Molecular Probes of Phylogeny and Biogeography in Toads of the Widespread Genus *Bufo*[1]

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Genetic relationships among 25 species of Central and South American *Bufo* and among representative North, Central, and South American, Asian, and African *Bufo* were probed, using the quantitative immunological technique of microcomplement fixation (MC'F) which indicated a clear separation of North, Central, and South American lineages of *Bufo*. The South American lineage likely diverged from the Central and North American lineages in the Eocene; the latter two lineages diverged later, probably in the mid-Oligocene. Some species groups of South American toads, defined on the basis of traditional morphological studies, are genetically quite similar within groups, whereas others are genetically divergent. The amount of albumin evolution does not appear to parallel the amount of karyotypic, morphological, ecological, or behavioral evolution documented. Comparisons suggest that the African lineages separated from the American and Asian lineages in the late Cretaceous, corresponding to the time of the final separation of Gondwanaland, the southern supercontinent including the modern continents of South America, Africa, Australia, Antarctica, and India. The Asian lineages diverged from the lineage giving rise to all of the American species in the early Paleocene.

Introduction

Comparative biochemical studies of albumin evolution over the past decade have greatly enhanced our understanding of evolutionary relationships in many interesting and diverse groups of vertebrates (Sarich and Wilson 1966; Gorman et al. 1971; Sarich 1973; Maxson and Wilson 1975; Sarich and Cronin 1976; Maxson et al. 1977, 1979, 1981*a*; Prager et al. 1980; Prager and Wilson 1980; Maxson and Heyer 1982). Such molecular analyses have provided new information and insights into many problematical areas of systematics, phylogenetics, and zoogeography. This has been particularly true in studies of the Amphibia because frogs, salamanders, and caecilians are each morphologically relatively uniform, and often there is a relative paucity of shared derived characters (apart from the molecular information) essential for rigorous phylogenetic resolution.

1. Key words: *Bufo* phylogeny, biogeography, albumin immunology (microcomplement fixation).

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There are some 3,000 described species of frogs living on Earth today—nearly as many species as there are described species of placental mammals. Frogs are an ancient lineage, their history going back some 200 Myr. Placental mammals, on the other hand, are much younger, their ancestry extending back only 75–90 Myr. Yet all frog species are classified into the single order Anura, whereas mammals are distributed among some 16–20 different orders—a tribute to their apparent morphological diversity. Largely because of amphibians' relative morphological uniformity and lack of abundance of diagnostic morphological characters, in many cases the new biochemical approaches of molecular systematics provide the only useful information for understanding relationships among them and for comparing their evolution with that within the mammals and other taxa.

MC'F analyses of albumin evolution within the Amphibia have included most major frog families, including the Leiopelmatidae (Daugherty et al. 1982), Discoglossidae (Maxson and Szymura 1984), Pipidae (Bisbee et al. 1977), Rhinophrynidae (Maxson and Daugherty 1980), Pelobatidae (Sage et al. 1982), Dendrobatidae (Maxson and Myers 1984), Myobatrachidae (Daugherty and Maxson 1982; Heyer et al. 1982), Leptodactylidae (Maxson and Heyer 1982), Bufonidae (Maxson 1981*a*, 1981*b*), Hylidae (Maxson and Wilson 1975; Scanlan et al. 1980), Pelodryadidae (Maxson et al. 1982), Ranidae (Wallace et al. 1973; Case 1978; Post and Uzzell 1981), and the Rhacophoridae (Wallace et al. 1973).

Toads of the genus *Bufo* are one of the best-studied anuran taxa. Nearly 200 species, having a worldwide distribution, have been described. A compilation of years of multidisciplinary systematic studies of the genus, "Evolution in the Genus *Bufo*" (Blair 1972*a*), addressed many aspects of biology and systematics in differing groups of *Bufo*, but an evolutionary synthesis of the entire genus was not achieved. My studies of evolutionary relationships in *Bufo* were initiated, shortly after the appearance of Blair's book, in an attempt to determine whether molecular systematic studies of albumin relationships in *Bufo* could provide answers to questions concerning current biogeographic patterns of the genus as well as shed light on relationships within this taxon.

Results of this molecular survey of phylogeny and biogeography of *Bufo* have appeared dealing with African *Bufo* (Maxson 1981*a*), Eurasian *Bufo* (Maxson 1981*b*), and North American *Bufo* (Maxson et al. 1981*b*). A South American origin was suggested for the genus, with subsequent dispersal to Africa, Eurasia, and North America (Blair 1972*a*). My work on African *Bufo* indicated that South American and African *Bufo* lineages diverged in the Cretaceous, at about the time of the final breakup of Gondwanaland (Maxson 1981*a*). Separate studies of North American and Eurasian *Bufo* (Maxson 1981*b*; Maxson et al. 1981*b*) identified major independent lineages in Eurasia since the Eocene, whereas most speciation events in North American *Bufo* appeared to be more recent, dating to the Miocene.

Among the African *Bufo*, pairs of cryptic species were detected. Despite nearly identical morphologies, distinct differences in albumins were detected between members of several cryptic pairs of toads. In one instance, the genetic differences were paralleled by marked differences in male mating call (table 1). In another species pair, with distinct morphology and mating calls, no difference in albumins was detectable. Similar examples of morphological differentiation with little or no accompanying biochemical differentiation were detected among pairs of American toads (table 1). The purpose of this paper is to extend the approach summarized above for North American and Old World *Bufo* to repre-

Table 1
Uncoupling of Albumin Evolution and Morphological Evolution in *Bufo*

	ID	Remarks	Reference
Morphologically very similar pairs:[a]			
B. regularis—B. gutteralis	10	Different calls	b
B. maculatus—B. pusillus	9	Very similar calls	b
B. oblongus—B. viridis	7	...	c
Morphologically distinct pairs:			
B. rangeri—B. kerinyagae	0	Calls distinct	b
B. cognatus—B. compactilis	0	...	d
B. cognatus—B. speciosus	1	...	d
B. marinus—B. arenarum	4	...	c
B. marinus—B. paracnemis	0	...	e

[a] Each pair, formerly a single species.
[b] Maxson 1981*a*.
[c] Maxson 1981*b*.
[d] Maxson et al. 1981*b*.
[e] This study.

sentatives of Neotropical *Bufo* and to summarize overall relationships among the major circumglobal lineages of this genus.

Material and Methods

Plasma from 24 Central and South American *Bufo* (listed in table 3), *B. boreas* (California), *B. cognatus* (Texas), *B. melanostictus* (Thailand), *B. stomaticus* (India), *B. regularis* (Nigeria), and *B. (Schismaderma) carens* (South Africa) were used as sources of albumin for this study.

Antisera were prepared by established procedures (Maxson and Szymura 1979) to albumins purified from plasma of *Bufo coccifer* (Costa Rica), *B. luetkeni* (Costa Rica), *B. marinus* (Mexico), *B. spinulosus* (Argentina), and *B. blombergi* (Colombia). These species were selected from available material to be representative of as many described species groups as possible. Antisera to North American, Eurasian, and African species are the same as used in earlier studies (Maxson 1981*a*, 1981*b*; Maxson et al. 1981*b*). Each albumin used to produce antisera was determined to be electrophoretically distinct prior to immunization.

Microcomplement fixation (MC'F) analyses were performed according to the methods described by Champion et al. (1974). Data are reported in immunological distance units (ID) that estimate the sequence difference of the albumins being compared (Wilson et al. 1977). For albumin, it has been estimated that one unit of ID is roughly equivalent to one amino acid difference between the albumins compared (Maxson and Wilson 1974) and that 10 such substitutions stochastically accumulate every 5.5–6 million years (Wilson et al. 1977).

The phylogenetic tree (fig. 1) was constructed from the averages of all reciprocal ID values (table 2), using a simple algorithm (Maxson et al. 1979) which is a modification of Farris's Wagner tree method (Farris 1972). This method is appropriate for MC'F data where the large number of amino acid positions in albumin permits one to assume there are very few parallel and back mutation contaminations. This makes the data robust, provided the branching nodes are separated by more than 5 ID units, as is generally the case.

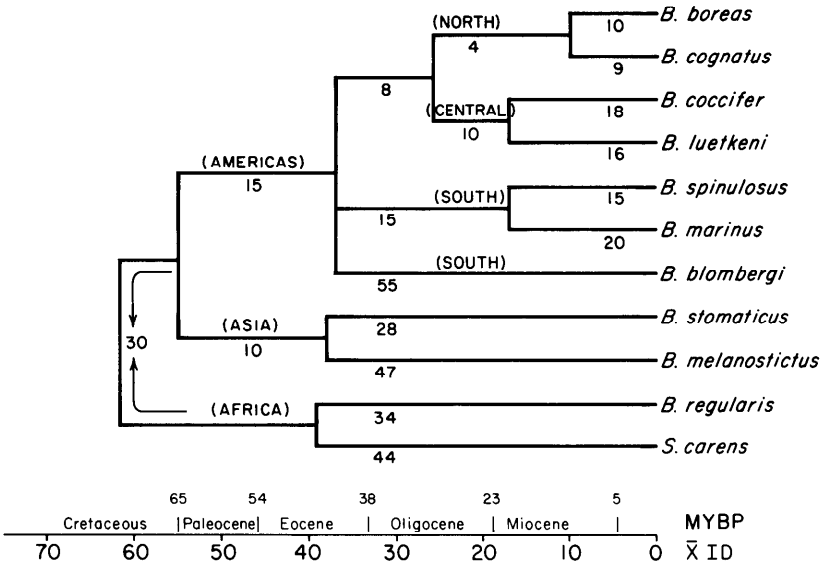

FIG. 1.—Phylogenetic tree representing relationships of representative *Bufo* lineages based on albumin comparisons. The scale indicates average ID's between species. The distance between *B. boreas* and *B. cognatus*, e.g., is 20 ID units. Geological epochs are indicated above corresponding ID, where 100 units of ID accumulate every 55–60 million years of lineage divergence, placing the *B. boreas*—*B. cognatus* separation in the Miocene (11–12 million years).

Table 2
Reciprocal Immunological Distances between Species of *Bufo*

	BO	*CO*	*CC*	*L*	*SP*	*MR*	*BL*	*ST*	*ML*	*R*	*CA*
B. boreas (*BO*)		19	47	36	43	72	89	78	88	99	118
B. cognatus (*CO*)	20		73	64	80	75	104	79	100	112	128
B. coccifer (*CC*)	41	76		34	77	100	98	64	88	106	117
B. luetkeni (*L*)	35	43	35		59	75	91	61	88	96	119
B. spinulosus (*SP*)	54	88	48	75		36	97	101	99	113	129
B. marinus (*MR*)	65	116	84	68	33		95	118	92	122	144
B. blombergi (*BL*)	62	109	102	84	94	89		108	122	141	155
B. stomaticus (*ST*)	59	63	57	59	89	116	113		72	83	105
B. melanostictus (*ML*)	108	100	95	103	113	150	150	78		130	160
B. regularis (*R*)	90	86	115	82	115	138	149	81	128		78
B. carens (*CA*)	94	88	108	97	119	127	140	94	153	79	

Different analysis algorithms lead to different trees most often when tree nodes are close together (differ by 0–5 ID units). In such situations, I prefer a conservative approach and "lump" such nodes, rather than present all possible trees, using different methods (Maxson and Wilson 1975). As others may wish to analyze such lumped nodes in greater detail, the raw data are presented.

Iterative data analysis to find the phylogenetic tree with the very lowest standard deviation is an overextension of the data base. There as yet has been no demonstration that the tree with the lowest standard deviation is the tree that is closest to the true phylogeny, nor should it be theoretically expected (Tateno et al. 1982). Because MC'F estimates have an increasing error with greater distances (Nei 1977), it is not optimal to use distant measures when close ID measures

are available for distributing distances along branches of the phylogeny. My rationale for the tree presented is that it has been constructed in such a way as to let the data that are the most robust do the most work. The data are presented in table 2; thus, anyone wishing to do further arithmetic manipulations of the data is able to do so.

Results

The 21-h titer for all 11 antisera averaged 3,800, and the slopes averaged 350, typical for anuran studies (Maxson et al. 1979). All antisera were judged to be directed solely to serum albumin as evidenced by a single precipitin arc in immuno-

[redacted]

Relationships between Major Lineages of *Bufo*

An 11 × 11 matrix of albumin cross reactions was constructed including all five Central and South American species for which antisera had been made, plus representative *Bufo* from studies completed on African, Eurasian, and North American lineages (table 2). No major nonrandomness in the data was detected, and the raw data were used in all calculations. The average standard deviation from reciprocity for these comparisons was 9.1%, comparable to that reported in other studies which range from a low of 0% (Daugherty et al. 1982) to a high of 31% (Uzzell 1982).

The Fitch-Margoliash standard deviation (Fitch and Margoliash 1967) for the tree presented is 17.6%; the Prager-Wilson *F* (Prager and Wilson 1976) is 11.6%. These values are typical for trees using large numbers of species which are relatively genetically differentiated from one another. Previous reported values for amphibian studies range from 2.5% to 24% and 2.1% to 16.5%, respectively (Scanlan et al. 1980; Maxson 1981*a*).

Relationships among Neotropical *Bufo*

With the use of antisera to the five species of Central and South American *Bufo,* comparisons of all available species of South American *Bufo* were performed, and the results are presented in table 3. Missing values were not determined as, for example, all members of the *marinus* group will have similar ID's. Therefore, only representative species of each species group were tested to antisera of non*marinus* group species.

Discussion

On the basis of morphological characters, two divisions of *Bufo* have been recognized in South America: a broad-skulled line occurring primarily in lowland habitats in tropical areas typified by toads of the *B. marinus* group, and a narrow-skulled lineage found at higher elevations and colder temperatures typified by members of the *B. spinulosus* group. Five major species groups, the *spinulosus, marinus, granulosus, typhonius,* and *guttatus* groups, have been proposed for the South American toads (Cei 1972).

The three species sampled in the *marinus* group were the closest genetic relatives of *B. marinus* paralleling studies reporting "a remarkable uniformity" in the patterns of serum proteins of these species (Cei and Cohen 1963). The next closest species were the *spinulosus* group at distances of 29–36 ID units. Several

Table 3
Albumin Cross Reactivity among Representative South and Central American *Bufo*

Species Group and Species	Locality	Antisera to *M*	*S*	*BL*	*C*	*L*
B. marinus (*M*):[a]						
B. marinus	Vera Cruz, Mexico	0	33	95	84	68
B. marinus	Iguitos, Peru	0	...	...	...	...
B. marinus	Costa Rica	0	...	...	...	...
B. marinus	Brazil	0	...	...	...	...
B. arenarum	Mendoza, Argentina	4	...	106	...	...
B. ictericus	São Paulo, Brazil	3	...	...	...	...
B. paracnemis	Argentina	0	...	106	...	...
B. spinulosus (*S*):[a]						
B. spinulosus	Jujuy, Argentina	36	0	97	48	75
B. spinulosus	La Paz, Bolivia	...	3	...	...	...
B. s. limensis	Lima, Peru	62	27	>84	68	44
B. trifolium	Palca, Peru	29	2	106	...	...
B. flavolineatus	Turin Plateau, Peru	33	2	...	...	...
B. variegatus	Barilochi, Argentina	>85	61	60	64	69
B. guttatus:[a]						
B. blombergi (*BL*)	Colombia	89	94	0	102	84
B. haematiticus	Costa Rica	>112	...	36	...	103
B. granulosus:[a]						
B. granulosus	Brazil	61	>80	85	57	67
B. typhonius:[a]						
B. typhonius	Salada, Ecuador	74	...	55	24	29
B. typhonius	Amazonas, Peru	77	65	64	24	...
B. crucifer	Rio de Janeiro, Brazil	22	54	>100	62	66
B. crucifer	São Punto, Brazil	20	52	...	63	...
B. coccifer (*C*):						
B. coccifer	Liberia, Costa Rica	100	77	98	0	35
B. coccifer	Esparta, Costa Rica	82	85	98	0	39
B. valliceps:						
B. luetkeni (*L*)	Costa Rica	75	59	91	35	0
B. luetkeni	Guatemala	...	...	...	37	2
B. valliceps	Texas	109	82	96	48	31
B. ibarrai	Guatemala	...	83	104	21	38
B. mazatlanensis	Sinaloa, Mexico	90	70	100	32	32
B. coniferus	Moravia, Costa Rica	82	>90	98	46	41
B. canaliferus:						
B. canaliferus	Guatemala	>80	62	>81	39	42
B. holdridgei:						
B. holdridgei	Costa Rica	>83	70	89	31	22
B. marmoreus:						
B. marmoreus	Tehuantepec, Mexico	90	79	95	36	35
B. perplexus	Tlaltzipan, Mexico	94	77	97	46	37
B. occidentalis:						
B. occidentalis	Oaxaca, Mexico	>86	>88	>94	64	43

Note.—Missing values were not determined; see text.

[a] South American species groups identified by Cei (1972).

populations of *B. marinus* sampled were all indistinguishable from the *B. marinus* antibody. The association of the *marinus* group with the South American rather than Central American lineages is not surprising (fig. 1). Although our antiserum was produced to a Mexican *B. marinus,* this species is widely distributed all over South America, Central America, and Mexico. More recently, *B. marinus* has been introduced in and spread widely over islands in the Caribbean and the Pacific and in Australia (Sabath et al. 1981). *Bufo* is believed to have originated in South America (Savage 1973; Tandy and Tandy 1976), and the widespread occurrence of *B. marinus* outside of South America attests to its capacity for dispersing and thriving.

Tests with *B. spinulosus* show similarly close relationships between allopatric populations of *B. spinulosus* and *B. trifolium* and *B. flavolineatus*. One population sampled from near Lima, Peru, has been both identified as a subspecies of *B. spinulosus, B. s. limensis,* and been recognized as a distinct species (Blair 1972*a*). Serological studies of members of the *spinulosus* group by Cei (1972) suggested considerable genetic isolation among allopatric populations of this species. *Bufo s. limensis* albumin is almost as distinct from that of our reference population as are the albumins of species of the *marinus* group. Thus this work indicates that the toads in Lima have been genetically isolated from the populations identified as *B. spinulosus, B. flavolineatus,* and *B. trifolium* since the Miocene (roughly 14–16 Myr). In instances such as exemplified by the toads of the *spinulosus* group, where external morphology provides few diagnostic characters, biochemical comparisons give information on reproductive isolation and subsequent species status of populations.

Bufo variegatus traditionally has been associated with the *spinulosus* group, because of its morphological similarities to *B. spinulosus*. Cei, however, suggested that *B. variegatus* was erroneously placed in this group and that its biogenic amine contents and spectrum differed from those of members of the *spinulosus* group (Cei et al. 1972). The albumin data show that this species is genetically distinct from the *spinulosus* group and moreover that it is not closely related to any of the lineages defined in our study.

The evolutionary relationships of the *guttatus* group (represented by *B. blombergi* and *B. haematiticus;* table 3) are unknown. Bioamine composition (Low 1972) suggests affinities of these toads lie with the broad-skulled toads of the African *B. regularis* complex. Comparisons of *B. blombergi* with all other South American toads show moderate affinities to *B. typhonius,* with all other comparisons being relatively distant. *Bufo blombergi* is quite distant from all of the representative lineages studied, including the African *Bufo*. Let us refer to figure 1; *B. coccifer* and *B. luetkeni* share a common lineage after diverging from the lineages leading to South American species. *Bufo spinulosus* and *B. marinus* also share common ancestry for a period, after separating from that line leading to the *guttatus* group. Without additional antisera, more detailed relationships of this lineage cannot be discerned.

The *B. typhonius* group consists of several widespread and problematical species. Blair (1972*c*) was forced to conclude that the evolutionary position of *B. typhonius* was uncertain. Hybridization data suggested close affinities of this species to both the *marinus* and *granulosus* groups. In appearance it is similar to *B. haematiticus* in the *guttatus* group. *Bufo typhonius* and *B. haematiticus* are also the only studied *Bufo* lacking bufotenine (Low 1972). Because of difficulties

in species identification, *B. typhonius* is the subject of a separate simultaneous study of morphology, ecology, and albumin evolution (Maxson and M. S. Hoogmoed, unpublished observations).

Bufo crucifer has been assigned to the *typhonius* group. Blair described it as possibly "the most similar of all living *Bufo* to the ancestral form of the genus." However, Low's studies of parotid gland secretion (1972) indicated that *B. crucifer*'s affinities were with members of the *marinus* group. Comparative studies of osteology and results of extensive hybridization tests by Blair (1972*b*) reached similar conclusions. Comparisons of *B. crucifer* albumin (table 3) also suggest that *B. crucifer* is most closely related to members of the *marinus* group. The measured distance of 20 ID units between *B. crucifer* and *B. marinus* is the smallest distance between *B. marinus* and any species not assigned to the *marinus* group.

Bufo luetkeni, representing the *valliceps* group, is as distant from members of the *valliceps* group as it is from many other species. Some species, including *B. holdridgei* and *B. typhonius,* members of two other groups, are even closer to *B. luetkeni* than are other members of the *valliceps* group. The species we studied from the *valliceps* group all exhibited similar patterns of albumin cross reactivity, clearly delineating a Central American and a South American lineage. These two lineages (fig. 1) have been separated since the Eocene. Representative toads of the Central American *canaliferus, holdridgei, marmoreus,* and *occidentalis* groups also exhibited patterns of albumin evolution similar to those of members of the *valliceps* group (table 3).

Analyses of all available information on distribution and relationships of living and fossil *Bufo* led Blair and others to conclude that the genus arose in South America and then dispersed to North America, Asia, Africa, and Europe in the late Tertiary (Blair 1972*c*). Our evidence, however, suggests that the morphological similarities are misleading and that the radiations within *Bufo* are much older. The albumin data (fig. 1) suggest that *Bufo* were already in Africa when the final separation of Gondwanaland was accomplished in the late Cretaceous.

The "albumin molecular clock" has permitted many inferences concerning lineage age and rates of evolution in amphibians (Maxson and Wilson 1975, 1979; Maxson 1981*a*). Because so few fossils are available for detailed calibration of an amphibian "albumin clock," an indirect calibration was made. Geological data from studies of geomagnetic polarity reversal, seafloor spreading, and plate tectonics on the time of separation of South America and Australia independently confirmed that albumin accumulates, on the average, 100 ID units every 60 million years of lineage independence (Maxson et al. 1975). Using the best available carnivore fossils led Wilson and colleagues (1977) to a calibration of 100 ID units every 55 million years as the best fossil-based estimate for mammalian albumins.

The present data are not inconsistent with this calibration. The average ID between the five African and South American *Bufo* (table 3) is 133 ± 14 ID units. The 100 ID units per 60-million-year figure suggest a separation of the *Bufo* albumins about 80 million years ago. Geophysical studies propose a Cretaceous opening of the Atlantic Ocean and separation of Africa and South America about 88–95 million years ago (Laurent 1979). This is fairly good agreement. It should be emphasized that even the theoretical minimal variance of the rate of molecular

evolution (a variance equal to the mean) is sufficient to make any closer agreement superfluous.

The striking similarities in size and coloration of the West African *B. superciliaris* and the South American *B. blombergi* have been noted by several workers (Blair 1972*c*). The degree of genetic differentiation indicated by albumin comparisons (ID = 114) suggests that the morphological similarities are a result of either retention of primitive characters or parallel evolution as a result of adaptation to similar niches on both continents.

The existence of Miocene fossils assigned to *Bufo* in Europe and Africa is not in conflict with the data suggesting separation of Asian and American lineages in the Paleocene—divergences can always predate fossil finds. On the basis of extensive hybridization studies among *Bufo* from all continents, Blair (1972*b*) concluded that *Bufo* from North and Central America evolved as a single radiation, a finding also congruent with the results of this study (fig. 1).

Analysis of relationships of Neotropical *Bufo* in terms of their belonging to the broad- or narrow-skulled lineages yields no consistent pattern. Both broad-skulled and narrow-skulled toads are found in all lineages depicted in figure 1. The African lineage is represented here by the broad-skulled *B. regularis* and narrow-skulled *S. carens*. In 1972, Tandy removed *carens* from *Bufo* and designated it the monotypic taxon *Schismaderma* in recognition of unique osteology and biochemical attributes. Our albumin data show *Schismaderma* genetically closer to all African *Bufo* than the African and Neotropical *Bufo* are to one another. Thus recognition of *Schismaderma* has made the genus *Bufo* paraphyletic.

On the basis of several biochemical studies of amphibians, it appears that, in groups such as *Bufo*, where morphological evolution is extremely conservative, paraphyly of taxa may be very common. During evolution in these groups, certain adaptive morphologies may stabilize and exist for millions of years. A sublineage may evolve an evolutionary novelty that will form the basis for new adaptive morphologies while the old adaptive morphology continues in the remainder of the assemblage. The clade showing the morphological changes is recognized as a new taxon. But these situations can be detected only when we have combinations of molecular and morphological data as presented here (Larson et al. 1981; Maxson 1981*a*, 1982).

There are also broad-skulled and narrow-skulled toads represented in each of the Asian and the three American radiations (see Maxson [1981*a*, 1981*b*]; Maxson et al. [1981*b*] for details of these radiations). Overall, phylogenetic separations in *Bufo* correspond more to historical geographic patterns than to skull osteology. It seems more likely that the skull morphology is a reflection of adaptive responses of the *Bufo* genome to differing environments.

In summary, these data reconfirm and add to the growing evidence of the independent evolution of proteins and the morphological characters traditionally used in systematic work. Because the distances between most groups based on morphological studies are substantial, more extensive work with additional key antisera would be needed to do any definitive phylogenetic reconstruction for the South American *Bufo*. This work is only indicative of the substantial genetic divergence among these toads and serves to point out areas in which further study could be productive.

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