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Patterns of Snake Evolution Suggested by Their Proteins

Herbert C. Dessauer
John E. Cadle
Robin Lawson

A Contribution in Celebration
of the Distinguished Scholarship of Robert F. Inger
on the Occasion of His Sixty-Fifth Birthday

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Patterns of Snake Evolution Suggested by Their Proteins

Abstract

Genetic variability and other data on snake proteins are reviewed in the context of population genetics, species relationships, and current phylogenetic hypotheses. Protein diversity in snakes is comparable to that reported in other vertebrates, and protein polymorphisms are useful for identifying individual snakes as well as for studies of breeding patterns, population genetics, and species formation. Such biochemical data suggest that many populations of natricine snakes, presently classified as subspecies, are already reproductively isolated or are "incipient" species. As molecular evolution is largely divergent and often regular over time, comparative protein evidence allows one to overcome many of the difficulties encountered in estimating branching patterns of organisms. Such comparisons support the following conclusions: (1) many major lineages of snakes include one or more highly speciose radiations of relatively recent origin; (2) some genera are relics of ancient radiations; (3) lizards are the closest relatives of snakes, which are probably monophyletic; (4) primitive snakes include a number of ancient lineages that are probably not monophyletic; (5) vipers are the sister group of other advanced snakes; (6) sea snakes are closely related to Australopapuan elapids; (7) natricines and colubrines are probably monophyletic but the xenodontine and lycodontine groups possibly are not; and (8) relationships among major clades of the Colubridae remain unresolved.

I. Introduction

Comparative protein studies have demonstrated that molecular information is capable of solving many previously intractable problems concerned with the evolutionary biology of snakes. Such problems arise because of the extreme convergence, parallelisms, and specializations that typify snake morphology (Underwood, 1967). The success of the molecular approach in giving insight where traditional methods have failed is due in part to the fact that evolutionary processes at the molecular and morphological levels are largely independent (Wilson et al., 1977). Morphological evolution is highly variable in rate, rapid within some groups but extremely slow in others, and often subject to homoplasy (Simpson, 1953; Gould & Eldredge, 1977). In contrast, protein evolution is largely divergent and evolutionary change can be measured in units of known quantity (amino acid substitutions) that are often regular over time. Because of this, evidence from proteins allows one to overcome many difficulties in evolutionary studies traceable to convergence, parallelism, and specialization (Fitch, 1982). Although many questions remain regarding the evolution of snakes, general patterns concerned with their genetic diversity, population structure, speciation, historical biogeography, and phylogeny are discernible in protein structure. In this paper we present an overview of current comparative protein evidence bearing upon evolution within and among lineages of living snakes.

We dedicate this paper to Robert F. Inger (fig. 1), who always has been highly supportive of such nontraditional approaches to evolutionary studies. Bob Inger and other biologists whose primary focus is field and comparative anatomical study have generally originated the phylogenetic hypotheses upon which molecular biologists base their experimental work. Realizing that many problems can be solved with molecular data, systematic biologists are collecting tissues in the field with increasing frequency for use in such research. Be-

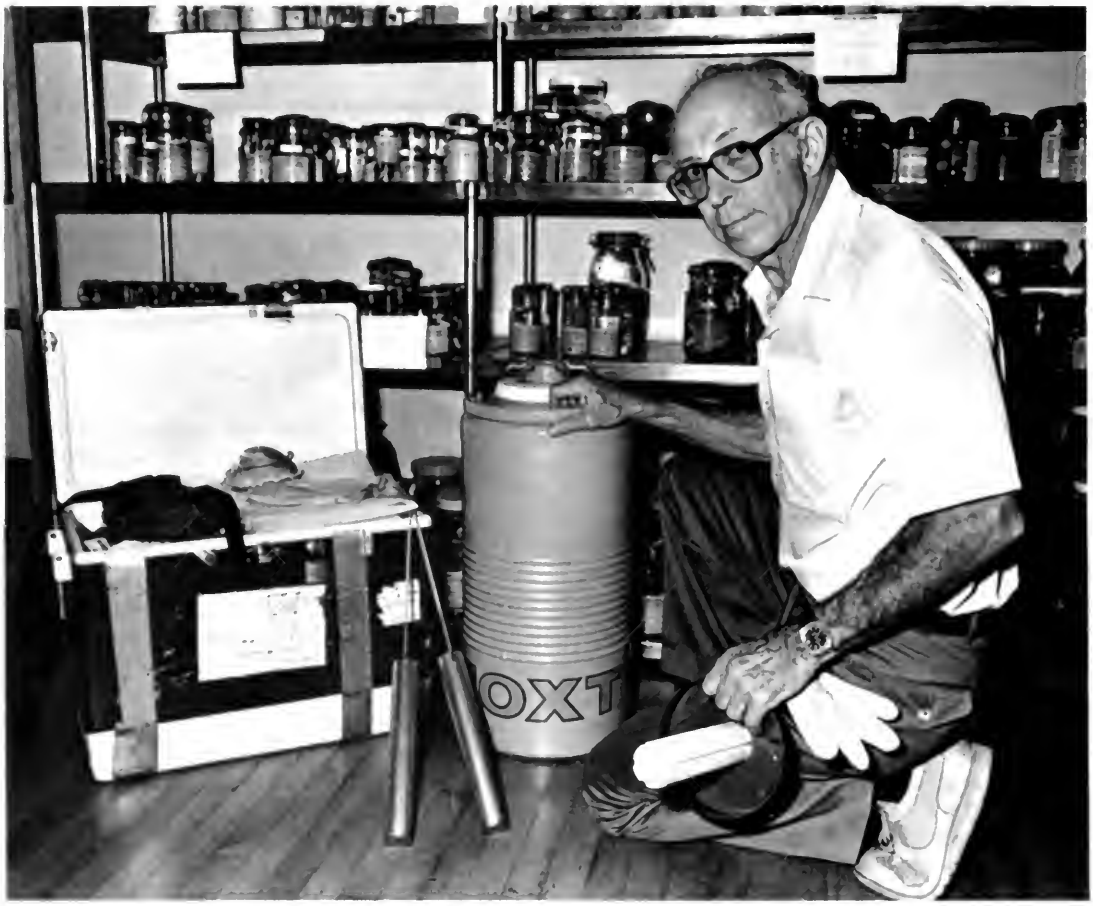


FIG. 1. Robert F. Inger preparing a liquid-N tank and other gear for storing tissues from specimens collected during a 1986 trip to Southeast Asia. (Photo by Harold Voris.)

sides the usual strenuous activities and logistic problems of fieldwork, the collector seeking tissues for molecular studies must transport liquid nitrogen tanks, centrifuges, and other unusual equipment into the field, many times into almost impenetrable areas (fig. 2). The frozen tissue collections that result from these activities provide a valuable resource for research in evolutionary biology and a variety of other disciplines. "Often, the principal contribution to a molecular study is the field work of the naturalist who provides the tissue upon which the study is based" (Dessauer & Hafner, 1984). We believe that collaborative interactions between field- and laboratory-oriented scientists (fig. 3) are producing more definitive phylogenies and a more comprehensive understanding of evolutionary processes.

II. Methods and Nature of the Evidence

Many biochemical techniques have been used to acquire comparative protein evidence regarding snake evolution. The majority of these data were obtained by means of electrophoresis (Smithies, 1959) of a wide variety of proteins, microcomplement fixation (Champion et al., 1974), or other immunological comparisons (Goodman & Moore, 1971) of transferrins and albumins, and peptide fingerprints (Canfield & Anfinsen, 1963) of hemoglobins. Amino acid sequences of comparative value on snake proteins are rare, consisting principally of those for neurotoxins of elapid venoms (Strydom, 1973, 1979; Hseu et al., 1977; Yang, 1978; Mebs, 1985; Tamiya, 1985).

The use of protein evidence in evolutionary

FIG. 2. Natives loading donkeys with liquid-N tanks for transport to a camp site of an L.S.U. Museum of Zoology expedition high in the Peruvian Andes. (Photo by J. P. O'Neill.)

FIG. 3. Field and laboratory scientists of the 1969 ALPHA HELIX Expedition to New Guinea, specifically organized to obtain tissues for molecular study (Dessauer, 1970). Standing, left to right: A. C. Wilson, W. Z. Lidicker, A. H. Brush, T. Gobble (physician), R. G. Zweifel, H. G. Cogger, V. M. Sarich; kneeling: R. Storez and H. C. Dessauer. (Photo by R. G. Zweifel.)

studies depends upon the fact that homologous genes among species diverge in a continuous fashion from the time of reproductive isolation. As a result, the sequence differences between the protein products of homologous genes among taxa represent an estimate of the degree of divergence among the organisms themselves (Zuckerkandl & Pauling, 1965). Furthermore, some genes and their protein products can be shown to diverge in a clocklike manner. The variation in rate for a particular protein is usually about twice that expected for a simple Poisson process such as radioactive decay (Wilson et al., 1977).

Although amino acid sequences offer the maximum evolutionary information encoded in the protein molecule, they are difficult to determine and require extensive expenditure of tissue, time, and money. The majority of questions confronting the evolutionary biologist can be answered sooner and far more economically with data sets on proteins based upon peptide fingerprinting and electrophoretic and immunological methods. Nuttall (fig. 4), for example, using the most primitive of immunological methods, had by 1904 successfully predicted the relative affinities of major lineages of primates (Nuttall, 1904).

Comparative immunological data sets on an homologous series of proteins are highly correlated with amino acid sequence differences between the proteins. Wilson and his colleagues (1977; see also Benjamin et al., 1984) have shown that immunological distances (IDs) obtained by microcomplement fixation titrations (MC'F) are directly proportional to sequence divergence of the same proteins. Antigenic distances obtained by immunodiffusion, in turn, are directly proportional to the MC'F IDs on the same protein (Goodman & Moore, 1971; Schwaner & Dessauer, 1982). When applied to closely related organisms, even genetic distances between taxa estimated from electrophoretic data sets on proteins relate roughly to the IDs for transferrins or albumins of the same taxa (Sarich, 1977; Wilson et al., 1977; Maxson & Maxson, 1979; Wyles & Gorman, 1980).

Each of these indirect methods differs in the nature of the evidence it furnishes and in the taxonomic levels at which it is most efficiently applied. Electrophoresis of proteins yields banding patterns of allozymes, the protein phenotype, that can be interpreted genotypically (Harris, 1975; Harris & Hopkinson, 1976; Dessauer et al., in press); consequently, the method has a wide variety of applications, especially in studies of inheritance involving closely related organisms. These include the detection of allelic variation at structural gene loci, determination of genotypes of individuals at polymorphic loci, estimation of levels of genetic diversity within populations, and genetic distances between populations and species (Avise, 1974; Smith et al., 1982). Currently, specific staining techniques are available to detect proteins determined by approximately 200 different loci, including nonenzymic proteins and enzymes of all major classes (Harris & Hopkinson, 1976; Hames & Rickwood, 1981).

Immunological estimates of protein divergence are measures of structural changes at antigenic sites (Benjamin et al., 1984). As rates of divergence of proteins coded by different structural genes vary more than one hundred-fold (Wilson et al., 1977), one of the factors to be considered in selecting a protein for use in a taxonomic study is its rate of evolutionary change. For example, transferrin is more sensitive than albumin for estimating the affinities of closely related taxa, as it usually diverges more rapidly than albumin, about twice as fast on the average in snakes (Cadle & Dessauer, 1985); however, albumin comparisons are valuable over a wider range of taxa. Quantitative precipitin and MC'F estimations of protein divergence can be used to construct phylogenetic trees showing the branching order of taxa and the amounts of protein divergence attributed to each lineage (Felsenstein, 1982).

The semiquantitative immunodiffusion method (Goodman & Moore, 1971) is simpler but less sensitive than MC'F. The immunodiffusion approach is ideal for survey studies and can also be used to construct phylogenetic trees (Dene et al., 1978), although their precision is generally less than with the more quantitative methods. Spur formation, the sign of protein divergence in the immunodiffusion reaction, does not occur with transferrins having MC'F IDs below 20 (Schwaner & Dessauer, 1982). It is generally not possible to use immunodiffusion to detect cross-reactions between transferrins or albumins with antibodies to their homologs from widely divergent species; however, by adding polyethylene glycol to the gel to lower the solubility of the resultant antigen-antibody complex, reactions can be visualized between antisera to snake albumins and albumins from the most distantly related snake taxa, even from albumins of some lizards (cf. fig. 8).

Comparisons of fingerprints of purified proteins can furnish estimates of the minimum number of sequence differences between homologous proteins. The proteins to be tested are hydrolyzed with

FIG. 4. Professor G. H. F. Nuttall, the pioneer worker in comparative immunology. (Photo from M. F. Shaffer.)

trypsin or some other enzyme with a high specificity for particular peptide bonds. The resultant peptide fragments are spread across sheets of filter paper by means of chromatography and electrophoresis and then visualized with chemical stains. The sequence divergence of the proteins is estimated on the basis of differences in positions and staining properties of peptide fragments (Sutton, 1969; Dessauer, 1974; Mao et al., 1978, 1984).

The relative timing of lineage separation can be inferred from quantitative estimates of sequence divergence between homologous proteins in different lineages, such as provided by immunological distances. Estimates of absolute times of sep-

aration of the taxa under study are possible if the rate of evolution of the protein can be estimated from fossil and/or biogeographic evidence (Maxson et al., 1975) and if the rate is relatively constant among the lineages, as shown by the relative rate test (fig. 5; Wilson et al., 1977). Although rates of albumin and transferrin evolution are approximately constant in most lineages, they can vary substantially within and especially among some vertebrate lineages (e.g., Sarich, 1985). Relative rate tests suggest that evolutionary rates for both albumins and transferrins differ somewhat among snake lineages (Cadle, 1982a,b; unpubl. data). For example, the rate of albumin evolution in some vipers appears to be at least 30% slower than in the majority of elapid and colubrid lineages (see sec. VI.B., 3a). Despite these differences in rate, however, the molecular clock concept can still be used in interpreting aspects of snake evolutionary history as long as rate differences are recognized and measured.

III. Biochemical Genetics and Population Structure

A. Protein Inheritance

High resolution electrophoresis of tissue homogenates followed by the identification of electromorphs for specific proteins has yielded considerable evidence on genetic diversity at structural gene loci, both within single populations and between populations presumably undergoing species formation. At such close levels of relationship, the majority of allelic differences at a specific locus is traceable to point mutations. If these result in amino acid substitutions in the polypeptide determined by the gene, the variant protein may be electrophoretically detectable.

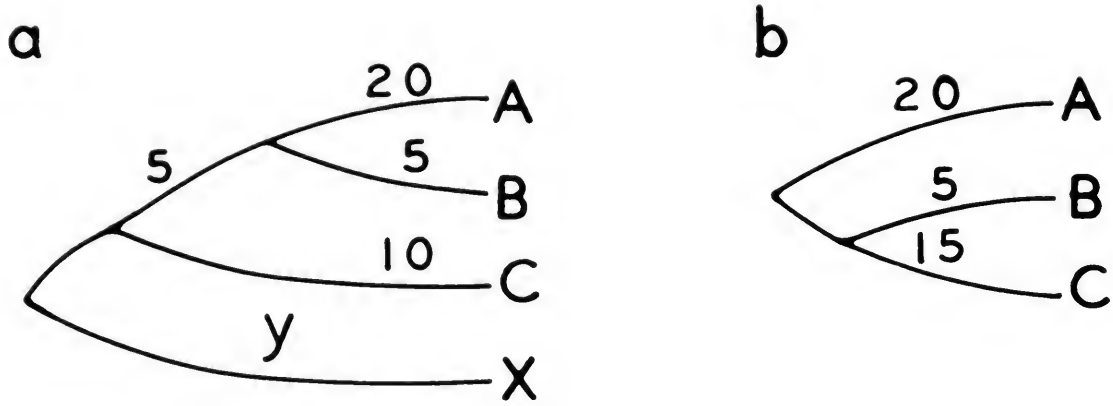
In snakes, as in other vertebrates, most proteins are inherited as the products of codominant alleles. Direct evidence for codominance as the mode of inheritance of proteins in snakes has been shown in studies involving the breeding colony of kingsnakes at the American Museum of Natural History (Dessauer & Zweifel, 1981) and in laboratory-bred rat snakes of known parentage (Lawson & Dessauer, unpubl. data). The maternal contribution to protein phenotypes of their offspring has been observed for rattlesnakes (Crabtree & Murphy, 1984; Murphy & Crabtree, 1985) and many species of natricine snakes (Schwaner et al., 1980; Dessauer & Lawson, unpubl. data). Considerable indirect evidence on snakes as well as on other vertebrates supports this general conclusion (Dessauer et al., in press).

Electrophoretic phenotypes for many proteins within a species or species group of snakes are identical, even in individuals from widely separated areas of the geographic range of a species. These invariant proteins are often useful markers for identifying species, or higher categories of snakes in a cladistic analysis. Most species, however, have some proteins that are polymorphic; in snakes these commonly include transferrin, phosphogluconate dehydrogenase, phosphoglucomutase, and esterase-D. Although most polymorphic loci are diallelic, three or more alleles are commonly observed at the transferrin locus in snakes of the same population (Dessauer et al., 1962; Gartside et al., 1977; Lawson & Dessauer, 1979). Amino acid oxidases (Jiménez-Porras, 1964a; Aird & Dessauer, 1977), proteases (Jiménez-Porras, 1964a,b) and toxins of venoms of viperid snakes (Schenberg, 1959) are so polymorphic that viperid venoms may have the most highly variable protein composition of any biological fluid (see Dessauer, 1974).

Polymorphic proteins have been used to identify individual snakes and to study their breeding patterns. Individuals in populations of *Bothrops neuwiedi* from southeastern Brazil could be identified by patterns of six venom antigens (Schenberg, 1963). Knowledge of transferrin and prolidase genotypes of individual kingsnakes (*Lampropeltis getulus*) in the American Museum of Natural History colony allowed Zweifel and Dessauer (1983) to plan matings that proved that kingsnake broods can be the result of inseminations by at least two males. Polymorphisms at the albumin, transferrin, superoxide dismutase, and esterase-D loci were utilized by Banks and Schwaner (1984) to show that a brood of Australian pythons, conceived and hatched at the Melbourne Zoo, were progeny of a mating between *Python spilotes* and *P. amethystinus,* and that the *P. amethystinus* female, coiled about the clutch of eggs during their incubation, was not the mother of the brood.

B. Population Genetics

Alleles responsible for polymorphic proteins may be rare to moderate in frequency, widespread, or



	A	B	C	X
A	0			
B	25	0		
C	35	20	0	
X	y + 25	y + 10	y + 10	0

FIG. 5. Illustration of a rate test. We are interested in the relationships among ingroup taxa A, B, and C. Observed molecular distances are given in the matrix. A straightforward apportionment of these distances would result in the estimated phylogeny illustrated in **b**. A rate test shows this to be in error. To perform the test, outgroup-X is chosen on the basis of nonmolecular evidence (e.g., morphology), and the distances between X and taxa A, B, and C are measured (matrix row 4; variable *y* is that portion of the distance between the outgroup and ingroup that is the same for all members of the ingroup). The rate test shows that taxa B and C are conservative relative to taxon A and, thus, the distances should be apportioned as in **a**. The resulting phylogenies **a** and **b** differ in branching order. In this example, the fact that taxa B and C are both conservative suggested their apparent phylogenetic association, but the rate test can be used to properly assess their relationships (Cadle, 1984a).

restricted to a specific population. The origin of geographic variation in allele distribution may be traceable to isolation by distance or by some natural barrier to gene flow. For example, garter snakes (*Thamnophis sirtalis*) from the northeastern and western coasts of North America are fixed for alternative alleles at the cytosolic superoxide dismutase locus (Lawson, 1978). Proteases have dif-

TABLE 1. Indices of genetic variability for snake populations.

Species	Geographic location	No. of specimens	No. of loci tested	P*	H†	Source‡
Rhinophis philippinus	Central SRI LANKA	34	26	19.2	4.3	1
Phyllorhynchus arenicolus	Isla San Marcos, Baja California, MEXICO	4	34	10.3	3.8	2
Thamnophis proximus	Vicinity of La Place, "St. John the Baptist" Parish, La.	40	26	23.1	3.8	3
T. couchii atratus	Isenberg Ranch, San Mateo County, Calif.	38	31	16.1	8.2	4
T. c. couchii	Feather River, Butte and Plumas counties, Calif.	13	31	12.9	3.4	4
T. c. hydrophilus	Applegate River, Jackson and Josephine counties, Ore.	14	31	22.6	7.8	4
T. c. hammondii	Picnic Lake Park, Potrero, San Diego County, Calif.	8	31	6.4	1.6	4
T. elegans terrestris	Samoa Peninsula, Humboldt County, Calif.	10	31	12.6	3.0	4
T. e. vagrans	Florida Mesa, La Plata County, Colo.	42	33	9.0	1.7	5
T. e. vagrans	Qualicum Beach, Vancouver Island, CANADA	12	26	3.9	2.6	6
T. ordinoides	Port Orford, Curry County, Ore.	23	33	24.2	8.9	5
T. ordinoides	Parksville, Vancouver Island, CANADA	37	33	21.2	5.3	5, 6
T. brachystoma	Allegheny River Valley, Warren County, Pa.	25	26	3.8	0.6	6
T. rufipunctatus	Río Papigochic, near Ciudad Guerrero, Chihuahua, MEXICO	8	26	7.7	1.0	6
T. sirtalis sirtalis	Bono, Ottawa County, Ohio	52	14	28.6	8.3	7
T. s. sirtalis	Islesboro Island, Waldo County, Maine	20	27	29.6	8.0	6
T. s. sirtalis	Baton Rouge, La.	17	27	25.9	8.2	6
T. s. sirtalis	Illinois	11	27	25.9	7.8	6
T. s. parietalis	Southwestern Illinois	13	27	25.9	7.7	6
T. s. parietalis	Inwood, Manitoba, CANADA	56	15	6.7	2.0	8
T. s. parietalis	Inwood, Manitoba, CANADA	14	27	18.5	4.4	6
T. s. pickeringi	Parksville, Vancouver Island, CANADA	11	27	11.1	2.7	6
T. s. dorsalis	Rio Grande Valley, N. Mex.	8	27	25.9	7.4	6
Nerodia fasciata confluens	Baton Rouge, La.	31	35	11.4	2.8	9
N. f. compressicauda	Boca Ciega Bay, Pinellas County, Fla.	35	35	8.6	1.0	9
N. f. clarkii	Grande Isle, Jefferson Parish, La.	16	35	17.1	4.8	9

* P = percent polymorphism where the frequency of the most common allele does not exceed 0.95.

† H = percent heterozygosity by direct count.

‡ Sources of data: 1 = Dessauer, Gartside & Gans (unpubl. data); 2 = Murphy & Ottley (1980); 3 = Gartside et al. (1977); 4 = Lawson & Dessauer (1979); 5 = Lawson (1978); 6 = Lawson (unpubl. data); 7 = Sattler & Guttman (1976); 8 = Bellemin et al. (1978); 9 = Lawson (1985).

ferent electromorphs in *Bothrops nummifer* populations from the Caribbean and Pacific slopes of the central mountain chain in Costa Rica (Jiménez-Porras, 1964b, 1967). Variations in frequencies of polymorphic amino acid oxidases and proteases distinguish populations of the fer-de-lance (*Bothrops asper*) from the two sides of these same mountains (Jiménez-Porras, 1964a). Geographic differences in venom proteins are so common that venomologists emphasize the importance of preparing regional types of antisera (Gonçalves & Vieira, 1950).

The magnitude of protein diversity in snakes (table 1) is similar to that observed in many other vertebrates (Selander & Johnson, 1973; Nevo et al., 1984). In populations of snakes that have been examined, polymorphism, the frequency of polymorphic loci relative to the number of loci ex-

amined, ranges from 0.04 to 0.30; and heterozygosity, the frequency of heterozygous phenotypes per individual over the number of loci examined, ranges from 0.01 to 0.09 (table 1). When data are obtained from different sources, much caution must be observed in comparing levels of polymorphism or heterozygosity across populations or between species. These indices of genetic variability are highly correlated with both sample size and the number and kind of loci tested. Exclusion of alleles found at less than the 5% level corrects for some disparities in sample size, but the inclusion or exclusion of such highly polymorphic loci as nonspecific esterases and transferrins can raise or lower these indices considerably (table 1; e.g., compare sets of data for *Thamnophis sirtalis* samples from Manitoba, Canada). Genetic diversity is not uniform across subspecies ranges. Snakes in zones of secondary contact between populations that have been disjunct for some time may have high levels of heterozygosity and often exhibit rare alleles not found in other areas of the species' range. Populations of *Nerodia f. fasciata* from the panhandle of Florida exemplify this phenomenon in snakes (Lawson, 1985), as it has been documented in population studies of other reptiles (Case & Williams, 1984; Murphy et al., 1984).

The majority of evidence on protein diversity in snakes concerns members of the genus *Thamnophis,* natricine snakes that are widely distributed throughout North America (Conant, 1975; Stebbins, 1985). The common garter snake (*Thamnophis sirtalis*) is one of the most widespread species; its range is continuous from the Atlantic to the Pacific and extends from Mexico into northern Canada. In a broad sense, this species can be divided into two color morphs. Those with lateral red markings occupy the western part of the range, and those without laterally distributed red pigment, the eastern part. The red-sided garter snakes along the West Coast have been divided into several subspecies, but a single subspecies (*Thamnophis s. parietalis*) occupies the extensive area between the western coastal states and the Mississippi Valley. In the East the subspecies *Thamnophis s. sirtalis* ranges from the Atlantic Coast westward to a narrow zone of contact with *T. s. parietalis* that generally follows a north-south line approximating the Mississippi Valley. The only subspecies that has a disjunct range is *Thamnophis s. dorsalis,* which is isolated in the Rio Grande Valley.

As an ecological generalist (Fitch, 1965), *Thamnophis sirtalis* has been able to occupy a vast geographic range and has adapted to all but the driest habitats. If the complicating influences of demographic factors and history on the genetics of a population could be eliminated, natural selection suggests that ecological generalists should possess above average genetic diversity, especially those populations from continuous areas of the species range (Nevo et al., 1984). Populations of *Thamnophis sirtalis* from inland areas do have heterozygosities of about 8%, relatively high for vertebrates (table 1; *Thamnophis s. sirtalis* from Illinois, Ohio, and Louisiana, and *T. s. parietalis* from Illinois).

In contrast, populations at the geographic periphery of a species' range, on islands, or in other distributional disjunctions might be expected to have lower levels of diversity. Although true for some populations, this hypothesis does not hold as a generalization for snakes. Bellemin and colleagues (1978) examined intrademic variability in *Thamnophis sirtalis* collected as they emerged from each of four hibernacula near Inwood, Manitoba, Canada. Of the 15 loci assayed, only one, xanthine dehydrogenase, was variable, with heterozygosities ranging from 1.1% to 2.8%. The authors posed two non-mutually exclusive hypotheses to explain these low indices: bottleneck effect due to periodic frost kills, and strong directional selection at the periphery of the species' range. While these factors may partially explain their results, choice of loci tested may be the more important factor. Lawson (1978; unpubl. data) has found that the 14 invariant loci of the Bellemin study are largely invariant throughout the range of this garter snake species. Transferrin and cytosolic superoxide dismutase were among 27 protein loci that Lawson examined in a *Thamnophis sirtalis* sample also taken near Inwood, although not necessarily from one of the same dens. The majority of the individual snakes were heterozygous at one or both of these loci. Thus, *Thamnophis sirtalis* from the vicinity of Inwood actually falls in the midrange for percent polymorphism and for percent heterozygosity, considering populations of *T. sirtalis* as a whole (table 1).

Probably because *Thamnophis sirtalis* is semiaquatic and a feeding generalist (Fitch, 1965; Kephart & Arnold, 1982), it is found on many coastal islands, both in eastern and western North America. Continuous recruitment from the mainland to continental islands should be the rule, so reduced genetic variability due to founder effect is not expected. Garter snakes from Islesboro Island in Penobscot Bay, Maine, are as variable as those from

inland populations; however, the population on Vancouver Island off the coast of western Canada does appear to have a low level of genetic diversity (table 1)

A variety of studies on snakes shows that morphological and protein polymorphisms are generally inherited independently. In laboratory-bred kingsnakes (*Lampropeltis getulus*), color pattern is highly polymorphic (Zweifel, 1981), but these morphological features are inherited independently of protein polymorphisms (Dessauer & Zweifel, 1981; Zweifel & Dessauer, 1983). In *Thamnophis ordinoides,* marked variability in ground color and in the pattern and color of dorsal striping is accompanied by high molecular variability (Lawson, 1978). On the other hand, *Nerodia fasciata compressicauda,* a snake of highly variable color pattern with melanistic and erythristic morphs in many populations, shows very low protein diversity (table 1).

Differentiation through isolation by distance appears to be a factor in producing and maintaining a number of color morphs in *Thamnophis*; many of these are recognized as subspecies. Differentiation that may in part be due to isolation by distance can also be demonstrated at the molecular level in *Thamnophis sirtalis.* Populations along the northeastern and western coasts of North America are fixed for alternate alleles at the cytosolic superoxide dismutase locus (Lawson, 1978), but in all inland populations except those of the Florida Peninsula both alleles are found in approximately equal frequencies (Sattler & Guttman, 1976; Lawson, unpubl. data). Factors acting to maintain fixation of these different alleles in the Atlantic and Pacific coast populations of *T. sirtalis* are unknown. Sattler and Guttman (1976) used electrophoresis in an attempt to determine whether reproductive isolation or localized natural selection is responsible for the maintenance of high levels of melanism found in some populations of garter snakes in Ottawa County, Ohio. Allelic frequencies at 14 loci from melanistic and normally colored garter snakes collected near the town of Bono on the southwestern shore of Lake Erie supported the view that these snakes are freely interbreeding. Based upon this finding, Sattler and Guttman hypothesized that selection for concealing coloration is responsible for the high frequency of the melanistic morph endemic to that general geographic area.

The high levels of genetic variability at the molecular level observed in *Thamnophis sirtalis* have not been found in all species of ecological generalists with wide geographical and altitudinal ranges (table 1). Populations of wide-ranging *T. elegans* sampled at sea level and at 10,000 feet all have relatively low variability indices. The converse of the correlation of high genetic variability with ecological adaptability and extensive range is that the ecological specialist with a small distributional range should have low genetic variability. For some snake species this certainly holds true. *Thamnophis brachystoma* and *T. rufipunctatus* each have very low indices of variability. However, not all specialization results in reduced genetic variability; the fossorial uropeltid *Rhinophis phillippinus* (Dessauer et al., 1976; unpubl. data) and the xenodontine *Carphophis amoenus* (Lawson & Axtell, unpubl. data) show average and higher than average genetic diversity, respectively.

Overall, these observations suggest that any relationship between levels of genetic diversity and ecological specializations is extremely tenuous at best; they also serve to focus attention on population structure and history as major determinants of genetic variation.

IV. Species Formation

Studies at the protein level are giving insight into many problems concerned with the processes of speciation. If unique alleles distinguish two taxa, genotypes of individuals in the geographic area of contact may offer irrefutable evidence for the presence or absence of gene flow between them. For example, such data suggest that the morphologically polymorphic and wide-ranging kingsnake *Lampropeltis getulus* is a single species. Eastern and western populations have different albumin and haptoglobin phenotypes; but where the eastern and western forms meet in western Texas intergradation is apparent, as proteins of both forms are present in snakes from that region (Dessauer & Pough, 1975). Additionally, *Elaphe bairdii* (see Olsen, 1977) and *E. obsoleta* are distinguished by unique esterase-D alleles as well as by frequency differences of alleles at other structural gene loci. Populations in a contact zone between the two forms include snakes heterozygous for the two marker esterase-D alleles (Lawson & Lieb, unpubl. data).

The evolutionary biology of the North American radiation of natricine snakes presents many speciation problems that have been most extensively studied in members of the *Nerodia sipedon*

complex and in various groups of *Thamnophis*. Three groups of water snakes have ranges that come into contact along the coastal region of southeastern United States: (1) *Nerodia sipedon*, adapted to freshwater streams; (2) *Nerodia fasciata*, adapted to other freshwater habitats; and (3) *Nerodia fasciata clarkii* and *N. f. compressicauda*, adapted to saline environments. Conant (1963) concluded that *Nerodia sipedon* and *N. fasciata* were distinct. Later investigators (Schwaner & Mount, 1976; Blaney & Blaney, 1979) interpreted color pattern similarities as signs of intergradation. Protein studies suggest that Conant probably was correct, at least for populations in contact zones along the Tchefuncte and Bogue Chitto rivers in southeastern Louisiana (Schwaner et al., 1980). Similarly, the freshwater and salt marsh forms of *Nerodia fasciata* are largely reproductively isolated across their long but narrow zone of contact along the coasts of the Gulf of Mexico and the Atlantic Ocean (Lawson et al., 1981; Lawson, 1985).

Unraveling the taxonomy of garter snakes of genus *Thamnophis*, the most speciose genus of North American natricines, has been especially baffling. The molecular evidence suggests that many members of the genus either have become reproductively isolated only recently or are presently in final stages of speciation. Transferrins of members of the genus differ by a maximum ID of only 15 (George & Dessauer, 1970; Mao & Dessauer, 1971); their albumins differ by a maximum ID of 10 (Dowling et al., 1983).

The ribbon snakes, *Thamnophis sauritus* and *T. proximus*, are sibling species. *Thamnophis s. sauritus* inhabits a large area, stretching from the eastern coast of the United States westward to a line running northward approximately from the Pearl River through western Indiana. Along its western boundary it contacts *T. proximus* to produce a narrow zone of parapatry. The Florida Peninsula is inhabited by *T. sauritus sackenii*, which may intergrade with *T. s. sauritus* in the region of the former Suwanee Straits (Rossman, 1962).

Thamnophis sauritus and *T. proximus* show little differentiation either morphologically (Rossman, 1962) or at the molecular level (Gartside et al., 1977). No marker alleles have been found, and the Nei genetic distance between them is only 0.023 (Lawson & Dessauer, 1979), a level usually indicative of conspecific populations. Extensive field observations and morphological evidence, however, convinced Rossman (1962) that gene flow does not occur between these taxa; similarly, morphological evidence suggests that *T. s. sauritus* and *T. s. sackenii* may also be reproductively isolated (Williamson & Moulis, 1979).

Additional insight into the evolution of these snakes, not apparent in the phenetic analysis of gene frequency data, is revealed by the distribution of a derived allele at the cytosolic malate dehydrogenase locus, based upon combined data on 250 individuals (Gartside et al., 1977; Lawson, unpubl. data). There are two common alleles at this locus in the ribbon snakes. One, representing the derived state as determined by outgroup comparison (Watrous & Wheeler, 1981), is apparently fixed in *Thamnophis s. sauritus*. The alternative, primitive allele predominates in *T. proximus* and *T. sauritus sackenii* as well as in other species of *Thamnophis*. Although interbreeding between *T. sauritus* and *T. proximus* in the zone of parapatry probably no longer occurs (Rossman, 1962), evidence for its occurrence in the recent past is provided by the presence of a step cline coincident with the zone of parapatry in Louisiana, showing penetration of the derived allele typical of *T. s. sauritus* into *T. proximus* populations as far west as western Texas. Similarly, gene flow from *T. s. sauritus* into *T. s. sackenii* populations has occurred with the derived malate dehydrogenase allele detectable in *sackenii* as far south as Tampa Bay in central Florida. Protein data are insufficient at present to estimate whether or not interbreeding is still taking place between these subspecies in the putative zone of intergradation.

Relationships among the West Coast garter snakes present another problem that has challenged herpetologists for many years (Rossman, 1979). Based on a series of classical studies, Fitch (1940) and Fox (1951) concluded that the many morphologically distinct forms comprised aquatic and terrestrially adapted groups of races of one species, *Thamnophis elegans*, distributed over most of California as a ring of races. Those subspecies adapted to aquatic conditions were thought to intergrade with subspecies adapted to terrestrial conditions along the Klamath River valley in northern California.

Protein electrophoretic studies have modified our concept of relationships within the complex. Phenotypes for marker transferrin alleles showed that gene flow does not occur between forms of the two ecological groups in the area that Fitch and Fox proposed as the site of intergradation (Fox & Dessauer, 1965; Lawson & Dessauer, 1979; see

also the morphological studies of Rossman, 1964, 1979). A matrix of Nei genetic distances between subspecies showed that the terrestrial and aquatic groups of subspecies were members of relatively widely divergent lineages. The terrestrial lineage, *Thamnophis elegans,* was found to consist of four very closely related subspecies. The aquatic lineage split into two subgroups: the *atratus* subgroup, consisting of the subspecies *atratus, hydrophilus, aquaticus,* and *gigas*; and the *couchii* subgroup, consisting of the subspecies *couchii* and *hammondii* (Lawson & Dessauer, 1979).

Relationships within the aquatic lineage have been further clarified by data on marker alleles for specimens collected from zones of contact between the subspecies. Apparently, gene flow in nature is rare between *hydrophilus* and *couchii.* Of several dozen snakes examined from the area of parapatry, only three individuals were identified as putative hybrids. Morphological studies on the same individual snakes provided a similar interpretation (Rossman & Stewart, 1979). Moreover, *couchii* appears not to intergrade with *hammondii* where they contact in the Tehachapi Mountains, a conclusion also drawn independently on morphological grounds by Rossman and Stewart (1982). In southwestern California where the ranges of the subspecies *atratus* and *hammondii* overlap extensively, occasional hybrids have been identified on both molecular and morphological criteria, again indicating that reproductive isolating mechanisms can break down occasionally between members of the aquatic subgroups. The California giant garter snake, *T. gigas,* was formerly thought to be derived from the *couchii* subgroup; however, protein evidence (Lawson & Dessauer, 1979; Lawson, unpubl. data) clearly shows that its affinities are with the *atratus* subgroup (recognition of *gigas* as a distinct species follows Rossman & Stewart, 1985).

A third species of garter snake endemic to the west coast of North America, *Thamnophis ordinoides,* has ecological preferences similar to coastal populations of *T. elegans.* Phenetic analysis of protein evidence suggested that its affinities were with the *atratus* subgroup (Lawson & Dessauer, 1979); however, cladistic analysis of the same allelic data has since shown that *T. ordinoides* is instead closer to *T. elegans* (Lawson, unpubl. data). Thus, current evidence taken in toto suggests that the complex of West Coast garter snakes may consist of six closely related species: *Thamnophis elegans, T. couchii, T. atratus, T. hammondii, T. gigas,* and *T. ordinoides.*

In general, genetic evidence suggests that many populations of natricine snakes, presently classified as subspecies, are either already reproductively isolated or have at least attained the "incipient" species level. Often these forms contact parapatrically without interbreeding. Each appears to be adapted to some unique feature or features of the environment (e.g., fresh vs. salt water); selection countering gene flow appears to maintain these habitat distributions. Yet some forms are so similar genetically that it is easy to visualize how changes in the environment due to geological or climatic events or to man-induced disturbances could easily alter population equilibria.

V. Differential Rates of Morphological Evolution

A. Radiations Illustrating Rapid Morphological Evolution

Many major lineages of snakes include one or more highly speciose radiations that appear to be relatively recent in origin. Such characterized groups have been identified in the natricine, colubrine, xenodontine, and elapid lineages. Each radiation includes species that are so distinct in morphology and/or ecology that they are classified in different genera. Yet the divergence of such rapidly evolving proteins as transferrin and albumin is so small that electrophoretically generated evidence on alleles has been required to assess affinities of members of each radiation. Examples from four well-studied groups are discussed here.

1. North American Natricines (fig. 3, top)—The tribe Thamnophiini (Rossman & Eberle, 1977), the natricine snakes of North America, is the most thoroughly studied of these radiations. Morphological divergence within the group is considerable; taxonomists recognize nine genera and about 45 species. These species are distributed from Central America to Canada and show terrestrial, semiarboreal, semiaquatic, aquatic, and semifossorial adaptations. The diets of the different species vary, encompassing invertebrates such as worms, slugs, and crayfish, as well as most classes of vertebrates (Wright & Wright, 1957).

Molecular divergence among thamnophiines is low. IDs among the transferrins of 22 species ranged from 4 to 28, with an average of 11 (George, 1969;

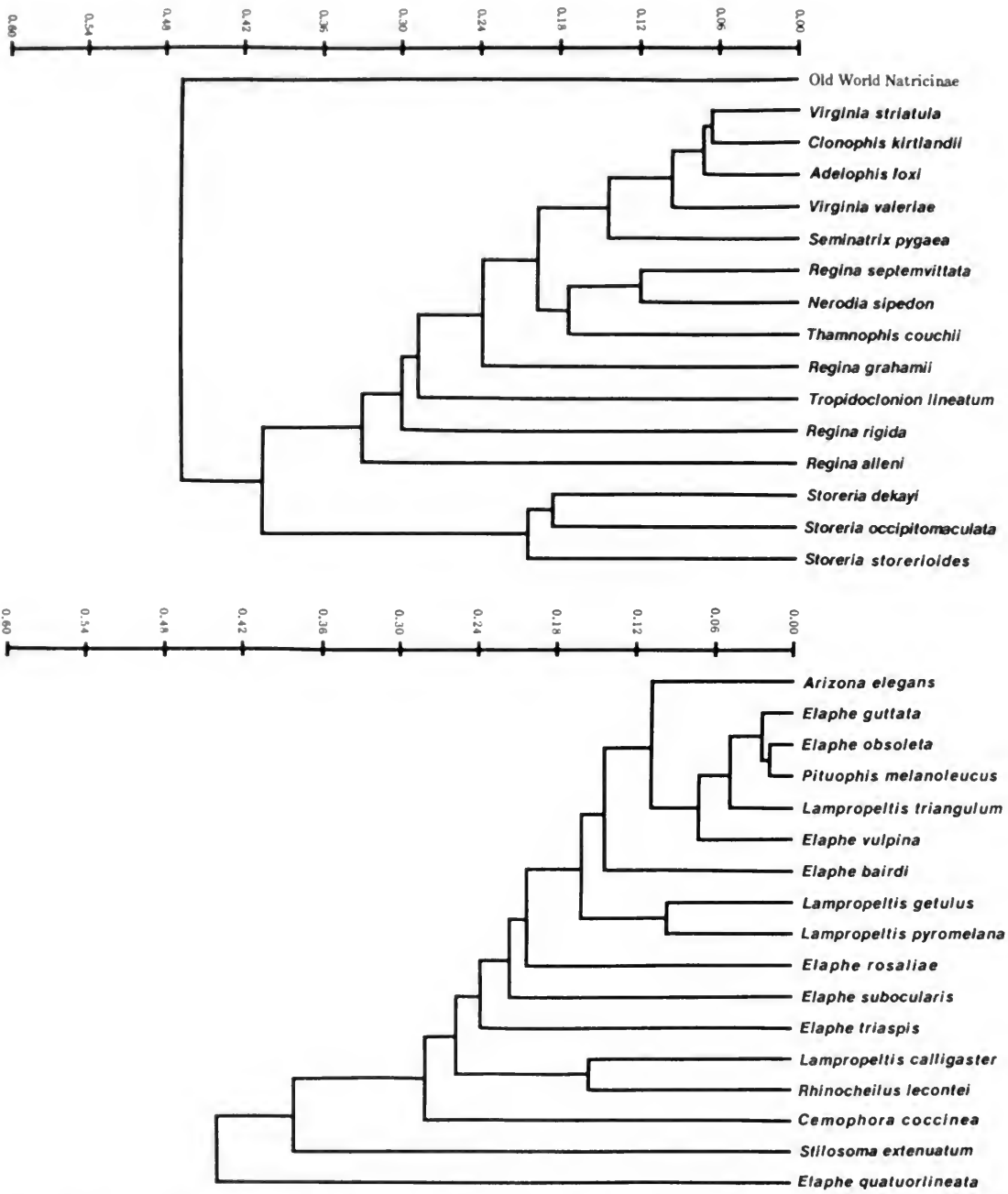


FIG. 6. Genetic differentiation among species within North American colubrid radiations. These are UPGMA phenograms (Sneath & Sokal, 1973) clustering Nei's unbiased genetic distances (Nei, 1978), which appear on the scale associated with each phenogram. These diagrams show only the relative degree of genetic differentiation among taxa and *should not* be interpreted as phylogenetic trees. **Top,** The New World natricine (Thamnophiini) radiation (Lawson, 1985); **bottom,** species of a North American colubrine radiation, along with Old World *Elaphe quatuorlineata* (Lawson & Dessauer, 1981).

Mao & Dessauer, 1971; Schwaner & Dessauer, 1982); IDs for albumins of 21 species ranged from 1 to 19,* with an average of 7 (Dowling et al., 1983). Chromosomal morphology of the different species is very similar (Baker et al., 1972; Eberle, 1972; Rossman & Eberle, 1977). The phenogram (fig. 6, top) showing relative degrees of genetic differentiation among the Thamnophiini is based upon an electrophoretic survey of 27 loci and also indicates the low degree of molecular divergence within this group. All species cluster within a Rogers's genetic distance of about 0.4 (Lawson, 1985).

2. North American Colubrines (fig. 6, bottom)—Molecular evidence is most extensive regarding the group that includes *Lampropeltis.* Minton and Salanitro (1972), using antiserum to plasma proteins of *Elaphe vulpina,* were unable to distinguish immuno-electrophoretic patterns of plasma proteins of *Elaphe vulpina, E. obsoleta, E. guttata, Pituophis melanoleucus, Lampropeltis getulus,* and *L. calligaster.* Immunodiffusion comparisons suggest that transferrins of species of these genera have IDs of less than 30 when compared to the transferrin of *Elaphe obsoleta* (Schwaner & Dessauer, 1982; Lawson & Dessauer, unpubl. data). Most MC'F IDs for albumins in these taxa, obtained with antisera raised to albumins of *Elaphe obsoleta* and *Lampropeltis getulus,* are less than 20 (Dowling et al., 1983). The organisms within this radiation are so close genetically that electrophoretic evidence was needed to assess affinities of individual species (fig. 6, bottom; Lawson & Dessauer, 1981, unpubl. data).

All North American colubrines examined, with the exception of *Elaphe subocularis,* have very similar karyotypes (Baker et al., 1972; Bury et al., 1970); even the unique karyotype of *E. subocularis* may be derived from the common colubrine pattern (Baker et al., 1971). Numerous instances of hybridization between New World *Elaphe guttata* and *E. obsoleta* in captivity and in the wild have been recorded (see Neill, 1949; Mertens, 1950; Lederer, 1950). On the other hand, an interspecific mating between *E. obsoleta* and Old World *E. schrenckii* produced an inviable clutch (Bröer, 1978).

3. Xenodontines (fig. 7)—Two groups of xenodontine snakes appear to comprise relatively recent radiations. One, the pseudoboines, comprising eight genera (*sensu* Bailey, 1967) and here excluding *Saphenophis* and *Tropidodryas* as proposed by Jenner and Dowling (1985), is molecularly the most cohesive and geographically one of the most widespread groups of South American xenodontines (Cadle, 1984a). Immunological comparisons of albumins and transferrins and multilocus electrophoretic comparisons (fig. 7, top) suggest that these snakes share a long period of common ancestry relative to other South American xenodontine genera. Despite the recent separation among these genera, they have radiated into habitats ranging from rain forests to savannas and deserts; representatives of four genera, *Clelia, Oxyrhopus, Tripanurgos,* and *Siphlophis,* have dispersed from South America into Central America (Cadle, 1985). This considerable geographic and habitat distribution has been achieved without extensive speciation (approximately 25 to 30 species among eight genera). There is some morphological diversity in body size and form, dentition, and skull structure (Bailey, 1939, 1967).

A different situation (fig. 7, bottom) is found in Central American xenodontines in which four "dipsadine" genera, *Dipsas, Sibon, Tropidodipsas,* and *Sibynomorphus,* plus *Ninia* and *Geophis,* form a closely related clade (Cadle, 1984b); all albumin IDs are less than 25. For such closely related genera, and in contrast to the pseudoboines, this group is remarkable for its diversity of morphological specializations: most *Geophis* species are modified for a fossorial existence, whereas *Ninia, Sibynomorphus,* and some *Tropidodipsas* are terrestrial, and *Sibon* and *Dipsas* are arboreal specialists. Dipsadines have developed various specializations related to their gastropod-feeding habits. For reviews of these feeding and habitat specializations see Downs (1967) and Peters (1960). This group provides the best example to date among snakes of the extraordinary morphological changes that may accrue with little molecular change among species. Indeed, the morphological specializations found in more highly modified species of *Dipsas* are extreme for snakes, and their origin has long been recognized as an intriguing evolutionary problem (Dunn, 1951). In addition, this group is very speciose (approximately 100 species) and has an extensive geographic distribution, encompassing the entire range of the Central American xenodontines (Cadle, 1985). From the biochemical data we may infer that the fossorial, arboreal, and trophic specializations of this group probably have arisen within the last eight to 15 million years (Cadle, 1982a).

4. Australian Elapids/Hydrophiids—Rapid morphological evolution has characterized the

* Exclusive of *Thamnophis mendax.*

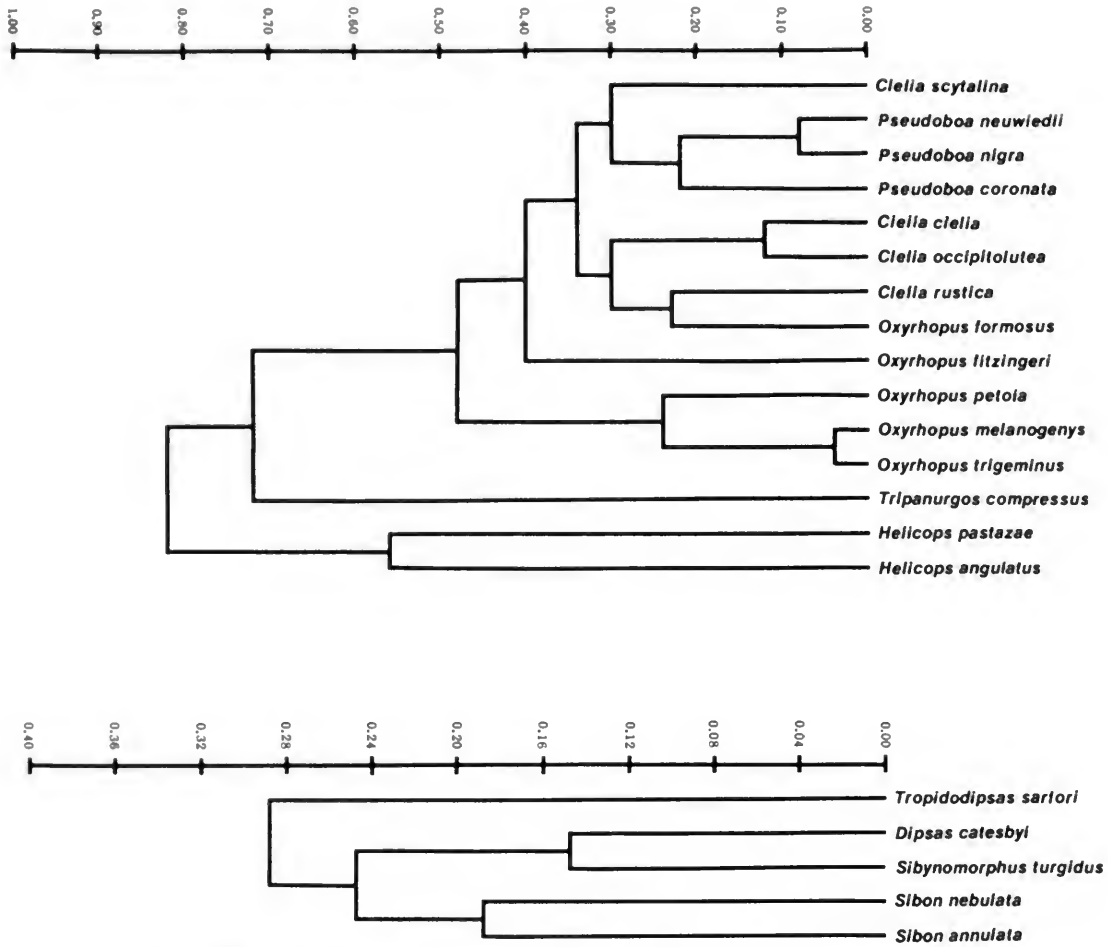


FIG. 7. Genetic differentiation among species within two xenodontine radiations. These are phenograms prepared as in Figure 3. **Top,** The South American pseudoboine radiation (Cadle & Dessauer, 1985); **bottom,** South and Central American dipsadines (Lawson, unpubl. data; see also Cadle, 1984b).

elapid snakes of Australia. Their radiation appears to have followed an invasion of precursors from Asia, perhaps beginning in the middle Miocene. Within Australia, species in 16 genera related to the tiger snake genus *Notechis* appear to comprise a remarkable radiation of even more recent origin. Immunological distances among transferrins of species within the group range between two and 20. As compared to the transferrin of *Notechis*, those of *Austrelaps, Echiopsis, Hemiaspis, Hoplocephalus, Suta, Tropidechis,* and *Unechis* have IDs of less than 10, differences close to the limit of sensitivity of the MC'F method and indistinguishable by immunodiffusion analysis. If species with transferrin IDs below 20 are included, members of *Cryptophis, Furina, Parademansia, Simoselaps, Vermicella,* and some species of *Drysdalia* and *Denisonia* also belong to the *Notechis* radiation (Schwaner et al., 1985). Branching sequences based upon preliminary electrophoretic analysis of tissue proteins are broadly concordant with the transferrin evidence (Mengden, 1985a). The close relationship of *Denisonia* and *Notechis* was suggested by Kellaway and Williams (1931) in one of the first comparative immunological studies. Divergence in karyology (Mengden, 1985a), behavior, ecology (Schwaner, 1985), and external morphology is very great within the presumptive tiger snake radiation. Terrestrial forms alone are presently classified as 32 species in 15 genera (Cogger, 1975), exclusive of the species of *Denisonia* and *Drysdalia* that have transferrin IDs above 20.

The protein evidence also shows that sea snakes, which are generally classified either as a subfamily

of the Elapidae or as a distinct family, the Hydrophiidae, are members of the Australian radiation of elapids (Minton & Da Costa, 1975; Minton, 1978; Cadle & Gorman, 1981; Mao et al., 1983), with at least some species possibly being members of the tiger snake group. The transferrin IDs between *Notechis* and sea snakes of two of the most speciose genera, *Aipysurus* and *Hydrophis,* are less than 20. The low albumin and transferrin IDs between sea snakes and Australian terrestrial elapids suggest that the morphological diversity within and between these two groups has arisen rapidly in geological time (Schwaner et al., 1985).

B. Taxa Illustrating Convergence or Slow Morphological Evolution

In contrast to those snake lineages showing great degrees of morphological differentiation relative to molecular divergence, others provide examples of conservative morphological evolution or homoplasy. We do not distinguish the latter two processes here, since more detailed phylogenetic hypotheses are required to assess their relevance. Our examples derive from the use of molecular data to evaluate systematic arrangements of particular snake taxa. Most cases involve genera that were traditionally considered monophyletic, but whose para- or polyphyletic nature was demonstrated by biochemical data. Presumably, convergence or retention of primitive character states were responsible for the inability of classical taxonomic procedures to partition these genera into natural units.

The most thoroughly documented example of the application of protein taxonomy to such a problem concerns the species composition of nominal genus *Natrix.* Prior to Malnate's (1960) study, 86 species were included in what he called this "unwieldly and confusing assemblage." Although external morphology of these snakes was too similar to classify them effectively, by focusing attention on tooth and hemipenial structures Malnate was able to partition the 86 species into five genera. With 26 species retained in *Natrix* (*sensu* Malnate, 1960), the genus still included snakes of North America, Asia, Europe, and Africa.

Immunological comparisons of transferrins proved that the placement of species from these different regions in the same genus was artificial. Immunological distances between the transferrins of species from the four regions were relatively great, ranging between 40 and 61. In contrast, those between species from the same region averaged about 10 and ranged from 2 to 23. Transferrins of the North American species, in fact, were more similar to those of other North American genera of natricines than to those of *Natrix* from other continents (George & Dessauer, 1970; Mao & Dessauer, 1971; Gartside & Dessauer, 1977; Schwaner & Dessauer, 1982).

Based upon the transferrin evidence, serological analyses of unfractionated plasma proteins (Pearson, 1966; Minton, 1976), karyological findings (Bury et al., 1970; Baker et al., 1972; Eberle, 1972), and several sets of morphological characters, Rossman and Eberle (1977) repartitioned the assemblage, retaining species from Europe and North Africa in *Natrix,* placing those from North America in *Nerodia* as a member of the tribe Thamnophiini, those from Asia in *Sinonatrix,* and those from Africa south of the Sahara in *Afronatrix.* Albumin immunological evidence (Dowling et al., 1983) and electrophoretic evidence on numerous proteins (Lawson, 1985, fig. 2a) have furnished additional support for considering thamnophiine snakes as a natural group.

Comparative protein studies suggest that the colubrine genus *Elaphe* is also not monophyletic. Currently, the more than 50 species assigned to the genus are found in North America, Europe, Asia, and the East Indies. Antiserum to plasma proteins of North American *Elaphe obsoleta* reacts more strongly with sera of other North American colubrine genera than with sera of Eurasian congeners (Minton, 1976). Immunological distances between the albumins of the different North American species of *Elaphe* and the North American species of *Cemophora, Lampropeltis,* and *Pituophis* averaged 16, whereas the ID between the North American *Elaphe* and *E. radiata* of Southeast Asia equalled 50 (Dowling et al., 1983). Immunodiffusion analyses, using antisera to transferrins of reference species from North America, Europe, and Asia, also attest to the wide divergence of forms from the different zoogeographical regions. Large spurs formed in cross-reactions involving serum and antiserum samples of species from the different regions (Lawson & Dessauer, unpubl. data).

Electrophoretic evidence on proteins determined by 15 structural gene loci also attest to the non-monophyletic nature of *Elaphe,* in concordance with the immunological findings. Genetic distances distinguishing North American species of *Elaphe* and other North American colubrine genera are usually less than those distinguishing New World and Old World *Elaphe* (see fig. 6,

bottom). Nei genetic distances between *Elaphe* from different geographic regions ranged from 0.24 to 0.78, the Asian *E. moellendorffi* being the least distinct and *E. scalaris* of Southern Europe the most distinct from *E. obsoleta* (Lawson & Dessauer, 1981; unpubl. data).

The genus *Coluber* is another unnatural assemblage of New and Old World species that has defied partition. The external morphological characters used traditionally in classification are either too uniform or too variable to offer useful character states for developing a more natural classification. Schätti (1985), in a preliminary report, noted that his observations on osteology, anatomy, and protein electrophoresis support the separation and distinction of Old and New World species and clearly demonstrate the polyphyletic nature of Palearctic *Coluber*. Serological analyses also show that *Coluber constrictor* of North America is more closely related to the North American colubrine genera *Masticophis* and *Drymarchon* than to *Coluber jugularis* of Israel (Minton, 1976).

Other examples where molecular data have suggested or confirmed the polyphyletic nature of genera include *Rhadinaea*, a widespread and speciose genus of Neotropical xenodontines. Using albumin immunological comparisons, Cadle (1984b) showed that members of the *R. brevirostris* species group were derived from South American xenodontines, whereas other species of *Rhadinaea* stem from a Central American stock. This provided strong evidence for earlier suspicions of their independent origins based upon morphological evidence (Myers, 1974).

Molecular data also suggest that several morphologically similar genera of the xenodontines are not close relatives. These include the rather distant relationship of *Geophis* to *Atractus*, despite considerable convergence in morphological features related to fossoriality (Downs, 1967; Cadle, 1984b), and the very distant relationship between *Heterodon* and *Xenodon* (Cadle, 1984a; unpubl. data), which are also similar in many aspects of morphology (e.g., Weaver, 1965).

C. Implications of Differential Rates of Evolution in Snakes

The examples discussed indicate that lineages of snakes vary considerably in rates of speciation and morphological evolution. Conservatively, we estimate that the lineages discussed here arose in the Middle Miocene or later, with much of the evolution within groups such as the thamnophiines and Australian elapid/sea snake radiations having occurred since the late Miocene. The reasons why particular lineages may show either rapid morphological evolution or stasis has been an important problem in evolutionary biology (Simpson, 1953). Rapid morphological evolution has usually been attributed to alterations in the control of gene expression or to changes in the sequence or timing of developmental events (Wilson et al., 1977; Alberch et al., 1979). The incorporation of changes produced by these mechanisms is influenced by selective pressures and aspects of population structure and history (e.g., see Larson, 1984). There has been little investigation of these parameters in snakes (but see Haluska & Alberch, 1983, for a possible example of heterochronic change). A fruitful area for future research on mechanisms of evolutionary change in snakes will be to analyze the distribution and developmental basis of morphological features in lineages for which detailed phylogenetic data are available.

In some groups of organisms, rates of chromosomal evolution are correlated with rates of speciation and morphological evolution (Wilson et al., 1977; Larson et al., 1984). This correlation appears to be only weakly supported in snakes. Although karyotypic evolution is slow in snakes as compared to many other vertebrates (Wilson et al., 1975), there is some variability in rates among snakes. The remarkable diversity of karyotypes observed among species of the Australian elapid radiation is accompanied by high rates of speciation and morphological evolution (Mengden, 1985a,b). On the other hand, karyotypes within North American natricines and colubrines, respectively, are extremely uniform (Baker et al., 1972; Eberle, 1972; Rossman & Eberle, 1977) despite a diversity of species and morphological types. Thus, superficially, there appears to be only a tenuous association between gross karyotypic evolution and evolution at the species level. Finer resolution of chromosomal structure and broader sampling among lineages will be necessary to evaluate this association more fully.

Based on the few lineages that have been extensively studied biochemically, rapid morphological divergence appears to occur commonly in snakes. Examples such as the Australian terrestrial elapids/sea snake radiation and the Central American genera allied to *Dipsas* demonstrate that dramatic trophic and habitat adaptations may occur among closely related forms during relatively brief periods of evolutionary time. This observation is

perhaps one reason why it has proven so difficult to estimate phylogenetic relationships among snakes; derived characters linking various taxa may be transformed rapidly into new states. Without detailed knowledge of character state transformations, which depends on an estimated phylogeny (see Lauder, 1981; Alberch, 1985), the use of such characters in phylogenetic reconstruction is difficult. Compounding the difficulties is an apparently high degree of homoplasy in snake morphology (Cadle, 1982a).

We believe that biochemical evidence, although not free of interpretative problems, can circumvent some of the difficulties inherent in the use of morphological data to reconstruct snake phylogeny. Ultimately, of course, any worthwhile phylogenetic hypopthesis must be evaluated with respect to all comparative data. For the remainder of this paper we discuss the comparative biochemical data bearing on snake systematics and phylogeny above the generic level.

VI. Higher Levels of Relationship

A. Position of Snakes Among Reptiles

Biochemical data support the traditional view that lizards and snakes, comprising the Order Squamata, are closest relatives among extant reptiles (see Dessauer, 1974). Serologists since Graham-Smith (1904) have obtained weak immunological cross-reactions in tests involving proteins from lizards and snakes but little or no cross-reaction in tests involving proteins of members of the Squamata and other orders of reptiles. Using MC'F, Gorman and colleagues (1971) demonstrated that heart lactate dehydrogenases of lizards and snakes were much more similar than were the lactate dehydrogenases of lizards compared to those of *Sphenodon,* crocodilians, turtles, or birds. Similarly, fingerprints of tryptic peptides of the hemoglobins of snakes and some lizards (e.g., *Iguana*) share numerous similarities, whereas few comparable peptide fragments of snake hemoglobins are detectable on hemoglobin fingerprints for crocodilians or chelonians (Sutton, 1969; Dessauer, 1974). Qualitative differences in the metabolic pathways involved in bile acid synthesis (Haslewood, 1978; Tammar, 1974) and nitrogen metabolism (Cohen & Brown, 1960) also distinguish the Squamata from other reptilian orders.

Although biochemical studies show that snakes and lizards are more closely related to each other than to other reptiles, these studies have contributed little evidence on the precise relationship among lizards and snakes (Dessauer, 1974). That is, does the divergence between lizards and snakes predate the separation of extant lineages within either of these groups, or are snakes derived from a particular lineage of lizards (e.g., the Anguimorpha; McDowell & Bogert, 1954)? Using antisera to snake albumins we have recently compared various lizard albumins in enhanced Ouchterlony double-diffusion tests (see sec. II). Strong cross-reactions were obtained with albumins of iguanids (fig. 8, sample Cr), anguids, amphisbaenids, and *Heloderma* (fig. 8, sample H); weaker reactions with albumins of agamids and teiids; and no reaction with albumins of *Varanus* (fig. 8, sample V), skinks, xantusiids, cordylids, chamaeleonids, pygopodids, or gekkonids. Because of the lack of rate tests for these albumins, results such as the strong reactions for *Gerrhonotus* and *Heloderma* and no reaction for *Varanus* (fig. 8, middle), all of which are anguimorphs, are difficult to interpret at present. The differential reaction of snake albumins with various lizard groups could reflect either variations in rates of albumin evolution among groups, or differences in their phylogenetic relationships. Because it is possible to obtain such cross-reactions, immunological and other molecular methods promise to offer valuable insights on the relationships of snakes and lizards.

Although the evidence is not clear on the relative placement of lizards and snakes, comparable molecular evidence supports the monophyletic status of snakes. In cross-reactions involving antisera to albumins of snakes, such as those for *Leptotyphlops* and *Boa* (fig. 8, samples L and B), precipitin arcs for snake albumins spur over reactions for lizard albumins, showing albumins of snakes to be more similar to each other than to albumins of lizards. This is true even for *Typhlops,* which McDowell and Bogert (1954) have considered to be a lineage distinct from snakes (fig. 8, sample T over Cr).

B. Relationships Within and Between Major Groups of Snakes

Snakes are divided into two major monophyletic groups, the Scolecophidia and Alethinophidia (McDowell, 1974; Rieppel, 1979a). The latter group includes both the Henophidia and the Caenophidia of Underwood (1967). Beyond this area

FIG. 8. Enhanced Ouchterlony double-diffusion tests of albumins. Central wells contain antiserum to albumins of: **top,** *Leptotyphlops humilis*; **center,** *Boa constrictor*; and **bottom,** *Rhinophis phillippinus*. Peripheral wells contain plasma of: L = *Leptotyphlops humilis*; T = *Typhlops* (*Rhamphotyphlops*) *braminus*; Cr = *Crotaphytus collaris*; Ag = *Agkistrodon bilineatus*; H = *Heloderma suspectum*; V = *Varanus varius*; G = *Gerrhonotus multicarinatus*; R = *Rhinophis phillippinus*; P = *Pseudotyphlops phillippinus*; Cy = *Cylindrophis rufus*; B = *Boa constrictor*; An = *Anilius scytale*; U = *Uropeltis liura*.

of comparative agreement, taxonomists have many different opinions concerning further taxonomic subdivisions, as well as on the generic composition and inter-relationships of major subdivisions. The monophyly of the Henophidia has also been questioned (see Groombridge, 1979a; McDowell, 1987; Cadle, 1987). Protein studies are offering new insights on such problems. Definitive evidence on xenodontine, natricine, and colubrine snakes illustrates the potential of molecular approaches for studies at the suprageneric level. Less conclusive but suggestive molecular data are also becoming available regarding affinities within and between other groups of advanced snakes and lineages of primitive snakes.

Many major unsolved problems in snake phylogeny concern the relationships among major clades. Although the molecular data available at this point are capable of resolving more recent separations among genera and, in some cases, subfamilial groups, there is a paucity of evidence bearing on the branching order of more ancient separations. At that level the resolving power of the most widely used molecular techniques is limited because few shared derived states at the amino acid level are likely to be conserved for such long periods of evolutionary time (Sarich, 1985). For these reasons, we feel that it is premature to present a phylogenetic tree estimating relationships among major clades. Concerted efforts are currently underway to gather such data. Thus, the discussions that follow concentrate on relationships within major clades and reflect our current interpretation of data bearing on intergroup relationships.

Immunological evidence on plasma proteins shows that the Scolecophidia (blind snakes), Henophidia (primitive snakes), and Caenophidia (advanced snakes) are the result of ancient radiations. Precipitin tests involving plasma proteins show low levels of cross-reactivity when antigens from a species of one infraorder are tested with antibodies raised to the plasma proteins of a member of a different infraorder (Graham-Smith, 1904; Pearson, 1968; Cadle, 1982a; Schwaner & Dessauer, 1982). In enhanced Ouchterlony tests using antisera to the albumins of *Boa* and *Rhinophis* (Henophidia), weak reactions were obtained with albumins of *Leptotyphlops* (Scolecophidia; fig. 8, sample L), *Boa* and *Cylindrophis* (fig. 8, samples B and Cy), and *Agkistrodon* (Caenophidia; fig. 8, sample Ag). Quantitative precipitin tests also suggest that the Henophidia (represented by *Boa* and *Python*) are more closely allied to the Caenophidia

than to the Scolecophidia (Pearson, 1968). Transferrins of species of the three infraorders are so different that they do not form precipitin lines in immunodiffusion tests with antibodies raised to the transferrin of a member of another infraorder (Schwaner & Dessauer, 1982).

1. SCOLECOPHIDIA—Comparative immunological evidence suggests that the Leptotyphlopidae and Typhlopidae form a lineage relative to other snakes, consistent with their grouping in the Scolecophidia. Pearson (1968), with whole-serum precipitin tests, found that proteins of the Typhlopidae have only weak affinities to those of boids, pythons, colubrids, and viperids. Antiserum to *Leptotyphlops* albumin in enhanced Ouchterlony immunodiffusion tests, reacts strongly with albumin of *Typhlops,* but yields only weak reactions with albumins of other snakes (fig. 8, top). In all comparisons of *Leptotyphlops* albumin with henophidian albumins, the *Typhlops* precipitin arc spurs over that for the henophidians. The precipitin line for *Leptotyphlops* albumin also spurs strongly over the arc for *Typhlops* albumin (fig. 8, top), illustrating that within the Scolecophidia the molecular divergence between the Typhlopidae and Leptotyphlopidae is substantial.

2. HENOPHIDIA—**a. Boidae/Pythonidae/Tropidophiidae**—Immunological comparisons of plasma proteins suggest that the Henophidia is a complex of ancient lineages. Immunological distances between albumins of a selection of henophidians, obtained with antiserum to the albumin of *Boa,* are given in Table 2. Rather than interpreting these data phylogenetically, as they have not been rate tested, we simply make the following observations: (1) albumin IDs within this group approach the technical limits of the MC'F technique, implying very ancient, perhaps Cretaceous, separations between several lineages; and (2) relative to *Boa,* several presumptive associations do not reflect the current systematic arrangement of these taxa. For example, the MC'F data suggest that the albumins of *Boa* and *Exiliboa* are more similar to each other than are those of *Boa* and *Tropidophis. Exiliboa* and *Tropidophis* are currently placed together in the family Tropidophiidae (McDowell, 1975). Also, the albumins of *Cylindrophis* and *Boa* are more similar to each other than are those of *Boa* and *Python. Cylindrophis* is classified either in the Aniliidae (Underwood, 1967) or the Uropeltidae (McDowell, 1987; Rieppel, 1979b), whereas *Boa* and *Python* are usually placed together in the Boidae (Underwood, 1967; but see Groombridge, 1979b, and McDowell, 1979, 1987).

TABLE 2. Immunological distances between the albumin of *Boa constrictor* and other henophidian albumins. The classification follows McDowell (1987).

Albumin	Antiserum to *Boa* albumin
Boidae (Booidea)	
Boa constrictor	0
Epicrates cenchria	37
Corallus caninus	58
Pythonidae (Booidea)	
Python molurus	135
Tropidophiidae (Tropidophoidea)	
Exiliboa plicata	54
Tropidophis greenwayi	126
Tropidophis haetianus	113
Uropeltidae (Anilioidea)	
Cylindrophis rufus	94
Loxocemidae (Anilioidea)	
Loxocemus bicolor	169
Acrochordidae (Acrochordoidea)	
Acrochordus javanicus	152

Precipitin tests involving plasma proteins (Pearson, 1966, 1968) and immunodiffusion studies of transferrins and unfractionated plasma proteins (Schwaner & Dessauer, 1981) also document the wide separation of boas and pythons. Both albumin IDs (table 2) and immunodiffusion evidence on plasma proteins show that the New World genera *Epicrates, Corallus, Charina,* and *Lichanura,* as well as *Candoia* of New Guinea, group closer to *Boa* than to *Python. Candoia,* however, is widely divergent from the New World boas (Schwaner & Dessauer, 1981).

b. Uropeltidae/Aniliidae—Molecular studies are supplying evidence on affinities of the Uropeltidae. Tests with antisera to albumins of *Rhinophis* and *Cylindrophis* appear to confirm a distant association of these genera, a conclusion consistent with some recent morphological evidence (Rieppel, 1979b; McDowell, 1987). Electrophoretic patterns of tissue proteins (Dessauer et al., 1976) and comparisons with these antisera also suggest that the uropeltids are a rather compact radiation. Immunodiffusion reactions with the *Rhinophis* antialbumin yield at most only weak spurs in cross-reactions with albumins of species of *Rhinophis, Pseudotyphlops,* and *Uropeltis* (fig. 8, samples R, P, and U). In contrast, the albumin of the aniliid genus *Anilius* reacts only weakly with the antialbumins of both *Rhinophis* (fig. 8, sample An) and *Cylindrophis.* Although the biochemical evidence suggests that *Anilius* is widely divergent from the

uropeltids, it is not yet clear whether or not *Anilius* shares a common lineage with the uropeltids relative to other primitive snakes.

c. Acrochordidae—Immunological comparisons confirm the distinctness of *Acrochordus* relative to other snakes, although current data offer no insight on whether or not *Acrochordus* is a sister group of colubroids (Groombridge, 1979a,b; Rieppel, 1979a). Using an antiserum to *Acrochordus* albumin, *Agkistrodon* (which has a conservative albumin) spurs over albumins of some henophidians (e.g., *Python, Tropidophis, Loxocemus*) but not others (*Boa, Cylindrophis*). The precise placement of *Acrochordus* among these lineages can be ascertained once relationships among henophidian lineages are better understood. We specifically reject hypotheses associating *Acrochordus* with either natricine or homalopsine colubrids (e.g., Dowling & Duellman, 1978; Dowling et al., 1983). In tests with antisera to transferrins of natricine and colubrine snakes, transferrins of *Acrochordus* gave MC'F IDs greater than 115 (George & Dessauer, 1970) and produced no detectable precipitin bands in Ouchterlony immunodiffusion analyses (Schwaner & Dessauer, 1982). Only weak immunodiffusion reactions were detectable between albumins of *Acrochordus* and antisera to albumins of species of natricines and homalopsines. In enhanced Ouchterlony tests using antiserum to *Acrochordus* albumin, only weak reactions were obtained with albumins of *Rhabdophis* (Natricinae) and *Homalopsis* (Homalopsinae), and the precipitin arc for *Boa* albumin spurred over both of these.

d. Conclusions—Collectively, the molecular evidence shows that the Henophidia is composed of a number of widely divergent lineages. Even a cautious interpretation of the data reveals several conclusions that are not concordant with current classifications. For example, the very large molecular distances between boas and pythons and relatively less between boas and some aniliids (*Cylindrophis*) are not predicted by most classifications. There exists much disagreement among taxonomists concerning the definition and composition of taxa within the Henophidia (see Rieppel, 1977, 1979a; Groombridge, 1979b; McDowell, 1987). Groups such as the Boidae of Underwood (1967, 1978) are placed together because of primitive morphological features. As knowledge of molecular evolution in these primitive snake groups increases, we expect that a reevaluation of many accepted phylogenetic hypotheses for the henophidians will be necessary and that this group will be recognized as a paraphyletic taxon, as suggested by Groombridge (1979b).

3. CAENOPHIDIA—Molecular comparisons involving all groups of advanced snakes (Viperidae, Elapidae, *Atractaspis,* and Colubridae) include whole-serum precipitin studies of Graham-Smith (1904) and Pearson (1966, 1968), MC'F comparisons of albumins (Cadle, 1982a,b), and an immunodiffusion survey of transferrins (Schwaner & Dessauer, 1982). These studies corroborate the monophyly of the colubroids and suggest that the Viperidae is the sister group of the three other clades (Cadle, 1982a; unpubl. data). In the following sections we discuss molecular data bearing on relationships within each of these groups.

a. Viperidae—Biochemical evidence on viperid relationships includes an immunoelectrophoretic study of venom proteins (Detráit & Saint-Girons, 1979), observations on bile acid synthetic pathways (Haslewood, 1978; Tammar, 1974), and immunological comparisons of plasma proteins (Kuwajima, 1953) and albumins (table 3). Current data are consistent with the view that the Viperinae and Crotalinae are monophyletic sister groups (Liem et al., 1971; Groombridge, 1979b, 1984). These two groups can be distinguished on the basis of bile acid synthetic pathways (Tammar, 1974) in which crotalines show a pattern common to many advanced snakes, whereas viperines are characterized by acids almost restricted to this group.

The interpretation of the albumin immunological evidence (table 3) is complicated by the fact that the rate of albumin evolution appears to be variable within vipers. For example, relative to the albumin of an outgroup represented by *Boa,* the albumin of *Bitis* has changed about 34 ID units more than that of *Crotalus* (table 3). Differential rates of albumin evolution are also evident in precipitin tests involving unfractionated serum, in which albumin-antibody complexes compose much of the precipitate (Pearson, 1966, 1968), and in MC'F tests using another outgroup, *Atractaspis* (table 4). Using the rate test, most of the albumin divergence between *Bitis* and *Crotalus* is attributable to the *Bitis* lineage. Not only do rates of albumin evolution appear to be variable within vipers, but as a group they have more conservative albumins than elapids, colubrids, and *Atractaspis* (table 3; Cadle, 1982a).

Additional reciprocal comparisons and rate tests for albumins from a wider selection of vipers are needed to support statements on intraviperid re-

TABLE 3. Immunological distances and rate tests concerned with the albumins of the Viperidae. The rate tests for advanced snake albumins used an antiserum to *Boa* albumin as an outgroup and are expressed as immunological distances relative to *Crotalus enyo* = 0. Note the marked conservatism shown by viperid albumins relative to those of elapids and colubrids.

Albumins	Antisera: Crotalinae — *Crotalus* albumin	Antisera: Crotalinae — *Bothrops* albumin	Antisera: Viperinae — *Bitis* albumin	Rate tests
	RECIPROCAL COMPARISONS			
Crotalus enyo	0	22	66	0
Bothrops atrox	24	0	76	− 2
Bitis nasicornis	72	79	0	+ 34
	UNIDIRECTIONAL COMPARISONS			
Viperinae	...			
Bitis arietans	...	77	70	...
Vipera aspis	...	53	77	+ 25
Vipera palestinae	...	62	67	...
Causus resimus	64	62	89	...
Causus maculatus	...	75	111	...
Echis ocellatus	47	51	70	+ 16
Echis coloratus	...	44	76	+ 14
Pseudocerastes fieldii	...	38	47	...
Cerastes cerastes	...	40	74	...
Crotalinae				
Lachesis muta	30	32	92	+ 4
Sistrurus catenatus	16	20	83	...
Sistrurus miliarius	...	33	96	+ 8
Agkistrodon piscivorus	...	28	93	...
Agkistrodon contortrix	...	34	...	...
Agkistrodon bilineatus	...	39	98	+ 19
Elapidae (5 species)	...	...	...	+ 86 ± 7
Colubridae (7 species)	...	...	...	+ 65 ± 16

lationships. It is apparent, however, from available comparisons that within crotalines *Sistrurus* and *Crotalus* are close relatives, and that divergence among the extant genera began early in the history of the lineage. Although the phylogenetic interpretation is not clear, Detráit and Saint-Girons (1979) determined that venoms of the African viperine genera *Bitis, Echis,* and *Cerastes* share more antigens with each other than with European *Vipera,* suggesting that there may be a basic phyletic separation between African and Eurasian viperine genera (Cadle, 1987). Without comparisons using additional antisera and rate testing, we cannot evaluate this hypothesis with respect to current albumin data (table 3).

b. Elapidae—Molecular evidence on elapid phylogeny comes from a variety of sources, including MC'F and immunodiffusion comparisons of transferrins (Mao et al., 1977; Schwaner et al., 1985), MC'F comparisons of albumins (Cadle & Sarich, 1981; Cadle & Gorman, 1981; Mao et al., 1983), peptide fingerprinting of hemoglobins (Mao et al., 1978, 1984), and venom protein sequences and antigenic structure (Strydom, 1973, 1979; Minton & Da Costa, 1975; Hseu et al., 1977; Coulter et al., 1981; Dufton, 1984; Tamiya, 1985; Mebs, 1985). Most of these studies have addressed questions concerning the phylogenetic position of sea snakes among elapids, and the relationships of the endemic Australian forms.

The extensive body of albumin and transferrin immunological comparisons and studies of venom proteins strongly support the close relationship between sea snakes (Laticaudinae + Hydrophiinae) and Australopapuan terrestrial elapids; in addition they suggest that these are derived clapid lineages. It is less clear as to whether the two sea snake groups were derived independently from different groups of terrestrial elapids (McDowell, 1969). Cadle and Sarich (1981), Cadle and Gorman (1981),

and Mao et al. (1983) concluded that there was no special association between laticaudines and New World *Micrurus* (McDowell, 1967, 1969), since the albumins of these groups were more distinct from one another than were albumins of laticaudines, hydrophiines, and Australian terrestrial elapids. Schwaner and colleagues (1985) interpreted transferrin immunological data as supporting a possible derivation of hydrophiines from the *Notechis* group of Australian elapids (sec. V.A.), whereas laticaudines were derived independently from an unspecified lineage. However, the transferrin immunological data have not been rate-tested, and the association between *Notechis* and hydrophiines could be due to rate differences among transferrins of these groups (as suggested for *Notechis* in fig. 2, the unrooted tree of Schwaner et al., 1985). In the absence of rate test data, the problem of independent origins for the two sea snake groups cannot be resolved.

Although all molecular studies confirm the association of sea snakes with Australopapuan terrestrial elapids, there has been little attention directed to the rest of the Elapidae. The phylogenetic position of New World coral snakes (micrurines) within the Elapidae was addressed by Cadle and Sarich (1981), and their general conclusions were supported by morphological studies (McCarthy, 1985). Subsequent immunological comparisons of albumins (Cadle, unpubl. data) have failed to demonstrate a close association between micrurines and specific Old World elapid groups, but many Old World lineages remain to be tested. Mao and his colleagues (Mao et al., 1977, 1978, 1983) have interpreted their albumin and transferrin immunological comparisons within a framework which, a priori, assumes a basic division of elapids into a "terrestrial" group and a "sea snake" group, the latter recognized in 1983 as including Australopapuan terrestrial elapids. Consequently, they concluded (Mao et al., 1983) that the albumin of *Naja* was highly divergent from those of other elapids (showing, for example, three times the rate of evolution of *Bungarus* albumin, see Mao et al., 1983, fig. 1). An elapid phylogeny constructed by using antisera to albumins of a variety of African and Asian elapids and sea snakes (Cadle, unpubl. data) shows that the assumption of "terrestrial" and "sea snake" groups is unwarranted; that is, the terrestrial elapids do not form a clade relative to the sea snakes. Our rate test data using appropriate outgroups (e.g., see table 4) indicate that, although albumin has changed somewhat more in *Naja* than in some other elapids, it has not changed to the degree suggested by Mao and colleagues. A tree analysis of albumin immunological comparisons using appropriate outgroups (Cadle, unpubl. data) shows that *Naja* is a derivative of an ancient lineage from the common elapid stock, and thus its albumin and transferrin are very dissimilar from those of other elapids.

Sequence data for elapid venom proteins have not been used extensively in addressing problems of elapid phylogeny. Sequences vary considerably within and between species and are subject to extensive length mutations and gene duplications; also, their interpretation may depend on specific models of toxin evolution (Hseu et al., 1977; Dufton, 1984). Three major classes of elapid venom toxins are recognized: long and short neurotoxins, and cytotoxins. These classes are apparently related by gene duplication events, but the relationship among the three classes is not clear (Strydom, 1979; Hseu et al., 1977). Short and long neurotoxins are found in all elapids examined to date, whereas cytotoxins have thus far been found only in cobras of the genera *Naja* and *Hemachatus* (Hseu et al., 1977). This suggests that, primitively, a single duplication gave rise to the long and short neurotoxins, and these have been retained in all elapids. Subsequently, a further duplication occurred in the lineage leading to *Naja* and *Hemachatus* and gave rise to the cytotoxins. The cytotoxins have not yet been found in the king cobra (*Ophiophagus*), suggesting that it might belong to a separate lineage from the other cobras or that the gene duplication giving rise to the cytotoxins occurred after the divergence of *Ophiophagus* from other cobras. Independent albumin immunological evidence (Cadle, unpubl. data) shows that the former interpretation is more likely. *Naja* is a very early branch of the elapid lineage, whereas *Ophiophagus* is a much later lineage, more closely related to several other genera of Asian elapids.

c. Atractaspis—Hypotheses concerning the relationships of this enigmatic African genus were summarized by Cadle (1982b). Although *Atractaspis* was long considered to be an aberrant viper, more recent comparative anatomical studies have suggested that it is an "aparallactine" colubrid (Bourgeois, 1965; McDowell, 1987) or has elapid affinities (Kochva et al., 1967; Kochva & Wollberg, 1970). MC'F comparisons of albumins (table 4) allow us to reject an association between *Atractaspis* and viperids (Cadle, 1982a). These results show that viperids are among those advanced snakes most distant from *Atractaspis*. In view of the conservative nature of many viperid (partic-

ularly crotaline) albumins, one would expect the *Atractaspis*-viperid distances to be less than those to other advanced snakes if there were a phylogenetic association between these groups, but this is not the case. The separation of *Atractaspis* from the viperid clade is also demonstrated by a tree analysis of albumin immunological data involving all major lineages of colubroids (Cadle, 1982a).

We are less confident in offering a definitive statement on the phylogenetic position of *Atractaspis* at present, because of difficulties concerning the development of a molecular phylogeny for all colubroids (see Cadle, 1987, for discussion). The available albumin immunological data indicate no special relationship between *Atractaspis* and some aparallactines (*Amblyodipsas* and *Aparallactus*; Cadle, 1982b); but others remain to be tested, and the "Aparallactinae" may not be monophyletic (Cadle, 1982b; McDowell, 1987). Dowling and colleagues (1983) placed *Atractaspis* among the lycodontines on the basis of an albumin ID of 80 from *Madagascarophis*; however, this distance is typical of that between *Atractaspis* and many lineages of colubrids (Cadle, 1982a,b; unpubl. data) and does not specifically support a lycodontine association.

Among all taxa to which *Atractaspis* has yet been compared, there is a possibly remote association with the elapids (Cadle, 1983). The data are also consistent with an independent origin for these lineages at about the same time from a common colubroid stock that later also gave rise to colubrids (Cadle, 1982a). The elapid association is apparent in Table 4, where immunological distances between *Atractaspis* and elapids are lowest among all comparisons. That this association is not due to conservativeness of either elapid or *Atractaspis* albumins has been confirmed by relative rate tests (Cadle, 1982a). Notably, the possible phylogenetic association between elapids and *Atractaspis* is also indicated by certain aspects of venom composition (Minton, 1968; Parnas & Russell, 1967; Kochva et al., 1982), venom gland structure (Kochva et al., 1967; Kochva & Wollberg, 1970), and ectopterygoid shape (Lombard et al., 1986).

d. Colubridae—Unraveling relationships within this large group poses many difficulties. Because homoplasy in morphological characters appears to be rampant in colubrids, molecular data, which generally do not exhibit strong convergence, ultimately will help solve many of the more difficult phylogenetic problems. Currently, molecular data bearing on colubrid relationships consist of comparisons of albumins and transferrins by MC'F and immunodiffusion. It is clear that the amount of molecular change separating major clades (e.g., natricines, colubrines) is relatively small for these two proteins, thus making it difficult to sort the lineages into a series of dichotomous branches. For example, Cadle (1982a, 1984a), using MC'F comparisons of albumins, could not resolve a trichotomy involving colubrines and two xenodontine lineages. Dowling and colleagues (1983) provided an estimated phylogeny for several colubrid lineages based on MC'F comparisons of albumins. However, these data were not rate-tested using suitable outgroups, and involved few representatives of each lineage; thus, we question the details of branching order among major lineages presented in that work.

In general, members of the various colubrid lineages are separated by about 70 to 90 albumin ID

TABLE 4. Immunological distances between the albumins of *Atractaspis* and other advanced snakes, using an antiserum to *Atractaspis bibroni* albumin.

Albumins	Antiserum to *Atractaspis bibroni* albumin
Atractaspis bibroni	0
Atractaspis dahomeyensis	16
Atractaspis microlepidota	32
Crotalinae	
Crotalus enyo	94
Bothrops atrox	98
Lachesis muta	97
Sistrurus catenatus	91
Sistrurus miliarius	104
Agkistrodon piscivorus	86
Agkistrodon contortrix	82
Agkistrodon bilineatus	100
Viperinae	
Bitis arietans	132
Vipera aspis	106
Vipera palestinae	126
Causus resimus	117
Causus maculatus	113
Echis ocellatus	122
Echis coloratus	119
Cerastes cerastes	112
Pseudocerastes fieldii	95
Elapidae	
Micrurus spixi	74
Bungarus fasciatus	73
Laticauda semifasciata	72
Hydrophis melanosoma	72
Dendroaspis polylepis	79
Elapsoidea semiannulata	91
Naja haje	99

units and 100 or more transferrin ID units (Cadle, 1982a,b; George & Dessauer, 1970; Mao & Dessauer, 1971; Cadle & Dessauer, unpubl. data). This is estimated to represent between 30 and 60 million years of separation for the lineages (Cadle, 1982a, 1987). In the discussion below, we concentrate on those areas where molecular data have contributed substantially to the phylogenetic analysis of particular groups of colubrids. We do not review previously published data in detail.

Xenodontinae—Albumins of numerous species of xenodontines have been compared by MC'F (Cadle, 1984a,b,c); multilocus electrophoretic studies and quantitative immunological comparisons of transferrins are in progress (Cadle & Dessauer, 1985, unpubl. data). The albumin immunological results are consistent with immunodiffusion comparisons of transferrins (Schwaner & Dessauer, 1982). The biochemical data suggest a major dichotomy between two speciose Neotropical lineages (Central and South American xenodontine lineages, using terminology in Cadle, 1984a) and several essentially monotypic lineages that are well differentiated from the major lineages and from each other: the North American genera *Farancia, Heterodon, Carphophis, Diadophis,* and *Contia*; the Central American *Conophis*; and the South American *Hydrops* (Cadle, 1984c; unpubl. data). Members of these lineages differ on average by approximately 70 albumin immunological units. The molecular data were instrumental in unraveling relationships among genera within this complex group, and in interpreting historical biogeographic patterns in the Neotropics (Cadle, 1985). Molecular studies will likely contribute substantially to the resolution of two major phylogenetic problems that still exist for xenodontines: (1) Are the various xenodontine lineages monophyletic relative to other colubrid lineages; and (2) what is the sister group or groups of the xenodontines?

Lycodontinae/Boodontinae—There has been little agreement concerning relationships of snakes in these groups (e.g., compare Underwood, 1967; Dowling & Duellman, 1978; and McDowell, 1987). The molecular work that has been done on them indicates that, like the xenodontines, several ancient lineages are involved. Semiquantitative immunological comparisons of transferrins (Schwaner & Dessauer, 1982) and MC'F comparisons of albumins (table 5; Dowling et al., 1983) suggest that lycodontines may not be monophyletic. Clearly, the magnitude of albumin IDs within the lycodontines is equivalent to that between lycodontines and other colubrid lineages. These data are consistent with McDowell's (1987) view that the lycodontine/boodontine group includes many primitive snakes that are not clearly linked to one another by derived characters. Our present molecular data suggest that the phylogeny of the lycodontines will prove to be a complex series of lineages such as is seen in the xenodontines.

Homalopsinae—This is a morphologically distinctive radiation of primarily estuarine and aquatic snakes (Gyi, 1970; McDowell, 1987). Although they have sometimes been considered relatives of the natricines (e.g., Dowling & Duellman, 1978), biochemical studies show that they are an independent lineage (George & Dessauer, 1970; Schwaner & Dessauer, 1982; Dowling et al., 1983). Dowling and colleagues (1983) compared the albumins of *Erpeton* and *Enhydris* to *Thamnophis* and *Madagascarophis* by MC'F and found the IDs separating these (approximately $\geq$ 70) equivalent to those generally separating colubrid lineages. Thus, homalopsines are molecularly well differentiated, but their relationship to other colubrid lineages is as yet unclear. We specifically exclude *Acrochordus* from the Homalopsinae (sec. VI.B., 2c).

Natricinae—Relationships among natricines have been extensively studied using immunological techniques (Pearson, 1966, 1968; Mao & Dessauer, 1971; Schwaner & Dessauer, 1982; Gartside & Dessauer, 1977; Dowling et al., 1983), peptide fingerprinting (Sutton, 1969; Dessauer, 1974), and electrophoresis (Lawson & Dessauer, 1979; Lawson, 1985, 1986). These studies demonstrate the monophyly of North American genera (the Thamnophiini) relative to Old World forms (see sec. V.A.). The albumins and transferrins of thamnophiines are so similar when compared immunologically (Mao & Dessauer, 1971; Dowling et al., 1983) that electrophoretic approaches are proving more useful in working out details of their relationships (fig. 3, top; Lawson & Dessauer, 1979; Lawson, 1985, 1986, 1987). Among Old World genera, transferrin immunological comparisons (Mao & Dessauer, 1971; Gartside & Dessauer, 1977; Schwaner & Dessauer, 1982) show four major groups (*Natrix, Afronatrix, Sinonatrix,* and *Xenochrophis-Amphiesma-Rhabdophis*). The transferrin and albumin IDs separating these groups are about 50 to 60 and 40 to 50, respectively; however, the branching order among the major lineages is not resolved by currently available biochemical data. Numerous other genera from Asia and Africa are possible natricines (McDowell, 1987), but most of these have not been examined

TABLE 5. Immunological distances involving Lycodontine/Boodontine albumins.

Albumins	Antisera (1)	(2)	(3)	(4)
Lycodontinae/Boodontinae				
(1) *Lamprophis fuliginosus*	0	...	...	...
(2) *Rhamphiophis oxyrhynchus*	79	0	...	...
(3) *Amblyodipsas polylepis*	88*	121	0	...
(4) *Mehelya crossi*	57	89	100	0
Aparallactus capensis	†	†	91*	†
Colubrinae	71 (12)	87 (11)	88 (4)	82 (10)
Xenodontines				
South American	103	100	†	89
Central American	92	133	111	108

All intralycodontine comparisons are means of reciprocal titrations, except those marked with an asterisk (*), which are unidirectional comparisons. For the interlineage comparisons, numbers in parentheses give the number of comparisons which were averaged to give the reported values; all other interlineage comparisons are based on a single titration.

† No value available.

biochemically (Dowling et al., 1983, included *Natriciteres* here on the basis of an albumin ID of 46, as compared to *Thamnophis*). Biochemical data specifically refute the association of *Acrochordus* and homalopsines (Dowling & Duellman, 1978) with the natricines (see sec. VI.B., 2c).

Colubrinae—A substantial body of albumin and transferrin immunological comparisons and electrophoretic studies indicate that members of this lineage are relatively closely related worldwide (George & Dessauer, 1970; Minton & Salanitro, 1972; Dowling et al., 1983; Cadle, 1984c; Lawson & Dessauer, 1981; Schwaner & Dessauer, 1982). Many of the large number of genera are closely related genetically (Lawson & Dessauer, 1981, unpubl. data; Dowling et al., 1983); others, such as *Elaphe* and *Coluber,* are clearly polyphyletic (see sec. V.B.). Relationships within the colubrines are not worked out in detail, but the available molecular evidence identifies two major generic groups in the North American fauna: group 1 includes North American *Arizona, Elaphe, Lampropeltis, Rhinocheilus, Pituophis, Stilosoma,* and *Cemophora*; and group 2 includes North American *Coluber, Masticophis,* and *Opheodrys* (Pearson, 1966; George & Dessauer, 1970; Minton & Salanitro, 1972; Lawson & Dessauer, 1981; Dowling et al., 1983). Other genera can be associated with each of these groups, based on additional electrophoretic and immunological evidence (see sec. V). Among African forms, species of Bourgeois's (1965) Dispholidinae, *Thrasops, Dispholidus,* and *Thelotornis* form a tight cluster (Cadle, unpubl. data; *Rhamnophis* has not been tested). The close relationships among genera within the Colubrinae indicated by the molecular studies draw attention to the fact that, more than any other lineage of the Colubridae, this one has apparently radiated explosively. The approximately 150 genera of colubrines are distributed among all habitable continents, posing interesting questions concerned with their biogeography and dispersal (Cadle, 1987). Further discussion of molecular evidence on this group may be found in George and Dessauer (1970), Minton and Salanitro (1972), Minton (1976), Dowling and colleagues (1983), and Cadle (1984c).

Although such molecular studies have been very effective in increasing our knowledge of relationships among the colubrines, it is clear that an enormous amount of work remains before a clear picture of their phylogeny will emerge. Given the large number of taxa involved and the limitations of the techniques employed, most colubrine genera are too distantly related for electrophoresis to be effective and too closely related for the resolving power of MC'F.

VII. Summary

1. Most electrophoretically studied structural genes of snakes are inherited as codominant alleles.

2. Protein polymorphism in snakes is similar to levels observed in other vertebrates.

3. Polymorphic proteins are useful markers for identifying individual snakes and for studying their breeding patterns, population genetics, and problems concerned with species formation.

4. Among North American species of *Nerodia* and *Thamnophis*, many populations presently classified as subspecies are either already reproductively isolated or have at least attained the "incipient" species level.

5. Comparative biochemical studies suggest that several widespread colubrid genera are not monophyletic (e.g., *Natrix, sensu* Malnate, 1960; *Elaphe*; *Coluber*; *Rhadinaea*).

6. Many major lineages of snakes include one or more speciose radiations characterized by marked morphological and ecological diversity and minimal protein evolution. The best-documented examples of these are the Australopapuan elapid/sea snake radiation and various groups of natricines, colubrines, and xenodontines.

7. A number of groups include some genera with few species that appear to be relics of ancient radiations (e.g., *Acrochordus, Loxocemus, Farancia, Heterodon, Carphophis*).

8. Among extant reptiles, the closest relatives of snakes are among the lizards. At present, the molecular evidence is not sufficient to determine the precise relationship between lizards and snakes.

9. Snakes are monophyletic, made up of at least two very ancient lineages, the blind snakes and the primitive snakes plus the advanced snakes. Their origins probably stem from the Mesozoic Era. The blind snakes and advanced snakes are monophyletic; the primitive snakes (Henophidia) very likely are not.

10. *Typhlops* is an ancient sister group of *Leptotyphlops*.

11. The uropeltids are a compact radiation and appear to be the sister group of *Cylindrophis*. The relationship of *Anilius* to this group is remote.

12. Pythons and boids are not each other's closest relatives among primitive snakes; however, most classifications group these snakes together as either the Booidea or Boidae. Limited biochemical evidence also conflicts with other aspects of henophidian classification, such as the monophyletic status of the Tropidophiidae.

13. *Acrochordus* is excluded from the Natricinae and Homalopsinae; its phylogenetic position cannot be resolved with present molecular data.

14. Vipers are the sister group to other lineages of advanced snakes. Albumin evolution is variable within vipers, and on the average is more conservative in this group than in other advanced snakes.

15. The sea snakes are a derived lineage of elapids, closely related to Australopapuan terrestrial elapids. *Atractaspis* is possibly a member of the elapid clade, but present data cannot exclude the possibility that it is the sister group of elapids and colubrids.

16. A major unresolved question in colubrid systematics is whether this group is monophyletic. Relationships among major clades is unresolved. Xenodontines and Lycodontine/Boodontines may not be monophyletic. Homalopsines and natricines do not clearly form a clade relative to other lineages. Colubrines are a highly speciose but molecularly very cohesive group.

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