 40, fig. 16; 1974, 1976: ibid.

Eglisia brunnea Habe et Kosuge. Habe, 1977: 28 (cited as of 1966a).

Bivalvia

Limidae

(54) *Mantellum perfragile* Habe & Kosuge, 1966a

Mantellum perfragile Habe et Kosuge. Habe & Kosuge, 1966a: 144, 177, pl. 68, fig. 6; 1966c, 1972, 1974, 1976: 144.

Mantellum perfragile Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 323, 338. (not figured; 1966a not referred to)

Limaria perfragile Habe et Kosuge. Habe & Kosuge, 1972, 1974, 1976: 177, pl. 68, fig. 6 (as *Mantellum* on p. 144).

Mantellum perfragile Habe et Kosuge. Habe, 1977: 95 (cited as of 1966a and placed in *Limaria* (*Platilimaria*)).

Ostreidae

(55) *Anomiostrea* Habe & Kosuge, 1966b

Anomiostrea pyxidata (Adams et Reeve, 1850). Habe & Kosuge, 1966a: 144, pl. 55, fig. 9; 1966c, 1972, 1974, 1976: ibid. (genus-group name = *nomen nudum*).

Anomiostrea Habe et Kosuge (gen. nov.). 1966b: 323, 338, with type designated as *Ostrea pyxidata* Adams et Reeve (with reference to 1966a).

Anomiostrea Habe et Kosuge. Habe, 1977: 23 (cited as of 1966b; 1966a not mentioned).

Taxonomic note: Listed under "*nomina dubia*" by Stenzel (1971: N1167, figs. J140a-c), who also showed the name of the type species to be preoccupied. Type species renamed *Anomiostrea coralliophila* Habe, 1975a (new name for *O. pyxidata* Adams & Reeve, 1848 [sic; = 1850] non Born, 1780 [sic; = 1778]).

Cardiidae

(56) *Laevicardium rubropictum* Habe & Kosuge, 1966a

Laevicardium rubropictum Habe et Kosuge. 1966a: 153, pl. 59, fig. 2; 1966c, 1972, 1974, 1976: ibid.

Laevicardium rubropictum Habe et Kosuge (sp. nov.). 1966b: 324, 339, pl. 29, fig. 20 (with reference to 1966a)

Laevicardium rubropictum Habe et Kosuge. Habe, 1977: 102 (cited as of 1966a).

(57) *Vasticardium nigropunctatum* Habe & Kosuge, 1966a

Vasticardium nigropunctatum Habe et Kosuge. Habe & Kosuge, 1966a: 154, pl. 59, fig. 9; 1966c, 1972, 1974, 1976: ibid.

Vasticardium nigropunctatum Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 324, 340, pl. 29, fig. 16 (with reference to 1966a).

Vasticardium nigropunctatum Habe et Kosuge. Habe, 1977: 84 (cited as of 1966a).

Mactridae

(58) *Macrotoma yamamurae* Habe & Kosuge, 1966a

Mictrotoma [sic] *yamamurae* Habe et Kosuge. Habe & Kosuge, 1966a: 166, pl. 65, fig. 8; 1966c: ibid.

Mactrotoma yamamurae Habe et Kosuge (sp. nov.). Habe & Kosuge, 1966b: 325, 340, pl. 29, fig. 15 (with reference to 1966a; original misspelling of genus noted).

Mactrotoma yamamurae Habe et Kosuge. Habe & Kosuge, 1972: 166, pl. 65, fig. 8.

Heterocardia gibbosula Philippi [sic; = Deshayes]. Habe & Kosuge, 1974: 166, pl. 65, fig. 8; 1976: ibid.

Mactrotoma yamamurae Habe et Kosuge. Habe, 1977: 133 (cited as of 1966a; stated to be a synonym of *Heterocardia gibbosula* Deshayes, 1855).

descriptions, and to Dr. M. G. Harasewych who, while in Japan, searched for and obtained for us a copy of the elusive 3rd printing of Habe & Kosuge. We also wish to thank two anonymous reviewers for their comments.

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ULTRASTRUCTURAL STUDY OF EUSPERMIOGENESIS IN *CLYPEOMORUS BIFASCIATA* AND *CLYPEOMORUS TUBERCULATUS* (PROSOBRANCHIA: CERITHIIDAE) WITH EMPHASIS ON ACROSOME FORMATION

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ABSTRACT

The ultrastructure of euspermiogenesis and euspermatozoa of *Clypeomorus bifasciata* and *C. tuberculatus* are almost identical. Early spermatids have oval to spherical nuclei, sparse endoplasmic reticulum, few mitochondria, and a well-developed Golgi complex with many vesicles in its vicinity. Acrosome differentiation occurs anywhere within the cytoplasm, and begins with a proacrosomal vesicle, which becomes cup-shaped and plugged at its edges with a dense interstitial granule. Microtubules are embedded in the matrix between the outer and inner acrosomal membranes. The acrosomal vesicle becomes aligned parallel to the antero-posterior nuclear axis, and changes into an inverted flask shape, with two external supporting structures at its basal margins. The interstitial granule becomes hat-shaped, separating the acrosome from the nucleus. The mature acrosome consists of a flat cone with microtubules in its core, an acrosomal rod-like material, and a basal plate. Nuclear shape changes from spherical to hammer-head to club-shape, with a posterior invagination enclosing the initial axonemal portion. The fine chromatin material of early spermatids changes to fibrillar, lamellar, and finally very compact material. The euspermatozoan midpiece originates from fusion of spermatid mitochondria into four large spheres, which are later organized into four non-helical mitochondrial elements, two of which are large and the other two are extremely small. A dense ring structure marks the junction between the midpiece and the glycogen piece. The latter consists of nine tracts of glycogen granules surrounding nine axonemal doublets. The results of this study suggest that acrosomal ultrastructure could be used to establish phylogenetic relationships in Cerithiacea at the generic level.

INTRODUCTION

Morphological diversity of spermatozoa in prosobranchs, as in other animal groups, has been considered as a tool that can be used to ascertain evolutionary paths, through building up phylogenetic and taxonomic affinities among species (Franzen, 1955, 1956, 1970; Nishiwaki, 1964; Healy, 1983a, 1988a; Koike, 1985). Based on ultrastructural studies of spermiogenesis and sperm morphology, mesogastropods as a part of caenogastropods (mesogastropods and neogastropods) are classified into two groups. Members of the first group have short nuclei with shallow basal invaginations, associated with conical or flattened acrosomes. The midpiece may show modification of cristae into parallel cristal plates, and it is separated from the glycogen piece by a dense ring structure. The glycogen piece consists of axonemal micro-tubules and nine tracts of glycogen granules, whereas the short end piece is composed of an axoneme surrounded only by a plasma membrane. This group of caenogastropods includes superfamilies Cerithiacea (Healy, 1982a, b, 1983a; Afzelius & Dallai, 1983; Koike, 1985), Viviparacea (Griffond, 1980; Koike, 1985), and Cyclophoracea (Selmi & Giusti, 1980; Healy, 1984; Kohnert & Storch, 1984a, b, Koike, 1985). All other superfamilies in Caenogastropoda are classified into the second group, which shares with the first group similar glycogen pieces, dense ring structures and end pieces. On the other hand, members of this group have apical acrosomal vesicles and accessory acrosomal membranes, whereas their short or long tubular nuclei may be completely invaginated by the axoneme (Healy, 1988a). The midpiece elements are helically coiled, with usually unmodified cristae (Healy, 1983a, 1986b; Max-

well, 1983; Kohnert & Storch, 1984a; Koike, 1985; Jaramillo et al., 1986). Furthermore, comparative sperm ultrastructure has been useful in establishing the affinities of many cerithiacean superfamilies of the Caenogastropoda (Healy, 1982a, b, 1983a, 1986a, b, 1988a, b, 1990a, b, 1993; Houbrick, 1988).

The present work deals with the ultrastructure of euspermiogenesis and mature eusperm (typical sperm) in two species of the superfamily Cerithiacea (family Cerithiidae) that inhabit the rocky shore of the Gulf of Aqaba (Houbrick, 1985; Hulings, 1986). These are: *Clypeomorus bifasciata* (Sowerby, 1855) [= *C. moniliferum* (Kiener, 1841), *auett.*], and *C. tuberculatus* (Linnaeus, 1758) [= *C. petrosa gennesi* (Fisher & Vignal, 1901)]. Comparative study of spermiogenesis and sperm morphology of the two cerithiid species as well as other reported cerithiids aims to emphasize species-specific characters between cerithiids from different geographical regions, and to establish the phylogenetic status of cerithiaceans among prosobranchs.

MATERIALS AND METHODS

Specimens were collected monthly for a year in the intertidal zone opposite to the Marine Science Station of the Gulf of Aqaba. The shell was gently broken, and the testis, removed by dissection, was immediately immersed in 2.5% glutaraldehyde in filtered sea water for 2 hours at room temperature. The tissue was rinsed thoroughly in filtered sea water, post fixed in 1% OsO_4 solution in filtered sea water, dehydrated in acetone and embedded in Spurr's (1969) medium. Blocks were cut with Sorval MT 2B ultramicrotome using glass knives, and ultrathin sections (50–60 nm) were stained with uranyl acetate and lead citrate. Electron microscopic examinations were done with a Zeiss EM 10B transmission electron microscope operated at 60 KV.

RESULTS

The various stages of euspermiogenesis in *Clypeomorus bifasciata* and *C. tuberculatus* are almost identical. Therefore, the following description applies for both species unless otherwise mentioned.

Early spermatids are spherical to ovoidal, with eccentric nuclei. The chromatin material is granular, with some local aggregations of no specific pattern. The granular cytoplasm contains few cisternae of endoplasmic reticulum, few mitochondria, and a well-developed Golgi complex with many vesicles at the extremities of its cisternae, indicating activity (Fig. 1). Nutritive cells can be seen in the intercellular space with many elongated pseudopodia (Fig. 1).

Acrosome development can be divided into two major phases; the pre-attachment acrosome and the post-attachment one, in reference to its attachment to the nucleus. Acrosomal genesis during the first phase begins with a single proacrosomal vesicle associated with Golgi complex, in addition to many nearby dense vesicles that are likely to be utilized in the production of the acrosomal elements (Fig. 2). Later, this vesicle attains an inverted U-shape due to posterior indentation, and a dense interstitial granule plugs the prospective subacrosomal space (Figs. 3, 4). The dispersion of dense material from this granule and its deposition on the inner and outer acrosomal membranes assist in the accentuation of the acrosome (Figs. 4, 9). Two dense internal supporting structures appear within the acrosomal body, and microtubules constitute the skeleton of the acrosomal cone between the inner and outer acrosomal membranes (Figs. 4, 8, 9).

The second phase of acrosomal development is demarcated by the attachment of the basal interstitial granule to a depression on the anterior pole of the fibrous nucleus, opposite to the site of axoneme development (Fig. 10). The acrosome rotates 90° to become aligned parallel to the antero-posterior axis of the developing spermatid (Figs. 10, 11). Following its attachment, the acrosome looks like an inverted flask due to a constriction at its posterior part (Figs. 11, 12). Two crescent-shaped external supporting structures can be seen at the basal margins of the acrosome near its attachment point to the nucleus. The post-attachment acrosome is further elongated, while the dense interstitial granule gives rise to a basal plate between the acrosome and the nucleus (Figs. 11, 12).

The acrosome of the mature euspermatozoon in *C. bifasciata* and *C. tuberculatus* consists of three structures; acrosomal cone, acrosomal rod-like material, and basal plate (Figs. 16 inset, 17). The tapering cone may occasionally show parallel plate-like substructures, and it is characterized by basal
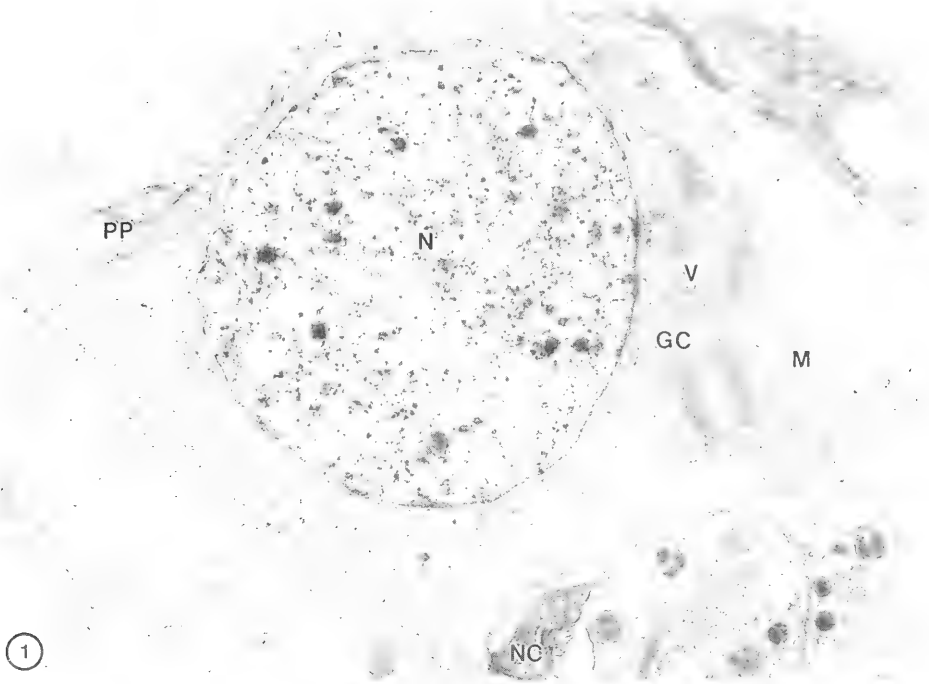
FIG. 1. *Clypeomorus bifasciata*. Early spermatid showing nucleus (N), mitochondrion (M), Golgi complex (GC) and associated vesicles (V). Notice pseudopodia (PP) of the nutritive cell (NC). ×11,500

FIG. 2. *C. bifasciata*. Early spermatid showing peripheral chromatin lining the nucleus (N), Golgi complex (GC), and interstitial granule (IG). ×31,250
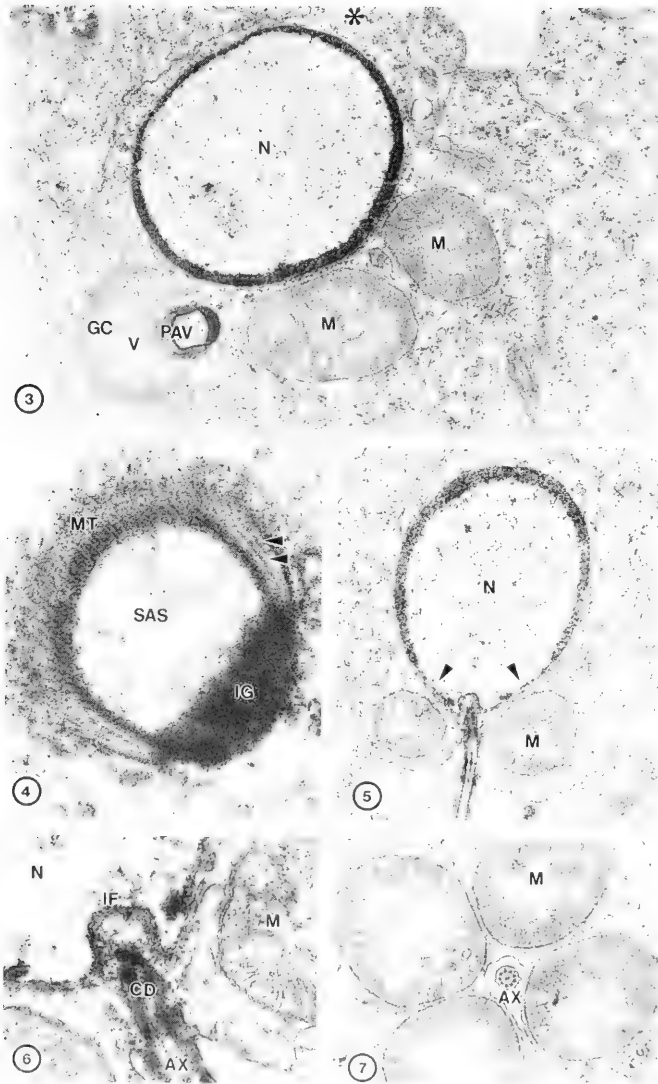
FIG. 3. *C. bifasciata.* Early spermatid showing nucleus (N), two mitochondrial (M) spheres at nuclear base, Golgi complex (GC), vesicles (V), and differentiating proacrosomal vesicle (PAV). Notice cytoplasmic bridge (asterisk). ×16,000

FIG. 4. *C. bifasciata* early spermatid. Section showing a differentiating acrosome with microtubules (MT) in its cone, internal supporting structures (arrows) subacrosomal space (SAS), and interstitial granule (IG). ×36,000

FIG. 5. *C. tuberculatus* early spermatid. Section showing nucleus (N), with sites of the attachment of mitochondrial (M) spheres (arrows). ×15,200

FIG. 6. *C. tuberculatus* early spermatid. Section showing nuclear (N) base with mitochondrial (M) spheres, implantation fossa (IF), centriolar derivative (CD), and axoneme (AX). ×56,700

FIG. 7. *C. bifasciata* early spermatid. Section showing four mitochondrial (M) spheres surrounding the axoneme (AX) as the first stage of midpiece development. ×15,000

bulges in the cone wall that cause a constriction in the subacrosomal space. The latter, which extends the whole length of the acrosomal cone, contains an acrosomal rod-like material (Figs. 16 inset, 17). A dense basal plate linking the acrosome and the nucleus can be seen as a straight dense layer between the two structures. Cross sections in the mature acrosome indicate its flatness, and microtubules assume a zipper-like structure in the matrix between the inner and outer acrosomal membranes (Fig. 18).

Chromatin condensation starts with the formation of a uniformly thick layer at the periphery of the nucleus (Figs. 2, 3). As spermiogenesis proceeds, the granular chromatin accumulates at the posterior nuclear pole, and the nucleus undergoes antero-posterior compression, leading to gradual increase in the nuclear width at the expense of its length (Fig. 8). In addition, the axoneme extends backwards from a centriolar derivative in the implantation fossa, so that vertical sections through a developing euspermatozoon at this stage present hammer-head and handle configurations (Fig. 10).

A second phase of chromatin condensation is evident in middle spermatids as the mid-anterior portion of the nucleus, opposite to the axoneme, begins a forward movement. During this phase, fibrils are arranged longitudinally parallel to the nuclear antero-posterior axis (Fig. 11). As fibrils increase in thickness, they start to fuse into fibers and subsequently into lamellae representing thereby the lamellar phase of chromatin condensation (Figs. 13-15). Chromatin condensation culminates in a homogenous, compact club-like nucleus with no distinct ultrastructure (Fig. 19).

Concomitant with nuclear condensation, a growing axoneme pushes the nucleus forward to increase its length, while the nuclear width is reduced under a force of lateral compression. This leads to progressive lengthening of the antero-posterior nuclear axis, thus reversing its trend in the previous stages (Figs. 10, 11). The mature nucleus in *C. bifasciata* and *C. tuberculatus* has a short posterior invagination accommodating the proximal portion of the axoneme (Figs. 16, 19).

The euspermatozoan tail in *C. bifasciata* and *C. tuberculatus* is composed of a middle piece, a glycogen piece and an end piece. The posterior nuclear envelope becomes indented at its center defining thereby the implantation fossa, which represents the point of axoneme development (Fig. 6). The genesis of the axoneme appears to be associated with a single dense structure (centriolar derivative), which does not seem to possess the common pattern of centriolar arrangement (Fig. 6). As nuclear condensation commences, mitochondrial fusion gives rise to four large spherical mitochondria at the posterior nuclear pole (Fig. 7). The association of these spheres with the nucleus is achieved by their attachment to four posterior nuclear depressions, and it represents the first step in midpiece development, which is concomitant with the granular phase of nuclear condensation. The mitochondrial cristae are modified into parallel cristal plates that have undergone considerable reorganization as mitochondria form a sheath around the typical 9 + 2 axoneme (Figs. 10, 11). Transverse sections through the midpiece reveal four non-helically arranged mitochondrial elements, two of which are semicircular large elements that are arranged perpendicular to the central pair of axonemal microtubules, and each reveals multiple cristal plates. The other two mitochondrial elements are extremely small and are aligned with this central pair, showing at most one cristal plate (Fig. 21). In addition, a ring of microtubules is observed surrounding the midpiece at late stages of its development (Fig. 21). Glycogen granules in the glycogen piece are organized in nine tracts; one per microtubular doublet (Fig. 24), and the transition zone between the midpiece and the glycogen piece is marked by a dense ring structure that is attached to the euspermatozoan plasma membrane (Fig. 22). The latter continues to encircle the axonemal microtubules, forming the end piece of the tail (Fig. 25), without a distinct transition structure between the glycogen piece and the end piece (Fig. 23). Cytoplasmic bridges connect adjacent developing spermatids throughout various stages of euspermiogenesis (Figs. 3, 11).

DISCUSSION

Euspermiogenesis as seen in *Clypeomorus bifasciata* and *C. tuberculatus* includes many common features that were reported in all other cerithiaceans (Giusti, 1971; Healy, 1982a, 1984; Koike, 1985; Afzelius et al., 1989, Hodgson & Heller, 1990; Minniti 1993) as well as other mesogastropods and neogastropods (Giusti, 1969; Buckland-Nicks &
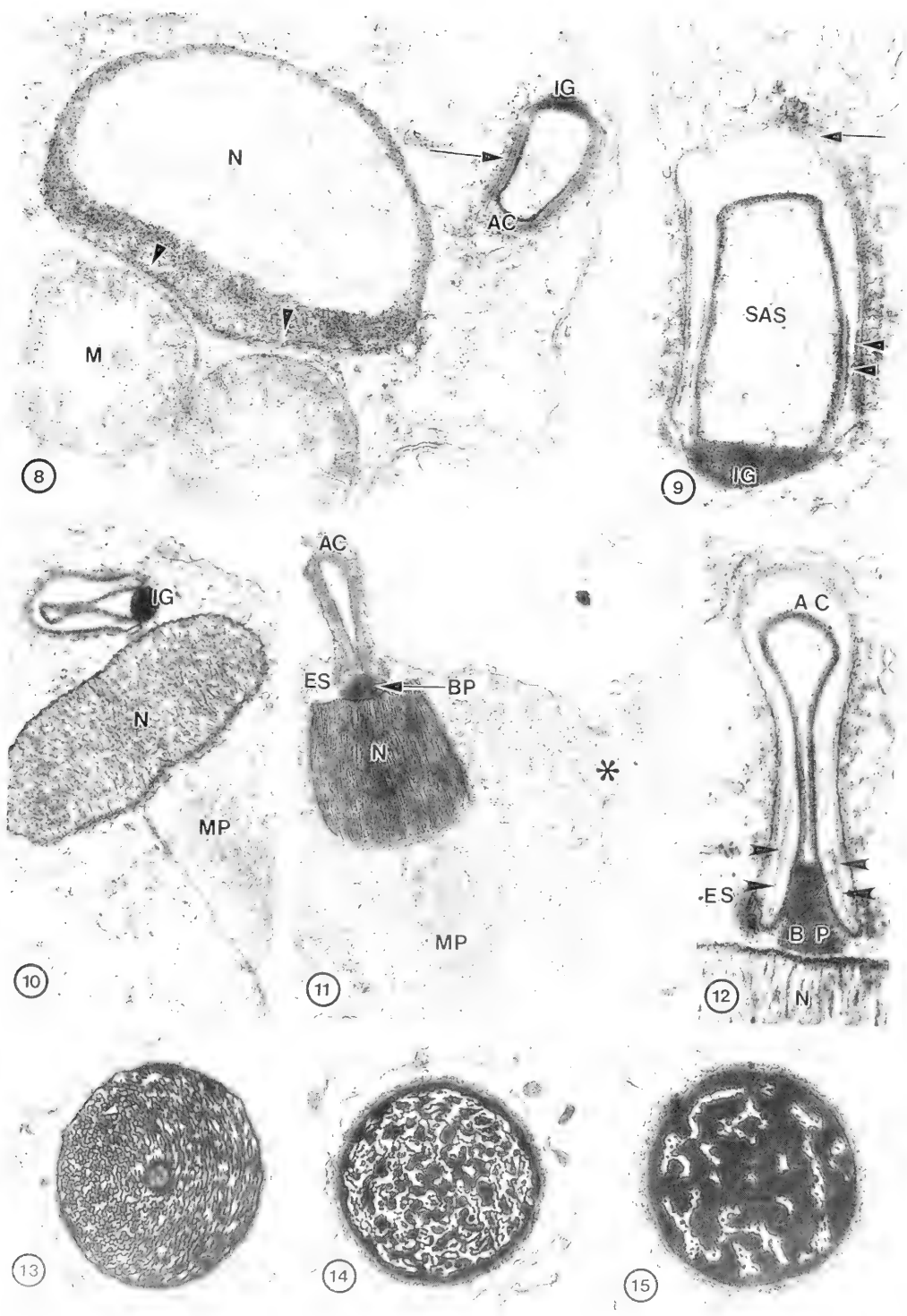
FIGS. 8–15.

Chia, 1976; West, 1978; Griffond, 1980; Kohnert, 1980; Healy 1983b; Koike 1985). In general, acrosome formation is associated with Golgi complex and involves the production of a proacrosomal vesicle, which occurs anywhere in the cytoplasm, because there is no definite route of acrosome migration from the posterior to the anterior pole of the developing spermatid. This situation was reported in other cerithiaceans (Healy, 1982a, 1986a; Minniti, 1993), in contrast to many other mesogastropods and neogastropods, in which such a route is marked and linked to various stages of nuclear shaping (Buckland-Nicks & Chia, 1976; West, 1978; Jong-Brink et al., 1977; Buckland-Nicks et al., 1983; Jaramillo et al., 1986, Gallardo & Garrido, 1989). Cerithiids, including those used in this study, are characterized by a high degree of development of the pre-attachment acrosome. Prior to its attachment to the nucleus, the acrosome bears an acrosomal cone, an acrosomal rod-like material and an interstitial granule, which gives rise to the basal plate; these constitute the elements of the mature acrosome.

Because spermiogenesis involves several complex processes of cellular shaping and remodeling, microtubules and microfilaments are expected to play a crucial rule in these processes. In contrast to other reported cerithiids, microtubules were seen within the developing acrosomal cones of *Clypeomorus bifasciata* and *C. tuberculatus*. This was strongly suggested by the longitudinal sections cutting through the developing acrosomes (Figs. 4, 9) as well as the hollow round structures seen in transverse sections (Fig. 18). Such an arrangement of microtubules within the cone is thought to provide it with a degree of rigidity, and aid in its elongation after it attaches to the nucleus, as was suggested in some non-cerithiacean mesogastropods and neogastropods (Walker & MacGregor, 1968; Buckland-Nicks & Chia, 1976; Giusti & Mazzini, 1973). Other cerithiids as well as other mesogastropods and neogastropods have a ring of microtubules surrounding developing acrosomes (Buckland-Nicks & Chia, 1976; Huaquin & Bustos-Obergon, 1981; Buckland-Nicks et al., 1983; Healy, 1983b). In addition, microtubules were seen around midpieces of *Clypeomorus bifasciata* and *C. tuberculatus* at late stages of development. Their appearance at such late stages in these two cerithiids as well as other mesogastropods (Jong-Brink et al., 1977; Kohnert, 1980; Griffond, 1980; Healy, 1982a, 1983a, b, 1988b; Buckland-Nicks et al., 1983; Afzelius et al., 1989; Al-Hajj & Attiga, 1995) strengthens the idea that they are important in sloughing the excess cytoplasm around midpieces as well as other parts of the euspermatozoon (Fawcett et al., 1971).

Furthermore, the ornamentation of the mature acrosomal cone with parallel plate-like substructures seen in *Clypeomorus bifasciata* and *C. tuberculatus* was also reported in

FIG. 8. *C. tuberculatus.* Early spermatid showing basal chromatin accumulation in nucleus (N), sites of mitochondrial (M) association with the nucleus (arrow heads), acrosomal cone (AC), interstitial granule (IG), and internal supporting structures (arrow) in acrosomal cone. ×27,200

FIG. 9. *C. tuberculatus* early spermatid. Section showing acrosomal cone with microtubules (arrow) and internal supporting structures (arrows), subacrosomal space (SAS), and interstitial granule (IG). ×50,000

FIG. 10. *C. bifasciata.* Middle spermatid showing interstitial granule (IG), hammer-like nucleus (N), and midpiece (MP). ×22,400

FIG. 11. *C. tuberculatus.* Middle spermatid with a cytoplasmic bridge (asterisk) showing acrosomal cone (AC), external supporting structure (ES), basal plate (BP), nucleus (N), and midpiece (MP). ×15,000

FIG. 12. *C. tuberculatus* middle spermatid. Section showing acrosomal cone (AC), internal supporting structure (arrow heads), external supporting structure (ES), basal plate (BP), and fibrillar nucleus (N). ×44,000

FIG. 13. *C. bifasciata* middle-late spermatid. Cross section in fibrillar nucleus. ×23,750

FIG. 14. *C. bifasciata* late spermatid. Cross section in nucleus showing islands of lamellae. ×35,000

FIG. 15. *C. tuberculatus* late spermatid. Cross section in nucleus with semi-fully condensed chromatin. ×31,500
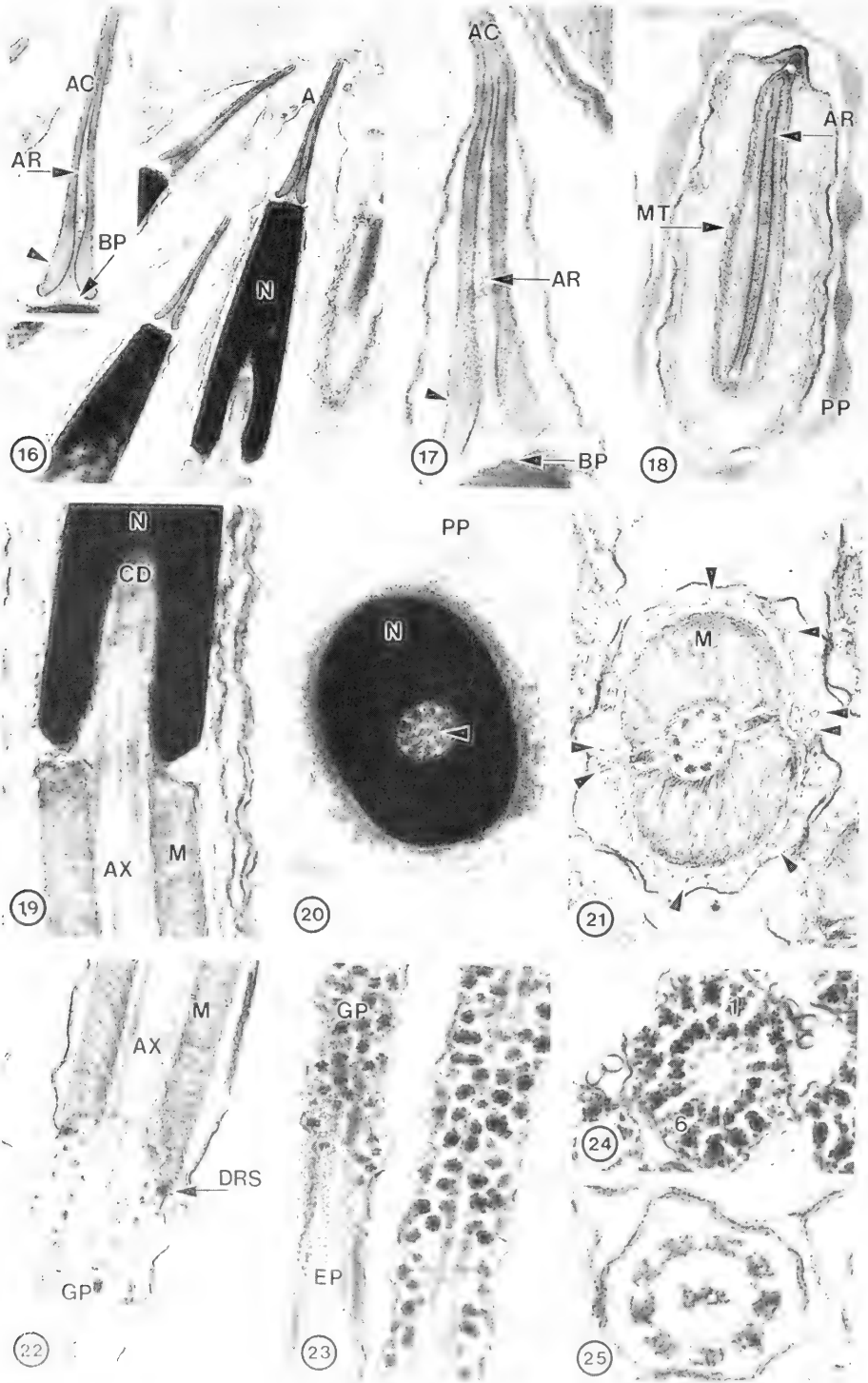
FIGS. 16–25.

other mesogastropods and neogastropods (Giusti & Mazzini, 1973; Healy, 1983a, 1986b; Jaramillo et al., 1986; Afzelius et al., 1989; Al-Hajj & Attiga, 1995). Acrosomal membranes in *Chorus giganteus* (Jaramillo et al., 1986), *Truncatella subcylindrica* (Giusti & Mazzini, 1973), and *Melanopsis* (Afzelius et al., 1989) were reported to have a scalloped appearance with regular periodicity. This ornamentation of the growing acrosome that is later hidden by electron-dense material seems to be of scarce occurrence. Giusti & Mazzini (1973) interpreted this periodicity as microtubular palisade, whereas Jaramillo et al. (1986) thought that it is due to the presence of actin crests, which may play a role in acrosome reaction and egg penetration. However, the lack of knowledge about acrosome reaction and fertilization in gastropods makes it difficult to conclude the nature or function of these structures, pending further investigation.

Chromatin condensation and nuclear shaping are two highly linked processes in spermiogenesis of mesogastropods and neogastropods. Chromatin condensation passes through granular, fibrillar and lamellar phases, culminating in a homogeneous compact nucleus with no ultrastructure (Walker & MacGregor, 1968; Buckland-Nicks & Chia, 1976; Feral, 1977; West, 1978; Huaquin & Bustos-Obergon, 1981; Healy, 1982a, b, 1983b, 1988b; Buckland-Nicks et al., 1983; Jaramillo et al., 1986; Gallardo & Garrido, 1989). The mature nucleus in the two cerithiids investigated in this study has a short basal invagination accommodating the proximal portion of the axoneme, which is a common nuclear shape seen in many other mesogastropods and neogastropods (Giusti, 1969, 1971; Giusti & Mazzini, 1973; Reader, 1973, Griffond, 1980; Kohnert, 1980; Koike & Nishiwaki, 1980; Healy, 1982a, b, 1983a, 1986b). On the other hand, some mesogastropods and many neogastropods have intranuclear canals that invaginate the nucleus completely up to its apex (Walker & MacGregor, 1968; Buckland-Nicks, 1973; Buckland-Nicks & Chia, 1976; West,

FIG. 16. *C. tuberculatus* mature euspermatozoon, with acrosome (A) and nucleus (N). ×48,000 Inset: Section showing acrosomal cone (AC) with plate-like substructure (arrow head), acrosomal rod-like material (AR), and basal plate (BP). ×57,500

FIG. 17. *C. bifasciata* semi-mature acrosome. Section showing acrosomal cone (AC) with plate-like substructure (arrow head), acrosomal rod-like material (AR), and basal plate (BP). ×63,000

FIG. 18. *C. bifasciata* semi-mature euspermatozoon. Section in acrosome showing acrosomal rod-like material (AR), microtubules (MT) in acrosomal cone, and surrounding pseudopodium (PP) of nutritive cell. ×40,000

FIG. 19. *C. tuberculatus* semi-mature euspermatozoa showing nucleus (N), centriolar derivative (CD) in nuclear basal invagination and midpiece with mitochondrial sheath (M) surrounding the axoneme (AX). ×60,000

FIG. 20. *C. tuberculatus* semi-mature euspermatozoon. Cross section in nucleus (N) showing the proximal portion of the axoneme (arrows) within nuclear basal invagination. Notice pseudopodia (PP) of nutritive cells. ×40,000

FIG. 21. *C. bifasciata* late euspermatozoon. Cross section in midpiece showing two large and two extremely small mitochondrial (M) elements surrounded by microtubules (arrows). ×48,000

FIG. 22. *C. tuberculatus* mature euspermatozoon. Section showing dense ring structure (DRS) at the junction between midpiece with mitochondrial sheath (M) around axoneme (AX), and glycogen piece (GP). ×38,000

FIG. 23. *C. bifasciata* mature euspermatozoa. Sections showing the junction between glycogen piece (GP) and end piece (EP). ×52,000

FIG. 24. *C. bifasciata* mature euspermatozoa. Cross section in glycogen piece showing nine tracts of glycogen granules associated with axonemal microtubular doublets. ×37,500

FIG. 25. *C. bifasciata* mature euspermatozoa. Cross section in end piece showing 9 + 2 pattern of microtubular arrangement surrounded by plasma membrane. ×128,000

1978; Huaquin & Bustos-Obergon, 1981; Buckland-Nicks et al, 1983; Healy, 1984; Jaramillo et al., 1986; Hodgson, 1993). Healy (1983a) interpreted this extreme structural diversity in the extent of nuclear invagination to factors in the reproductive environment, especially because some superfamilies include both types of nuclei among their species (Buckland-Nicks, 1973; Healy, 1988a).

Centrioles were not seen in developing euspermatids in *C. bifasciata* and *C. tuberculatus,* and the proximal portion of the axoneme was attached to the posterior nuclear invagination through a single dense structure that has no apparent microtubules. This centriolar derivative was reported in other caenogastropods (Buckland-Nicks, 1973; Healy, 1982a, 1983a, 1986a, b, 1988b).

The midpiece in euspermatozoa of *C. bifasciata* and *C. tuberculatus* is consistent with those of other cerithiids and members of subgroup 1(i) of Healy's (1983a) classification of mesogastropods as a group of caenogastropods. Midpieces in these animals are characterized by four non-helically arranged elements, two of which are extremely small, whereas the other two are large showing multiple cristal plates. The non-helical arrangement of mitochondria around the axoneme in Cerithiacea is considered a primitive character compared to the helical mitochondrial sheath seen in other mesogastropods and neogastropods (Walker & MacGregor, 1968; Giusti, 1969, 1971; Anderson & Personne, 1970; Buckland-Nicks, 1973; Giusti & Mazzini, 1973; West, 1978; Koike & Nishiwaki, 1980; Kohnert, 1980; Griffond, 1980). This feature supports Healy's (1982a) proposition that cerithiaceans represent ancestral mesogastropods, acting as a linkage group between primitive spermatozoa of Archaeogastropoda on one hand and modified spermatozoa of higher mesogastropods and neogastropods on the other. Such a position makes it an interesting group to study spermatozoan evolution.

In cerithiids, including those investigated in this study, the glycogen piece consists of nine tracts of glycogen granules associated with the nine doublets of the axonemal microtubules. This arrangement, which is seen also in other mesogastropods and neogastropods (Giusti, 1969, 1971; Buckland-Nicks, 1973; Reader, 1973; West, 1978; Koike & Nishiwaki, 1980; Kohnert, 1980; Huaquin & Bustos-Obergon, 1981; Healy, 1982a, 1986a, b, 1988a, c; Jaramillo et al., 1986; Gallardo &

Garrido, 1989; Al-Hajj & Attiga, 1995), is thought to be linked to the euspermatozoan motility, because the nine glycogen tracts in the mature euspermatozoa obscures nine radial links between the axonemal doublets and the plasma membrane in the immature spermatid (Healy, 1983a).

Many investigators have suggested that acrosomal ultrastructure of gastropods could provide useful taxonomic data by being species-specific. In Cerithiacea, such a species-specific acrosome ultrastructure is not well presented. Species-specific features were seen when comparing acrosomes of *Cerithium vulgatum* (Giusti, 1971), *C. nodulosum* (Koike, 1985), *C. rupestre* (Minniti, 1993), *C. caeruleum* (Al-Hajj & Attiga, 1995), whereas *Clypeomorus bifasciata, C. tuberculatus* (this study), *C. moniliferus* (*bifasciata*), and *C. breviculus* (Healy, 1983a) have almost identical acrosomes. In addition, differences in the ultrastructure of the acrosome were established at the generic level between *Cerithium, Rhinoclavis, Australaba* (Healy, 1983a), and *Clypeomorus* (Healy, 1983a; this study), but were not seen between euspermatozoa of *Conomurex* and *Lambis,* which possess very similar acrosomes (Koike & Nishiwaki, 1980).

In conclusion, comparative studies of sperm ultrastructure have proved to act as an acceptable guide for determining the affinities between major groups in gastropods as well as in many other animal groups.

ACKNOWLEDGMENTS

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CA REGULATION IN THE FRESHWATER BIVALVE *ANODONTA IMBECILIS:* I. EFFECT OF ENVIRONMENTAL CA CONCENTRATION AND BODY MASS ON UNIDIRECTIONAL AND NET CA FLUXES

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ABSTRACT

The present paper reports unidirectional and net Ca fluxes of a freshwater bivalve, *Anodonta imbecilis,* as a function of external Ca concentration and body mass. Larger animals were better able to maintain Ca balance than smaller animals, which experienced net loss of Ca. External Ca concentration had no significant effect on net Ca flux. Unidirectional Ca influx decreased with body mass and increased with external Ca concentration. The relationship between external Ca concentration and unidirectional Ca influx follows the Michaelis-Menten equation. The estimated half saturation Ca concentration for unidirectional Ca influx and the maximum unidirectional influx were 0.213 mM and 4.329 µmol g dry mass^{-1}h^{-1}, respectively. External Ca concentration did not affect unidirectional Ca efflux of the animals. Unidirectional Ca efflux decreased with body mass.

Key words: Calcium flux, calcium concentration, body mass, bivalve.

INTRODUCTION

While calcification has been relatively well studied in molluscs (reviewed by Watabe, 1983), the contribution made by whole body unidirectional Ca flux to Ca regulation in bivalves has not been well established.

In molluscs, various epithelia can take up external Ca (Van der Borght, 1963; Van der Borght & Van Puymbroeck, 1964; Greenaway, 1971; Thomas et al., 1974), especially the mantle surface facing the mantle cavity (Jodrey, 1953; Horiguchi, 1958) and the gill (Horiguchi, 1958). The active transport of Ca was demonstrated in the freshwater gastropods *Lymnaea stagnalis* (Van der Borght & Van Puymbroeck, 1964; Greenaway, 1971) and *Biomphalaria glabrata* (Thomas et al., 1974). Based on models for active Ca uptake into other freshwater species (for example teleost fish, Flik et al., 1985), the epithelial uptake occurs in two stages: diffusion down an electrochemical gradient across the apical membrane into the cytosol, and active transport across the basolateral membrane into the haemolymph. If, as in other freshwater species, the Ca pump in molluscan epithelia is Ca-activated ATPase (Watabe, 1983), the rate of Ca uptake should be correlated with the Ca concentration in the ambient water.

Previous studies showed that this is true for some molluscs (Greenaway, 1971; Thomas et al., 1974; Russell-Hunter, 1978; Pynnonen, 1991) but not others (Hunter, 1975; Russell-Hunter et al., 1981).

Allometry refers to the scaling of physiological function/morphological parameters to body mass. Interspecific allometry of captive aquatic molluscs described scaling of water flux to body mass (Nagy & Peterson, 1988). Significant relationships between dry body mass and shell length, shell height, gut-passage time, gut content or metabolic fecal loss have also been reported (Hawkins et al., 1990). The relationship between dry mass and oxygen consumption had a negative correlation (Dietz, 1974).

The present study uses radiotracer techniques to determine the unidirectional Ca fluxes in a freshwater bivalve, *Anodonta imbecilis,* as a function of external Ca concentration and body mass.

MATERIALS AND METHODS

Experimental Animals and General Holding Conditions

The freshwater bivalves *Anodonta imbecilis* (6–58 g) were collected 60 km from Gainesville, Florida, from a canal along the

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TABLE 1. Wet and dry mass of animals used to determine the effect of body mass on unidirectional and net Ca flux.

	group 1	group 2	group 3	group 4
wet mass (g)	11.15 ± 0.89	15.88 ± 0.56	25.98 ± 1.40	44.02 ± 2.03
dry mass (g)	0.362 ± 0.041	0.511 ± 0.064	1.061 ± 0.076	1.929 ± 0.171

Mean ± SEM. N (groups 2, 3, 4) = 10. N (group 1) = 9.

Suwannee River at Fanning Springs. The animals collected were kept in aquaria with aged and well-aerated 21°C Gainesville tap water with the following cationic composition (in mM): Na^+, 0.55; K^+, 0.04; Ca^{2+}, 0.60; Mg^{2+}, 0.42; and Cl^-, 0.73. The pH was 7.7. Food was withheld for the holding period (up to two months), and animals were used within two months of collection. Animals were acclimated in aquaria for at least 10 days before measurements were made. Only healthy animals (indicated by relatively heavy weight, active ventilation and powerful water ejection upon disturbance) were used in the experiments. All experiments were conducted at 21°C.

Unidirectional and Net Ca Fluxes—Effect of External Ca Concentration

Four groups of animals were used in the experiment. The Ca concentrations of the experimental media were 0.27, 0.60, 1.00 and 2.00 mM, respectively. The outer surface of the shell of the experimental animals was covered with wax to prevent direct Ca loss from the shell/water interface. Animals were acclimated in the experimental water for 3 days before conducting the experiment. Media with Ca concentration of 1.00 and 2.00 mM were made by adding $CaCl_2$ to Gainesville tap water (0.6 mM Ca). The medium with Ca concentration of 0.27 mM was made using the following recipe (in mM): NaCl, 0.4; $CaCl_2$, 0.27; $NaHCO_3$, 0.2; and KCl, 0.04. Animals were placed individually in experimental flux chambers containing 300 ml medium and acclimated for more than 12 hours. At the beginning of a flux measurement, the water was drained from the chamber and 200 ml fresh medium were added. An initial water sample was taken from each chamber and then 1 μCi of ^{45}Ca ($CaCl_2$ in water, 10 mCi ml^{-1}, Du Pont) was added to each chamber. Water samples were taken from each chamber at t = 0 h and t = 6 h for determination of radioactivity and Ca concentration. These samples were used to estimate net and uni-directional Ca fluxes. At the end of the experiment, animals were sacrificed by cutting the adductor muscles using a dissecting knife. Soft tissues of each animal were then dissected out and dried to constant weight to determine dry mass. In a parallel experiment, empty shells (the valves sealed together and covered with wax on the outer surface) were bathed in an identical chamber to estimate the possible accumulation of ^{45}Ca by the shell surface. Throughout the paper, dry mass means the dried mass of the soft tissues (excluding shells), wet mass refers to the whole wet mass of the animals (including shells).

Unidirectional and Net Ca Flux—Effect of Body Mass

Bivalves with wet mass of 6–58 g (N = 39) and dry mass of 0.2–2.7 g were used in the experiment. Animals were divided into four groups according to wet mass (Table 1). Groups were numbered 1 to 4 (small to large). For each group, the flux volume and isotope addition were as follows: group 1, 100 ml and 0.5 μCi ^{45}Ca; group 2 and 3, 150 ml and 0.8 μCi ^{45}Ca; group 4, 200 ml and 1 μCi ^{45}Ca. The experimental method was the same as described above. The Ca concentration of the media was 1 mM.

Analytical Methods

Water samples (3 ml) were mixed with ScintiVerse fluor (3 ml) and then radioactivity was measured using a liquid scintillation counter (LSC, Beckman LS5801). The Ca concentration of experimental water or extrapallial fluid (EPF) was measured after appropriate dilution (0.2 ml sample + 2 ml 2% $LaCl_3$ + 1.8 ml distilled water) using an atomic absorption spectrophotometer (Perkin Elmer 2100).

Calculation

The flux equation described by Wheatly (1989) was used to calculate unidirectional Ca influx:

$$Jin = \frac{(Ri - Rf)V}{SAtW} \quad (1)$$

where Jin is unidirectional Ca influx (μmol g dry mass^{-1}h^{-1}), Ri and Rf are the initial and final radioactivity (cpm ml^{-1}) of respective water samples, V is the flux volume (ml), SA is the medium mean specific radioactivity (cpm μmol^{-1}) calculated as the mean radioactivity divided by the mean Ca concentration, t is the elapsed time (h), and W is the dry mass of the animal (g).

Net flux was calculated as:

$$Jnet = \frac{(Ci - Cf)V}{tW} \quad (2)$$

where Jnet is Ca net flux (μmol g dry mass^{-1} h^{-1}), Ci and Cf are the initial and final medium Ca concentrations (mM), V is the flux volume (ml), t is the elapsed time (h), and W is the dry mass of the animal (g). A positive value for net flux indicates net Ca influx while a negative value indicates net Ca efflux.

Unidirectional efflux was calculated using the conservation equation:

$$Jout = Jin - Jnet \quad (3)$$

Statistical Analysis

Data were expressed as mean and standard error ($\pm$ SEM). The statistical analysis was performed using StatView 4.01 and Super ANOVA computer packages. Correlation analysis was performed by calculating correlation coefficients (r values) and using Fisher's r to z method to test the significance of correlation. One-factor ANOVA was used to analyze differences between groups and then Fisher's PLSD test was used when necessary to compare the means. ANCOVA was used to compare the slopes and intercepts of different linear relationships. The significance level for all statistical analyses was set at 0.05.

RESULTS

Empty shells showed no significant accumulation of ^{45}Ca on the waxed outer shell surface. Ca net flux was affected by the Ca concentration in the medium (Fig. 1). Animals in medium containing 0.27 mM Ca showed a significant net Ca efflux of -1.63 ± 0.24 μmol g dry mass^{-1}h^{-1} (N = 9) compared to those
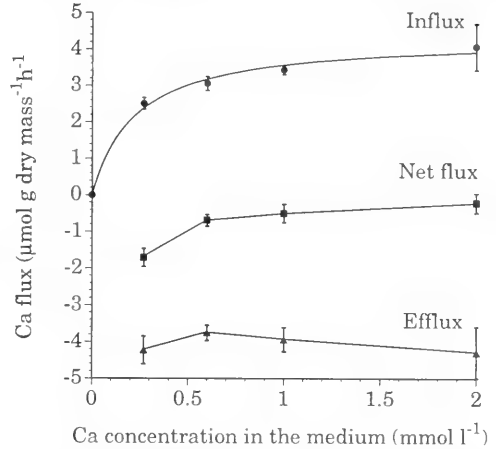

FIG. 1. Unidirectional and net fluxes of Anodonta imbecilis in media of different Ca concentrations. Points represent mean and standard errors. The wet mass of animals used were as follows: group 1 (Ca = 0.27 mM), 41.59 $\pm$ 2.30 g (N = 9); group 2 (Ca = 0.6 mM), 40.14 $\pm$ 2.41 g (N = 10); group 3 (Ca = 1.0 mM), 44.02 $\pm$ 2.03 g (N = 10); group 4 (Ca = 2.0 mM), 43.59 $\pm$ 2.07 g (N = 8). The equation of the curve fitting the influx data is: influx 4.329C/(0.213 + C), where C is the Ca concentration of the medium; r = 0.996.

of the animals in media containing 0.60 mM (Fisher's PLSD, p = 0.0036), 1.00 mM (Fisher's PLSD, p = 0.0007) and 2.00 mM Ca (Fisher's PLSD, p = 0.0001). There were no significant differences in Ca net flux in Ca concentrations of 0.60, 1.00, 2.00 mM (Fisher's PLSD, p = 0.1500 for the largest difference).

There was a nonlinear relationship between the unidirectional Ca influx and the external Ca concentration (Fig. 1). The unidirectional influxes for animals in media containing 0.27, 0.60, 1.00 and 2.00 mM Ca were significantly different (one-factor ANOVA, p = 0.0132). The mean unidirectional influx increased as external Ca concentration increased, was partially saturable and could be approximately described by the Michaelis-Menten equation:

$$Influx = K \frac{C}{Km + C} \quad (4)$$

where K is the maximum rate of unidirectional Ca influx, Km is the Ca concentration in the medium at which half saturation is attained, and C is the Ca concentration in the
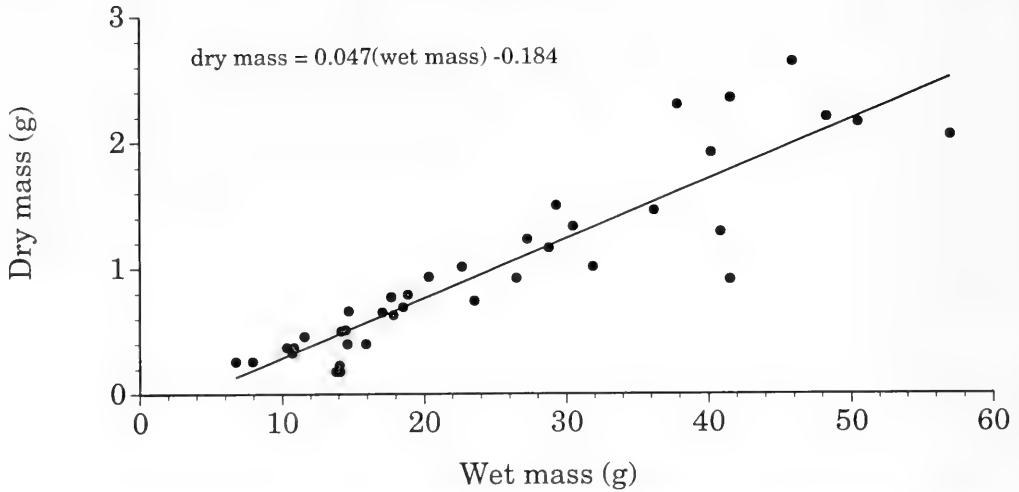
FIG. 2. Relationship between dry mass and wet mass of *Anodonta imbecilis*. Points represent the mass of individual animals; r = 0.909 for regression. Fisher's r to z, p < 0.0001, N = 39.

medium. The calculated half saturation Ca concentration for unidirectional Ca influx (K_m) was 0.213 mM. The maximum unidirectional influx (K) was 4.329 µmol g dry mass^{-1} h^{-1}. Thus, the following equation can be used to describe the relationship between unidirectional influx and external Ca concentration:

$$\text{Influx} = 4.329 \, \frac{C}{0.213 + C} \qquad (5)$$

Unidirectional Ca effluxes showed no significant difference among animals in media with different Ca concentration (one factor ANOVA, p = 0.7807; Fig. 1).

The dry mass of bivalves (excluding shell) was positively correlated with wet mass (r = 0.909, Fisher's r to z, p < 0.0001, Fig. 2). Larger animals generally maintained Ca balance as indicated by the negligible net flux (Fig. 3). However, animals smaller than 0.5 g tended to exhibit a negative Ca balance as indicated by a net efflux (Fig. 3). Both the unidirectional Ca influx and efflux decreased with increase in dry body mass. Negative linear relationships were derived between log unidirectional Ca influx and log dry mass (r = −0.800, Fisher's r to z, p < 0.0001), and log unidirectional efflux and log dry mass (r = −0.862, Fisher's r to z, p < 0.0001; Fig. 4). The slopes and intercepts respectively were as follows: −4.03, 6.52 (unidirectional influx) and −8.34, 7.54 (unidirectional efflux). The

slope and intercept for unidirectional efflux were both significantly higher than those for unidirectional influx (ANCOVA, p = 0.0001).

DISCUSSION

Active unidirectional Ca influx that follows enzyme saturation kinetics has been previously demonstrated in freshwater snails. Greenaway (1971) found in the snail *Lymnaea stagnalis* that active uptake of Ca was necessary below external levels of 0.5 mM. The uptake mechanism was half-saturated and near-saturated in external media containing 0.3 and 1.0–1.5 mM Ca, respectively, and snails showed a positive Ca balance in media containing more than 0.062 mM Ca. For the snail *Biomphalaria glabrata,* the half and near saturated Ca concentration for Ca uptake were 0.267 and 1.0–2.0 mM, respectively, and the minimum equilibrium concentrations were 0.012–0.025 mM for a closed system and 0.25 mM for an open system (Thomas et al., 1974). Both animals exhibited a high affinity Ca uptake mechanism. In the present study, the unidirectional Ca influx of *A. imbecilis* seems to display the same kinetics as a function of external Ca concentration. The half saturation Ca concentration in the medium was 0.213 mM, lower than the value estimated for the freshwater snail *L. stagnalis* (0.3 mM; Greenaway, 1971) and the value es-
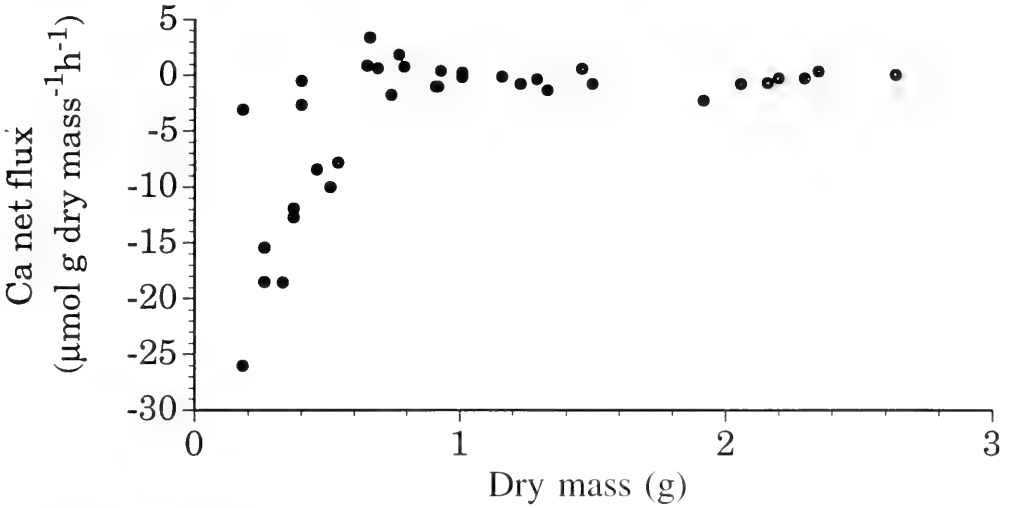
FIG. 3. Relationship between Ca net flux and body dry mass of *Anodonta imbecilis*. Points represent Ca net flux of individual animals. The Ca concentration of the medium is 1 mM. N = 36.
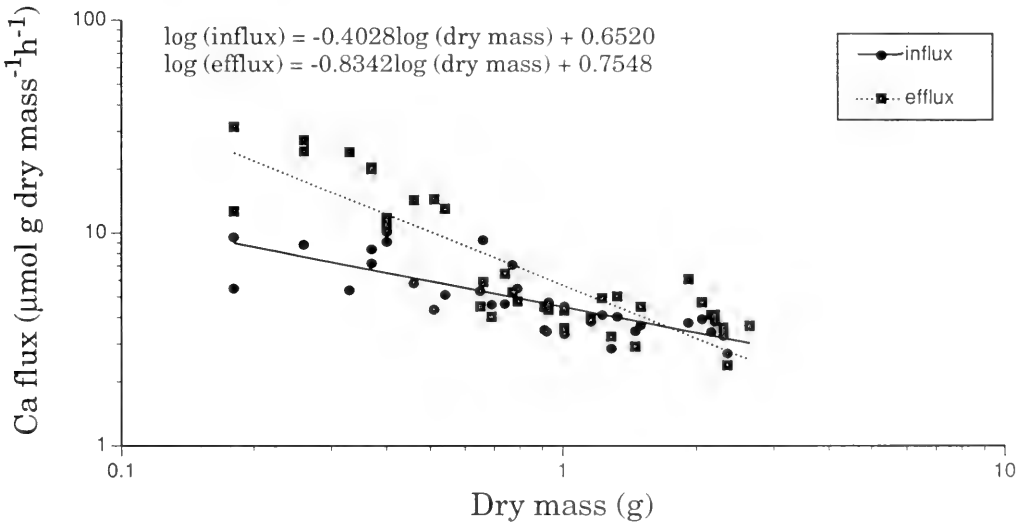

$$\log(\text{influx}) = -0.4028\log(\text{dry mass}) + 0.6520$$
$$\log(\text{efflux}) = -0.8342\log(\text{dry mass}) + 0.7548$$

FIG. 4. Relationship between log unidirectional Ca influx and log dry mass (r = 0.800, Fisher's r to z, p < 0.0001), and between log unidirectional Ca efflux and log dry mass (r = 0.862, Fisher's r to z, p < 0.0001) of *Anodonta imbecilis*. Points represent the unidirectional influx or efflux of individual animals. The slopes for log unidirectional influx and log unidirectional efflux are significantly different (ANCOVA, p = 0.0001). N = 36.

timated for the freshwater snail *Biomphalaria glabrata* (0.267 mM; Thomas et al., 1974).

If the unidirectional Ca efflux is purely by passive diffusion, one would expect unidirectional efflux to decrease as the external concentration is raised. Because unidirectional Ca efflux was unaffected by change in external Ca concentration, other mechanisms may be involved. Greenaway (1971) suggested that part of the unidirectional Ca influx in *L. stagnalis* is due to exchange diffusion and that this component increases when external Ca concentration increases following enzyme-saturation kinetics. If the unidirectional

Ca efflux in *A. imbecilis* is attributable to exchange diffusion, then unidirectional efflux would increase as external Ca rises. Any portion of unidirectional Ca efflux not attributed to exchange diffusion ("routine loss") would decrease with the increase in external Ca concentration because of the reduction in concentration gradient. The combined effect might be that unidirectional Ca efflux is unchanged. The net Ca flux of animals in media of different Ca concentration mirrored the change in unidirectional Ca influx because unidirectional Ca efflux remained constant. This pattern of Ca net flux is similar to the Ca net uptake pattern of *L. stagnalis* in media of different Ca concentration (Greenaway, 1971). The difference between these two animals is that in *A. imbecilis* Ca net flux was negative as opposed to the net uptake exhibited by *L. stagnalis*.

The relationship between wet and dry mass of *A. imbecilis* is linear, indicating the proportional increase of soft body tissue with shell and water content. A similar relationship was found between shell weight and fresh tissue weight in the freshwater snail *L. stagnalis* (Greenaway, 1971).

Few animals in this study exhibited a significant net uptake of Ca from the medium, suggesting that considerable Ca is obtained from dietary sources. The freshwater snail *Lymnaea stagnalis* was found to obtain 20% of its calcium from food (Van der Borght & Van Puymbroeck, 1966). This is also consistent with previous work, which demonstrated that freshwater bivalves obtain part of their Ca from food (Pynnonen, 1991).

Larger animals are better able to maintain their Ca balance with the environment than smaller animals, which tend to lose Ca to the medium. This was due to the fact that unidirectional Ca efflux is larger than Ca influx in small animals and decreases at a greater rate with increase in body mass. This implies that smaller animals depend more on dietary Ca than larger animals, possibly since they calcify their shell more rapidly.

Allometric regression showed that unidirectional Ca fluxes decreased with dry body mass of *A. imbecilis*. Thus, smaller animals exchange Ca with their environment more rapidly commensurate with an increased surface area to volume ratio. This result is similar to a recent study on crayfish, which revealed that the diffusional and active ion flux rates are both greater in small crayfish (Wheatly et al., 1991). An allometric study of Na fluxes in

amphibia (Pruett et al., 1991) showed that regression lines for unidirectional Na influx and efflux had the same slope and intercept confirming Na balance in animals of all size. In the present study, efflux decreased more than influx with increase of body dry mass resulting in significantly greater net efflux of Ca in smaller animals.

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MICROSCULPTURES OF CONVERGENT AND DIVERGENT POLYGYRID LAND-SNAIL SHELLS

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ABSTRACT

Polygyrid evolution has produced five pairs of closely convergent shell forms, four of which occur in sympatry. Scanning electron microscopy of the apertural parietal and basal denticles (or regions) (at about 500×) in those ten species, and of the body whorl (at about 100×) in those and eleven more polygyrid species, reveals possible new microsculptural characters, homologies, and radiations. Twelve informative new character states are tentatively proposed, of which half support, without homoplasy, previous shell-free phylogenetic hypotheses based on anatomy and allozymes. Two of the homoplastic characters actually enhance shell-form convergences, which are nonetheless distinguishable using other microsculptural features. Further SEM studies are warranted to test these proposed characters, to add others, and to test the hypothesis that shell micromorphology is much more informative than shell macromorphology for land-snail phylogenetics.

Key words: Gastropoda: Stylommatophora, morphology, systematics, phylogenetics, cladistics, SEM.

INTRODUCTION

Polygyrid shell-form evolution is unique for its multiple close convergences in sympatry and is also noteworthy for its sudden divergences; shell-sculpture evolution in polygyrids is also of great interest for its repeated convergences on periostracal hairs and its divergences among sister taxa (Pilsbry, 1940; Solem, 1976; Emberton, 1988, 1991a, 1994, 1995a, 1995b; Asami, 1988, 1993). These combinations of close convergences and rapid divergences make it virtually impossible to reconstruct polygyrid phylogeny from gross shell morphology, even when developmental characters are viewed by x-ray (Emberton, 1995b). Polygyrid shell convergences and divergences, however, have so far been compared only macroscopically, at magnifications no greater than 50×.

The purpose of this paper is a preliminary assessment of the the microsculptures of selected polygyrid shell convergences and divergences, using scanning electron microscopy (SEM).

Four species of polygyrids have previously been examined under SEM for microsculptural features of the apertural lip: *Stenotrema barbatum* (Clapp) (Solem, 1972: figs. 23, 24 = Solem, 1974: fig. 5), as well as *Daedalochila*

auriformis (Bland), *Millerelix mooreana* (Binney), and *M. doerfeuilliana sampsoni* (Wetherby) (Solem & Lebryck, 1976: figs. 33–46). All four species had fields of hexagonal to rounded crystalline plates, uplifted on one side, and those plates varied in size and distributional pattern among species. Intraspecific variation was studied in *D. auriformis*, with the important discoveries that the parietal and the palatal apertural denticles differed in microsculpture, and that a gerontic shell had more strongly developed microsculpture than a younger adult shell (Solem & Lebryck, 1976).

Only a single polygyrid specimen has previously been examined under SEM for shell periostracal microsculpture. *Stenotrema barbatum* exhibited at 195× and 360× a regular array of gradually tapered, sharp-pointed hairs, seemingly round in cross-section and projecting perpendicularly from a surface field of subparallel, slightly anastomosing ridges (Solem, 1974: fig. 6).

The present study is a preliminary survey, based on only one shell per species (although including several pairs of sister species), so the microsculptural characters discovered herein must be considered tentative. In order to minimize the known sources of intraspecific variation (Solem & Lebryck,

1976), only gerontic shells were used and both parietal and palatal apertural denticles (or regions) were examined.

MATERIALS AND METHODS

Twenty-one polygyrid species were chosen for examination; Figure 1 presents their phylogenetic relationships as hypothesized from anatomical and biochemical data (Emberton, 1988, 1991a, 1994a, 1995b). The species include North America's four most extreme cases of shell-form convergence in sympatry (Emberton, 1995b: fig. 1): globose *Neohelix major* and *Mesodon normalis,* umbilicate *Allogona profunda* and *Appalachina sayana,* flat *Xolotrema fosteri* and *Patera laevior,* and tridentate *Triodopsis fallax* and *Inflectarius inflectus.* A fifth shell-form convergence (Emberton, 1991b) was also included: "lipped" *Neohelix dentifera* and *Inflectarius ferrissi.*

Additional polygyrid species were included for their periostracal-microsculpture divergences and convergences. *Xolotrema denotata* and *X. obstricta* are sister species (Emberton, 1988) that can hybridize in the field (Vagvolgyi, 1968) and in the laboratory (Webb, 1980), but their differences in shell-whorl shape and sculpture are extreme. *Xolotrema obstricta* has a strongly keeled periphery and is sculpted with large, strongly raised ribs, whereas *X. denotata* has a rounded periphery and is sculpted with hair-like processes (Pilsbry, 1940; Emberton, 1988). The keeled, ribbed shell of *X. obstricta* is closely paralleled by that of *Patera sargentiana* (Pilsbry, 1940; Emberton, 1991a), with which it is sympatric in northern Alabama. Species of the *Patera* radiation (Emberton, 1991a) have diverged primarily in their shell surface sculpture: *P. laevior* is smooth, *P. sargentiana* is ribbed, *P. perigrapta* bears incised spiral grooves, and *P. appressa sculptior* is pustulose (Pilsbry, 1940).

Hair-like periostracal processes on the shell surface have arisen independently and convergently (Emberton, 1995b) in *Xolotrema denotata,* in some *Vespericola* such as *V. columbiana pilosa,* in the *Stenotrema* clade, and in the *Inflectarius* clade (Pilsbry, 1940). *Stenotrema*'s radiation is marked by extreme divergence in shell hairs, the variation of which includes short and dense (e.g. *S. maxillatum*), and long and sparsely distributed (e.g. *S. barbigerum*) (Pilsbry, 1940). To a

much smaller degree, the general shapes of shell hairs also seem (at 50×) to differ among species of *Inflectarius:* broad-based and sharp-tipped in *I. inflectarius* and *I. magazinensis,* acutely triangular in *I. smithi,* obtusely triangular in *I. subpalliatus,* and lost in *I. ferrissi,* the sister species of *I. subpalliatus* (Emberton, 1991a).

All studied shells are in the collection of the Academy of Natural Sciences of Philadelphia (ANSP). Species authors and ANSP catalog numbers of vouchers are given in the figure captions.

Shells were prepared for SEM using methods modified slightly from Solem (1970): soaking overnight in a weak solution of detergent (Alconox), immersing for five to 20 seconds in an ultrasonic cleaner, rinsing in distilled water, air-drying, and mounting—in standard position—on stubs using some combination (depending on the size of the shell) of double-sided conductive tape, carbon paint, carbon cement, carbon paste, and custom-bent paper clips. Mounted shells were gold-coated and photographed with a Cambridge Stereoscan 200 SEM in one or more of the following views: (a) whole shell (or as much as possible, including the entire aperture) in apertural view; (b) edge of parietal denticle (or callus) at about 1,000×; (c) edge of basal denticle (or lamellum or lip) at about 1,000×; and (d) body-whorl sculpture at about 200× and at <100× if necessary.

The resulting photographs were descriptively compared, then subjected to a standard phylogenetic character analysis (Wiley, 1981; Brooks & McLennan, 1991: chapter 2). The states of each character were parsimoniously mapped by hand on the polygyrid phylogenetic hypothesis (Emberton, 1988a, 1991a, 1994a, 1995b).

RESULTS

Four Convergences in Sympatry

Figures 2–5 show SEM photographs of the four pairs of convergent species that occur in sympatry, with triodopsins and *Allogona* on the left, and mesodontins on the right: a, b, *Neohelix major* and *Mesodon normalis;* c, d, *Xolotrema fosteri* and *Patera laevior;* e, f, *Triodopsis fallax* and *Inflectarius inflectus;* and g, h, *Allogona profunda* and *Appalachina sayana.* Apertural views at lowest possible magnifications (Fig. 2) indicate extremely close convergences in apertural dentition between
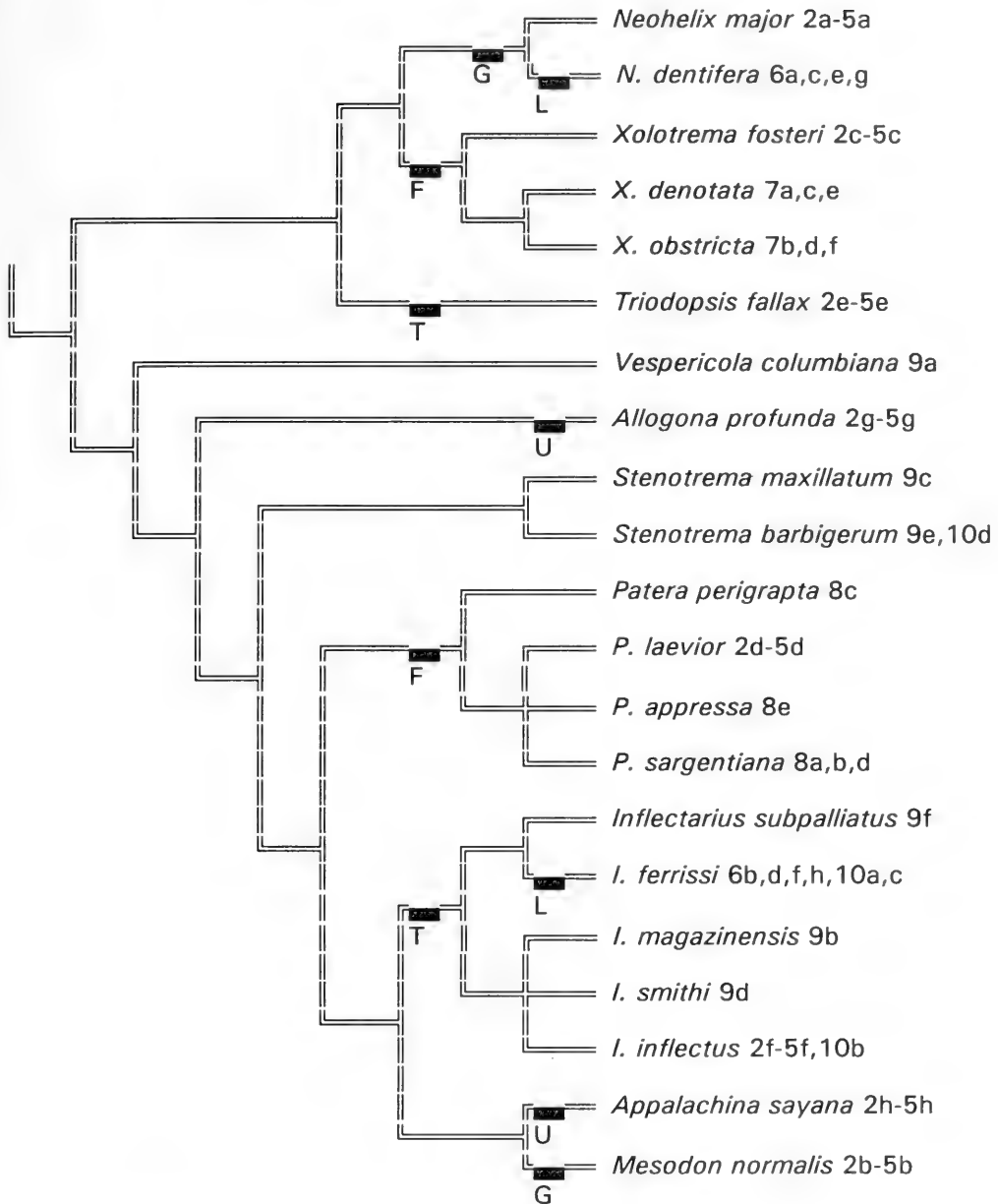
FIG. 1. Phylogenetic relationships of the 21 species examined for this study, as hypothesized from allozymes and reproductive morphology and behavior (Emberton, 1988, 1991a, 1994a). Principal convergences are designated by five abbreviations for shell shape/size: G, globose; F, flat; T, tridentate; U, umbilicate; L, lipped (Emberton, 1991b, 1995b). Figure numbers of SEM photos are given for each species name; "2a–5a" = 2a, 3a, 4a, 5a.

N. major and M. normalis (without dentition) and between X. fosteri and P. laevior (blade-like parietal denticles and basal lamellae); moderately close convergence between T. fallax and I. inflectus (parietal denticles similar but more curved in T. fallax, palatal denticles nearly identical, basal denticles similar but with a columellar buttress in T. fallax); and
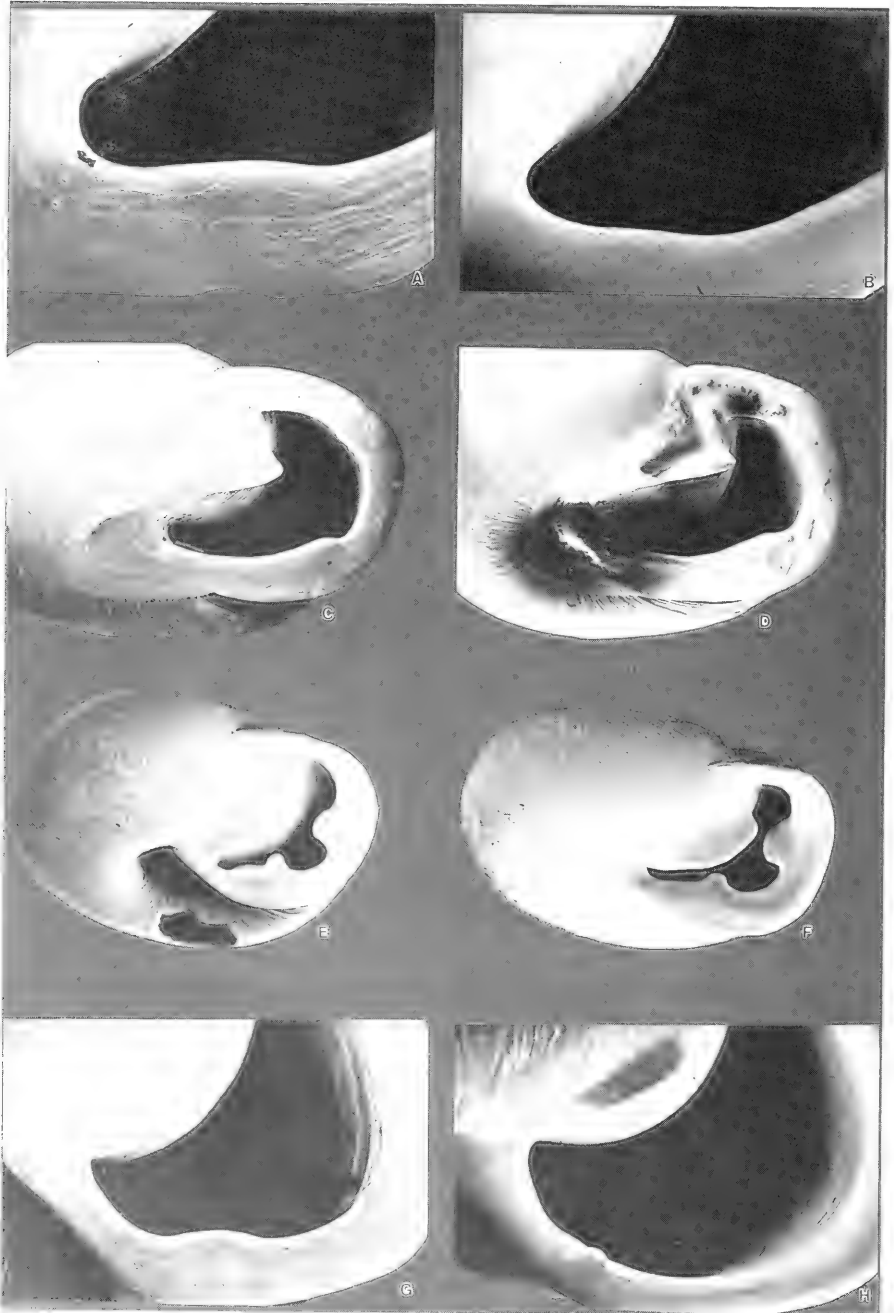
FIG. 2. Apertural features of North America's four most extreme cases of polygyrid shell-form convergence in sympatry (Emberton, 1995b: fig. 1). A, *Neohelix major* (Binney), ANSP uncataloged, 5.65×. B, *Mesodon normalis* (Pilsbry), ANSP uncataloged, 5.50×. C, *Xolotrema fosteri* (F. C. Baker), ANSP 117483, 4.43×. D, *Patera laevior* (Pilsbry), ANSP 186465, 3.98×. E, *Triodopsis fallax* (Say), ANSP 192768, 4.95×. F, *Inflectarius inflectus* (Say), ANSP 91616, 6.30×. G, *Allogona profunda* (Say), ANSP 77867, 4.90×. H, *Appalachina sayana* (Pilsbry), ANSP 264654, 5.35×.
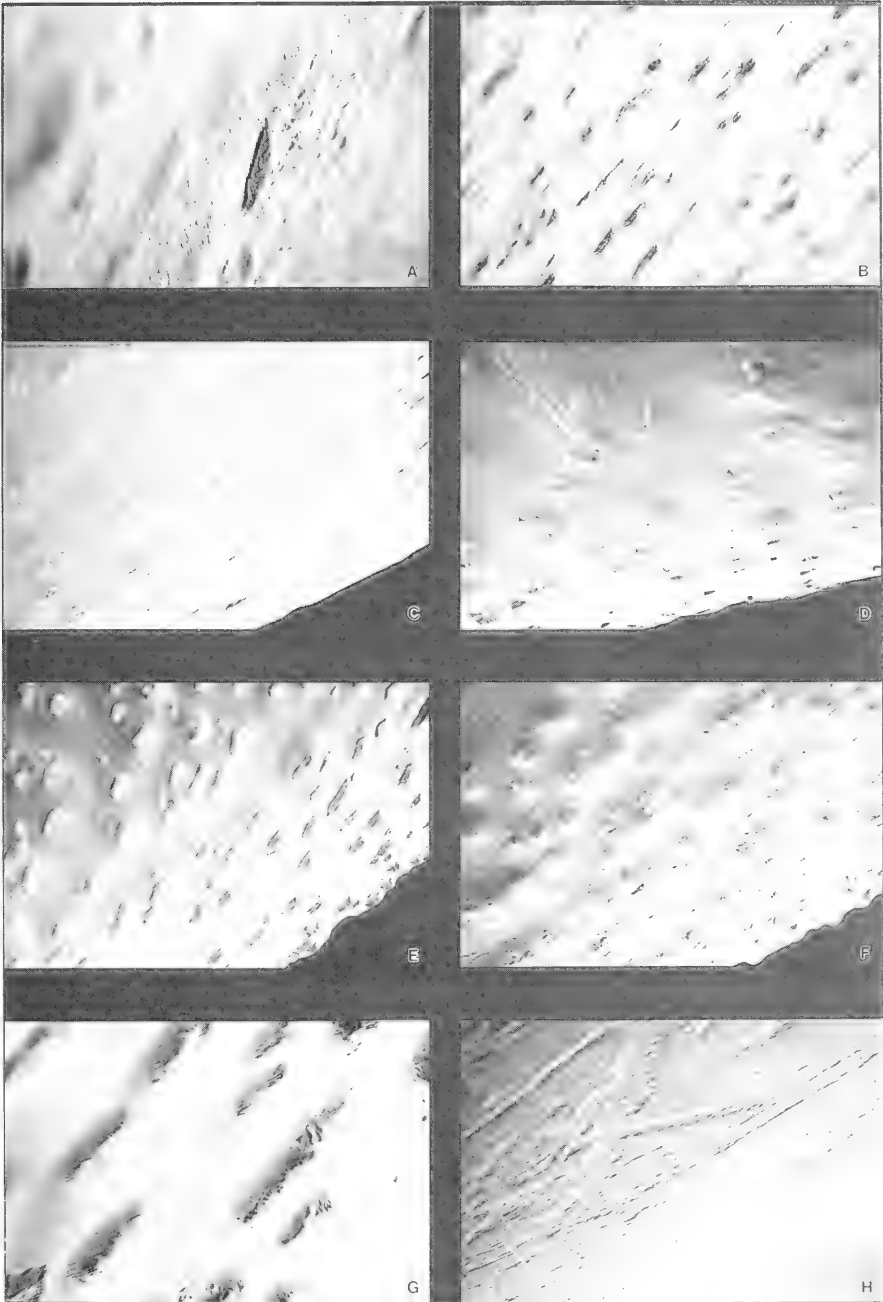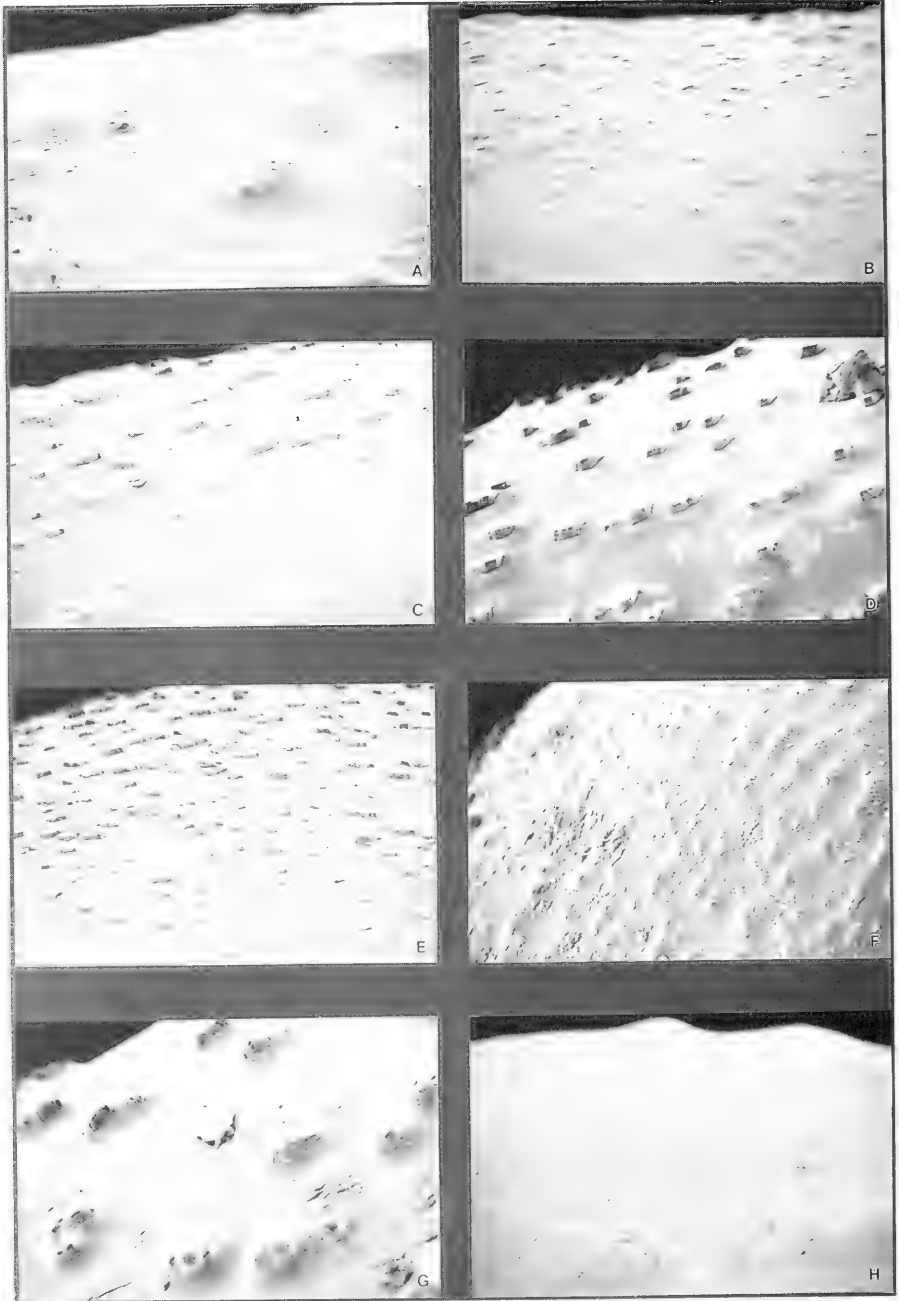
FIG. 3. The parietal denticles or parietal regions of the same specimens as Fig. 2, at about 500× magnification. A, *Neohelix major* (Binney), ANSP uncataloged, 505×. B, *Mesodon normalis* (Pilsbry), ANSP uncataloged, 500×. C, *Xolotrema fosteri* (F. C. Baker), ANSP 117483, 510×. D, *Patera laevior* (Pilsbry), ANSP 186465, 500×. E, *Triodopsis fallax* (Say), ANSP 192768, 550×. F, *Inflectarius inflectus* (Say), ANSP 91616, 565×. G, *Allogona profunda* (Say), ANSP 77867, 520×. H, *Appalachina sayana* (Pilsbry), ANSP 264654, 515×.

FIG. 4. The basal denticles or basal regions of the same specimens as Fig. 2, at about 500× magnification. A, *Neohelix major* (Binney), ANSP uncataloged, 515·. B, *Mesodon normalis* (Pilsbry), ANSP uncataloged, 500·. C, *Xolotrema fosteri* (F. C. Baker), ANSP 117483, 520·. D, *Patera laevior* (Pilsbry), ANSP 186465, 510∧. E, *Triodopsis fallax* (Say), ANSP 192768, 540·. F, *Inflectarius inflectus* (Say), ANSP 91616, 540×. G, *Allogona profunda* (Say), ANSP 77867, 510×. H, *Appalachina sayana* (Pilsbry), ANSP 264654, 520×.

FIG. 5. The body-whorl sculptures of the same specimens as Fig. 2, at about 100× magnification. A, *Neohelix major* (Binney), ANSP uncataloged, 102.0×. B, *Mesodon normalis* (Pilsbry), ANSP uncataloged, 101.5×. C, *Xolotrema fosteri* (F. C. Baker), ANSP 117483, 104.54ts. D, *Patera laevior* (Pilsbry), ANSP 186465, 102.5×. E, *Triodopsis fallax* (Say), ANSP 192768, 99.5×. F, *Inflectarius inflectus* (Say), ANSP 91616, 102.5×. G, *Allogona profunda* (Say), ANSP 77867, 104.0×. H, *Appalachina sayana* (Pilsbry), ANSP 264654, 102.5×.

only slight convergence between *Al. pro-funda* and *Ap. sayana* (basal node much broader in *Al. profunda,* parietal denticle lacking in *Al. profunda.* Convergences between iterated pairs are consistently close in overall shell size and shape, and apertural size and shape (Fig. 2).

Flat Shell Forms: On the parietal denticle or parietal region, convergences disintegrate at high magnification (Fig. 3), with the notable exception of *X. fosteri* and *P. laevior,* both of which have a smooth surface dotted with low mounds bearing wide, shallow, rough-bottomed craters; the mounds are virtually identical between species in their sizes, densities, apparently random distributions, and structural details (Fig. 3c, d). This microstructural convergence is all the more remarkable given the great variation in this region among the other six species. Background surfaces range from smooth (Fig. 3b, e, g, h) to flocculent (Fig. 3a) to randomly pitted (Fig. 3f). Secondary structures range from large, straight escarpments both sparse (Fig. 3a) and dense (Fig. 3g), to small dense escarpments both straight (Fig. 3b) and polygonal (Fig. 3e), to rounded pustules (Fig. 3f), to shallow canals that are randomly sized and directed (Fig. 3h).

On the basal denticle or basal region, microsculpture in each sympatric-convergent species (Fig. 4) is similar to that on its parietal denticle or region, with the exceptions of *X. fosteri* and *P. laevior.* These two species share, on a smooth background, a pattern of polygonal escarpments (Fig. 4c, d) that are similar in density and dispersion to, but very different in structure from, the cratered mounds they share on the parietal denticles (Fig. 3c, d). These polygonal escarpments are similar in size and shape, but are slightly more uptilted in *P. laevior* than in *X. fosteri.* The other six species have virtually identical backgrounds on their basal and parietal denticles or regions, except that the flocculence in Figure 4a is less pronounced than in Figure 3a. In secondary structures, Figures 4f and 3f are virtually identical, as are Figures 4e and 3e, and Figures 4b and 3b. Figure 4a is similar to Figure 3a, but with the significant addition of a new structure: a smooth-surfaced puddle overlying a low, bumpy knob. Figure 4h has the canals of Figure 3h, but they are much smaller and sparser, and there is the addition in Figure 4h of low mounds. Figure 4g differs from Figure 3g only in that its es-

carpments are generally slightly shorter and less straight.

Globose Shell Forms: The shell body-whorl surface at high magnification (Fig. 5) shows additional features previously undetected. The microsculptural convergence between *Neohelix major* and *Mesodon normalis,* indistinguishable under the dissecting microscope at 50× (Emberton, 1995a), is readily detectable under SEM. Although both have a matte-like surface produced by a pattern of large transverse ridges crossed by smaller spiral cords, the cords in *N. major* (Fig. 5a) are even in width, whereas the cords in *M. normalis* are variable in width and generally much wider than in *N. major.* Both have uneven, transverse micro-wrinkles, which are more pronounced in *N. major,* however. Furthermore, *N. major* has an even pattern of parallel, spiral microstriae that are totally lacking in *M. normalis.*

These spiral microstriae appear in all three members of the Triodopsinae (Fig. 5a, c, e), as well as in *Allogona* (Fig. 5g), but in none of the four members of the Mesodontini (Fig. 5b, d, f, h). The microstriae are periostracal structures only, as they do not appear in the underlying calcium carbonate layer in those patches where the periostracum has flaked off (Fig. 5a, c, g).

Tridentate Shell Forms: Periostracum also seems to be the sole source of the unique pattern of jumbled wrinkles or folds that tend toward a spiral direction in *Inflectarius inflectus* (Fig. 5f), the hairs of which (broken in this specimen) are simple outward extensions of arcuate periostracal folds, as well as of the unique network of transverse-trending wrinkles in *Xolotrema fosteri* (Fig. 5c). The spirally oriented pustules of *Patera laevior,* on the other hand, are also part of the calcium carbonate shell matrix, as evidenced by the pustules over which the periostracum had flaked off (Fig. 5d). The same seems to be true of the unusual nodulose spiral cords of *Allogona profunda* (Fig. 5g).

The remaining outstanding features of comparative shell body-whorl microsculpture in Figure 5 are the transverse ridges, which vary in size, shape, density, and angle. In the convergent pair *N. major* and *M. normalis* (Fig. 5a, b), they are equally large (only one complete ridge appears in each photograph), low, and rounded in profile, but in *N. major* they are slightly more angled from the vertical

than in *M. normalis.* In the convergent pair *X. fosteri* and *P. laevior* (Fig. 5c, d), the transverse ridges are about equal in angle, size, and density (three ridges in each photograph), but they are extremely weak in *P. laevior.* The shell convergence between *T. fallax* and *I. inflectus* breaks down entirely at microsculptural level (Fig. 5e, f): *T. fallax* has strong, dense, well-separated, sharply angled transverse ridges, whereas *I. inflectus* nearly lacks them entirely. The transverse ridges of *Al. profunda* (Fig. 5g), although equal in density and angle to those of *N. major* (Fig. 5a), are about twice as broad, so broad in fact that they are adjacent, separated by only narrow gutters. Thus, they are quite different from the convergent *Ap. sayana,* whose pronounced, narrow, well separated, mildly angled ridges are more like a stronger version of *P. laevior* (Fig. 5d).

The *N. dentifera*—*I. ferrissi* Convergence

The extreme shell-shape convergence between *Neohelix dentifera* and *Inflectarius ferrissi* (Fig. 6a, b; Emberton, 1991b) carries strong clues of its origins in its apertural and body-whorl microsculptures (Fig. 6c-h). In the case of *N. dentifera,* these clues conflict slightly with anatomical and allozymic evidence (Emberton, 1988, 1991b). Thus, although *N. dentifera*'s parietal denticle (Fig. 6c) shares a flocculent-textured background surface with its congener *N. major* (Fig. 3a), the dominant sculpture of short, polygonal escarpments is much closer to that of the related *Triodopsis fallax* (Fig. 3e), from which it differs only in the much steeper angles of its escarpments. The discrepancy is similar for *N. dentifera's* basal apertural region (Fig. 6e), which has a flocculent ground similar to that of *N. major* (Fig. 4a), but has a sculpture of steep, polygonal escarpments which are similar in density and distribution to those of *Xolotrema fosteri* (Fig. 4c) and which are similar in their small size and steep polygons to those of *Triodopsis fallax* (Fig. 4e). In the distinctly exaggerated degree of their steepness, however, the basal-lip polygons of *Neohelix dentifera* are convergently more similar to *Patera laevior* (Fig. 4d). Despite these discrepancies, apertural microsculpture clearly agrees with nonconchological data (Emberton, 1988) in placing *N. dentifera* in the Triodopsini. Body-whorl microsculpture, on the other hand, is entirely concordant: *N. dentifera* (Fig. 6g) is nearly identical

to its congener *N. major* (Fig. 5a), from which it differs only in its broader spiral cords and weaker transverse wrinkles.

The phylogenetic affinities of *I. ferrissi* (Fig. 6b) based on shell microsculpture are clear and concordant with anatomical and allozymic data (Emberton, 1991a, b). Its parietal denticle has the same randomly pitted background surface and the same rounded pustules as *I. inflectus* (Fig. 3f), except that the pustules are arrayed in unevenly parallel rows instead of distributed randomly as in *I. inflectus. Inflectarius ferrissi*'s basal apertural region (Fig. 6f) also seems homologous with the basal denticle of *I. inflectus* (Fig. 4f), with a similar randomly pitted background surface (less evident in Fig. 6f due to low contrast), and with similar rounded pustules, which are more sparsely distributed in *I. ferrissi.* The unique body-whorl microsculpture of *I. ferrissi* (Fig. 6h) consists of the same jumbled pattern of spiral-trending wrinkles as in *I. inflectus* (Fig. 5f), but on a finer and lower size scale.

Thus, shell body-whorl microsculpture and, to a lesser extent, shell apertural microsculpture tend to bear out generic relationships in *Neohelix* and *Inflectarius,* despite extreme divergences in shell size and shape.

Divergences Within the Flat-Shell Clades

Intrageneric divergence in body-whorl microsculpture is extreme in *Xolotrema* (Figs. 5c, 7) and in *Patera* (Figs. 5d, 8), with structural homologies difficult if not impossible to decipher. Nevertheless, these two genera, which closely converge on each other in shell size and shape (Figs. 2c and 2d; Figs. 7a and 8a) and in apertural microsculpture (Figs. 3c and 3d; Figs. 4c and 4d), are always clearly distinguishable in body-whorl microsculpture, as discussed below.

The Xolotrema Clade: Shell divergence between the presumably hybridizing sister species *Xolotrema obstricta* and *X. denotata* is extreme, not only on a gross scale (Fig. 7a, b), but also at high magnification (Fig. 7c, d) and at very high magnification (Fig. 7e, f), at which they also can be seen to diverge from their congener *X. fosteri* (Fig. 5c). *Xolotrema obstricta*'s peripheral keel and transverse ribbing are lacking in *X. denotata,* which bears periostracal hairs (all broken in this specimen) lacking in *X. obstricta. Xolotrema fosteri* lacks all these features, except transverse
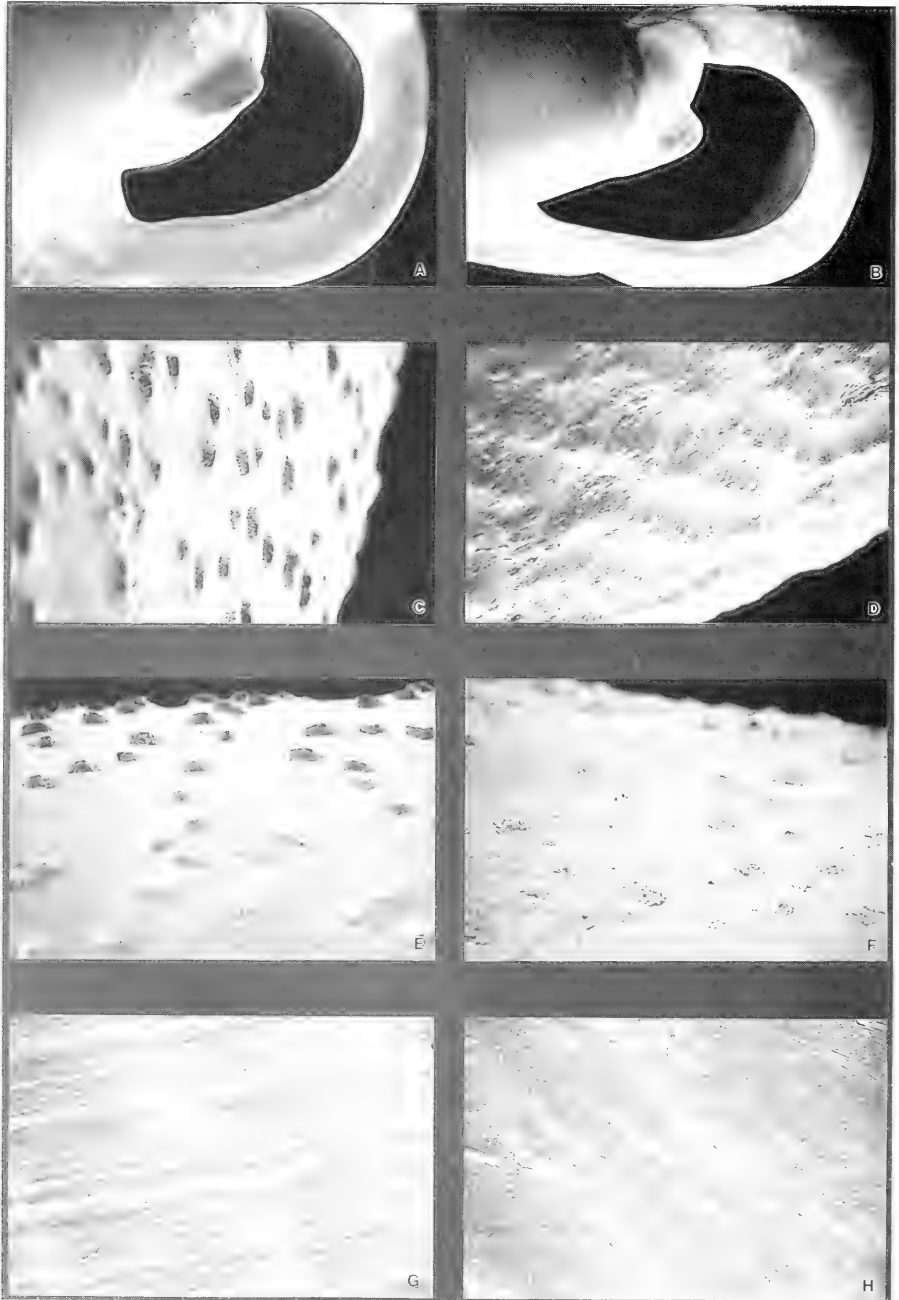
FIG. 6. Two conchologically convergent species that are also are ecologically parallel and convergent (Emberton, 1991b). A, C, E, G, *Neohelix dentifera* (Binney), ANSP 90119: A, aperture, 4.56×; C, parietal denticle, 540×; E, basal apertural lip, 505×; G, body-whorl sculpture, 104.0×. B, D, F, H, *Inflectarius ferrissi* (Pilsbry), ANSP 98085: B, aperture, 4.56×; D, parietal denticle, 505×; F, basal apertural lip, 510×; H, body-whorl sculpture, 102.5×.
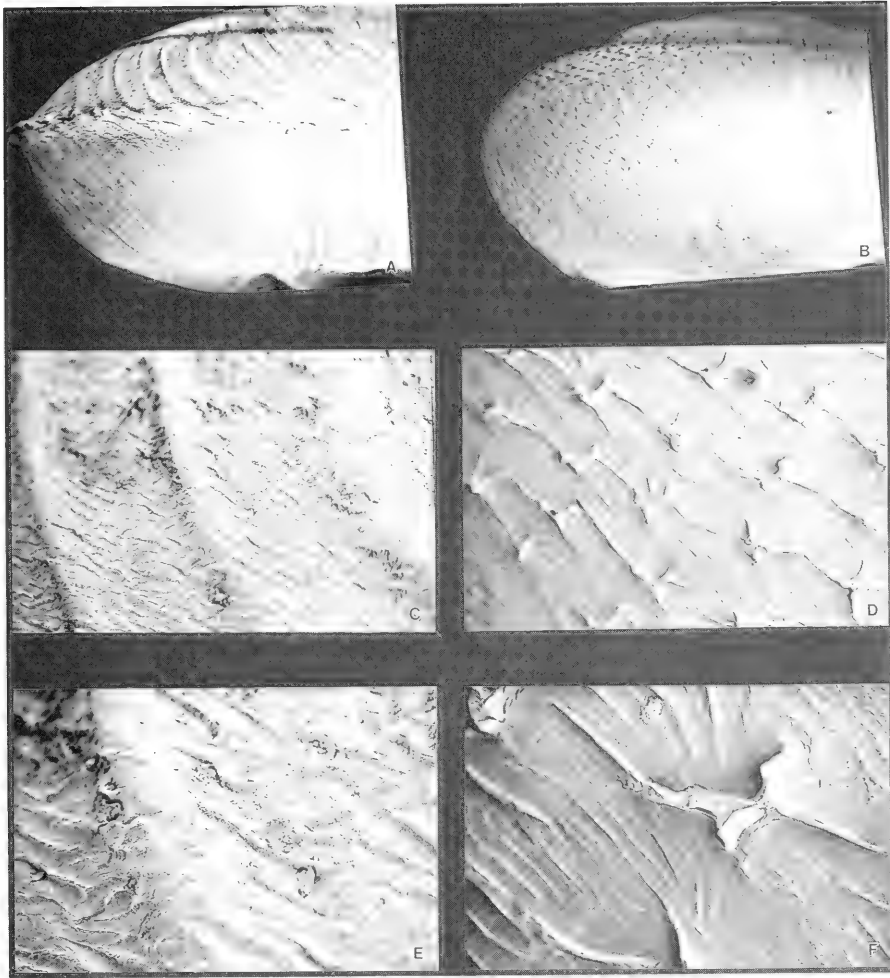
FIG. 7. Two conchologically divergent but hybridizing sister species. A, C, E, *Xolotrema obstricta* (Say), ANSP 68553: A, body whorl to the left of the aperture, 6.25×; C, body-whorl sculpture, 39.0×; E, body-whorl sculpture, 101.0×. B, D, F, *Xolotrema denotata* (Férussac), ANSP 172721: B, body whorl to the left of the aperture, 6.3×; D, body-whorl sculpture, 38.7×; F, body-whorl sculpture, 104.0×.

ribs, which are nonetheless lower, denser, and more angled than those of *X. obstricta. Xolotrema fosteri*'s spiral microstriae are entirely lacking in both *X. obstricta* and *X. denotata*. The strongest candidate for homology among the three species is in the periostral wrinkles, but these vary enormously. In *X. fosteri*, the wrinkles are small and appear only sporadically in dense reticulate patterns oriented transversely in the gullies between transverse ribs. In *X. obstricta*, the wrinkles are very large and appear universally in a dense, somewhat reticulate pattern oriented at an angle between transverse and spiral. The wrinkles in *X. denotata* are medium to large and universally distributed in a sparse, somewhat reticulate pattern generally oriented transversely, and regularly punctuated at right angles by short, thick hairs that form cross patterns on locally thickened transverse wrinkles. Thus, these three types of wrinkles share a generally reticulate pattern, but even in this factor, their differences are so great (Figs. 5c, 7e, 7f) as to
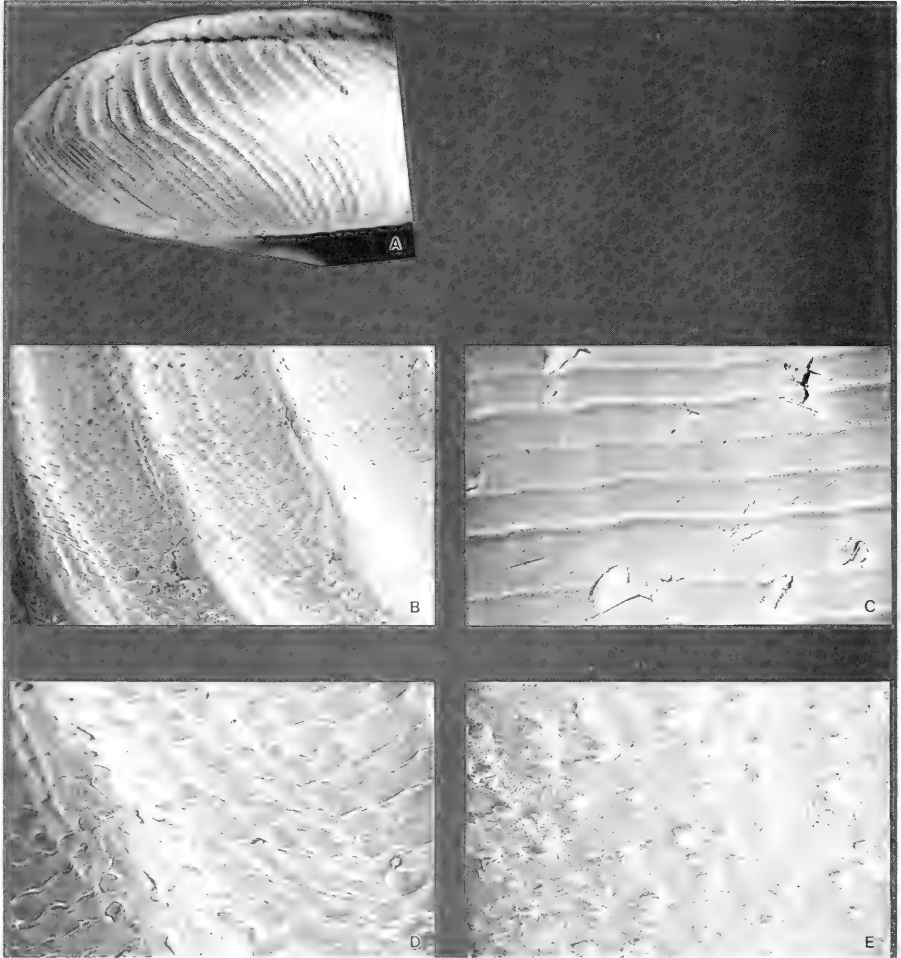
FIG. 8. A species convergent on and sympatric with *Xolotrema obstricta* (Fig. 7a, c, e), and two of its congeners. A, B, D, *Patera sargentiana* (Johnson & Pilsbry), ANSP 150249: A, body whorl to the left of the aperture, 6.30×; B, body-whorl sculpture, 39.0×; D, body-whorl sculpture, 101.0×. C, *Patera perigrapta* (Say), ANSP 160543, body-whorl sculpture, 104.5×. E, *Patera appressa sculptior* (Chadwick), ANSP 128954, body-whorl sculpture, 102.5×.

defy detection of homology. In fact, the periostracal wrinkles of *X. denotata* (Fig. 7f) are much more similar to those of *Inflectarius inflectus* (Fig. 5f), a close convergence that differs slightly in the general orientation of the wrinkles (spiral in *I. inflectus* and transverse in *X. denotata*) and that differs greatly in the form of periostracal hairs (broad upward extensions of arcuate folds in *I. inflectus;* narrow, buttressed blades at right angles to straight folds in *X. denotata*). The perio-

stracum is quite thick in *X. denotata,* as evidenced by the depth of the cracks appearing in Fig. 7f).

The *Patera* Clade

The intersubfamilial convergence in sympatry between *Patera sargentiana* (Fig. 8a, b, d) and *Xolotrema obstricta* (Fig. 7a, c, e) is rather close. Both have large, flat, heavily ribbed shells with peripheral angulations

(Figs. 7a, 8a), but in *X. obstricta* the angulation is a pronounced keel. The transverse ribs are equal in density and form (Figs. 7c, 8b), but are more raised in *P. sargentiana*. Both have spirally oriented ridges (Figs. 7e, 8d), but in *X. obstricta* these are periostracal wrinkles or folds in a vaguely reticulate pattern, whereas in *P. sargentiana* they are shell-matrix pustulose cords in regularly parallel pattern, with an additional underlying substructure of weak transverse cords.

Patera sargentiana's parallel spiral rows of micro-pustules (Fig. 8d) are clearly homologous with those of *P. laevior* (Fig. 5d) described above. Another congener, *P. perigrapta* (Fig. 8c), lacks pustules entirely, but bears parallel spiral grooves equal in density to the pustular rows of *P. laevior*. A fourth congener, *P. appressa sculptior* (Fig. 8e), combines the grooves of *P. perigrapta* (but weaker than in that species) with the pustules of *P. laevior* (but stronger than in that species): its microsculpture is one of pustules equally spaced within shallow, parallel, spiral grooves. Thus, it appears that spiral grooves, spiral rows of pustules, and spiral cords of pustules are all homologous in *Patera*, and concomitantly that the spiral gullies in *P. sargentiana* (Fig. 8d) are not homologous with the spiral grooves of *P. perigrapta* (Fig. 8c). Transverse ribbing in *P. laevior, P. perigrapta,* and *P. appressa sculptior* is similarly weak and variable, entirely unlike the strong ribs of *P. sargentiana,* but possibly homologous with the secondary, weak transverse ribs of that species.

Shell Hairs: Convergence and Divergences

Convergent periostracal hairs were described and compared above for *Inflectarius inflectus* (Fig. 5f) and *Xolotrema denotata* (Fig. 7f), although incompletely because the hair tips were broken off in both specimens. The periostracal hairs of *Vespericola columbiana pilosa,* which are shown unbroken and at the same high magnification in Figure 9a, are entirely different in structure. These hairs are thick, rigidly curved, and columnar, arising from shallow, socket-like depressions, and are relatively unbuttressed. They arise from a unique background surface sculpture of both spirally and transversely oriented patterns of periostracal wrinkles, punctuated by large but weak transverse ribs and by small shelf-like protrusions (Fig. 9a).

In *Stenotrema* (Figs. 9c, e; Fig. 10d), the periostracal hairs and surface sculptures of the two examined species are so different that they seem entirely non-homologous. *Stenotrema maxillatum* (Fig. 9c) has relatively small, dense, regularly arranged, backward-directed, elongate-conic hairs that are slightly buttressed transversely and that are marked by short, forward, spiral wrinkles in an otherwise transversely wrinkled, smooth background surface. *Stenotrema barbigerum* (Fig. 9e), on the other hand, bears relatively large, moderately dense, regularly arranged, transverse folds that arc with the concave side forward, that have tiny, low spines on their forward surfaces, and that lie in a background surface of minute, densely packed, parallel, transverse ridges overlying an uneven system of shallow, spiral grooves. The conspicuous, micro-spinose, transverse folds of *S. barbigerum* vary in length and shape, depending on position (Fig. 10d): on the upper shell whorls they are low in profile, on the lower shell whorls their central regions are drawn outward and backward into thorn-like hairs, and on the shell's keeled periphery they extend outward into long, unevenly blade-like hairs (Fig. 10d; Fig. 9e: upper right).

Unlike in *Stenotrema,* homologies among periostracal hairs of the genus *Inflectarius* (Figs. 5f; 6h; 9b, d, f) are much more evident, despite extensive morphological radiation. The hairs of *I. subpalliatus* (Fig. 9f) seem to be enlarged versions of the hairs described above of *I. inflectus* (Fig. 5f): both are long, arcuate, high-standing folds rising from a background of smaller, variously oriented wrinkles. *Inflectarius subpalliatus*'s sister species, *I. ferrissi* (Fig. 6h), lacks hairs entirely, and carries only vestigial traces of folds and wrinkles in its relatively featureless body-whorl microsculpture. In stark contrast to this effacement, the hairs of *I. magazinensis* (Fig. 9b) show increased complexity: the bottom of the arcuate fold is abruptly curled forward in a scoop-like fashion, the central high-point of the fold is thickened and curled over like a cresting wave, and extending from this crest is a downward arching secondary fold that continues onto the background surface as a rear-support buttress. These same modifications are developed even further, to a remarkable degree, in *I. smithi* (Fig. 9d). In this species, the entire arcuate fold is relatively deeply arched and scoop-like; the central high-point extends forward as a long, blunt, club-like structure with a surface sculpture of
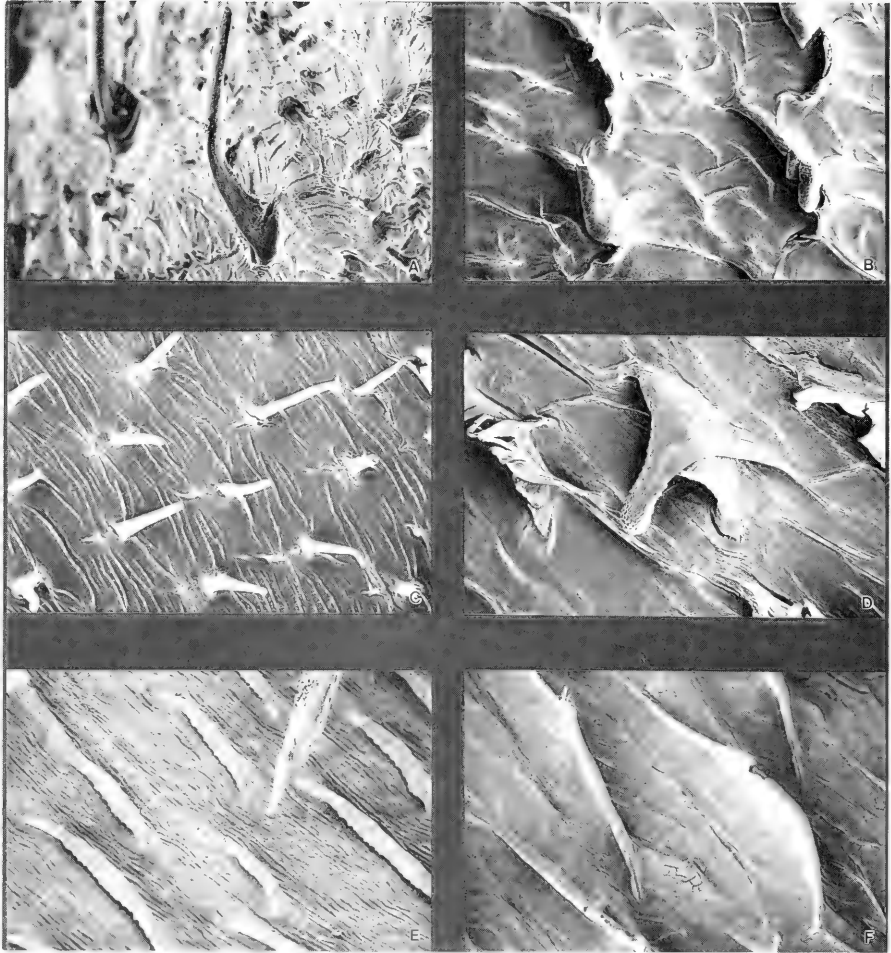
FIG. 9. More periostracal hairs (body-whorl microsculpture). A, *Vespericola columbiana pilosa* (Henderson), ANSP 158355, 100.0×. C, *Stenotrema maxillatum* (Say), ANSP 170141, 99.0×. E, *Stenotrema barbigerum* (Redfield), ANSP 170110, 102.0×. B, *Inflectarius magazinensis* (Pilsbry & Ferriss), ANSP 395865, 102.5×. D, *Inflectarius smithi* (Clapp), ANSP 160055, 100.5×. F, *Inflectarius subpalliatus* (Pilsbry), ANSP 171134, 103.0×.

minute, regular, adjacent pits; and the secondary, downward-arching, rear-buttress fold is high-standing and strongly developed.

Character Analysis

All of the ten species that were examined for apertural microsculpture have patterns of escarpments, nodules, or mounds (their absence in Fig. 3h is considered an artifact of the sparse distributions of mounds in that species: Fig. 4h). These microprojections seem to be homologous, with a basic morphology of inclined, crystalline platelets (Figs. 3e, 4c-e, 6c, e) that is modified by various coating surfaces. A good example is the highly modified surface of the parietal denticle of *Inflectarius ferrissi* (Fig. 6d), which shows little evidence of crystalline platelets. At lower magnification, however, this surface can be seen to coat only the leading edge of the parietal denticle (Fig. 10a), the uncoated interior of which has a standard pattern of microplatelets (Fig. 10c). Similarly, in the closely related *I. inflectus,* the rounded nod-
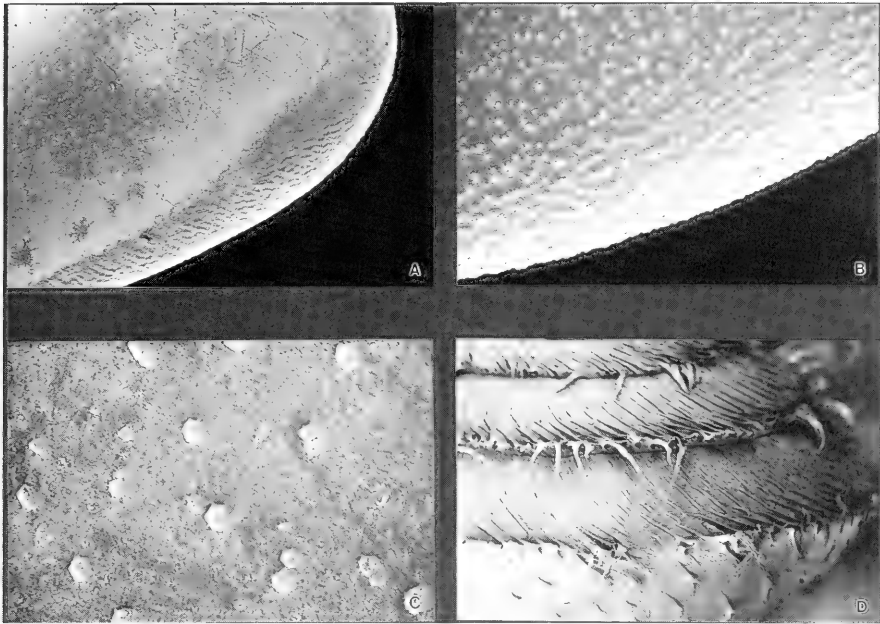
FIG. 10. A, C, apertural parietal denticle of *Inflectarius ferrissi* (Pilsbry), ANSP 98085: A, tip region, 43.05×; C, central region, 515×. B, edge of apertural parietal denticle of *Inflectarius inflectus* (Say), ANSP 91616, 127.5×. D, mid region of the shell of *Stenotrema barbigerum* (Redfield), ANSP 170110, 12.35×.

ules (Fig. 3f) are seen at a lower magnification to be crystalline microplatelets as they lose some of their coating away from the edge of the parietal denticle (Fig. 10b).

Similar crystalline microprojections have been found in a wide variety of pulmonates, and in other gastropod groups as well (Solem, 1970, 1972, 1973; Solem & Lebryk, 1976). They seem therefore to be constructional aspects of apertural deposition (Wilbur & Saleuddin, 1983; Watabe, 1988), although it has been argued that in some cases their shape is modified by natural selection for defense (Solem, 1972).

The relative sizes and distributions of microprojections seen in this study do not seem to be reliable systematic characters, because they correlate with shell size. Thus, three of the four large shells (Fig. 2a, g, h) have the largest and sparsest microprojections (Figs. 3a, g, h; 4a, g, h); the two small shells (Fig. 2e, f) have the smallest and densest microprojections (Figs. 3e, f; 4e, f); and the four intermediate-sized shells (Figs. 2c, d; 6a, b) and one of the large shells (Fig. 2b) have microprojections that are intermediate in size and density (Figs. 3b–d; 4b–d; 6c–f).

Based on this analysis, a single, multi-state character can be proposed:

Character 1. Apertural coating.
    State a. No coating on parietal or basal denticle/region; surface very smooth and featureless; microprojections crystalline and clean. *Triodopsis fallax.*
    State b. Thin, flocculent coating on both parietal and basal denticles/regions; surface flocculent; microprojections clean to partially coated. *Neohelix major* and *N. dentifera.*
    State b'. Same as state b, but on the basal region, the microprojections and their surroundings have an additional coating of thin, smooth material. *N. major.*
    State c. Smooth-surfaced coating on the parietal denticle/region only; flanks but not tips of parietal microprojections coated; no coating on basal denticle/region. *Xolotrema fosteri* and *Patera laevior.*
    State d. Medium-thick, minutely pitted coating on both parietal and basal denticles/regions; microprojections entirely covered on denticle edges, but with tips exposed away from denticle edges. *Inflectarius inflectus* and *I. ferrissi.*

State d'. Same as state d, but the coating covers only the edge of the parietal denticle, where it is thick and forms parallel rows. *I. ferrissi.*

State e. Thick coating scored with shallow canals of random size and orientation, on both parietal and basal denticles/regions; microprojections thickly and entirely covered. *Appalachina sayana.*

State f. Thin, smooth coating on both parietal and basal denticles/regions; microprojections completely to partially covered. *Mesodon normalis* and *Allogona profunda.*

Analysis of body-whorl microsculpture is based on the 21 species studied. Intrageneric variation is so great in transverse ribs that they seem unreliable as systematic characters. The same must be said for the patterns of (but not necessarily for the presence of) periostracal wrinkles. The periostracum forms as a flexible, curtain-like sheath that later is sclerotized by quinone tanning (Saleuddin & Petit, 1983; Waite, 1983), so the pattern of wrinkles may be influenced by a number of environmental and constructional factors other than phylogenetic constraints. Periostracal hairs are always associated (in this sample) with periostracal wrinkles, however.

The following characters seem reliable.

Character 2. Spiral microstriae.

State a. Present. *Neohelix major, N. dentifera, Xolotrema fosteri, Triodopsis tridentata, Allogona profunda.*

State b. Absent. All other examined species.

Character 3. Spiral cords, smooth unless traversed by spiral microstriae.

State a. Present. *Neohelix major, N. dentifera, Mesodon normalis.*

State b. Absent. All other examined species.

Character 4. Spiral cords, nodulose.

State a. Present. *Allogona profunda.*

State b. Absent. All other examined species.

Character 5. Spiral grooves/pustules/pustulate cords.

State a. Present. *Patera laevior, P. sargentiana, P. perigrapta, P. appressa.*

State b. Absent. All other examined species.

Character 6. Periostracal wrinkles.

State a. Present. *Xolotrema fosteri, X. obstricta, X. denotata, Vespericola columbiana, Stenotrema maxillatum, S. barbigerum, Inflectarius inflectus, I. ferrissi, I. smithi, I. magazinensis, I. subpalliatus.*

State b. Absent. All other examined species.

Character 7. Periostracal hairs.

State a. Thin, straight, cruciform base. *Xolotrema denotata.*

State b. Thick and round, recurved, socket-like base. *Vespericola columbiana.*

State c. Thick, straight, simple base. *Stenotrema maxillatum.*

State d. Thick to thin, straight to curved, long arcuate sculpted base. *Stenotrema barbigerum.*

State e. Thin, curved, long arcuate smooth base. *Inflectarius inflectus, I. subpalliatus, I. magazinensis, I. smithi.*

State e'. Same as state e, but with arched medial buttress and thickened central extension. *Inflectarius magazinensis, I. smithi.*

State e". Same as state e', but with medial buttress very large and central extension very long, clubbed, and sculpted. *I. smithi.*

State f. Absent. All other examined species.

Figure 11 maps the informative character states onto the phylogenetic hypothesis previously shown in Figure 1. There are 12 informative character states, of which seven appear homoplastic in Figure 11. One of these homoplasies (state 7e') is spurious, because it actually resolves a trichotomy by providing a new synapomorphy uniting *Inflectarius magazinensis* with *I. smithi.* Two of the homoplasies microsculpturally enhance the general shell convergences between *X. fosteri* and *P. laevior* (state 1c) and between *N. major* and *M. normalis* (state 3a). The homoplasies in state 6a involve multiple origins of wrinkles in the periostracum, yet this state is still informative in uniting the three species of *Xolotrema,* for example. The loss of periostracal hairs in *I. ferrissi* (state 7f) accompanied its great evolutionary shifts in shell size and in ecology (Emberton, 1991b). The remaining homoplasies involve the apertural coatings of *Al. profunda* and *M. normalis* (state 1f) and the spiral microstriae of most triodopsins and *Al. profunda* (state 2a). Thus, six of the twelve informative microstructural character states (1b, 1d, 2b, 5a, 7e, 7e') sup-

port the phylogenetic hypothesis without homoplasy.

DISCUSSION

Although this study is preliminary, it offers hope that the shells of polygyrids—and by inference the shells of other land-snail families—are not entirely useless for hypothesizing phylogeny. Thus, although polygyrid gross shell morphology and ontogeny yielded virtually no phylogenetic resolution among subgenera (Emberton, 1995b: fig. 16), polygyrid microsculptural shell morphology has so far yielded potential new informative characters with a 50% (6 of 12) "success rate" in resolving a previously, robustly hypothesized phylogeny (this paper: Fig. 11). Verifying these characters will require much more work, which will also undoubtedly disclose many new microsculptural characters.

Of the new characters tentatively proposed, some are particularly intriguing. Spiral microstriae, a possible new synapomorphy for the tribe Triodopsini, may finally provide a means of distinguishing fossils of this tribe from those of the iteratively convergent tribe Mesodontini (Emberton, 1994a, 1995b). The homology among adult-shell spiral pustules, spiral pustular ridges, and spiral grooves proposed here for *Patera* is extreme, but is in line with Pilsbry's (1940: 576) remark that, in the embryonic sculpture of many polygyrid species, "many stages in the transition from striae to granules are found."

The remarkable radiation of periostracal hairs in *Inflectarius* was unsuspected and raises questions concerning the function of such complex hairs as in *I. smithi.* Likewise, the great discrepancy in hair microstructure between *Stenotrema maxillatum* and *S. barbigerum* raises many questions regarding the origin(s), radiation(s), functions, and phylogenetic-information content of shell hairs in this large genus, almost all species of which have hairs.

Periostracal hair-like or scale-like processes on the shell (Kaiser, 1966; see Saleuddin & Petit, 1983, on the periostracum) have evolved numerous times within the Polygyridae. They evolved at least three times within the Mesodontini alone, for example (Emberton, 1991a). Polygyrid shell hairs date back to at least the Miocene (Roth & Emberton, 1994) and display a wonderful variation in size, disposition, microsculpture, and fra-

gility (Pilsbry, 1940; Solem, 1974: fig. 6; Emberton, 1995b: fig. 4; this paper).

Thus, polygyrids provide an excellent system for testing functional hypotheses regarding shell hairs. These hypotheses, none of which have been tested, include the functions (a) "to repel moist particles" (Solem, 1974) and prevent wet leaves from adhering to the shell; (b) to defend against predators (Webb, 1950); and (c) to camouflage the shell by trapping soil and debris (Pilsbry, 1940: p. 761). Apparently, different functions are served by different shaped hairs (Fig. 9), but this remains to be investigated.

The different types of coatings on the aperture found here were a marked addition to previous SEM discoveries (Solem, 1970, 1972, 1973; Solem & Lebryk, 1976); the compositions and functions of these coatings are unknown. There is evidence, however, that coatings may change with shell age. Thus, in *Daedalochila auriformis,* the parietal denticle of a non-gerontic adult had a coating identical to that of *Inflectarius inflectus* (Fig. 3F), but the parietal dentical of a gerontic *D. auriformis* was smooth and coating-free (Solem & Lebryk, 1976: figs. 33, 37). Although such coatings could possibly be preservational artifacts in the form of dried mucous films, they do not in any way resemble the mucous films illustrated in Solem (1970: figs. 12–15). Clearly, future studies should fully assess intraspecific variation in aperture microsculpture if such characters are to have any value for phylogenetics.

Two proposed microsculptural convergences are remarkable for actually enhancing gross shell-form convergences in sympatry. Thus, *Neohelix major* and *Mesodon normalis* (Emberton, 1994b, 1995a, 1995b: fig. 1) also converge in their body-whorl spiral cords, and *Xolotrema fosteri* and *Patera laevior* (Emberton, 1995b: figs. 1, 17) also converge in their uncoated apertures, revealing nearly identical microsculptures of crystalline projections from a smooth surface. Both these pairs of species are separable, however, by other shell microsculptural features (Fig. 11).

For future work, particularly on periostracal hairs, it can be recommended to use very fresh, live-collected material, preferably alcohol preserved. Dried periostracum can be quite brittle, and thus no unbroken hairs could be found on the shells of *I. inflectarius* or *X. denotata* selected for this study. On the positive side, however, most microstructures
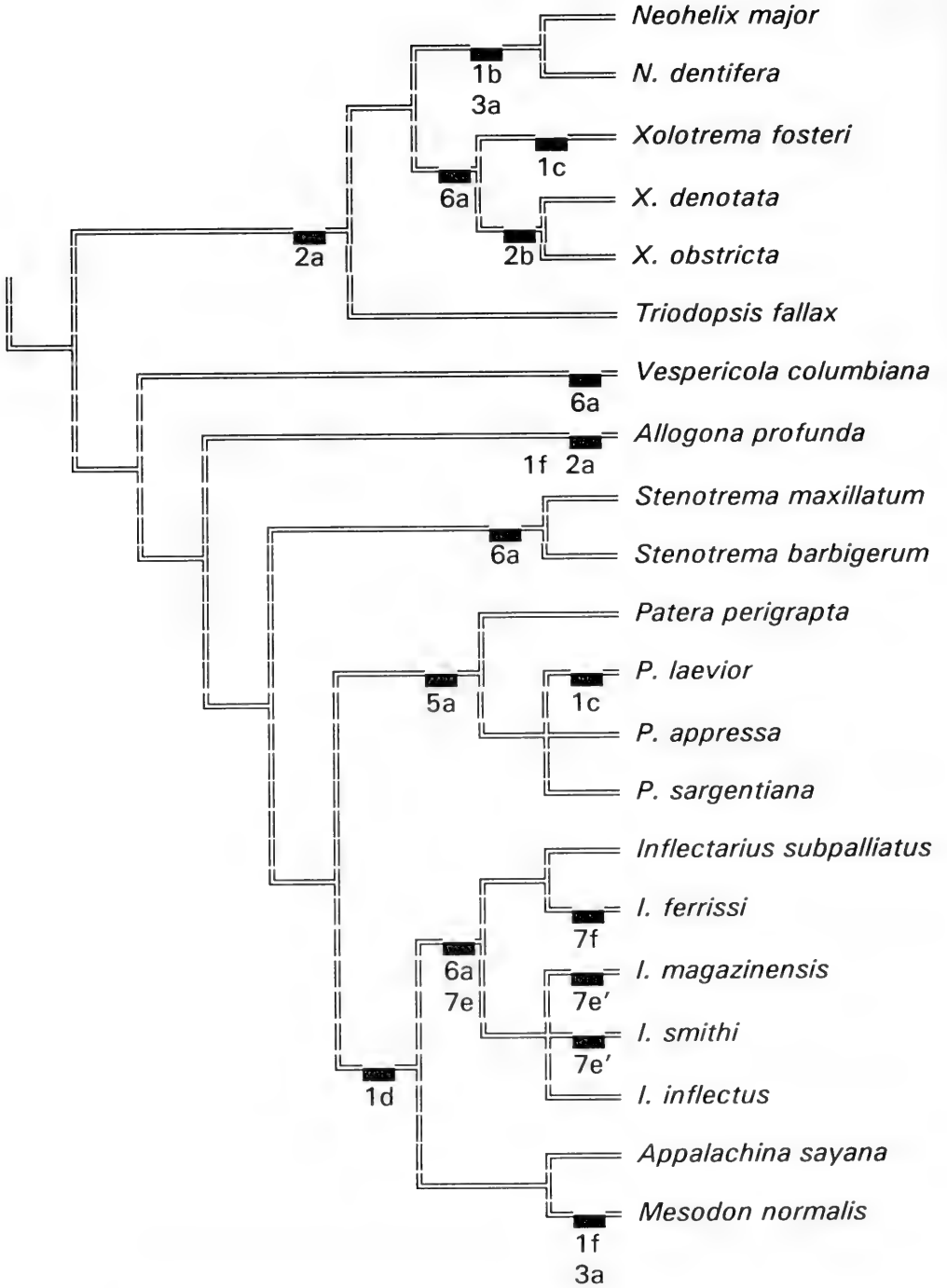
FIG. 11. Map of tentatively proposed microsculptural character states onto the cladogram of Fig. 1. See text for definitions.

remained intact despite the relatively great age of many of the specimens used in this study. Regarding apertural microsculpture, Solem & Lebryk (1976) found clear, uneroded details in subfossil pupillid shells. In preparing shells for SEM, Solem (1970) cautioned against the difficulty or impossibility of removing dried mucous films, which are not a problem in alcohol-preserved material. No such films were noticed in this study.

Polygyrid shell-form evolution may be unique for the sympatry of its convergences (Emberton, 1995a, 1995b), but certainly not for the convergences themselves, which parallel shell-form evolution in other stylommatophoran groups, such as the Helicidae *sensu lato,* Bradybaenidae, and Camaenidae (Zilch, 1959–1960). For phylogenetics of these and other land-snail groups, it can be hypothesized (from this study and Emberton, 1995b) that shell micromorphology is much more informative than shell macromorphology.

ACKNOWLEDGEMENTS

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ANATOMY AND SYSTEMATICS OF *BUCCINANOPS GRADATUS* (DESHAYES, 1844) AND *BUCCINANOPS MONILIFERUS* (KIENER, 1834) (NEOGASTROPODA, MURICOIDEA) FROM THE SOUTHEASTERN COAST OF BRAZIL

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ABSTRACT

A morphological revision of species of the genus *Buccinanops,* endemic to South America, begins with the description of *B. gradatus* and *B. moniliferus.* In an attempt to obtain data to resolve systematic problems from the family to the specific level in this group, a detailed anatomical description of the head-foot, pallial organs, digestive system, including odontophoral muscles, and genital system are given. These animals are blind, have a vestigial valve of Leiblein and, in the case of *B. moniliferus,* there is sexual dimorphism, males being about half of the size of females.

INTRODUCTION

The systematic concepts on the South American neogastropod species *Buccinanops gradatus* (Deshayes, 1844) and *B. moniliferus* (Kiener, 1834) are confused at almost every level.

There a controversy about their at the family-level placement; some authors (e.g., Abbott & Dance, 1983; Rios, 1994) have considered these species to be Nassariidae, whereas others (e.g., Rios, 1985) have included the genus in the Buccinidae. Meanwhile, Ponder (1973: 325) noted that anatomical characters for the separation of these two families have not been established.

At the generic level, *B. moniliferus* was considered to belong to *Dorsanum* Gray, 1847, by several authors (e.g., Carcelles & Parodiz, 1939; Rios, 1994) and *Buccinanops* Orbigny, 1841, by Calvo (1987) and Rios (1985), based on radular characters, and by Pastorino (1993) because of differences from the type species of the genus *Dorsanum, D. miran* (Bruguiére). Both species—*B. moniliferus* and *B. gradatus*—were included in the South African genus *Bullia* Gray, 1834, in early literature (e.g., Reeve, 1846) and by Abbott & Dance (1983) and Allmon (1990).

At the species level, *B. moniliferus* in contrast, is well established, due its distinctive conchological characters. *Buccinanops gradatus,* on the other hand, is a variable species with several synonyms according to some authors (e.g., Rios, 1975), whereas oth-

ers consider these synonyms to be valid species. No convincing arguments have been given to support either position. The available species-group names are: *B. lamarckii* (Kiener, 1834), *B. cochlidius* (Dillwyn, 1817), *B. uruguayensis* (Pilsbry, 1897), and *B. deformis* (King & Broderip, 1832). Aggravating these problems is the fact that neither *B. gradatus* or *B. moniliferus* were described with a specific type locality.

A step in solving these systematic problems may be an anatomical analysis of well localized and identified specimens. This paper includes anatomical descriptions of *Buccinanops moniliferus* and *B. gradatus,* which will serve as the basis for future comparisons.

The specific names are changed to masculine gender herein, following Art. 30(a)ii of the ICZN *Code* for generic names ending in *ops.*

MATERIAL AND METHODS

Part of the studied material belonged to Museu de Zoologia da Universidade de São Paulo (MZUSP) and part was collected by otter trawl by fishermen in Praia Grande, São Paulo, Brazil, and has been deposited in MZUSP, fixed in 70% ethanol.

The anatomical dissections were made using standard techniques. Some anatomical parts, such as the genital organs and anterior region of the digestive system, were dehy-

drated in ethanol series, stained in carmine, cleared and fixed in creosote. Radulae and protoconch were also examined using SEM in the Laboratório de Microscopia Eletrônica do Instituto de Biociências da USP. All drawings were made with the aid of a camera lucida.

The musculature of the odontophore was studied by means of dissection of three specimens of each species preserved with an extended proboscis. The jugal muscles and peroral muscles are not described in detail. For the most part, the muscles are named according to the terminology of Wilsmann (1942).

The synonymic list of *B. gradatus* is not given here, because studies on possible synonymy are continuing.

Abbreviations

aa	anterior aorta
af	anterior furrow of the foot
ag	albumen gland
an	siphoned anus
ao	anterior oesophagus
au	auricle
bm	mantle border
cg	capsule gland
cm	columellar muscle
cv	ctenidial vein
da	duct to anterior digestive gland
dl	duct of the gland of Leiblein
dp	duct to posterior digestive gland
ft	foot
ga	inner gland near anus
gd	gonopericardial duct
gi	gill
gk	glandular part of the kidney
gl	gland of Leiblein
go	gonad
gp	female genital pore
in	intestine
lc	left cartilage
m1 to m14	odontophoral muscles
me	mid oesophagus
mf	muscular fibers
mo	mouth
ne	nephrostome
ng	nephridial gland
nn	nuchal node
nr	nerve ring
nv	nephridial vessel
nw	nephridial wall
od	odontophore

oe	posterior oesophagus
os	osphradium
pa	posterior aorta
pc	pericardic walls
pe	penis
pn	proboscis nerve
pp	penial papilla
ps	penial sinuses
pv	proximal vertex of the cartilages
pw	proboscis wall
ra	radula
rc	right cartilage
rm	radular membrane
rn	radular nucleus
rt	rectum
sd	salivary gland duct
sg	salivary gland
si	siphon
st	stomach
sv	seminal vesicle
te	tentacles
ty	gastric typhlosoles
uc	union between both cartilages
va	vas deferens aperture to pallial cavity
vd	vas deferens
ve	ventricle
vl	valve of Leiblein
vm	visceral mass
vp	villous part of the kidney

Buccinanops gradatus (Deshayes, 1844)
(Figs. 1–3, 6, 9, 12–30)

Diagnosis

Shell generally homogeneous beige in color; subsutural carina generally present, without spines. Osphradium about 2/3 of gill length. Radular rachidian teeth with eight well-spaced cusps that are heterogeneous in size; two well-developed median cusps on lateral teeth. Odontophore with only one pair of "m9" muscles; and with double radular protractor muscle (m14). Both stomach typhlosoles longitudinal. Penis long, with a well-developed papilla. Female genital pore papillate, surrounded by two folds.

Description

Shell: Up to 60 mm in length, homogeneous beige, with up to 8 convex whorls (Figs. 1–3). Protoconch of about 2.5 whorls; first whorl glassy-smooth, semi-spherical; others with strong axial ridges. Limit between protoconch and teleoconch not conspicuous. Teleoconch to 5 whorls; two first whorls with
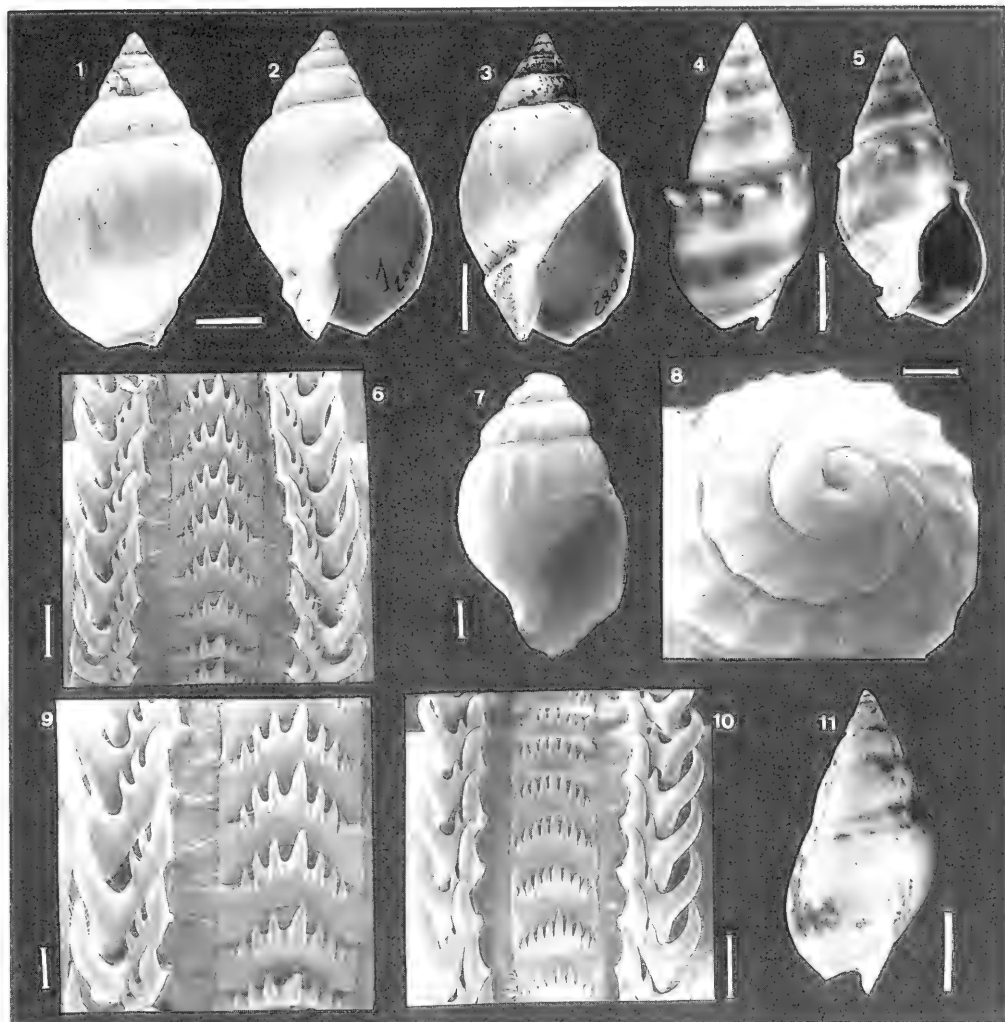
FIGS. 1–11. Shells and radulae: 1, 2, dorsal and frontal view of female of *Buccinanops gradatus* (MZUSP 28079), scale = 10 mm; 3, frontal view of a male of *B. gradatus* (MZUSP 28078), scale = 10 mm; 4, 5, dorsal and frontal view of two specimens of *B. moniliferus* (MZUSP 28191), scale = 10 mm; 6, radula of *B. gradatus*, SEM, scale = 0.2 mm; 7, profile of the protoconch and first teleoconch whorl of *B. moniliferus*, SEM, scale = 1 mm; 8, the same in apical view, scale = 0.5 mm; 9, detail of Fig. 6, scale = 0.1 mm; 10, radula of *B. moniliferus*, SEM, scale = 0.2 mm; 11, dorsal view of a specimen of *B. moniliferus* without developed spines on the subsutural carina (MZUSP 28181), scale = 10 mm.

axial ridges, similar to those of protoconch, gradually disappearing on subsequent whorls. Subsutural carina generally present, low, rounded (Fig. 3). Periostracum very thin, dark-brown, lost on body whorl. Aperture elliptic; outer lip arched, sometimes notched by carina; inner lip concave, covered by thin callus. Canal short, broad, bordered externally by well-developed carina.

There is considerable shell variation; the most common form is shown in Figures 1–3, but specimens with a shorter or taller spire are common. The subsutural carina is lacking in some specimens, resembling *B. cochlidius* and *B. uruguayensis,* whereas others have a well-developed carina and resemble *B. deformis.* The lot MZUSP 28080 has specimens showing both conditions. Several specimens
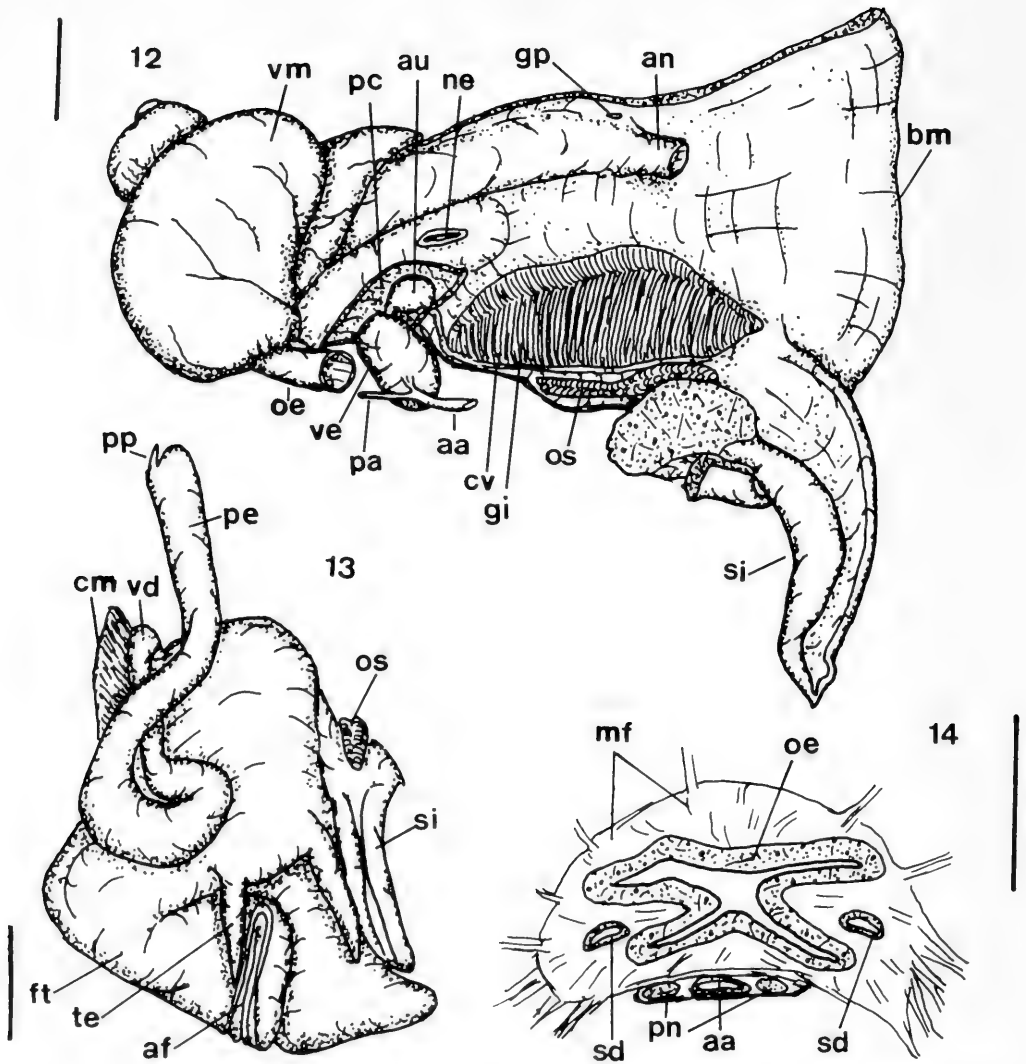
FIGS. 12–14. *Buccinanops gradatus* anatomy: 12, visceral mass and pallial cavity organs of a female, scale = 5 mm; 13, frontal view of a male head-foot, mantle removed, scale = 5 mm; 14, transversal section of the mid region of the anterior oesophagus, scale = 1 mm.

have the spire without a carina and a well-developed carina on the last whorl. No notable shell differences between males and females were found.

Operculum: Corneous, ovate-unguiculate, with terminal nucleus, partially sealing shell aperture. Muscle scar elliptic, near inner border. Operculum deformation very common.

Head-Foot: Homogeneous pale-beige in color. Head somewhat projecting. Tentacles long, lateral, without eyes (Figs. 13, 15). Foot

large, with furrow along anterior edge for anterior pedal glands (Fig. 13: af). Males with large penis, behind right tentacle (Fig. 13). Small posterior metapodial tentacle present.

Mantle Border: Simple, slightly thick. Siphon developed, with smooth borders (Figs. 12, 13). Without pigment or with scanty dark spots.

Mantle Cavity: About one whorl in length (Fig. 12). Osphradium bipectinate, narrow, long (about 2/3 of the total gill length), with

several leaflets on both sides. Gill monopectinate, somewhat elliptic, with numerous triangular, low leaflets. Hypobranchial gland a thin glandular mass covering mantle between gill and rectum.

Circulatory and Excretory Systems: Heart at posterior-right side of pallial cavity (Fig. 12); auricle fusiform; ventricle spherical, very-large. Anterior and posterior aorta as normal for caenogastropods (Fig. 12). Kidney large, behind posterior-left side of pallial cavity (Fig. 12). Internally, kidney with villous and glandular parts (Fig. 28); nephridial gland covering pericardial wall of kidney lumen (Fig. 28: ng). Nephrostome a slit surrounded by muscle fibers, in mid region of kidney wall at posterior end of pallial cavity (Figs. 12, 28: ne).

Digestive System: Proboscis pleurembolic, thick-muscular (Fig. 15), very-long (about same length as shell when extended). Buccal mass about half length of proboscis. Proboscis opening surrounded by thick muscular sphincter. Mouth a vertical slit at distal end of proboscis. Proboscis structure (Fig. 15): odontophore in anterior half attached to inner ventral wall; muscles at posterior odontophore edge running posteriorly and attaching to ventral half of inner proboscis surface up to ventral face of rhynchodeal cavity (Fig. 15: mf). Aorta, paralleled in both sides by a pair of nerves, runs in mid line of ventral surface covering these muscles; oesophagus lies above all these structures, connected to proboscis by tridimentional net of thin muscle fibers.

Odontophore muscles (Figs. 20–26): (m1) dorsal jugal muscles—origin: outer-proximal dorsal wall of odontophore; insertion: inner-dorsal peribuccal wall; (m2) transversal muscle—uniting dorsally outer edge of both cartilages, involving dorsally other muscles of odontophore; (m3) pair of lateral retractor muscles of radula (retractor of pharynx)—origin: in dorsal region of foot, running attached to inner-ventral wall of proboscis; insertion: proximal vertex of each cartilage (pv); (m4) medial retractor muscle of radula—origin: partly in dorsal region of foot, between m3 muscles, running attached to inner-ventral wall of proboscis also between the m3, and partly in ventral face of proximal vertex of each cartilage (mid tensor); insertion: mainly on ventral edge of radula; (m5) dorsal protractor muscle of radula—origin: joined with

medial retractor muscle (m4), bifurcating in mid region of odontophore; insertion: dorsally on both sides of peroral wall; (m6) pair of tensor lateral muscles—lying on anterior half of the outer edge of both cartilages; (m7) pair of small muscles—origin: on outer edge of cartilages just proximal to m6 origin; insertion: on peribuccal wall just proximal to m5 insertion; (m8) small muscle—origin: on outer edges of both cartilages just proximal to m7 origin; near mid region of muscle both branches unite for a short distance and after they separate inserting on ventral region of peribuccal wall near mid line; (m9) pair of small muscles—origin: outer edge of cartilages just proximal to the m8 origin; insertion: dorsal edge of radula; (m10) pair of large lateral tensor muscles of radula—origin: dorsal face of proximal vertex of cartilages; insertion: mainly lateral-dorsal margin of radula, uniting with medial retractor muscle (m4) for about 2/3 of their length (Fig. 22); (m11) horizontal muscle—uniting ventrally inner edge of both cartilages; (m12) ventral jugal muscles—origin: outer-proximal-dorsal wall of odontophore; insertion: inner-ventral peribuccal wall, some muscular fibers more developed (Fig. 24); (m13) pair of large tensor ventral muscles—origin: ventral face of posterior vertex of each cartilage just at medial retractor muscle (m4) origin; insertion: ventral edge of radula; (m14) pair of small protractor muscles of radula—origin: mixed with medial retractor muscle (m4), distinguishable only near horizontal muscle (m11); insertion: ventral edge of radula between tensor ventral muscle (m13) insertion.

Radula (Figs. 6, 9)—Rachidian flattened, arched, with eight well-spaced cusps that are smaller towards outer edges; lateral teeth oblique, each with four cusps, marginal cusp largest, middle two cusps smallest.

Anterior oesophagus lumen "X" in section (Fig. 14), covered by net of radial and oblique muscles uniting oesophagus with inner surface of proboscis wall; salivary gland ducts running on either side of oesophagus (Fig. 14: sd) and discharging into peroral chamber.

Valve of Leiblein vestigial, anterior to nerve ring, poorly visible on outer surface of oesophagus (Fig. 15), marked internally by suddenly change of inner longitudinal folds, forming a low valve (Fig. 16: vl).

Two salivary glands clustered around nerve ring (Fig. 15: sg), their ducts on outer side of nerve ring, running to posterior half of anterior oesophagus and within muscular net
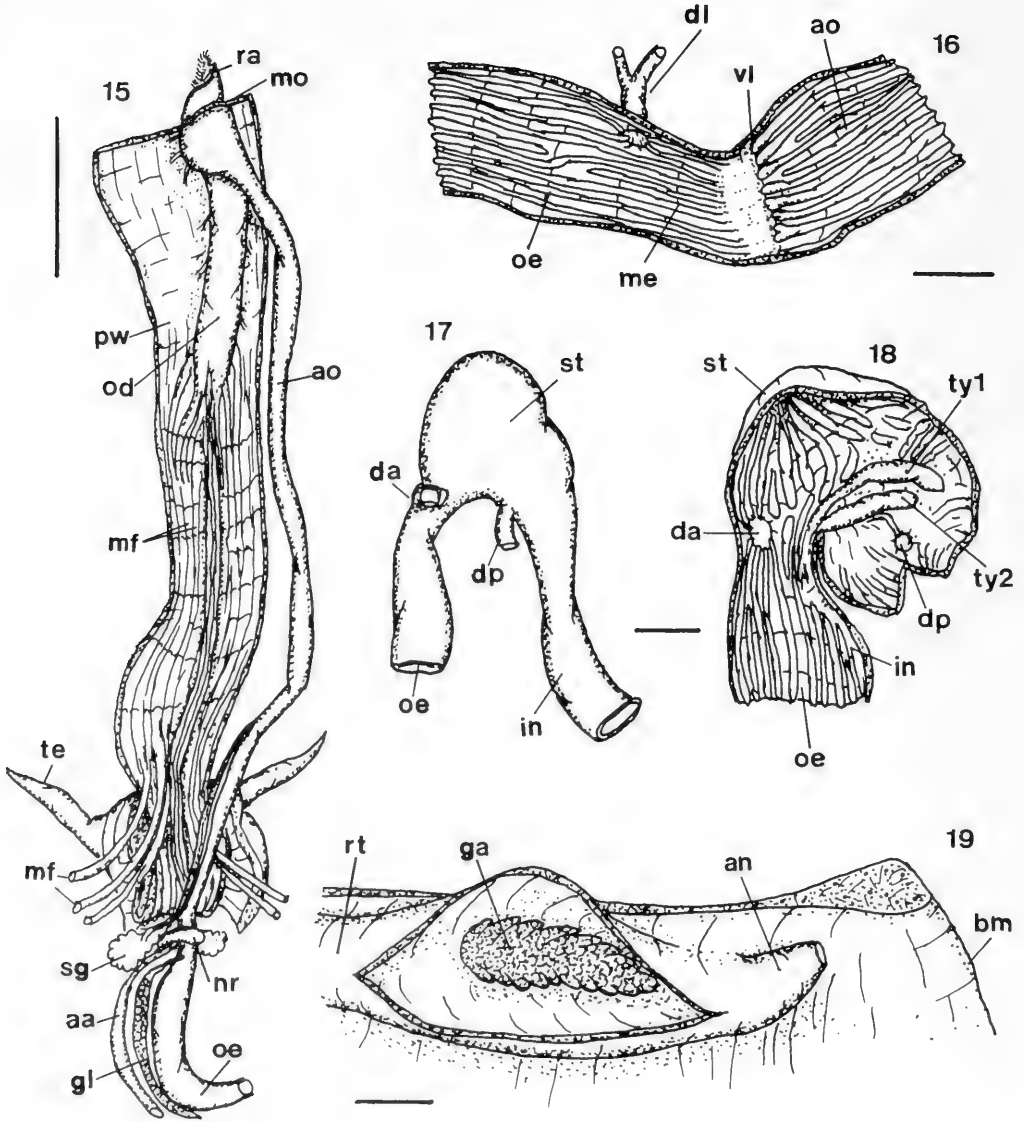
FIGS. 15–19. *Buccinanops gradatus* anatomy: 15, anterior region of the digestive system and proboscis opened longitudinally along dorsal mid line and head mid line, scale = 10 mm; 16, detail of the region of mid oesophagus opened longitudinally, scale = 2 mm; 17, stomach in ventral view, scale = 2 mm; 18, the same opened longitudinally, scale = 2 mm; 19, detail of the anal region, terminal region of the rectum partially opened longitudinally to expose an inner gland, scale = 1 mm.

of anterior half of anterior oesophagus (Fig. 14). No accessory salivary glands present.

Entire oesophagus a long, somewhat uniform, thick muscular walled tube without crop (Figs. 15–17); internally with several longitudinal folds (Fig. 16). Mid-oesophagus very short (Fig. 16: me). Gland of Leiblein

long, thin, with short duct, running posteriorly close to posterior oesophagus (Figs. 15, 16), yellowish-brown in color.

Stomach well developed; walls somewhat thick; two ducts to digestive glands, one dorsal near insertion of oesophagus, the other ventral near opening to intestine (Fig. 17). In-
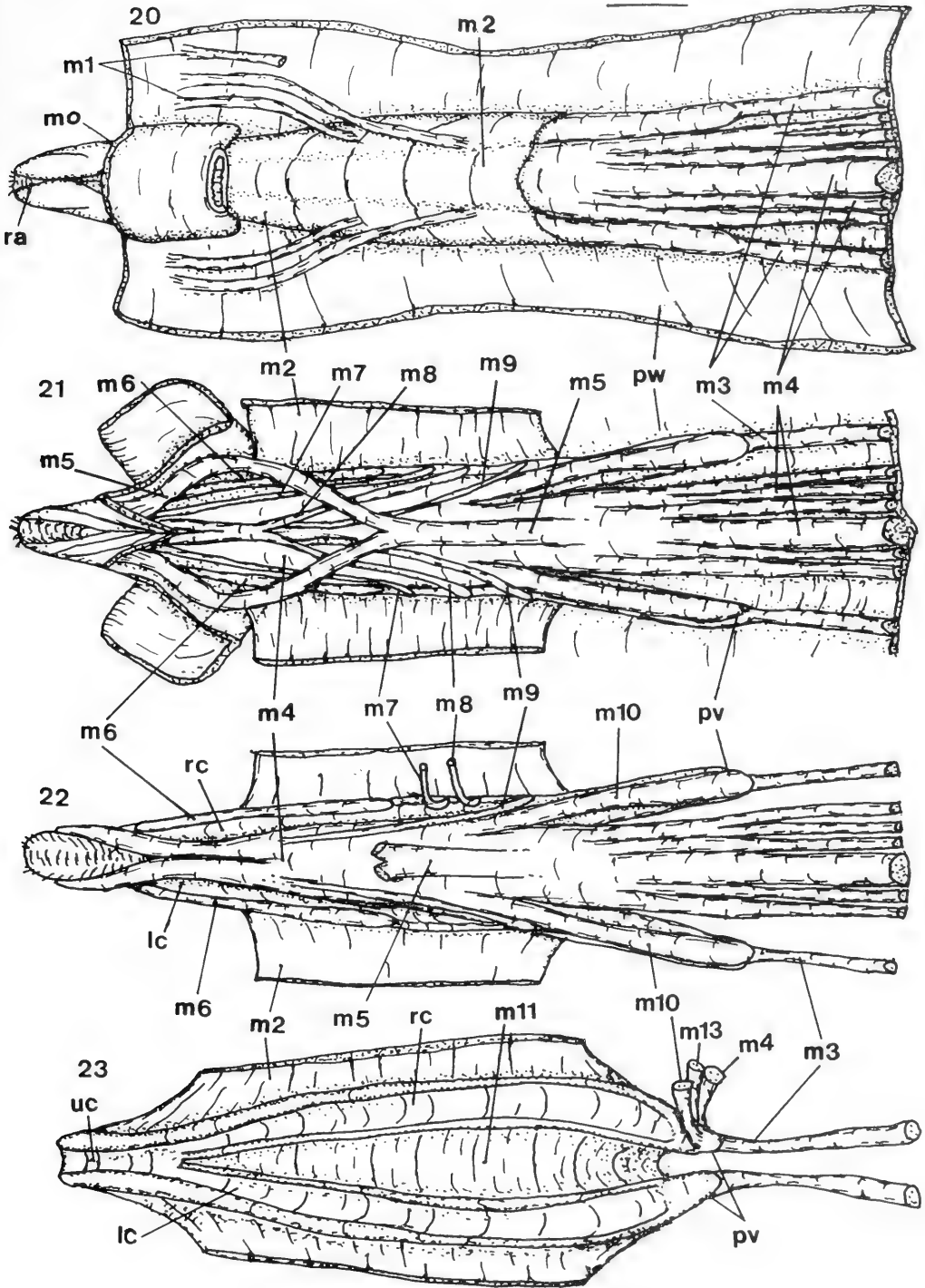
FIGS. 20–23. Odontophore of *Buccinanops gradatus:* 20–23, successive dissection in dorsal view. 20, only proboscis wall opened and oesophagus removed. 21, the outer layer of muscles removed. 22, second layer of muscles removed exposing the inner muscles. 23, most muscles removed to show the cartilages.
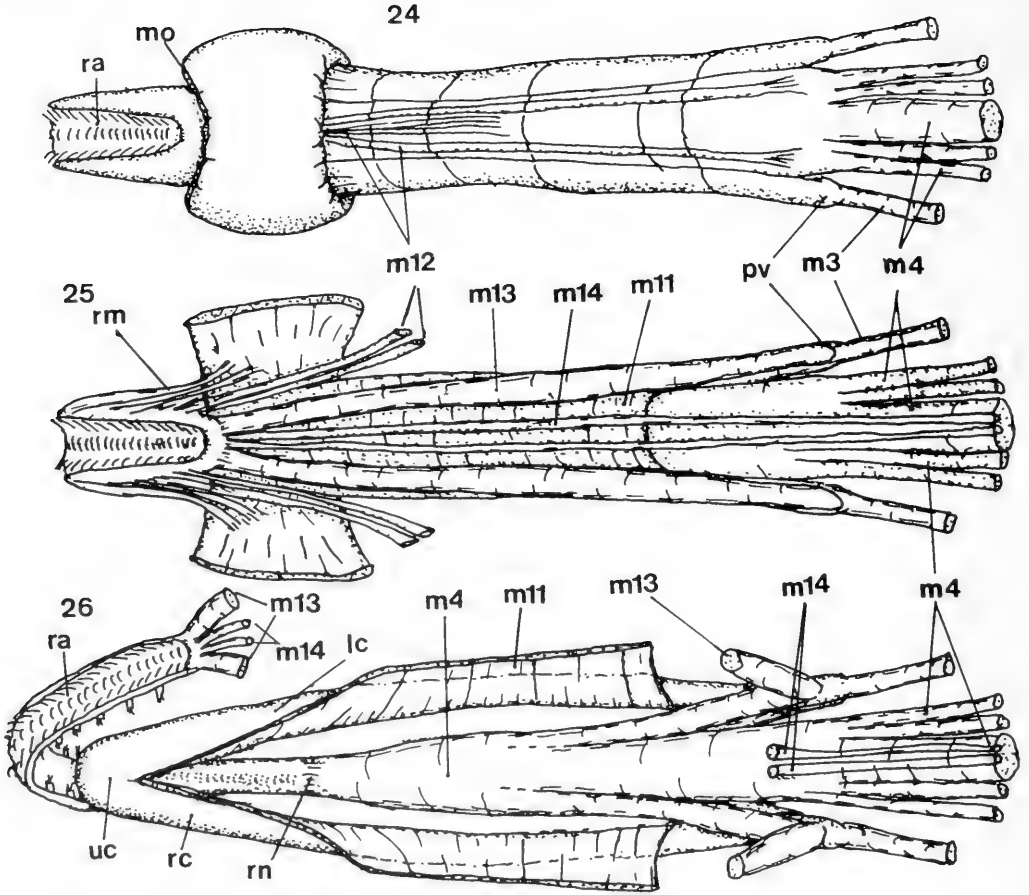
FIGS. 24–26. Successive dissection in ventral view, proboscis entirely removed. 24, outer view of the odontophore; 25, same with first layer of muscles removed. 26, second layer of muscles removed, horizontal muscle (m11) opened longitudinally exposing a part of the dorsal muscles. Scales = 2 mm.

ner stomach surface rich in folds; opposite to digestive gland ducts these folds converging; two ventral typhlosoles present between these ducts (Fig. 18).

Intestine thin-walled, lying anteriorly to kidney (Fig. 28), in right side of pallial cavity in males or close left side of pallial oviduct in females (Fig. 12). Anus siphoned, slight back of mantle border (Figs. 12, 19: an). Internally, a sub-terminal glandular mass present (Fig. 19).

Genital System: Male. Testis in visceral mass near columella; vas deferens initially a narrow duct. Seminal vesicles greatly convoluted just posterior to pallial cavity (Fig. 29). Gonopericaldial duct present, small (Fig. 29: gd). In floor of pallial cavity, vas deferens a closed duct thickened by prostate gland, except in its posterior extremity, where there is a small aperture (Fig. 29: va). Penis narrow, long, internally with a convoluted vas deferens and well-developed sinuses on both sides (Fig. 29); tip rounded, with a small pointed papilla on right side in which the vas deferens opens (Figs. 13, 29).

Female. Ovary in visceral mass mixed with digestive gland, mainly concentrated near columella. Oviduct extremely narrow, on right side of pallial cavity, with well-developed albumen-capsule glands (Fig. 27), both difficult to differentiate from one another, occupying about half of pallial cavity length (Fig. 27). Vestibule thin-walled, somewhat long. Female genital aperture papillated, surrounded
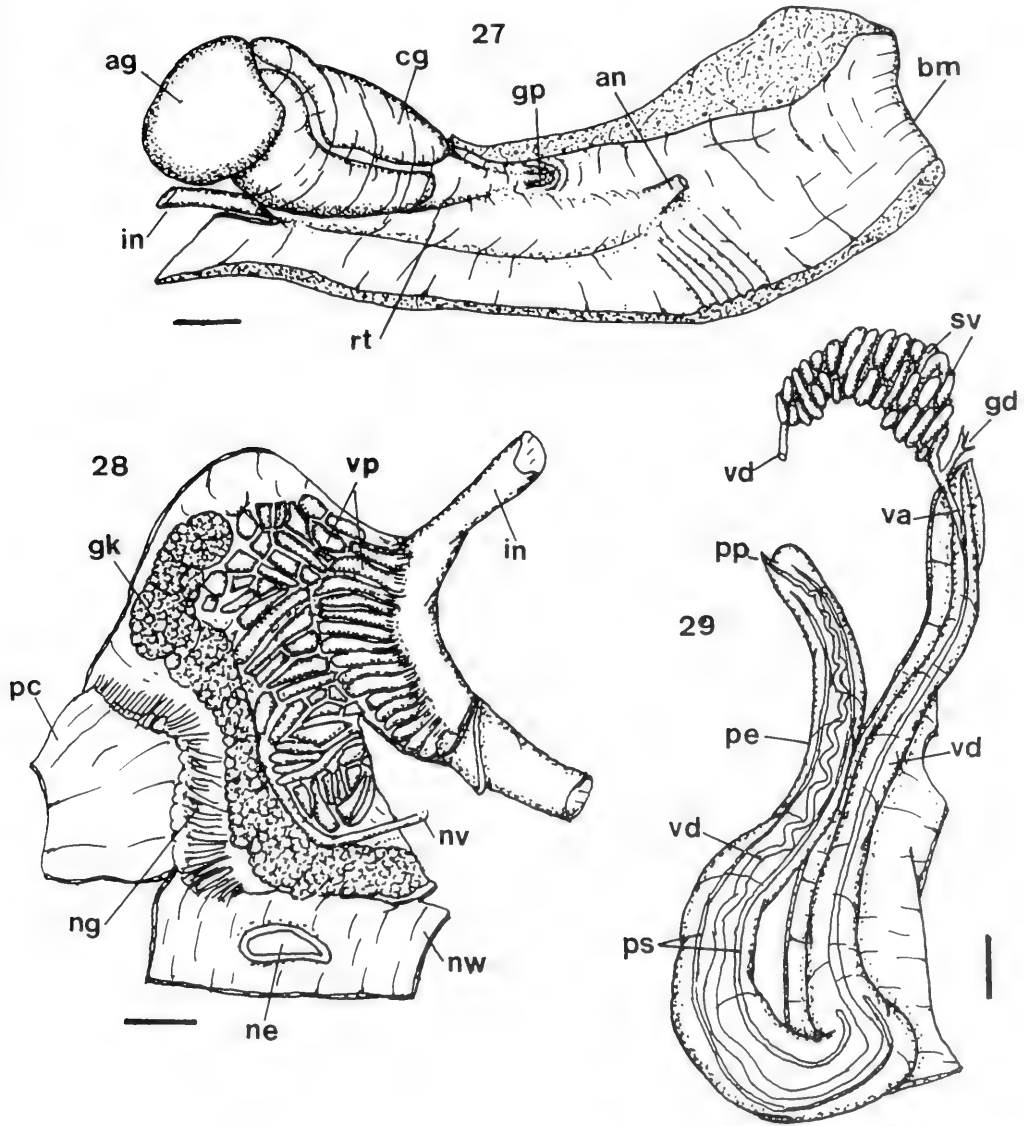
FIGS. 27–29. *Buccinanops gradatus* anatomy: 27, detail of the right side of the pallial cavity in inner view to show the pallial oviduct; 28, kidney chamber opened ventrally; 29, detail of a cleared penis and right side of the pallial cavity floor showing the mid and anterior regions of the male reproductive system. Scales = 2 mm.

by two folds, right fold thin, left fold larger broad (Fig. 30), sited in the posterior-right side of anus (Figs. 12, 27, 30).

Habitat. Sandy-mud bottoms, from 5 to 25 m depth. For data on posture and capsules, see Penchaszadeh (1973).

Range. With certainty from Rio de Janeiro to São Paulo coast; specimens from other regions still under study.

Examined specimens. BRAZIL, otter trawl. Rio de Janeiro: MZUSP 28184, 1 specimen, Cabo de São Thomé (11/ii/1969); MZUSP 15295, 1 specimen, Atafona, São João da Barra. São Paulo: Ubatuba: MZUSP 28080, 8 specimens, Itaquá Beach (i/1971, Montouchet col.); MZUSP 28185, 2 specimens, Cabras Is., Anchieta Is. (28/vi/1978); MZUSP 28186, 2 specimens, Anchieta Is. (4/viii/1960,
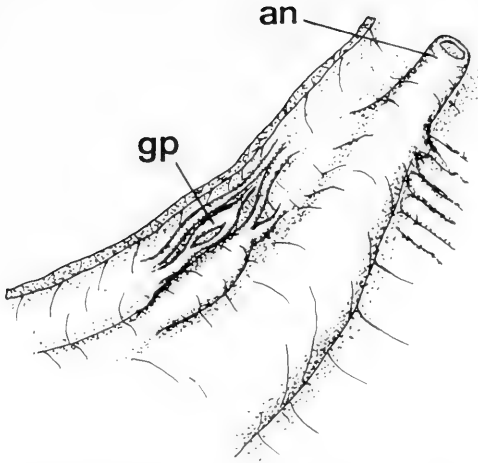
FIG. 30. *Buccinanops gradatus* anatomy: detail of Fig. 27, showing the female genital pore, scale = 1 mm.

Clarimundo col.); MZUSP 28081, 1 specimen, same (21/v/1979). Baixada Santista: MZUSP 28183, 5 specimens, from Barra de Santos to Guarujá (vii/1969, Instituto de Pesca col.); MZUSP 28192, 20 specimens, Perequê Beach, Guarujá (6/vi/1985); MZUSP 28193, 8 specimens, Santos Bay (2/ix/1970); MZUSP 28187, 2 specimens, from Moela Is. to Ponta Perequê (17/v/1962, Clarimundo col.); MZUSP 28188, 5 specimens, Goes Beach, Guarujá (17/viii/1970, Colella col.); MZUSP 28189, 2 specimens, from Barra de Santos to Farol da Moela (vii/1969, Instituto de Pesca col.); MZUSP 28078, 28079, 28082, 25 specimens, Barra de Santos (21/ix/1970, Colella col.); MZUSP 28183, 14 specimens, Moela Is., 15 m deep (17/v/1962, Clarimundo col.). Praia Grande, off Boqueirão Beach: MZUSP 28190, 12 specimens (i/1994, Simone col.); MZUSP 27319, 12 specimens (10/i/1990, Simone col.). Total: 117 specimens.

Buccinanops moniliferus (Kiener, 1834)

(Figs. 4, 5, 7, 8, 10, 11, 31–42)

Buccinum moniliferum Kiener, 1834: 2, pl. 3, fig. 8; Reeve, 1842: 234, pl. 268, fig. 4; Deshayes, in Lamarck, 1844: 191.
Bullia armata Gray, 1854: 26; Reeve, 1846: pl. 1, fig. 2 [Hab. ?]; Adams & Adams, 1858: 113; Kobelt, 1877: 290; Tryon, 1882: 14,

pl. 6, figs. 82, 83; Paetel, 1888: 116; Morretes, 1949: 98.
Buccinanops moniliferum: Orbigny, 1845: 199; Rios, 1985: 103, pl. 35, fig. 456; Calvo, 1987: 143, fig. 122 (radula); Pastorino, 1993: 160–165, figs. 1–3 (radula).
Buccinum (Buccinanops) maniliferum: (err.) Orbigny, 1846: 434.
Buccinanops cochlidium: Gray, 1854: 40 (*non* Dillwyn, 1817).
Buccinum armatum: Küster, 1858: 90, pl. 15, fig. 20.
Bullia (Buccinanops) moniliferum: Chenu, 1859: 160, fig. 750; Abbott & Dance, 1983: 117 (fig.).
Dorsanum armatum: Cossmann, 1901: 218.
Dorsanum moniliferum: Carcelles & Parodiz, 1939: 747, figs. 1, 2; Carcelles, 1944: 249; Rios, 1970: 92, pl. 28; Rios, 1975: 95, pl. 27, fig. 398; Penchaszadeh, 1971a (posture and capsules); Penchaszadeh, 1971b: 480; Figueiras & Sicardi, 1972: 179, pl. 13, fig. 176; Martorelli, 1991 (parasite); Castellanos, 1994: 89, 96, fig. 31–4 (capsule); Rios, 1994: 130, pl. 41, fig. 557.

Diagnosis

Shell generally with two spiral purple bands on each whorl; subsutural carina with regular-spaced spines. Osphradium about half of gill length. Radular rachidian teeth with nine cusps of homogeneous size; generally only one mid cusp on lateral teeth. Odontophore with two or three pairs of m9 muscles; and with single radular protractor muscle (m14). Typhlosoles of stomach perpendicular one another. Male about half of female size. Penis somewhat long, with a small node in tip. Female genital pore single, bordered by bulged thick muscular walls.

Description

Shell: Up to 50 mm in length, with up to seven convex whorls, generally pale-cream, with two broad spiral bands brown-purple on each whorl (Figs 4, 5). Protoconch of about 2.5 whorls; first whorl smooth, others with strong axial ridges and subsutural furrow (Figs. 7, 8). Limit between protoconch and teleoconch not conspicuous. First two whorls of teleoconch with axial ridges, similar to those of protoconch, disappearing on subsequent whorls. Subsutural carina present, with short, uniform, somewhat spaced, triangular

spines turned distally and dorsally (Figs. 4, 5). Periostracum very thin, black, lost on body whorl. Aperture elliptic; outer lip arched, notched by carina, with a low anal sinus; inner lip concave, covered by a thin white callus. Canal short, broad, bordered externally by well-developed carina.

Shell variation is low compared with the preceding species, as shown in Figure 42. In rare specimens, absence of spines in subsutural carina were observed (e.g., MZUSP 28181; Fig. 11). In other specimens, there is a homogeneous purple color, in contrast to the common two spiral bands per whorl. Albino and sinistral specimens are also known.

Operculum: Corneus, ovate-unguiculate, with terminal nucleus, partially sealing shell aperture; muscle scar elliptic near inner border. Operculum deformation very common, rarely lost. One female (MZUSP 28151) has two well-developed opercula side by side, in the normal position.

Head-Foot: Homogeneous pale-beige in color. Head somewhat projecting; tentacles long, lateral, without eyes. Foot large, with furrow along anterior edge for anterior pedal glands (Figs. 31, 32: af). Small posterior metapodial tentacle present.

Mantle Border: Simple, slightly thick (Fig. 33). Siphon developed, with smooth borders (Figs. 31, 33), pigmented by dark-brown irregular spots. Siphon with well-developed muscular root.

Mantle Cavity: About 1.5 whorls in length (Fig. 33). Osphradium bipectinate, narrow, long, with several short leaflets in both sides, lying along about half of gill length. Gill monopectinate, elliptic, long, with numerous triangular, low leaflets. Hypobranchial gland thin, poorly developed, near and anterior to anal region.

Circulatory and Excretory Systems: As described for preceding species (Fig. 33).

Digestive System: Radular rachidian teeth with nine cusps that are somewhat uniform in size and close one-another (Fig. 10); marginal teeth with only one mid cusp (Fig. 10) or rarely with two smaller cusps, the inner cusp longer.

In odontophore, most part of muscles and other structures very similar to that of *B. gradatus*, except that in *B. moniliferus* the small

muscles originating on the outer edge of cartilages and inserting on the dorsal edge of radula (called "m9" in preceding species) are multiple and vary from 2 to 3 successive similar-sized pairs. The small muscle that originates with medial retractor muscle of radula (m14) and inserts on ventral edge of radula near mid line is single (Fig. 34: m14a) and has a part of its fibers inserting ventrally in beribuccal wall also near mid line (Fig. 34: m14b).

Stomach (Fig. 37) similar to that of preceding species, except one typhlosoles is longitudinal, from the oesophagus to the intestine (fig. 38: ty1), whereas the other is transversal, lying duct to posterior digestive gland (Fig. 38: ty2).

All other studied characters of the digestive system of *B. moniliferus* are closely similar to preceding species (Figs. 35, 36), including characters of valve and gland of Leiblein (Fig. 35) and anus (Fig. 33).

Genital System: Male. Testis in visceral mass near columella. Seminal vesicles greatly convoluted (Fig. 39) just posterior to pallial cavity. A small aperture where vas deferens enters floor of pallial cavity (Fig. 39: va); remainder closed, thickened by prostate gland (Fig. 40). Penis narrow, long (Fig. 32), internally a convoluted vas deferens and two well-developed sinuses in both sides (Fig. 40: ps); rounded tip with a very small vesicle on right side in which vas deferens opens (Fig. 40).

Female. Ovary in columellar side of visceral mass not mixed with digestive gland (Fig. 33). Oviduct very narrow. Albumen and capsule glands well developed, difficult to distinguish from one another, occupying about half of pallial cavity length (Fig. 41); vestibule thin-walled, very short. Female genital aperture small, bordered by bulged thick muscular walls (Fig. 41). Two specimens (39.8 mm and 33.0 mm length, MZUSP 28176) have a small node where penis occurs in males (Fig. 31).

Sexual dimorphism. Mature males notably smaller than mature females. Mature male length: 20.3–27.8–36.8 mm. Mature female length: 31.0–43.5–49.5.

Habitat. Sandy-mud bottoms, from 5 to 25 m depth.

Range. From Rio de Janeiro, Brazil, to San Matías Gulf, Argentina.

Examined specimens. BRAZIL, otter trawl. Rio de Janeiro: MZUSP 19591, 1 specimen, sta. IV, 22°06'S, 41°04'W, off Cabo de São
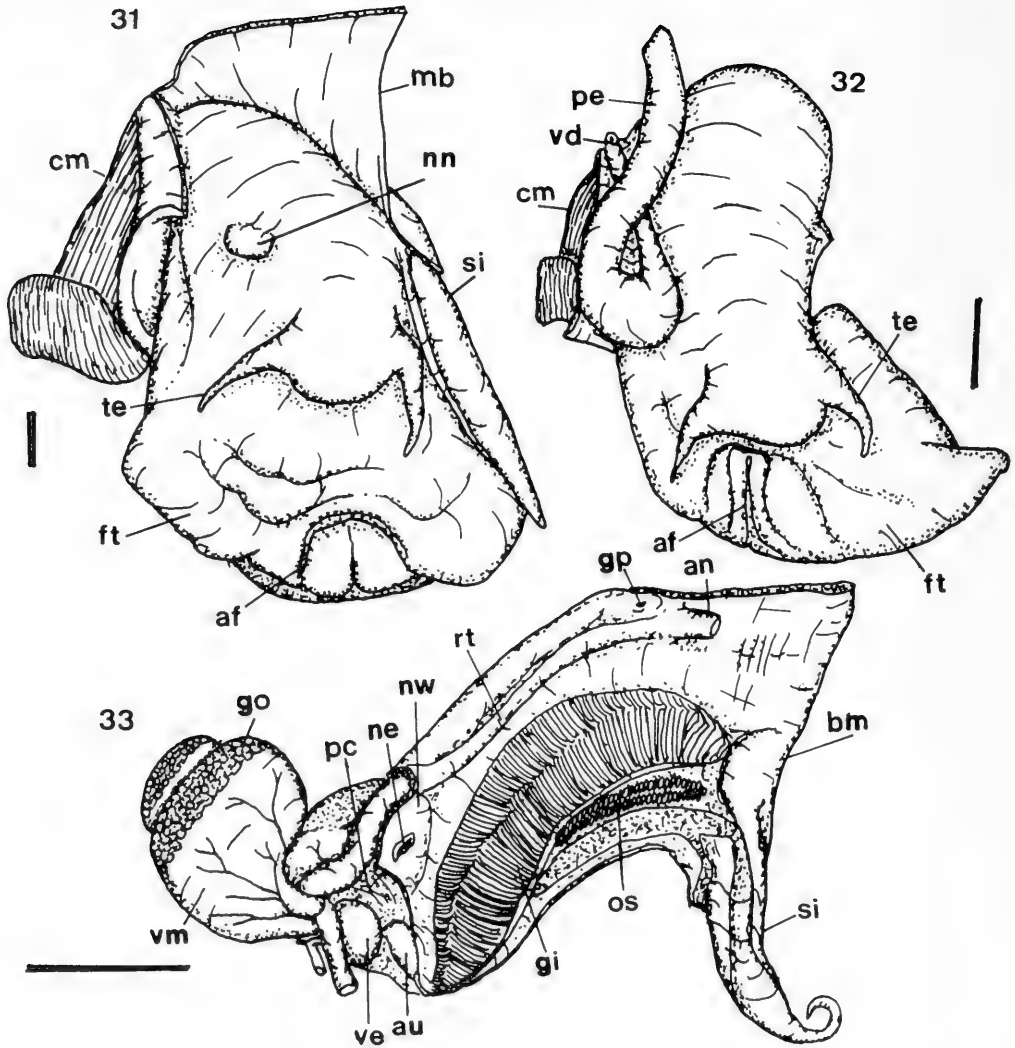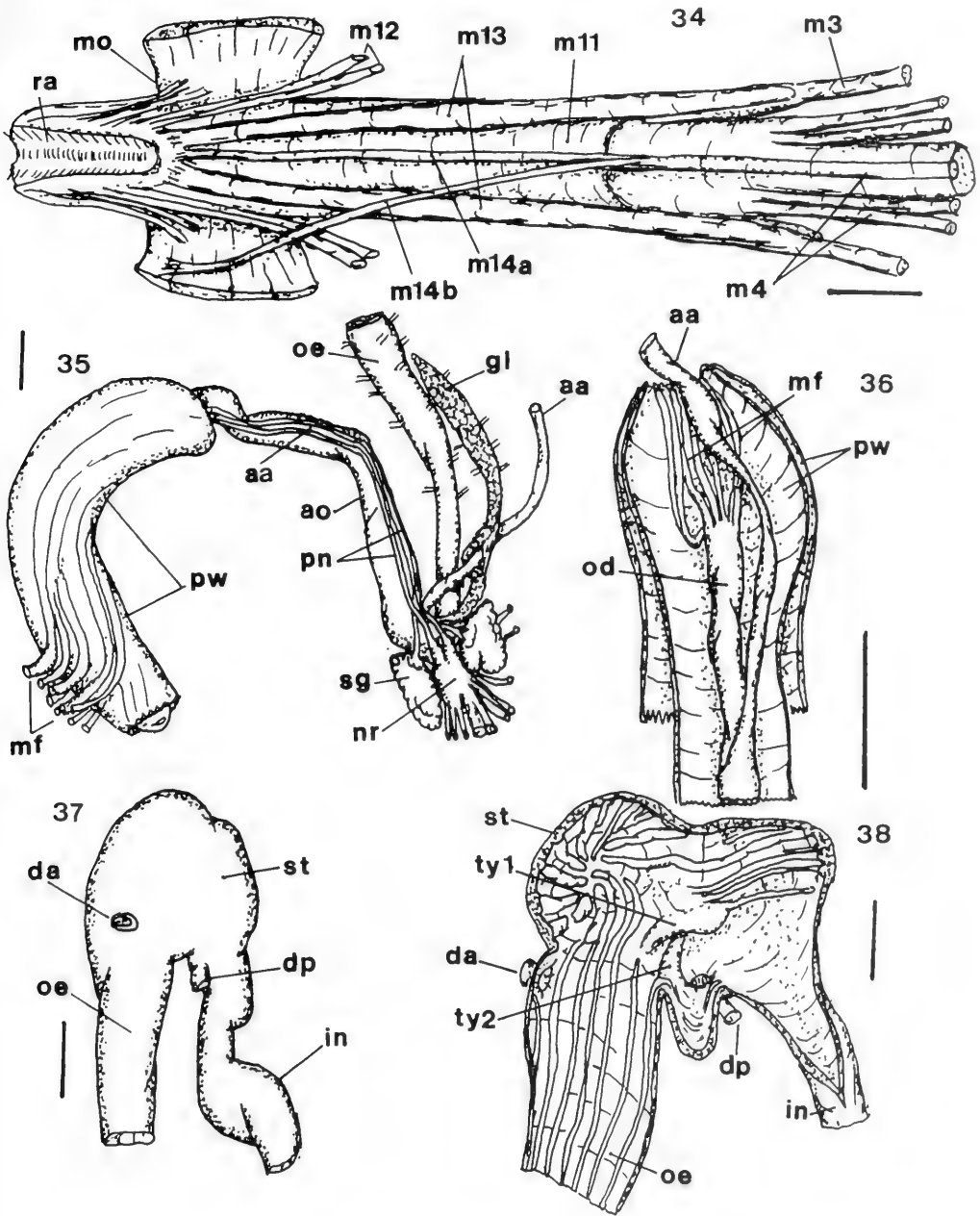
FIGS. 31–33. *Buccinanops moniliferus* anatomy: 31, frontal view of a female (MZUSP 28176) with nuchal node, mantle partially opened, scale = 2 mm; 32, frontal view of the head-foot of a male, mantle and siphon removed, scale = 2 mm; 33, visceral mass and pallial cavity organs of a female in inner view, scale = 10 mm.

Thomé, 16 m (11/ii/1969, "W. Besnard" col.). São Paulo: off Ubatuba: MZUSP 28124, 17 specimens, 22°05'50"S, 41°04'12"W, 10 m (vii/1991); MZUSP 28125, 19 specimens, 22°06'07"S, 41°04'08"W, 13 m (3/1992). Baixada Santista: MZUSP 28179, 1 specimen, from Barra de Santos to Guarujá (vii/1969, Instituto de Pesca col.); MZUSP 28181, 2 specimens, Goes Beach, Guarujá (17/viii/1970, Colella col.); MZUSP 28084, 10 specimens, Perequê Beach, Guarujá (6/vi/

1985). Praia Grande, off Boqueirão Beach: MZUSP 28191, 11 specimens (i/1994, Simone col.); MZUSP 26865, 2 specimens (10/xi/1970, Ribas col.); MZUSP 28175, 20 specimens (i/1994, Simone col.); MZUSP 28176, 46 specimens (i/1990, Simone col); MZUSP 27320, 2 specimens (10/i/1990, Simone col.); MZUSP 28177, 5 specimens (summer, 1994, Simone col); MZUSP 28151, 86 specimens (xii, 1991, Simone col); MZUSP 28152, 17 specimens (summer, 1987, Simone col);

FIGS. 34–38. *Buccinanops moniliferus* anatomy: 34, odontophoral muscles exposed by dissection (compare with the fig. 25), scale = 2 mm; 35, left view of the anterior region of the digestive system, scale = 2 mm; 36, region of the proboscis opened longitudinally in dorsal mid line, scale = 8 mm; 37, stomach in ventral view, scale = 2 mm; 38, the same opened longitudinally, scale = 2 mm.

MZUSP 28153, 18 specimens (i/1987, Simone col). Itanhaém: MZUSP 28180, 1 specimen, Prainha Beach (18/ii/1970, Vaz col.); MZUSP 28178, 13 specimens, Prainha Beach (18/ii/1970, Vaz col.). Total: 271 specimens.
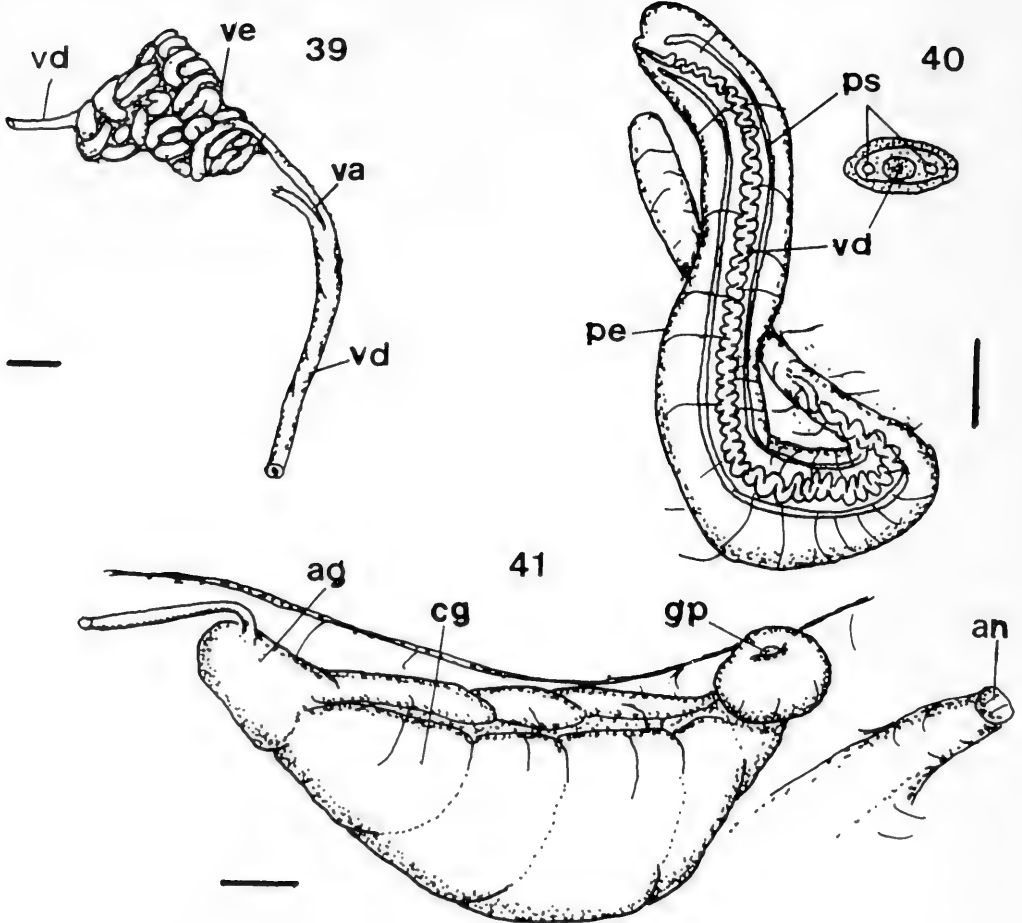
FIGS. 39–41. *Buccinanops moniliferus* anatomy: 39, mid region of the male genital duct in ventral view; 40, dorsal view of a cleared penis and right side of the pallial floor with a detail of a section in mid region of the penis; 41, detail of the right side of the pallial cavity showing the pallial oviduct, scales: 1 mm.

DISCUSSION

Buccinanops gradatus differs anatomically from *B. moniliferus* in having: (1) the osphradium proportionally longer; (2) the rachidian teeth of the radula with fewer, more widely spaced cusp that are less uniform in size; (3) the lateral teeth with two well-developed intermediate cusps (*B. moniliferus* generally has only one or two smaller cusps, see fig. 1 of Pastorino, 1993); (4) only one pair of odontophoral "m9" muscles; (5) double "m14" muscle; (6) stomach with the typhlosoles parallel one another; (7) absence of sexual dimorphism—in *B. moniliferus,* the mature male is smaller than the mature female; (8)

penis proportionally longer, and with the papilla more developed; (9) the female genital pore in form of a small papilla surrounded by two folds, whereas in *B. moniliferus,* it bulges, has thick walls, and is without papilla.

Analysis of the anatomical characters of other species of *Buccinanops* is necessary for any systematic interpretation of the above-cited differences. Probably, based on number and degree of differences, both studied species may belong to close, but different genera. *Buccinanops moniliferus* is maintained in the genus *Buccinanops,* but the generic attribution may change in future. Pastorino (1993) gave a strong argument in favor to the separation of this species from
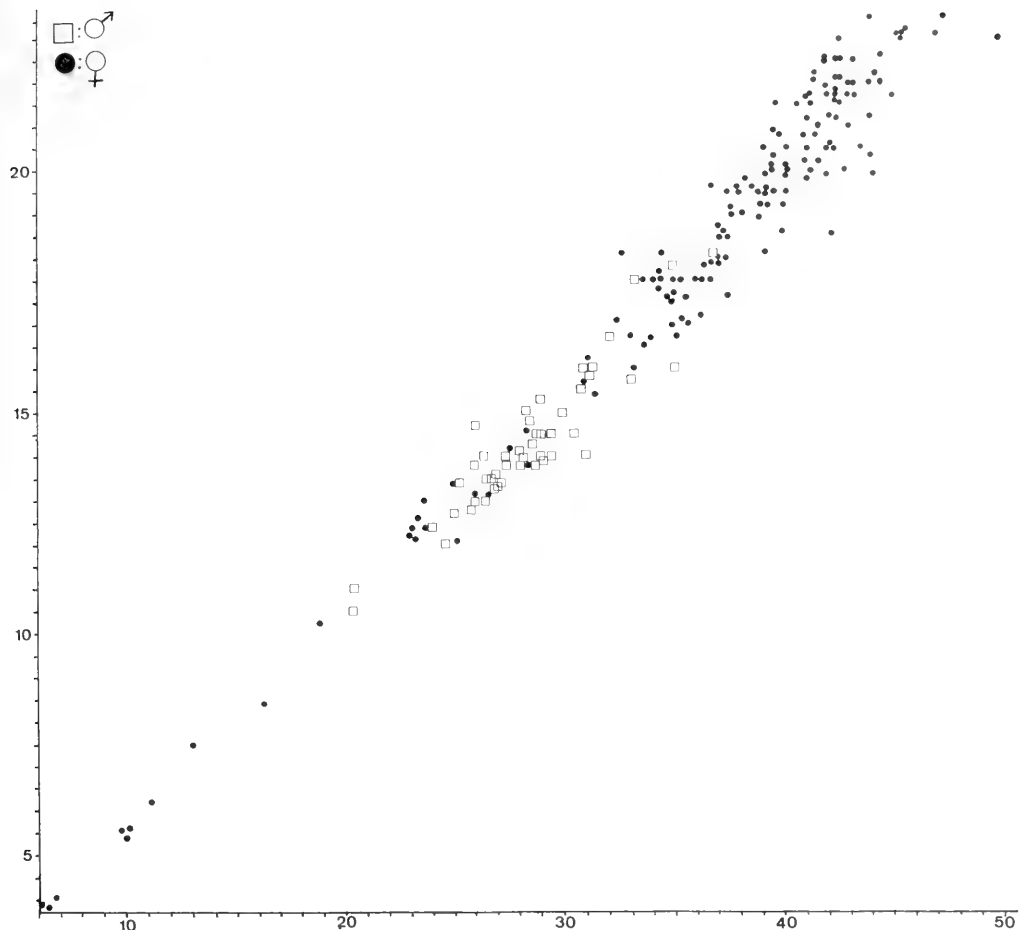
FIG. 42. Graph length × width based on 203 specimens of *Buccinanops moniliferus,* 156 females (dark circles) and 48 males (squares).

the genus *Dorsanum,* based on differences from its type species, *D. miran* from Africa (Allmon, 1990).

The radula of *B. moniliferus* is similar to that of *B. cochlidius* (see Pastorino, 1993: 162, figs. 4–6), but differs in having more cusps on the rachidian, and its largest cusp on the right, not the left side.

Both studied species have some morphological similarity to the European *Buccinum undatum* (Buccinidae) and *Nassarius reticulata* (Nassariidae) (Fretter & Graham, 1962: 214–5, figs. 115–116), differing mainly in hav-

ing tentacles without eyes and by reduction of the valve of Leiblein. The odontophoral muscles of both studied species are similar to those of *Buccinum undatum* (see Wilsmann, 1942), differing mainly in having: (1) the horizontal muscle (m11) shorter, (2) the dorsal protractor of the radula (m5) thinner, (3) the lateral tensor muscle (m10) stronger, and (4) the minor dorsal muscles (m7, 8 and 9) differently arranged. No studies with this level of detail of the *Nassarius* odontophore exists. The ongoing comparative study on the arrangement of the odontophoral muscles of

other *Buccinanops* and *Nassarius* species may add data useful in family-level distinctions.

ACKNOWLEDGMENTS

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SYSTEMATICS, BIOGEOGRAPHY AND EXTINCTION OF CHIONINE BIVALVES (BIVALVIA: VENERIDAE) IN TROPICAL AMERICA: EARLY OLIGOCENE-RECENT

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ABSTRACT

The genus *Chione* ranges from the Early Oligocene of the tropical western Atlantic to the Recent of the tropical western Atlantic and eastern Pacific. The genus is generally considered to comprise the subgenera *Chione s.s., Chionista, Chionopsis, Iliochione, Lirophora, Panchione,* and *Puberella.* A phylogenetic analysis of the subgenera suggests that the genus is paraphyletic, because its current definition excludes the related genera *Anomalocardia, Protothaca* and *Timoclea.* This problem is resolved by converting the taxonomic classification to a phylogenetic one, constituting *Puberella, Chionista, Chionopsis, Iliochione* and *Lirophora,* newly elevated to full generic status.

The first genera to appear in the fossil record are *Lirophora* and *Puberella* in the Early Oligocene of the western Atlantic. The genus *Chionopsis* appears next, in the Late Oligocene of the western Atlantic. *Chione* and *Panchione* first occur in the Early Miocene, also of the western Atlantic. By the Early Pliocene, *Chione, Chionopsis, Lirophora* and *Panchione* were present in the eastern Pacific. During the Pliocene, diversities and distributions of these genera changed dramatically. In the Early-middle Pliocene (5.2-2.5 Ma), both *Lirophora* and *Panchione* suffered severe extinction in the western Atlantic. All supraspecific taxa present in the western Atlantic suffered elevated extinction during the Late Pliocene. Conversely, diversities increased in the eastern Pacific during the Pliocene, added to by the evolution of *Chionista,* and *Iliochione.* The net result is much higher Recent diversity in the eastern Pacific compared to the western Atlantic.

Key words: *Chione,* extinction, Neogene, systematics.

INTRODUCTION

The reasons for diversification and extinction of a lineage are the results of complex interactions between phenotype and environment. While phylogenetic and morphological analyses can lead to an understanding of patterns of phenotypic change, the impetus for such change must come from the organism's biotic and abiotic environments. The potential therefore exists for understanding the evolution of a clade, given knowledge of the relationships among its inclusive taxa, and an understanding of the ecological conditions under which the clade evolved.

This approach is utilized in a phylogenetic and paleoecological study of the Cenozoic tropical American marine bivalve genus *Chione* (von Mühlfeld, 1811). Species assigned to this genus are among the most abundant members of many shallow soft-bottom communities throughout Atlantic and Pacific tropical America (North, Central and South), and have been so since at least the Early Miocene. The genus has remained restricted to tropical America since its first appearance in the Early Oligocene, and occurs today in the Atlantic from South Carolina to Brazil, and in the Pacific from southern California to northern Peru. The purpose of this study is to present an analysis of phylogeny, and a taxonomic revision, of *Chione* subgenera using shell morphological characters. A biogeographic history, focusing on the late Neogene, is also presented using fossil occurrences. The geographic range of the genus is within a region that was subject to several significant geological and oceanographic changes during the late Neogene, notably the uplift of the Isthmus of Panama, and the initiation of Northern Hemisphere cooling. These events are likely to have affected the diversities and distributions of *Chione* taxa. Many other molluscan taxa were affected adversely (Stanley, 1986; Vermeij & Petuch, 1986; Vermeij, 1993), namely by extinction in the Western Atlantic and subsequent restriction to the tropical Eastern Pacific.

The phylogenetic analysis was performed at the subgeneric level, because while mono-

phyly of *Chione* is questionable (as discussed below), species within each *Chione* subgenus share well-defined, discrete character states with little interspecific variation (see, for example, Roopnarine, 1995). The ease with which species can be assigned to subgenera using the combination of the states present within them argues strongly for the monophyly of the currently defined subgenera. The phylogenetic analysis facilitated a taxonomic revision of *Chione,* because the current taxonomy is not consistent with the analysis. The new taxonomy will be more accurate phylogenetically, and therefore offer a more accurate depiction of relationships among the subgenera.

Analyzing the distribution and diversity of *Chione* subgenera revealed patterns of change in geographic ranges. Placing the geological and geographic ranges of the subgenera in a phylogenetic framework allowed changes in biogeography and diversity to be applied to understanding patterns of diversification and extinction. Diversity and extinction of *Chione* taxa were documented by examining the available records of all *Chione* species of the late Neogene in both the Western Atlantic and the Eastern Pacific. These data will be used to test various hypotheses constructed to explain the late Neogene molluscan extinctions in the tropical Western Atlantic. Prior to hypothesis testing, though, it will be essential to present some information explaining the situation of tropical America during the Neogene.

Late Neogene Extinctions in
Tropical America

Tropical America during the late Neogene was the site of dramatic oceanographic changes, coupled with changes in faunal composition and diversity. The two most commonly cited causes of diversity changes are decreasing temperature (Stanley, 1986) and decline or disruption of planktonic productivity levels (Vermeij, 1978, 1987; Allmon et al., 1993; see also Jones & Hasson, 1985). Both mechanisms, however, are plausibly linked to two geological events, the uplift of the Isthmus of Panama, and the initiation of intense Northern Hemisphere cooling.

Uplift of the Isthmus of Panama may have begun as early as the early Middle Miocene (>15.2 mya) (Duque-Caro, 1990). Termination of surficial circulation and final closure probably did not occur, however, until the Early Pliocene (approximately 3.5 mya) (Coates et al., 1992). Separation of the oceans has long been associated with Plio-Pleistocene changes in faunal composition in both the tropical Western Atlantic and Eastern Pacific (Vermeij & Petuch, 1986; Jackson et al., 1993).

Severe late Neogene Northern Hemisphere cooling and glaciation, documented in the deep-sea stable isotopic record (Shackleton & Hall, 1985; Krantz, 1991), the stratigraphic record of North Atlantic coastal deposits (Krantz, 1991; Cronin, 1993) and microfaunas (Cronin, 1991), began about 2.5–2.4 Ma (Shackleton & Hall, 1985; Stanley, 1986; Cronin, 1991, 1993; Allmon et al., 1993). This event, along with later Late Pliocene-Early Pleistocene cooling events, has been linked to molluscan extinctions in the southeastern United States and the Caribbean region (Stanley, 1986). Weyl (1968) hypothesized that closure of the Panama seaway would have intensified the northward flowing Gulf Stream current. The resultant change in the heat distribution and precipitation in the Northern Hemisphere may have initiated the buildup of Arctic ice and the subsequent glaciations. Therefore, the two events may be linked.

Initial observations of the changes in faunal composition were interpreted as a large-scale decline in the diversity of the Western Atlantic molluscan fauna, compared to the Eastern Pacific (Woodring, 1966; Vermeij, 1978; Vermeij & Petuch, 1986). Recent compilations of Pliocene to Recent molluscan faunas in the Caribbean and the southeast United States have modified this interpretation by noting that the Recent molluscan faunas of the tropical Western Atlantic are actually as diverse, or more than their Pliocene counterparts (Allmon et al., 1993; Jackson, 1993). Therefore, extinction must have been matched by speciation and/or invasions (Vermeij, 1993; Vermeij & Rosenberg, 1994). Such observations do not, however, negate the fact that numerous molluscan taxa that were once widespread in the tropical Western Atlantic are now restricted to the Eastern Pacific (Vermeij & Petuch, 1986), or to a few "refuges" in the Caribbean Sea (Petuch, 1982).

A necessary step in describing the late Neogene biological history of this region is to explore plausible reasons why some molluscan taxa suffered declines in diversity in the Western Atlantic during the Late Pliocene,

while others were either unaffected or increased in diversity in the Western Atlantic or Eastern Pacific. One possible approach to this problem is to document the changing diversities within a phylogenetic context or framework, and observe if the nature of diversity change is uniform throughout the clade. Analysis of geographic and character distributions with respect to diversity change may subsequently yield some clues to whether extinction was phylogenetically random, or if it followed a character-based pattern.

A basis for selective extinction is not necessarily recognizable in the results of a phylogenetic analysis if the pattern of extinction is related to a non-phylogenetic property (strictly speaking, for example, geographic distribution). This reason deserves attention because it implies that the basis for extinction could be an environmental perturbation with effects that transcend patterns of evolutionary relationship. The two causes of extinction suggested above, cooling and decline of planktonic productivity, could belong potentially to this class of environmental perturbation. To demonstrate the action of this type of variable, one could document relevant physical (geological) evidence, or more important, predict and test the effect(s) of these agents on the organisms under study. Tests of cooling and declining planktonic productivity as agents of selection are explored more fully in the following sections.

Cooling and Extinction

Stanley (1984) argues that the decimation of tropical bivalves relative to cooler-water species would be an effect of a cooling-based extinction mechanism. This hypothesis requires that an established latitudinal temperature gradient exists at the time of cooling. The gradient would, prior to cooling, have determined the development of recognizable temperate and tropical faunal provinces. Temperate biotas can migrate equatorward during times of global cooling, but tropical biotas have no refuges. Moreover, the provinces would presumably be composites of stenothermal and eurythermal taxa, the differential survivals of which indicate the occurrence of a cooling-related extinction. For example, Stanley (1984, 1986) suggests that the relatively higher survival of eurythermal bivalves from the Upper Pliocene Pinecrest (Upper) Beds of Florida is the result

FIG. 1. Geographic ranges of Recent species of *Chione*.

of a middle-Late Pliocene cooling event. The recognition of the provinces is crucial to the hypothesis, for only then can comparisons be made between provinces located in different thermal regimes. Such a comparison could possibly be made in the late Neogene between the tropical Pacific and Atlantic Gatunian province, and the more northerly, subtropical Caloosahatchian province (Petuch, 1982; Jones & Hasson, 1985) (Fig. 1). Recognizing the provinces has depended traditionally on the identification of resident and endemic species and their ranges (Woodring, 1966; Petuch, 1982), and inferences about the thermal tolerances of these species (Stanley, 1986).

The middle Pliocene (~4.0–2.5 Ma), though not a formal stratigraphic subdivision, is recognizable by indications of a period of global warming following Late Miocene and Early Pliocene cooling events (summarized in Cronin, 1991, and Krantz, 1991). The end of the interval may be marked by a major regression associated with Northern Hemisphere glaciation, and quite evident in coastal deposits of the North Atlantic, for example in the southeast United States (Krantz, 1991). Despite data indicating that the middle

Pliocene was a time of relative global warmth, the Gatunian and Caloosahatchian provinces were unexpectedly cooler than other contemporaneous regions, and were only as warm or slightly cooler than they are today (Jones & Hasson, 1985; Cronin, 1991). Gatunian waters were, however, absolutely warmer than Caloosahatchian during this time, by as much as 10°C in the winter, and 6°C in the summer (Cronin, 1991).

Cronin's (1991) observations do not support Stanley's (1986) cooling hypothesis, because they imply a reduced latitudinal thermal gradient in the Western Atlantic (Cronin, 1991) at a time when the hypothesis would require a highly developed gradient. For example, during the middle Pliocene, summer temperatures south of Cape Hatteras, North Carolina (Fig. 1) were approximately 2.6°C warmer than temperatures north of the cape. The temperature difference today is 8.6°C (Cronin, 1991). Similarly, summer temperatures differed between the southern Caribbean and Florida during the middle Pliocene by approximately 3.8°C, but as much as 4.3°C today (Cronin, 1991).

The cooler temperatures in the Caloosahatchian and Atlantic Gatunian regions during a time of global warmth seem anomalous, but it has been suggested that they could be explained by the existence of upwelling zones (Vermeij, 1978; Jones & Hasson, 1985; Vermeij & Petuch, 1986; Cronin, 1991). According to Cronin's (1991) data, winter temperatures in the Caribbean were an average 1.1°C cooler than today, and in the Caloosahatchian an average of 1.2°C cooler. The cooling hypothesis, however, does not require that middle Pliocene temperatures be absolutely warmer than Pleistocene and Recent temperatures, simply that temperatures declined during the time of extinction.

A simple, faunally-based test of the explanatory power of the cooling hypothesis would be to document the geographic distributions of Chione subclades in the Gatunian and Caloosahatchian provinces during the Pliocene and post-Pliocene. The test depends on the ability to distinguish between the provinces on the basis of faunal composition (in this case excluding Chione taxa). Petuch (1982) demonstrated that the two provinces maintained high levels of endemism, with respect to gastropods, throughout the Early Miocene to Pleistocene. Catastrophic cooling would, in accordance with Stanley's (1984) argument, affect taxa that were restricted to the more southerly Gatunian province more severely than taxa resident in the Caloosahatchian province. In addition, one should expect to observe the migration of Caloosahatchian taxa to Gatunian waters as temperatures in the Gatunian approached pre-cooling temperatures of the Caloosahatchian.

Modern species of Chione are at least partially restricted in their latitudinal distributions by temperature. No species range beyond tropical and sub-tropical regions (Fig. 2), and it can be demonstrated that species' ranges have changed in response to changing thermal regimes. For example, the species Chione undatella (Sowerby, 1835), is abundant in the Upper Pleistocene, interglacial Millerton Formation of northern California. The northernmost extent of the species today is southern California, approximately 640 km to the south. The modern boundaries of molluscan faunal provinces are frequently associated with temperature gradients (Vermeij, 1978), but also with barriers to circulation, for example Cape Hatteras.

Productivity and Extinction

Unlike the cooling hypothesis, a hypothesis of extinction resulting from declining planktonic productivity is not based on any well-documented geological event. Evidence for higher levels of planktonic productivity in the tropical Western Atlantic during the Pliocene is mostly indirect. Petuch (1982) identified Recent "primary" relict molluscan faunas of Mio-Pliocene systematic affinities, off the coast of Venezuela, in cool, upwelling areas of high planktonic productivity. He inferred that the survival of these communities in upwelling areas was indicative of the widespread occurrence of these areas in the Miocene, when the Caribbean was dominated by such communities. The presence of systematically related "secondary" relict communities off the Yucatan Peninsula and Roatan also support this contention (Petuch, 1982). Stanley (1986) hypothesized that a zone of strong upwelling off the southern extent of the Florida peninsula during the Early Pliocene could explain faunal differences between the Caloosahatchian province and the nearby Bahamanian fauna, an idea supported by Cronin's (1991) ostracod paleotemperature data. More recently, Jones & Allmon (1995) have suggested the occurrence of extensive upwelling off the west
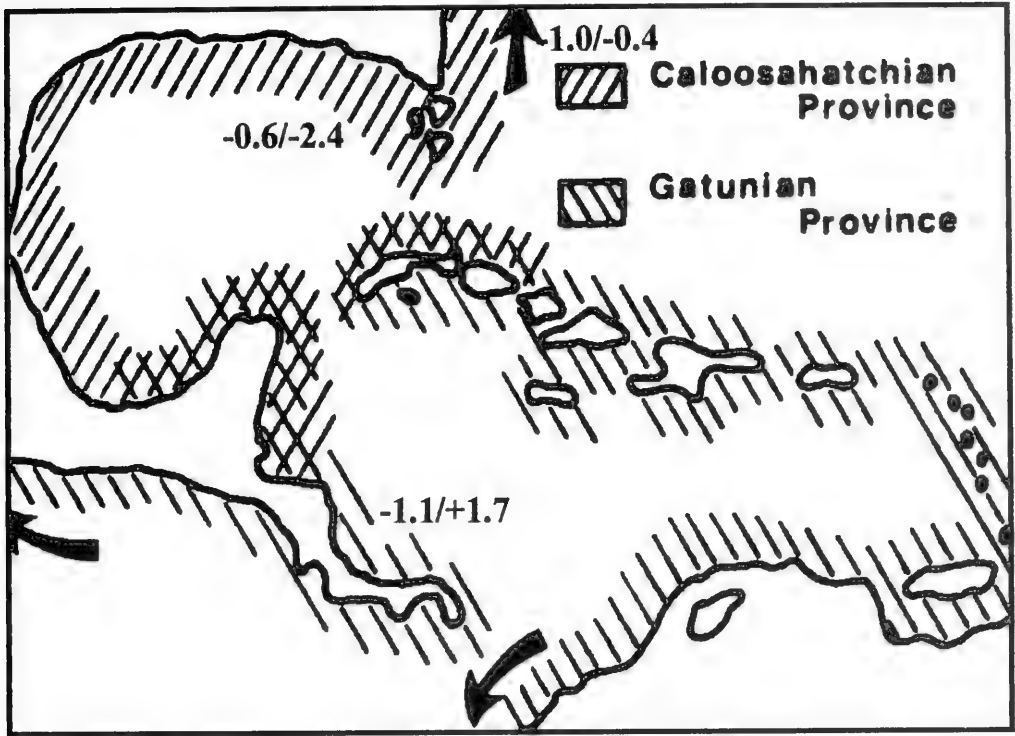
FIG. 2. Petuch's (1982) provincial configuration of tropical America during the middle Pliocene. Arrows indicate that the provinces extend further in those directions. Numbers indicate winter and summer paleotemperature estimates (Cronin, 1991). Map adapted from Jones & Hasson (1985).

coast of Florida during the Early-middle Pliocene, as evidenced by the ontogenetic stable isotopic records of various molluscan taxa. Cronin's (1991) data suggest strongly the existence of extensive upwelling systems off the southeast United States and Central America, during the Pliocene. Cronin (1991) (see also Raymo et al., 1990) speculated that during the middle Pliocene, overall global warmth and reduced amounts of sea ice resulted in a relatively stronger Gulf Stream gyre, which in turn caused upwelling along much of the east coast of North America. Moreover, given the westward direction of surficial flow from the Atlantic to the Pacific, during the Early and middle Pliocene, through what is now Panama, it is conceivable that extensive upwelling existed in surrounding shelf areas (Stanley, 1986). Allmon et al. (1993) proposed that declines in productivity were responsible for the decimation of the rich Pliocene molluscan fauna of southern Florida.

Declining levels of planktonic productivity would result in different levels of extinction among different biogeographic regions if the ecological crisis was more severe in one region than in another, or altogether absent in one or more regions. This, for example, would be the case if the Caribbean in the Early Pliocene was a region with extensive upwelling, and has suffered a subsequent decline in the number of upwelling areas and therefore planktonic productivity (Stanley, 1986; Cronin, 1991). Distinguishing the action of this agent of extinction from others is difficult, because there is no reliable, direct method for assessing levels of productivity in fossil communities. Changes in planktonic productivity could, however, affect community composition. For example, more productive habitats may comprise more species than less productive ones (Brown, 1975; Pianka, 1975), perhaps because higher production provides more resources for successful utilization by more species (MacArthur, 1965).

This type of observation or speculation however, cannot generally be measured in fossil communities, because many organisms and materials (for example, organic detritus) that form the resource bases of ecological communities have low fossilization potentials (Dodd & Stanton, 1990).

One possibly relevant parameter that can be measured easily in the fossil record is the distribution of body sizes within a community. Under conditions of declining suspended food supply, a reasonable prediction is that all suspension-feeding bivalves would be affected. A further prediction is the disproportionately higher extinction of large bodied bivalves. In support of this prediction are observations that: (1) animal body-size scales negatively with population size (Peters, 1983), making larger animals more susceptible to extinction (Vermeij, 1987) under conditions of ecological duress, (2) the rate of nutrient intake scales positively with body size, and (3) conditions of limited resource supply may lead to communities dominated by small bodied taxa (Thiel, 1975; Peters, 1983). Vermeij (1978) also points out that molluscs inhabiting cool, upwelling waters off Venezuela are significantly larger than conspecifics elsewhere in the Caribbean. He attributes the differences to higher juvenile growth rates afforded molluscs in more productive waters, but evidence in support of this remains circumstantial.

This paper presents analyses that demonstrate that the *Chione* subgenera collectively reveal a pattern of greater extinction in the late Neogene of the Western Atlantic relative to the Eastern Pacific, coupled with higher rates of origination in the Eastern Pacific. Phylogenetic revision of the complex, however, further demonstrates that the extinctions were not distributed equally among the subgenera, but seemingly at random. Several closely related subgenera were affected more severely than others, while other subgenera had higher rates of origination in the Eastern Pacific.

A summary of the current taxonomic information of *Chione* is presented in the following section. A compilation of the geological histories of the subgenera is also presented. Hypotheses of extinction caused by cooling or declining planktonic productivity were tested by first examining paleobiogeographic distribution patterns, and then evaluating body size distributions of late Neogene species.

Taxonomic Status of *Chione*

There are approximately 67 described extinct and extant species of *Chione* (although some of these are undoubtedly synonyms) (Table 1). The species have been assigned traditionally, correctly or not, to one of seven subgenera composing the genus-*Chione* s.s., *Chionopsis* Olsson, *Puberella* Fischer-Piette, *Lirophora* Conrad, *Panchione* Olsson, *Iliochione* Olsson, *Timoclea* Brown, and *Chionista* Keen. While ambiguity of taxonomic ranks confuses the relationships among subgenera, this paper will demonstrate that the genus is paraphyletic. The paraphyletic nature of the genus can be resolved by revising *Chione,* and changing the definition and composition of the genus.

The above subgenera are all recognized as members of the subfamily Chioninae Frizzell, 1936, but various authors have argued that at least some subgenera are different enough from the definition of *Chione* (see Palmer, 1927; Olsson, 1961; Keen, 1971, for definitions of *Chione*) to deserve status as separate genera. Therefore, Olsson (1961) regarded *Chionopsis* as a distinct genus, and both Woodring (1982) and Ward (1992) treat *Lirophora* similarly. The latter authors also consider *Panchione* a subgenus of *Lirophora,* contrary to Keen (1969). In addition, it is unclear from a morphological perspective whether some taxa, such as *Chionista,* are members of the *Chione* clade (as currently defined), or are nested within other taxa, such as the related genus *Protothaca* Dall (Keen, 1971). From a taxonomic viewpoint, the boundaries between *Chione* and *Protothaca* become obscured when such subgenera as *Chionista* and *Leukoma* Römer are considered. These problems underscore the value of a phylogenetic approach, in which unique characteristics of taxa are emphasized less, and relationship among them stressed instead. Recently, Harte (1992a, b) has emphasized, using morphological and immunological distance data, the close relationship of *Chione* to the other chionine genera *Mercenaria* Schumacher, and *Anomalocardia* Schumacher. Because of these complications, the *Chione* subgenera will be referred to throughout the rest of the paper collectively as the *Chione* complex.

Other *Chione* subgenera are also problematic from both taxonomic and biogeographic perspectives. For example, *Timoclea,* if it were indeed a subgenus of *Chione* (see for-

TABLE 1. List of documented and described species assigned originally to *Chione* subgenera. Abbreviations for Localities/Range refer to geological formation, and are explained in Table 3. Asterisked species were personally examined by the author. Species that have been reassigned to new higher taxa are noted as such under Comments. Sources consulted frequently were Gardner (1926), Palmer (1927), Grant & Gale (1931), Parker (1949), Hertlein & Strong (1948), Olsson & Harbison (1953), Olsson (1961, 1964), Perrillat (1963), Jung (1969), Keen (1971), Abbott (1974), Woodring (1982) and Ward (1992).

Species	Age	Localities/Range	Author(s)	Comments
Chione				
araneosa*	E.—L. Pliocene	EM, BU	Olsson, 1942	Described from the Burica Fm. (U. Pliocene) (Olsson, 1942), but also present in Esmeraldas Fm. (L. Pliocene)
californiensis*	L. Pliocene—Recent	Baja Sur, Mexico, L. Pleistocene; Pt. Mugu, Calif.—Panama, Recent	Broderip, 1835	
cancellata*	E. Pliocene—Recent	Most shallow water deposits in tropical western Atlantic from m. Pliocene on.	Linnaeus, 1767	
chipolana*	E. Miocene	CH	Dall, 1903	
compta*	Recent	G. of Calif.—Bayovar, Peru	Broderip, 1835	
erosa*	m.—L. Pliocene	CA, JB, PB	Dall, 1903	
guatulcoensis*	Recent	Pt. Guatulco, Mexico—Panama Bay	Hertlein & Strong, 1948	
mazyckii*	Recent	N. Carolina—Cape San Roque, Brazil	Dall, 1902	
pailasana	E.—m. Pliocene	Venezuela	Weisbord, 1964	
primigenia	E. Pliocene	Dominican Rep.	Pilsbry & Johnson, 1917	= *cancellata?*
quebradillensis	E. (?) Pliocene	Puerto Rico	Maury, 1920	= *cancellata?*
santodomingensis	E. Pliocene	Dominican Rep.	Pilsbry & Johnson, 1917	= *cancellata?*
undatella*	L. Pliocene—Recent	SD; s. Calif.—Paita, Peru, Recent	Sowerby, 1835	*allisoni* (Hertlein & Grant, 1972) = *undatella*
Chione				
subimbricata*	Recent	G. of Calif.—Paita, Peru	Sowerby, 1835	
tumens*	L. Pliocene—Recent	Baja Calif., Plio.; MZ, Pleist.; Baja Calif., Pacific coast and G. of California	Verrill, 1870	
vaca*	E.—m. Pliocene	EM, BU	Olsson, 1942	
Chionista				
cortezi*	Recent	Pacific coast Baja Calif., and Gulf of Calif.	Carpenter, 1864	
fluctifraga*	L. Pliocene—Recent	Upper Pliocene, Baja Calif.; s. Calif.—Gulf of Calif.	Sowerby, 1853	
Chionopsis				
amathusia*	Recent	G. of Calif.—Mancora, Peru	Philippi, 1844	
eurylopas	M. Miocene	BO	Woodring, 1982	
gnidia*	Recent	G. of Calif.—Peru	Broderip & Sowerby, 1829	
jamaniana	Pliocene—Recent	Pliocene, Ecuador; Punta Pasado, Ecuador, Recent	Pilsbry & Olsson, 1941	
ornatissima	Pliocene—Recent	Pliocene, Ecuador; Panama—Ecuador, Recent	Broderip, 1835	
posorjensis	L. Oligocene	UB	Olsson, 1931	
procancellata*	m.—L. Pliocene	PB, JB, CA	Mansfield, 1932	formerly *Chione*
propinqua	M. Miocene—E. Pliocene	BO, ZO, DA	Speiker, 1922	

(continued)

TABLE 1. (*Continued*)

Species	Age	Localities/Range	Author(s)	Comments
rowleei	L. Miocene	GT	Olsson, 1922	
tegulum[*]	L. Miocene	GT	Brown & Pilsbry, 1911	
Chionopsis				
walli	Miocene(?)	Manzanilla, Trinidad	Guppy, 1866	formerly *Chione*
woodwardi[*]	E.—L. Pliocene	BW, GB, AX; Cumana, Venezuela	Guppy, 1866	formerly *Chione*
Iliochione *subrugosa*[*]	L. Pliocene—Recent	Baja Sur, Mexico, L. Pliocene; G. of Calif.—Peru, Recent	Wood, 1828	
Lirophora				
athleta[*]	E.—L. Pliocene	PB, CA	Conrad, 1862	
alveata[*]	L. Miocene	SM	Conrad, 1831	
ballista	E. Oligocene	SI	Dall, 1903	
carlottae	E. Pliocene	CE, GB	Palmer, 1927	
caroniana	L.(?) Miocene	Springvale, Trinidad, W.I.	Maury, 1925	
chiriquiensis	E. Pliocene	LI	Olsson, 1922	
clenchi[*]	L. Pleistocene— Recent	L. Pleist., Louisiana; Texas —G. de Campeche, Mexico, Recent	Pulley, 1952	
dalli	L. Miocene	ES	Olsson, 1914	
discrepans	Recent	Nayarit, Mexico—Islay, Peru	Sowerby, 1835	
ebergenyi	E. Pliocene	PN	Bose, 1906	
falconensis	L. Miocene— E. Pliocene	AL, AN, GT, UR	Hodson, 1927	
hendersoni[*]	L. Pliocene	BW	Dall, 1903	
latilirata[*]	E. Miocene— Recent	BE, CA, CV, JB, PB, WA, YT, ZO; N. Carolina—Brazil, Recent	Conrad, 1841	numerous morphs probably representing different species
mariae[*]	Recent	G. of Calif.—Guayaquil, Ecuador	d'Orbigny, 1846	
obliterata	Recent	Pacific Mexico—Panama(?)	Dall, 1902	
paphia[*]	Recent	West Indies—Brazil	Linnaeus, 1767	
quirosensis	M. Miocene	CU. BO	Hodson, 1927	
riomaturensis	E. Pliocene	MA	Maury, 1925	
sellardsi[*]	E. Miocene	CH	Gardner, 1926	
tembla	L. Miocene	AN	Olsson, 1964	
victoria	E. Oligocene	Vicksburg Group, RB, MS, Mississippi	Dall, 1903	
vrendenburgi	L. Miocene	ES	Ward, 1992	
Panchione				
burnsii[*]	E. Miocene	CH	Dall, 1900	formerly *Lirophora*
funiakensis[*]	E. Miocene	CH	Gardner, 1926	formerly *Lirophora*
holocyma[*]	E. Miocene— L. Miocene	CH, GT; Chesapeake region	Brown & Pilsbry, 1911	formerly *Lirophora*
hotelensis	L. Miocene	GT	Olsson, 1922	formerly *Lirophora*
kellettii[*]	E. Pliocene— Recent	PN, G. of Calif.— Peru	Hinds, 1845	formerly *Lirophora;* not *Mercenaria* (as in Harte, 1992a)
mactropsis[*]	L. Miocene	AN, GT	Conrad, 1855	formerly *Lirophora*
parkeria	E.—M. Miocene	CV, PM	Glenn, 1904	formerly *Lirophora*
Panchione				
trimeris[*]	E. Miocene	CH	Gardner, 1926	formerly *Lirophora*
ulocyma[*]	m. Pliocene	JB, PB	Dall, 1895	formerly *Lirophora*
Puberella				
bainbridgensis[*]	E. Oligocene	E. Oligocene of Mississippi, Alabama, Georgia and Florida	Dall, 1916	= *spenceri,* Cooke, 1919; formerly *Chione*
cortinaria[*]	E. Miocene	CH; Murfreesboro Stg., Virginia	Rogers, 1835	formerly *Chione*
cribraria[*]	m.—L. Pliocene	PB, CA; Duplin Stg., N. Carolina	Conrad, 1843	formerly *Chione*

TABLE 1. (*Continued*)

Species	Age	Localities/Range	Author(s)	Comments
intapurpurea*	Recent	N. Carolina—Brazil	Conrad, 1843	formerly *Chione*
montezuma*	Recent	Costa Rica—Panama	Pilsbry & Lowe, 1932	formerly *Chionopsis*
morsitans*	L. Pliocene	CA	Olsson & Harbison, 1953	formerly *Chione*
olssoni	Recent	Ecuador	Fischer-Piette, 1969	
pubera	L. Miocene (?)—Recent	L. Miocene, Trinidad(?); West Indies—Brazil, Recent	Valenciennes, 1827	
pulicaria*	Recent	G. of Calif.—Tumaco, Colombia	Broderip, 1835	
purpurissata	Recent	G. of Calif.—Ecuador	Dall, 1902	
sawkinsi*	E.—L. Pliocene	CE, BW	Woodring, 1925	formerly *Chione*

example, Keen, 1971), would be the only sub-genus to range beyond the Americas, being found also in the Indo-Pacific and eastern Atlantic. However, there are several fundamental morphological differences between *Timoclea* and other members of the *Chione* complex; the sculpture is almost entirely radial, except for irregularly raised points on the radial lines, which together form an apparent cancellate pattern. Also, the pallial sinus tends to be deeper than the normal condition for *Chione,* and the hinge plate is not bowed ventrally, an uncommon condition in *Chione.*

Similarly, the species *C. (Chione) subimbricata* (Sowerby) and *C. (Chione) tumens* (Verrill), were originally classified in the Western Atlantic and Indo-Pacific genus *Anomalocardia* (Hertlein & Strong, 1948), but were reclassified in *Chione s. s.* (Olsson, 1961). Olsson (1961) argued correctly that *C. subimbricata* possesses the hinge characteristics and some of the sculptural characters of *Chione.* *Chione tumens* is essentially a larger version of *C. subimbricata,* and has been considered by some to be a subspecies of *C. subimbricata* (for example Keen, 1971). Olsson (1942) also described a closely related species from the Lower Pliocene Burica Formation of Panama, *Chione vaca* Olsson. Phylogenetic analyses reported later in this paper support Olsson's (1961) assignment of these taxa to *Chione s. s.,* despite the obvious and unique nature of their sculpture.

Given the obvious taxonomic confusion of *Chione* and related genera, it is doubtful that the examination of diversity patterns and adaptation within the current genus could yield evolutionarily meaningful results. Such an examination presumes that the genus is monophyletic, but this presumption cannot be legitimized until a phylogenetic analysis of the taxa (subgenera) within the genus is undertaken, and the taxonomic relationships and character transformations within the clade established.

Geological History

The *Chione* complex ranges geologically from the Early Oligocene (Rupelian Stage) (Dockery, 1982) to the Recent, and appears to be restricted to tropical, primarily shallow, New World waters (Olsson, 1961). The earliest occurrences are of *Puberella* and *Lirophora* in the Lower Oligocene Byram Formation of Mississippi (Dockery, 1982), and *Puberella* in Lower Oligocene strata of Antigua (Dockery, 1982). Dall described one of the Byram species as *Chione (Chione) bainbridgensis* (= *spenceri* Cooke). The type of surface sculpture and the depth of the pallial sinus, however, suggest that this species is more closely related to *Puberella pubera* (Saint-Vincent). *Lirophora* had extended its range to the Eastern Pacific by at least the Late Miocene, occurring in the Zorritos Formation of Peru (Woodring, 1982; see Duque-Caro, 1990, for age of formation). *Puberella,* on the other hand, while diverse today in the Eastern Pacific, has no documented fossil record in that region.

Chionopsis first occurs in the Late Oligocene (Chattian Stage?) Upper Bohio Formation of Panama (Caribbean side) (Woodring, 1982). It was widespread in both the Eastern Pacific and Western Atlantic during the Miocene, but is today a paciphilic taxon, having become extinct in the Western Atlantic by the end of the Pliocene. The earliest documented occurrence of *Chione* s.s. is in the species-rich Lower Miocene (Burdigalian Stage) Chipola Formation of northwestern Florida (*Chione chipolana* Dall, 1903). The taxon reached the Eastern Pacific by at least the Early Pliocene (Roopnarine, unpub-

TABLE 2. Species used in the phylogenetic analysis. *Chione* subgenera are listed first. Specimens belonging to all species, with the exception of *Lirophora victoria,* were examined by the author.

Genus/subgenus	Species	Locality/Formation
Chione	*cancellata*	Jamaica (Recent)
	chipolana	Chipola Fm., Florida (Lower Miocene)
Chionisata	*fluctifraga*	Gulf of California (Recent)
Chionopsis	*amathusia*	Pacific Panama (Recent)
Iliochione	*subrugosa*	Pacific Panama (Recent)
Lirophora	*victoria*	Lower Oligocene (Dockery, 1982)
	athleta	Caloosahatchee Fm., Florida (Upper Pliocene)
Panchione	*mactropsis*	Gatun Fm., Panama (Upper Miocene—Lower Pliocene)
	ulocyma	Lower Pinecrest Beds, Florida (middle Pliocene)
Puberella	*cribraria*	Waccamaw Fm., South Carolina (Upper Pliocene)
Chione	*tumens*	Gulf of California (Recent)
Anomalocardia	*auberiana*	Florida, Recent
	flexuosa	Brazil (Recent)
Mercenaria	*mercenaria*	South Carolina (Recent)
Protothaca	*asperrima*	Pacific Panama (Recent)
Timoclea	*marica*	Guam (Recent)

lished), and is today widespread in both the tropical Western Atlantic and Eastern Pacific.

The Chipola Formation is also the first occurrence of *Panchione,* even though *Panchione* species have at times been classified as *Lirophora* species (Gardner, 1926). Woodring (1982) however, likens the type of *Panchione, P. mactropsis* (Conrad) (Late Miocene, Gatun Formation, Panama), to another species, *P. ulocyma* (Dall) from the Chipola Formation. It is clear from the description of *Panchione* (Olsson, 1964) that the earliest species occur in the Chipola Formation. Woodring (1982) incorrectly states that *Panchione* persists in the Western Atlantic only until the Late Miocene, for species similar (if not identical) to *P. ulocyma* occur in the Upper Pliocene Caloosahatchee Formation of Florida. *Panchione* is today paciphilic, being represented by a single Eastern Pacific species *P. kelletii* (Hinds) (Woodring, 1982).

The two remaining subgenera, *Chionista* and *Iliochione* have brief fossil records. Both have their earliest documented occurrences in Upper Pliocene deposits of Baja California (Durham, 1950). Also, the earliest documented occurrence of the *Chione vaca-subimbricata-tumens* species trio is in the Lower Pliocene Esmeraldas Formation of Ecuador (*C. vaca,* personal observation). All three taxa are today restricted to the Eastern Pacific.

MATERIALS AND METHODS

Phylogenetic Analyses

Twenty-seven morphological characters were described for species from all the sub-

genera discussed above. Type species were used whenever specimens were available. Type species examined include: *Chionopsis amathusia* (Philippi), *Chione cancellata* (Linnaeus), *Panchione mactropsis* (Conrad), *Iliochione subrugosa* (Wood), *Chionista fluctifraga* (Sowerby), *Mercenaria mercenaria* (Linnaeus), *Anomalocardia flexuosa* (Linnaeus), and *Protothaca (Leukoma) asperrima* (Sowerby). The type species of *Timoclea, T. ovata* (Pennant), was not available, so the Indo-Pacific species *T. (Glycydonta) marica* (Linnaeus) was used instead. A complete list of the species used in the analysis is given in Table 2. Characters were obtained from both left and right valves, utilizing the asymmetry typical of chionine valves, and are discussed in more detail in Appendix I. The numerically coded data set is presented in Appendix II. Specimens from the following collections were examined: California Academy of Sciences, Field Museum of Natural History, Florida Museum of Natural History, Tulane University Geological Collections (the collections of Drs. Emily and Harold Vokes), University of California Berkeley (Museum of Paleontology), the private collection of Dr. Geerat Vermeij, and the author's own collection.

The data were analyzed, and phylogenetic hypotheses constructed, using PAUP 3.11 (upgrade of PAUP 3.0, Swofford, 1991). The branch-and-bound algorithm was used to provide an exact solution to the search for a most parsimonious cladogram. All equally most parsimonious solutions were retained. Non-binary characters were unordered and scaled to equal weight based on the number of states per character (PAUP option

WEIGHT SCALE; Swofford, 1991). This method of weighting ensures that characters with three or more states do not dominate the resulting trees (Swofford, 1985).

The ingroup comprises the subgenera *Chione, Chionopsis, Puberella, Lirophora, Panchione, Iliochione, Chionista* and the *Chione tumens* group. The genera *Mercenaria, Anomalocardia, Protothaca* and *Timoclea* were treated as outgroup taxa. The outgroup taxa were not constrained to be outgroups with respect to the ingroup (i.e., the cladograms were not rooted with the outgroups), nor was an *a priori* hypothesis of relations among the outgroup taxa included in the analysis. In fact, preliminary analyses (Roopnarine, unpublished) constraining these genera to be outgroups indicated that the ingroup cannot be monophyletic with respect to the outgroup taxa. The cladograms of the present analysis are unrooted.

Optimal character state trees were reconstructed after analysis according to the ACCTRAN and DELTRAN criteria. ACCTRAN maximizes character reversals, and minimizes convergences (Swofford, 1991). This criterion is therefore a conservative test of parallel and convergent evolution, which is suspected for many chionine character states. DELTRAN forces character transformations in the opposite direction, favoring convergence over reversals. A comparison of the transformations formulated by each algorithm allows a comparison of alternative evolutionary pathways of chionine characters. Character transformations that are supported by both algorithms could be considered particularly robust. Finally, the results of the analysis were used as a basis to revise the current taxonomic arrangement of *Chione* taxa.

Biogeographic Analysis

The purpose of this analysis was to examine the geological and geographic distributions of species within each of the *Chione* subgenera, and thereby arrive at conclusions about subgeneric survival and restriction during the late Neogene. The primary focus was a consideration of differences between the Eastern Pacific and the Western Atlantic, and between the Gatunian and Caloosahatchian provinces. Based on previous work (Woodring, 1966; Vermeij & Petuch, 1986; Stanley, 1986), higher levels of extinction are predicted to occur in the Western Atlantic compared to the Eastern Pacific. More recent work (Allmon et al., 1993; Jackson et al.,

1993) suggests that the extinctions in the Western Atlantic might be matched by speciation, whereas the Eastern Pacific should have a higher overall rate of origination.

A list (Table 1) was compiled comprising all species assigned to the following taxa: *Chione, Chionopsis, Puberella, Lirophora, Panchione, Iliochione,* and *Chionista.* The genus *Anomalocardia* was also included, based on the results of the phylogenetic analyses. Several species were re-assigned to new subgenera based on a reconsideration of subgeneric definitions (Table 1), and some species names were synonymized on the basis of the information gathered by specimen examination and literature descriptions. All these changes are noted on the species list for each subgenus (Table 1). Of the 90 species listed in Table 1, specimens belonging to 47 of them were examined by this author. Thirty-nine of the 47 species examined originated after the Miocene. Literature sources listed in Table 1 were used to obtain information pertaining to the time of first appearance, and geological and geographical ranges of each species. A complete listing of geological formations considered is given in Table 3, along with ages, and source of age information.

Many Neogene deposits in tropical and sub-tropical America remain poorly dated, due to a combination of poor stratigraphic resolution, the lack of continuous sequences with index fossils, and discontinuous geological study. Several recent advances have started to resolve the situation, but on primarily regional scales (Duque-Caro, 1990; Jones et al., 1991; Krantz, 1991; Coates et al., 1992; Jones, 1995). Therefore, "consensus" ages were assigned to many of the formations listed, based on faunal characteristics and the most recent age estimates available.

The paleobiogeographic data cover the Early Oligocene to the Recent. In order to summarize the general biogeographic history of the subgenera, each chronological epoch was divided into sub-epochs, according to Harland et al. (1990). Chronological stages were not used because that resolution is simply not yet available for many formations. In each sub-epoch interval, the number of species in each subgenus was listed, along with the geographic locations or range of the species (Table 1). Each location or range was assigned to one or both of two general regions: the tropical Western Atlantic or Eastern Pacific. Central American deposits and the species therein which pre-date Isthmian uplift (middle Pliocene) were considered to

TABLE 3. List of all geological deposits and formations considered in this study. Abbreviations are used throughout the text. References were used as sources of most current age assignments or reevaluations.

Formation	Abbreviation	Age	Location	Reference
Aguequexite	AX	middle Pliocene	Atlantic Mexico	Jackson et al., 1993
Alhajuehla	AL	Late Miocene	Pacific Panama	Woodring, 1982
Angostura	AN	Late Miocene	Ecuador	Duque-Caro, 1990
Bermont	BM	Early Pleistocene	Florida	Lyons, 1991
Bowden	BW	Late Pliocene	Jamaica	Stanley, 1986
Burica	BU	middle Pliocene	Pacific Panama	Coates et al., 1992
Byram	BY	Early Oligocene	Mississippi	Dockery, 1982
Caloosahatchee	CA	Late Pliocene	Florida	Lyons, 1991
Calvert	CV	E.—Middle Miocene	Maryland	Ward, 1992
Cercado	CE	Early—middle Pliocene	Dominican Republic	Saunders et al., 1986
Chipola	CH	Early Miocene	Florida	Bryant et al., 1992
Culebra	CU	Middle Miocene	Atlantic Panama	Duque-Caro, 1990
Daule	DA	L. Miocene—E. Pliocene	Ecuador	Duque-Caro, 1990
Duplin	DU	Early—middle Pliocene	South Carolina	Krantz, 1991
Eastover	ES	Late Miocene	Virginia	Ward, 1992
Esmeraldas	EM	Early Pliocene	Ecuador	Duque-Caro, 1990
Gatun	GT	Late Miocene	Atlantic Panama	Coates et al., 1992
Gurabo	GB	Early—middle Pliocene	Dominican Republic	Saunders et al., 1986
Jackson Bluff	JB	middle Pliocene	Florida	Lyons, 1991
La Boca	BO	Middle Miocene	Pacific Panama	Woodring, 1982
Limon Group	LI	middle—Late Pliocene	Atlantic Costa Rica	Coates et al., 1992
lower Pinecrest Beds	PB	middle Pliocene	Florida	Jones et al., 1991
Matura	MA	Early Pliocene	Trinidad, W. I.	Jung, 1969
Mint Spring	MS	Early Oligocene	Mississippi	Dockery, 1982
Montezuma	MZ	Early Pleistocene	Pacific Costa Rica	Coates et al., 1992
Murfreesboro Stage		Early Miocene	Maryland	Ward, 1992
Penita	PN	Early—middle Pliocene	Pacific Panama	Coates et al., 1992
Red Bluff	RB	Early Oligocene	Mississippi	Dockery, 1982
Rio Banano	RN	Early—middle Pliocene	Costa Rica	Coates et al., 1992
San Diego	SD	Late Pliocene	California	Hertlein & Grant, 1972
Silex Beds	SI	Early Oligocene	Florida	
St. Mary's	SM	Late Miocene	Virginia	Ward, 1992
Upper Bohio	UB	Late Oligocene	Pacific Panama	Woodring, 1982
Upper Pinecrest Beds	PB	Late Pliocene	Florida	Jones et al., 1991
Urumaco	UR	M(?)—L(?) Miocene	Venezuela	
Waccamaw	WA	Late Pliocene	South Carolina	Krantz, 1991
Yorktown	YT	Early—middle Pliocene	Virginia	Krantz, 1991
Zorritos	ZO	Late Miocene	Peru	Duque-Caro, 1990

belong to a region based on characteristics of faunal composition and whether they are located on the Pacific or Atlantic sides of the isthmus. Contemporaneous Neogene deposits on opposite sides of the isthmus are often very different faunistically (Duque-Caro, 1990; Coates et al., 1992), possibly reflecting the action of oceanographic barriers, the age of initial isthmus uplift, and differences in tectonic and sedimentary histories. The diversity within each region was then documented by summing the number of species in each of the two regions for successive sub-epoch intervals. Levels of speciation and extinction were assessed as the numbers of first and last occurrences per region per sub-epoch. Note that by adopting this approach, extinctions can only be constrained to the sub-epoch following a last occurrence. Biostratigraphic discontinuity for a subgenus does not necessarily represent true extinction, but is also dependent on stratigraphic continuity, which as already noted is problematic for much of Neogene tropical America.

Testing the hypothesis that cooling caused the late Neogene extinctions requires a fine-scale latitudinal resolution of biogeographic distributions. Due both to the absence of widespread, stratigraphically continuous late Neogene sections in tropical America, and the coupled uncertainties in paleobiogeographic boundaries, latitudinal resolution is limited. Using Petuch's (1982) scheme, I have divided the entire region into three areas (Fig. 2): the tropical Pacific Gatunian Province, which was roughly equivalent to the Recent Panamic Pacific region; the Atlantic Gatunian Province, which encompassed the modern day Caribbean Sea; and the Caloosahatchee Province, which extended from the Florida peninsula to South Carolina. The Florida peninsula during the Pliocene may have represented a zone of transition between the tropics and sub-tropics (Stanley, 1986). Species from Florida were assigned, at least initially, to the Caloosahatchian category, a decision supported in Roopnarine (1995).

The last occurrences of species within each subgenus were placed within this framework for the Early-middle (5.2–2.5 mya) and latest Pliocene (2.4–1.6 mya). A general analysis of extinction levels was performed by summing the number of species within each province during each sub-epoch. The analysis was also performed using time intervals based on the latest age estimates of relevant deposits. A hypothesis of cooling, as formulated by Stanley (1984), cannot be rejected if a higher level of extinction is noted in the tropical Atlantic Gatunian Province compared to the subtropical Caloosahatchian Province, after 2.4 Ma.

Body Size

As noted earlier, declining levels of planktonic productivity in the late Neogene tropical Western Atlantic has been cited as a proximal cause of the extinctions. Changing the nutrient supply to a community should alter the trophic composition of the community. Hypothetically, larger-bodied species are affected more adversely than smaller, trophically equivalent (all suspension-feeders) species. This statement is based on the following observations: large-bodied species, while numerically inferior with respect to smaller species, nevertheless account for comparable quantities of biomass (Stanton & Nelson, 1980; Stanton et al., 1981; Staff et al., 1985); and while smaller poikilotherms ingest relatively larger quantities of food than do larger ones (the amount of food ingested by poikilotherms is roughly four times their metabolic rates), the *rate* of nutrient intake scales positively with body size (Peters, 1983).

$$I = 0.78W^{0.82}$$

where I = ingestion rate and W = body weight (Peters, 1983).

In order to test a hypothesis of declining levels of planktonic primary production, maximum body size was documented for as many Atlantic Pliocene, and Atlantic and Pacific post-Pliocene chionine species as were available. Body size was defined simply as maximum valve height, and was measured with digital calipers to the nearest 0.01 mm. The number of specimens examined for each species was noted (see Results, Table 4), to caution that this type of analysis is prone to sample-size bias. Articulated valves were counted as single individuals. Species collections with many specimens may have increased chances of containing very large specimens (due purely to sampling bias), analogous to the rarefaction relationship between species richness and sample size observed in ecological studies (Sanders, 1968). This potential source of bias is compensated for, however, by the observation that small-bodied species are more abundant in deposits than larger species, are therefore generally represented by larger samples, and hence have an increased probability of containing specimens near the maximum size of the population from which the sample was derived. Empirical rarefaction curves could not be used in this instance to verify the observation quantitatively, because the derived curves would not be independent of time (Raup, 1975).

The maximum body size of a species was defined as the body size of the largest specimen measured. In cases when a species was not represented adequately in one of the museum or author's collection, maximum size was taken from a literature description of the species, primarily from Abbott (1974), Keen (1971) and Palmer (1927).

Many fossil species are known from single localities or very restricted geographic areas. A review of Table 1, however, will show that many extant species have very large geographic ranges, and therefore undoubtedly exhibit ecologically based variation. Therefore, when documenting body size for Recent
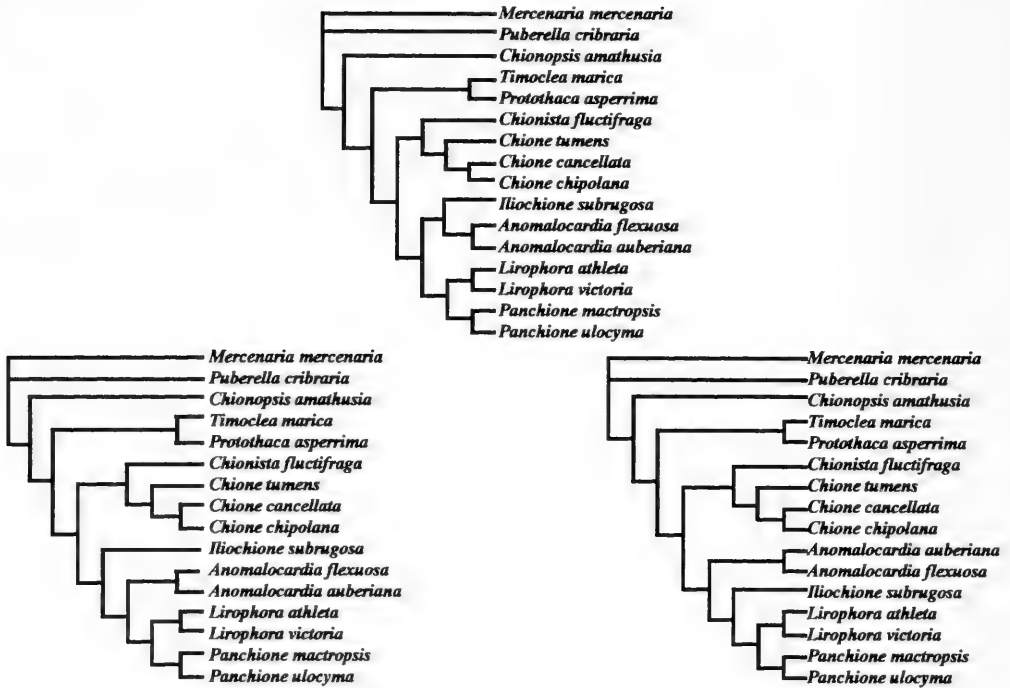
FIG. 3. Cladograms resulting from analysis of *Chione* shell characters. Incongruency among the cladograms is entirely dependent upon the placement of *Iliochione*.

species, geographic range and location were considered.

RESULTS

Phylogenetic Analyses

Analysis of the character data by PAUP resulted in three equally parsimonious cladograms (Fig. 3), each 106 steps in length, and with consistency indices of 0.552, homoplasy indices of 0.448 and retention indices of 0.602. The cladograms are similar in topology, differing only in the placement of the species *Iliochione subrugosa.* The species *Iliochione subrugosa* and *Lirophora athleta* (Conrad) have zero branch lengths. They could thus be identified as potential ancestors, or as possessing the same combination of character states as an ancestor. The zero branch length for *Iliochione subrugosa* results, however, from the exclusion of character #26 (radial indentation of posterior valve surface), which is autapomorphic in *I. subrugosa,* from the analysis. The character was included in the data matrix because it may

become informative if more characters or taxa are added at a later time, or if the data set is later analyzed at a higher level of universality (Wiley, 1981; S. Carlson, personal communication). It also indicates that *I. subrugosa* possesses ancestral states for many characters, but is most likely not an ancestral taxon. *Lirophora athleta* has a zero branch length, possibly because its sister species *L. victoria* (Dall) has been coded with several missing character states. *Lirophora victoria* is the earliest documented species of *Lirophora* (Early Oligocene), and not all character states could be coded with confidence. The results of the analysis could therefore be interpreted to indicate *L. athleta* as a potential ancestor. No autapomorphies could be identified to distinguish the two species, reflecting a common problem when dealing with morphological "species" of *Lirophora* (see, for example, Ward, 1992). All three cladograms identify species in the same subgenus as sister species: *Chione cancellata* and *C. chipolana; Lirophora athleta* and *L. victoria; Panchione ulocyma* and *P. mactropsis;* and *Anomalocardia auberiana* and *A. flexuosa.*
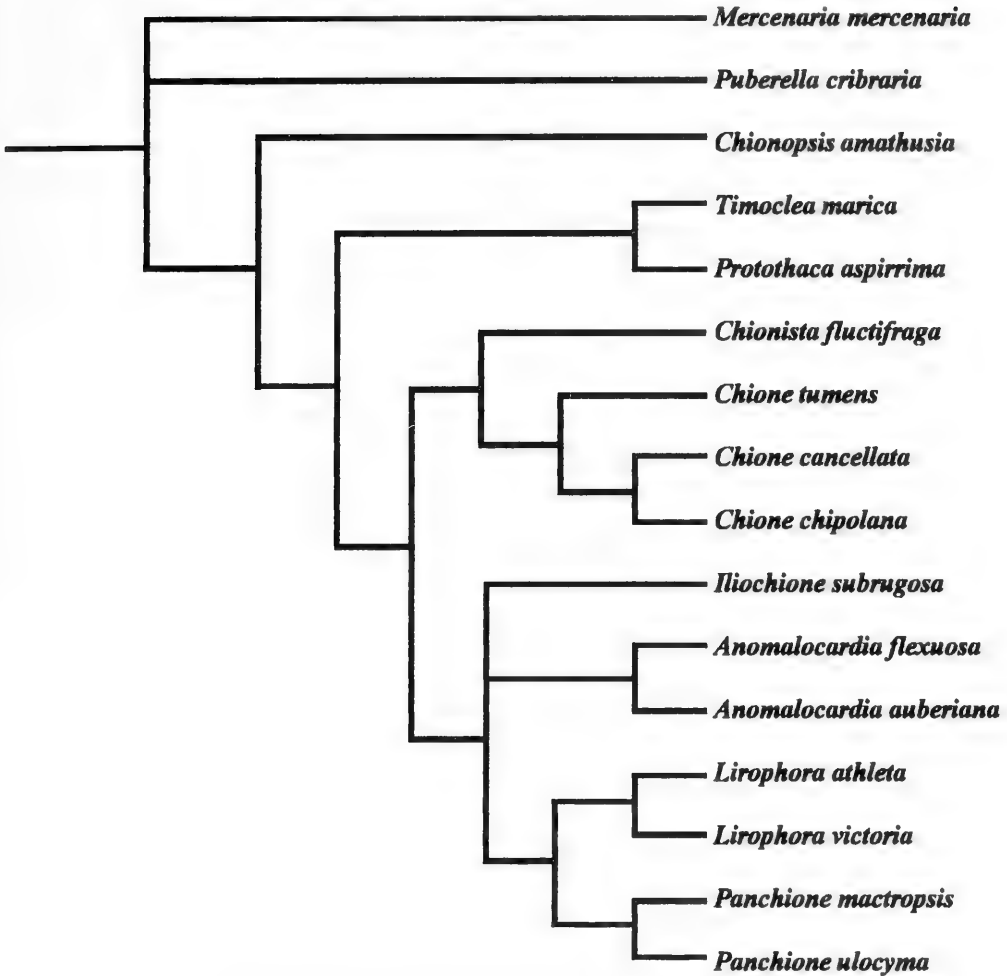
FIG. 4. Strict consensus tree of cladograms illustrated in Fig. 3.

Strict and 50% majority rule consensus trees were used to summarize the information of the three cladograms (Fig. 4, 5). Both trees are well resolved and identical in topology because of the high congruency among the source cladograms. The incongruencies among the cladograms result in a polytomy from which branch *Anomalocardia, Iliochione,* and the sister taxa *Lirophora* and *Panchione.* The ingroup is divided into two sister clades containing the following species: first, *Chionista fluctifraga, Chione tumens, C. cancellata* and *C. chipolana;* and secondly, *Anomalocardia auberiana, A. flexuosa, Iliochione subrugosa, Lirophora atheleta, L. victoria, Panchione mactropsis,* and *P. ulocyma.* The inclusion of *Anomalocardia* in the large *Chione* clade, and the surprising exclusion of *Chionopsis amathusia* and *Puberella cribraria,* indicate that *Chione,* as currently defined, is a paraphyletic genus. The branching position of *Timoclea* confirms its status outside the main clade of *Chione* subgenera. The structure of the cladograms and the consensus trees suggest strongly the need for a thorough taxonomic revision of *Chione.* Revising the taxonomy of *Chione* on the basis of a phylogenetic analysis presumes that the phylogenetic hypotheses are more informative about the interrelationships of the *Chione* subgenera than is the traditional taxonomy.
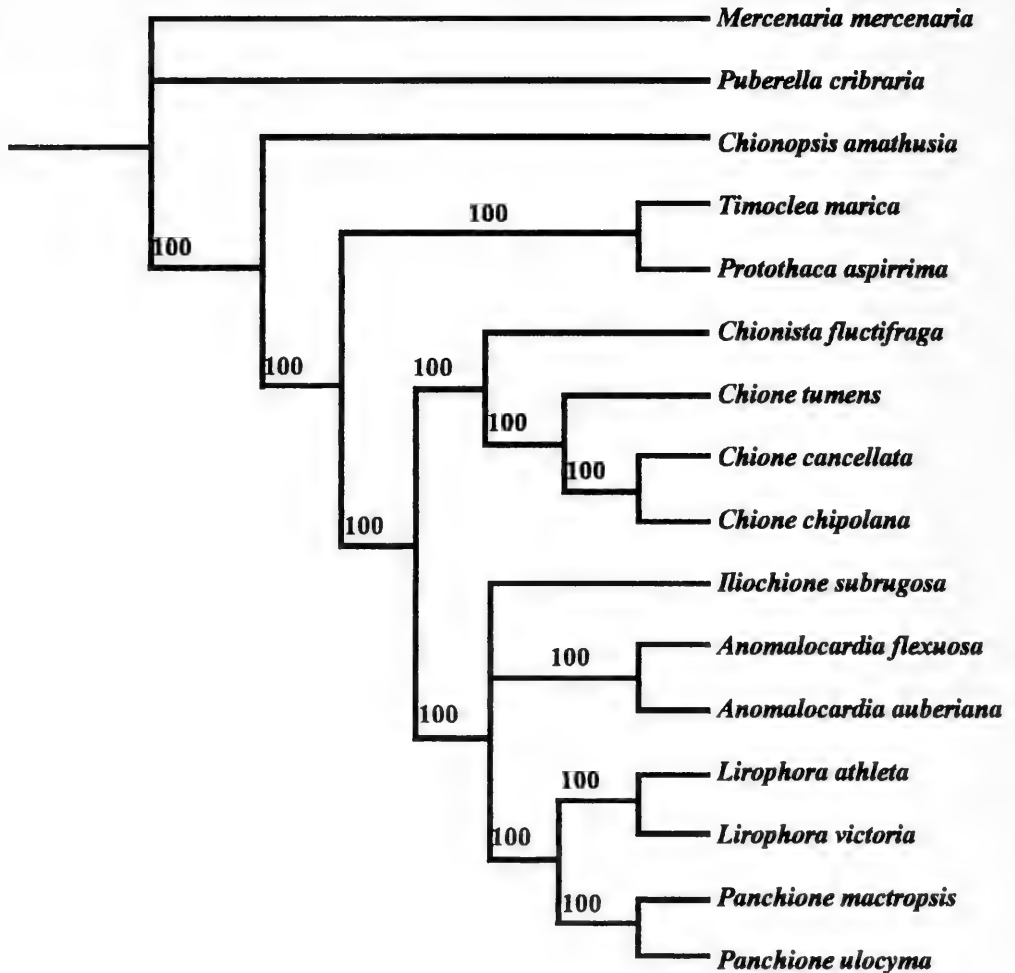
FIG. 5. 50% majority rule consensus tree of cladograms illustrated in Figure 3. This tree is identical in topology to the strict consensus tree (Fig. 4).

Character Optimization and Evolution

The three cladograms are very similar in topology, but the ACCTRAN and DELTRAN reconstructions differ for several significant characters. Perhaps a useful manner in which to view the data will be to examine the support for the three major clades that are a consistent feature of all the cladograms; the overall *Chione* clade (including *Anomalocardia,* but excluding *Chionopsis* and *Puberella*), the *Chione-Chionista* clade (hereafter *Chione* subclade) and the *Lirophora-Panchione-Anomalocardia-Iliochione* clade (hereafter *Lirophora* subclade). Character transformations supported by several cladograms can

be regarded as robust, but unresolved differences have to await the addition of more character information to the analysis. The following character transformations are all illustrated on cladogram 1. Interior nodes are labelled on Figure 6, and the synapomorphies uniting taxa are listed in Appendix III.

Chione Clade

Two characters are apomorphic at the ancestral node of the *Chione* clade on all cladograms, and support the monophyly of the clade. Two additional characters are apomorphic on the basis of ACCTRAN and DELTRAN reconstructions respectively.
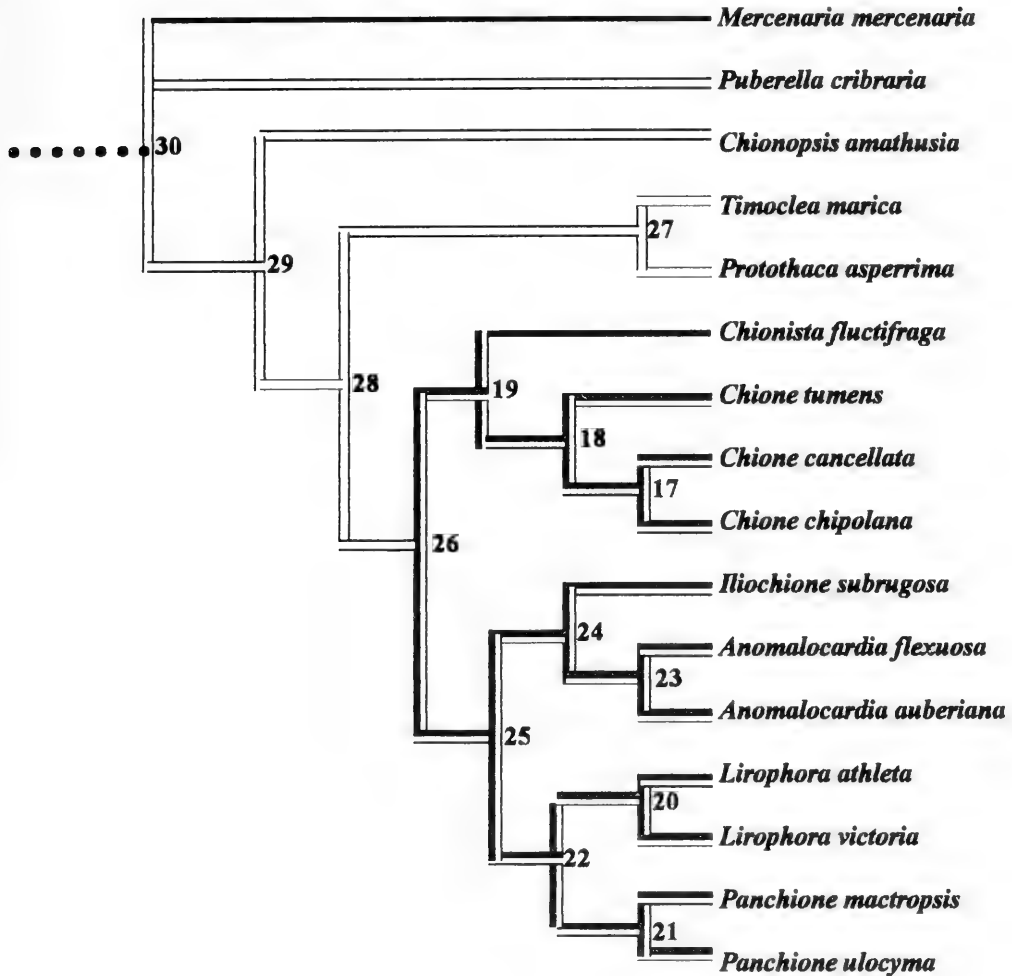
FIG. 6. Character state tree of Character #1 (depth of pallial sinus). Key to character states: (0) solid line—reduced; (1) double line—short; (2) double line, upper (left) thick—deep; (3) double line, lower (right) thick—very deep. Transformations at labelled interior nodes are listed in Appendix III.

Character #20 (condition of right middle cardinal tooth) (CI = 0.667) is apomorphic at the ancestral node on all three cladograms (node 26) and is reconstructed identically by the ACCTRAN and DELTRAN algorithms. The character is mapped onto cladogram 1 to illustrate the changes (Fig. 6). The plesiomorphic state is a tooth with a shallow, surficial groove. At node 26, the tooth becomes smooth. The only exception is *Chionista fluctifraga*, which evolves a bifid tooth. This condition is apparently convergent with the bifid tooth of *Mercenaria*.

Character #7 (distal edge of concentric sculptural lamellae) (CI = 0.500) is apomor-phic at node 26 on all three cladograms, but is reconstructed differently by ACCTRAN and DELTRAN. Based on the ACCTRAN reconstruction (Fig. 7), the plesiomorphic state is a concentric sculptural element with a sharp distal edge. At the *Chione* ancestral node, the sculptural edges become smoother, but revert (converge in the DELTRAN reconstruction) to a sharp morphology at the ancestral node of the *Chione* subclade. Both *Chione chipolana* and *Puberella cribraria* have sculptural elements with reinforcing ridges on the distal edges. This similarity is indicated to be a homoplasy.

Character #8 (orientation of concentric
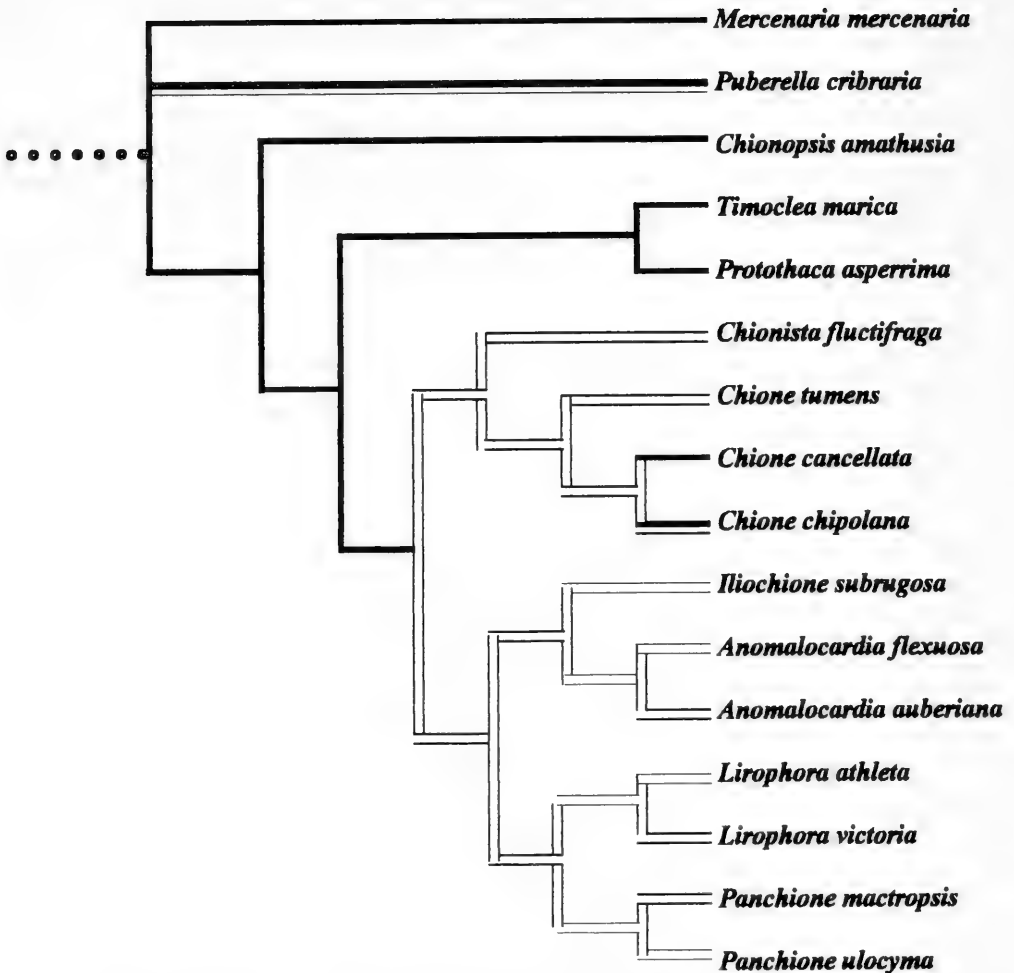
FIG. 7. Character #7 (summit of concentric sculpture). Key (lines same as for Fig. 6): (0)—sharp; (1)—smooth; (2)—summit reinforced.

sculptural elements) (CI = 0.667) is apomorphic at the *Chione* clade's ancestral node (Fig. 8), but is reconstructed differently by the ACCTRAN and DELTRAN algorithms. The plesiomorphic state is vertical sculpture, which reappears in the *Chione (Chione)* subclade. The apomorphic state is folded sculpture which is flattened in the dorsal direction. This type of concentric sculpture is typical of the *Lirophora* subclade, and is useful in defining the group. *Chione chipolana*, however, also has sculpture that appears to be partially folded. The sculpture is vertical at its base, but is foliaceous and becomes folded toward the summit. This variation may be synapomorphic with the ancestral sculpture of the

Lirophora subclade, but such a hypothesis cannot be demonstrated with the material currently available. The rounded nature of the sculpture in *Chione tumens* is shared only by *C. vaca* and *C. subimbricata* (not included in this study).

Lirophora Subclade

There are four characters that strongly support the *Lirophora* subclade; #1, depth of pallial sinus, CI = 0.600; #2, type(s) of sculpture on valve surface, CI = 0.800; #8, orientation of concentric sculptural lamellae, CI = 0.667 (discussed above); #11, morphology of nymph, CI = 0.500. All these were recon-
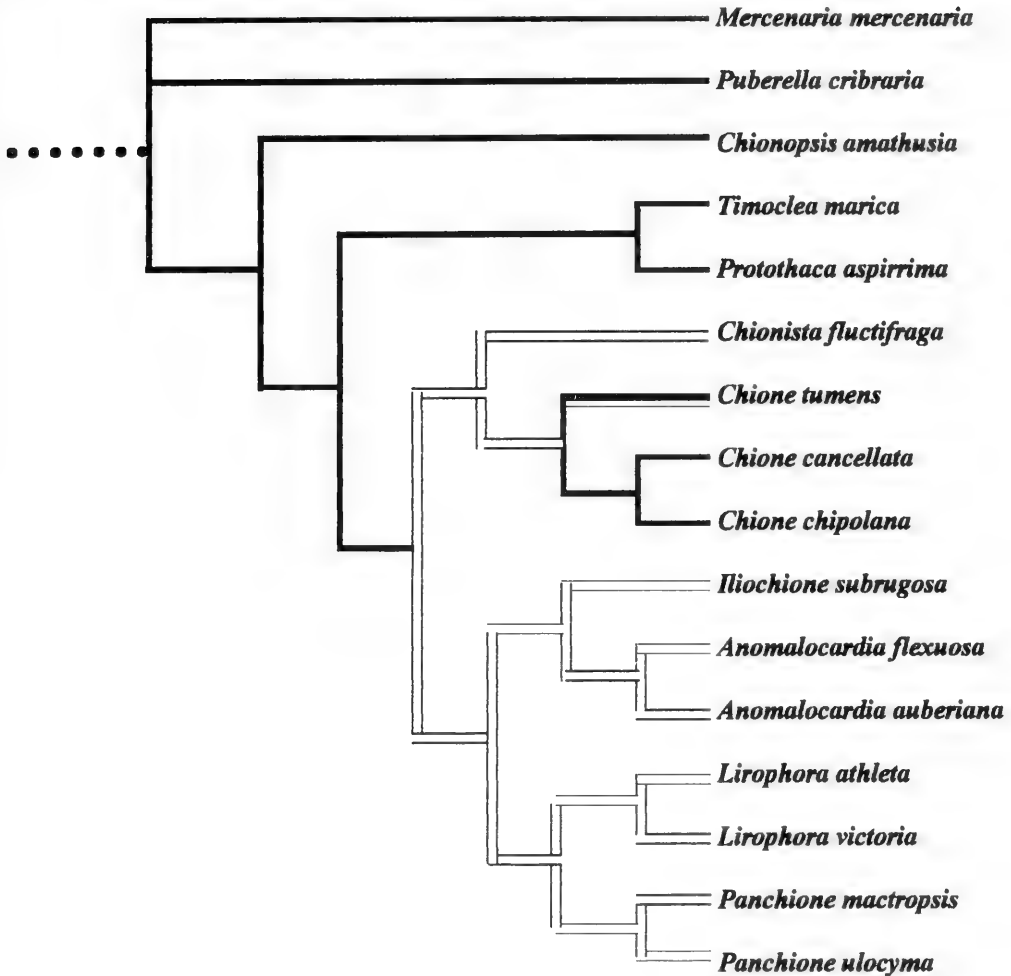
FIG. 8. Character #8 (orientation of concentric sculpture). Key (as in previous figures): (0)—vertical; (1)—folded dorso-ventrally; (2)—rounded.

structed identically by ACCTRAN and DEL-TRAN (Figs. 9, 10), with the exception of character #8. Character #8 was already discussed as being apomorphic at the ancestral node of the overall *Chione* clade, but can be used to distinguish between the *Lirophora* subclade and the *Chione* subclade.

The pallial sinus is generally reduced in all chionine taxa, compared to other venerid subfamilies. It is further reduced in the *Lirophora* subclade, being present but extremely small. This is probably a reflection of the shallow burial of these clams during life. The pallial sinus is completely absent in *Anomalocardia flexuosa,* a species used in this analysis, but is present in other species assigned

to *Anomalocardia,* for example *A. auberiana.* The state in these other species is identical to the state in the other taxa of the *Lirophora* subclade. The presence of much reduced or absent pallial sinuses in the *Chione* subclade is convergent with the states in the *Lirophora* subclade.

Perhaps the character most diagnostic of the *Lirophora* subclade is the morphology of the nymph. This is a binary character, the nymph being either smooth, or roughened, a condition described as "rugose." All taxa nested within the *Lirophora* subclade have rugose nymphs. The only other taxon with a rugose nymph is *Mercenaria,* but this is apparently a convergent condition.
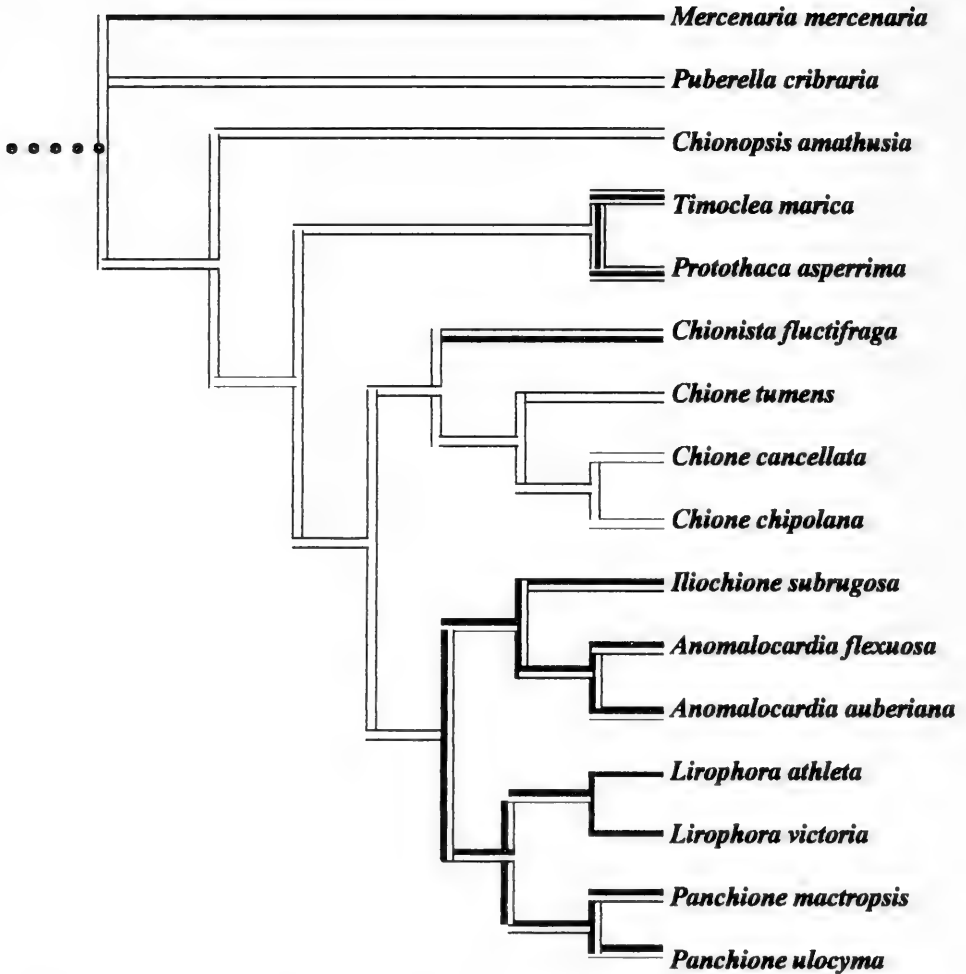
FIG. 9. Character #2 (type of sculpture present). Key (as in previous figures): (0)—concentric only; (1)—radial and concentric, concentric dominant; (2) both, radial sub-obsolete; (3) triple line—both sub-obsolete; (4) double line, upper thick—both, radial dominant.

Like nymph morphology, the morphology of the sculptural elements is diagnostic of the *Lirophora* subclade. Taxa comprising the *Lirophora* subclade have both radial and concentric sculptural elements, but the radial elements tend to be faint to obsolete. *Lirophora* itself has no radial elements present, but this can be viewed as a complete loss of radial sculpture, the concentric sculpture being synapomorphic with the rest of the subclade. The absence of radial sculpture in both *Lirophora* and *Mercenaria* should therefore be recognized as homoplastic, both states being the result of the loss of a character.

Chione Subclade

The *Chione* subclade is supported strongly by at least three characters, #1, #8 and #10 (anterior cardinal tooth of left valve, CI = 0.500) (Fig. 11), two of which (1 and 10) are reconstructed identically by ACCTRAN and DELTRAN. The pallial sinus is altogether absent in all species of *Chione s.s.*, and *Chionista*, with the exception of *Chione chipolana*. *Chione chipolana*'s pallial sinus appears to be convergent with the state in the *Lirophora* subclade. The plesiomorphic condition of character #10 is a relatively wide tooth, but the tooth is noticeably narrow in both the
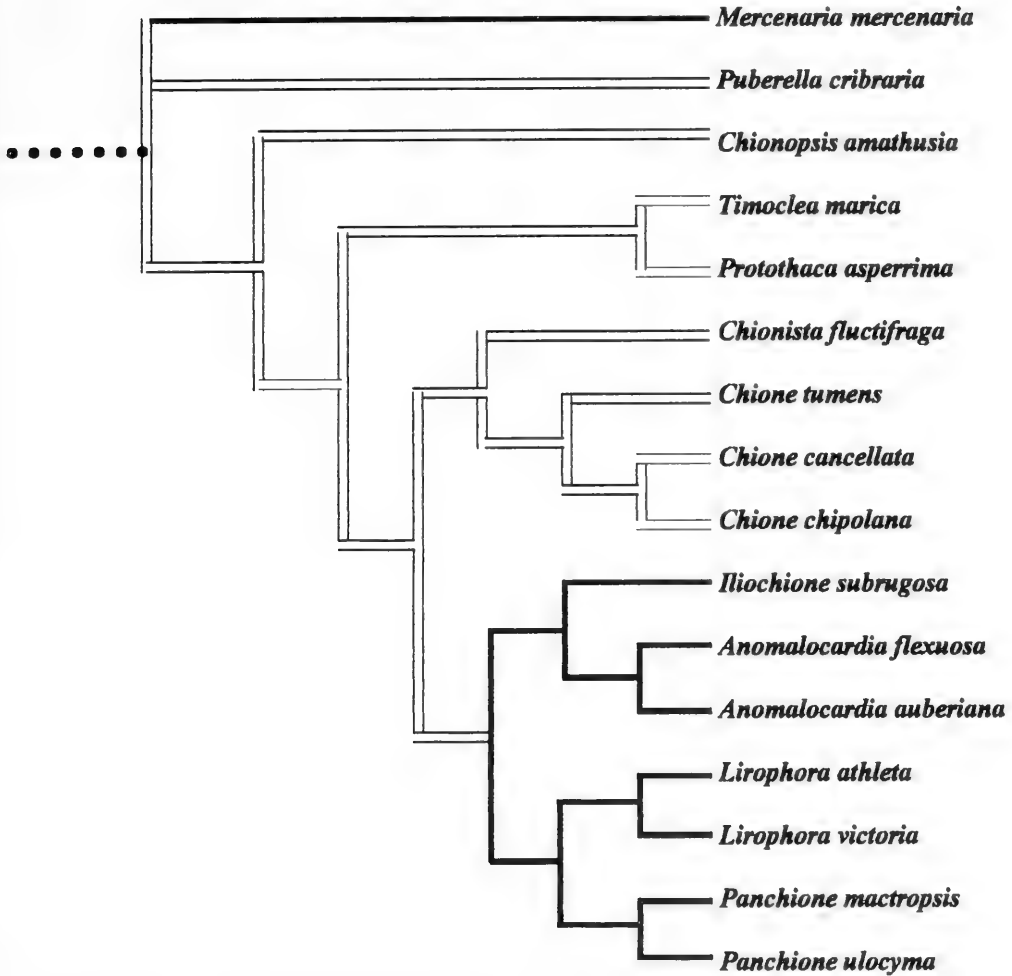
FIG. 10. Character #11 (condition of nymph). Key as in previous figures. (0)—rugose; (1)—smooth.

Chione subclade and *Mercenaria* (the result of convergence).

The orientation of the concentric lamellae is vertical in *Chione s.s.*, and this is probably the retention of a plesiomorphic character state (contrary to the ACCTRAN reconstruction). *Chione tumens* has a truly unique type of sculpture, and it is unclear how it is derived from any of the character states in the Chioninae.

Biogeographic Analyses

All the *Chione* subgenera (with the exception of *Chionista*) first appear in the Western Atlantic. The number of species described from the Early Oligocene through the Middle Miocene (35.4–16.3 Ma) is low (Fig. 12). The Early Miocene is an exception, but most of the species recorded here (85.7%) are from the very rich Chipola Formation of northwest Florida. Woodring (1982) suggested that some *Panchione* species documented there may be synonyms and require revision. Three of the four Chipola *Panchione* species however were examined by this author, and all are consistently recognizable. It should also be noted that the number of well-described Oligocene and Lower Miocene soft-sediment deposits in tropical America is relatively small, and accurate chronological and biostratigraphic dating is problematic. The num-
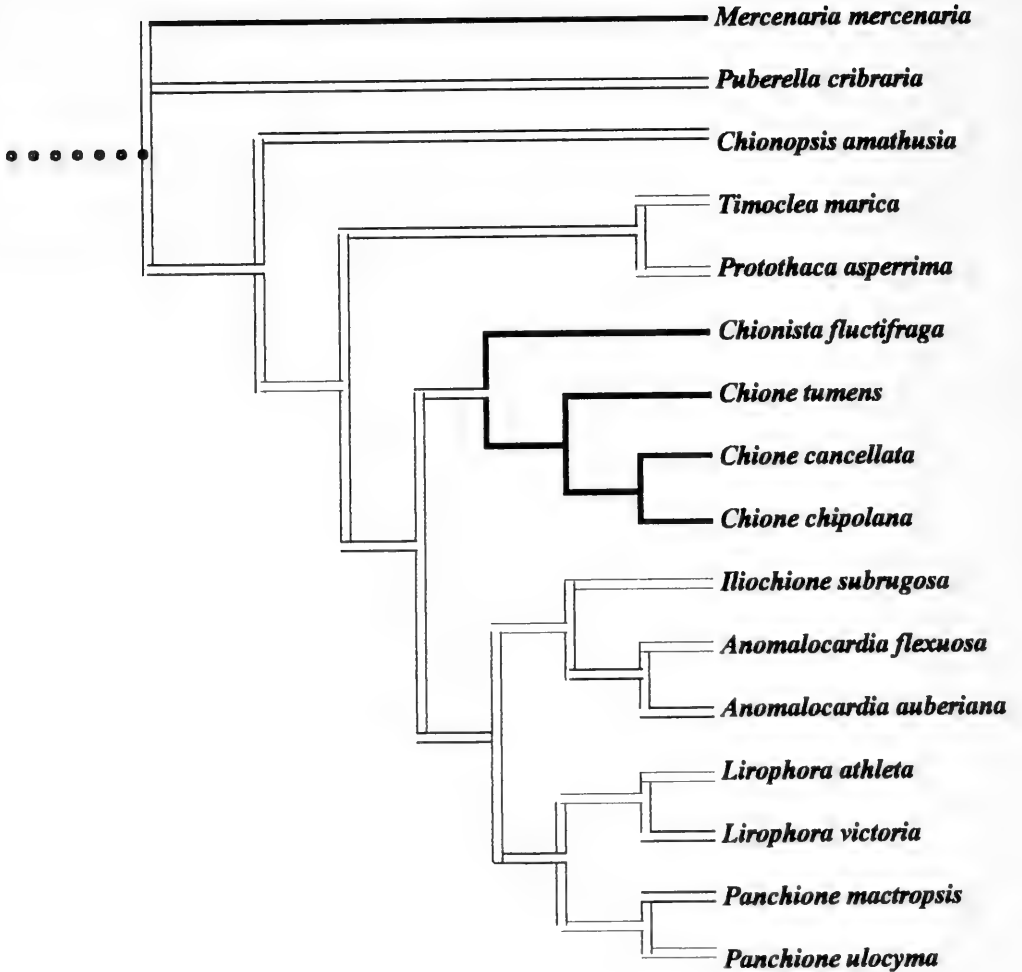
FIG. 11. Character #10 (condition of left valve anterior cardinal tooth). Key as in previous figures. (0)—tooth wide; (1)—tooth narrow.

ber of species reported here for this period is therefore probably not a dependable reflection of actual diversity.

There is a steady increase in diversity from the Middle Miocene to the Early Pliocene (16.3–5.2 Ma) in both the Western Atlantic and Eastern Pacific (Fig. 12), corresponding in part to an increase in the number of deposits available for sampling. During this period, increasing diversity in the Western Atlantic is accounted for primarily by the appearance of new species, which is accompanied by an increasing level of last appearances (Fig. 12). In the Eastern Pacific on the other hand, increasing diversity is the result of first appearances, coupled with a low rate

of extinction, and therefore higher species longevities.

The level of extinction in the Western Atlantic continues to increase into the Late Pliocene, and the proportion of species that last appear in the Late Pliocene greatly exceeds the number of first appearances (Fig. 13). The result is a dramatically lower diversity in the Recent. In contrast, the rate of extinction in the Eastern Pacific decreases during the late Neogene (Fig. 13). The proportion of new species, though also decreasing, is never exceeded by the proportion of species going extinct. There are many species (17) which first appear in the Eastern Pacific during the Pleistocene and Recent; this results in
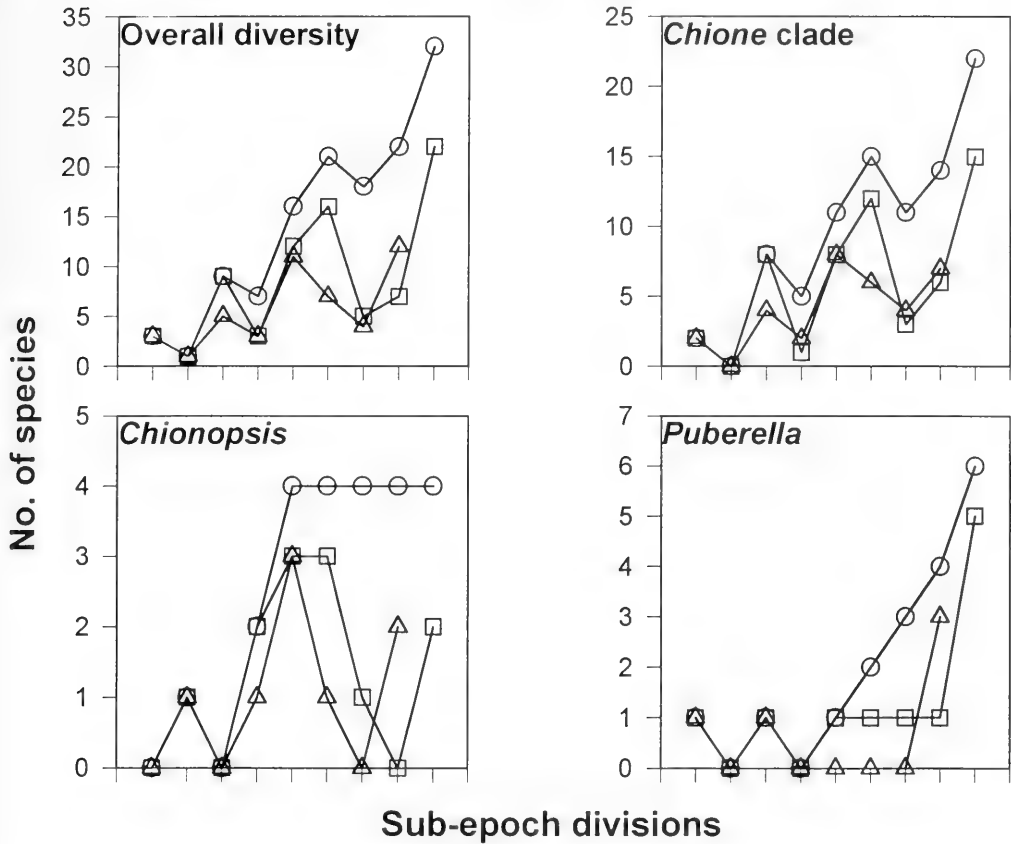
FIG. 12. Total species diversity of *Chione* subgenera since the Early Oligocene. Chronological (horizontal axis) intervals are geological sub-epochs. Graphs on upper left represents sum diversity of traditional subgenera. Upper right—*Chione* clade (as defined in this paper); lower left—*Chionopsis;* lower right—*Puberella.* Circles—total number of species present; squares—number of first appearances; triangles—number of last appearances.

a higher diversity than in the Western Atlantic. The Pliocene is also the time of first appearance of the strictly Eastern Pacific subgenera, *Chionista, Iliochione,* and the *Chione vaca-subimbricata-tumens* trio.

In summary, these data agree well with observations that the Western Atlantic suffers heavier extinction during the Pliocene (82.6%) than does the Eastern Pacific (38.5%) (Woodring, 1966; Stanley, 1986; Vermeij & Petuch, 1986) (Fig. 13). The Eastern Pacific exhibits a higher rate of origination during the post-Pliocene compared to the Western Atlantic, resulting in a higher diversity in the Eastern Pacific (17 vs. 7 new species). The overall pattern is reflected by the individual subgenera. All exhibit higher levels of extinction in the Western Atlantic relative to the Eastern Pa-

cific, and higher levels of origination in the Eastern Pacific during the post-Pliocene. Extinction within these *Chione* subgenera in the Western Atlantic was not matched by speciation.

These results cover the entire Pliocene though, obscuring the relative timing of the disappearances, and hence the action of an extinction agent such as cooling. In order to focus on the time of the extinctions, it becomes necessary to assign estimated ages to sampled deposits. Given the contentious nature of aging Neogene tropical American deposits, the most current age estimates available were relied upon, coming primarily from the following references; Coates et al. (1992), Duque-Caro (1990), Jones et al., (1991) and Krantz (1991). These works either
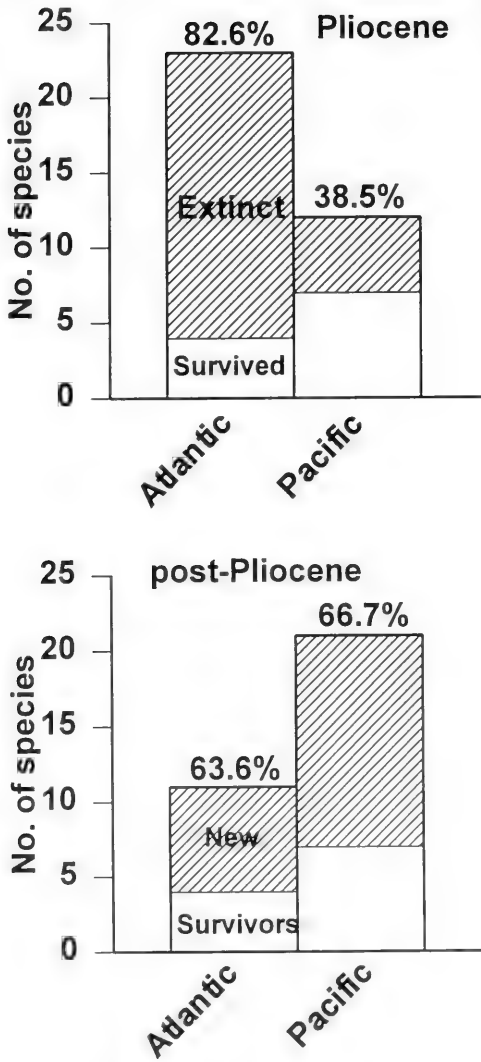
FIG. 13. Late Neogene changes in diversity. Upper graph illustrates levels of extinction during the Pliocene in the western Atlantic and the eastern Pacific. Shaded areas represent number of extinct species, unshaded represent survivors. Lower graph illustrates levels of origination in the Pleistocene and Recent. Shaded areas represent new species.

re-evaluate and assign ages, or summarize current estimates. The Early Pliocene (5.2–3.4 Ma) of the Atlantic Gatunian is covered in this set of data by species from the Agueguexquite Formation of Mexico (Perrillat, 1963), the Cercado and Gurabo deposits of the Dominican Republic (Saunders et al.,

1986), the Rio Banano Formation of the Limon Group in Costa Rica (Coates et al., 1992) and the Tubara Formation of Colombia (Duque-Caro, 1990). All these formations probably pre-date the initiation of Northern Hemisphere cooling (~2.4 Ma), or end shortly thereafter. They could therefore be listed as Early-"middle" Pliocene. The Lower Pliocene deposits of the Caloosahatchian Province, namely the Jackson Bluff Formation, the lower Pinecrest Beds, the Duplin Formation, the Raysor Formation and the Yorktown Formation, all range past the official stage boundary of the Late Pliocene (Jones et al., 1991; Krantz, 1991), 3.4 Ma, but terminate about 2.5 Ma. Therefore they too could be considered technically as Early-middle Pliocene. The Atlantic Gatunian Bowden Formation is Late Pliocene in age (Stanley, 1986), as are the Caloosahatchian Caloosahatchee Formation, upper Pinecrest Beds, the Murfreesboro Stage (Ward, 1992) and the Waccamaw Formation (Lyons, 1991; Jones et al., 1991). These formations are all < 2.5 million years old. Early-middle Pliocene Eastern Pacific deposits comprise formations such as the Burica, Esmeraldas and Progreso formations, while the Late Pliocene is represented primarily by deposits from Baja and southern California.

Placed in a temporal framework of Early-middle and Late (latest) Pliocene categories, the pattern of extinction is striking. Only 50% of all species in the Early-middle Pliocene of the Western Atlantic were extinct by the Late Pliocene, but 73.3% of Late Pliocene species are absent from the Pleistocene (Fig. 14). On the other hand, extinction levels in the Eastern Pacific are 57.1% and 11.1% respectively for the Early-middle and Late Pliocene. The extinctions would therefore seem to be concentrated in the Late Pliocene of the Western Atlantic. This result is consistent with the compilations of Allmon et al. (1993) and Jackson et al. (1993). At the subgeneric level however, a different pattern emerges (Fig. 15). In the Western Atlantic, the subgenera *Lirophora* and *Panchione* experience much higher levels of extinction (60% and 100% respectively) during the Early-middle Pliocene, than in the Late Pliocene. The subgenera *Chione, Chionopsis* and *Puberella* do not exhibit heightened levels of extinction until the Late Pliocene.

The difference of timing among the subgenera is not explained easily. The data could be biased biogeographically if the sub-
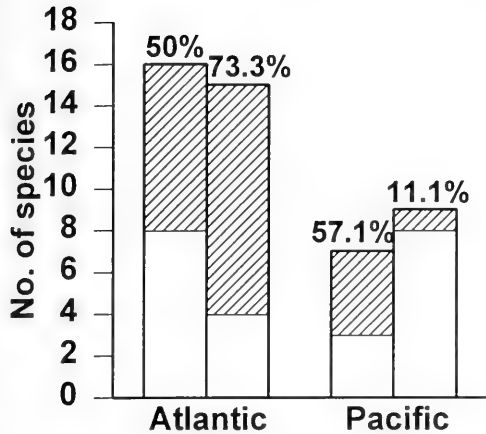
FIG. 14. Timing of Pliocene extinctions in Western Atlantic and Eastern Pacific. In each category, bar on the left summarizes data for the Early-middle Pliocene, bar on right the Late Pliocene. See text for explanation of chronological intervals. Percentages refer to percent of diversity extinct in next chronological interval. Shading as in Fig. 13.

genera were not distributed evenly between the Atlantic Gatunian region and the Caloosahatchian Province, and if one of the two regions experienced more severe extinction. The extinctions are probably not related to phylogenetic history. It is interesting to note that *Lirophora* and *Panchione* occupy a separate subclade that could have been decimated by a selective extinction agent (Fig. 4). If the extinction was biased against *Lirophora* and *Panchione* however, then *Anomalocardia* should exhibit the same pattern. It does not, and it seems therefore that any hypothesis of phylogenetic selectivity of the extinction can be rejected.

In order to examine the role of biogeographic distribution in the extinction, it is necessary to assign species to one or both of two biogeographic regions, the Atlantic Gatunian region and the Caloosahatchian Province. Interestingly, all Pliocene species of *Chione* were endemic to one of the two regions, with the possible exception of *Chione cancellata.* Even this species, however, may in fact be two separate taxa (Roopnarine, 1995). Species can therefore be placed easily into one or two of the following categories: Early-middle Pliocene Atlantic Gatunian; Early-middle Pliocene Caloosahatchian; Late Pliocene Atlantic Gatunian; Late Pliocene Caloosahatchian. The results indicate that extinction of Early-middle Pliocene species in

the Atlantic Gatunian region was 53.8%, while in the Caloosahatchian Province it was only 18.2% (Fig. 16). By the latest Pliocene, however, extinction declined slightly in the Atlantic Gatunian to 50%, but climbed to 58.3% in the Caloosahatchian.

The high level of extinction in the Atlantic Gatunian during the Early-middle Pliocene is due almost entirely to the extinction of species assigned to *Lirophora* and *Panchione* (Fig. 17). Both subgenera also experience higher levels of extinction at this time, in the Caloosahatchian Province, relative to other subgenera. Furthermore, it is apparent that *Lirophora,* with its numerical dominance of the species diversity of both the Atlantic Gatunian and Caloosahatchian, contributes the most to the extinction. During the latest Pliocene all surviving subgenera experience high levels of extinction in both geographic regions, with the exception of *Anomalocardia.*

Body Size

Early-middle Pliocene species of the Caloosahatchian Province all exceed 35 mm in height (Table 4). The largest species is *Chionopsis procancellata* (Mansfield) from the lower Pinecrest Beds, the largest specimen of which measured 57.75 mm. *Lirophora athleta* from the lower Pinecrest Beds reached a height of at least 35.5 mm. Species from the same deposit include *Chione erosa,* which reached a height of 47.35 mm, and *Panchione ulocyma*, which reached a height of 43.30 mm. *Puberella cortinaria* (Rogers) from the Jackson Bluff Formation was measured at 37.03 mm. Species from the northern regions of the Atlantic Gatunian Province tend to be much smaller. There are no records of Early-middle Pliocene specimens of *Chionopsis woodwardi* (Guppy) or *Puberella sawkinsi* (Woodring) from the Cercado or Gurabo Formations exceeding 30 mm in height, nor of specimens of *Chione primigenia* Pilsbry & Johnson or *C. santodomingensis* Pilsbry & Johnson (Palmer, 1927). Interestingly, there is some evidence that species from the southern portion of the province, for example the Rio Banano Formation of Costa Rica, were larger. Woodring (1982) recorded a specimen of *Panchione mactropsis* of 51.5 mm height from the Rio Banano. *Chionopsis tegulum* (Brown & Pilsbry), while not measured for this study, is a large species, attaining heights in
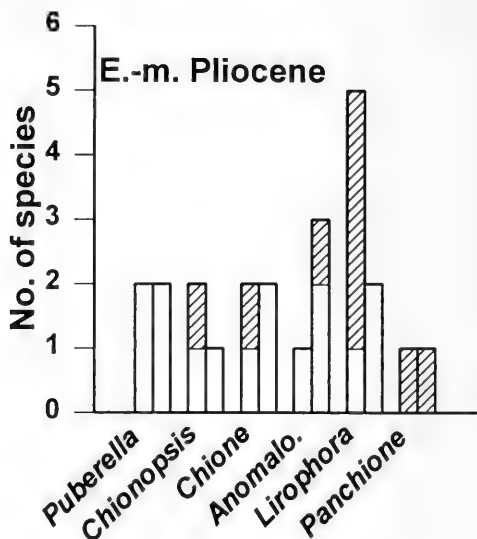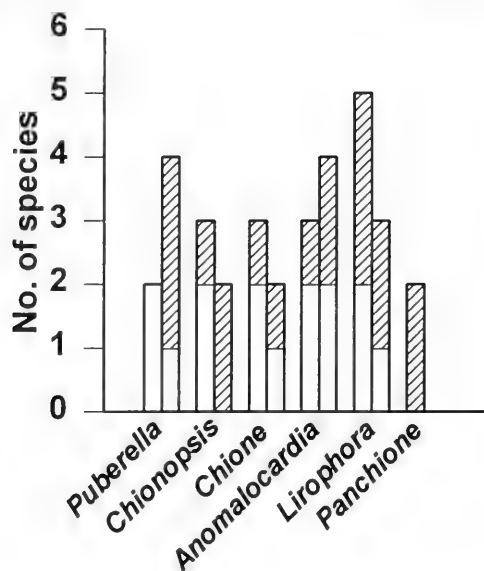
FIG. 15. Subgeneric breakdown of data presented in Fig. 14.
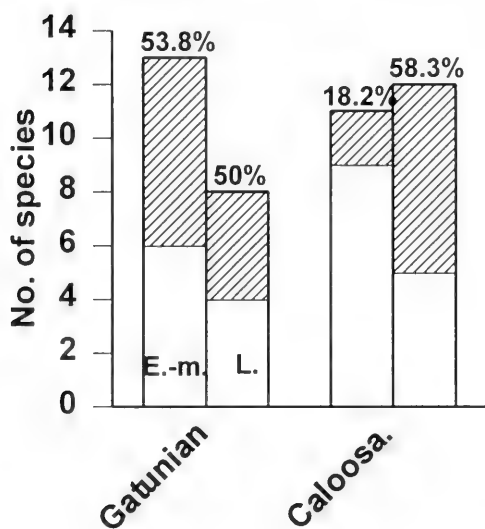
FIG. 16. Temporal and biogeographic categorization of Pliocene extinctions in the western Atlantic. Shading represents extinct species. Abbreviations: E.-m.—Early and middle Pliocene; L.—Late Pliocene; Gatunian—Atlantic Gatunian region; Caloosa.—Caloosahatchian Province.
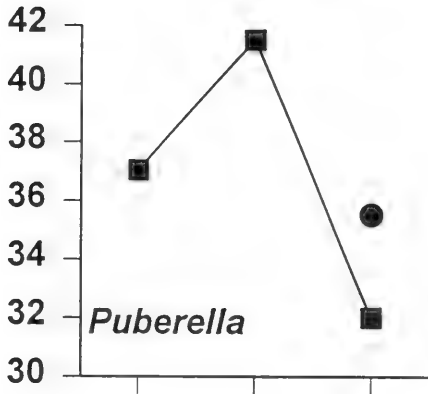

FIG. 17. Subgeneric breakdown of data presented in Fig. 16. Per subgenus, bars on right represent the Caloosahatchian Province, and on left the Atlantic Gatunian region. Key to bars as in previous figures.

excess of 45 mm in the Upper Miocene Gatun Formation. It also persists into the Lower-middle Pliocene Rio Banano Formation. The only Early Pliocene sizes available from the Eastern Pacific are of the species *Chionopsis amathusia* and *Chione vaca* (Olsson) from the Esmeraldas Formation of Ecuador. Both exceeded 30 mm in valve height.

The situation alters significantly in the lat-

TABLE 4. Valve heights measured for samples of chionine bivalves. Maximum valve height recorded for each sample.

Species	Age	Locality/Formation	No. of specimens	Max. height (mm)
Chione erosa	E.—middle Pliocene	Raysor Fm., South Carolina	7	39.83
	E.—middle Pliocene	Pinecrest Beds, Bird Rd., Florida	68	41.46
	E.—middle Pliocene	Pinecrest Beds, Collier Co., Florida	27	38.07
	E. Pliocene	Pinecrest Beds, Sarasota, Florida	11	47.34
	L. Pliocene	Caloosahatchee Fm., Palm, Beach Co., Florida	100	32.41
	L. Pliocene	Caloosahatchee Fm., Palm Beach Co., Florida	34	36.86
Chione cancellata (United States)	L. Pliocene	Waccamaw Fm., South Carolina	15	34.48
	E. Pleistocene	Bermont Fm., Highlands Co., Florida	39	38.37
	M. Pleistocene	Ft. Thompson Fm., Collier Co., Florida	100	26.91
	L. Pleistocene	Anastasia Fm., Palm Beach Co., Florida	38	31.58
	Recent	Sanibel Is., Florida	50	26.87
	Recent	Palm Beach Co., Florida	100	36.24
	Recent	South Carolina	9	38.76
Chione cancellata (West Indies)	Recent	Bahamas Islands	30	22.07
	Recent	Jamaica	58	27.49
	Recent	St. Thomas, U.S. Virgin Is.	34	26.03
	Recent	Venezuela	40	35.35
Chione mazyckii	Recent	South Carolina	3	12.97
Chione undatella	Recent	Laguna San Ignacio, Baja California, Mexico	5	37.43
	Recent	Bahia San Luis, Baja California, Mexico	27	47.99
	Recent	San Carlos, Baja California, Mexico	16	52.17
Chionopsis procancellata	E. Pliocene	Lower Pinecrest Beds, Sarasota Co., Florida	5	57.75
	L. Pliocene	Highlands Co., Florida	13	52.31
	L. Pliocene	Palm Beach Co., Florida	4	55.84
	L. Pliocene	Okeechobee Co., Florida	11	52.14
Chionopsis woodwardi	L. Pliocene	Bowden Fm., Jamaica	9	21.33
Chionopsis amathusia	E. Pliocene	Esmeraldas Fm., Ecuador	1	34.53
	Recent	Baja California, Mexico	6	41.61
Chionopsis gnidia	Recent	Guaymas, Mexico	11	75.15
Lirophora athleta	E. Pliocene	Sarasota Co., Florida	5	35.5
	E. Pliocene	Collier Co., Florida	18	27.72
	L. Pliocene	Collier Co., Florida	32	27.86
	L. Pliocene	Caloosahatchee Fm., Florida	1	30.83
	L. Pliocene	Caloosahatchee Fm., Florida	10	24.05
Lirophora latilirata	L. Pliocene	Waccamaw Fm., North Carolina	13	28.99
	E. Pleistocene	Bermont Fm., Palm Beach Co., Florida	35	32.65
	Holocene	Mississippi delta, Louisiana	42	31.68
Lirophora hendersoni	L. Pliocene	Bowden Fm., Jamaica	42	24.46
Lirophora paphia	Recent	Jamaica	19	22.47
Panchione ulocyma	E.—m. Pliocene	Pinecrest Beds, Sarasota Co., Florida	10	43.30
Puberella cribraria	L. Pliocene	Waccamaw Fm., South Carolina	40	40.56
	L. Pliocene	Waccamaw Fm., North Carolina	8	38.91
Puberella intapurpurea	Recent	Bahamas	3	30.09
Puberella morsitans	L. Pliocene	(Olsson and Harbison, 1953)	1	41.50
Puberella sawkinsi	L. Pliocene	Bowden Fm., Jamaica	14	22.37
Puberella pulicaria	Recent	San Felipe, Baja California, Mexico	1	35.50
Chionista cortezi	Recent	San Felipe, Baja California, Mexico	12	64.70
Chionista fluctifraga	Recent	Gulf of California, Baja California, Mexico	8	49.04
Iliochione subrugosa	Recent	Panama	29	34.26
Chione tumens	Recent	Gulf of California, Baja California, Mexico	35	39.01
Chione raca	E. Pliocene	Esmeraldas Fm., Ecuador	1	33.94

est Pliocene. Caloosahatchian descendants of Early Pliocene conspecifics remain quite large, all exceeding 30 mm in height. New species, for example *Puberella morsitans* (Olsson & Harbison), are also large. In the Atlantic Gatunian, however, species from the Upper Pliocene Bowden Formation do not exceed 25 mm in height. The species in this formation, *Chionopsis woodwardi, Puberella sawkinsi* and *Lirophora hendersoni* (Dall), range in size from 20–25 mm.

Species in the Caribbean today remain relatively small. The two most common species, *Chione cancellata* and *Lirophora paphia* (Linnaeus) rarely exceed 30 mm in height. Notable exceptions occur in upwelling areas (Vermeij, 1978), for example off the coast of northern Venezuela. This region yields large specimens of *C. cancellata*. The largest one measured in this study was 35.35 mm in height. Species from the coastal waters of the United States, however, are comparable in size to Early and Late Pliocene Caloosahatchian species. All species examined had specimens over 30 mm. Palmer (1927) describes a specimen of the Caribbean species *Puberella pubera* with a valve height of 51 mm, but does not give detailed locality information.

It is noteworthy that no Recent species in neither the Atlantic Gatunian nor the Caloosahatchian Provinces exceeds 40 mm in height. Table 4 indicates that there were at least four Early Pliocene species (*Chione erosa* Dall, *Chionopsis tegulum, Chionopsis procancellata,* and *Panchione ulocyma*), and three Late Pliocene Caloosahatchian species (*Chionopsis procancellata, Puberella cribraria* (Conrad), and *P. morsitans*) which did exceed 40 mm in height. All these species are extinct, and the subgenera *Chionopsis* and *Panchione* are today paciphilic. In contrast, Recent Pacific chionine species measured for this study are very large. *Chione californiensis* Broderip and *C. undatella* both attain heights in excess of 50 mm, while the species *Chionopsis amathusia* and *C. gnidia* (Broderip & Sowerby) have maximum sizes of 41.61 mm and 75.15 mm respectively. One specimen of *Puberella pulicaria* (Broderip) from the Gulf of California measured 35.50 mm. Moreover, the subgenera that have evolved in, and are restricted to the Eastern Pacific, *Chionista, Iliochione* and *Chione tumens* are also quite large; for example, *Chionista cortezi* (Carpenter), 64.70 mm; *Chionista fluctifraga,* 49.04 mm; *Iliochione*
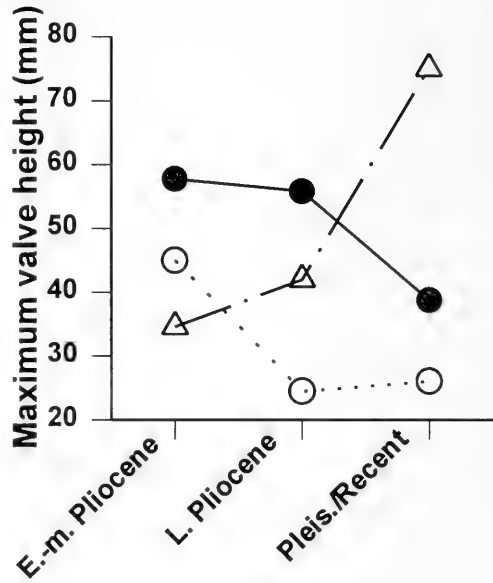
FIG. 18. Maximum valve heights of chionine species during the late Neogene. Open circles—Atlantic Gatunian region; filled circles—Caloosahatchian Province; open triangles—Pacific Gatunian region.

subrugosa, 34.26 mm; *Chione tumens,* 39.01 mm. In general, Pacific species are larger than their Atlantic congeners. Figure 18 summarizes these data, and Figure 19 presents the data for individual subgenera.

DISCUSSION

Evolution of *Chione*

Assuming that the paleontological record of chionine species is reasonably well documented, the common ancestor of the *Chione* subgenera, as well as *Anomalocardia,* had evolved by the Late Eocene, possibly earlier. Some subgenera bear morphological resemblances to others, for example *Lirophora* and *Panchione,* but all appear in the fossil record essentially fully developed, with taxon-defining synapomorphies present.

Within the entire clade (comprising *Anomalocardia, Chione, Chionista, Iliochione, Lirophora,* and *Panchione*) there are examples of convergent and parallel evolution exhibited
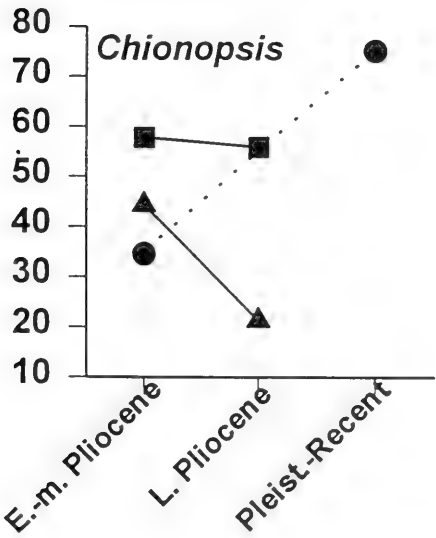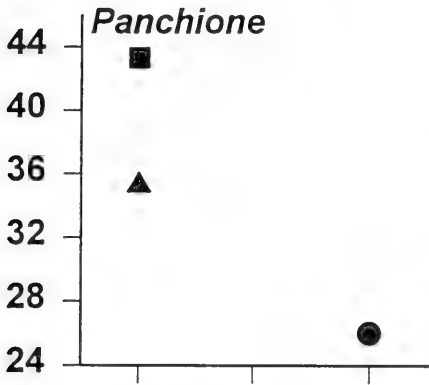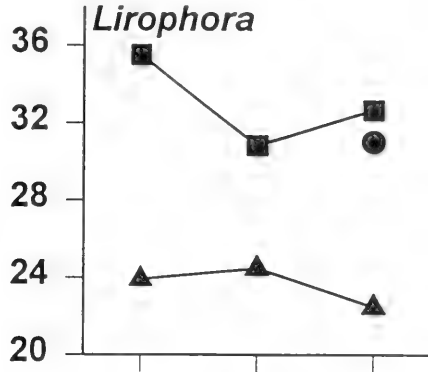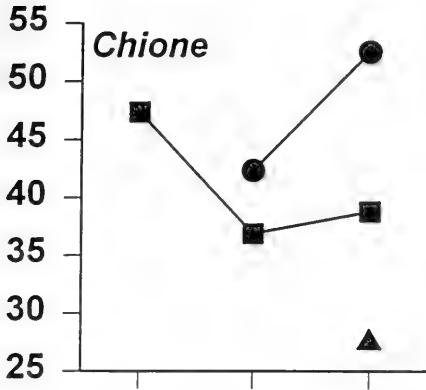
FIG. 19. Subgeneric breakdown of data presented in Fig. 18. Circles—Pacific Gatunian region; triangles—Atlantic Gatunian region; squares—Caloosahatchian Province. Note extinction of *Chionopsis* and *Panchione* in Atlantic regions.

by related taxa inhabiting similar habitats. Two characters, however, define the clade strongly and consistently. The smooth condition of the right middle cardinal tooth distinguishes the clade from the outgroups *Mercenaria, Protothaca* and *Timoclea,* as well as *Chionopsis* and *Puberella.* These outgroup taxa have teeth that are grooved, with the exception of *Mercenaria*, which has a bifid tooth. That condition is convergent with the bifid tooth of *Chionista.* The concentric sculpture is also diagnostic of the clade. The distal edge of the concentric lamellae tend to be smooth in all members of the clade, except *Chione chipolana,* which has a reinforcing ridge along the lamellar edge, and *C. cancellata*, which has a sharp edge (the common condition in *Chione s.s.*). The common outgroup condition (plesiomorphic) is sharp edged lamellae. *Puberella* however, also has distally reinforced lamellae.

The two subclades are likewise well defined. The *Chione* subclade is defined primarily by three characters; an extremely shallow pallial sinus, vertical orientation of the concentric lamellae, and a narrow left anterior cardinal tooth. Taxa of the *Lirophora* subclade on the other hand, have four strong, diagnostic synapomorphies; a short pallial sinus, the dominance of concentric sculpture relative to radial sculpture, folding of the concentric sculpture, and the possession of a rugose nymph. The characters that define the two subclades contrast strongly between them, but it is interesting to note that only two characters are the common strong apomorphic definitions of these two clades. Considering the levels of homoplasy in characters that distinguish the subclades, it would be imprudent to make inferences of relationship and clade membership based on analysis of a single or few characters.

A note of caution must be expressed at this point against the use of seemingly consistent, functionally significant single characters as descriptors of phylogeny or taxonomic classification. The possession of a functionally significant character state by several taxa could be indicative of common ancestry, but might also signify the homoplastic nature of a trait that is in "high demand." Fisher (1981, 1985) argued this with respect to the multiple, independent evolution of functionally significant traits. An example relevant to this study is the condition of the nymph. The nymph as described above is a platform on the valve margin, just posterior to the poste-

rior cardinal tooth. It is an attachment site for the elastic ligament between both valves, and is typically smooth. In several chionine taxa (*Mercenaria* and the entire *Lirophora* subclade), however, the nymph is noticeable rugose. The distinct difference between the two states of this character has made it very useful in defining species and subgeneric boundaries. Moreover, its role as an attachment site for the ligament has lent it some notion of functional significance, though how smoothness and rugosity may affect ligament biomechanics is unknown. Harte (1992a) used the roughened nymph of *Lirophora kellettii* (revised in this study to *Panchione kellettii*) as the basis for assigning the species to *Mercenaria.* Implicit in her study was the untested assumption that the traditional taxonomic genus *Chione* is a monophyletic clade, provided that the genus *Mercenaria* is nested within it. The phylogenetic analysis presented here does not support the nesting of *Mercenaria* with *Chione* subgenera, and has demonstrated therefore that *Chione* is not a monophyletic genus, and that the roughened nymphs of *Lirophora* and *Mercenaria* are most likely homoplastic.

Overall, the entire *Chione* clade has undergone tremendous diversification since the Early Oligocene. There are 90 species recorded in this paper. Perhaps even more striking is the number of distinct morphologies, or "bauplans" (as defined by Hall, 1992), that are nested within the clade. Though the entire clade is supported by several characters, for example prominent concentric sculpture, the subgenera as mentioned earlier can all be distinguished easily from each other. These terminal taxa are not necessarily distinguished by autapomorphic characters, but more often by possessing unique combinations of synapomorphic character states. Moreover, the taxa, or the synapomorphic combinations that they represent, appear in the fossil record abruptly and fully defined. In general, the original synapomorphic combinations still define the subgenera in the Recent, and allow the easy assignment of species to proper subgeneric clades. It would seem therefore that above the species level the rate of character evolution in the *Chione* clade was initially very high, probably during the Late Eocene, but character innovation has since stabilized or fallen to near zero. To-date there is very little fossil evidence to shed light on the origins of individual subgenera.

Taxonomic Revision

Revision of a taxonomic classification on the basis of phylogenetic analysis should meet at least two important criteria. First, the new classification must reflect the sister-group relationships implied by the analysis (deQueiroz & Gauthier, 1992). Secondly, revisions of the existing classification should be minimized, with the only alterations of rank and taxon membership being those necessitated by the phylogenetic analysis (Wiley et al., 1991). This criterion ensures that rank-based studies are affected as little as possible, but are consistent with the phylogenetic analysis. Rank taxa would therefore represent monophyletic clades, and as such would remain convenient, phylogenetically meaningful units for use in studies of diversification and extinction. The simplest method for achieving these goals is to convert the taxonomic classification to the tree that it supports, and then evaluate the logical consistency of the taxonomic tree with the phylogenetic tree. Changes made to the taxonomic tree to make it logically consistent with the phylogenetic tree are subsequently translated to modifications of the rank taxonomic classification (Wiley et al., 1991).

The three cladograms and the resulting consensus trees all show that the genus *Chione* is paraphyletic because of the recognition of *Anomalocardia* as a separate genus. If the prevailing classification is converted to a phylogenetic tree (Fig. 20), it is immediately obvious that it is logically inconsistent with the consensus tree. Not only does the taxonomic classification not imply relationships among the *Chione* subgenera, but there are no hypotheses concerning the relationships among the chionine genera. The simplest resolution of the problem is the revision of *Chione*. Harte (1992a) suggested that *Mercenaria* be subsumed under *Chione* as a new subgenus. That solution, however, would disturb the long-standing understanding of *Mercenaria* as a genus. For reasons of consistency, it would also necessitate the inclusion of *Anomalocardia* as a *Chione* subgenus. Such alterations would violate the criterion of minimal changes outlined above.

Alternatively, several *Chione* subgenera could be elevated to genus rank. Based on the treatment of several of these subgenera as genera by previous authors, the elevations would minimize the changes necessary to convert the taxonomic classification to a phy-
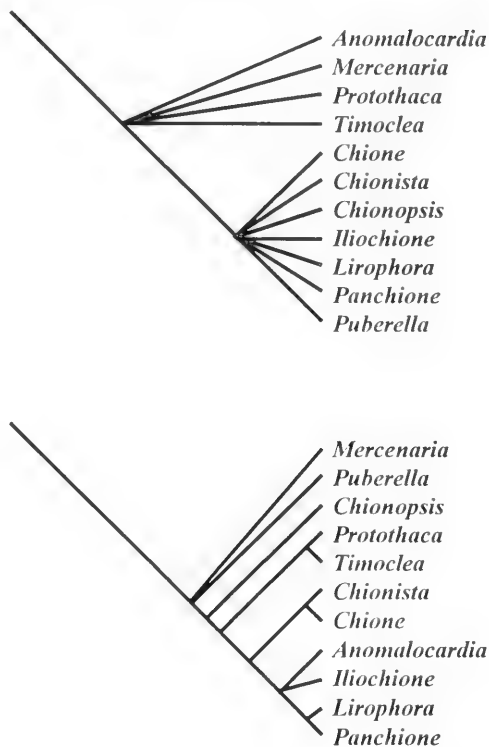
FIG. 20. Upper figure illustrates the maximum amount of phylogenetic information that can be derived from the traditional rank classification. Lower figure is the consensus tree of this study, showing genera only.

logenetic one. As discussed earlier, Woodring (1982) and Ward (1992) both considered *Lirophora* to be of genus rank, and Olsson (1964) treated *Chionopsis* similarly. Keen (1969) also elevated *Panchione* to genus rank. Elevations of these subgenera to genus rank would therefore not be unconventional.

The changes required to convert the taxonomic classification to a phylogenetic classification begin with the exclusion of *Timoclea* from consideration as a subgenus of *Chione* and recognition of it as a distinct genus. Next, the subgenera *Chione*, *Lirophora*, *Iliochione*, and *Panchione* are elevated to genus rank. *Puberella*, originally designated a subgenus of *Chionopsis* (Fischer-Piette & Vukadinovic, 1977), is elevated to generic rank. In the monophyletic clade comprising *Lirophora*, *Anomalocardia*, *Iliochione* and *Panchione*, both *Iliochione* and *Panchione* must be considered separate genera, due primarily

TABLE 5. Comparison of traditional classification (left columns) to new phylogenetically based classification. Subgenera listed on right are genera sensu stricto.

OLD CLASSIFICATION		NEW CLASSIFICATION	
GENUS	SUBGENUS	GENUS	SUBGENUS
Chione	Chione	Chione	Chione
	Chionista		Chionista
	Chionopsis	Iliochione	
	Iliochione	Lirophora	
	Lirophora	Panchione	
	Panchione	Puberella	
	Puberella	Chionopsis	
Anomalocardia		Anomalocardia	
Mercenaria		Mercenaria	
Protothaca		Protothaca	
Timoclea		Timoclea	

to the uncertain position of *Iliochione.* The alternative would require the subsuming of both genera, plus either *Lirophora* or *Anomalocardia,* into a large genus classified as either *Lirophora* or *Anomalocardia,* on the basis of historical precedence. The subgeneric rank of *Chionista* is supported by the reclassification. Table 5 compares the old taxonomic classification with the new phylogenetic one. It is important to note here the number of taxa (genera) which were formerly subsumed in the paraphyletic *Chione,* and the exclusion of other genera on the basis of autapomorphies, not patterns of relationship.

Pliocene Extinctions

Since Woodring's (1966) initial claim, the general picture of late Neogene molluscan diversity in tropical America has been one of high Pliocene extinction in the tropical Western Atlantic compared with the Eastern Pacific (Vermeij, 1978; Vermeij & Petuch, 1986). Coupled with this is the documentation of extensive contemporaneous extinctions in the subtropical waters off the southeast United States (Stanley, 1986). Several mechanisms have been suggested to explain the extinctions, the most commonly cited ones being disruption of planktonic productivity levels (Vermeij, 1978; Vermeij & Petuch, 1986; Allmon et al., 1993) and Northern Hemisphere cooling (Stanley, 1986). It has also been argued that the extinctions were in fact faunal turnovers, and that losses of diversity have been overcompensated for by new originations and invasion (Allmon et al., 1993; Jackson et al., 1993; see also Vermeij & Rosen-

berg, 1993). Jackson et al.'s (1993) data (comprising subgeneric rank taxa) also support the initiation of the extinctions at 2.4 Ma, well into the Late Pliocene.

The history of the chionine taxa considered in this paper agree in general with the scenario constructed by Jackson et al. (1993), with several exceptions. During the entire Pliocene, extinction of Atlantic chionine species (restricted from here on to species belonging only to the former *Chione* subgenera and *Anomalocardia*) exceeded origination (Fig. 12). Origination decreases in the Pacific during the Pliocene, but is never outweighed by extinction. This observation, and a high origination rate in the Pacific during the Pleistocene and Holocene, result in a higher total diversity of chionine species in the Eastern Pacific today compared to the Western Atlantic. Two new supraspecific taxa, *Chionista* and *Iliochione,* also first appear in the Eastern Pacific during the Pliocene. Overall, the extinction level of chionine species during the entire Pliocene was 82.6% in the tropical Western Atlantic, but only 38.5% in the Pacific Gatunian (Fig. 13). Today there are 21 chionine species in the Eastern Pacific, versus 13 in the Western Atlantic (*Chione cancellata* is considered to comprise two distinct "morphological" species, based on Roopnarine, 1995). Of the five subgenera present in both regions during the Pliocene, all survive in the Eastern Pacific today, but only three in the Western Atlantic. The conclusions to be drawn from this study therefore, are that extinction of chionine species during the Pliocene was significantly higher in the Western Atlantic than in the Eastern Pacific,

and the loss of diversity was not compensated for by new originations.

The timing of a large portion of the extinction remains ambiguous. Of Early-middle Pliocene *Chione* species in the Western Atlantic 45% were absent from the Late Pliocene (Fig. 15). Extinction in the Eastern Pacific at that time was a slightly higher 57% (though species diversity was lower than in the Western Atlantic). During the Late Pliocene, however, extinction in the Western Atlantic increased to 66.7%, but fell in the Eastern Pacific to 11.1%. Dating the extinction to the Late Pliocene on the basis of these summary calculations would be incorrect, though, because the pattern is not reflected by all the individual subgenera. *Lirophora* and *Panchione* exhibit much higher extinction in the Early-middle Pliocene (60% and 100% respectively) than do the other subgenera (Fig. 16). It therefore seems that there were at least two episodes of extinction.

The first extinction episode, in the Early-middle Pliocene, was associated primarily with the subgenera *Lirophora* and *Panchione.* The extinctions were also concentrated in the Atlantic Gatunian region. The reason(s) for the differential extinction among the subgenera is not obvious. *Lirophora* and *Panchione* are nested within a subclade separate from the subclade to which *Chione* belongs, but the characters distinguishing the two subclades currently have limited functional interpretations. There are furthermore no obvious ecological differences among the subgenera. All inhabit fairly shallow, coarse to medium-grained sediments, and are sympatric today. *Lirophora* and *Panchione* probably dwell deeper in the sediment than does *Chione,* as evidenced by their deeper pallial sinuses. *Anomalocardia,* however, disrupts any phylogenetic pattern because it experiences minimal levels of extinction. The greater proportion of *Anomalocardia* diversity resided in the Caloosahatchian Province, not the Atlantic Gatunian region, unlike *Lirophora* and *Panchione,* and is suggestive of and consistent with a geographic extinction pattern. This possibility is explored more fully below.

Mechanisms of Extinction

Two expectations of extinction caused by cooling would be more severe extinction of Atlantic Gatunian species relative to Caloosahatchian species, and the higher survival of eurythermal (biprovincial) species (Stanley, 1984, 1986). According to Cronin's (1991, 1993) data, the Northern Hemisphere cooling event reported by Stanley (1986) actually began 2.6–2.4 Ma. This event coincided with, and was probably causative of, a major regression recorded on the Atlantic Coastal Plain, and allows the categorization of the Pliocene deposits into Early-middle and Late Pliocene groups. Of the species in the Atlantic Gatunian during the Early-middle Pliocene 40% did not survive into the Late Pliocene (Fig. 17). The extinction level in the Caloosahatchian Province at that time was only 18.75%. Stanley's prediction would therefore seem to be supported. Of the Late Pliocene species in the Atlantic Gatunian and Caloosahatchian 36.4% and 50% respectively were extinct by the Early Pleistocene. At this level of the analysis, the data cannot reject Stanley's cooling hypothesis. However, the possibility that the surviving species were eurythermal cannot be entertained, because there were no biprovincial chionine species in the Late Pliocene. A possible exception would be *Chione cancellata,* except that, as noted above, the Caloosahatchian and Caribbean forms may be separate species. The Caloosahatchian form first appears in the Upper Pliocene/Pleistocene Waccamaw Formation of South Carolina, and has not been documented further south than the Florida peninsula. Therefore, no evidence exists to support the higher survival of eurythermal species, nor the survival of Caloosahatchian species by southward migration.

The complete pattern of Pliocene extinction of chionine species does not support a hypothesis of cooling as a mechanism. Extinction is higher in more tropical areas during or soon after the initiation of cooling, but the higher level is due to the non-random extinction of *Lirophora* and *Panchione* species. By the Late Pliocene, extinction was distributed fairly evenly among all the subgenera. It is interesting to note, however, that the two Recent paciphilic chionine genera, *Chionopsis* s.s. and *Panchione,* were distributed throughout the Atlantic Gatunian, but only as far north as Florida during the Pliocene. These are the only two taxa to suffer complete extinction in the Atlantic. Their extinctions do not appear to support a hypothesis of cooling though, because these taxa failed to find refuge by migrating equatorward.

The indirect test of declining planktonic productivity as a mechanism of extinction

yields more intriguing results. All Early-middle Pliocene Atlantic Caloosahatchian species exceeded 35 mm in maximum valve height. Only two of eight contemporary Atlantic Gatunian species, *Chionopsis tegulum* and *Panchione mactropsis,* exceeded 35 mm, and these in fact exceeded 40 mm. Only one of the Caloosahatchian species did not survive beyond the middle Pliocene. Three of the Atlantic Gatunian species survived into the Late Pliocene, but they were all small, being less than 25 mm in height. Caloosahatchian species, survivors and new Late Pliocene species on the other hand remained relatively large, all exceeding 30 mm in maximum valve height, and three of six exceeding 40 mm. Recent Caloosahatchian species regularly exceed 30 mm in valve height, but none are known to attain heights of 40 mm or more. Caribbean species have remained small, generally not exceeding 25 mm. Notable exceptions occur in areas of upwelling, and hence relatively high planktonic productivity. Recent Pacific species commonly attain heights in excess of 50 mm (Fig. 19); *Chionopsis gnidia* is the largest described chionine species dealt with in this paper. In summary, size distributions have not changed very much in the Caloosahatchian Province since the Early Pliocene, despite changes in species composition. In the Gatunian Province though, the change has been more dramatic. Large species in the Atlantic Gatunian region did not survive into the Late Pliocene. Species size distribution there remains small because of the differential extinction of the larger Early-middle Pliocene species, and apparently because no large (> 35 mm) species have evolved in that region since. The opposite is true of the Pacific Gatunian, or Panamic Province. Post Early-middle Pliocene species there tend to be very large, perhaps a consequence of the widespread coastal upwelling in that region.

Given the equivocal nature of the results of the above tests, any hypothesized cause(s) of chionine extinction during the Pliocene in the Western Atlantic is speculative. Moreover, the data are limited to only those genera examined, and the availability of material documented in the field, museum collections, and the literature. Regardless, the pattern of extinction can be explained tentatively by current geological data which appear to support a decline in productivity, at least in the Early-middle Pliocene.

The loss of Early-middle Pliocene large-and small-bodied species from the Atlantic Gatunian, representing 40% of the species, contrasts strongly with the contemporary Caloosahatchian. Only 18.75% of Early-middle Pliocene Caloosahatchian species did not survive into the Late Pliocene, and there is no reduction in overall body size. There is therefore an indication of an Early-middle Pliocene episode of extinction in the Atlantic Gatunian that did not have a great impact on the Caloosahatchian Province. The Late Pliocene extinctions in the Atlantic Gatunian are matched in severity, however, by the Late Pliocene Caloosahatchian extinctions, but there is no change in overall body size in either province. It is therefore possible that there were two episodes of extinction in the tropical Western Atlantic during the Pliocene (Petuch, 1995).

The earlier extinction in the Atlantic Gatunian probably followed the final closure of the Panama seaway (~3.5 Ma). Final closure occurred during the earliest Pliocene when the shallowing of the seaway was already dramatic (< 100 m depth, Duque-Caro, 1990; see also Coates et al., 1992). The shallowing undoubtedly resulted in the fragmentation of once contiguous and widespread populations, but perhaps more importantly, it also changed the oceanographic configuration of the Caribbean region. It has not yet been determined how changes in circulation and the decline and eventual termination of flow from the Atlantic to the Pacific may have affected local diversity. The presence of seasonal cool water in the Caribbean during the middle Pliocene (Cronin, 1991) unlike today, the existence of relict communities in the Caribbean in areas of upwelling today (Petuch, 1982), and the loss of large bodied species from the Atlantic Gatunian (this paper) however, all suggest a decline in planktonic productivity. Interestingly, though, Lower-middle Pliocene deposits in the Caloosahatchian Province show no signs of significant extinction or loss of large bodied species. This would suggest that the extinctions did not extend to, or did not affect the Caloosahatchian Province significantly.

The Late Pliocene extinctions of the Atlantic Gatunian and Caloosahatchian provinces coincide with the initiation of Northern Hemisphere cooling (2.5–2.4 Ma) (Stanley, 1986; Cronin, 1991, 1993). Neither extinction, however, exhibits pattern expected of cooling scenarios. The only noticeable difference between Late Pliocene and Recent faunas, be-

sides the lower diversity of modern faunas, is the absence of very large-bodied species (> 40 mm) in the Recent. Large species, or large specimens (30–35 mm), survive in the Caloosahatchian region, and in areas of upwelling in the Caribbean. This observation suggests that declining planktonic productivity may again have been a factor, but perhaps not the only, or primary one.

SUMMARY

(1) Phylogenetic analysis shows that the genus *Chione* as currently defined is paraphyletic. A monophyletic clade comprises the genera *Chione* (excluding the subgenera *Chionopsis* and *Puberella*), and *Anomalocardia*. *Chione* has been revised in order to construct a taxonomic classification that is consistent with the results of the phylogenetic analysis. The genus was revised and replaced by the genera (formerly subgenera) *Chione (s.s.), Chionopsis, Lirophora, Iliochione, Panchione* and *Chionista*. The treatments of *Chionopsis* as a genus (Olsson, 1964), similarly *Lirophora* (Woodring, 1982; Ward, 1992), and the consideration of *Panchione* as a subgenus separate from *Lirophora* (Keen, 1969), support the revision.

(2) Sculptural characters, primarily the retention of radial sculpture, and the marginal elaborations of concentric sculpture, are diagnostic of the "*Chione* subclade" (comprising the genera *Chione* and *Chionista*). The "*Lirophora* subclade" is defined more strongly by a combination of characters describing internal as well as external valve morphology, most notably aspects of concentric sculpture and nymph rugosity.

(3) During the late Neogene the origination of new chionine species rose steadily in the Eastern Pacific. The number of last appearances increases briefly during the Pliocene, but never exceeds the number of first appearances. Of Early-middle Pliocene species in the Atlantic Gatunian Province 40% do not survive into the Late Pliocene, compared with 18.75% of Early-middle Pliocene Caloosahatchian species. The higher extinction in the Atlantic Gatunian region is accounted for primarily by the seemingly non-random extinction of species assigned to the genera *Lirophora* and *Panchione*. During the Late Pliocene however, 36.4% and 50% of species in both the Atlantic Gatunian and Caloosahatchian Provinces respectively are

last appearances, and these extinctions seem to be distributed randomly among the surviving genera. This wave of extinction began at least 2.5 million years ago.

(5) A hypothesis of cooling as a mechanism of extinction is not supported by these data. Extinctions caused by cooling would have been restricted to the Late Pliocene because of the late date of the onset of Northern Hemisphere refrigeration (Cronin, 1991, 1993). Extinction is not more severe in the fully tropical Atlantic Gatunian Province than the sub-tropical Caloosahatchian. Moreover, there is no evidence that Caloosahatchian species survived the crisis by southward migration. Some equivocal support might be provided by the observation that neither of the two paciphilic genera examined, *Chionopsis* (s.s.) and *Panchione*, extended further north than Florida during the Pliocene.

(6) The loss of all large bodied (> 35 mm) species from the Early-middle Pliocene Atlantic Gatunian Province is consistent with a hypothesis of declining planktonic productivity. During the Late Pliocene, however, body size did not seem to be a very discriminating factor, since small bodied Atlantic Gatunian species also suffered extinction. Modern species have maximum valve heights, in the Caloosahatchian and Caribbean, ranging from 25 to 35 mm. On the other hand, Late Pliocene Caloosahatchian species exceeding 40 mm in maximum valve height suffered 100% extinction. Therefore, it seems that large bodied species did suffer differentially higher levels of extinction during the Pliocene. The mechanism responsible is hypothesized to be a disruption of planktonic productivity patterns and levels. Additional, indirect evidence is provided by the Late and post-Pliocene evolution of very large chionine species in the upwelling-rich Panamic Province.

ACKNOWLEDGMENTS

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APPENDIX I

Left Valve

1. Depth of pallial sinus: 0 = greatly reduced to absent; 1 = present but short; 2 = pallial sinus deep, anterior end to beneath posterior cardinal tooth; 3 = very deep, past beneath posterior cardinal.

   The most common condition of the pallial sinus in chionine bivalves is moderately to very deep. Examples can be found in the genera *Mercenaria* and *Timoclea.* Many other taxa, for example *Lirophora,* have very short sinuses. An intermediate condition can be found in *Puberella* and *Chionopsis.* Neither *Chione* nor *Petenopsis* have a recognizable pallial sinus.

2. Types of sculpture present: 0 = concentric elements only; 1 = radial and concentric elements, co-dominant; 2 = radials and concentrics, concentrics dominant, radials sub-obsolete; 3 = radials and concentrics, both sub-obsolete; 4 = radials and concentrics, radials dominant.

   There are two types of sculptural elements in the Chioninae, radial and concentric. Both forms are present and co-dominant in *Chione* and *Chionopsis* (including *Puberella*). The concentric elements in these genera are thin, raised lamellae, whereas the radials are raised and cordlike. The radials are absent in *Mercenaria,* but this appears to be a derived autapomorphic condition, not homologous with the "concentric-dominant" sculpture of *Lirophora, Anomalocardia, Iliochione* and *Panchione; Mercenaria*'s concentrics are of the *Chione* and *Chionopsis* type. *Lirophora, Anomalocardia, Iliochione* and *Panchione* all have greatly reduced or absent radial elements, and the development of thick, folded concentric lamellae. *Leukoma* and *Timoclea,* on the other hand, lack concentric elements almost entirely, but have radial sculpture very similar to the *Chione* and *Chionopsis.* The sculptures of *Chionista* and *Petenopsis* seem to be autapomorphic.

3. Spacing of concentric elements: 0 = close (narrow spacing); 1 = widely spaced; 2 = wide spacing on older, juvenile shell, but tending to become more narrowly spaced with increasing age.

4. Posterior development of concentric elements: 0 = flared in ventral direction; 1 = weakly flared, but flattened; 2 = no flare.

   The posterior end of concentric elements is highly developed in many chionine taxa, possibly for functional reasons associated with shallow burrowing.

5. Anterior development of concentric elements: 0 = no anterior projection; 1 = developed anteriorly; 2 = weak anterior development.

   The concentric elements are developed anteriorly in many chionine taxa, and these may aid during burrowing, probably acting as ratchets, but this has not been rigorously tested.

6. Structure of concentric elements: 0 = concentric elements smooth; 1 = ventral surface of concentric elements bearing closely packed vertical ribs; 2 = ventral surface of concentric elements bearing widely spaced vertical ribs; 3 = concentric elements present only as raised structures on radial elements.

7. Distal edge of concentric elements: 0 = sharp; 1 = smooth; 2 = smooth and reinforced.

   The distal edges of the concentric elements are either thin and sharp, or thickened

and smooth. Some, as in *Puberella,* are thin, but have a thickened, reinforced summit.

8. Orientation of concentric elements: 0 = vertical; 1 = folded; 2 = step-like and rounded.

This character describes the orientation of concentric elements relative to the surface of the valve.

9. Definition of escutcheon: 0 = escutcheon developed and set off from rest of valve by obvious ridge ("keel"); 1 = escutcheon developed but weakly keeled; 2 = escutcheon not developed.

10. Width of anterior cardinal tooth: 0 = wide; 1 = narrow.

The anterior cardinal is either well developed and wide, as in *Chione,* or thin and blade-like, as in *Chionopsis.*

11. Nymph: 0 = rugose; 1 = smooth.

The nymph, a platform posterior to the posterior cardinal tooth, houses the ligament in venerid species. The nymph is generally smooth, but can have a roughened or "rugose" surface. The state of this character is sometimes given predominant weight in determining chionine relationships (for example, Harte 1992a), but nymph rugosity, of variable morphology, is present in non-chionine taxa, for example *Pitar (Lamelliconcha)* Dall, 1902. Nymph rugosity is therefore homoplastic at some levels.

12. Ventral margin crenulation: 0 = large; 1 = fine; 2 = intermediate between 0 and 1, and regular.

13. Lunule sculpture: 0 = numerous concentric elements; 1 = few concentric elements; 2 = smooth; 3 = concentric elements with subdominant radial ribs; 4 = sub-obsolete concentric and radial elements; 5 = numerous radial ribs; 6 = lunule not developed.

14. Middle cardinal tooth morphology: 0 = bifid; 1 = smooth; 2 = smooth, except for dorso-ventral groove.

15. Posterior cardinal tooth shape: 0 = straight; 1 = curved; 2 = weakly curved.

16. Shape of hinge plate margin: 0 = plate very bowed beneath anterior cardinal tooth; 1 = plate weakly bowed, not obvious; 2 = plate

bowed, not exaggerated as in 0; 3 = plate straight.

Many chionine species have hinge plates that are noticeably bowed, or curved, beneath the anterior cardinal tooth. An extreme example of this can be found in *Mercenaria.* Other taxa have straight margins, and there is a continuum between the two states.

Right Valve

17. Definition of escutcheon: 0 = escutcheon developed, but not demarcated noticeably from rest of valve surface; 1 = escutcheon developed and demarcated from rest of valve by keel; 2 = escutcheon not developed.

18. Sculpture of escutcheon: 0 = concentrically sculptured; 1 = escutcheon smooth; 2 = sub-obsolete concentric sculpture; 3 = escutcheon absent;

19. Width of middle tooth: 0 = tooth narrow; 1 = tooth wide.

20. Condition of middle cardinal tooth: 0 = tooth bifid; 1 = tooth grooved on dorsoventral axis; 2 = tooth smooth.

21. Condition of posterior cardinal tooth: 0 = tooth bifid; 1 = tooth grooved on dorsoventral axis; 2 = tooth smooth.

22. Orientation of groove on posterior margin: 0 = groove just overlaps ventral tip of posterior cardinal tooth; 1 = groove distal to posterior cardinal tooth; 2 = groove abuts posterior cardinal tooth; 3 = groove overlaps posterior cardinal tooth significantly; 4 = groove absent.

This groove is present in many chionine taxa on the right valve, and houses the posterior margin of the left valve when the shell is closed.

23. Condition of radial ribs between concentric sculpture: 0 = no radial ribs present; 1 = radial ribs prominent; 2 = radial ribs present but fine, tending to become obsolete.

General Shell Morphology

24. Escutcheon symmetry: 0 = escutcheon symmetric between valves; 1 = escutcheon asymmetric between valves; 2 = escutcheon absent.

The escutcheon is generally of different

morphology between valves in chionine taxa, but is sometimes identical.

25. Development of valve surface near posterior margin: 0 = identical to rest of valve surface; 1 = sculpture and valve surface differentially developed as posterior margin approached.

Some taxa have significant changes of sculpture towards the posterior margin. Much of the change is in concentric sculptural morphology, as in *Panchione mactropsis,* where the folded concentric sculpture becomes lamellar. *Iliochione* has an indentation of the valve.

APPENDIX II

(see APPENDIX I for explanation of numerical codes)

"9"= missing data

Chione cancellata	0120210000103221001212110
Chione chipolana	1100212000106121001212110
Chionopsis amathusia	2110110011101011110101101
Puberella cribraria	2100112001100020000110210
Lirophora athleta	1002001101012121010213001
Lirophora victoria	1000001101912121019919001
Panchione mactropsis	1201211101012221011210201
Panchione ulocyma	1202211101012223011210201
Iliochione subrugosa	1202011101012101011223201
Chionista fluctifraga	2302021120125002231004121
Petenopsis tumens	0112011200112121011222100
Mercenaria mercenaria	2000000000000000000000010
Anomalocardia flexuosa	0202001111012103020221001
Protothaca asperrima	2402030001114201011112111
Timoclea marica	3401030001104103121124100
Anomalocardia auberiana	1202021111011101021221201

APPENDIX III

Synapomorphies for interior nodes, cladogram #1. Results of both ACCTRAN and DELTRAN routines are listed. Interpret listings as (x:y) = (character:state). ACCTRAN

Synapomorphies

Node	ACCTRAN	DELTRAN
17	4:0, 5:2, 7:0, 13:3, 18:0, 24:1	4:0, 5:2, 18:0, 24:1
18	1:0, 8:0, 15:2, 25:0	1:0, 15:2, 25:0
19	10:0	10:0
20	2:0, 6:0, 19:0, 23:0	2:0, 6:0, 23:0
21	5:2, 14:2, 22:0	5:2, 14:2, 22:0
22	15:2	15:2
23	6:0, 9:1, 18:2, 22:1	9:1, 18:2, 22:1
24	21:2	21:2
25	1:1, 2:2, 11:0, 12:1, 22:3, 23:2	1:1, 2:2, 8:1, 11:0, 12:1, 22:3, 23:2
26	7:1, 8:1, 20:2	7:1, 13:2, 20:2
27	2:4, 6:3, 13:4	2:4, 6:3, 13:4
28	4:2, 13:2, 14:1, 19:1, 21:1, 22:2	4:2, 14:1, 19:1, 21:1, 22:2
29	13:1, 16:1, 18:1, 22:1, 23:1, 24:0, 25:1	16:1, 18:1, 23:1, 24:0, 25:1

THE GENITAL SYSTEM OF *ACOCHLIDIUM FIJIENSE* (OPISTHOBRANCHIA: ACOCHLIDIOIDEA) AND ITS INFERRED FUNCTION

Martin Haase[1] & Erhard Wawra[2]

ABSTRACT

The genital system of the adolescent-gonochoric freshwater opisthobranch *Acochlidium fijiense* is described from histological serial sections of five individuals and dissection of a sixth animal in full detail. The penis has a characteristic armature consisting of an ascending spiral of chitinous spines on the edge of the glans. The basal finger in association with the paraprostate probably functions as stimulatory organ analogous to the gypsobelum of pulmonate gastropods. The presence of sperm in the haemocoel and the kidney of one specimen and the penial armature suggest that *A. fijiense* transfers sperm through hypodermic impregnation. The most peculiar feature is the connection of the genital system with the digestive system. A duct with unknown function connects the digestive gland with the distal gonoduct. In addition, in one individual the ampulla which stores autosperm had an opening into the digestive gland. This opening is interpreted as a temporary structure established only when required in order to digest excess autosperm, thus compensating the lack of a gametolytic gland. However, it cannot be ruled out that this connection seen in a single individual was an abnormality.

Key words: *Acochlidium,* genital system, hypodermic impregnation, Opisthobranchia, sperm transfer, stimulatory organs

INTRODUCTION

The vast majority of opisthobranch gastropods are marine. Up to now only seven species are known from freshwater habitats. These seven species all belong to the order Acochlidioidea. All marine acochlidioidean species are smaller than 5 mm. All but one species of the freshwater forms, on the other hand, exceed 15 mm. The exception is the Caribbean *Tantulum elegans* Rankin, 1979, which lives interstitially (Rankin, 1979). The large species—*Strubellia paradoxa* (Strubell, 1892) and five species of the genus *Acochlidium* Strubell, 1892 [Following Wawra (1989), we use a conservative classification and reject Rankin's (1979) taxonomic splitting.]—occur on islands in the Pacific region (Haynes & Kenchington, 1991). Despite the size of the Pacific freshwater species, which would make anatomical investigation and maintenance and observation in aquaria rather easy compared to small, interstitial snails, relatively little is known on both their anatomy and biology. The present study gives a detailed anatomical description of the genital system of *A. fijiense* Haynes & Kenchington, 1991, and allows inferences on its function and the reproductive biology of this species.

MATERIALS AND METHODS

Five individuals from the series of paratypes of *A. fijiense* from Vanua Levu, Fiji, deposited in the mollusc collection of the Museum of Natural History in Vienna by Haynes & Kenchington (1991) under the inventory number 84901 were embedded in Paraplast and serially sectioned, one specimen at 7 µm and the remaining four at 10 µm. The series were stained with Heidenhain's Azan. The fixed (Bouin) snails measured 6.1 mm, 7.4 mm, 7.9 mm, and 8.03 mm respectively. One specimen could not be measured because it had the visceral hump turned down. These individuals belonged to the largest among the series of paratypes. In the following, they will be referred to as snails number 1 to 5 beginning with the smallest animal. We do not proceed on the assumption that these snails represent a developmental sequence, because they might have had contracted to differing degrees at fixation. The genital system was reconstructed using the computer program PC3D of Jandel Scientific. The penis of a sixth paratype from the same lot was dissected, critical point dried and investigated by scanning electron microscopy (SEM).

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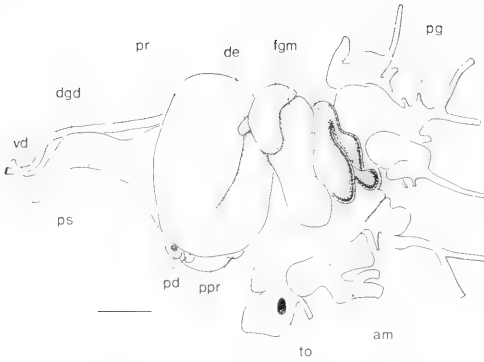
FIG. 1. Reconstruction of the genital system except gonad from dorsal. am = ampulla; de = ductus ejaculatorius; dgd = distal gonoduct; fgm = female gland mass; pd = paraprostatic duct; pg = praeampullary gonoducts; ppr = paraprostate; pr = prostate; ps = penial sheath; to = [presumptive (see Discussion)] temporary opening of the ampulla into the digestive gland; vd = vas deferens. Scale bar = 500 μm.

FIG. 3. Schematic representation of the genital system and its connection with the digestive gland. Accessory organs not drawn. am = ampulla; dd = duct connecting digestive gland and distal gonoduct; de = ductus ejaculatorius; dg = digestive gland; dgd = distal gonoduct; fgm = female gland mass; go = gonad; in = intestine; oe = oesophagus; pg = praeampullary gonoduct; po = postampullary gonoduct; pr = prostate; st = stylet; to = [presumptive (see Discussion)] temporary opening of the ampulla into the digestive gland; vd = vas deferens.

FIG. 2. Distal genital system from latero-dorsal. dd = duct connecting digestive gland and distal gonoduct; de = ductus ejaculatorius; dgd = distal gonoduct; fgm = female gland mass; pr = prostate; ps = penial sheath; vd = vas deferens. Scale bar = 500 μm.
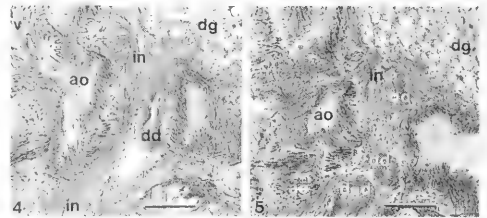
FIGS. 4, 5. Origin of duct connecting digestive gland and distal gonoduct. Increment between Figs. 4 and 5 = 30 μm. ao = aorta; dd = duct connecting digestive gland and distal gonoduct; dg = digestive gland; dgd = distal gonoduct; in = intestine; v = ventricle. Scale bars = 100 μm.

RESULTS

The description of the genital system follows the route the gametes take from their place of origin in the gonad to the genital openings, that is from posterior to the anterior end of the snail. The gonad is covered by the lobes of the digestive gland and consists of a large number of acini (Fig. 16). Oocytes and spermatocytes mature in the same acinus. The gametes produced in these acini are collected through a branching net of ciliated preampullary gonoducts, which open into the ampulla (Figs. 1, 16, 17). This ampulla consists of a number of communicating chambers. Its epithelium lacks cilia. The glandular postampullary gonoduct connects the ampulla with the large female gland mass. The duct enters the gland mass ventrally on the left side. This gland mass has two histologically distinct portions, which probably function as albumen and mucous gland, respectively. At the right side, the ciliated distal gonoduct leaves the gland mass and traverses the body wall to the anterior end, where it opens close to the mouth (Figs. 1, 2).

There are two ducts branching off the distal gonoduct. Proximally, a short duct connects the gonoduct with the digestive gland.
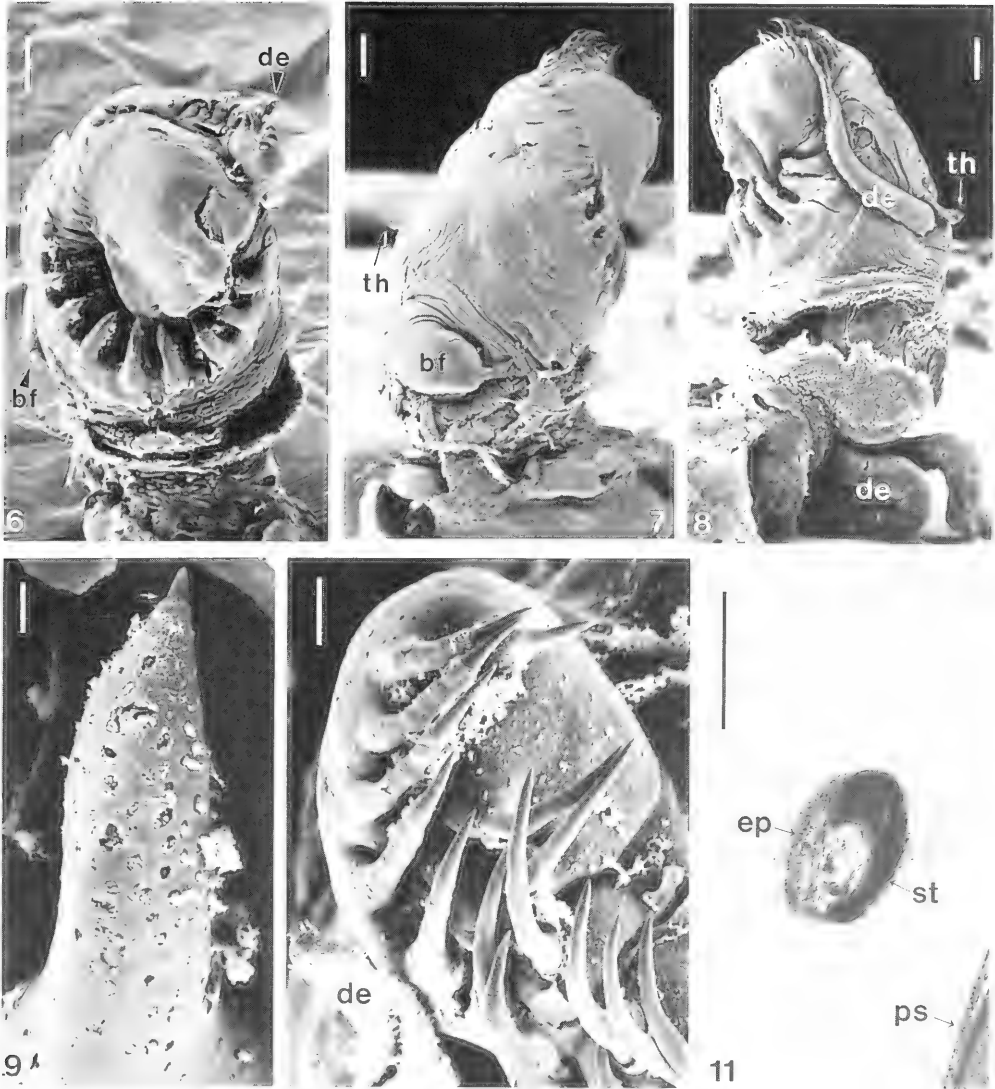
FIGS. 6–11. Penis. 6. Apical view. Scale bar = 100 μm; 7, 8. Lateral views. Scale bars = 100 μm; 9. Large spine in epidermal sheath. Arrow indicates distal end of the sheath. Scale bar = 10 μm; 10. Bulge of the edge of the penial glans with small spines. Arrow indicates distal end of an epidermal sheath. Scale bar = 20 μm; 11. Cross-section through the tip of the ejaculatory duct. Scale bar = 50 μm. bf = basal finger; de = ductus ejaculatorius; ep = epithelium; ps = penial sheath; st = stylet; th = thorn.

The junction of this short duct with the digestive gland is close to the origin of the intestine (Figs. 2–5). Distally, the vas deferens, ventrally attached to the penial sheath, leads backwards to the prostate (Figs. 1–3, 14).

The muscular ductus ejaculatorius originates in the middle of the ventral side of the prostate (Fig. 2). It enters the muscular penis at its base after several coils between the lobes of the prostate and the paraprostate (see below) and around the penis (Figs. 1, 8, 14, 15). Distally, the ductus ejaculatorius leaves the penis at the left side and rests on its external wall (Figs. 6, 8, 12, 13) (In snail no. 3 the ductus ejaculatorius was completely retracted into the penis.). The opening through

FIG. 12. Schematic representation of the penis. Chitinous elements are solid black. bf = basal finger; de = ductus ejaculatorius; ip = intrapenial gland; pd = paraprostatic duct; ppr = paraprostate; pr = prostate; th = thorn.

distal-most part where the edge of the penial glans is broadened to a bulge (Figs. 6, 10, 12). All spines, the stylets of the ductus ejaculatorius and the basal finger, and the thorn at the opening through which the ductus ejaculatorius leaves the penis are partly covered by an epidermal sheath (Figs. 7, 9, 10, 13), which, in the case of the spines on the penial glans, bears bundles of presumably sensory cilia (Fig. 9). The penis can be protruded through a sheath (Figs. 1, 2, 13, 14), which opens behind the right rhinophore.

The gonad of snail no. 1 contained only spermatogonia and spermatozoa. Snails nos. 4 and 5 had in addition yolk material, but no oocytes, whereas spermatognia, spermatozoa, yolk material and oocytes were found in snails nos. 2 and 3. Specimen no. 3 had significantly more oocytes than no. 2. In these latter two individuals, the digestive gland contained spermatozoa (Fig. 17). The ampulla of snail no. 2 had a distinct opening into the digestive gland (Fig. 17). No such opening was found in the remaining specimens. This opening in snail no. 2 is no preparatorial artefact as indicated by the extension of the mass of sperm in the digestive gland far in front of and behind the opening. A bundle of spermatozoa lay in a fold of the foot close to the anus of animal no. 3 (Fig. 18). These spermatozoa were obviously expelled through the intestine at fixation. Spermatozoa were also found in the posterior third of the visceral hump in the kidney and in the haemocoel of snail no. 2 (Fig. 19).

DISCUSSION

Acochlidium fijiense was described as hermaphroditic (Haynes & Kenchington, 1991). However, from the different states of gonadal maturation we conclude that adolescent gonochorism, that is beginning as a male (or female, which does not apply in this case) and then becoming a simultaneous hermaphrodite (Ghiselin, 1987), may be a more precise characterization of the reproductive strategy of *A. fijiense*.

Our findings of the penial morphology differ in several aspects from the description of Haynes & Kenchington (1991). These authors observed neither the basal finger nor the true course of the ductus ejaculatorius. Their statement on number and position of the spines on the edge of the penial glans varies as well. These discrepancies are probably

which the ductus ejaculatorius exits the penis is guarded by a chitinous thorn (Figs. 7, 8, 12, 13). This thorn is associated with a gland lying in the penis, which is hereafter referred to as intrapenial gland (Fig. 12). At its tip, the ductus ejaculatorius bears a chitinous stylet which is in fact a groove closed by epithelium (Fig. 11). Basally, at the dorsal side, the penis bears a finger, the basal finger, armed with a corneous stylet (Figs. 6, 7, 12). This hollow stylet is connected with a gland, which we call paraprostate because of its position ventral of the actual prostate, by the paraprostatic duct (Figs. 1, 14, 15). The glans penis is armed with chitinous spines, too (Figs. 6–10, 12). Two types of spines in an ascending spiral on the edge of the glans can be distinguished. This spiral comprises almost an entire whorl. In the specimen dissected, 12 large spines formed the lower semi-circle and 24 finer spines completed the spiral. The spines stand in a single row except in the

FIGS. 13–15. Male genital organs. 13. Ductus ejaculatorius leaving penis; 14. Distal region of prostate and paraprostate, penis and sperm conducting ducts. Arrows indicate spines, arrow heads the penial sheath; 15. Proximal region of prostate and paraprostate with ejaculatory duct. de = ductus ejaculatorius; dg = digestive gland; oe = oesophagus; p = penis; ppr = paraprostate; pr = prostate; ps = penial sheath; sg = salivary glands; th = thorn; vd = vas deferens. Scale bars = 100 μm.

due to different states of contraction of the penis after fixation. Besides, Haynes & Kenchington (1991) neither had the opportunity of SEM investigations nor did they section the penis.

For similar reasons, comparison with other species of the genus *Acochlidium* are difficult. What Bücking (1933) described as an oviduct in *A. amboinense* Strubell, 1892, is clearly the paraprostatic duct with the basal finger. And in *A. sutteri* Wawra, 1979 (Wawra, 1979), the smaller thorn appears to represent the armature of the basal finger or its homologue. The descriptions of *A. bayerfehlmanni* Wawra, 1980 (Bayer & Fehlmann, 1960; Wawra, 1980) and *A. weberi* (Bergh, 1896) (Bergh, 1896) are too superficial to allow a detailed comparison.

Organs similar in structure and position to the paraprostate and the basal finger of *A. fijiense* have been described in another acochlidioidean species, the interstitial *Pseudunela cornuta* (Challis, 1970) (Challis, 1970: 37). The penial gland and the "complex muscular organ equipped with a single, hollow, curved spine" of *P. cornuta* are probably respective homologues.

The basal finger probably functions as a stimulatory organ analogous to the dart (gypsobelum) of some stylommatophoran land snails or other organs in some other pulmonates (Tompa, 1984). Adamo & Chase (1988, 1990) found that in *Helix aspersa* O. F. Müller, 1774, dart shooting decreased courtship duration. In that species, the active substance is secreted by the digitiform glands which produce a mucus that coats the dart. This mucus is only effective if it is injected into the body cavity (Adamo & Chase, 1990; Chung, 1986). We assume that in *A. fijiense* the basal finger in association with the paraprostate has a similar function during copulation.

The three strategies of sperm transfer occurring in the order Acochlidioidea, namely copulation, injection, and transfer by sper-
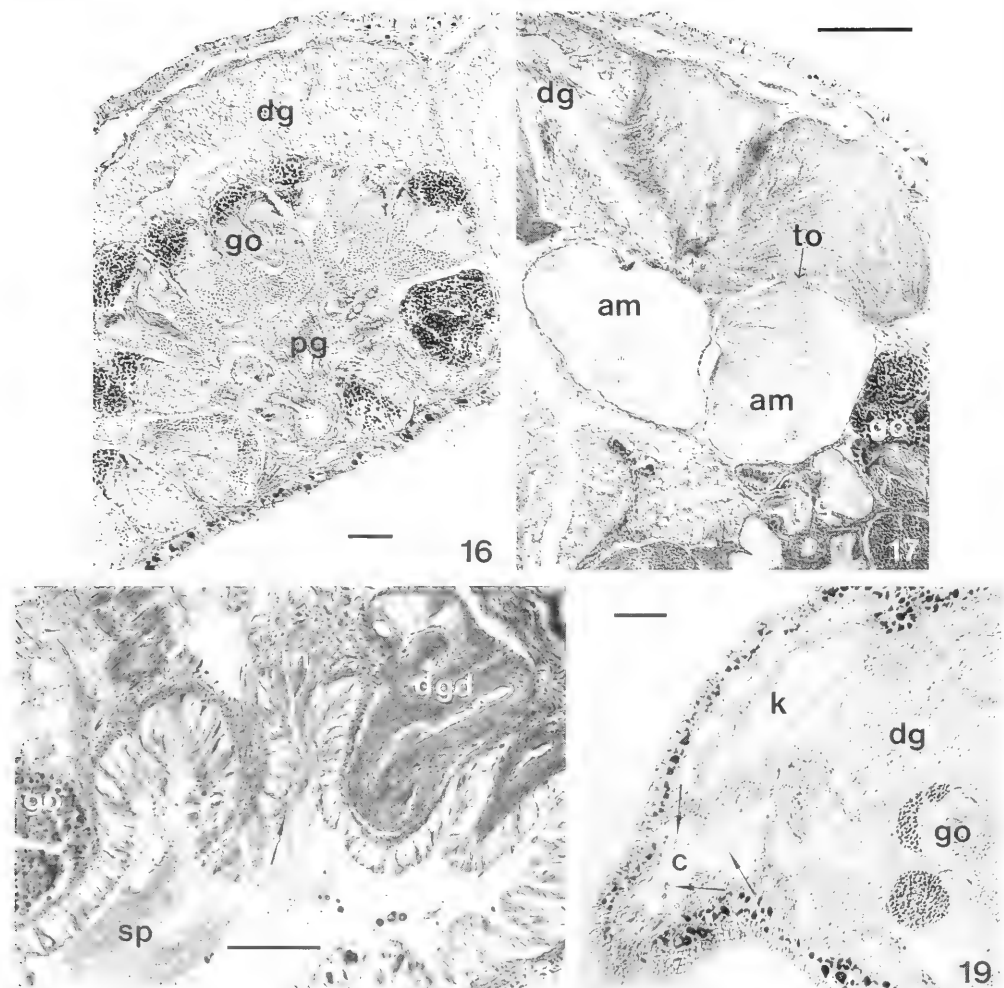
FIGS. 16–19. Spermatozoa. 16. Gonad; 17. Ampulla opening into digestive gland; both organs are filled with sperm; 18. Sperm in a fold of the foot. Arrow indicates the anus; 19. Sperm in haemocoel and kidney. Arrows indicate blood cells; Figs. 16, 17 and 19 are interference contrast photographs. am = ampulla; c = haemocoel; dg = digestive gland; dgd = distal gonoduct; go = gonad; in = intestine; k = kidney; pg = praeampullary gonoducts; sp = spermatozoa; to = [presumptive (see Discussion)] temporary opening of the ampulla into the digestive gland. Scale bars = 100 μm.

matophores, are briefly discussed by Wawra (1992). In *A. fijiense,* the penial armature, the stylet-bearing ductus ejaculatorius and the fact that we found spermatozoa in the haemocoel and in the kidney of snail no. 2 indicate that sperm are injected into the haemocoel at copulation. The injection of sperm into the kidney was probably an accident.

Hypodermic impregnation is the presumptive mode of sperm transfer in another aco-

chlidioidean species, the interstitial *Hedylopsis spiculifera* (Kowalewsky, 1901) (Wawra, 1989). Hypodermic injection of sperm is further known in some Sacoglossa (Baba & Hamatani, 1970; Gascoigne, 1956, 1975, 1976, 1978, 1993; Hand & Steinberg, 1955; Jensen, 1986; Marcus, 1973; Reid, 1964; Trowbridge, 1995) and two nudibranch species (Rivest, 1984). Gascoigne (1993) distinguished between precise and imprecise hypodermic injection. In the first, more common

mode, sperm are injected through the body wall directly into parts of the genital system, while in the imprecise mode, spermatozoa are released into the haemocoel. Imprecise hypodermic impregnation is only reported for the sacoglossans *Elysia maoria* (Powell, 1937) (Reid, 1964), *E. subornata* Verrill, 1901 (Jensen, 1986), *Bosellia corinneae* Marcus, 1973 (Marcus, 1973), and *Alderja modesta* (Lovén, 1844) (Hand & Steinberg, 1955). *Hedylopsis spiculifera* and our study organism, *Acochlidium fijiense,* probably practice the imprecise mode, too. In none of the imprecisely injecting species is the fate of the transferred sperm known. That holds also for those acochlidioideans in which sperm transferred in spermatophores attached to the body wall enter the haemocoel through lysis of the recipient's epidermis (Doe, 1974; Hadfield & Switzer-Dunlap, 1984; Morse, 1976; Swedmark, 1968a, b).

In the nudibranchs *Palio zosterae* (O'Donoghue, 1924) and *P. dubia* (Sars, 1824), sperm must be injected into the gonadal acini. Sperm that are released into the haemocoel are phagocytosed by blood cells (Rivest, 1984). In the specimen of *A. fijiense* impregnated with sperm, we found accumulations of blood cells, too. But because these spermatozoa are presumably intended to fertilize eggs, the blood cells have probably a different function such as nourishment or guidance to the fertilization site.

Like most acochlidioideans, *A. fijiense* lacks both a seminal receptacle for storage of allosperm and a gametolytic gland (bursa copulatrix) to digest excess allo- and autosperm and other surplus substances and products of the genital system (Hadfield & Switzer-Dunlap, 1984). Because these sperm-receiving organs are typical of the *Bauplan* of genital systems of opisthobranchs (e.g., Salvini-Plawen, 1991), we consider their loss to be secondary (see below). The loss of the gametolytic gland appears to be compensated by the digestive gland. In the five specimens that we sectioned, only the ampulla of snail no. 2 had an opening into the digestive gland. Both this snail and individual no. 3 had sperm in the digestive gland. Based on the fact that ampulla and digestive gland were connected in only one individual but two snails had sperm in the digestive gland, and supported by the consideration that both digestion of food and release of gametes would be hampered by a permanent opening of the ampulla into the digestive gland, we conclude

that this connection is transient, established only when required. Whether the digestive gland also digests allosperm cannot be told. This might be the case if sperm were accidently injected into the digestive gland at copulation. If *A. fijiense* copulated through the genital pore, sperm could reach the digestive gland through the duct connecting distal gonoduct and digestive gland. However, the distal gonoduct has no vaginal characteristics which would indicate reception of a copulatory organ and sperm. Circumstantial evidence (penial armature, sperm in haemocoel and kidney of snail no. 2) suggests that the mode of sperm transfer is hypodermic injection.

Because the connection of ampulla and digestive gland was seen in only a single individual, one might argue that this opening was an abnormality, and consequently, the sperm in the digestive gland of individual no. 3 would be allosperm (see above). But the fact that this opening was seen in only a single snail is not a strong argument against the presumed regularity of the temporary connection of digestive gland and ampulla, simply because of the improbability to detect a transient structure. Until further evidence we intuitively prefer the first interpretation of the findings in snail no. 2.

The loss of seminal receptacle and gametolytic gland in the genital system of acochlidioidean species appears to be correlated with the mode of sperm transfer. *Strubellia paradoxa* is the only acochlidioidean species possessing both organs (Wawra, 1988). Only the gametolytic gland is present in *Pseudunela cornuta* (Challis, 1970). In both species, allosperm must enter the genital system through the genital opening in order to get to the receptacle or to the gametolytic gland. All other species for which the genital anatomy is described, including those of the genus *Hedylopsis* Thiele, 1931 (contra Odhner, 1937, and Rankin, 1979, see Wawra, 1989), have lost both the receptacle and gametolytic gland. In all these species, sperm are or are presumed to be transferred either by hypodermic impregnation or through spermatophores attached to the body wall of the mating partner. All these species are either small, interstitial forms or relatively large freshwater snails of the genus *Acochlidium* (not sensu Rankin, 1979; the species are listed in Haynes & Kenchington, 1991). Because of the poor state of knowledge on the majority of the species of the order Acochlid-

ioidea, it is too early to speculate how often these modes of sperm transfer accompanied by the loss of the receptacle and the gametolytic gland evolved in this group. This holds also for the question whether form and function of the genital system of *Acochlidium* are autapomorphies of the genus or inherited from a marine, possibly even interstitial, ancestor.

The bundle of sperm observed in a fold of the foot near the anus of snail no. 3 was obviously expelled from the intestine at fixation. In this way, the animal decreased its volume and thus could contract more efficiently. The decrease of volume of the digestive gland could in addition be achieved through the bypass to the distal gonoduct. However, the true function of the duct connecting the digestive gland and the distal gonoduct remains a matter of speculation. A noteworthy analogy exists in some turbellarians where the ductus genitointestinalis connects the genital system with the digestive system (e.g., Reisinger, 1968). The true function of this ductus genitointestinalis is also unclear.

The peculiar course of the vas deferens was described earlier for *A. sutteri* (Wawra, 1979) and *A. bayerfehlmanni* (Wawra, 1980), but it remained unclear through which duct eggs are laid. In *A. fijiense*—and we assume that the same holds for the above mentioned species—the distal gonoduct continues beyond the branch to the vas deferens to open anteriorly. It thus provides the passage for the eggs to the exterior. Egg masses and larvae of *A. fijiense* were described by Haynes & Kenchington (1991).

Some of our conclusions as to the function of the genital system of *A. fijiense* are necessarily speculative. Observations of and experimentation with living animals have to complement our findings and will confirm or falsify our hypotheses.

ACKNOWLEDGEMENTS

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QUANTIFICATION OF THE DEVELOPMENT OF THE CEPHALIC SAC AND PODOCYST IN THE TERRESTRIAL GASTROPOD *LIMAX MAXIMUS* L.

G. M. Kuchenmeister[1], D. J. Prior[2] & I. G. Welsford[3]

ABSTRACT

During embryonic development in the terrestrial gastropod *Limax maximus* L., an anterior cephalic sac and a posterior podocyst are elaborated. At 4 ± 1.2 d (X ± SD; n = 25), rhythmic contractions began in the podocyst. By 6.3 ± 0.8 d of development, the cephalic sac began rhythmic contractions in antiphase to those of the podocyst; a behavior we have termed cephalopedal pumping. Pumping preceded regular heart activity, which began at 8 ± 1.8 d of development, and progressively decreased in frequency throughout embryonic development, ceasing by hatching. Cephalopedal pumping was capable of redistribution of dye throughout the embryo. The two structures were capable of sustained, independent, rhythmic contractions. Because pumping precedes regular heart activity and can, presumably, redistribute hemolymph, cephalopedal pumping may serve as a primordial circulatory system, separate from the developing cardiovascular system.

Key words: circulatory system, development, *Limax maximus,* slug, gastropod, heart

INTRODUCTION

Terrestrial gastropods such as slugs undergo direct development and are easily raised in the laboratory. Due to the combination of these characteristics, slugs drew early attention as model developmental systems (e.g., Laurent, 1837; Jourdain, 1884; Henchman, 1890; Cuenot, 1892; Kofoid, 1895; Cardot, 1924). It has been known for over 100 years that slug embryos elaborate an anterior cephalic sac (= anterior vesicle, Laurent, 1837; Jourdain, 1884; = vesicle, Kofoid, 1898; = ectodermal sac, Simpson, 1901) and a posterior podocyst during development. It has also been widely reported that, once formed, these structures undergo rhythmic contractions. It has been proposed that the rhythmic contractions serve a circulatory function in embryos (e.g., Jourdain, 1884). Alternatively, the contractions of the cephalic sac and podocyst have been suggested to control movement of the embryo within the egg (Laurent, 1837; Jourdain, 1884), a circulatory function (e.g., Cuenot, 1892; Cardot, 1924) and/or serve a respiratory or osmoregulatory function (Laurent, 1837; Jourdain, 1884; Kofoid, 1895).

Although the development of the podocyst and cephalic sac has been used as a quali-

tative component of a staging scheme for some species of slugs (Carrick, 1938), the development of these structures has yet to be quantified. Furthermore, there exists very little data on the potential physiological significance of these structures and/or their contractile behavior. We have initiated investigations into the development of the cephalic sac and podocyst of the terrestrial slug *Limax maximus* L. and compared this with the development of heart in an attempt to quantify both the morphology and contractile behavior of these structures during development. A preliminary report of these data has appeared in abstract form (Welsford & Prior, 1988).

MATERIALS AND METHODS

Animals

Sexually mature (according to the criteria of Sokolove & McCrone, 1978) *L. maximus,* which had either been collected from the field or raised from eggs in a laboratory culture, were kept in vented containers lined with water-saturated paper towels under a regulated light cycle (L:D 14:10 or 13:11), a regulated temperature cycle (18°C during lights on and

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12°C during lights off) and approximately 100% RH. Slugs were fed *ad libitum* on laboratory food pellets (Purina Rat Chow). *Limax maximus* lays its eggs in discrete clumps or masses of 20–250 eggs, thus facilitating identification of eggs laid by an individual animal (Prior, 1983). Egg masses were collected daily from containers and placed on water-saturated filter paper-lined petri dishes at a constant temperature (either 15°C or 20°C) under a light cycle (L:D 14:10).

Morphological Measurements

Every 2 d, embryos were decapsulated (i.e., removed from the eggs) in a dish of sterile slug saline (55.6 mM Na$^+$, 4.2 mM K$^+$, 7 mM Ca^{++}, 4.6 mM Mg^{++}, 80.3 mM Cl$^-$, 0.2 mM H$_2$PO$_4^-$, 5.0 mM HCO$_3$, 5.0 mM Dextrose, pH 7.3–7.41, 139–145 mOsm/kg H$_2$O; Prior & Grega, 1982). Decapsulated embryos were transferred by pipette to a depression slide and viewed under a compound microscope fitted with an ocular micrometer (Nikon Optiphot II or Olympus Bmax). The following measurements were taken (in μm): (1) maximal width of the cephalic sac; (2) maximal length of the cephalic sac; (3) maximal length of the foot; (4) maximal length of the podocyst; (5) maximal width of the podocyst; (6) maximal width of the pericardial chamber (Fig. 1A,B). To allow for comparisons of relative structural dimensions at varying embryonic stages, all measurements were normalized to the length of the foot because this structure is readily identifiable at all developmental stages.

To determine whether cephalopedal contractions could distribute dye throughout the embryo, a microcapillary tube was filled with 2% Blue Dextran (MW approximately 2 X 10^6; Sigma Chemical Co., Inc.) and inserted into either the cephalic sac or podocyst with the aid of a micromanipulator (Leitz or WPI inc.). Dye was injected using low pressure (not greater than 0.5 atm). To control for potential damage during injection, only data from embryos which exhibited normal contraction activity for at least 20 min after injection were reported. To more clearly delineate the regions throughout which contractions were distributing dye, a saturated carmine solution was injected into either the cephalic sac or podocyst. Following injection with carmine, embryos were placed in a saturated solution of chlorobutanol for 30 min to ensure muscular relaxation (Kempf, personal communi-

cation), fixed in paraformaldehyde, dehydrated, embedded in paraffin, sectioned at 10 μm thickness and mounted onto slides. Sections were stained with Harris' Hematoxylin and counter stained with Eosin Y following standard protocols (Schleicher, 1953). Stained sections were observed under light microscopy and the distribution of carmine particles was determined. Sections were drawn with the aid of a drawing tube attachment to a Nikon Optiphot II.

Physiological Measurements

To ensure that decapsulation had no effect on the contractions of the cephalic sac, podocyst or (in later stages) heart, the rate of heart and/or cephalopedal contractions was determined prior to and following decapsulation. Contractile activity was measured by placing the embryo on a modified depression slide which allowed the embryo to be continuously superfused with saline. Embryo behavior was recorded on VHS videotape using a videomicroscope (Zeiss or Olympus).

Statistical Analysis

Comparisons between developmental times at varying temperatures and between heart rate and cephalopedal contraction rate were performed using a T-test for independent samples. Trends for heart and cephalopedal contraction during development were determined by calculating linear regressions. Statistical analyses were performed using either PsiPlot (PsiPlot Inc.) or DataDesk (DataDesk Inc.), and graphs were constructed using either PsiPlot or Kaleidograph (Kaleidograph Inc.). In all comparisons, a probability values of less than 0.05 was considered significant.

RESULTS

Development of the Cephalic Sac, Podocyst and Heart

Although there was considerable variation in the developmental time of different animals within a clutch and between clutches, embryonic development in *L. maximus* averaged 26.6 ± 13.6 (X ± SD; n = 15 clutches) d at 20°C. Development was temperature-dependent, taking significantly longer at 15°C (35.6 ± 19.2 d; t = 4.97, p = 0.00011). The cephalic

A

Cephalic Sac

Heart

Tentacles

Mouth

Podocyst

Foot

100 μm

B

1

2

5

6

4

3
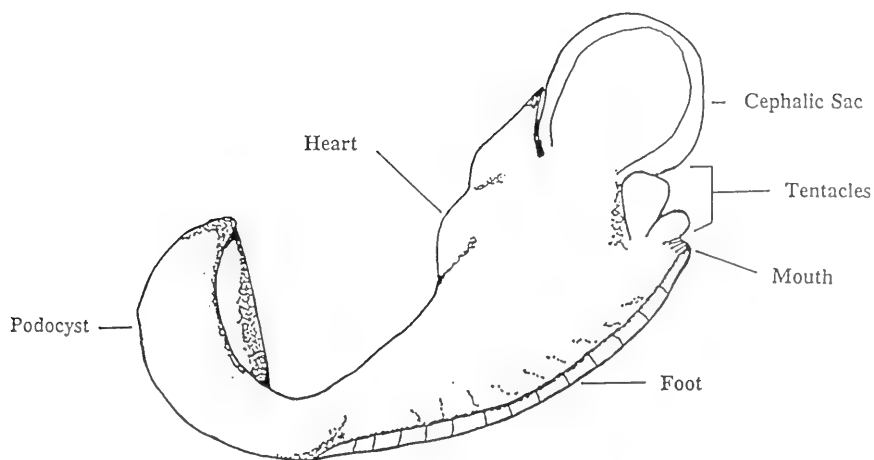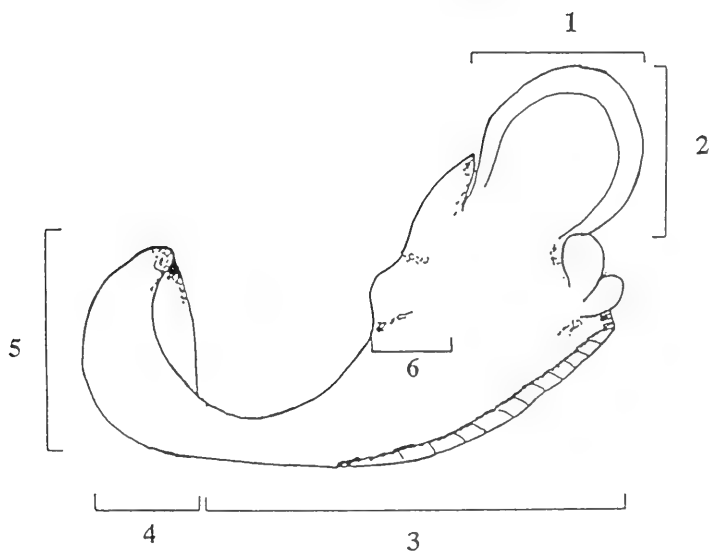
1 = Maximum Width of Cephalic Sac
2 = Maximum Length of Cephalic Sac
3 = Maximum Length of Foot
4 = Maximum Length of Podocyst
5 = Maximum Width of Podocyst
6 = Maximum Width of Pericardium

FIG. 1. A: Drawing of a slug embryo at 15 d of development indicating the relative positions of the podocyst, foot, cephalic sac and pericardial area. B: Indicates the morphological measurements taken at each embryonic stage. Scale bar is 100 μm.

sac developed within 4 d of egg laying and decreased progressively in size relative to the foot throughout embryonic development (Fig. 2A). The cephalic sac invaginated within 22–26 d of development and, consistent with the observations of Carrick (1938) for *Agriolimax agrestis,* was observed to form components of the internal organs including the albumin glands and reproductive structures. The sac was absent by hatching in all animals observed (n = 350).

The podocyst also developed within the first 4 d, and increased in size relative to the foot throughout the first 20–22 d of development. The podocyst decreased in size after this time, usually disappearing prior to hatching (Fig. 2B). Occasionally a hatchling slug retained a remnant of the podocyst after hatching, but this was lost in all observed cases within 3 d post-hatch (n = 350).

The heart first appeared between 10–12 d of development from a dorsal evagination of the embryo just caudal to the cephalic sac. The heart increased in size relative to the foot until approximately 18 d of development then decreased in relative size thereafter (Fig. 2C).

Cephalopedal Contractions

By 4 ± 1.2 (X ± SD; n = 25) d of development, the podocyst initiated irregular, low frequency contractions (16.2 ± 5.4 beats per minute [BPM]; X ± SEM; n = 45; Fig. 3A). By day 5 (56.4 ± 1.8 d), the cephalic sac began contractions in antiphase to the podocyst. Cephalopedal contractions peaked in frequency at approximately 4 d of development and decreased in frequency throughout development ($r^2 = -0.932$; p < 0.0001; Fig. 3A). After 18 d of development, there was a marked increase in the variance of the cephalopedal contractions (Fig. 3A.).

The rate of cephalopedal contractions was unaffected by decapsulation (Table 1). The antiphasic nature of the cephalopedal contractions led us to investigate the nature of the oscillatory control of contractions. The contractions of both the podocyst and cephalic sac were unaffected by rupture or removal of the other structure (Table 1) or, indeed, by complete transection of the embryo, with the exception that the structures ceased to contract in antiphase to one another (Table 1). The independent contractions in each structure in completely transected embryos lasted for an average of 2 h (2 ± 8.7 h; n = 15) and in one instance, continued for 20 h after transection.
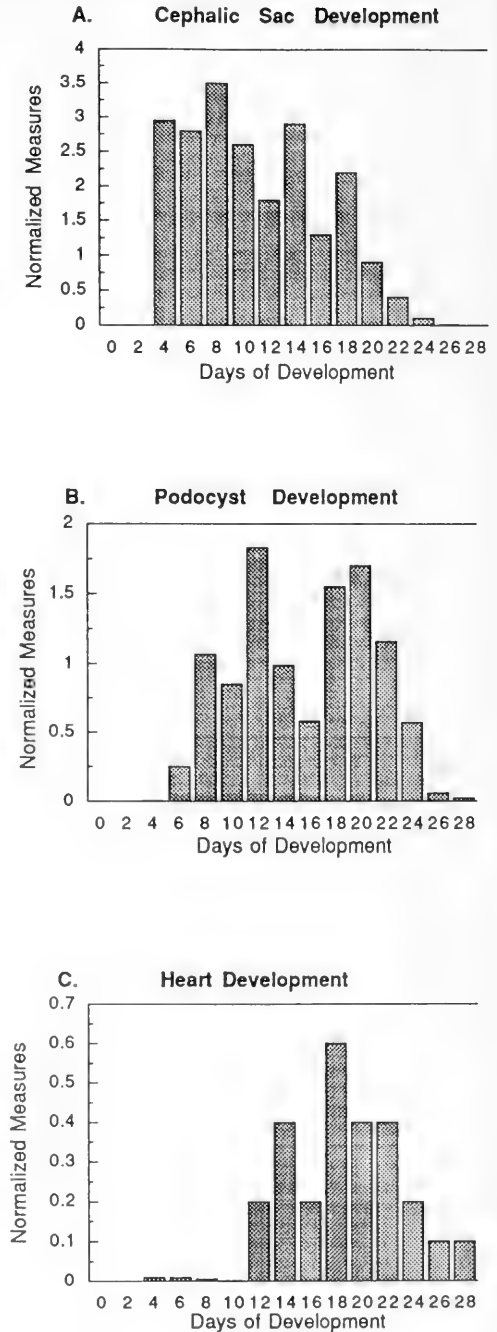
FIG. 2. Means of normalized measures of cephalic sac width (A), podocyst width (B) and pericardial area width (C) during embryonic development. Each bar represents the mean value of 55 animals.

A.

B.

Days of Development
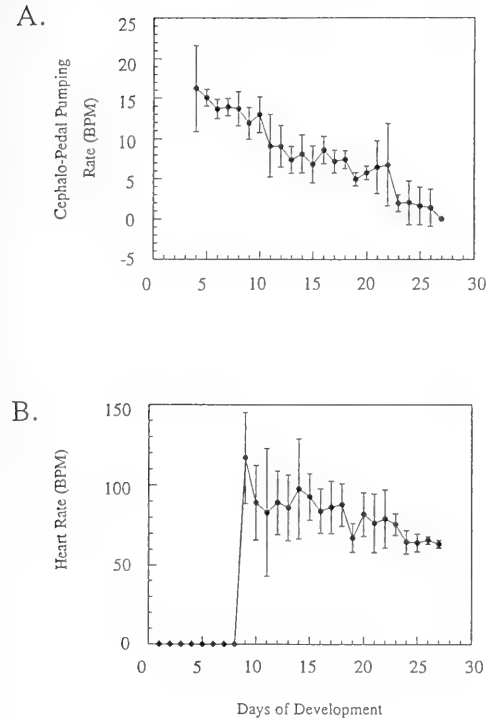
FIG. 3. A: Frequency (in BPM) of cephalopedal pumping in embryos of varying ages (measured at 20°C). Each point represents the mean (± SD) response of 45 animals. B: Frequency (in BPM) of heart activity in embryos of varying ages. Each point represents the mean (± SD) response of 45 animals.

Heart Contractions

In contrast to the cephalopedal activity, heart activity did not begin until 9.2 ± 1.4 d of development (Fig. 3B). Heart rate was significantly greater than cephalopedal contractions at all stages (mean across all stages for heart was 57.4 ± 156.7 BPM vs. 7.1 ± 25.7 BPM; t = 6.545; p < 0.0001). Heart rate peaked at 9 d of development and decreased progressively throughout development (r^2 = 0.7; p < 0.0001), but with a significantly different slope than that of cephalopedal contractions (−2.0 BPM/d for heart vs. −0.64 BPM/d for cephalopedal contractions; Fig. 3A,B). Initially, heart rate was strongly coupled to pumping activity, cyclically increasing and decreasing with each cephalopedal contraction (Fig. 5A). After 14 d of development, heart activity became independent of cephalopedal activity (Fig. 5B). Heart activity

was highly variable throughout the first 18 d of development, but the variance of heart activity decreased from 18–20 d of development to hatching (Fig. 3B).

Staining of Intra-Embryonic Regions

Cephalopedal contractions were capable of redistributing dye throughout the embryo (Fig. 6). However, the portion of the embryo that was stained by this procedure depended upon the age of the embryo. Injection into the podocyst in embryos younger than 10 d, resulted in staining of primarily the entire posterior region of the embryo, including a region surrounding the CNS and heart (summarized in Fig. 6). The cephalic sac was stained by podocyst injections only lightly and only after prolonged injections (>5 min). Injections into the cephalic sac in embryos younger than 10 d resulted primarily in staining a restricted internal space distinct from that stained with the podocyst injection and continuous with the stomach, reproductive organs and hepatopancrease (Fig. 6). Injection into the cephalic sac only lightly stained the podocyst and heart and only after prolonged injections. In embryos older than 12 d, injection into the podocyst or cephalic sac did not stain the heart. In addition, in embryos older than 12 d, injections into the podocyst in excess of 10 min failed to stain the regions of the embryo continuous with the cephalic sac (Fig. 6). At all stages of embryonic development tested, histological sections confirmed the dye separation of the internal regions continuous with either sac or podocyst in embryos older than 12 d (Fig. 6).

DISCUSSION

The observations in the present study are consistent with qualitative observations on the development of *Agriolimax agrestis* (by Carrick, 1938) and *Limax maximus* (by Simpson, 1901) and thus support the utility of the use of Carrick's (1938) scheme (developed for use with embryos of *A. agrestis*) for the staging of embryos of *Limax maximus*. Because preliminary observations suggest that this scheme also holds for the slugs *Lehmania valentiana* and *Agriolimax (=Deroceros) reticulatus,* this scheme may be generalizable to all slug species. The availability of a staging scheme for slugs may aide in the approximate aging of embryos for which the date of

TABLE 1. The effect of rupture or removal of either the cephalic sac or podocyst on the rate of cephalic sac, podocyst and heart contractions is shown, In addition, the effect of complete embryo transection on cephalic sac and podocyst contractions is shown. Data are mean ($\pm$ SD) response of 15 animals.

Structure	Rate in Egg Case (BPM)	Rate in Saline (BPM)	Rate 60 min Post-Rupture of Opposing Structure (BPM)	Rate 60 min Post-Transection (BPM)
Podocyst	25 ± 12.6	23 ± 13 (91%)	22 ± 14 (96%)	20 ± 16 (97%)
Cephalic Sac	23 ± 14.3	22 ± 18 (98%)	19 ± 17 (86%)	21 ± 19 (110%)
Heart	134 ± 23	122 ± 28 (91%)	119 ± 47 (98%)	121 ± 59 (101%)

A.   Heart Activity at 10d of Development

B.   Heart Activity at 20d of Development
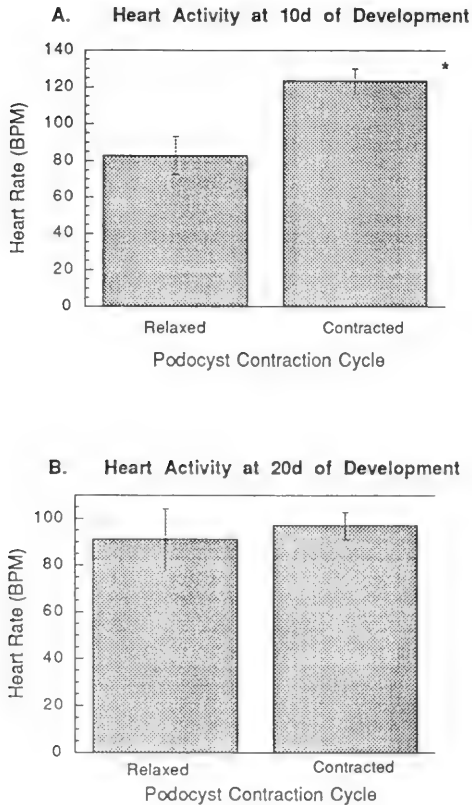
FIG. 4. Differences in sensitivity of heart activity to cephalopedal activity at 10 d of development (A) and 22 d of development (B). In each figure, the mean ($\pm$ SD) instantaneous heart rate is shown (expressed in BPM) during podocyst contraction and relaxation. Each bar represents the mean ($\pm$ SD) response of 45 animals.

laying of a clutch is unknown and/or which have developed under variable thermal conditions, because the development is markedly temperature sensitive.

The cephalic sac and podocyst developed earlier than the heart and began rhythmic, antiphasic contractions prior to the onset of heart activity. The frequency of cephalopedal contractions was significantly lower than that of heart and the rate of decrement in activity throughout development between heart and cephalopedal contractions differed significantly. In addition, heart and cephalopedal contractions demonstrated differing patterns of variance, with heart becoming more regular as cephalopedal contractions became less regular. Because podocyst and cephalic sac contractions were capable of redistributing dye throughout the embryo, contractions of these structures could accomplish the circulation of hemolymph throughout various regions of the embryo prior to maturation of the cardiovascular system. We have thus termed the rhythmic antiphasic contractions of the cephalic sac and podocyst, cephalopedal pumping.

The fact that heart activity was significantly affected by cephalopedal pumping at early stages suggests that, early in development, it may be continuous with the cephalic sac and podocyst, but that this connection is lost as the embryo matures. This hypothesis was supported by dye injections into the cephalic sac and podocyst, which stained the lumen of the heart in early stages, but not in later stages, of *L. maximus* embryos.

Both the cephalic sac and the podocyst can contract independently of one another. Thus, each structure may be driven be separate oscillators that are coupled to one another. Because the CNS reportedly develops within approximately 9 d in embryos of *L. maximus* (Henchman, 1890), the nature of such putative oscillators remains uncertain as does their fate in hatchling slugs. Work on these oscillators is ongoing.

Gastropod mollusks have served as useful model systems for the study of the development of the central nervous system and behavior due to the relatively small number of neurons present within the CNS and the fact
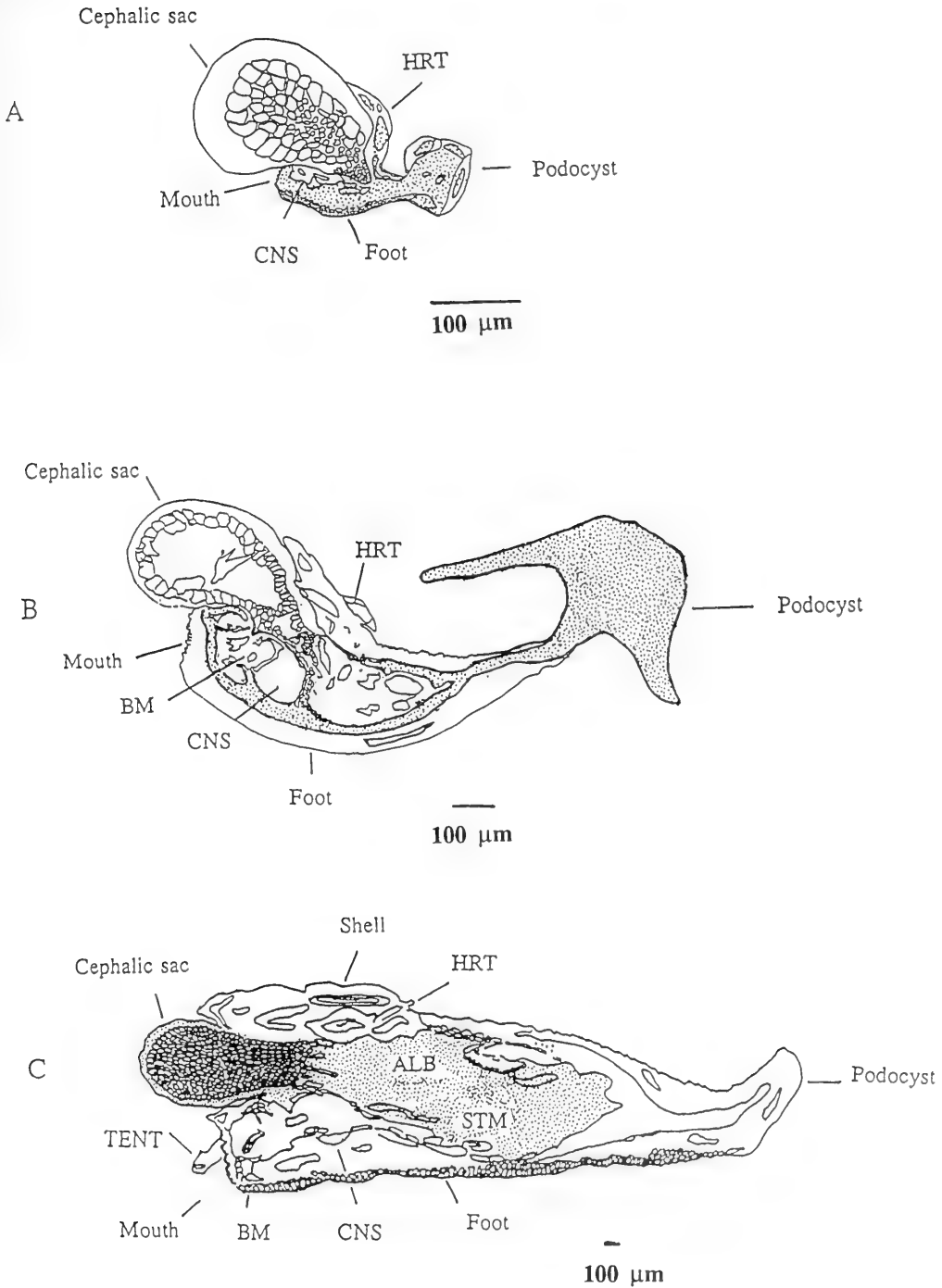
FIG. 5. Drawings of histological sections of embryos at 5 d of development (A), 15 d of development (B) and 25 d of development (C). In each figure, the stippled areas denote the regions stained by injection of carmine solution into the podocyst (A and B) or cephalic sac (C). Scale bar is equal to 100 μm. STM = stomach, ALB = albumin gland, BM = buccal mass, CNS = central nervous system, TENT = tentacles, HRT = heart.

that neurons are frequently large and easily identifiable from individual to individual. However, considerably less information is available on the development of peripheral structures in these organisms. Investigations of the development of the peripheral structures are hampered in many mollusks by a metamorphosis that includes a veliger stage and can entail dramatic alterations in central and peripheral morphology and physiology. Furthermore, the housing and maintenance of these organisms frequently requires mariculture facilities. Terrestrial gastropods are easily raised the laboratory and thus may serve as useful model systems for certain developmental investigations.

ACKNOWLEDGMENTS

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LOCAL PATTERNS OF LAND SNAIL DIVERSITY IN A KENYAN RAIN FOREST

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ABSTRACT

Terrestrial molluscs were sampled in indigenous forest and plantation plots in Kakamega Forest, western Kenya, which is the eastern-most patch of Guineo-Congolian rain forest in Africa. Fifty species (one slug and 49 snails) were recorded from 27 indigenous forest plots, and the mean species per plot was 23.4. The majority of the species present in the fauna were small, litter dwellers, with 52% having a major shell dimension of less than 5 mm. Overall, species richness and faunal composition were relatively uniform throughout the forest system. However, forest edge plots, including plots located along large rivers and in smaller blocks of forest, had a deficiency of some minute, litter-dwelling species but supported a higher frequency of some large-shelled taxa. The four plantations sampled supported fewer species per plot (15.25 species/plot) and also lacked several of the small, litter-dwelling species found in the indigenous forest.

Many other species of mollusc have been previously reported from Kakamega Forest. The reported mollusc fauna of Kakamega Forest represents about 5.8–9.5% of the total known East African forest mollusc fauna, thus suggesting that there must be considerable taxonomic replacement of species throughout the region. The recorded molluscan diversity in Kakamega Forest is high in a worldwide context. Kakamega Forest is not old in geological terms, the Lake Victoria basin having received a much more arid climate during periods of extended glaciation at higher latitudes. Its forest fauna must have colonised since the last glacial maximum in Africa, approximately 14000 years BP; the composition of the recorded fauna supports the view that recolonisation was mainly from forest refugia in central Africa. The conservation implications of the findings are discussed.

Key words: land snails, Gastropoda, biodiversity, rain forest, Africa, Kenya, Kakamega Forest.

INTRODUCTION

Comparisons of land snail diversity at a regional scale indicate that there are sometimes large differences between tropical and temperate zones (Cameron, 1995). At the local scale, faunas from temperate sites are often rich in species but quite uniform over large geographical areas. However, there is little comparable information about local diversity patterns in tropical areas. Solem (1984) proposed a model to account for world-wide land snail diversity levels, but noted that much further information is required about levels of sympatric diversity in many parts of the world, especially in the tropics. This lack of information clearly has implications when trying to assess the impact of habitat loss on molluscan biodiversity, and consequently for conservation planning (Cameron, 1995).

Much has been written about the East African terrestrial mollusc fauna, but there have been few investigations on molluscan as-semblages in different habitat types. This study investigates the patterns of land snail diversity in a relatively restricted area of rain forest habitat in western Kenya. It examines areas of forest that have been subjected to varying levels of human disturbance and exploitation and also surveys the mollusc fauna in plantations of both exotic and indigenous tree species.

THE SITE

The Kakamega Forest complex (about 0°15′N, 34°54′E) (Fig. 1) lies mostly to the west of Kakamega town in west Kenya, about 40 km north of Lake Victoria. It comprises several separate blocks of forest (Muriuki & Tsingalia, 1990), of which Kakamega Forest itself is by far the largest; the smaller, isolated areas of Kisere and Mlaba forests lie to the north of the main forest block. Bunyala, Maragoli and Kaimosi forests are situated to the northwest, south and southwest of Kaka-
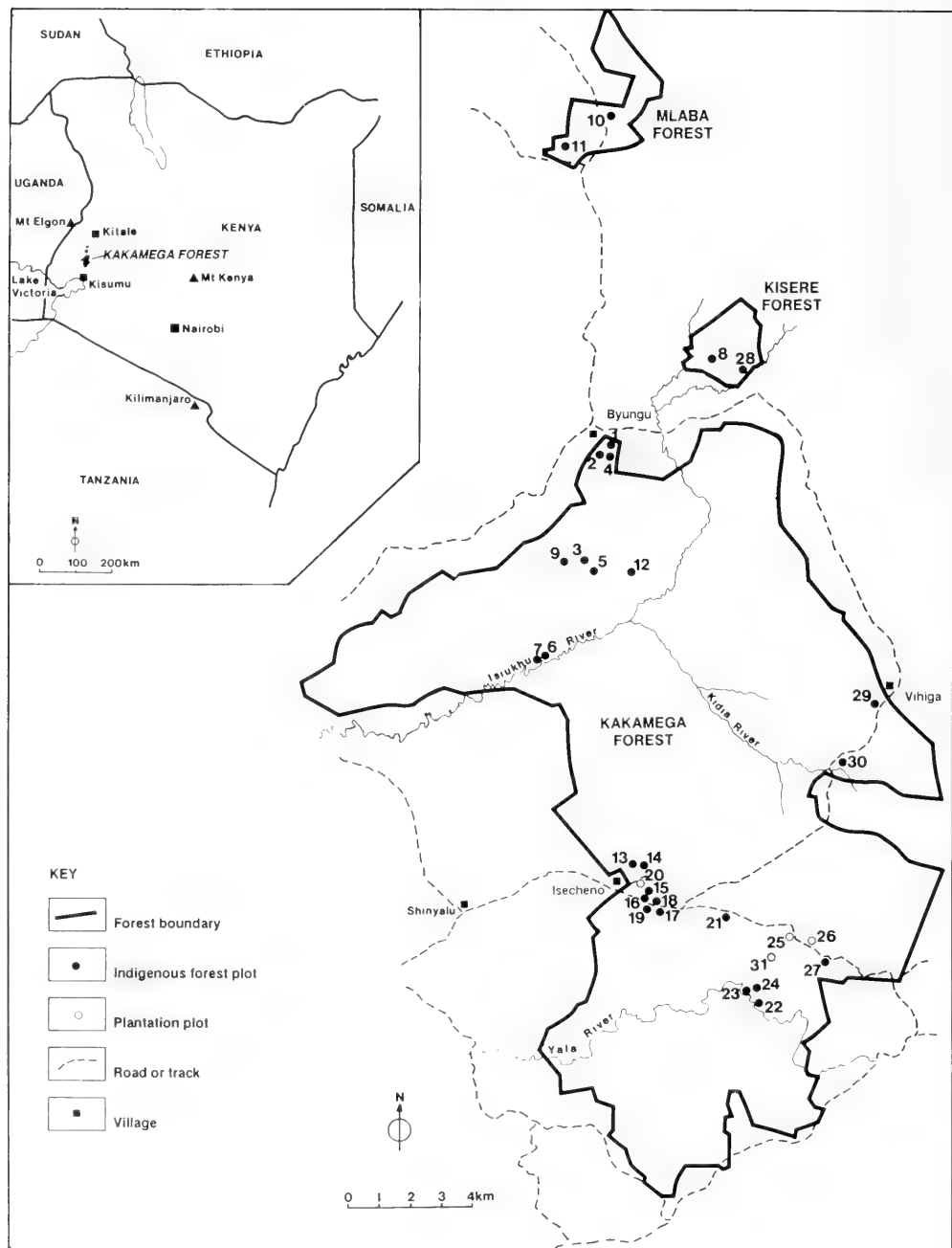
FIG. 1. The location of Kakamega Forest and the sampling plots.

mega Forest, respectively. The area has a relatively flat or gently undulating topography at an altitude of approximately 1500–1680 m. Two major rivers, the Yala and Isi-

ukhu, as well as many smaller watercourses, run through the forest.

The forest complex covers an area of approximately 265 km². About 45 km² is pro-

tected for wildlife as national reserve and nature reserve, and some of the area is currently proposed as a national park. The remainder is gazetted as forest reserve and is managed by the Forestry Department; current policy is to encourage indigenous forest rather than plantation. According to Muriuki & Tsingalia (1990), about 48% of the forest complex supports indigenous forest stands, the remainder containing plantations and grassland clearings of both natural and anthropogenic origin.

Parts of the forest were selectively logged in the 1930s, 1940s, late 1970s and early 1980s (Tsingalia, 1990). The region contains some of the densest population in Kenya, and until a presidential decree in 1986, many parts of the forest were subject to shifting cultivation by local people. Kokwaro (1984) noted a rapid decline in the extent of indigenous forest. Such activities have now been stopped, but other forms of illegal exploitation still take place, especially the removal of plant products for medicinal use and firewood, stock grazing and poaching for small game. If caught, offenders face a stiff punishment, with fines for a first offence equivalent to one month's income or three months in prison.

The climate can be described as relatively hot and humid, although rainfall is seasonal, with most falling between March and July and again in October and November. Muriuki & Tsingalia (1990) report mean annual rainfall as 2216 mm, although Zimmerman (1972) recorded over 3500 mm during 1963, indicating substantial fluctuation from year to year; mean monthly maximum temperature ranges from 18–29°C (Muriuki & Tsingalia, 1990).

The site is biogeographically important, being situated at the edge of several regional vegetation zones. White (1983) classifies it as transitional rain forest, noting that it supports several Guineo-Congolian plant species at their easternmost African limit. The fauna also has strong central and west African affinities (Faden, 1970; Zimmerman, 1972). Lucas (1968) describes Kakamega Forest as "the most easterly point of the West African-Congo type forest." It supports many plant and animal species which are not found elsewhere in Kenya (Faden, 1970).

METHODS

Plot Selection and Description

Thirty one plots (1–31), each approximately 40 m × 40 m, were sampled in Kakamega, Kisere and Mlaba forests; 27 of the plots were in mixed, indigenous forest, the other four being in plantations (Fig. 1). Maximum plot separation was approximately 30 km. Forest edges were avoided during selection although three plots were situated at the edge of the Yala and Isiukhu rivers (Plots 6, 7, 22) and contained marginal, riverine forest vegetation locally dominated by light demanding tall herbaceous species; plot 23 lay within about 50 m of the Yala River but did not contain forest edge.

Twenty three of the indigenous forest plots contained mature, mixed stands (canopy height generally about 20–30 m). All had been exploited for wood, timber or other resources to some degree, although observations on forest structure and other signs, such as the presence of saw pits, freshly cut wood and evidence of cattle grazing indicated that five of these plots (Plots 10, 11, 14, 16 and 29) were substantially more disturbed than the others. The other four indigenous forest plots (5, 9, 12, 30) contained relatively young, mixed, stands. These plots were all characterised by a relatively low canopy (about 10–20 m), high light penetration at ground level, and a mixed and generally well-developed grassy herbaceous field layer. Some of them contained *Acanthus arborea* Forsskal, a characteristic plant species of disturbed sites requiring high illumination. Details of former land use are unknown, but it is possible that these areas had been cut and subsequently regrown or been recolonised by indigenous forest.

All four plantations (Plots 20, 25, 26 and 31) were probably about 20-40 years old and all had a well-developed canopy. The trees were well spaced and had presumably been thinned. Two contained monoculture stands of the non-indigenous *Bischofia javanica* Blume, one had been planted with mixed, non-indigenous conifers (*Pinus patula* Schlecht & Chamisso and *Cupressus lusitanica* Mill.) and the other contained a monoculture of the indigenous, Guineo-Congolian lowland rain forest tree species *Maesopsis eminii* Engl. The detailed history of these areas is not known, although it is probable that they were formerly covered in indigenous forest, possibly with a period of cultivation prior to conversion to plantation.

Physical plot characteristics were recorded including topography, inclination, aspect, and presence and relative abundance of such potential molluscan microhabitats as dead wood, fallen trees, and rocks. Forest

structure was described by estimating canopy height and the percentage cover of different vegetation strata (tall, medium and low tree components and scrub, herb and liane categories) for each site. Many of the indigenous plots had a dense understorey shrub layer of *Dracaena afromontana* Mildbr. Plant species were not generally recorded, although notes were made on common dominant species where they could be identified. Mutangah et al. (1992) provide further information about the vegetation of Kakamega Forest.

Mollusc Sampling

Sampling for molluscs was undertaken by a combination of direct search and litter sieving methods. Each plot was searched for at least 30 minutes, ensuring that all potential microhabitats, such as dead wood, rocks, tree trunks, leaf litter and living vegetation, were examined. It was not possible to survey the forest canopy directly, but fallen trees and canopy branches supporting epiphytic orchids, mosses and lichens were examined when available. All molluscs found were collected. Up to three local guides assisted with the direct searching and therefore sampling effort varied amongst the plots. However, the mean number of species per plot does not differ significantly between the sites sampled by one or three people ($F_{1,23 \text{ d.f.}} = 3.70$, $P > 0.05$), and thus this variation in sampling effort does not appear to affect the assessment of diversity levels. About 4 litres of surface leaf litter and soil were taken from each plot and passed through a coarse sieve (4 mm mesh size). Large species retained in the sieve were removed. The fine fraction was then dried and passed through two further sieves (mesh sizes 2 mm and 0.5 mm). These sieve fractions were searched separately under good illumination until no further molluscs could be found (generally about 30–45 minutes). Material passing through the 0.5 mm mesh for the first few sites was searched for snails, but because none were found, this fraction was discarded for subsequent samples. Some specimens were preserved in 70% ethanol, the others were stored dry. These sampling methods are similar to those used in other studies (e.g., De Winter, 1995).

Identification and Analysis

Most molluscs have been identified to species level and nomenclature is provided in Appendix I. A reference collection has been sent to the National Museums of Kenya, Nairobi, and the remainder of the material will be deposited in the National Museum of Wales, Cardiff (NMW Z 1993.062). Several species in the urocyclid genus *Thapsia* are present in the samples. Two of these (*Thapsia microleuca* Verdcourt and *T. eucosmia* Pilsbry) are distinctive, but the other species, of which there are at least two, are difficult to separate and have been aggregated for the purposes of analysis. Further notes on identification are provided in Appendix I.

The number of individuals (separated into living and dead specimens based on the presence of body tissues and shell condition) has been recorded for each of the samples. However, since sampling effort varied amongst the plots, it does not provide a measure of absolute species abundance or allow direct comparisons between the plots. Most of the analyses have therefore been based on presence and absence data. Nevertheless, the number of individuals does represent a measure of the relative abundance of species in the fauna and is therefore of some interest. The analysis of diversity patterns has followed Cameron's (1992) methods. Two measures have been adopted, Whittaker's (1975) Index (*I*), the ratio of overall species number (*S*) to the mean number of species per plot (∝), provides a measure of between plot differences. An index of 1 reveals identical faunas, whereas higher values demonstrate increasing differentiation. High values of *I* can either result from the geographical replacement of taxa within the same habitat or along habitat gradients (Cody, 1986). Following Cameron's (1992) methods, these effects have been examined by calculating the ratio of the variance of the number of sites per species to the maximum variance possible for the same values of *S* and ∝. Where replacement effects are important, as opposed to random effects due to sampling error, the achieved variance is low compared with the maximum possible.

RESULTS

Table 1 lists the 53 species recorded from the 31 plots during the survey. These consist of one slug and 52 snail species. Seven species were recorded only as dead shells and one of these, *Cecilioides* species, has been excluded from the analyses because it was only found on Plot 7, which lies adjacent to

TABLE 1. Species of molluscs recorded in the 31 sampling plots in Kakamega Forest.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Elgonocyclus koptaweliensis							+			+								
Maizania elatior						+	+	+		+	+		+	+				
Succinea sp.						+	+	+									+	+
Truncatellina ninagongonis	+		+				+	+	+				+			+	+	+
Nesopupa bisulcata	+		+					+		+				+	+	+		+
Pupisoma harpula	+			+	+					+	+				+	+		
Pupisoma orcula						+				+					+	+		
Pupisoma sp. A	+		+					+		+			+					
Pupisoma sp. B	+	+								+	+				+			
Acanthinula sp.	+					+												
Rhachidina chiradzuluensis var. *virginea*						+	+											
Conulinus rutshuruensis major						+		+		+	+							
Cerastua trapezoidea lagariensis						+		+		+	+							
Micractaeon koptawelilensis	+	+		+	+	+	+	+		+	+	+	+	+	+	+	+	+
Nothapalus sp.																		
Subulona clara		+				+		+	+					+				
Oreohomorus iredalei								+			+							
Pseudoglessula elegans	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudopeas cf. *yalaensis*	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Curvella sp. A	+	+	+	+	+			+	+	+		+		+			+	+
Curvella cf. *babaulti*	+	+	+	+	+		+	+	+	+		+	+	+	+		+	+
Achatina stuhlmanni	+	+				+		+										
Limicolaria cf. *saturata*																		
Punctum ugandanum	+	+	+	+			+		+		+			+	+	+	+	
Punctum sp. A	+		+	+	+		+	+	+	+						+		+
Punctum sp. B	+						+	+		+	+							+
Trachycystis iredalei	+		+	+	+	+	+	+	+		+	+	+			+		
Trachycystis ariel							+					+				+		
Prositala butumbiana	+			+	+		+		+		+				+	+	+	+
Kaliella barrakporensis	+	+	+	+	+	+	+	+	+		+	+	+	+		+	+	
Kaliella iredalei	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
carinate species	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Guppya quadrisculpta	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Afroconulus iredalei		+				+	+		+		+							+
Trochozonites cf. *medjensis*		+				+												+
Thapsia eucosmia					+	+	+											
Thapsia microleuca	+	+		+		+		+			+		+		+		+	
Thapsia spp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Gymnarion aloysiisabaudiae		+	+	+		+	+			+	+	+	+	+	+		+	+
Chlamydarion oscitans						+	+	+		+					+			
urocyclid slug																		
Halolimnohelix percivali																		
Halolimnohelix plana	+							+		+								+
Gonaxis elgonensis		+				+	+				+				+		+	+
Gulella woodhousei	+	+	+		+	+	+	+	+		+	+		+				
Gulella osborni	+	+	+	+	+	+	+	+		+	+		+	+	+	+	+	+
Gulella impedita	+	+	+	+		+	+	+					+	+	+		+	+
Gulella ugandensis						+		+		+	+							
Gulella lessensis	+			+		+	+	+		+		+				+		
Gulella handeiensis						+												
Gulella disseminata	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptostele bacillum	+	+	+	+			+	+	+	+	+		+	+	+	+		+
Cecilioides sp.						+												
Site totals	30	22	24	20	19	33	27	27	22	24	30	18	19	19	23	20	23	24

TABLE 1. Species of molluscs recorded in the 31 sampling plots in Kakamega Forest. (*continued*)

Species	19	20	21	22	23	24	25	26	27	28	29	30	31	Species totals
Elgonocyclus koptaweliensis														2
Maizania elatior				+	+	+	+		+					12
Succinea sp.	+													6
Truncatellina ninagongonis	+		+			+			+					13
Nesopupa bisulcata	+								+	+	+	+		13
Pupisoma harpula	+		+			+				+			+	12
Pupisoma orcula	+		+	+							+		+	9
Pupisoma sp. A	+		+			+			+					9
Pupisoma sp. B										+		+		7
Acanthinula sp.														2
Rhachidina chiradzuluensis var. *virginea*														2
Conulinus rutshuruensis major				+		+		+						7
Cerastua trapezoidea lagariensis			+	+		+								7
Micrataeon koptawelilensis	+		+	+	+	+			+	+		+		24
Nothapalus sp.				+										1
Subulona clara			+	+	+	+		+	+	+	+	+		14
Oreohomorus iredalei				+				+	+					5
Pseudoglessula elegans	+	+	+	+		+	+	+		+	+	+	+	29
Pseudopeas cf. *yalaensis*	+		+	+	+		+	+			+		+	24
Curvella sp. A	+	+	+			+	+		+		+	+		20
Curvella cf. *babaulti*	+		+				+	+	+	+	+	+	+	24
Achatina stuhlmanni					+		+		+					7
Limicolaria cf. *saturata*				+				+					+	3
Punctum ugandanum	+								+	+	+	+		16
Punctum sp. A	+	+		+	+	+		+	+	+			+	19
Punctum sp. B									+	+				8
Trachycystis iredalei						+					+	+	+	16
Trachycystis ariel												+		4
Prositala butumbiana	+		+				+				+	+		14
Kaliella barrakporensis	+		+			+		+	+	+	+	+	+	24
Kaliella iredalei	+		+	+		+	+	+	+	+	+	+		27
carinate species	+		+		+	+			+	+	+	+		26
Guppya quadrisculpta	+		+			+		+	+	+	+	+	+	27
Afroconulus iredalei	+		+				+	+			+	+		12
Trochozonites cf. *medjensis*									+					4
Thapsia eucosmia				+	+	+					+	+		8
Thapsia microleuca			+				+			+				12
Thapsia spp.	+	+	+	+	+	+	+	+	+	+	+	+	+	31
Gymnarion aloysiisabaudiae	+		+	+	+	+	+	+	+		+			22
Chlamydarion oscitans	+					+		+	+		+		+	11
urocyclid slug									+					1
Halolimnohelix percivali									+					1
Halolimnohelix plana	+						+	+	+					8
Gonaxis elgonensis	+		+		+	+	+	+	+			+	+	16
Gulella woodhousei	+				+	+	+	+		+	+	+	+	20
Gulella osborni	+		+	+	+	+			+	+	+	+	+	26
Gulella impedita	+		+			+			+	+	+	+		19
Gulella ugandensis			+	+		+		+	+			+	+	11
Gulella lessensis	+									+				11
Gulella handeiensis														1
Gulella disseminata	+		+			+	+		+	+	+	+		26
Streptostele bacillum		+	+							+	+	+	+	20
Cecilioides sp.														1
Site totals	29	5	26	18	13	26	16	22	27	21	24	24	18	

the Isiukhu River. *Cecilioides* is an open-country genus, and the shells were probably deposited onto the plots by the river. The other species only recorded as dead shells were *Cerastua trapezoidea lagariensis, Gulella handeiensis, Nothapalus* sp., *Trachycystis*
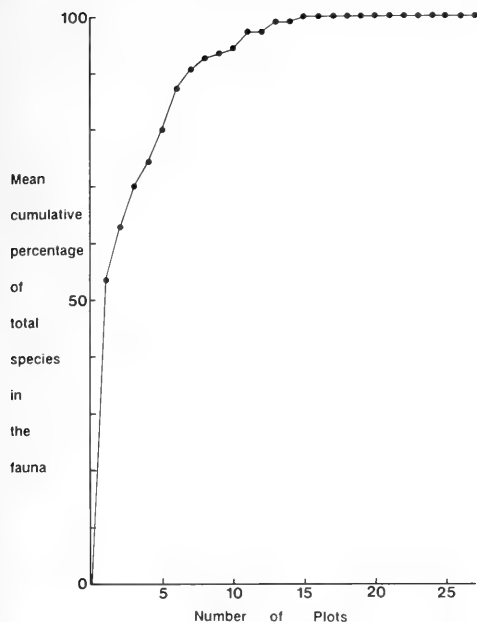
FIG. 2. Proportion of total indigenous forest fauna (50 species) as a function of the number of plots sampled.

ariel, *Pupisoma* sp. A and *Rhachidina chiradzuluensis* var. *virginea;* these have been included in the analyses because they are forest-dwelling species. Two species are confined to the plantation plots and thus, overall species number (S) in the indigenous forest is 50. A plot of cumulative species number against plot number (three randomized plot orderings) reaches an asymptote after 15 sites (Fig. 2). Because 27 indigenous forest plots were sampled, it is clear that the sampling detected the great majority of the species present in the plots, unless some important micro-habitats containing specialist species were overlooked.

The Fauna in Indigenous Forest

Possible groupings of plots have been investigated using Reciprocal Averaging Ordination (RAO) based on the presence and absence data from all 31 plots. Species distribution and diversity levels have also been examined in geographically restricted groups of plots. RAO (Hill, 1973) is an ordination method which arranges the plots along artificial axes according to their species complements. The ordination shows (Fig. 3) that the faunas of most of the indigenous forest plots are closely similar, except for a group of seven plots which includes the Yala and Isiukhu river plots (Plots 6, 7, 22, 23 and 24) and Plots 8 and 10, which lie in Kisere and Mlaba forests respectively. This smaller group therefore appears to represent a forest-edge fauna, which is either found in riverine forest or in the smaller forest blocks; it is referred to as the "riverine forest" group. Six species are more frequent in the riverine forest group, whereas four are more frequent in the larger subset of indigenous forest plots (Table 2). Plot 22 is particularly isolated on the ordination; it contains the only record for *Nothapalus* sp. and is the only indigenous forest plot to contain *Limicolaria* cf. *saturata.* The species occurring in excess in the riverine forest group are relatively large species, whereas the four found in excess in the main forest group are small litter-dwellers. The plots containing disturbed or young indigenous forest fall within the main cluster of indigenous forest plots on the ordination, suggesting that their fauna does not differ substantially from that of the more mature and less disturbed stands. However, one species, *Gulella impedita,* is significantly less frequent in the young forest than in the mature, undisturbed category (Fisher's Exact Test, $P < 0.05$).

Species distribution throughout the study area has also been investigated by plotting site occupancy for each species on maps of the forest, and by examining species frequency in seven geographical groups of plots (Table 3). These analyses essentially confirm the findings of the ordination and show that most species are widespread throughout the forest. However, one species, *Gulella ugandensis,* is widespread in the survey area, occurring from Mlaba to the Yala River, but is apparently absent from the eight plots near Isecheno; this pattern does not obviously relate to any of the environmental factors recorded and the reason for it is not known.

Patterns of Diversity in the Indigenous Forest

Total species number varies amongst the indigenous forest plots, but does not relate obviously to geographical position or any of the habitat or other environmental factors recorded. Mean species per plot (α diversity) does not differ significantly amongst the seven geographical groups (Table 3; $F_{6,18 \text{ d.f.}}$
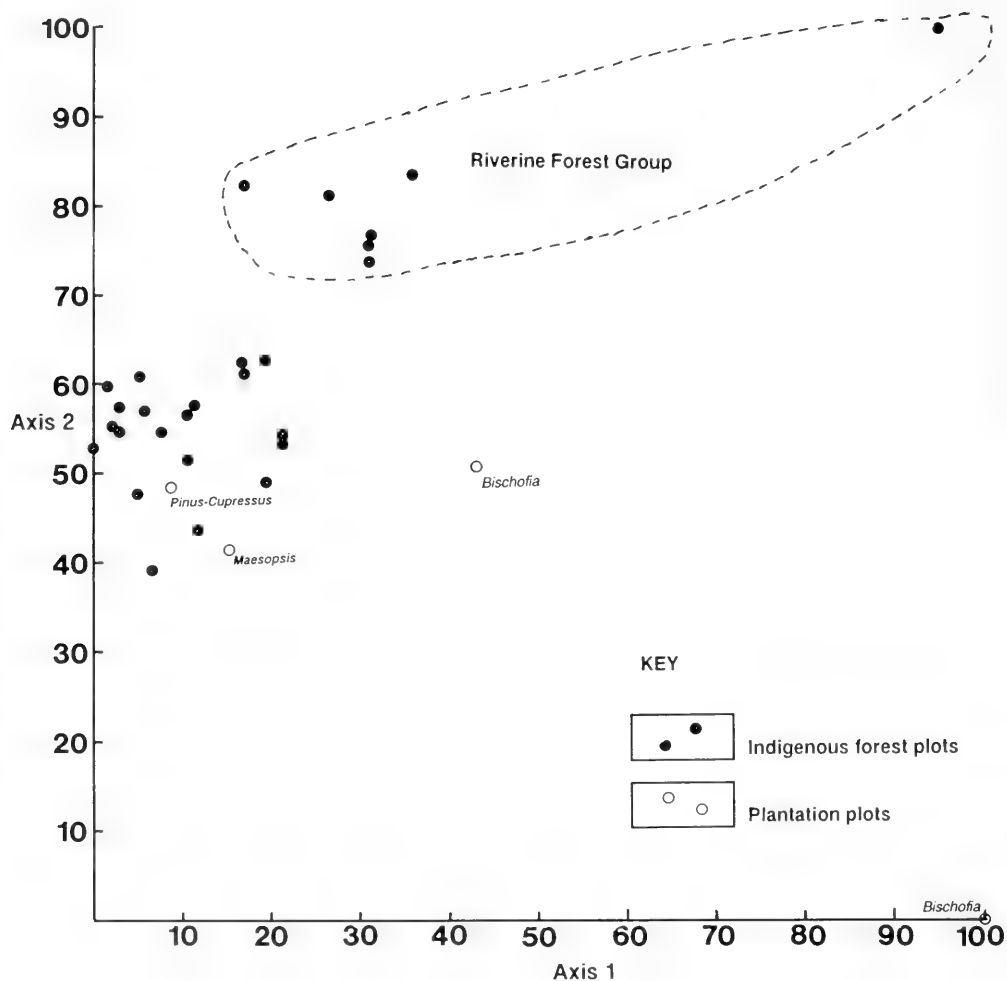
FIG. 3. Reciprocal averaging ordination of the plots.

= 1.22, P = 0.34), between the riverine forest group and the main indigenous forest groups identified by the ordination (Table 3; $F_{1,25\ d.f}$ = 0.16 P = 0.69) or between the undisturbed mature indigenous forest, the young forest and the highly disturbed indigenous stands (Table 4; $F_{2,24\ d.f.}$ = 0.85, P = 0.44). There is thus no evidence of significant variation in alpha diversity levels throughout the survey area or between the different age and disturbance categories of indigenous forest. Table 4 also gives values for Whittaker's Index, I, and the proportion of maximum variance achieved for the sites per species statistic. Neither of these measures suggests that

there are large differences between the plots or that there is significant geographical replacement of taxa across the forest system.

Other Characteristics of the Fauna

The majority of the species recorded are small litter-dwellers and were retrieved by the sieving. Few species appeared to be restricted to specific microhabitats. However, *Thapsia eucosmia* was found almost exclusively on living vegetation and on tree trunks, *Nesopupa bisulcata* was most frequently recorded on the underside of dry, large, fallen leaves on the forest floor, and *Maizania elatior*

TABLE 2. Species recorded in significant excess in the riverine forest and other indigenous forest groups identified by the ordination

	Number of Plots		
Species	Riverine forest (Max = 7)	Other indigenous (Max = 20)	Probability*
More frequent in riverine forest group			
Maizania elatior	7	4	0.000
Cerastua trapezoidea lagariensis	5	2	0.005
Chlamydarion oscitans	5	4	0.023
Thapsia eucosmia	5	3	0.011
Conulinus rutshuruensis major	5	1	0.006
Gulella ugandensis	5	4	0.023
More frequent in other indigenous forest group			
Nesopupa bisulcata	0	13	0.006
Curvella cf. babaulti	2	19	0.001
Curvella sp. A	2	16	0.023
Punctum ugandanum	1	15	0.009

*Probability based on Fisher's Exact Test. Null hypothesis that frequencies are equal in both groups.

TABLE 3. Mean number of species in geographical groups of plots and in two groups identified by the ordination

Group	Plots	N	Mean	S.E.
Geographical Groups				
Mlaba	10, 11	2	27.00	3.00
Kisere	8, 28	2	24.00	3.00
Byungu[1]	1, 2, 3, 4, 9	5	23.60	1.72
Isiukhu	6, 7	2	30.00	3.00
Isecheno	13, 14, 15, 16, 17, 18, 19, 21	8	22.88	1.25
Yala	22, 23, 24, 27	4	21.00	3.34
Vihiga	29, 30	2	24.00	0.00
Ordination Groups				
Riverine Forest	6, 7, 8, 10, 22, 23, 24	7	24.00	2.49
Other indigenous forest	1, 2, 3, 4, 5, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 21, 27, 28, 29, 30	20	23.20	0.83

[1]Excludes the young forest plots 5 and 12

was found on the surface of the forest floor and appeared to have a highly aggregated distribution. No snails were found amongst epiphytes or on fallen tree branches, and no evidence was detected that molluscs live in the forest canopy.

About 73% of the species in the indigenous fauna have shell sizes (maximum dimension) of 10 mm or less and 52% are less than 5 mm. About 17% have shell size exceeding 15 mm; Achatina stuhlmanni is the largest species in the fauna with a shell length in excess of 100 mm. In total, 3,723 specimens were collected from the 27 indigenous plots, of which 1,504 (approximately 40%) were classified as living. Guppya quadrisculpta constituted 10% and 11.25% of the living and overall totals respectively and was the commonest species. Only six species (12% of the total recorded fauna) exceeded 5% of all the specimens (i.e., living plus dead) recorded (Table 6) and these collectively represented 46.23% of the overall total. Twenty five species (50% of the fauna) each contributed less than 1% to the total number of specimens. The numbers of dead and living specimens were very roughly equivalent for most species, except for Subulona clara and the aggregated Thapsia species, of which many more dead shells than living ones were found. During the fieldwork, it was noted that substantial accumulations of mostly dead Subulona clara shells were

TABLE 4. Estimates of mollusc diversity in indigenous forest and plantation

| | Indigenous Forest | | | | |
	Undisturbed, mature	Disturbed, mature	Young, recent colonisation	All indigenous	Plantation
No. Plots	18	5	4	27	4
Total species, S	—	—	—	50	33
Species per site					
Mean ± SE, α	24.0 ± 1.1	23.4 ± 1.9	20.75 ± 1.4	23.4 ± 0.9	15.25 ± 3.6
Range	13–33	19–30	18–24	13–33	5–22
Whittaker's Index, *I*	—	—	—	2.1	2.2
Sites per species					
Variance	—	—	—	60.64	—
Maximum var.	—	—	—	167.84	—
% achieved	—	—	—	36.1%	—

TABLE 5. Species recorded in significant excess in the plantation and indigenous forest categories

| | Number of Plots | | |
Species	Plantation (Max = 4)	Indigenous Forest (Max = 27)	Probability*
More frequent in plantation			
Limicolaria cf. *saturata*	2	1	0.037
More frequent in indigenous forest			
Micractaeon koptawelilensis	0	24	0.001
Punctum ugandanum	0	16	0.043
Gulella osborni	1	25	0.008
Gulella disseminata	1	25	0.008
Gulella impedita	0	19	0.016
carinate species	0	26	0.000

*Probability based on Fisher's Exact Test. Null hypothesis that frequencies are equal in both plantation and indigenous forest groups.

sometimes found in the leaf litter beneath rotting and fallen logs.

Plantation Faunas

The small number of plantations sampled inevitably means that conclusions are tentative. However, mean species per plot (Table 4) is significantly lower in the plantations than in the indigenous forest (all 27 plots combined) ($F_{1,29 \text{ d.f.}} = 10.05$, $P = 0.004$). Total species number is exceptionally low in the *Pinus-Cupressus* plantation. However, neither the *Pinus-Cupressus* nor the *Maesopsis* plantations ordinate separately from the main cluster of forest plots, although the two *Bischofia javanica* plantations do (Fig. 3). Table 4 gives Whittaker's Index (*I*) for the plantation plots; it does not differ substantially from the value for the indigenous forest.

Six species are significantly more frequent in the indigenous forest than the plantations (Table 5), and all of these are small, litter-dwellers; they are all also relatively abundant in the indigenous forest (all being represented by at least 60 shells, Table 6). One large species, *Limicolaria* cf. *saturata,* is significantly more frequent in the plantations than indigenous forest, and two others, the

TABLE 6. Number of specimens (living plus dead), proportion of total specimens collected and rank order of each species in indigenous forest and plantation habitats

Rank	Species	Indigenous forest		Plantation		Rank
		Number	%	Number	%	
1	*Guppya quadrisculpta*	419	11.25	15	4.5	6
2	*Thapsia* spp.	393	10.56	36	10.81	4
3	*Gonaxis elgonensis*	248	6.66	47	14.41	2
4	*Pseudoglessula elegans*	238	6.39	7	2.1	14
5	*Subulona clara*	215	5.78	3	0.9	22
6	*Pseudopeas* cf. *yalaensis*	208	5.59	10	3	7
7	*Kaliella barrakporensis*	174	4.67	21	6.31	5
8	*Kaliella iredalei*	159	4.27	4	1.2	20
9	carinate species	153	4.11	0	—	—
10	*Maizania elatior*	140	3.76	1	0.3	29
11	*Curvella* cf. *babaulti*	129	3.47	8	2.4	10
12	*Gulella disseminata*	100	2.69	5	1.5	18
13	*Thapsia microleuca*	96	2.58	3	0.9	22
14	*Punctum ugandanum*	76	2.04	0	—	—
15	*Gulella impedita*	75	2.02	0	—	—
16	*Micractaeon koptawelilensis*	73	1.96	0	—	—
17	*Gulella ugandensis*	71	1.91	49	16.72	1
18	*Punctum* sp. A	63	1.69	9	2.7	8
19	*Gulella osborni*	62	1.67	1	0.3	29
20	*Gymnarion aloysiisabaudiae*	59	1.59	7	2.1	14
21	*Gulella woodhousei*	58	1.56	8	2.7	8
22	*Curvella* sp. A	57	1.53	8	2.4	10
23	*Nesopupa bisulcata*	52	1.4	0	—	—
24	*Thapsia eucosmia*	50	1.35	0	—	—
25	*Streptostele bacillum*	44	1.18	3	0.9	22
26	*Trachycystis iredalei*	33	0.89	1	0.3	29
27	*Prositala butumbiana*	22	0.59	2	0.6	26
28	*Conulinus rutshuruensis major*	21	0.56	4	1.2	20
29	*Chlamydarion oscitans*	20	0.54	8	2.4	10
29	*Pupisoma harpula*	20	0.54	2	0.6	26
31	*Afroconulus iredalei*	19	0.51	6	1.8	16
32	*Punctum* sp. B	17	0.46	0	—	—
32	*Gulella lessensis*	17	0.46	0	—	—
32	*Truncatellina ninagongonis*	17	0.46	0	—	—
35	*Pupisoma* sp. A	15	0.43	0	—	—
36	*Cerastua trapezoidea lagariensis*	14	0.38	0	—	—
36	*Halolimnohelix plana*	14	0.38	6	1.8	16
36	*Pupisoma orcula*	14	0.38	5	1.5	18
39	*Elgonocyclus koptaweliensis*	10	0.27	0	—	—
39	*Achatina stuhlmanni*	10	0.27	3	0.9	22
39	*Pupisoma* sp. B	10	0.27	0	—	—
42	*Succinea* sp.	9	0.24	0	—	—
43	*Oreohomorus iredalei*	7	0.19	1	0.3	29
44	*Limicolaria* cf. *saturata*	6	0.16	39	11.71	3
45	*Trachycystis ariel*	4	0.11	0	—	—
45	*Trochozonites* cf. *medjensis*	4	0.11	2	0.6	26
47	*Rhachidina chiradzuluensis*	3	0.08	0	—	—
48	*Acanthinula* sp.	2	0.05	0	—	—
49	*Gulella handeiensis*	1	0.03	0	—	—
49	*Nothapalus* sp.	1	0.03	0	—	—

urocyclid slug and *Halolimnohelix percivali*, are confined to the *Bischofia* plantation (although in one plot only). The abundance of several species also differs substantially be- tween the plantations and indigenous forest (Table 6). In particular, *Gulella ugandensis* is relatively much more abundant in the plantations, whereas *Guppya quadrisculpta* and

Pseudoglessula elegans rank higher in the indigenous plots than in the plantations.

DISCUSSION

The Fauna of Kakamega Forest

The analysis of cumulative species number suggests that the sampling has provided an accurate picture of indigenous forest diversity levels. However, many additional species of terrestrial molluscs have been reported previously from the Kakamega Forest area (Germain, 1923; Pickford, 1995; Pain, 1957; Verdcourt, 1983, 1988). A comprehensive appraisal of these species would require critical examination of all the available original material and is beyond the scope of this study. However, some species can be eliminated from the list for the indigenous forest on the grounds of conspecificity (e.g., see *Pseudopeas yalaensis* in Appendix I), and others have almost certainly been misidentified. For example, Germain (1923) lists *Trachycystis planulata* Preston from "les bords de la rivière Yala," but because this species was described from 9,000–10,000' on Mount Kenya, it seems highly improbable that it occurs at Kakamega. Verdcourt (1962) discusses another error made by Germain. Some of these species may be associated with non-forest habitats or ecotones and are therefore not part of the indigenous forest fauna. Furthermore, the need for major revisions of some taxa, such as the genus *Thapsia,* means that it is not possible to make precise estimates of *S* for the forest. However, by taking these factors into account as far as possible, the total list for the forest might be estimated at roughly 70–80 species. This substantially exceeds the total species number (S) of 50 found in the indigenous forest plots.

Local Patterns and Levels of Diversity

Solem (1984) has reviewed the evidence on worldwide land snail diversity and has shown that sympatric levels in most parts of the world are low, with most sites typically supporting less than ten species. However, a few places are known to support much richer faunas. The highest site diversity reported to date is on the Manukau Peninsula, North Island, New Zealand, where more than 60 species have been found to exist microsympat-

rically in lowland patches of relict forest (Solem et al., 1981). Solem (1984) also cites the work of Fred Thompson and John Stanisic, who have recorded 25–30 species and 40 species from sites in the Greater Antilles (Hispaniola and Jamaica) and from rain forest in Queensland, New South Wales, respectively. At least 50 species are present in some Tanzanian coastal and upland forests (K. Emberton, pers. comm., and Tattersfield, unpublished). Alpha diversity can also be high in rich temperate sites. Cameron (1986) reports a median species number of 15 in coniferous forest with mull humus soils in British Columbia; Wäreborn (1969) assessed there to be a mean snail species number of 16.58 per plot in his richest Swedish woodlands; and Waldén (1981) reported mean snail species as 25.2 in five broad-leaved woodland site on calcareous moraine in Sweden. Old woodlands in the Pennines (Cameron, 1978a) support a mean snail number of 28.25 species. Values of S can also be high in optimum temperate forests. Coney et al. (1982) reported 57 species from 37 forest sites in Tennessee; Tattersfield (1990) found 31 species in English woodland sites on both limestone and acidic geologies; and Branson & Batch (1970) recorded a total of 45 species in Kentucky. Based on these studies, it is therefore apparent that the mean plot diversity of 24 species/site and the overall total of 50 species from the indigenous Kakamega stands are relatively high in a worldwide context. Microhabitat specializations can help account for high snail diversity in some faunas (Cameron, 1978b), but further work would be needed to assess whether this is important in the Kakamega fauna. However, the current study revealed very few examples of possible microhabitat specializations.

Calculation of the Shannon-Weaver diversity index (H) (Poole, 1974) and index of evenness ($J = H/Hmax$), which take into account the number of specimens contributed by each species as well as *S,* also indicates that the Kakamega fauna is more diverse than almost all other woodland/forest faunas that have been studied (Table 7); indeed, these indices show that it is on a par with the richest known fauna described from New Zealand (Solem et al., 1981).

Verdcourt, cited in Solem (1984), considered that "the wet forests of East Africa may yield up to 20–25 species from a small area although more frequently such a collection yields only 10–15." The only systematically

TABLE 7. Overall species number (S) and Shannon-Weaver diversity (H) and evenness (J) indices for molluscan faunas from Kakamega Forest and other forest systems

Area/Forest type	S	H	J	Source
Kakamega Indigenous Forest				
Live snails	43	3.23	0.86	This study
All snails	50	3.27	0.84	
New Zealand				
Live snails	45	3.22	0.85	Solem et al. (1981)
All snails	56	3.26	0.81	
Tennessee, U.S.A.	57	2.93	0.73	Coney et al. (1982)
British Columbia*				
Mull litter sites	26	2.62	0.80	Cameron (1986)
Intermediate litter sites	16	2.17	0.78	
Mor litter sites	9	1.72	0.78	
Finland (islands)				
Deciduous woodland Site 9	21	2.60	0.85	Valovirta (1984)
Site 8	21	2.35	0.77	
Site 6	19	2.26	0.77	
Site 5	20	2.25	0.75	
Sweden				
Moist meadow woods	31	2.31	0.67	Wäreborn (1969)
Drier mixed woods	9	1.41	0.64	
Moist mixed woods	9	1.23	0.56	
Shropshire, United Kingdom	12	1.75	0.64	Cameron (1982)

*excludes slugs

collected information about mollusc diversity patterns in African forests is from De Winter's (1995) series of 20 lowland rain forest plots (from a total area of approx. 48 km^2) in the Ogoou, Maritime region of Gabon, West Africa. Using similar methods to those adopted here (snails extracted from approx. 4 l of litter), he reported mean species number per litter sample as 3.4 (range 0–7, median 3), with the number of specimens ranging from 0–20 (mean 7.9). It was estimated that the litter zone supported 28 (± 2.2) species. A similar number of species (about 25) in the Kakamega fauna can be classified as small (shell size < 5 mm) litter dwellers. Large species were not sampled in plots so it is not possible to compare directly alpha diversity levels, but it is evident that many more species and individuals were found in the Kakamega litter samples than in Gabon; the average number of shells recovered from the Kakamega indigenous forest plots was 137, most of which were small litter-dwellers.

De Winter (1995) recorded a total of 32 species (including four freshwater species) and estimated that the whole forest might support 39 terrestrial species by taking into account previous records; these values of S are also lower than at Kakamega. Taking into account the litter dwellers only, which form 82% and 51% of the recorded fauna in Kaka-

mega Forest and Gabon respectively, the ratio S/∝ is substantially greater in Gabon than at Kakamega. This indicates that there is substantially more variation in the fauna amongst the Gabonese plots than in Kakamega Forest, but it is not possible to establish whether this is related to habitat variation or to the geographical replacement of sister species. Such a conclusion was also tentatively made by De Winter (1995), who noted that there were few species in common from similar forest types over a distance of less than 50 km, but that a fair number of additional species were found. Solem (1984) predicted that the median total linear range of all land snails worldwide would be found to be less than 100 km and probably less than 50 km, and Cameron (1992) has reported taxonomic replacement effects over short distances in semi-arid habitats in Western Australia. The commonest of De Winter's (1995) species was found in only half of the litter samples, whereas 18 (36.7%) of the 49 indigenous forest species in Kakamega Forest were found in more than 50% of the plots; this also suggests that the fauna of the Gabonese Forest is very much more heterogeneous than at Kakamega. In Kakamega Forest, both *I* and the proportion of the maximum variance of the sites per species statistic achieved are broadly similar to the val-

ues reported by Cameron (1992) for faunas from woodland in the English South Downs (maximum separation 75 km) and from rock habitats in the Pennines (separation 30 km). They differ from British Columbian coastal forests (maximum site separation of 300 km), which have a more homogeneous fauna, and from faunas from the Oscar and Napier ranges of Western Australia (about 160 km maximum separation), where both *I* (9.82) and the proportion of variance achieved (8%) revealed strong replacement effects in large camaenid taxa (Cameron, 1992).

Origins of the Kakamega Forest Fauna

The African climate has been unstable during the Pleistocene, and this had a strong influence on forest cover. Many parts of equatorial Africa were dry and cool during the last ice age, and Lake Victoria was almost non-existent at around 14000 BP, during the Last Glacial Maximum in Africa (Kendall, 1969; Livingstone, 1980). With the absence of moisture-laden convection currents from the Lake Victoria waterbody, the Kakamega area would not have supported forest cover. There is evidence (reviewed in Hamilton, 1982) that forest in equatorial Africa became confined to a relatively small number of discrete areas during times of extended glaciation and that the current distribution patterns of many groups of forest species can be accounted for by subsequent expansion from these refugia (Hamilton, 1982; Kingdon, 1990). Former forest refugia are now often rich in endemic species. The Gabonese forest studied by De Winter (1995) falls within or close to such a refugium at the Gabon-Cameroon border (Kingdon, 1990), whereas Kakamega Forest does not. The mollusc fauna of Kakamega Forest must have recolonised after the climatic amelioration and redevelopment of forest. Palynological evidence (Hamilton, 1972; Kendall, 1968; Livingstone, 1967) shows that at 12000–10000 years BP, forest spread from a refugium in eastern Zaïre (possibly extending into west Uganda) across what is now Uganda and the Kakamega area of west Kenya. Furthermore, studies on the avifauna (Zimmerman, 1972) and tree flora (Hamilton, 1982) of Kakamega Forest have shown that they are impoverished versions of the Central African biotas, which also strongly suggests that recolonisation was from west or central Africa. The mollusc fauna of Kakamega Forest would suggest a similar route of recolonisa-

tion because Pain (1957) has shown that *Achatina stuhlmanni* is commonest in Zaïre west of the Upper Ituri River and the genera *Prositala, Pseudoglessula* (*Ischnoglessula*), *Oreohomorus, Nothapalus, Conulinus, Trochozonites* (*Zonitotrochus*), and *Gulella* (sect. Silvigulella) all have west and central African affinities (Verdcourt, 1972).

Regional Patterns and Levels of Diversity

The mollusc fauna of East Africa (Kenya, Uganda and Tanzania) contains about 1,015 terrestrial species of which about 844 (83%) are forest dwellers (Verdcourt, 1972). Based on the current survey results plus other records it can therefore be assessed that the fauna of Kakamega Forest supports about 5.8–9.5% of the potential forest fauna of the region. This low proportion indicates that there must be substantial geographical replacement of taxa throughout the region. As discussed above, this situation is probably not unusual worldwide; Solem (1984) summarised the available evidence which suggests that allopatric diversity is exceptionally high amongst land snails. The regional levels and patterns of terrestrial snail diversity are very different in temperate northwest Europe, where the fauna is relatively homogeneous over large geographical areas (Cameron, 1995). Kerney & Cameron (1979) cover a land area about 38% larger than East Africa, but it supports only 279 terrestrial molluscs (Kerney & Cameron, 1979), of which about 152 or 54.5% are forest or woodland species. Rich sites in Britain may support a large proportion of the national gastropod fauna; for example, Whitcombe Wood on Jurassic limestone in Gloucestershire (Boycott, 1934) supports 37 gastropods (28 snails and nine slugs), which represent approximately 33% of the total British land gastropod fauna (or 32% of the snail fauna).

Bernard Verdcourt (in Rogers & Homewood, 1982) lists 111 terrestrial gastropod species and subspecies known from the Usambara Mountains in northern Tanzania. These mountains support lowland and intermediate rain forest communities and have rainfall (1919 mm/year at Amani (911 m asl)) and temperature (21.7–28.3°C mean maximum at Amani) regimes broadly rather similar to Kakamega Forest. However, the list in Rogers & Homewood (1982) has only seven species in common with the total reported fauna (i.e., all records) from Kakamega For-

est. Unlike Kakamega Forest, the Usambaras are thought to have have supported forest cover for millions of years, probably since before the Miocene, and contain very high numbers of endemic species (Rogers & Homewood, 1982). The richer overall fauna in the Usambaras may be related to this long period of forest stability, plus its greater extent and more diverse physical geography and habitats. However, at individual sites in the East Usambara, values of both S and $\propto$ are broadly similar to those found in Kakamega Forest (Tattersfield, unpublished).

Plantation Faunas

Impoverished snail faunas and low molluscan diversity levels have been reported from plantations elsewhere (Cameron, 1978a). The very small number of plantations examined restricts firm conclusions, but there is evidence that both the *Maesopsis* and *Bischofia* plantations also have impoverished faunas, and that the latter also appears to be compositionally different from the indigenous forest. Differences in the diversity and species composition in several groups of soil arthropod have been reported between primary forest and *Maesopsis eminii* plantation in the East Usambaras in Tanzania (Mahunka, 1989). *Maesopsis eminii,* which is not an indigenous species in northeast Tanzania and where it is considered to be an ecological weed, has also been shown to alter radically the characteristics of the litter and topsoil (Hamilton, 1989) and to be associated with a loss of the organic soil horizons (Macfadyen, 1989; Binggeli & Hamilton, 1993). Whether *Maesopsis* itself or other factors, such as canopy loss, drainage or disturbance, is responsible for organic horizon loss is not clear (Macfadyen, 1989), although such changes might be expected to have a large effect on the small, litter-dwelling snails, in line with that reported here. The six species (Table 5) that are less frequent in the plantations may thus, tentatively, be regarded as indicators of indigenous forest, in the same way that some species can be used to assist in the differentiation of ancient and secondary woodland in Britain (Kerney & Stubbs, 1980). Lovejoy et al. (1986) have demonstrated that substantial changes occur in microclimate at the edges of recently cleared rainforest. It is interesting in this context that the species that are significantly less frequent in both the riverine (forest edge) indigenous plots and in the plantations are all small, litter dwellers that might be expected to be more suceptible to such changes.

Conservation Implications

The mollusc fauna of Kakamega Forest does not contain the high numbers of endemic species found in some other forest systems in East Africa (for example the Tanzanian Usambara ranges (Rogers & Homewood, 1982)). However, in common with the bird and butterfly faunas and the flora, its malacofauna does support central and west African elements, which are scarce or absent from most of Kenya and Tanzania. The molluscs of Kakamega Forest are therefore of some biogeographical interest, and they supplement this previously acknowledged conservation importance of the Kakamega Forest system.

Further information is required about local diversity patterns in other African forests, but if faunas from other forest systems are relatively uniform like in Kakamega Forest, then this, and the high level of allopatric diversity throughout East African forests, have several potential conservation implications. Notwithstanding the probable importance of edge effects in forest fragments, the majority of mollusc species found during the study could probably be conserved in a relatively small area of forest. Of course, there are many important reasons why the size of protected areas should be maximised, but based on these conclusions and from a solely molluscan perspective, it is apparent that the protection of a large number of widely distributed, small forest blocks might be more effective at conserving regional molluscan biodiversity than would the retention of a smaller number of large forest areas of equal extent. The degree to which regional mollusc biodiversity could be maintained by conserving the endemic-rich forest systems needs further survey and analysis.

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APPENDIX I. SPECIES NOMENCLATURE AND NOTES ON IDENTIFICATION

Museums are referred to in this appendix as follows:

BMNH — Natural History Museum, London, United Kingdom

NMW — National Museum of Wales, Cardiff, United Kingdom

AMNH — American Museum of Natural History, New York, U.S.A.

MCZH — Museum of Comparative Zoology, Harvard, Cambridge, Mass., U.S.A.

MNHN — Muséum Nationales d'Histoire Naturelles, Paris, France

Systematic List

Elgonocyclus koptaweliensis (Germain) — Verdcourt (1982a, 1991a).

Maizania elatior (von Martens) — Verdcourt (1964).

"*Succinea*" sp. — A revision of African Succineidae is required before the genera present can be elucidated or the species named consistently. The genus *Succinea* Drap. probably does not occur in Africa (Verdcourt, 1972).

Truncatellina ninagongonis (Pilsbry) — The Kakamega material matches the holotype (MCZH 77268) collected from Mt. Ninagongo, 9000 ft. (north of Lake Kivu), Zaïre (Tattersfield, 1995).

Nesopupa bisulcata (Jickeli) — Adam (1954) and Bruggen & Verdcourt (1993).

Pupisoma (Salpingoma) harpula (Reinhardt) (= *Pupisoma japonicum* Pilsbry) — Identification by Dr. A. C. van Bruggen (Leiden). Also Adam (1957). This widespread species is not listed in Verdcourt (1983).

Pupisoma orcula (Benson) — Adam (1957) and material in NMW from Bekar, India.

Pupisoma sp. A — Shell almost globular (c. 2.2 × 2 mm), brownish olive-green, with fine radial sculpture.

Pupisoma sp. B — Probably a *Pupisoma* but matches none of the species in Adam (1954, 1957). However, the species (approx. 2 × 1.4 mm) has a moderately large umbilicus and traces of lamellae on the periphery, which make it rather endodontoid in appearance.

Acanthinula sp. — The Kakamega material has only a few small spines. It lacks the spiral striation on the first whorl of both Preston's *expatriata* Preston (holotype, BMNH 1937.12.30.2085) and Pilsbry's *azorica;* it may be underscribed.

Rhachidina chiradzuluensis var. *virginea* (Preston) — Matches Preston's cotypes (BMNH) from Mount Kenya.

Conulinus rutshuruensis major Verdcourt — Matches paratype (BMNH 196731, Nandi Forest, Kenya).

Cerastua trapezoidea lagariensis (E. A. Smith)

Micractaeon koptawelilensis (Germain) (= *kakamegaensis* Verdcourt) — Verdcourt (1990).

Nothapalus sp. — Shell yellow, 19.5 × 6.4 mm. It seems unwise to name this species on the basis of the single specimen. The shell of Preston's *iredalei* is narrower and his *suturalis* is larger. However, it is not disimilar in shape to either of these species. It has not been possible to compare the shell with material of *N. babaulti* (Germain) or *N. paucispirus* (von Martens).

Subulona clara Pilsbry — The Kakamega

species matches material of *clara* in BMNH.

Oreohomorus iredalei Preston — The size and shape of the Kakamega specimens match material from the Belgian Congo and Mount Elgon in NMW, although shells of the former have more white colouration. This species is conspecific with *O. nitidus* (von Martens) (Verdcourt, 1983). Examination of a syntype of *O. albini* Germain (MNHN), which was described from Kakamega Forest, indicates that it is close or identical to the material collected in 1993, although it has lost its periostracum. The strong crenulations and spiral sculpture on the early whorls described by Germain (1923) and shown on the illustration are not visible even though the syntype examined is clearly the illustrated shell.

Pseudoglessula (Ischnoglessula) elegans (von Martens) — Kakamega material matches syntypes of *elegans* (BMNH), although all the types are in very poor condition with bleached or missing periostracum. The material from Kakamega also appears identical to the illustration of *P. subfuscidula* Pilsbry, 1919, which is probably conspecific (Verdcourt, 1983). Verdcourt (1983) lists *P. mutandana* Connolly from Kakamega Forest but this species (syntype in BMNH) is larger and has much finer ribbing on the first four whorls than *elegans;* it was not found during the survey.

Pseudopeas cf. *yalaensis* Germain — The Kakamega specimens match syntype material of *yalaensis* (MNHN); the types have very faint and barely perceptible spiral sculpture on the first whorl. *Opeas euschemon* Connolly may be conspecific (Verdcourt, 1983); however, the six shells of this species (NMW, Melvill and Tomlin coll., Mt. Mikeno) are larger than the types of *yalaensis,* and there is no trace of spiral sculpture. Their general shape is however similar and further material would be needed to confirm whether they are the same species.

Curvella sp. A — shell broadly conic, thin, transluscent white, approx. 7 × 3.5 mm. Apical whorls smooth, remainder with irregular, arcuate growth lines. Outer lip curved in profile, arching forward in the centre. Columella curving smoothly into the basal margin of the shell mouth, not truncate.

Curvella cf. *babaulti* Germain — The Kakamega specimens appear identical to the syntype (MNHN). The Kakamega material also matches *Pseudopeas kekumeganum* Connolly (syntype in BMNH), which Verdcourt (1983) suggests may be conspecific. The Kakamega shells have faint spiral microsculpture indicating that the species belongs in *Pseudopeas.*

Achatina stuhlmanni von Martens — Pain (1957).

Limicolaria cf. *saturata* Smith — The Kakamega material has a similar shape to the *saturata* holotype although the shells are smaller. Material of *saturata* in NMW also generally has larger shells than the Kakamega specimens and further investigations are desirable to confirm identification.

Punctum ugandanum (E. A. Smith) — Verdcourt (1988).

Punctum sp. A — The shells (approx. 1.2 × 0.8 mm) have rather regular ribbing and a characteristic spiral micro-sculpture suggesting that the species is in *Punctum.* Possibly close to *hottentotum* (Melvill & Ponsonby) but spire more elevated.

Punctum sp. B — Shell (c. 1.6 × 0.9 mm) with lamellae and possibly in *Trachycystis.* Smaller, less elevated spire and without the very broad lamellae of E. A. Smith's *lamellifera.*

Trachycystis iredalei Preston — Verdcourt (1991b, c).

Trachycystis ariel (Preston) — Agrees with paratype in BMNH. Also illustration in Bruggen (1969).

Prositala butumbiana (von Martens) — Verdcourt (1991b, c).

Kaliella barrakporensis (Pfeiffer)

Kaliella iredalei Preston

Carinate species (undescribed) — This distinctive but undescribed, minute species is distributed widely across Africa (Malawi, Zaïre, Angola, Ghana and various other west African countries) (pers. comm., A. C. van Bruggen). The shell is discoid and has six, spiral lamellae. The Kakamega material appears to be polymorphic for shell colour with both white/transparent and red-brown shells.

Guppya quadrisculpta (Connolly)

Afroconulus iredalei (Preston) — Microscopic and larger-scale shell sculpture and shape agree very well with cotype of

iredalei (BMNH, Mt. Kenangop, Aber-
dares, Kenya). However, the Kakamega
material also does not differ significantly
from *urguessensis* (Connolly), which may
therefore be conspecific. The type of *A.
diaphanus* (Connolly) could not be found
in BMNH.

Trochozonites (Zonitotrochus) cf. *medjensis*
Pilsbry — The angle of the shell apex is
smaller and the ribbing stronger in the
Kakamega material than in the holotype
(AMNH, Medje, Belgian Congo). How-
ever, overall shape and size agree well.
The holotype shell of *expatriata* Preston
(BMNH, Mt. Mikeno, Belgian Congo) has
a much flatter base than both *medjensis*,
and the Kakamega species and is clearly
different.

Thapsia eucosmia Pilsbry — Agrees well with
the holotype (AMNH, Medge, Belgian
Congo). This *Thapsia* has a "nipple-like"
apex to its shell and shouldered whorls,
which appear effectively to separate it
from the other species collected during
the study.

Thapsia microleuca Verdcourt — Verdcourt
(1982b).

Thapsia spp. — Preston figured many spe-
cies in this genus, which appear to be
barely separable even with type material
side by side for comparison. In the ab-
sence of a full revision of the genus, it
seems unwise to assign names to the
Kakamega material. In addition to *eu-
cosmia* and *microleuca,* the following
species have been recorded from Kaka-
mega forest previously:

Thapsia elgonensis (Preston)
Thapsia cinnamomeozonata Pilsbry
Thapsia densesculpta (Preston)
Thapsia karamwegasensis Germain
Thapsia yalaensis Germain
Thapsia gerstenbrandti (Preston) ?= elgonen-
sis (Prest.)
Thapsia mime (Preston)

There appears to be at least two unidentified
species in the Kakamega material. One
has strong spiral striae and tight whorls
without any evidence of a shell band.
The other has faint spiral microsculpture
and a brown shell with a faint band; this
species is close to *mime* (Preston).

Gymnarion aloysiisabaudiae (Pollonera)
Chlamydarion oscitans (Preston)

Urocyclid slug

Halolimnohelix percivali (Preston) — Con-
firmed by dissection by B. Verdcourt.
Both holotype and paratypes (BMNH)
are juvenile and lack a reflected peris-
tome, but the shells otherwise agree with
the Kakamega material.

Halolimnohelix plana Connolly — Agrees
with holotype in BMNH; also Verdcourt
(1981).

Gonaxis elgonensis (Preston) — material
agrees well with paratypes in BMNH.

Gulella woodhousei (Preston), = babaulti
Germain, ?= perturbata Preston.

Gulella osborni Pilsbry — Illustrated by Pils-
bry (1919).

Gulella impedita Connolly — agrees perfectly
with holotype in BMNH.

Gulella lessensis Pilsbry — Illustrated by Pils-
bry (1919).

Gulella handeiensis Verdcourt

Gulella disseminata (Preston) — The Kaka-
mega material agrees well with the holo-
type of var. *kekumegaensis* Connolly
(BMNH).

Gulella ugandensis (E. A. Smith) — See Verd-
court (1970).

Streptostele bacillum Pilsbry — The Kaka-
mega material has spiral microsculture
on the apical whorls. It matches perfectly
with the holotype (AMNH, Bequaert
Coll.) collected from Ituri Forest, Penge,
Zaïre (Pilsbry, 1919).

MOLECULAR GENETIC IDENTIFICATION TOOLS FOR THE UNIONIDS OF FRENCH CREEK, PENNSYLVANIA

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ABSTRACT

A molecular genetic key to the unionids of French Creek, Pennsylvania, an Allegheny River tributary, is presented here. The key is an integral part of a new approach to identifying unionid glochidia larvae attached to host fishes in the drainage. Working with tissue from adult union-ids, we used the polymerase chain reaction (PCR) followed by restriction enzyme digests to find species-specific genetic "fingerprints" for the 25 species in the drainage. We have demon-strated the utility of the key by using it to identify 70 glochidia attached to fishes collected in the French Creek drainage.

Key words: Unionoidea, glochidial identification, PCR, RFLP analysis, ITS regions.

INTRODUCTION

North America's freshwater mussels (Bi-valvia: Unionoidea) are declining precipi-tously in richness and abundance (e.g., Den-nis, 1987; Anderson et al., 1991; Nalepa et al., 1991; Williams et al., 1992, 1993). Sizable gaps in knowledge of unionid reproductive requirements hamper current preservation efforts. Information on the identities of the host fishes upon which unionid glochidia lar-vae are obligate parasites is especially in-adequate. Traditional methods of gathering such data have a variety of drawbacks.

To date, lists of unionid host fishes have been derived primarily in two ways. The first, which has its roots in artificial propagation efforts (e.g., Lefevre & Curtis, 1910, 1912; Coker et al., 1921), involves inoculating pu-tative hosts with glochidia taken from gravid females of the unionid species of interest. Fishes in aquaria that ultimately contain meta-morphosed juveniles are considered suitable hosts (e.g., Zale & Neves, 1982; Waller & Hol-land-Bartels, 1988). Unsuitable hosts launch immune responses that thwart glochidial en-cystment, preventing further development and causing glochidia to be shed (Arey, 1923a, 1932).

As the completion of metamorphosis re-quires a week to several months of attach-ment (Zale & Neves, 1982), this approach is often time-consuming. It is also ill-suited to systems with large numbers of potential host fishes. Moreover, drawing inferences from in-oculation studies can be complicated by the fact that "suitable" host fishes can appar-ently acquire immunity to glochidia with re-peated exposure, the duration and species specificity of which are poorly established (Reuling, 1919; Arey, 1923b; Fuller, 1974). To obtain unambiguous results, it is often nec-essary to collect putative hosts from unionid-free streams or to inoculate naïve fishes bred and raised in the laboratory. Finally, while artificial inoculation methods are appropriate if laboratory propagation of unionids is the only goal, the results of such studies might be inapplicable to organisms in their natural environments. Such studies disregard micro-habitat preferences and specialized mor-phologies and behaviors (e.g., the waving of fish-like mantle flaps by gravid female *Lamp-silis* species; Ortmann, 1911; Kraemer, 1970) that might modulate unionid-fish interactions *in situ*.

To circumvent these problems, several in-vestigators (e.g., Wiles, 1975; Stern & Felder, 1978) have attempted morphology-based identification of glochidia attached to fishes. Such determinations have thus far entailed identifying the glochidia using dissecting mi-croscopes or compound light microscopes.

There are drawbacks to this approach as well. Glochidia are less than 1 mm in diameter. Encystment makes them difficult to observe and might influence their shapes in unpredict-able ways (Wiles, 1975). Closely related spe-

cies, such as *Villosa nebulosa* (Conrad) and *Villosa vanuxemensis vanuxemensis [vanuxemi]* (I. Lea) (Zale & Neves, 1982), are difficult to distinguish from each other and are easily misidentified. Hoggarth (1992) reported that glochidia photographed by Wiles (1975) and identified by the author as *Pyganodon [Anodonta] cataracta* (Say) were actually *Alasmidonta undulata* (Say). Clarke (1981, 1985), Rand & Wiles (1982), and Hoggarth (1988) demonstrated that scanning electron microscopy can be used to distinguish among glochidia taken from gravid females. Whether their techniques can be adapted for species-level identification of glochidia from host fishes remains to be investigated, however.

The objective of the research described herein was to develop a new method for identifying glochidia attached to fishes, a method that exploits genetic differences among unionid species. The method utilizes restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR) products. In combination, PCR and RFLP analysis are useful for performing sensitive analyses of minute quantities of DNA (e.g., Whitmore et al., 1992; Simon et al., 1993), such as those present in single glochidia. In short, a diagnostic suite of restriction sites (or "genetic fingerprint") is sought for each unionid species in the drainage of interest. Glochidia on host fishes are then identified on the basis of the "fingerprints" they possess.

MATERIALS AND METHODS

Study Site

The aquatic system for which the glochidial identification method was developed is the French Creek drainage, in southwestern New York and northwestern Pennsylvania (Fig. 1). French Creek is a fourth-order tributary to the upper Allegheny River. It drains approximately 3,000 km^2. Twenty-five unionid species (C. Bier, pers. comm.) and 53 fish species (J. Stauffer, unpubl. data) have been collected from the French Creek drainage recently, making its fish and molluscan faunas the richest in Pennsylvania. Two of the drainage's unionid species, *Epioblasma torulosa rangiana* (I. Lea) and *Pleurobema clava* (Lamarck), are federally endangered and have no known hosts. Two additional species are considered globally threatened and seven

are of special concern (Williams et al., 1993); of these nine, five have no known hosts.

LeBoeuf Creek is thought to harbor higher densities of *P. clava* than any other part of the drainage (A. Bogan, pers. comm.). To assess the utility of the identification technique, fishes were collected from LeBoeuf Creek at Moore Road bridge, just east of Route 19, 3 km south of LeBoeuf Gardens, Pennsylvania (Fig. 1). Full descriptions of the site and collection procedures are given by White (1994).

Specimen Collection and Preservation

Adult unionids. Adult unionids were collected throughout the French Creek drainage (Fig. 1, Table 1) in 1991, 1992, and 1993. Numbers of unionids collected ranged from one to 23 per species, with a median of six. Adult *Lasmigona costata* (Rafinesque), *Amblema plicata* (Say), and *Lampsilis siliquoidea* (Barnes) specimens were also collected from West Virginia (Dunkard Creek) and Ohio (lower Muskingum River, Little Muskingum River, and Big Darby Creek), so that their genetic "fingerprints" could be compared with those of French Creek specimens to evaluate the key's applicability to other drainages.

Adult unionids were collected using masks and snorkels or Plexiglas-bottomed buckets. Nonendangered species were transported to the laboratory either alive (wrapped in cheesecloth in chlorine-free ice water) or frozen on dry ice. In the laboratory, live unionids were either killed and frozen at −80°C, or maintained in aquaria in which currents were established. Two small (5- to 100-mg) pieces of foot tissue were excised from each individual in the laboratory using a sterile scalpel blade or scissors. Both samples were frozen at −80°C, one for nucleic acid extraction and the other for voucher material. The remaining tissue was preserved with the valves in 70% ethanol, also as voucher material. To facilitate future molecular genetic examination, the latter tissue was not fixed in formalin. All voucher material was deposited into the mollusc collection of the Academy of Natural Sciences in Philadelphia upon completion of the research (Dry Catalog # 398499-398500; Alcohol Catalog # A18354-A18438; Frozen Catalog # F100-F118).

For endangered unionids, a single tissue sample was obtained from each specimen at streamside by relaxing its adductor muscles in soda water and clipping off a 5- to 50-mg
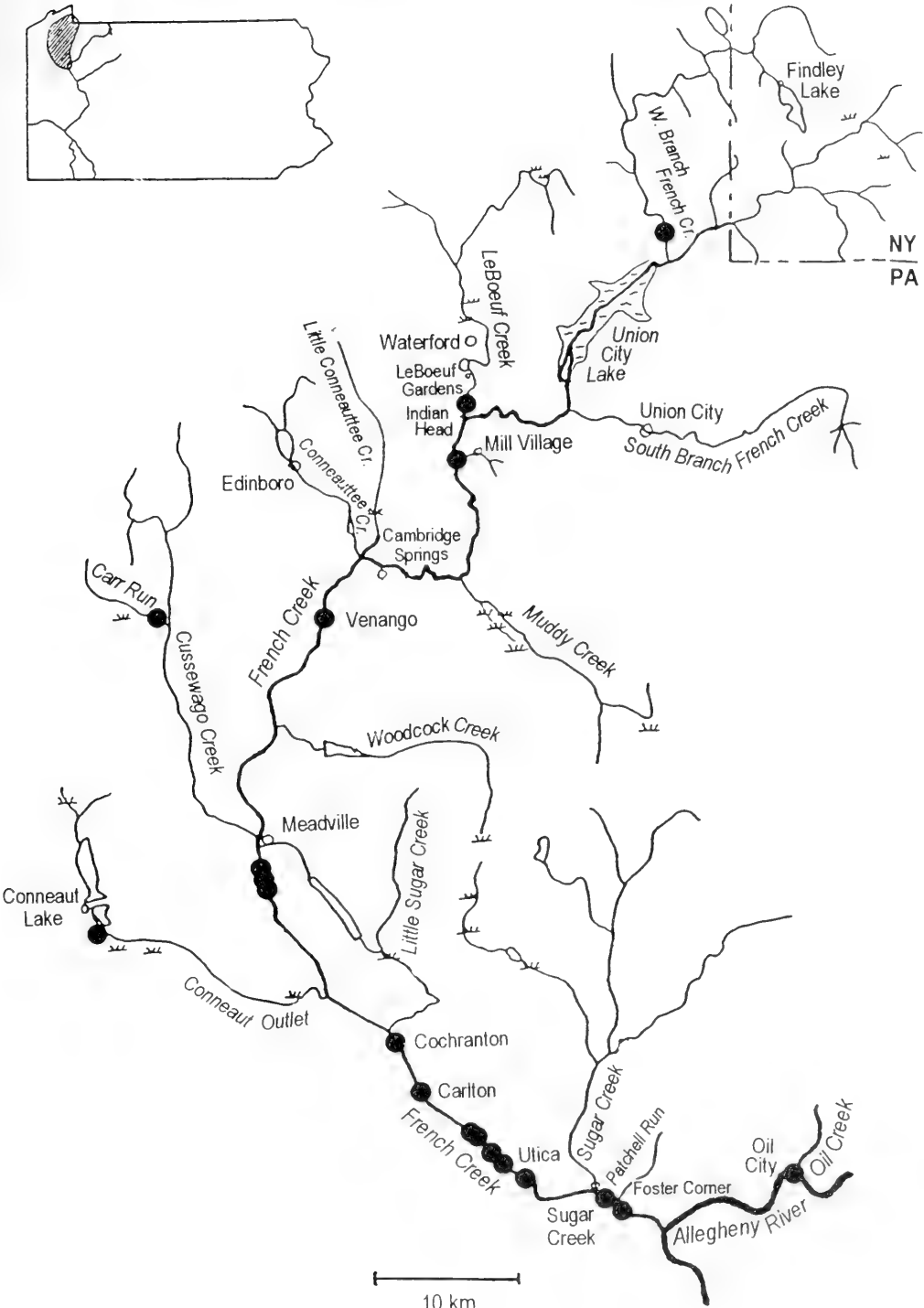
FIG. 1. Location of French Creek drainage collection sites. (For site descriptions, see Table 1 and White, 1994).

TABLE 1. Number of unionids collected, by species and site.

Species	French Creek drainage																	Dunkard Creek			Ohio		
	WB	LB	MV	VN	CR	MD	CO	CN	CA	U1	U2	U3	U4	U5	SC	FC	OC	MM	PN	BL	MU	LM	BD
Actinonaias ligamentina				4		8			1	2							3						
Alasmidonta marginata				4		3			1	1													
Amblema plicata									2	4						1		11	4		18		
Anodontoides ferussacianus	3																						
Elliptio dilatata				7						4		1	1				1						
Epioblasma torulosa				2								1	1										
Epioblasma triquetra			1	1										1									
Fusconaia subrotunda											5	1											
Lampsilis cardium				4+1?		1?					1+1?				1								
Lampsilis fasciola				1		1				1													
Lampsilis ovata				1+1?		1				2+1?	1?	1	1										
Lampsilis siliquoidea				3		1				1								5	2	10		5	1
Lasmigona complanata							1																
Lasmigona compressa	1				3		1																
Lasmigona costata				9		7	11		2	1						1		22	2			2	6
Ligumia nasuta																	1						
Ligumia recta				1					1	1													
Pleurobema clava		2					1																
Pleurobema sintoxia				6		1				1	3					1							
Ptychobranchus fasciolaris								1		3	1					1							
Pyganodon grandis			1				4																
Quadrula cylindrica				2					5	3	4												
Strophitus undulatus				10				1		3		4											
Villosa fabilis				1						11		3	3	1									
Villosa iris													6										

BD = Big Darby Cr., Ohio; BL = Dunkard Cr. at Blacksville, West Virginia; CA = French Cr. at Cochranton; CN = French Cr. at Carlton; CO = Conneaut Outlet; CR = Carr Run; FC = French Cr. at Foster Corner; LB = LeBoeuf Cr.; LM = Little Muskingum R., Ohio; MD = French Cr. downstream of Meadville; MM = Dunkard Cr. near Mt. Morris, Pennsylvania; MU = lower Muskingum R., Ohio; MV = French Cr. at Mill Village; OC = Allegheny River at Oil City; PN = Dunkard Cr. near Pentress, West Virginia; SC = French Cr. near town of Sugar Cr.; U1 = French Cr. 2.7 km upstr. of Utica; U2 = French Cr. 2.8 km upstr. of Utica; U3 = French Cr. 1.1 km upstr. of Utica; U4 = French Cr. at Utica; U5 = French Cr. 1 km downstr. of Utica; VN = French Cr. at Venango; WB = West Br. French Cr.; ? signifies specimen of questionable identity

piece of foot using a sterile scalpel blade or scissors (Pennsylvania Fish and Boat Commission permit number 142 (Type I); procedure reviewed prior to permitting by the United States Fish and Wildlife Service). Tissue samples were frozen immediately on dry ice for transportation to the laboratory, where they were kept at $-80°C$ pending nucleic acid extraction. After a 10- to 15-min recovery period in a bucket of streamwater, the specimens were photographed and returned to natural positions in the substrate as close to their original locations as possible.

Fishes. Fishes were collected throughout French Creek by kick-seining and were transported to the laboratory on dry ice. In the laboratory, a 5- to 100-mg piece of muscle was excised from the body wall of each and was frozen at $-80°C$ prior to nucleic acid extraction. The remainder of each specimen was also frozen at $-80°C$ as voucher material.

Glochidia. Glochidia of known identity were obtained from marsupia of gravid nonendangered female unionids collected and frozen as described above. Glochidia of unknown identity were obtained from fishes collected throughout French Creek by kick-seining. The fishes were transported to the laboratory alive, maintained in an aquarium for one week, then killed and frozen at $-80°C$; unencysted glochidia were presumed to have been shed during the holding period. Encysted glochidia were removed as described below.

Laboratory Techniques

Nucleic acid extraction. For adult unionids, unattached glochidia, and fishes, a standard phenol-chloroform extraction protocol (after Kocher et al., 1989) was used to isolate total nucleic acids. Each tissue sample was minced over ice using a sterile scalpel blade, then transferred to a 1.5-ml microfuge tube and homogenized in 500–800 µl of extraction buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA; 125 mM NaCl; 0.1% SDS; 50 mM DTT; 5 µg/µl proteinase K) using a flame-sealed 1000-µl pipette tip; different scalpel blades and pipette tips were used for each sample, to prevent cross-contamination. Homogenized samples were incubated 2–24 hrs at 37°C, then extracted sequentially with equal volumes of Tris-buffered phenol, 50%

phenol-50% chloroform, and chloroform (= 24 chloroform: 1 isoamyl alcohol, v:v; Sambrook et al., 1989). Samples were centrifuged 4–5 min at $16,000 \times g$ during each extraction to separate the phases. After the final extraction, 0.05 volume of 5 M ammonium acetate and two volumes of cold absolute ethanol were added to each sample. Samples were placed at $-80°C$ for 15–30 min, then spun 15–45 min at $16,000 \times g$ at $4°C$. Supernatants were decanted and pellets were dried in a Savant SpeedVac Concentrator. Pellets were resuspended in 10–25 µl of sterile distilled water, depending on their size, and stored at $-20°C$. Even when no pellet was visible in a tube, 10 µl of sterile distilled water was added and the sample was stored at $-20°C$. Extractions were assayed on 0.8%-agarose minigels stained with ethidium bromide and were diluted 0–1000× depending upon estimated DNA concentration.

For glochidia attached to fishes, an extraction protocol similar to that described by Martin et al. (1992) for fish oocytes was used. Each glochidium was removed from its host over ice using sterile forceps and a dissecting light microscope, then transferred with a 200-µl pipette tip to a 1.5-ml microfuge tube containing 30 µl of buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1 µg/ml proteinase K; 1 µg/ml bovine serum albumin). Nonidet P-40 was added to a final concentration of 1%. Solutions were heated to $95°C$ for 5 min in a thermal cycler, diluted to a final volume of 50 µl with sterile distilled water, and stored at $4°$ or $-20°C$. Extractions were not assayed prior to amplification, as they contained too little DNA to be visualized with ethidium-bromide staining (data not shown).

Amplification. Reaction volumes of 50 or 100 µl were used. Reaction mixtures consisted of 0.5–2.0 µl of diluted template DNA; 1 µM of each primer (0.2 µM of each RAPD primer); 0.1 mM each of dATP, dCTP, dGTP, and dTTP; 2.0–2.5 units of Perkin-Elmer Cetus Taq polymerase; and manufacturer-supplied buffer at 1× final concentration (10 mM Tris-HCl, pH 8.3; 50 mM KCl; 15 mM $MgCl_2$; 0.01% (w:v) gelatin). For glochidia from host fishes, 1–10 µl of undiluted template was used.

Primer sequences were as follows. ITS-1 of nuclear rDNA: 5′-TAACAAGGTTTCCG-TAGGTG-3′ (18S region) and 5′-AGCTRGC-TGCGTTCTTCATCGA-3′ (5.8S region); ITS-1 through ITS-2: 5′-TCCGTAGGTGAACCTGC-

GG-3' (ITS1 of Lee & Taylor, 1992; 18S region) and 5'-TCCTCCGCTTATTGATATGC-3' (ITS4 of Lee & Taylor, 1992; 28S region); 12S mitochondrial rDNA: 5'-TAATAATAAGAGCGA-CGGGCGATGTGT-3' (adapted from H1478 of Kocher et al., 1989 using sequence data for *Drosophila yakuba* Burla (Clary & Wolstenholme, 1985)) and 5'-TAATAAAAAACTAGG-ATTAGATACCCTATTAT-3' (adapted from L1091 of Kocher et al., 1989); RAPD primer A-02: (5'-TGCCGAGCTG-3'; Operon Technologies, Inc., Alameda, CA). Rationales for primer choices are discussed in White (1994) and White et al. (1994).

Thirty-four amplification cycles were performed (1 min at 93°C, 1 min at 50°C, and 2 min at 72°C) followed by one cycle with increased extension time (9 min). For RAPD PCR, 45 amplification cycles of 1-min denaturation at 94°C, 1-min reannealing at 36°C, and 2-min extension at 72°C were performed. Reaction products were assayed on 0.8–2.0% agarose minigels stained with ethidium bromide.

Restriction Enzyme Digestion. Restriction enzyme digests were performed in 10- to 20-μl reaction volumes consisting of 8–12 μl of PCR product, 5–15 units of restriction enzyme, and the manufacturer-supplied buffer at a final concentration of 1×. Digests were conducted at the manufacturer-recommended temperature (usually 37°C) for 4–48 hrs. Restriction fragments and uncut PCR products were assayed on 2.0%-agarose gels stained with ethidium bromide. Efforts to separate poorly-resolved fragments with 6–10% polyacrylamide or 2–4% MetaPhor high-resolution agarose met with limited success and were ultimately abandoned.

RESULTS

Key to the Unionids of French Creek

The following key was developed for identification of French Creek unionid glochidia. One proceeds through the key by amplifying the genomic region indicated in **bold text,** digesting the PCR product with the restriction enzyme listed after the *x,* and assigning a letter to the resulting restriction fragment pattern (by referring to the accompanying figure and/or to the fragment size data in Appendix 1). Assaying undigested PCR products along-side digested products facilitates pattern interpretation and is highly recommended. Superscripts refer to notes that follow the key. While the key likely reflects phylogeny to some extent, the data from which it was constructed are insufficient for testing specific hypotheses about relationships; thus, the key should be considered artificial.

1. **ITS-1** × MspI   (Fig. 2)
   A . . . . 2[a]
   B . . . . *Ligumia nasuta* (Say)
   C . . . . 9[b]
   D . . . . *Amblema plicata* (Say)[c]
   E . . . . *Quadrula cylindrica* (Say)
   F 12
   G . . . . *Strophitus undulatus* (Say)[d]
   H . . . . *Alasmidonta marginata* Say
2. **ITS-1** × Sau96I   (Fig. 3)
   A 3
   B 8
3. **12S** × HaeIII   (Fig. 4)
   A 4
   B . . . . *Actinonaias ligamentina* (Lamarck)
   C . . . . . . *Lampsilis siliquoidea* (Barnes)[e]
4. **ITS 1–2** × MspI[f]   (Fig. 5)
   A 5
   A' 7
   B . . . . *Lampsilis fasciola* Rafinesque
5. **12S** × RsaI   (Fig. 6)
   A . . . . *Villosa iris* (I. Lea)
   0 . . . . *Epioblasma* spp. . . . . 6[g]
6. **ITS 1–2** × MboII   (Fig. 7)
   A . . . . *Epioblasma torulosa rangiana* (I. Lea)[h]
   A' . . . . *Epioblasma triquetra* (Rafinesque)
7. **ITS-1** × AvaI   (Fig. 8)
   A . . . . . . *Lampsilis cardium* Rafinesque, *Lampsilis ovata* (Say)[i]
   0 . . . . *Ligumia recta* (Lamarck)
8. **ITS-1** × AccI   (Fig. 9)
   A . . . . *Ptychobranchus fasciolaris* (Rafinesque)
   0 . . . . *Villosa fabalis* (I. Lea)
9. **ITS-1** × BstEII   (Fig. 10)
   A . . . . *Elliptio dilatata* (Rafinesque)
   0 10
10. **ITS 1–2** × MspI[f]   (Fig. 11)
    A 11
    A' . . . . *Fusconaia subrotunda* (I. Lea)
11. **RAPD A-02**   (Fig. 12)
    A . . . . *Pleurobema clava* (Lamarck)[h]
    B . . . *Pleurobema sintoxia* [= *coccineum*] (Rafinesque)
12. **ITS-1** × BamHI   (Fig. 13)
    A . . . . . *Lasmigona costata* (Rafinesque)
    0 13

13. **ITS-1** × HinfI (Fig. 14)
  A . . . *Anodontoides ferussacianus* (I. Lea)
  B 14
14. **ITS-1** × MboII (Fig. 15)
  A . . . . *Lasmigona compressa* (I. Lea)

0 15
15. **ITS 1–2** × MspI[f] (Fig. 16)
  A . . . . . *Pyganodon* [= *Anodonta*] *grandis*
  (Say)
  A′ . . . . . *Lasmigona complanata* (Barnes)

FIG. 2. ITS-1 PCR products from 25 French Creek unionid species digested with MspI. Restriction fragment patterns (A-H) separate species into their respective tribes. Tribe Am = Amblemini, An = (subfamily) Anodontinae, L = Lampsilini, P = Pleurobemini. Tribe Lampsilini (patterns A, B): 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Lampsilis cardium*, 4 = *Lampsilis fasciola*, 5 = *Lampsilis ovata*, 6 = *Lampsilis siliquoidea*, 7 = *Villosa fabalis*, 8 = *Villosa iris*, 9 = *Actinonaias ligamentina*, 10 = *Ptychobranchus fasciolaris*, 11 = *Ligumia recta*, 12 = *Ligumia nasuta*; tribe Pleurobemini (pattern C): 13 = *Elliptio dilatata*, 14 = *Pleurobema clava*, 15 = *Pleurobema sintoxia*, 16 = *Fusconaia subrotunda*; tribe Amblemini (patterns D, E): 17 = *Amblema plicata*, 18 = *Quadrula cylindrica*; subfamily Anodontinae (patterns F, G, H): 19 = *Anodontoides ferussacianus*, 20 = *Pyganodon grandis*, 21 = *Lasmigona compressa*, 22 = *Lasmigona costata*, 23 = *Lasmigona complanata*, 24 = *Strophitus undulatus*, 25 = *Alasmidonta marginata*. Tribe designations follow Vaught (1989). Gels shown throughout key are 2.0% agarose. Size marker used throughout key is 100-bp ladder.

a. uncut

b. *Sau*96I-cut

FIG. 3. ITS-1 PCR products from "1-A" species digested with Sau96I. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Actinonaias ligamentina*, 4 = *Lampsilis cardium*, 5 = *Lampsilis fasciola*, 6 = *Lampsilis ovata*, 7 = *Lampsilis siliquoidea*, 8 = *Ligumia recta*, 9 = *Villosa iris*, 10 = *Villosa fabalis*, 11 = *Ptychobranchus fasciolaris*.
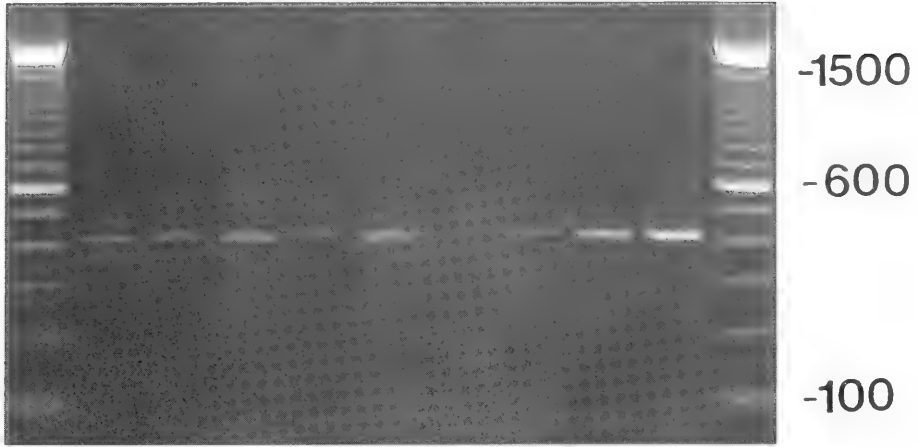
a. uncut   1 2 3 4 5 6 7 8 9
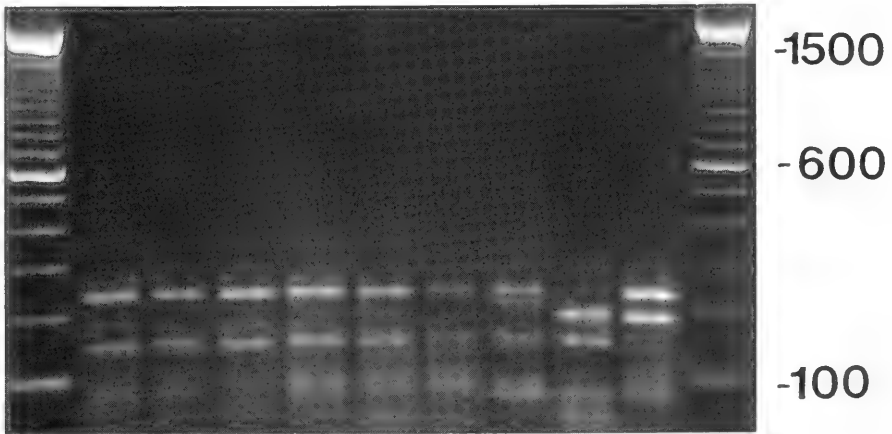
b. *Hae*III-cut   A   B C

FIG. 4. 12S PCR products from "2-A" species digested with HaeIII. 1 = *Lampsilis cardium*, 2 = *Lampsilis fasciola*, 3 = *Lampsilis ovata*, 4 = *Ligumia recta*, 5 = *Epioblasma torulosa rangiana*, 6 = *Epioblasma triquetra*, 7 = *Villosa iris*, 8 = *Actinonaias ligamentina*, 9 = *Lampsilis siliquoidea*.

Notes to Accompany the Key

[a]Includes *Ptychobranchus fasciolaris,* in contradiction to White et al., 1994; the specimen identified in White et al. (1994) as *P. fasciolaris* is almost certainly *Elliptio dilatata.*

[b]The *Pleurobema sintoxia* specimen from Foster Corner exhibited a unique pattern (Fig. 17).

[c]One of the 18 *Amblema plicata* specimens from the lower Muskingum River, Ohio, exhibited a unique pattern quite similar to that of *Ligumia nasuta* (Fig. 18).

[d]In contradiction to White et al., 1994; the specimen identified by White et al. (1994) as *Strophitus undulatus* was subsequently re-identified as *Pyganodon grandis* by A. E. Bogan.

[e]Two *Lampsilis siliquoidea* specimens from the French Creek drainage (one of the three
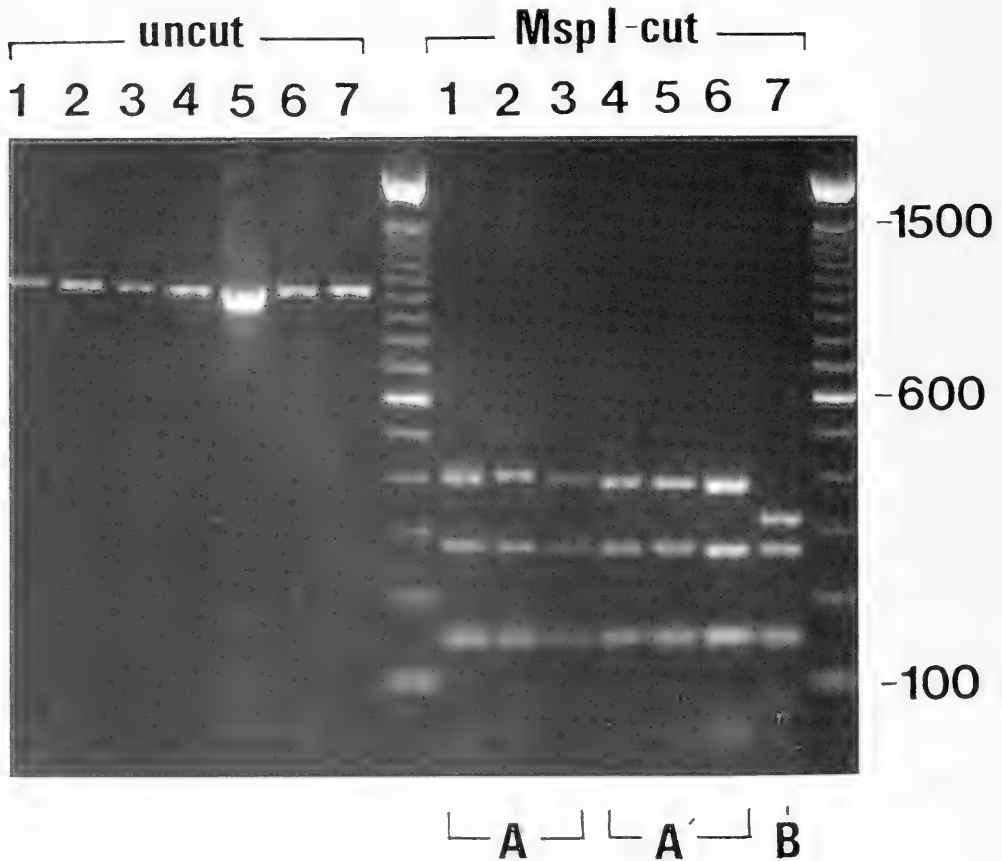
FIG. 5. ITS 1-2 PCR products from "3-A" species digested with MspI. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Villosa iris*, 4 = *Lampsilis cardium*, 5 = *Lampsilis ovata*, 6 = *Ligumia recta*, 7 = *Lampsilis fasciola*.

from Venango and the one from Conneaut Outlet) exhibited patterns with three bands instead of two (Fig. 19).

[f]A and A′ are most reliably distinguished by digesting samples of known DNA and assaying them in lanes adjacent to the unknown DNA. Digesting several samples of each known and unknown DNA is recommended, as it allows one to intersperse samples of each type on a single gel for easier detection of subtle length differences. Assays should be run on at least a 2%-agarose gel, for as long as possible, to achieve maximal separation.

[g]Couplet 6 reliably separates two of the four *Epioblasma torulosa rangiana* specimens examined (one of the two from Venango and the one from Utica) from the three *Epioblasma triquetra* specimens exam-

ined. The broader utility of this couplet is uncertain; it should be used with caution. Also see note f.

[h]federally endangered species

[i]*Lampsilis cardium* and *Lampsilis ovata* specimens could not be distinguished from each other using any of the primers and restriction enzymes tried (White, 1994: appendix B2). It is conceivable that these species hybridize in French Creek; some specimens exhibited intermediate shell morphologies and could not be identified to species with certainty on the basis of external characters (A. E. Bogan, pers. comm.).

Reliability of the Key

The key was tested extensively using adult unionids identified morphologically. In its annotated form, it proved valid for all French
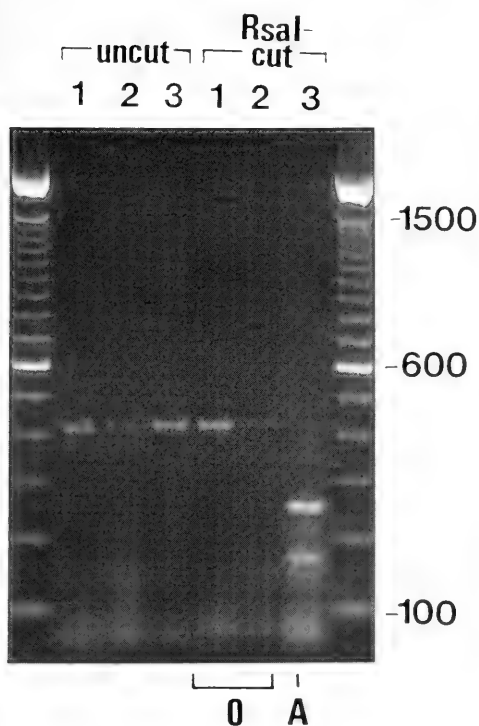
FIG. 6. 12S PCR products from "4-A" species digested with RsaI. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *Villosa iris*.

FIG. 7. ITS 1-2 PCR products from "5-0" species digested with MboII. 1 = *Epioblasma torulosa rangiana*, 2 = *Epioblasma triquetra*, 3 = *E. triquetra*.

Creek specimens examined. It was also valid for all Ohio and West Virginia *A. plicata* (Fig. 20), *L. siliquoidea,* and *L. costata* specimens examined. Moreover, glochidia obtained from a gravid French Creek female *L. costata* followed the key, exhibiting restriction fragment patterns identical to those of adult *L. costata* specimens, as expected (data not shown).

Identification of Unknown Glochidia with the Key

Four unknown glochidia from the gills of a tippecanoe darter (*Etheostoma tippecanoe* Jordan & Evermann) collected 20 July 1993 in French Creek downstream of Utica, Pennsylvania, exhibited restriction fragment patterns identical to those of adult *V. fabalis* specimens (unpubl. data). In a larger-scale test of the technique's utility, all glochidia found on fishes collected 6 June 1994 at the LeBoeuf Creek site were analyzed. Of the 115 glochidia

FIG. 8. ITS-1 PCR products from "4-A'" species digested with AvaI. 1 = *Lampsilis cardium,* 2 = *Lampsilis ovata,* 3 = *Ligumia recta.*
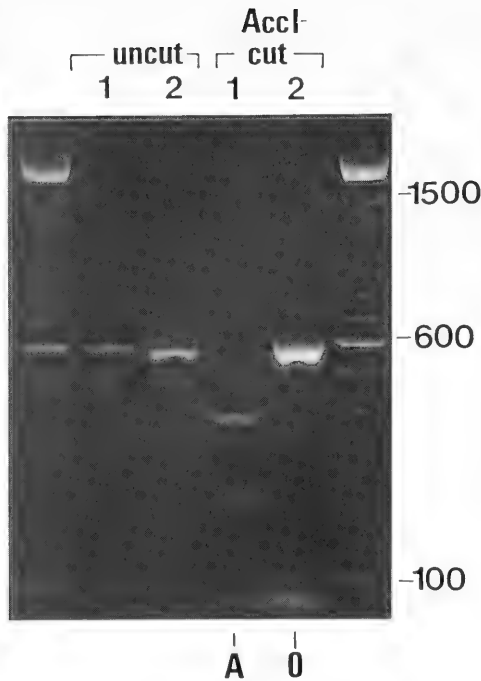
FIG. 9. ITS-1 PCR products from "2-B" species digested with AccI. 1 = *Ptychobranchus fasciolaris*, 2 = *Villosa fabalis*.

FIG. 10. ITS-1PCR products from "1-C" species digested with BstEII. 1 = *Elliptio dilatata*, 2 = *Fusconaia subrotunda*, 3 = *Pleurobema clava*, 4 = *Pleurobema sintoxia*.

DISCUSSION

Distinguishing Unionid DNA from Host Fish DNA

processed, 72 (63%) were amplified successfully (i.e., their ITS-1 PCR products were visible on an agarose gel stained with ethidium bromide). Of these, 66 (92%) were identifiable; the other six yielded restriction fragments too faint to be seen. Fourteen of the 43 glochidia not amplified successfully were in the first set of samples, extracted using a protocol that differed slightly from that ultimately adopted. Disregarding this flawed first attempt, the amplification success rate was 72 out of 102 (71%).

All 66 glochidia identified exhibited the restriction fragment patterns characteristic of *Ptychobranchus fasciolaris* (Fig. 21), a species for which no hosts are currently known (Hoggarth, 1992). Four *Etheostoma blennioides* Rafinesque, three *Etheostoma flabellare* Rafinesque, five *Etheostoma nigrum* Rafinesque, and one *Etheostoma zonale* (Cope) harbored the glochidia. These four darter species are therefore suggested tentatively to be *P. fasciolaris* hosts, pending verification through laboratory inoculation studies.

Without exception, the ITS-1 regions of the fishes examined are markedly different in length from those of the unionids. For single individuals of five of the six darter species examined (*E. blennioides, E. flabellare, E. tippecanoe, Etheostoma variatum* Kirtland, and *E. zonale*), the product is approximately 690–710 bp; for the sixth darter, *Etheostoma maculatum* Kirtland, the product is approximately 410 bp long (White, 1994: Fig. 2.3). Among most of the unionids, the ITS-1 product ranges from approximately 580 to 625 bp; for *Alasmidonta marginata* Say and *Strophitus undulatus* (Say), it is approximately 950–1,050 bp long (see uncut products in Fig. 2). Because the length ranges for fishes and unionids are non-overlapping, any host-fish DNA contaminating glochidial DNA is easily recognized as such. Furthermore, when ITS-1 PCR products of the six darter specimens are digested with MspI, they yield restriction fragment patterns different from all unionid patterns. Hence, even if the glochid-
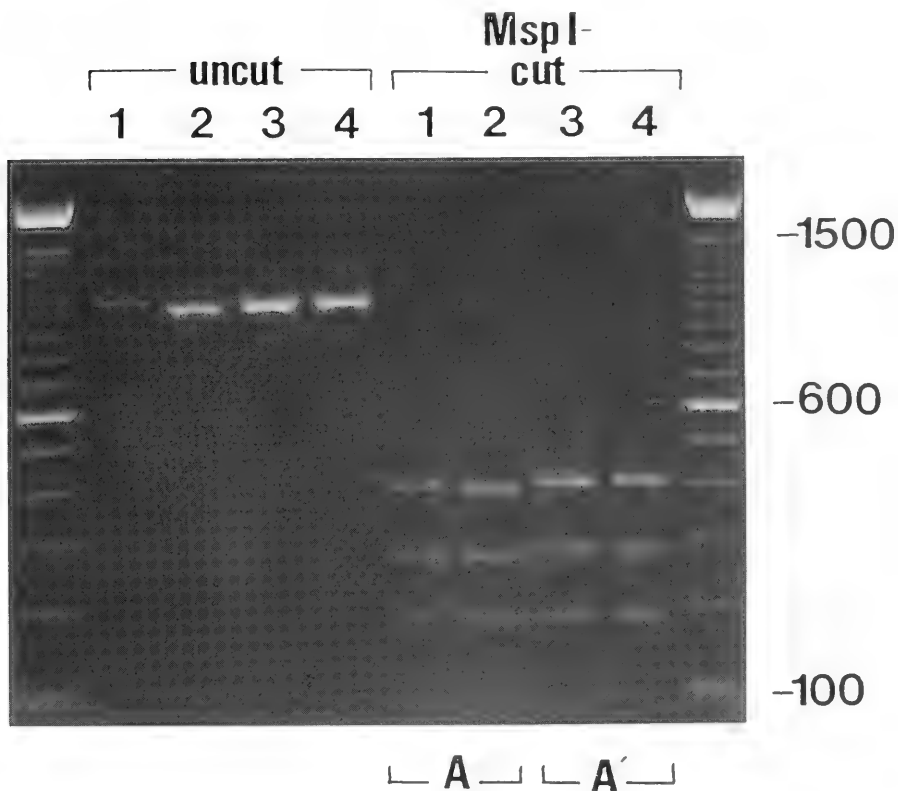
FIG. 11. ITS 1-2 PCR products from "9-0" species digested with Mspl. 1 = *Pleurobema clava*, 2 = *Pleurobema sintoxia*, 3 = *Fusconaia subrotunda*, 4 = *F. subrotunda*.

ial identification method described herein were applied to fishes (e.g., salmonids) whose ITS-1 regions are close to the unionids' in length (Pleyte et al., 1992), contamination could be detected reliably by digesting the host fish's ITS-1 product and assaying it alongside the digested products of the glochidia it harbored. The contaminating DNA could be factored out of the RFLP analyses by disregarding restriction fragments present in both gel lanes.

Current Limitations of the Technique

Identifying glochidia on naturally infected fishes is a hit-or-miss approach to discovering hosts of a particular unionid species of interest. To maximize the chances of succeeding, it is important to collect fishes from sites where the unionid species of interest is abundant relative to other species (or at least where it is abundant relative to other sites). As the preliminary LeBoeuf Creek study
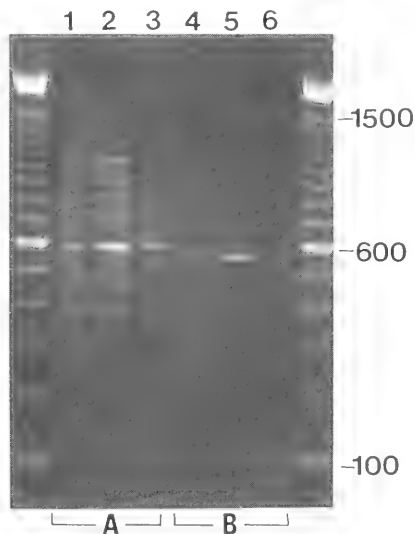
FIG. 12. RAPD A-02 PCR products from "10-A" species. 1–3 = *Pleurobema clava*, 4–6 = *Pleurobema sintoxia*.
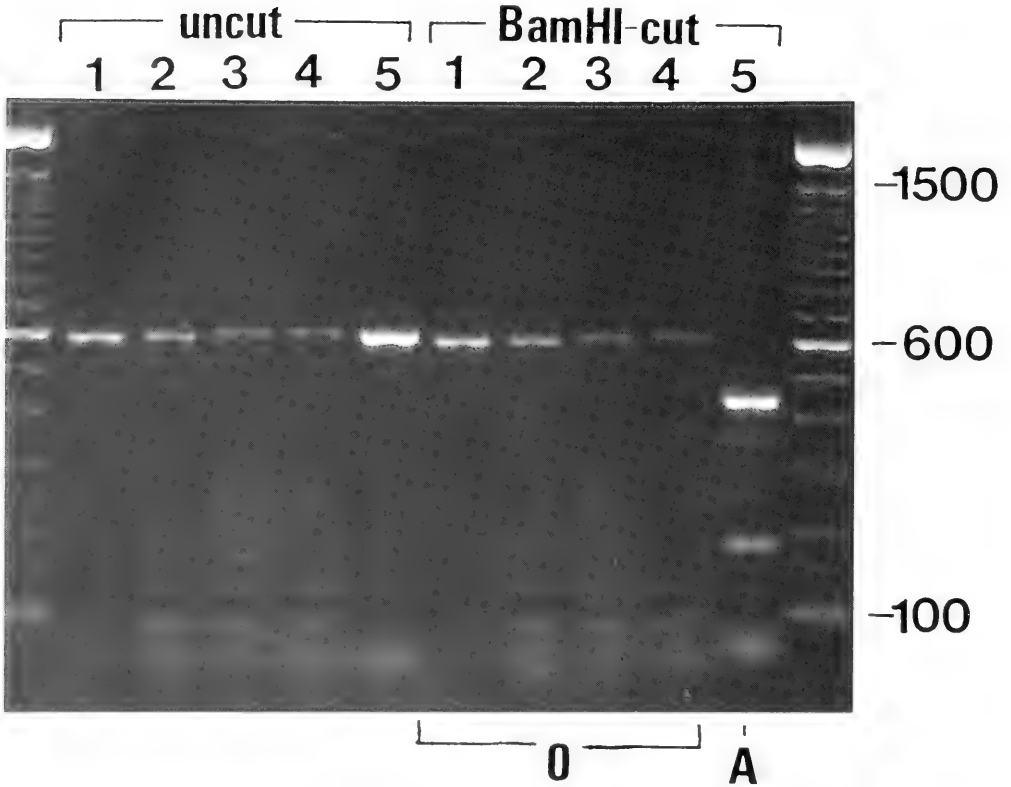
FIG. 13. ITS-1 PCR products from ''1-F'' species digested with BamHI. 1 = *Anodontoides ferussacianus*, 2 = *Pyganodon grandis*, 3 = *Lasmigona complanata*, 4 = *Lasmigona compressa*, 5 = *Lasmigona costata*.
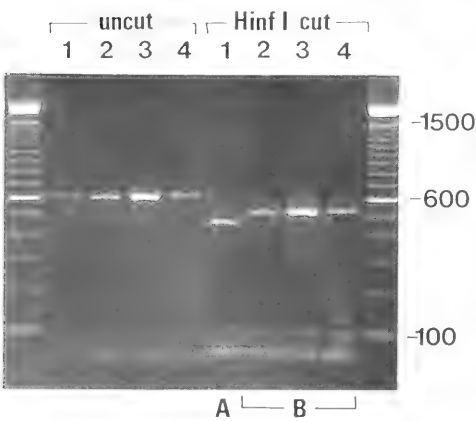
FIG. 14. ITS-1 PCR products from ''12-0'' species digested with Hinfl. 1 = *Anodontoides ferussacianus*, 2 = *Pyganodon grandis*, 3 = *Lasmigona complanata*, 4 = *Lasmigona compressa*.
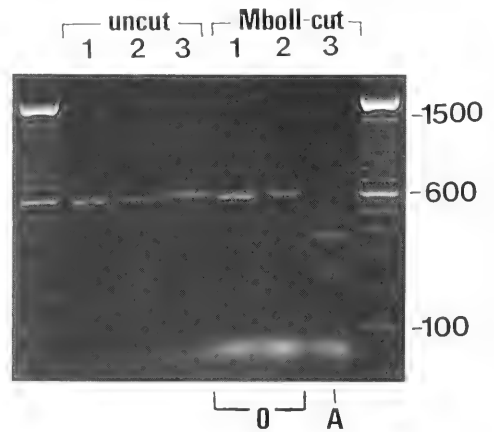
FIG. 15. ITS-1 PCR products from ''13-B'' species digested with MboII. 1 = *Pyganodon grandis*, 2 = *Lasmigona complanata*, 3 = *Lasmigona compressa*.
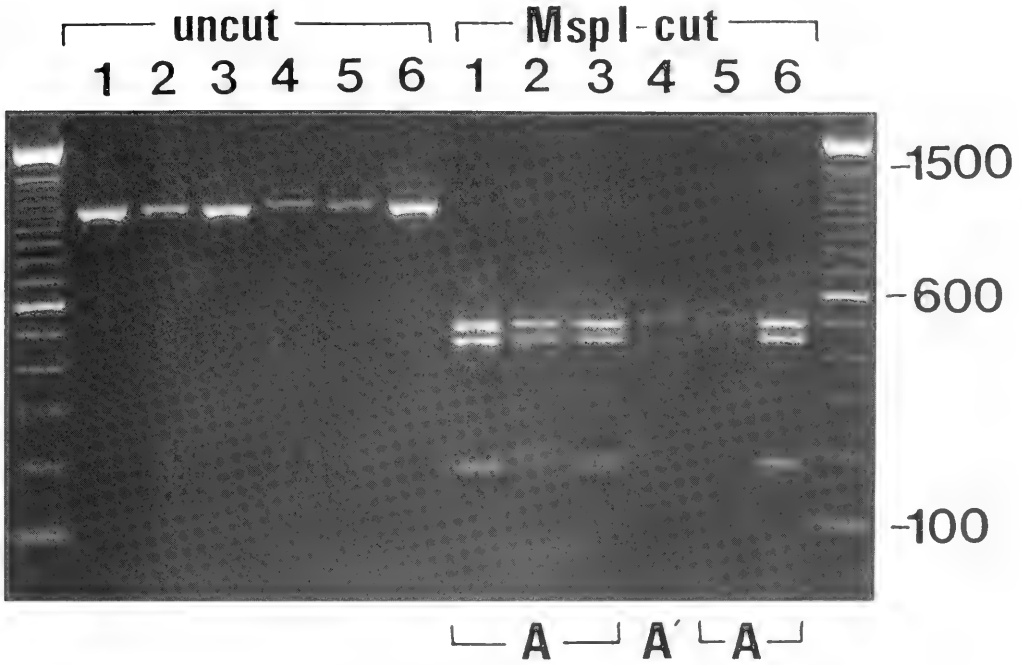
FIG. 16. ITS 1-2 PCR products from "14-0" species digested with Mspl. 1–3, 5, 6 = *Pyganodon grandis;* 4 = *Lasmigona complanata.*
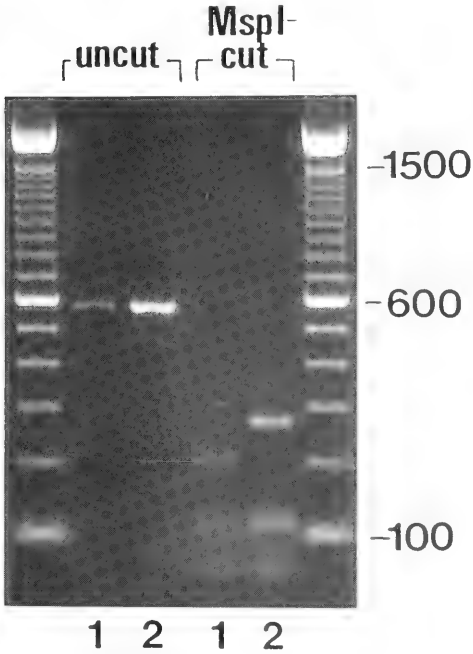
FIG. 17. ITS-1 PCR products from anomalous and standard *Pleurobema sintoxia* specimens digested with Mspl. 1 = anomalous pattern, 2 = standard pattern.
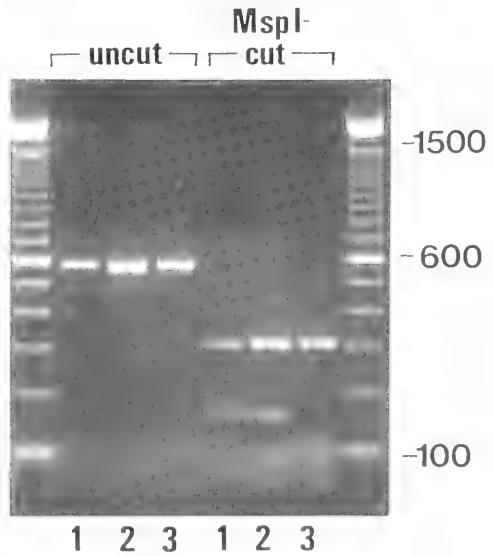
FIG. 18. ITS-1 PCR products from anomalous and standard *Amblema plicata* specimens digested with Mspl. 1 = *Ligumia nasuta,* 2 = anomalous *Amblema plicata,* 3 = standard *A. plicata.*
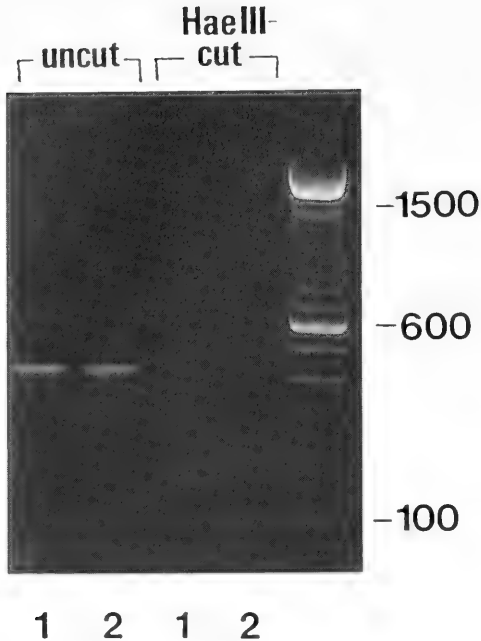
FIG. 19. 12S PCR products from anomalous *Lampsilis siliquoidea* specimens digested with HaeIII. Specimens exhibit 3-banded pattern instead of standard 2-banded pattern.

demonstrated, this will not guarantee success, however. Additionally, fishes should be collected repeatedly throughout the full duration of the unionid's breeding period.

The glochidial amplification procedure currently has a success rate below 100%. Most unsuccessful amplification attempts were likely the result of glochidia being lost during transfer from host to extraction buffer; once excised from the host, glochidia are extremely difficult to see. Improvements in the transfer technique could increase the amplification success rate dramatically. The identification success rate, already quite high, could probably be increased by gel-purifying and reamplifying very faint PCR products prior to restriction enzyme digestion.

Extending the Key Beyond French Creek

To apply the method to an aquatic system other than the French Creek drainage, some preliminary work is required. First, tissue samples must be obtained from several individuals of each unionid species found in the study system. Ideally, each species should be represented by specimens collected at a variety of sites.

Next, the reliability of the key, for the study-system species included in it must be assessed. All specimens of each such species should be analyzed using the key, to see whether they yield the expected restriction fragment patterns for each enzyme (as did the West Virginia and Ohio specimens we examined). If they do not, the key will have to be modified accordingly.

The key will also have to be extended to include any study-system species not found in French Creek. This is most easily accomplished as follows: first, analyze a single specimen of each new species, using the French Creek key. If a specimen yields a novel restriction fragment pattern for a certain couplet, test all individuals of the species to see if they share the pattern; if they do, modify the key accordingly. If a specimen yields no novel patterns, proving indistinguishable from a species already included in the key (or from another new species), screen single individuals of the indistinguishable species pair (or group) with a variety of primers and restriction enzymes until a diagnostic difference is found. (Consulting Appendix B of White, 1994, might prove useful in this regard.) Alternately, sequence a moderately variable region of the genome of each species and scan the sequence data for restriction site differences. Finally, verify that the differences found apply to all individuals of the species, then modify the key accordingly. Publish modified versions of the key promptly to save other investigators precious time and resources.

Overall Assessment of the Technique

Using a molecular genetic key to identify glochidia attached to fishes has distinct advantages over traditional means of identifying putative unionid hosts (White et al., 1994). The laboratory procedures are relatively fast and easy to perform. Once a key has been developed, glochidia can be identified in one or two days; the techniques involved can be learned (if not mastered) in a week. The method is also relatively inexpensive, particularly if one has access to a laboratory already equipped for molecular genetic research (see White, 1994: appendix C, for cost analysis).

The data generated to develop keys are potentially valuable to unionid systematists,

a. uncut

b. *Msp*I-cut
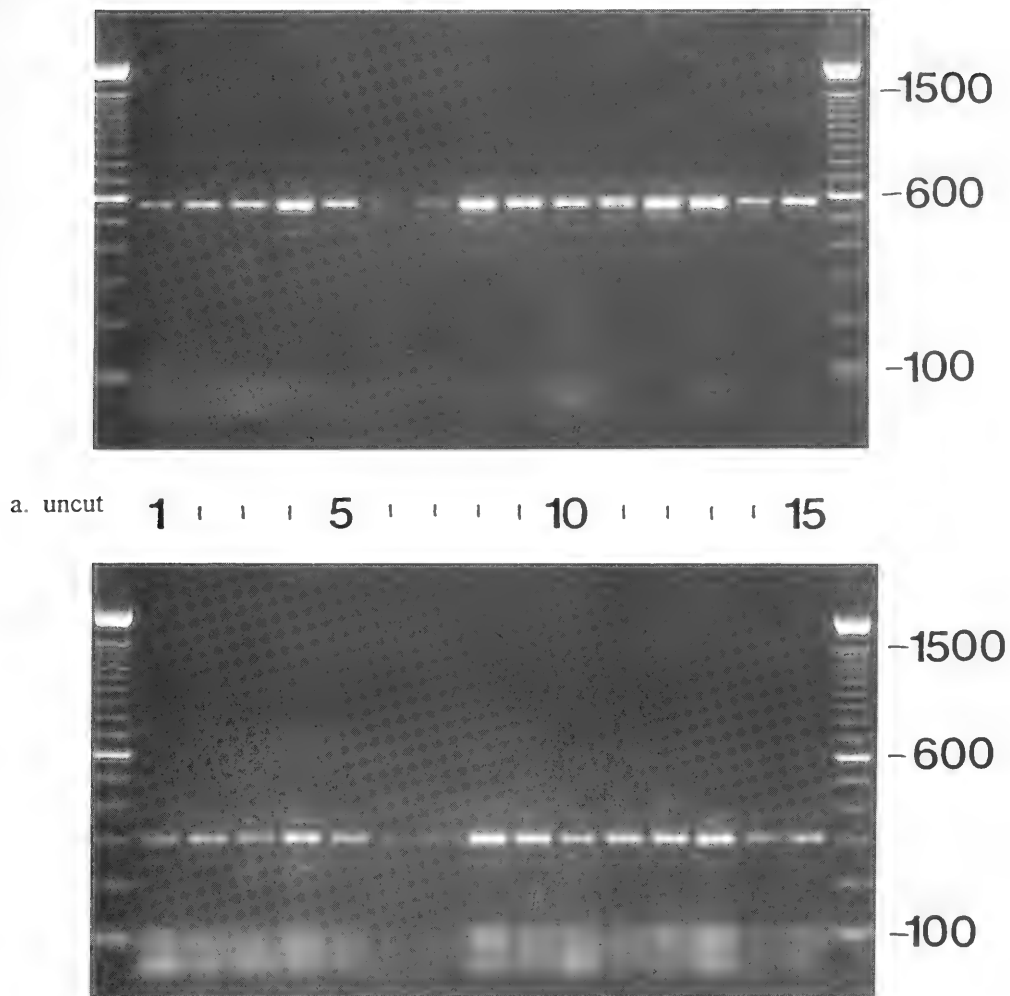
FIG. 20. ITS-1 PCR products from 15 *Amblema plicata* specimens from three drainages, digested with MspI. 1–5 = French Creek specimens, 6–10 = Dunkard Creek specimens, 11–15 = Muskingum River specimens.
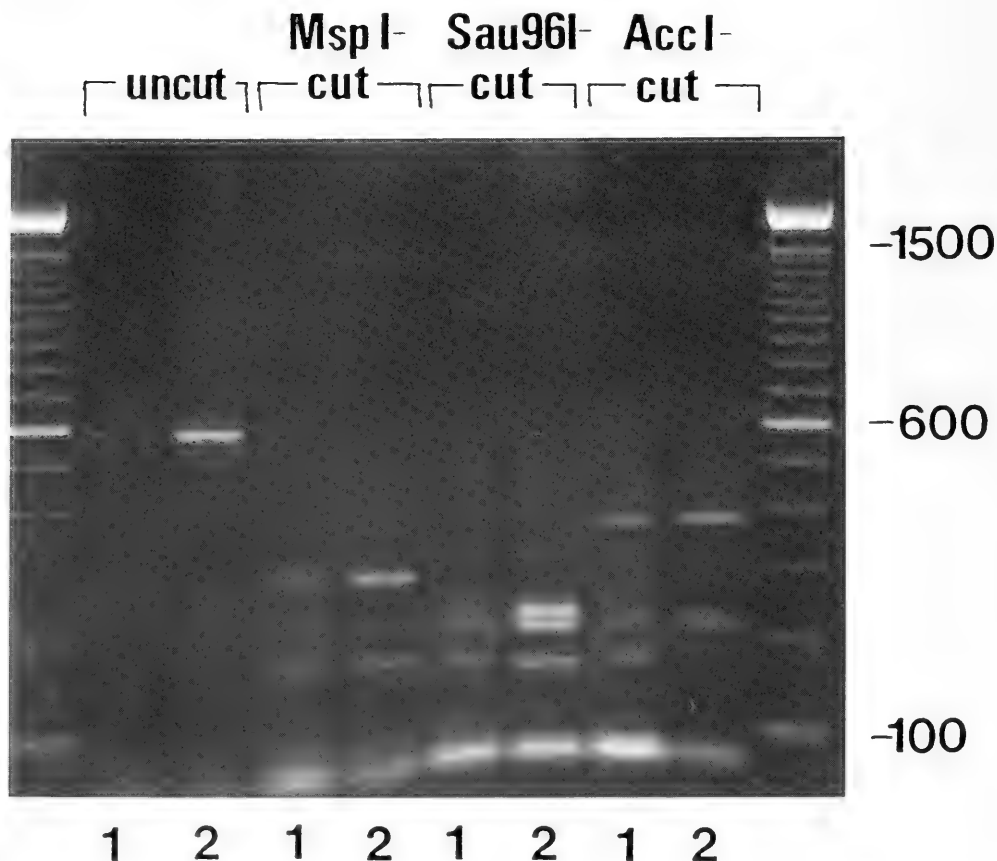
FIG. 21. ITS-1 PCR products from LeBoeuf Creek glochidium and adult *Ptychobranchus fasciolaris,* digested with MspI, Sau96I, and AccI. The glochidium exhibits restriction fragment patterns identical to those of the adult *P. fasciolaris.* The glochidium was removed from the gills of an *Etheostoma flabellare* specimen. 1 = glochidium, 2 = adult *P. fasciolaris.*

as well. For example, the RFLP analysis of ITS-1 shown in Figure 2 suggests that patterns of site gain and loss could demarcate tribal boundaries. In many organisms, this sort of information has been used to reconstruct phylogenetic relationships (reviewed in Avise, 1994). Our study was not designed to provide the complete matrix necessary to analyze this question, but our data (summarized in Appendix 2) do provide a starting point for systematists wishing to pursue the issue of higher relationships. (Note that many of the results presented in Appendix 2 are unreplicated and/or based on small numbers of specimens.)

The method is well suited to conservation work. It does not entail killing adult unionids and hence can be used with endangered species. It yields results that are relevant to natural communities. It can even furnish insights into subtle ecological matters, such as patterns of host-fish partitioning among unionids. Finally, it can be applied to diverse systems with large numbers of fish and unionid species.

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APPENDIX 1. Estimated sizes of restriction fragments used in the key (excluding fragments shorter than 100 bp).

Couplet	Pattern	Fragment Size (bp)	Couplet	Pattern	Fragment Size (bp)
1	A =	(275 − 285) + 185	5	0 =	415
	B =	305 + 185		A =	250 + 180
	C =	275 + 140*	6	A =	575 + 280 + 205
	D =	305 + 140**		A′ =	575 + 290 + 195
	E =	465 + 140	7	0 =	615
	F =	(495 − 505) + 140		A =	510
	G =	960	8	0 =	575
	H =	895		A =	370 + 225
	*anomalous *P. sintoxia* =		9	0 =	565
		205 + 140		A =	340 + 225
	**anomalous *A. plicata* =		10	A =	385 + 270 + 195
		305 + 170		A′ =	400 + 275 + 195
2	A =	350 + 230	11	A =	585
	B =	240 + 225 + 185		B =	555
3	A =	240 + 165	12	0 =	(590 − 620)
	B =	200 + 165		A =	430 + 190
	C =	235 + 195*	13	A =	455
	*anomalous *L. siliquoidea* =			B =	520
	1 =	245 + 205 + 170	14	0 =	(600 − 610)
	2 =	275 + 245 + 200		A =	370 + 240
4	A =	390 + 270 + 170	15	A =	500 + (445 − 460) + 195
	A′ =	375 + 265 + 170			
	B =	310 + 265 + 170		A′ =	515 + 460 + 195

APPENDIX 2. Partial matrix of restriction fragment patterns, by species.

Species	[PCR Product] Restriction Enzyme											
	[ITS-1]								[ITS 1-2]		[12S]	
	AccI	AvaI	BamHI	BstEII	HinfI	MboII	MspI	Sau96I	MboII	MspI	HaeIII	RsaI
Subfamily Anodontinae												
Alasmidonta marginata			O (3)		C (8)	O (3)	H (9)	D (2)		I (1)		
Anodontoides ferussacianus			O (1)		A (3)	O (1)	F (3)	C (3)	G' (1)			
Lasmigona complanata			O (4)		B (1)	A (4)	F (1)	C (1)		G'' (1)		
Lasmigona compressa					B (4)		F (4)	C (4)		G'' (1)		
Lasmigona costata			A (51)		B (1)	B (2)	F (51)	C (7)		G (1)		
Pyganodon grandis		O (1)	O (5)		B (5)	O (5)	F (5)	C (2)		G (5)		
Strophitus undulatus		O (1)	O (1)		D (18)	B (1)	G (22)			H (1)		
Subfamily Ambleminae												
Tribe Amblemini												
Amblema plicata		A (1)	O (1)		O (1)		D (39)/B(1)	A (1)		D' (1)		
Quadrula cylindrica			O (1)		O (1)	O (1)	E (9)	B (9)		F (1)		
Tribe Pleurobemini												
Elliptio dilatata	A (4)	A (1)	O (1)	A (13)	O (1)	O (1)	C (13)	B (13)		E (1)		
Fusconaia subrotunda	A (3)			O (6)		O (1)	C (6)	B (3)		E' (6)		
Pleurobema clava	A (1)	A (1)		O (3)		O (1)	C (3)	B (1)		E (3)	A (1)	
Pleurobema sintoxia	A (4)	A (1)		O (5)		O (2)	C(4)/I(1)	B(4)/E(1)		E (5)	A (1)	
Tribe Lampsilini												
Actinonaias ligamentina	A (2)	A (10)	O (2)	O (1)	O (1)	O (1)	A (18)	A (18)	A (1)	A (1)	B (18)	O (4)
Epioblasma torulosa	A'? (2)	A (2)	O (3)	O (1)	O (1)	O (1)	A (4)	A (4)	A(2)/A'(2)	A (4)	A (4)	O (3)
Epioblasma triquetra	A (2)	A (2)	O (3)	O (2)	O (1)		A (3)	A (3)	A' (3)	A (3)	A (3)	O(5)/A(1)
Lampsilis cardium	A (1)	A (8)	O (2)	O (2)	O (2)	O (4)	A (8)	A (8)	A (2)	A' (6)	A (8)	A (5)
Lampsilis fasciola		A (2)	O (2)	O (1)	O (2)	O (3)	A (5)	A (5)	A (1)	B (2)	A (5)	O(6)/A(1)
Lampsilis ovata	A (1)	A (7)	O (2)	O (1)	O (2)	O (4)	A (7)	A (7)	A (2)	A' (7)	A (7)	
Lampsilis siliquoidea	A (1)	A (2)	O (2)	O (2)	O (2)	O (3)	A (30)	A (30)	A (1)	A' (1)	C(28)/D(2)	
Ligumia nasuta					O (2)	O (4)	B (11)	A (2)		D (1)		
Ligumia recta		O (4)	O (1)		O (2)	O (2)	A (4)	A (4)		A' (4)	A (4)	O(3)/B(1)
Ptychobranchus fasciolaris	A (13)						A (13)	B (13)		C (1)		
Villosa fabilis	O (17)	A (1)	O (1)	O (1)			A (17)	B (17)		A (1)		
Villosa iris	A (2)	A (2)			O (2)	O (4)	A (6)	A (6)	A (1)	A (6)	A (6)	A (6)

A, B, . . . = restriction fragment patterns; O = no restriction sites present; (#) = sample size
Note: Many results presented in Appendix 2 are unreplicated.

QUANTITATIVELY SAMPLING LAND-SNAIL SPECIES RICHNESS IN MADAGASCAN RAINFORESTS

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ABSTRACT

Land-snail species richness in tropical rainforests tends to be high but difficult to assess because of low densities and often small shell sizes. We tested three quantitative sampling methods in primary rainforests of southeastern Madagascar. Timed searching yielded seven times as many micro-snail species (species that during at least part of their life have shells < 5 mm maximum dimension) per person-hour as either litter sampling or soil-plus-litter sampling. The number of species found in 20 m × 20 m during three person-hours of searching, however, was boosted a maximum of 38% by one eight-liter sample each of litter and soil-plus-litter. Litter sampling and timed searching both yielded more than 1.5 times the proportion of live-collected species as soil-plus-litter sampling. Sampling method was unbiased toward 12 of the 20 commonest species, but three large, presumed arboreal species were favored by timed searches; two minute, presumed burrowers by soil-plus-litter sampling; and three minute, cryptically colored species by both litter and soil-plus-litter sampling. A 1.2-mm sieve caught at least 78% of the total specimens and passed adults of 7% of species, of which the smallest adult dimension was 1.0 mm. These results suggest that the best sampling strategy is timed searching for micro-snails, while incidentally collecting macro-snails and litter-plus-soil for later picking of the 5.5–1.2 mm and the 1.2–0.85 mm, dry-sieved fractions. This strategy should be transferable to other tropical-rainforest land-snail faunas.

Key words: Gastropoda, tropical biodiversity, leaf-litter biota, soil biota.

INTRODUCTION

Land snail faunas of tropical rainforests tend to be quite diverse (maximum reported: 52 species per 4 ha) despite often low densities (Emberton, 1995a [and citations therein]; Tattersfield, 1994, in prep.; F. Thompson, pers. commun.; despite Solem's [1984] undocumented statement to the contrary). Much of this diversity consists of micro-gastropods (< 5 mm greatest dimension), the collection of which can be extremely labor-intensive (Emberton, 1994, 1995a, 1996; DeWinter, 1995; F. Thompson, pers. commun.; P. Tattersfield, pers. commun.; R. Ramirez, pers. commun.). Because most tropical rainforests are vastly undercollected for micro-gastropods and are undergoing irreversible deforestation, great urgency attaches to collecting these mostly undiscovered, undescribed molluscs as efficiently and thoroughly as possible. Because of the prime importance in land-snail systematics of preserving anatomies and DNA in ethanol, sampling methods should maximize live collections. Because land snails are generally so patchily distrib-

uted, even within seemingly uniform forest, sampling should probably avoid random-quadrat methods (Emberton, 1995a).

Timed searches by experienced collectors are a well-proven method of quantitatively sampling patchily distributed organisms (Coddington et al., 1991). One of us has recently advocated timed searches as the most efficient collecting method for tropical rainforest micro-snails (Emberton, 1995a), and has applied such data toward assessing conservation priorities (Emberton, 1996). The efficacy of timed searches for collecting all or a substantial portion of the micro-gastropod fauna, however, has never been tested, to our knowledge.

Collection of measured quantities of selected leaf litter is another quantitative sampling method that has proven effective for tropical-rainforest land-snail communities (Tattersfield, 1994). Soil-plus-litter samples also often yield species that are collected in no other way (F. Thompson, 1995). Some species may be soil specialists, other species may take refuge in soil from drying litter, and soil can accumulate dead shells of litter spe-

cialists (pers. observ.; Burch & Pearce, 1990). Processing of soil-plus-litter samples, however, is more labor-intensive than processing of litter samples.

The purpose of this paper is to compare the performances of (a) timed searching, (b) litter sampling, and (c) soil-plus-litter sampling for determining the species richness of and obtaining live material of the micro-land-snail fauna of Madagascan rainforests, and to arrive thereby at the most efficient overall sampling strategy.

METHODS AND MATERIALS

We sampled 48 plots, each 20 m × 20 m, at 16 stations on three widely separated mountains in southeastern Madagascar (Fig. 1, Table 1). Localities and stations were chosen to serve both for this study and for testing diversity patterns between the Vohimena and Anosy mountain chains (Emberton, 1996, Emberton et al., in review). Stations were at 100 m elevation intervals from 100 m to 500 m and at 200 m elevation intervals above 500 m, with a station at the highest or a local summit.

Stations were restricted to primary forest that had no more than limited selective cutting. For each station, we recorded the elevation (average of two Thommen Altitrek altimeters, calibrated from topographic maps), latitude and longitude (from topographic maps), and the topography (summit, ridge, slope, or valley). For more extensive data on these stations, see Emberton (in review).

At each station, we sampled three adjacent 20 m × 20 m replicate plots, each marked off with flagging tape. We sampled 25 January to 7 February 1995, during the rainy season, within one week of heavy rains, when snails and slugs seemed likely to be most active and therefore perhaps easier to find. We included only micro-snails, which for the purposes of this study we defined as those species that during at least part of their life have shells that are smaller than 5 mm maximum dimension (the vast majority remain below this size as adults).

Timed searching was for three person-hours per plot: one-half hour by six collectors. Three of these collectors (RR and two assistants who had been trained by all three authors) were constant over all stations and plots, and the other three were hired locally and trained by RR. As incentives, small cash prizes were offered for the most snails and the smallest snail collected in each plot. Micro-molluscs were hand-collected into 30-ml, snap-cap vials, drowned overnight, then fixed and preserved in 70–90% ethanol.

Litter samples and soil-plus-litter samples were each eight l in volume per plot, collected over a 30-minute period by KCE and TAP, respectively. Both types of sampling were from moist, sheltered microhabitats such as beside logs, between buttress roots of trees, within *Asplenium* and *Pandanus* rosettes, under and near piles of *Ravenala* and palm fronds, and in moist depressions (Emberton & Arijaona, in press: fig. 2). Litter and litter-plus-soil samples were collected into four-mill plastic bags and kept as cool as possible until processing, a maximum of three days later, with daily opening of each bag for aeration.

All litter and soil-plus-litter samples were wet-sieved through three mesh sizes: 11.5 mm, 5.5 mm, and 1.2 mm. We used wet sieving (i.e. washing the samples with water) in order to process quickly samples wet from recent or current rains, and to assure live recovery of slugs, semislugs, and thin-shelled species. Sieve boxes for the first three size fractions consisted of large plastic storage boxes (55 × 48 × 35 cm) from which the bottoms had been cut (leaving a 3.8-cm margin), then covered with hardware cloth (11.5 mm), hardware mesh (5.5 mm), or hardware screen (1.2 mm) (the latter two supported by hardware cloth) held in place with duct tape. The three sieve boxes were nested over an intact box to catch effluent during washing of a litter sample and were transferred to a second box if the first filled. Whenever the litter or soil-plus-litter samples were not too wet, as much dry-sieving as possible was performed prior to wet-sieving. The first two sieve fractions were picked immediately for all invertebrates by the authors, aided by teams of local workers, each of whom was carefully trained and monitored by at least one of the authors. The third fractions (retained by the 1.2-mm sieve) were fixed and stored for no longer than three weeks in an equal or greater volume of 90% ethanol (the resulting ethanol concentration averaged about 60%). The effluent was caught by pouring all sieved wash water from the bottom box or boxes through two nested nylon stockings, from which excess water was squeezed gently, then which were fixed and stored in an equal or greater volume of 90% ethanol.
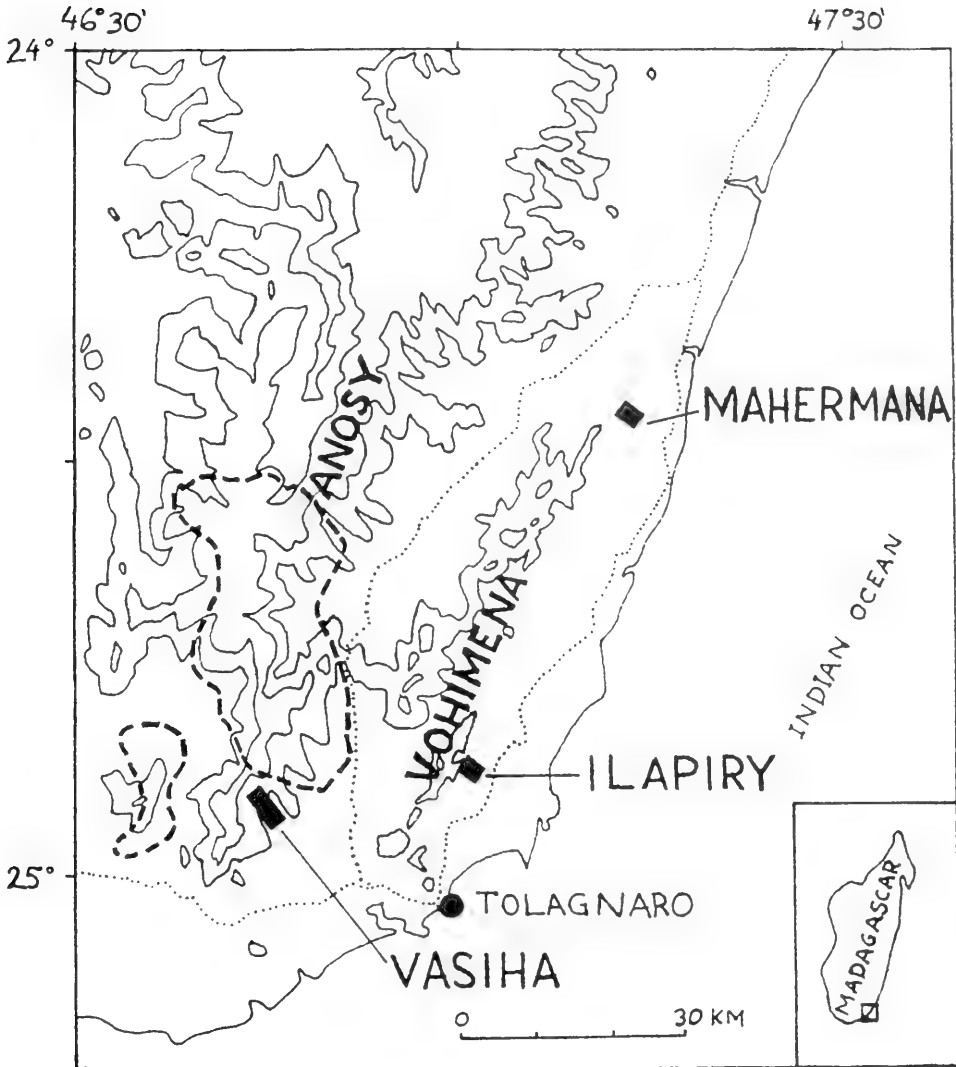
FIG. 1. The three mountains sampled in the Anosy and Vohimena chains, southeastern-most Madagascar (see inset). Contours are shown at 500 m and 1,000 m. The dashed line indicates Andohahela Reserve. The dot indicates the city of Fort Dauphin (= Tolagnaro).

All > 1.2-mm sieve fractions were picked for all invertebrates by RR and six assistants, each of whom was trained by all three authors and monitored by RR. Non-molluscan invertebrates are being distributed among interested specialists. Only molluscs are analysed in this paper.

To test the efficiency of the 1.2-mm sieve at catching snails, the sieving effluent (i.e., all that passed through the 1.2-mm sieve) from one plot per station (the plot whose upper

sieve fractions yielded the greatest number of species) was further sieved through U.S.A. Standard Testing Sieves Nos. 20 and 30 (0.85 mm and 0.60 mm). Both these fine fractions were picked for snails and shells by RR and four trained, monitored assistants, wearing Optivisor magnifying lenses of 2x magnification. Picking of all sieve fractions was performed on a white or light-gray, hard surface. Those snails from the < 1.2 mm fraction were used only for testing the sieve effi-

TABLE 1. Stations sampled for land snails in southeastern Madagascar. Elv = elevation in meters, r/s/v = ridge, slope, and valley.

#	Mountain	Elv	Latit. S	Long. E	Topogr
1	Mahermano	340	24.26.12	47.13.13	summit
2	Mahermano	300	24.26.17	47.13.10	slope
3	Mahermano	200	24.26.15	47.13.04	slope
4	Mahermano	100	24.26.22	47.12.41	valley
5	Ilapiry	540	24.51.40	47.00.20	summit
6	Ilapiry	500	24.51.33	47.00.27	ridge
7	Ilapiry	400	24.51.27	47.00.38	r/s/v
8	Ilapiry	300	24.51.36	47.00.40	slope
9	Ilapiry	200	24.51.39	47.00.46	slope
10	Vasiha	860	24.55.18	46.44.19	summit
11	Vasiha	700	24.55.23	46.44.27	slope
12	Vasiha	500	24.55.19	46.44.45	slope
13	Vasiha	400	24.55.25	46.44.45	valley
14	Vasiha	300	24.55.37	46.44.49	slope
15	Vasiha	200	24.56.13	46.45.13	slope
16	Vasiha	100	24.56.20	46.46.07	slope

ciency, and were not included in the main data matrix or data analysis.

All snails and shells were sorted and identified to morphospecies by KCE. For each morphospecies, a relatively intact adult representative was chosen and was photographed in two to five diagnostic views at standard magnifications, using a Polaroid camera mounted on a Wild dissecting microscope. The resulting reference collection and file of photographs were used to identify all specimens, both adults and juveniles, except for some juveniles of the most minute sieve fractions, which were identified only to genus or family. Systematic treatments of the morphospecies, 85% of which are new, are in progress; vouchers are in the collection of the Molluscan Biodiversity Institute, with types and references to be placed in the Madagascar national museum (Parc Botanique et Zoologique de Tsimbazaza, Antananarivo) and in the Academy of Natural Sciences of Philadelphia. (Patterns of diversity, distribution, and abundance of the morphospecies are treated in a separate paper [Emberton, in review].)

To compare efficiencies of the three methods, we calculated the number of person-hours required to collect and—in the case of litter and litter-plus-soil—to wet-sieve and to pick an average sample (for all invertebrates). We then computed the mean numbers of molluscan specimens and of species obtained per person hour by each method. We were not able to calculate the percent of picking time devoted to molluscs alone, so our person-hour calculations were overestimates.

We used analysis of variance (ANOVA) by least-squares estimation (Wilkinson, 1990) to evaluate differences among the three sampling methods in (a) number of species collected per plot, (b) percent of the total species that were found in each plot, and (c) percent of species collected live. For the percent of the total species collected within each plot, we used the entire data set in a one-way ANOVA. For species number and percent live, however, we factored out the effects of locality (mountain) and elevation by including them in a three-way ANOVA on the largest possible subset of the data including all three mountains (see Emberton et al., in review: fig. 2), which had to be limited to 200 m and 300 m elevations (Table 1).

For each species representing at least one percent of the total specimens, we used chi-square analysis to test among the three sampling methods for equal numbers of specimens. Predicted frequencies were based on the total number of specimens resulting from each method. Probability estimates were Bonferroni-adjusted to allow for multiple tests.

RESULTS

Including the macro-snail species that showed up in the upper sieve fractions, we collected a total of 87 species (also see below). Taxonomically, these species were dis-

TABLE 2. Average time investments and productivities of three sampling methods. Collect = collecting within a 20 m × m plot, Sieve = wet sieving of an eight-liter sample from a 20 m × 20 m plot, Pick = picking all invertebrates (not just gastropods) from the > 1.2-mm sieved sample, Total hours = total person-hours per plot sample, Specm./p-hr = mean number of specimens obtained per person hour, Spp./p-hour = mean number of species obtained per person hour, Spp./specm. = proportion of mean species to mean specimens.

Method	Person-Hours per Task			Total hours	Specm. p-hr	Spp./ p-hr	Spp./ specm.
	Collect	Sieve	Pick				
Timed search	3.0	0.0	0.0	3.0	9.36	3.03	0.32
Litter sample	0.5	4.8	4.7	10.0	0.88	0.46	0.52
Soil-plus-litter	0.5	4.8	9.8	15.1	0.91	0.41	0.45

tributed as follows, with higher classification following Abbott & Boss (1989) for "Prosobranchia" and Gymnomorpha and Nordsieck (1986) for Pulmonata:

"Subclass PROSOBRANCHIA"
  Order MESOGASTROPODA
    Superfamily CYCLOPHOROIDEA
      Cyclophoridae
        Boucardicus 17
        Cyathopoma 1
        Hainesia 1
      Diplommatinidae
        Malarinia 1
    Superfamily LITTORINOIDEA
      Pomatiasidae
        Tropidophora 3
    Superfamily RISSOOIDEA
      Assimineidae
        Omphalotropis 2
  Subclass GYMNOMORPHA
    Order SOLEOLIFERA
      Veronicellidae 1
  Subclass PULMONATA: Order
STYLOMMATOPHORA
    Suborder ORTHURETHRA
      Superfamily CHONDRINOIDEA
      Orculidae
        Fauxulus 2
    Suborder SIGMURETHRA
    Infraorder ACHATINIDA
      Superfamily ACHATINOIDEA
        Subulinidae 3
      Superfamily STREPTAXOIDEA
        Streptaxidae 14
      Superfamily ACAVOIDEA
      Acavidae
        Ampelita 1
        Clavator 1
        Helicophanta 1
      Superfamily PUNCTOIDEA
        Charopidae 9

Infraorder HELICIDA
    Superfamily HELICARIONOIDEA
      Helicarionidae: Sesarinae
        Kaliella 1
      Helicarionidae: Microcystinae
        Microcystis 10
      Helicarionidae: Ariophantinae
        Kalidos 7
        Malagarion 1
      Helicarionidae: Macrochlamydinae
        Sitala 9

We excluded from analysis all specimens of the one slug species (Veronicellidae) and of the six snail species that were considered always too large, even as juveniles, to qualify as micro-molluscs (< 5 mm): the one *Hainesia,* two of the three *Tropidophora,* and all three acavids.

Distributions of the 80 analyzed species among samples, totalling 2,430 specimens, are archived at the Molluscan Biodiversity Institute (MBI) and the Academy of Natural Sciences of Philadelphia (ANSP).

The three sampling methods required drastically different investments of time to acquire gastropods (Table 2). Timed search was by far the most efficient, yielding about ten times the number of specimens and seven times the number of species per person-hour as either litter sampling or soil-plus-litter sampling. These advantages are inflated somewhat, however, because we took time to pick all invertebrates.

The litter and soil-plus-litter methods were more diverse than timed search, yielding about half again as many species per specimen (also see below).

Table 3 gives ANOVA results for number of species collected per 20 m × 20 m plot. Sampling method had a highly significant effect when the less significant effect of elevation

TABLE 3. Analysis of variance in the number of species collected per 20 m × 20 m plot, with least-squares estimates of means. Independent variables are sampling method (timed search vs. litter sample vs. soil-plus-litter sample), elevation (200 m vs. 300 m), and location (one of three mountains).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-Ratio	Probability of Equality
Sampling	172.0	2	86.0	16.83	0.000***
Elevation	29.6	1	29.6	5.80	0.021*
Locality	28.3	2	10.2	1.99	0.152
Sam × Elv	16.1	2	8.1	1.58	0.220
Sam × Loc	38.3	4	9.6	1.88	0.136
Elv × Loc	0.9	2	0.5	0.09	0.914
S × E × L	10.6	4	2.7	0.52	0.722
Error	184.0	36	5.1		

	Number of Species		
	Mean	Std. Error	N
Sampling:			
Timed	9.0	0.5	18
Litter	4.7	0.5	18
Soil-Lit	6.3	0.5	18
Elevation:			
200m	7.4	0.4	27
300m	5.9	0.4	27
Locality:			
Mahermano	5.9	0.5	18
Ilapiry	7.4	0.5	18
Vasiha	6.6	0.5	18

*p < 0.05, *** p < 0.001.

was partitioned out (see Emberton et al., in review, concerning elevational variation). Timed searching within 20 m × 20 m for three person-hours averaged 9.0 species. This was about twice as many species as occurred in an eight-liter sample of litter selected from the same area (4.7 species), and was about half again as many species as occurred in an equivalent soil-plus-litter sample (6.3 species). Thus, this timed searching method produced more species than the other two sampling methods. When considered in the context of time invested, the productivity of timed searching by this method was even more pronounced (see above).

Timed searching alone, however, fell far short of assessing total number of species collected. ANOVA results in Table 4 indicate that timed searching produced on average only 72% of the species sampled within a 20 m × 20 m plot. Thus, the number of species found in a plot during three person-hours of searching was boosted 39% (28%/72%) by one eight-l sample each of litter and soil-plus-litter. Most of these additional species occurred in soil-plus-litter samples, which yielded half of the total, as opposed to the litter samples, which yielded only somewhat over a third of the total sampled species.

On the other hand, Table 5 shows that for sampling live-collected individuals, litter sampling was equivalent to timed searching (51.6 ±5.6 = 46.4 ±5.2) and significantly more efficient than soil-plus-litter sampling. Thus, nearly half of the litter-sample and timed-search species were represented by at least one live-collected individual, whereas only somewhat over a fourth of the soil-plus-litter-sample species were. This result was not surprising because soil can accumulate dead shells of snails living in litter or trees (pers. observ.; Burch & Pearce, 1990). In other words, litter sampling and timed searching both yielded more than 1.5 times the proportion of live-collected species as soil-plus-litter sampling.

Table 6 shows the total live-plus-dead number of each species collected by each of the three sampling methods. Twenty species (25%) were represented by at least 1% (> 24) of the total specimens. Chi-square tests on these species indicated that 12 (60%) of them had equal (not significantly different) representation among sampling methods. Of

TABLE 4. Analysis of variance in the percent of species that were collected within each replicate plot, with least-squares estimates of means. the independent variable is sampling method (timed search vs. litter sample vs. soil-plus-litter sample).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-Ratio	Probability of Equality
Sampling	30,530.9	2	15,265.4	49.1	0.000***
Error	43,868.3	141	311.1		

	Percent of Species		
	Mean	Std. Error	N
Sampling:			
Timed	72.4%	2.5%	48
Litter	37.1%	2.5%	48
Soil-Lit	50.1%	2.5%	48

*** $p \ll 0.001$.

TABLE 5. Analysis of variance in the percent of species represented by at least one live-collected individual, with least-squares estimates of means. Independent variables are sampling method (timed search vs. litter sample vs. soil-plus-litter sample), elevation (200 m vs. 300 m), and location (one of three mountains).

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-Ratio	Probability of Equality
Sampling	5057.0	2	2528.5	5.24	0.010**
Elevation	165.1	1	165.1	0.34	0.562
Locality	922.9	2	461.5	0.96	0.394
Sam × Elv	381.0	2	190.5	0.40	0.677
Sam × Loc	1446.5	4	361.6	0.75	0.565
Elv × Loc	712.9	2	356.4	0.74	0.485
S × E × L	2627.2	4	656.8	1.36	0.268
Error	16405.0	34	482.5		

	Percent Live Species		
	Mean	Std. Error	N
Sampling:			
Timed	46.4%	5.2%	18
Litter	51.6%	5.6%	16
Soil-Lit	28.4%	5.2%	18
Elevation:			
200m	43.9%	4.5%	25
300m	40.3%	4.2%	27
Locality:			
Mahermano	47.8%	5.4%	17
Ilapiry	41.3%	5.2%	17
Vasiha	37.3%	5.4%	17

** $p = 0.01$.

the remaining eight species, *Boucardicus* sp. 9 and *Microcystis* sp. 4 were significantly more prevalent in both litter and soil-plus-litter samples than in timed-search samples, and *Sitala* sp. 7 was present in the litter samples in greater proportions than expected in the chi-square test. All three of these are both dark brown in color (matching the color of litter and soil) and minute in size (adult greatest dimensions 2.2 mm, 2.2 mm, and 1.8 mm, respectively).

Two species—Streptaxidae spp. 9 and 13—were predominant in soil-plus-litter samples and notably scarce in litter only samples. Both these species are high-spired (height/diameters 2.5 and 2.2), very small (adult

TABLE 6. Numbers of snails of each of 80 species collected using three different sampling methods: t = timed search, l = litter sample, s = soil-plus-litter sample. Chi-square tests for equal frequencies among sampling methods were calculated for each species with > 24 specimens: * p < 0.05, Bonferroni adjusted. GnSp = genus or family and numbered morphospecies. Genera and families in taxonomic order are: BO, *Boucardicus;* CY, *Cyathopoma;* MN, *Malarinia;* TR, *Tropidophora;* OM, *Omphalotropis;* FA, *Fauxulus;* SU, Subulinidae; ST, Streptaxidae; CH, Charopidae; KL, *Kaliella;* MI, *Microcystis;* KD, *Kalidos;* MG, *Malagarion;* and SI, *Sitala.*

| GnSp | Number | | | | Chi-Sq | GnSp | Number | | | | Chi-Sq |
	t	l	s	Total			t	l	s	Total	
BO01	131	35	43	209	5.4	CH01	28	4	3	35	9.0
BO02	47	11	15	73	2.5	CH02	75	34	59	168	8.2
BO03	3	0	2	5	—	CH03	6	0	3	9	—
BO04	11	5	8	24	—	CH04	14	1	12	27	5.9
BO05	2	0	1	3	—	CH05	14	2	5	21	—
BO06	6	3	0	9	—	CH06	18	2	8	28	2.1
BO07	32	10	9	51	2.4	CH07	3	0	0	3	—
BO08	2	0	3	5	—	CH08	1	0	0	1	—
BO09	1	39	33	73	102.3*	CH09	4	0	0	4	—
BO10	1	1	1	3	—	KL01	8	6	1	15	—
BO11	7	0	5	12	—	MI01	15	3	4	22	—
BO12	1	0	1	2	—	MI02	7	0	1	8	—
BO13	5	5	6	16	—	MI03	31	9	14	54	0.1
BO14	2	2	2	6	—	MI04	1	9	20	30	34.6*
BO15	0	1	0	1	—	MI05	1	0	2	3	—
BO16	1	1	0	2	—	MI06	2	1	0	3	—
BO17	0	1	0	1	—	MI07	0	1	0	1	—
CY01	13	9	12	34	4.3	MI08	2	4	2	8	—
MN01	0	0	1	1	—	MI09	3	2	0	5	—
TR01	195	20	56	271	33.0*	MI10	2	0	1	3	—
OM01	0	1	2	3	—	MI11	1	0	0	1	—
OM02	1	7	1	9	—	MI12	1	0	0	1	—
FA01	1	2	1	4	—	KD01	105	9	21	135	27.7*
FA02	0	0	1	1	—	KD02	22	2	0	24	—
SU01	104	18	54	176	6.3	KD03	7	1	0	8	—
SU02	3	2	2	7	—	KD04	6	4	0	10	—
SU03	2	5	6	13	—	KD05	1	0	0	1	—
ST01	9	0	1	10	—	KD06	7	1	1	9	—
ST02	6	1	3	10	—	KD07	9	11	1	21	—
ST03	11	2	0	13	—	MG01	9	3	1	13	—
ST04	9	3	8	20	—	SI01	11	5	3	19	—
ST05	2	0	0	2	—	SI02	7	0	3	10	—
ST06	88	25	53	166	2.0	SI03	1	1	4	6	—
ST07	20	2	7	29	2.9	SI04	5	1	3	9	—
ST08	13	1	4	18	—	SI05	49	0	2	51	34.2*
ST09	13	1	25	39	27.8*	SI06	4	0	0	4	—
ST10	2	11	7	20	—	SI07	102	70	78	250	27.8*
ST11	2	2	5	9	—	SI08	1	0	0	1	—
ST12	6	1	3	10	—	SI09	0	0	1	1	—
ST13	10	8	27	45	26.7*	Tot	1348	420	662	2430	
ST14	3	0	1	4	—						

heights 3.9 mm and 3.6 mm), and with glossy, fusiform, small-apertured shells suggestive of a soil-burrowing niche. In contrast, *Tropidophora* sp. 1, *Kalidos* sp. 1, and *Sitala* sp. 5 all occurred predominantly in timed searches and were significantly under-represented in litter and soil-plus-litter samples. All three of these species are relatively large (adult greatest dimensions 13.1 mm, 33.5 mm, and 7.3 mm). *Tropidophora* sp. 1 is often if not exclusively arboreal, and *K.* sp. 1 juveniles are at least partially arboreal, as they frequently show up in vegetation-beating samples (Emberton, unpublished); *S.* sp.

5 has a fragile, light-colored shell that is high-spired for the genus (height/diameter 1.0), all suggestive of arboreality.

A total of 101 specimens passed through the 1.2-mm sieve. (Distributions of these specimens among species and samples are archived at MBI and ANSP.) Thus, the 1.2-mm sieve caught a minimum of 78% of the specimens in the litter and litter-plus-soil samples of each plot.

The 1.2-mm sieve caught representatives of all species in the samples, however, except for one: Streptaxidae sp. 15. This is a minute, high-spired species (adult height 2.4 mm, diameter 1.0 mm), of which only two specimens were obtained. In addition, the sieve passed at least one adult of the five smallest species of *Boucardicus,* some in substantial numbers. Thus, adults of six species (8% of total) passed through the sieve at least in part. The smallest adult dimension of any of these species was 1.0 mm.

DISCUSSION

Sieving of litter and litter-plus-soil may at first seem superior to timed searches for sampling land-snail diversities because it yields higher ratios of species to individuals. Practically, however, timed searches are the most expedient by far, yielding species at 6.6 times the rate per person hour of either sieved sampling method. The degree of this advantage is surely an overestimate, because of our labor-intensive method of wet-sieving then picking for all invertebrates; nevertheless, even if we could halve or quarter our litter-processing time, time searching would be 3.3 or 1.7 times as efficient. Timed searching also requires minimal equipment and minimal weight and volume of samples to transport (critical factors in expeditions that require extensive backpacking).

Nevertheless, our method of timed searching yielded fewer than three-fourths of the total species collected per plot. A more thorough sampling strategy must, therefore, include some litter or soil-plus-litter sampling. Both these methods were roughly equivalent in their species richness and number of specimens per person-hour of effort. There were different advantages to each. Litter samples were 50% faster to process and yielded more live-represented species, whereas soil-plus-litter samples collected burrowing species

that were otherwise missed. Thus a sample of litter-plus-soil seems preferable.

Thus, for greatest efficiency in assessing species number and obtaining live specimens, a good strategy seems to be collecting litter-plus-soil samples during timed searches, taking them from places that are yielding good numbers of micro-molluscs. To be quantifiable, samples should be taken to a constant or measurable volume.

Because only minute, cryptic or burrowing species were missed by timed searching, because the 1.2-mm sieve passed both adults and identifiable juveniles, and because 1.0 was the smallest adult dimension we encountered, we recommend in processing the supplemental litter-plus-soil samples that the 5.5-mm sieve fractions be discarded, and that both the > 1.2-mm and the > 0.85-mm fractions be retained and picked for micro-molluscs. Only a few of the minutest juveniles will be missed, at least for these Madagascar samples. Because wet-sieving is very labor-intensive (Table 2) and logistically difficult, we recommend dry-sieving, either on-site when litter and soil are dry enough, or later when the samples have been stored in, for example, muslin bags long enough to dry sufficiently without dehydrating slugs and semi-slugs.

Macro-molluscs (young juveniles > 5 mm) tend to comprise only a small part of the Madagascan rain-forest land-snail fauna, in this case 7% (6/88) of the species. Also, macro-snails have been the most extensively collected in the past (Emberton, 1995b), so are least likely to yield new biogeographic or systematic information. Therefore, for greatest efficiency in sampling total species richness, we recommend emphasizing the collection of micro-snails, and collecting macro-snails only as they are encountered during micro-snail searches.

Thus, in sum, timed searches for micro-snails, incidentally collecting macro-snails and litter-plus-soil for dry-sieving and picking the > 1.2-mm and > 0.85-mm fractions, seem best for quantitatively sampling Madagascan rainforest land-snails. This strategy should be transferable, with local modifications, to other tropical rainforests.

ACKNOWLEDGMENTS

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SEQUENCING METHODOLOGY AND PHYLOGENETIC ANALYSIS: CYTOCHROME b GENE SEQUENCE REVEALS SIGNIFICANT DIVERSITY IN CHINESE POPULATIONS OF ONCOMELANIA (GASTROPODA: POMATIOPSIDAE)

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ABSTRACT

The evolution of the snail species *Oncomelania hupensis,* a vector for transmission of *Schistosoma japonicum* in Asia, is tightly linked to the evolution of its parasite. We report here on studies of the evolution of *O. hupensis* on the mainland of China, using sequence divergence of the mitochondrial cytochrome *b* gene. The cytochrome *b* gene was amplified by PCR, cloned in pBluescript, and sequenced; these methods are described in detail. The sequences for three populations of two subspecies of this prosobranch gastropod were aligned and used in reconstructing phylogenetic trees. The phylogenetic analyses confirm the divergence of Chinese *Oncomelania* into subspecies and provide a finer tool for further genetic discrimination. Comparison of *Oncomelania* cytochrome *b* sequences to published sequences for two pulmonate gastropods shows a greatly increased divergence rate in the pulmonates relative to that of *Oncomelania* and of other metazoan groups.

Key words: *Oncomelania,* sequencing methodology, phylogenetics, PCR, cloning, cytochrome *b,* infraspecific diversity

INTRODUCTION

The rissoacean genus *Oncomelania* is of particular importance to the field of tropical medicine because one of its two species, the polytypic *Oncomelania hupensis,* is involved in the transmission of *Schistosoma japonicum* in Asia.

Oncomelania minima is restricted to northwestern Honshu, Japan. Polytypic *O. hupensis,* in contrast, is distributed from northern Burma (fossil) throughout southern China, Japan, the Philippines and Sulawesi. The polytypic status of *Oncomelania* has been reviewed (Davis, 1994) with the following subspecies recognized: *O. h. hupensis* (China mainland); *O. h. formosana* and *O. h. chiui* (Taiwan); *O. h. nosophora* (Japan); *O. h. quadrasi* (Philippines); *O. h. lindoensis* (Sulawesi). More recently, Davis et al. (1995) recognized three subspecies on the mainland of China on the basis of allozyme molecular genetics, shell morphology and biogeography: *O. h. hupensis; O. h. robertsoni* and *O. h. tangi.*

A coevolved relationship between snail lineages and the genus *Schistosoma* extends back to the Gondwanaland origin of these taxa. Davis (1992), in reviewing the patterns and processes of this coevolution, made the point that transmission of the parasite now is population-specific in many instances. The hypothesis is that as populations of *Oncomelania* have dispersed and diversified in the direction from Burma-Yunnan, China, throughout China to Japan and the Philippines (Davis, 1979), the parasite has had to modify genetically with the genetically changing snail populations or become regionally extinct. This hypothesis predicts that genetic distances among parasite populations parallel genetic distances among snail populations.

Allozyme electrophoretic data demonstrate strong population divergence among populations of *Oncomelania* throughout China (Davis et al., 1995). The problem with the electrophoretic approach, however, is that as one increases the number of populations compared, errors in assigning the homology of alleles increase. Because one must always run a control population as a standard for determining the identity of alleles at each of 30 or more loci, one needs exponentially increasing numbers of cross-comparisons among populations; the experimental labor and need to keep many fresh frozen populations becomes prohibitive.

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TABLE 1. Localities and collecting information for three populations of *Oncomelania hupensis* in China. Latitudes and longitudes are given. Catalog numbers given are for the Chinese Institute of Parasitic Diseases (CIPD) and the Academy of Natural Sciences of Philadelphia (ANSP).

1. Sichuan (SC):	Sichuan Province; TianQian County; Xing Hua District; Xia Len Village 2nd group. 102°46.0′E; 30°5.02′N; CIPD 0338
2. Yunnan (DA):	Yunnan Province; Dali City; Da Jin Ping Zi Ran Village. Ditch. 100°12.4′E; 25°27.6′N; CIPD 0349
3. JiangXi (JX):	JiangXi Province, Pengze County, WangLing District, JingWang Village. 116°30.0′E; 29°55.0′N; ANSP 399275 collected 7 Dec. 1993 by Zhang Jian Guo and Guo Gang Qiang

Gene sequencing provides an alternative method for reconstructing and evaluating phylogenetic relationships among a group of organisms. Once a gene is sequenced, that sequence is permanently available for comparisons, and does not need to be repeated as taxa are added to the analysis. We have looked for a gene that evolves rapidly enough to distinguish populations of *Oncomelania* to the same as or a greater degree than allozymes do. We have not used RFLD (restriction fragment length differences) methods because they are too crude and because they also rely on cross-comparisons of electrophoretic mobility. We have not used microsatellites because they give too fine a resolution: they provide excellent discrimination at the population level and below, but are too sensitive a tool for inter-population and interspecific comparisons. Sequences from the appropriate gene potentially provide the most powerful set of discrete character data for phylogenetic analysis.

This paper presents detailed methods and preliminary results of amplifying and sequencing the mitochondrial cytochrome *b* gene from *Oncomelania hupensis,* and comparing the results of a three-taxon analysis with results from the allozyme study by Davis et al. (1995). Problems encountered in PCR amplification of cytochrome *b* from *Oncomelania* are stressed. Numerous systematic studies of cytochrome *b* gene sequence document that the rate of evolution of this gene is appropriate to demonstrate differences among species and infraspecific taxa. Sufficient phylogenetically informative characters are present in such comparisons so that phylogenetic analyses are very robust. In contrast to ribosomal RNA genes, there are few or no insertions and deletions among cytochrome *b* genes, and thus no problems with alignment even among taxa as divergent as molluscs, insects, and mammals. Because correct alignment is essential to obtaining the correct phylogeny (Thorne & Kishino, 1992), comparison of genes coding for proteins allows one to reconstruct phylogenies that are more likely to reflect true evolutionary relationships for those genes.

MATERIALS AND METHODS

Specimens Studied

Snails were collected from three localities in China: in Sichuan and Yunnan Provinces in northwestern China, and in JiangXi Province in the east (Table 1). Snails were brought to the United States alive in an estivating state. Once in the laboratory, snails were activated by placing them on moist filter paper in Petri dishes and kept at 4°C. Immediately prior to isolation of DNA, the snails were quick-frozen at −80°C by placing them individually in the wells of a ceramic depression plate previously chilled to −80°C.

DNA Preparation

The methods used for preparing DNA from individual snails were modified from those of Spolsky & Uzzell (1984, 1986) and of Doyle & Doyle (1987). Briefly, a frozen snail (4–9 mm shell length) was crushed, the whole individual immediately dropped into 600 ul of lysis buffer (0.02 M Tris, 0.1 M EDTA, 0.5% Sarkosyl) containing 200 ug/ml proteinase K, and incubated at 55°C overnight. One hundred ul of each of 5 M NaCl and CTAB extraction solution (5% CTAB, 0.5 M NaCl) were added, and the resulting solution extracted with an equal volume of chloroform. 800 ul of CTAB precipitation buffer (1% CTAB, 50 mM Tris pH 8.0, 10 mM EDTA) were added to the aqueous phase, mixed, and placed at room temperature for 30 min. The CTAB-DNA precipitate was pelleted (15 min at 14K rpm), redissolved in NTE (1.2M

NaCl, 10 mM Tris, 1 mM EDTA) containing 100 ug/ml RNase, and again precipitated by addition of two volumes of ethanol. The DNA pellet was washed with 70% ethanol in TE, then redissolved in 100–200 ul of water or 0.1× TE. Aliquots (3 ul) of each DNA were subjected to electrophoresis through a 0.8% agarose gel in TBE and stained with ethidium bromide to obtain a rough estimate of the DNA concentration and quality. The amount of DNA was more precisely quantified using a Hoefer TKO100 fluorometer. Concentration of each DNA preparation was adjusted to 50–100 ng/ul. Using this protocol, we obtained between 5 and 65 ug of high molecular weight DNA per individual snail.

DNA Amplification

PCR was used to amplify the mitochondrial cytochrome *b* gene using the primer pair On5L (forward: 5′-CATTTAGGTCTGCGGTC-CAC) and On6H (reverse: 5′-GGCGTAAC-TAGTGGGTTAGCTGG). These *Oncomelania*-specific primers define a fragment 610 bp in length. Preliminary sequence for *O. hupensis* from Sichuan was obtained using molluscan primers SUP1 and SUP2 (a gift from T. Collins). These primers, although not optimal, provided sufficient sequence to enable design of On5L and On6H. Optimal sequence for the latter primers was determined using PRIMER version 0.5 (Lincoln et al., 1991) and was based on the preliminary *Oncomelania* sequence in combination with comparisons of conserved cytochrome *b* regions for a number of molluscan, echinoderm, and vertebrate taxa. Each PCR reaction contained approximately 50–100 ng of template DNA, 200 µM of each dNTP, 30 pmole of each primer, and two units of Taq polymerase (Promega), in 50 ul of supplier-provided buffer at a magnesium concentration of 2.5 mM. The PCR conditions consisted of 40 cycles of denaturation at 94°C for 45 sec, annealing at 43°C for 1 min, and extension at 72°C for 1 min 20 sec on an M-J Research model PTC-100 thermal controller.

Cloning, Screening and Sequencing

Amplified DNA products were separated on a 1% agarose gel. Bands corresponding to fragments of the correct size were cut out, purified using Geneclean (Bio 101) glass beads, and quantified by fluorometry. A 50-ng aliquot of the purified PCR product was used for ligation into the polycloning region of the plasmid vector pBluescript SK (Stratagene), previously prepared for ligation by linearizing with EcoRV and ddT-tailing using a modification of Holton & Graham's (1991) method. For the latter protocol, the linearized vector was incubated with ddTTP and terminal transferase at 37°C for one hour; 20 ng of this ddT-tailed vector was used per ligation. Each ligation reaction contained, in addition to PCR product and prepared vector, 4% polyethylene glycol 8000 and 0.2–0.5 unit of T4 DNA ligase (Promega) in the appropriate buffer. Ligations were allowed to proceed overnight at 15°C, then drop-dialyzed by placing each reaction on a Millipore type VS25 membrane floating in a Petri dish on 0.1× TE. One third of a ligation was used for transformation via electroporation (BioRad pulser) of the *E. coli* host cell line XL1Blue. Bacterial colonies with recombinant plasmids were identified by plating on selective Luria agar plates containing 100 mg/ml ampicillin, 40 ug/ml X-gal, and 40 ug/ml IPTG. Putative positive colonies were grown overnight at 37°C in 2 ml of LB + ampicillin. Minipreps (Sambrook et al., 1989) of these growths were screened for the presence of inserts of the correct size by cutting the insert out of the recombinant plasmid with HindIII and PstI, followed by electrophoretic analysis. Confirmed positive colonies were grown in larger scale liquid cultures (15 ml), and plasmid isolated from them on Qiagen tip-100 columns following the manufacturer's protocol. Sequences of the cloned fragments were determined by automated cycle sequencing on an ABI 373A sequencer with Stretch upgrade, using commercially available vector primers T3 and T7. Using the automated sequencer, these two primers provide completely overlapping sequence for each strand of the 610 bp cytochrome *b* fragment. To prevent incorrect nucleotide calls caused by occasional random misincorporation of nucleotides during amplification, at least three clones of each ligation were sequenced.

Data Analyses

Sequences for each individual were assembled by visual inspection using the sequence editor ESEE version 1.09e (Cabot & Beckenbach, 1989). ESEE was also used to align *Oncomelania* sequences with each other and with cytochrome *b* sequences

available from Genbank. Aligned sequences were formatted appropriately for phylogenetic analyses using EAT (Cabot, 1993). Pairwise maximum-likelihood distances were calculated using program DNADIST of the phylogenetic analysis package PHYLIP version 3.57 (Felsenstein, 1989, 1993); these distances were estimated under the Felsenstein maximum-likelihood model, which takes into consideration unequal frequencies of nucleotides, unequal rates of transitions and transversions, and multiple substitutions at individual sites. Distance, parsimony, and maximum-likelihood trees were calculated using programs FITCH, DNAPENNY, and DNAML of PHYLIP, programs that do not assume equal rates of change along the branches of a tree. Optimal FITCH and DNAML trees were found by running 20 repetitions of each program with randomized input order and optimization by global branch rearrangement. Bootstrap and delete-half-jackknife estimates (1,000 replicates) of confidence intervals for the maximum-likelihood analyses were made using program SEQBOOT, in conjunction with DNAML and CONSENSE.

RESULTS

Relationships Among the Populations of *Oncomelania hupensis*

Figure 1 presents aligned cytochrome *b* sequences for individual *O. hupensis* from three populations in China (Sichuan, Yunnan, and JiangXi); each sequence represents the consensus from at least three clones for that individual. Sequences were obtained for two individuals from the locality in Sichuan; divergence between the two specimens was less than 0.4% (two site differences). This low intrapopulation divergence is consistent with low intrapopulational variability in morphology as well as in other genetic measures. Given the low intrapopulation relative to interpopulation variability, we concentrated in this preliminary study on obtaining measures of cytochrome *b* divergence among, rather than within, populations. *Oncomelania* sequences were also aligned with published sequences for the gastropods *Albinaria coerulea* and *Cepaea nemoralis*. Nucleotides corresponding to the primer regions have been trimmed from the sequences, resulting in alignment of 572 nucleotides. In this region

of cytochrome *b*, *Cepaea* and *Albinaria* share an extra nucleotide triplet at positions 109–111; in addition, *Cepaea* alone has an insertion of two nucleotides at positions 521–522. For the phylogenetic analyses, all nucleotide positions were included. The three *O. hupensis* sequences have relatively few changes among them; a total of 70 variable sites were detected in this region of the cytochrome *b*. In contrast, *Albinaria* and *Cepaea* each differs from *Oncomelania* at numerous nucleotide positions. Sequence divergence estimates are given in Table 2. Divergences between the closest pair, Sichuan and Yunnan, are 3.8%, whereas between either of these and JiangXi, distances are 10.2 and 11.9% respectively. Distances between *Oncomelania* and *Albinaria* average 53%, between *Oncomelania* and *Cepaea*, 58.8%.

Phylogenetic Analyses

For the phylogenetic analyses, the three *Oncomelania hupensis* populations were compared to *Albinaria coerulea, Cepaea nemoralis,* the sea urchin *Strongylocentrotus purpuratus,* and the mammal *Homo sapiens.* The stylommatophoran pulmonate gastropods *Albinaria* and *Cepaea* are the closest relatives to *Oncomelania* for which published cytochrome *b* sequence is available. *Strongylocentrotus* and *Homo* were included in the analyses to provide rooting for the gastropod clade. The optimal transition/transversion ratio, that is, the ratio which minimizes the likelihood measure, was determined empirically using DNAML (P. Beerli, pers. comm.); for the set of taxa used, the optimal ratio was 1.1. For comparison, phylogenies were also reconstructed using distances and parsimony. The topology of the phylogenetic tree obtained by each of the three methods was the same (Fig. 2). The validity of each tree was tested by both bootstrap and jackknife resampling of the data. For both resampling methods with all tree building strategies, each node of the tree was strongly supported by high bootstrap values (minimum of 95% confidence level).

DISCUSSION

Although cytochrome *b* sequencing has been used in numerous systematic studies in organisms ranging from vertebrates to arthropods to echinoderms, it was not a simple matter to apply those techniques to molluscs. Because of the very ancient branching

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Sichuan    ATCTCTCGTG  ATGTAAACTA  TGGTTGACTT  TTACGGGCAC  TTCATGCAAA  TGGGGCCAGC  TGATTTTTTA
Yunnan     ..........  .......T..  ..........  .....A....  ..........  ......A..T  ..........
JiangXi    ..T.......  .......T..  C.........  .....A....  ..........  ......T...  ..........
ALBINARIA  ..TAT.....  .....CCTGG  A..A...T..  C.T..TTT..  ........T..  .......TCT  CTT.....CT
CEPAEA     ..TATG..C.  .C...CCAGG  C..G..G...  G.T..A...T-  CC........  ...C..ATCG  ATG.......

Sichuan    TCTGCATTTA  TTTTCATATT  GGGCGGGGGA  TGTATTAT--  -GGGTCATTT  ATATACCACC  ACACATGAAA
Yunnan     ..........  ..........  ........T.  ........--  -..A......  ........T.  ..........
JiangXi    ..........  ..........C  ..A..A....  .A........  -.........  ......TA..  ..........
ALBINARIA  .G.TT..G..  .GCC.....C  ..A..T..AC  .A..C...CA  AA...ATA..  ..CC.T.CA.  GTGT.....T
CEPAEA     .GCTT..G..  .GCC......  ..T..T..AG  .A..C...CA  AA.A.ATA..  T.GC.G.CA.  GG......CT

Sichuan    TATTGGCGTA  ATTCTTTTAT  TTATGACTAT  GGGGACAGCT  TTTCTAGGGT  ACGTTTTACC  GTGAGGTTAG
Yunnan     ......T...  ..........  ..........  ..........  ..C.....A.  ....C.....  ..........
JiangXi    ......T...  ....C.....  ....A.....  ...A.....C  ..........  ..........  A........A
ALBINARIA  GG.A..T...  .CAA....T.  .GG.T.GC..  ..CT..G...  ...T......  .T..AC.T..  A..G.....A
CEPAEA     CG.A..G.GT  .ACGA...T.  .AC.A.G...  ..CC..T..A  .....T..?.  .T..C.....  T.....A..A

Sichuan    ATATCTTTCT  GGGGTGCCAC  TGTAATTACT  AATTTATTAT  CGGCTATTCC  GTATGTGGGA  AAAAATATTAG
Yunnan     ..........  ..........  ..........  ..........  ..........  T.........  .........C...
JiangXi    .......T.  .A.....G..  ..........  ......C...  .A........  ......T...  ..........
ALBINARIA  .....A..T.  .A.....T..  .........A  ..C.......  .A..CG.G..  C...T.T..C  CCC.G.A.G.
CEPAEA     .......AT.  .A..C..T..  G..T.....A  ..C..GC.CA  GT...G....  A..CT.A..T  G.T.GGC.C.

Sichuan    TTGAATGGGT  TTGAGGTGGT  TTTGCTGTTG  ATAATGCAAC  ATTAACTCGA  TTTTTTACTC  TTCATTTTGT
Yunnan     ......A...  ..........  ..........  ..........  ..........  ......R.C.  ..........
JiangXi    ..........  .....G..A  ..........  ..........  ..........  ..C.......  .C........
ALBINARIA  .......A..  ...G..A..G  ..CT......  GGC....T..  CC.G.A...T  .....CT.A.  .A......T.
CEPAEA     .GAC...A..  G..G...CT.  ...T....AA  ..C.A..C..  .....A....  ....A.T..T  ........T.

Sichuan    ACTACCTTTC  GGCATTGCAG  CATTGACTGT  ATTACATTTG  CTATTCCTAC  ACGAGTCCGG  CTCAAATAAC
Yunnan     ..........  ..........  ....A.....  ..........A  ........W.  .T........  ..........
JiangXi    GT........  ..........  ....A.....  ......C.A  T.........  .T..A..T..  A.........
ALBINARIA  .T....C..T  CTT...AGG.  GG..AG.AC.  ..C.T...A.T  A.T..T..T.  .T..TAAG..  G...TC....
CEPAEA     G..C..C...  .TA...TT..  T...TGTGT.  .G.C..C...  ..T.A.....  .T..CAAA..  T..GTC....

Sichuan    CCATTAGGAT  TAAATAGAGA  CGGAGAAAAG  GTACCATTTC  ATTCTTATTA  CAGCTTTAAA  GATTTAGTTG
Yunnan     ...C.G..G.  ...T......  ..........  ..G.......  ..........  ..........  ..........
JiangXi    ..........  ....C.....  T........A  ..........  ..........  T........G  ........G.
ALBINARIA  ........TA  ATTTATTTC.  .TT.AGG..A  AA........  .?C.A....T  ..CAA.....  ...AGG....
CEPAEA     ..G..G...A  AT.TGTCCC.  T.TGTC...A  ..CAG.....  .CC......T  ..CA.GG..G  AT...GTGG.

Sichuan    GATTTATTAT  TTTACTTTTT  ATACCTTCAT  --TACTAGATA  CTATTTGCAG  CCCAAATACT  AACAGACCCA
Yunnan     ..........  ..........  ..........  --........  ..........  ..........  ..........
JiangXi    ..........  ..........  ..G..C..T.  .....G...  T.........  .T.....G..  G........G
ALBINARIA  .....T.A..  AG..T..GG.  G.T..G.T.A  ---..A.CAC.  T.T..AGC.  .TTCTT....  .CT...T..C
CEPAEA     TG...G.AT.  GC..TG....  T......ATG  TT.TG.T..G  ....A.CAC.  .TA.T...T.  T..G.....C

Sichuan    GAAAATTTCA  TT
Yunnan     ..........  ..
JiangXi    .......T..  ..
ALBINARIA  .......A..  ..
CEPAEA     .....C..TT  A.
```

FIG. 1. Alignment of sequences of a 572 nucleotide fragment of cytochrome *b* from three populations of *Oncomelania hupensis* in China and from two pulmonate gastropods. The top line contains sequence for cytochrome *b* of *Oncomelania hupensis* from Sichuan Province, China. For the remaining sequences (*O. hupensis* from Yunnan and JiangXi Provinces, *Albinaria coerulea, Cepaea nemoralis*), nucleotides were given only for sites that differed from the Sichuan sequence. A dot (.) indicates the presence of the same nucleotide as in the top sequence; a dash (-) indicates the absence of a nucleotide at that position. Sequences have been deposited with Genbank.

of molluscs from the basal phylogeny, their cytochrome *b* sequences are divergent enough so that the so-called "universal primers" for cytochrome *b* (Kocher et al., 1989) do not amplify this gene from molluscs. In fact, there is enough divergence within the molluscs to make designing a universal primer for Mollusca difficult. The PCR primers we designed, On5L and On6H, work for most, but not all, populations of Chinese *Oncomelania,* and variably well for the related *Tricula.* Because of the variable yield and purity of the product from different taxa, we cloned all amplified products prior to sequencing. Although more labor-intensive, this procedure provided such excellent quality sequence that in the long run time was saved by not having to do multiple repetitions
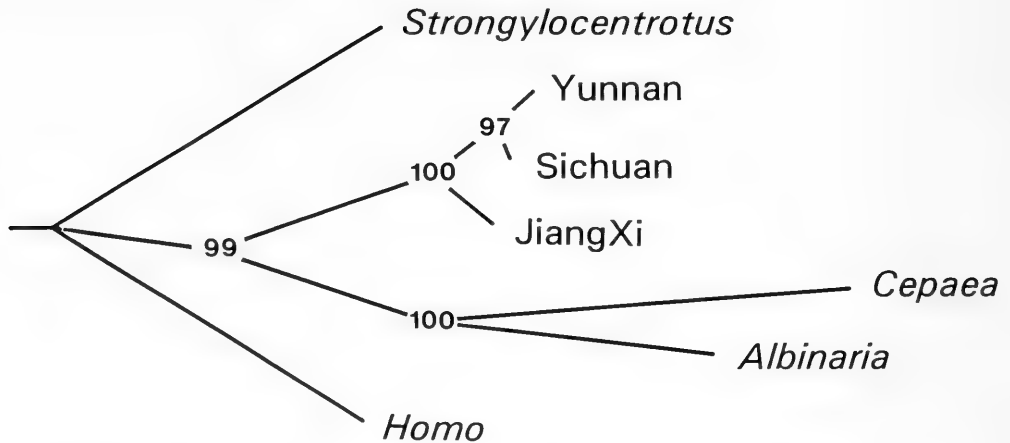
FIG. 2. A maximum-likelihood tree of relationships among three populations of the mesogastropod proso-branch *Oncomelania hupensis* and the stylommatophoran pulmonates *Albinaria coerulea* and *Cepaea nemoralis*. Both *Homo sapiens* and *Strongylocentrotus purpuratus* were used as outgroups for the analysis in order to provide rooting for the gastropod node and allow for calculation of a bootstrapping value for the node. Bootstrap values are listed to the left of each node; these indicate the number of times that node occurred among 1,000 bootstrap replicates.

and by the clarity and thus accuracy of the sequence obtained.

The three populations of *Oncomelania hupensis* form, as expected from divergence levels, a very closely related monophyletic group. Previous conchological, biogeographic, and electrophoretic analyses (Davis et al., 1995) support division of *O. hupensis* into three subspecies: the Sichuan and Yunnan populations belong to the subspecies *O. h. robertsoni,* whereas the JiangXi population belongs to the subspecies *O. h. hupensis.* The phylogenetic analyses of nucleotide sequence of cytochrome *b* presented here are consistent with this subspecies concept: the Sichuan and Yunnan populations are the most closely related (Fig. 2, Table 2). These populations are geographically close, and have smooth shells with no varix. Distances of either of these populations to the JiangXi population, ribbed and with a strong varix, are almost threefold more.

The two pulmonate gastropods, *Albinaria* and *Cepaea,* form a separate group that diverged over 300 million years ago from the prosobranch *Oncomelania.* Both gastropod groups, *Oncomelania* and pulmonates, share a common node relative to non-molluscan taxa. One striking feature of the phylogenetic tree is the very long branch lengths for the pulmonates, particularly for *Cepaea* (Fig. 2), suggesting many more changes along the branches leading to *Albinaria* and *Cepaea*

than along any other branches. This is also evident in pairwise distance comparisons (Table 2). In comparisons using either *Cepaea* or *Albinaria,* results are what one would expect: distances to *Oncomelania* are less than to *Homo* or *Strongylocentrotus;* on the other hand, using *Oncomelania,* pairwise distances to *Homo* and to *Strongylocentrotus* are less than to the more closely related gastropod taxon *Cepaea.* This is not a phenomenon restricted to the cytochrome *b* gene: phylogenetic analyses of cytochrome oxidase (Hoeh et al., 1996) have also demonstrated a longer branch length for *Albinaria.* In agreement with the large divergences we find for the pulmonates, the mitochondrial gene order for these two taxa also appears to have changed extensively, both from that of other molluscs as well as from other metazoan groups (Lecanidou et al., 1994; Hatzoglou et al., 1995). An increased rate of evolution of the mitochondrial genome and of its gene order has also been observed in the bivalve mollusc *Mytlius edulis* (Hoffmann et al., 1992; Hoeh et al., 1996), but not in the polyplacophoran mollusc *Katharina tunicata* (Boore & Brown, 1994).

The increased cytochrome *b* divergence in pulmonates is puzzling. Some of the extra length of the pulmonate branches may possibly be a result of frameshift-causing misreadings of the nucleotide sequence. For example, if we translate the nucleotide se-

TABLE 2. Pairwise comparisons of sequence divergence over 572 nucleotide positions in the cytochrome *b* gene. Sequence divergences were estimated using the program DNADIST of PHYLIP version 3.57, under Felsenstein's (1989) maximum likelihood method.

	S	Y	JX	CEP	ALB	HOMO	STR
Sichuan	—						
Yunnan	0.0382	—					
JiangXi	0.1021	0.1186	—				
CEPAEA	0.5969	0.5853	0.5899	—			
ALBINARIA	0.5364	0.5427	0.5256	0.5148	—		
HOMO	0.5158	0.5032	0.5067	0.7180	0.6190	—	
STRONGYLO	0.5409	0.5304	0.5324	0.7225	0.6177	0.4663	—

quence of *Cepaea* downstream from the point of the two-nucleotide insertion (Fig. 1), and compare it to the Sichuan *Oncomelania* sequence, we find virtually no homology among the 14 amino acids coded (Fig. 3); however, if we delete these two nucleotides from the *Cepaea* sequence, homology of the translated sequences becomes significantly higher. Possible sequence misreadings resulting in sequential insertions and deletions relative to other sequences may be difficult to detect if the sequence eventually goes back into alignment. If such shifts are not allowed for, however, this would result in significantly longer pairwise distances between taxa. Alternatively, if the apparent frameshift mutations reflect the real sequence rather than misreadings of the sequence, then the mitochondrial cytochrome *b* sequences in these two cases appear to behave as if they were nuclear pseudogenes (cf. Collura & Stewart, 1995). Because these sequences were obtained from cloned mitochondrial genomes, it is unlikely that these do represent nuclear pseudogenes. In this case, the apparent increased rate of evolution of the mitochondrial cytochrome *b* in the pulmonates suggests a decrease in functional constraints for this protein.

Some workers have avoided the apparent high labor costs of gene sequencing by using RFLD[1] analyses instead. This technique, however, has many of the same problems as

does genetic analysis of allozyme mobility differences, and it often provides even less information than allozyme analysis does. Two additional serious weaknesses of RFLD analysis, not present in allozyme studies, are: (1) the inability to identify genetic loci and therefore to know what is allelic to what; because of this, one does not know which characters are independent, a requirement for phylogenetic analysis. One therefore can at best get only a very crude measure of similarity; (2) the paucity of characters on which to base a phylogenetic analysis. A valid analysis requires a larger number of variable characters than the number of taxa; this is not the case for many RFLD studies. These weaknesses probably account for the discrepancy between the conclusions of Hope & McManus (1995) based on RFLD analyses and our conclusions based on phylogenetic analyses of cytochrome *b* gene sequence: among populations from a number of *Oncomelania* subspecies from China, the Philippines, and Japan, the largest difference Hope & McManus (1995) found is between the Sichuan and Yunnan populations of Chinese *Oncomelania.* Not only is this at variance with our electrophoretic and sequencing results, but it is also in conflict with conchological and biogeographic data. Our conclusions, on the other hand, are strongly supported: there is strong correspondance between our sequence data and biogeographic, conchological, and extensive allozyme data.

Ribosomal RNA sequencing has been used extensively in determining phylogenetic status among molluscs. In *Oncomelania,* sequences from the D6 domain and the 5′ terminus of 23S-like rRNA were useful in determining the phylogenetic relationship of the genus to a number of molluscan taxa, but were not helpful in resolving finer differences at the species level (Emberton et al., 1990; Rosenberg et al., 1994). The present report

[1]Although the abbreviation RFLP is often used to refer to restriction fragment analysis of sequence variation, we consider this inappropriate usage of the term polymorphism as applied to genetic analysis. Genetic polymorphism has a very specific meaning (cf. Ford, 1965) involving discontinuous variation, that is, distinct alleles, of specific genes (genetic loci). In the restriction fragment analysis method commonly termed RFLP, fragment mobility differences cannot be assigned to specific loci. We therefore intentionally use the term RFLD (restriction fragment length difference) for this method.

Sichuan	LVLFAPQMLTDPENFI
CEPAEA	LLCYITL.YLRTPKTF
CEPAEA-2	V..YH.N.F......Y

FIG. 3. Alignment of translated sequences for a portion of the cytochrome b gene 3′ from position 523 of Fig. 1. The invertebrate genetic code was used for translation. Amino acids were given only for sites that differed from the Sichuan amino acid sequence; a dot indicates the presence of the same amino acid as in the Sichuan sequence. *Cepaea* = amino acid sequence translated from nucleotide sequence stored in Genbank, accession U23045; *Cepaea*-2 = two "extra" nucleotides, at positions 521 and 522 of the amplified cytochrome b sequence, were removed prior to translation.

confirms the utility of nucleotide sequencing of cytochrome *b;* this gene provides the resolution necessary to determine intraspecific relationships among populations of *Oncomelania,* and to cluster the populations according to subspecies status. Work is in progress, using cytochrome *b* gene sequencing, on the degree and patterns of genetic divergence among *Oncomelania hupensis,* other species of *Oncomelania,* and *Tricula.* This work will serve to establish patterns of divergence, to substantiate the subspecies concepts for *Oncomelania* in China, to place the differentiation of *Oncomelania* in a phylogeographic context, and to corroborate the existence of two diverging subfamilies in the pomatiopsid assemblage, that is, the Pomatiopsinae (*Oncomelania*) and Triculinae (*Tricula*).

ACKNOWLEDGEMENTS

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LETTERS TO THE EDITOR

CRITERIA FOR THE DETERMINATION OF TAXONOMIC BOUNDARIES IN FRESHWATER UNIONOIDS (BIVALVIA: UNIONOIDA): COMMENTS ON STIVEN AND ALDERMAN (1992)

Walter R. Hoeh[1] & Mark E. Gordon[2]

The southern Atlantic Slope region of the United States is an area characterized by high species richness and considerable local endemism in the freshwater fauna (e.g., freshwater mussels [Johnson, 1970; Burch, 1975; Davis et al., 1981; Kat, 1983; Hoeh, 1990], snails [Burch & Tottenham, 1980; Thompson, 1968, 1984], crayfish [Hobbs, 1989], and fish [Lee et al., 1980]). Kat (1983) demonstrated species-level divergence within the regional *Lampsilis radiata* (Gmelin 1791) complex (i.e., *L. radiata s.s., L.* sp. [now *L. fullerkati* Johnson 1984], and *L. splendida* [Lea, 1838]). Using allozymic and morphological comparisons, Stiven & Alderman (1992; hereafter referred to as SA) examined nine populations of unionoids from North Carolina in order to, among other objectives, reassess and resolve the taxonomic status of three nominal taxa of *Lampsilis: L. radiata radiata, L. radiata conspicua* (Lea 1872), and *L. fullerkati* (taxa as listed by Turgeon et al., 1988). SA concluded, based primarily on genetic distance criteria, that the former two taxa, and probably *L. fullerkati* as well, should "be considered simply as allopatric 'populations' of *Lampsilis radiata.*"

A critical reading of SA reveals inconsistencies and errors in methodology and data interpretation. We believe that the following detailed discussion of SA is necessary for two reasons: (1) The taxonomic revisions suggested by SA, if implemented, would likely have a significant impact on the level of legislative protection afforded at least two of the taxa in question, and (2) the high visibility of this paper among malacologists and environmental resource professionals may lead to its use as an exemplar for studies of pop-ulation structure, taxonomic boundaries, and phylogenetic relationships in unionoid bivalves. Thus, because of the potential impact of this paper, aspects of the research presented therein should be carefully re-evaluated.

Morphological Analyses

The data presented in SA (table 2) suggest that there are statistically significant differences in length, height, and width among *Lampsilis r. conspicua, L. r. radiata,* and *L. fullerkati* shells. However, similarity in slope and y-axis intercept for plots of length versus height for *L. r. conspicua* and *L. r. radiata* (specimens of *L. fullerkati* not plotted) was used as an indicator of conspecificity in SA (fig. 2). Furthermore, the statistically significant mensurable differences among the above three *Lampsilis* taxa were downplayed by reference to "site effects" in *Lampsilis cariosa* and *Leptodea ochracea* (SA: 366).

Appeals to site effects (phenotypic plasticity) as explanations for the observed conchological differences among unionoid populations, without the appropriate substantiating data, are simply hypotheses to be tested. Contrary to statements in the text, the Deep River *Lampsilis cariosa* population is not allozymically identical to the other two populations of *L. cariosa* (SA: table 3). Therefore, the data presented in SA cannot discount genetic effects for the size differences among populations of *L. cariosa*. Furthermore, because nine of the alleles detected in *Leptodea ochracea* were found only in one of the two populations analyzed (again contrary to the text; see SA: table 3), genetic effects can-

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not be ruled out in this instance either. Even if two populations of a single species are allozymically identical, distinct genetic determinants for conchological morphology may be present.

We wonder if the "site effect analyses" presented in SA controlled for age and/or gender (sexual dimorphism). These potentially confounding factors were not discussed in SA (no explanation of the methodology utilized in the conchological analyses was presented in the "Methods" section). For example, assuming no genetic component for the observed size variation, were the Deep River individuals of *Lampsilis cariosa* larger because of site effects or because they were older? The Deep River ". . . population is comprised of only large old specimens and is thought to be declining" (SA: p. 356). We believe this statement to be suggestive of an age effect that could have confounded the conchological analyses. In summary, the significant mensurable differences between *L. r. conspicua, L. r. radiata,* and *L. fullerkati,* combined with no substantiation for site effects, are consistent with the hypothesis that these three taxa are distinct evolutionary lineages. The similarity in slope and y-axis intercept for plots of length versus height for *L. r. conspicua* and *L. r. radiata* is irrelevant given the significant morphological differences between these taxa.

Sampling Strategy for the Allozyme Study

"We note also that when distance measures are relatively large between pairs of species, and heterozygosity is low, the construction of phenograms can be carried out fairly reliably with only a few representative individuals for a species (Nei, 1978)" (SA: 357–358). However, the particular sampling strategies used in SA for organisms and loci will often produce unreliable estimates of genetic distances. Three problems are outlined below.

Although only single populations of *Lampsilis r. conspicua* and *L. r. radiata* were examined allozymically in SA, it is desirable to use multiple populations to represent each taxon in analyses of taxonomic boundaries and among-taxa relationships (e.g., Baverstock & Moritz, 1990). This is especially true for groups, such as the Unionoida, that are known to contain cryptic species (e.g., Davis

et al., 1981; Davis, 1983, 1984). Furthermore, representing species by single populations can produce phylogenetically misleading results (e.g., Smouse et al., 1991).

Species of *Drosophila* are typically considered to have "high" heterozygosities (15–20%; e.g., Gorman & Renzi, 1979). Therefore, the heterozygosities reported in SA (table 3) for the nine unionoid populations are not generally "low" (range: 5.1% to 31.8%, mean = 18.3%). Thus, estimates of genetic distances reported in SA may be inaccurate due to the small number of individuals used in certain comparisons (e.g., LAP was assayed for a single individual of *Lampsilis fullerkati* [SA: table 3]).

Nei (1978) makes it clear that a relatively large number of assayed loci are required for accurate estimates of genetic distance. Nei (1978: 583) stated the following: "Nei and Roychoudhurry (1974) concluded that for estimating average heterozygosity and genetic distance a large number of loci rather than a large number of individuals per locus should be used. . . ." What quantity does Nei imply with the phrase, "a large number of loci"? "In fact, less than 30 loci were studied in most recent protein surveys. This number is small; ideally, more than 50 loci should be used . . ." (Nei, 1978: 587). The import of a relatively large number of loci for estimation of genetic distances has been empirically substantiated (e.g., Gorman & Renzi, 1979). Both Nei's and Roger's distances are subject to large standard errors especially at relatively small distances (as is potentially the case among *Lampsilis fullerkati* and the two populations representing *L. r. conspicua* and *L. r. radiata* analyzed in SA), and the major factor influencing the standard errors is the number of loci sampled (e.g., Nei, 1978, 1987; Nei & Chesser, 1983; Richardson et al., 1986; Chakraborty & Leimar, 1987; Baverstock & Moritz, 1990). The sampling of eleven loci, as was the case in SA, does not give rigorous estimates of genetic distance. Therefore, the estimates of absolute genetic divergence presented in SA should be considered tentative as should any taxonomic revision based on those estimates.

Lack of Reference to Types

Neither *Lampsilis r. radiata* nor *L. r. conspicua* from their respective type localities or type locality drainages (Potomac and Yadkin

rivers, respectively) were allozymically analyzed in SA. Not utilizing topotypic or near-topotypic material for molecular evaluation compounded with the lack of reference to type material of any sort should preclude the taxonomic revisions suggested in SA. Taxonomic revisions must be based on reference to "types." Molecular analyses do not obviate this necessity. As it now stands, the specimens of *L. r. radiata* and *L. r. conspicua* utilized in SA cannot be confirmed as actually representing the taxa implied.

Taxonomic Concepts

Although often referring to conchological data, SA displays a strong reliance on levels of genetic distance for the determination of taxonomic boundaries. However, cogent arguments, based on theoretical and operational criteria, have been made against the use of genetic distances for delimiting taxa (e.g., Frost & Hillis, 1990). A major operational problem discussed in Frost & Hillis (1990) is the arbitrary nature of genetic distance measures. Because allozyme loci evolve at different rates (e.g., Sarich, 1977; Skibinski & Ward, 1982), genetic distance estimates are sensitive to the particular loci analyzed. Even if identical loci are scored, distance estimates may differ dramatically from one analysis to another due to the use of different electrophoretic conditions (e.g., Singh et al., 1976). The use of non-identical suites of allozyme loci combined with different electrophoretic conditions for some of the loci in common between studies may partly explain the discrepancy in reported genetic distance estimates between *Lampsilis fullerkati* and *L. radiata radiata* (SA: Nei's D = 0.049; Kat, 1983: Mean Nei's D = 0.129). Moreover, genetic distances are not appropriate measures of taxonomic status for recently diverged unionoid populations (e.g., Davis et al., 1981). "In no case is the species concept based on genetic distance alone" (Davis, 1983). A simplistic reliance on genetic distance-based taxonomic concepts should be abandoned.

Regarding genetic identity levels, SA (p. 366) states that ". . . Davis et al. (1981) argued that values > 0.9 could be found among sympatric freshwater mussel species that recently underwent speciation. However, these two so-called subspecies of *L. radiata* are currently not sympatric. . . ." This statement,

combined with SA's emphasis on genetic distance, implies that allopatric populations, in order to be recognized taxonomically, must be more divergent allozymically than sympatric populations. Can there not be distinct allopatric species with absolute genetic identities greater than 0.9? Theoretically, any number of assayed allozyme loci could indicate genetic identity between two distinct species. This may be expected for relatively recently diverged taxa (e.g., Johnson et al., 1977; Woodruff & Gould, 1980; Davis et al., 1981; Carson, 1982; Kat, 1983). However, genetic differences could still exist at non-allozyme loci. How are these potential differences evaluated? They are evaluated by reference to morphological, ecological, and phenological data. "The following species concept is used here: a species of unionid is a single lineage comprised of one or more populations that diverge from other lineages. Divergence is shown by significant morphological, cytological, reproductive biological and/or ecological differences. . . . The case for species status is strengthened if reproductive isolation is highly probable due to drainage system differences . . ." (Davis, 1983). We believe that the lack of reference to genetic distance in this unionid species concept is appropriate.

The concepts of (1) divergence (and, therefore, diagnosability) and (2) a lineage of populations (both following Davis, 1983) should be incorporated in evaluations of unionoid taxonomic boundaries. The limited data available in SA (tables 2 & 3, range and habitat data provided in the text) suggest that both *Lampsilis r. conspicua* and *L. fullerkati* are diagnosable from *L. r. radiata*. Despite the small number of loci assayed and minimal genetic distances reported in SA, the allozyme data set does suggest that there are diagnostic alleles (i.e., those alleles found in only one of the three taxa of interest here: *L. r. radiata,* one diagnostic allele; *L. r. conspicua,* eight diagnostic alleles; and *L. fullerkati,* four diagnostic alleles). The presence of unique genetic elements in a particular population is not consistent with a hypothesis of current genetic interchange with the other populations. Therefore, if these alleles remain diagnostic after adequate sampling of populations of the three nominal taxa, this would be strong evidence consonant with the distinctness of these populations. This sugges-

tion of distinctness is further evinced by the morphological and ecological data presented in SA.

CONCLUSIONS

The significant conchological, habitat, and range differences reported in SA (e.g., both in the text and table 2), suggest that the populations identified as *L. fullerkati, L. r. radiata* and *L. r. conspicua* represent distinct evolutionary lineages. Apparent non-identity of allozymic composition for these populations (SA: table 3) is consonant with this hypothesis. We believe that SA's "suggestion . . . of regrouping the two and possibly three previously recognized allopatric subspecies/species into one species complex, based upon very high levels of genetic identity as well as similar conchologies, is probably an uncommon event, yet appropriate until more distinctive biological species properties become evident" (SA: 367) is not appropriate at this time. Given the above discussion and the rapid decline of North American unionoid populations (e.g., Bogan, 1993), we believe that it is a scientifically justifiable and conservative course of action to continue to recognize *L. r. conspicua* (*sensu* SA) and *L. fullerkati* as taxa distinct from *L. r. radiata*. Data indicating need for further revision within the *L. radiata* group may become available; however, any revisions should necessarily be based on rigorous analyses of all the available characteristics of individuals selected by appropriate sampling criteria.

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A CALL FOR A NEW INTERNATIONAL CONGRESS OF ZOOLOGY

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We are looking for response concerning the feasibility of a New International Congress of Zoology, possibly to be convened in Athens, Greece, sometime during 1999 or 2000.

The First International Congress of Zoology was held in Paris, in 1889. Seventy years later, the XVIth Congress in Washington recommended the discontinuation of the congresses because of the feeling that zoology had split into too many unrelated, specialized fields. Nonetheless, a last XVIIth rump Congress was held in 1972 in Monte Carlo. The relatively few participants of this meeting unanimously, but in vain, asked for the continuation of the congresses. The idea was advanced that the International Conferences on Systematic and Evolutionary Biology would replace the defunct Zoological Congresses at a higher, integrative level. After several meetings of the ICSEB, it became evident that they did not live-up to this expectation. In contrast, the International Congresses of Botany have continued undisturbed and successfully.

One of the unhappy consequences of the cessation has been the fact that the International Commission of Zoological Nomenclature, once accountable to the congresses, came under the formal responsibility of the General Congresses of International Union of the Biological Sciences (IUBS). But the more painful and long-lasting consequence was a general depreciation of zoology in the academic world as such, and the replacement of this discipline by a plethora of euphemistically more fashionable designations.

However zoology at the end of this century is more alive than ever and rich in new ideas and achievements. A multispeciality exchange of views is more necessary than ever before. Not unexpectedly, this is also the consequence of the extreme parochialism of the different splinter fields and the ignorance of general zoological issues which it generated. There is a long list of such issues that cut across the lines of all the zoological specialities, some of them of important philosophical and practical significance.

The widely circulated "Systematics Agenda 2000" emphasizes our present incapacity to describe scientifically a zoological biodiversity that suddenly appears to be one order of magnitude larger than envisaged in the 1970s. This is not only a matter of quantities or of time needed, but a matter that calls for the restructure of zoological research world over. A critically depleted and weakened community of zoological systematics cannot live up to the task to investigate and possibly protect the heritage of the animal world.

On the positive side, there have been many developments during the last three decades that need to be appreciated by an international forum of all the zoologists. Confined to the pages of specialized journals, these important developments often do not reach the attention of peers in other zoological specialities. In the field of more classical zoology, it would be useful to acquaint our colleagues with such topics as the recently discovered new animal phyla and classes, new concepts of vertebrate evolution, zoology of clonal animals, and present views on the Protozoa. A sample of subjects of wider implications are sociobiology, cladistics, molecular taxonomy, modern embryology, the new vision on the Cambrian revolution, the neo-catastrophism, vicariance zoogeography, in situ and ex situ conservation, cryopreservation, and cloning. This is a different zoology from that which ended with a whimper in Monte Carlo.

We are ready to try to bring forward again the rich and unifying aspects of zoology and to reassert its general global, human and philosophical role. We are hoping for the approval and support of the zoological diaspora. The best encouragement will be to send us suggestions regarding the themes and the structure of the proposed New International Congress of Zoology. More importantly, we need personal commitments to

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help organizing symposia, workshops and hints of possible funding sources. We shall also need to establish an active and representative Action Committee. Understandably, we shall be able to appeal for funding only after having obtained convincing public support and after having a prestigious committee in place.

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