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Divergent Lineages within the *Bufo margaritifera* Complex (Amphibia: Anura; Bufonidae) Revealed by Albumin Immunology[1]

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ABSTRACT

The toads belonging to the *Bufo margaritifera* complex (Amphibia: Anura; Bufonidae) are widely distributed in the Neotropics. A molecular analysis, using the quantitative immunological technique of micro-complement fixation, assessed the degree of divergence in a plasma protein, serum albumin, among representative Central and South American toads currently placed in this complex. This analysis revealed a surprisingly large amount of genetic diversity. Comparisons of estimates of albumin sequence evolution in representatives of the *Bufo margaritifera* complex from 32 localities in one central and seven South American countries indicate that this complex includes more than three lineages that are very distinct genetically but show relatively little morphological variation. The degree of albumin divergence implies that some of these lineages have been isolated for at least 30 million years. This study also suggests that the amount of genetic diversity within other Amazonian species or species groups could be substantial.

Key words: *Amazonia;* Bufo "typhonius"; *micro-complement fixation; serum albumin; species diversity.*

CURRENT ESTIMATES OF GLOBAL BIOLOGICAL DIVERSITY range from 10 million to 100 million species (Hoogmoed & de Jong 1992, Wilson 1992); of these, fewer than 2 million have been named (May 1988). The majority of species occur in tropical areas (Wilson 1992) and recently these areas have become the intense focus of plans to catalog the vast number of organisms that face extinction due to habitat destruction and human encroachment (Raven & Wilson 1992). The Brazilian Amazon Basin has been at the center of these efforts due to the increasing rate of deforestation in that region (Skole & Tucker 1993) and the concomitant, often severe, effects on biological diversity that accompany deforestation in the tropics (Jordan 1986, Myers 1986). However, while attempts are being made to document the variety of species that occur in tropical areas, the degree of genetic diversity within described taxa remains virtually unknown (Thomson *et al.* 1991). There is a high probability that many of the known species are composites of morphologically cryptic species that are genetically unique. To address the question of genetic variation within an Amazonian taxon, we have evaluated divergence in the protein serum albumin within a wide-ranging complex of Neotropical toads.

Animals that have been recognized as *Bufo "typhonius"* are typically inhabitants of the tropical forest floor, and are characterized by a pointed snout, sometimes enlarged cephalic crests, a distinct tympanum, vertebral spines in some taxa, lateral tubercles, and a leaf-like coloration pattern (Hoogmoed 1990). The species (*Rana*) *typhonius* was first described by Linnaeus in 1758. Hoogmoed (1989a) pointed out that Anderson (1900) had shown that the name *typhonius* should be applied to the Indian species currently known as *Rana tigerina,* but Anderson's recommendation has not been followed by subsequent workers. Therefore, Hoogmoed (1989a, 1990) recommended doing away with the name *typhonius* for nomenclatural purposes in South America. Thus, in this paper we refer to all of the taxa that previously have been assigned to *B. "typhonius"* together with other species placed in the *B. "typhonius"* species group (see below) as members of the *B. margaritifera* complex; the name *B. margaritifera* (Laurenti 1768) is the oldest available for a species in this complex.

Toads currently allocated to this complex occur from central Panama through the Amazon Basin south to Bolivia and southern Brazil; additional disjunct populations are found in Pacific Ecuador

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and Chocoan Colombia, and in the Atlantic coastal forest belt in southeastern Brazil (Hoogmoed 1990). While *B. "typhonius"* has served as a catchall for many specimens of brownish, leaf-like, forest-dwelling toads, there is some morphological variation among specimens that have been assigned to this taxon. Variable characters include adult body size (30 to 104 mm snout/vent length), degree of pronouncement of the cephalic crests and vertebral spines, color, pattern, and texture of the skin, color of the iris, mating call, and ecological preference (Hoogmoed 1986).

Despite the external similarity of members of the *B. margaritifera* complex and *B. haematiticus* in the *guttatus* species group, Blair (1972) concluded that the evolutionary position of the members of the *B. margaritifera* complex (his *typhonius*) relative to other *Bufo* was uncertain. Although a molecular phylogenetic study of Central and South American *Bufo* included some toads assigned to this complex, the phylogenetic placement of the *B. margaritifera* complex was not addressed (Maxson 1984). Hoogmoed (1989a, b, 1990) redefined the *B. margaritifera* complex (his *"typhonius"* group), removing or synonymizing 13 of the 17 species names which had been associated with that group, leaving *B. ceratophrys, B. dapsilis, B. margaritifera, B. nasicus,* and *B. roqueanus* as its only members. Two additional species, *B. iserni* (but see Hoogmoed 1990) and *B. sternosignatus,* were placed in this complex (their *"typhonius"* species group) by Duellman and Schulte (1992). In addition, morphological analyses of animals in the *B. margaritifera* complex from all over South America indicate that this complex may contain as many as twelve species, most having small, allopatric distributions (Hoogmoed 1986, 1990). Therefore, the *B. margaritifera* complex currently contains six of the above seven named species (excluding *Bufo iserni*) and all of the as yet unnamed taxa that were previously placed in *B. "typhonius"*; we will refer to these unnamed toads as *B.* sp. in this paper.

Throughout the past two decades, immunological studies of protein evolution have provided insights into phylogenetic relationships and zoogeography of many vertebrates (Maxson & Myers 1985; Maxson & Maxson 1990 and references therein; Hedges *et al.* 1992). Molecular analyses have been valuable in studies of amphibians because their highly conservative morphologies offer relatively few characters that can be used to resolve phylogenetic relationships. In particular, molecular studies have been useful in discerning morphologically cryptic species that are differentiated genetically (Scanlan *et al.* 1980, Donnellan & Aplin 1989, Hedges & Thomas 1991). Thus, a molecular approach appears to be ideally suited for assessing levels of genetic variation within the *B. margaritifera* complex. Molecular studies have the advantage of providing divergence time estimates as well as patterns of divergence (Maxson 1992). This study extends an earlier molecular analysis of the genus *Bufo* (Maxson 1984) to evaluate albumin evolution among representative toads assigned to the *B. margaritifera* complex and to assess relationships among suspected cryptic species within this enigmatic taxon.

MATERIALS AND METHODS

The quantitative micro-complement fixation (MC'F) assay (Maxson & Maxson 1990) was used to estimate amino acid sequence differences among albumins. Antisera were prepared by established procedures (Maxson & Maxson 1990) to serum albumins purified from the plasma of representatives of the *B. margaritifera* complex from three localities. The taxon from Montagne de Kaw (SE of Cayenne), French Guiana has been recognized as the species *B. margaritifera* (Hoogmoed 1989a); females of this species have hypertrophied cephalic crests. The remaining two taxa, one from Reserva Ducke (25 km E of Manaus), Amazonas, Brazil and the other from Gigante Ridge, Panama, currently are placed in the *B. margaritifera* complex but are not assigned to species. In addition, antisera previously prepared to albumins of toads from two other South American groups, *B. marinus* (*marinus* group), and *B. spinulosus* (*spinulosus* group) were used (Maxson 1984). Due to limited amounts of antigen, only one rabbit per antiserum was used for *B.* sp.-Brazil and *B.* sp.-Panama. Although using more than one rabbit per antigen ensures maximum antibody diversity, single-rabbit antisera generally perform satisfactorily (Hutchinson & Maxson 1986). The antiserum against *B. margaritifera* was produced in two rabbits and these individual rabbit antisera were pooled in inverse proportion to their MC'F titers. Plasma or plasma preserved in PPS (Nakanishi *et al.* 1969) from *B. cognatus, B. diptychus, B. luetkenii, B. marinus, B. poeppigii, B. spinulosus,* and 41 representatives of the *B. margaritifera* complex were used as sources of albumin for comparison with these antisera. Localities for the populations examined are indicated in Figure 1; specific locality information for all animals used in this study is included in the Appendix.

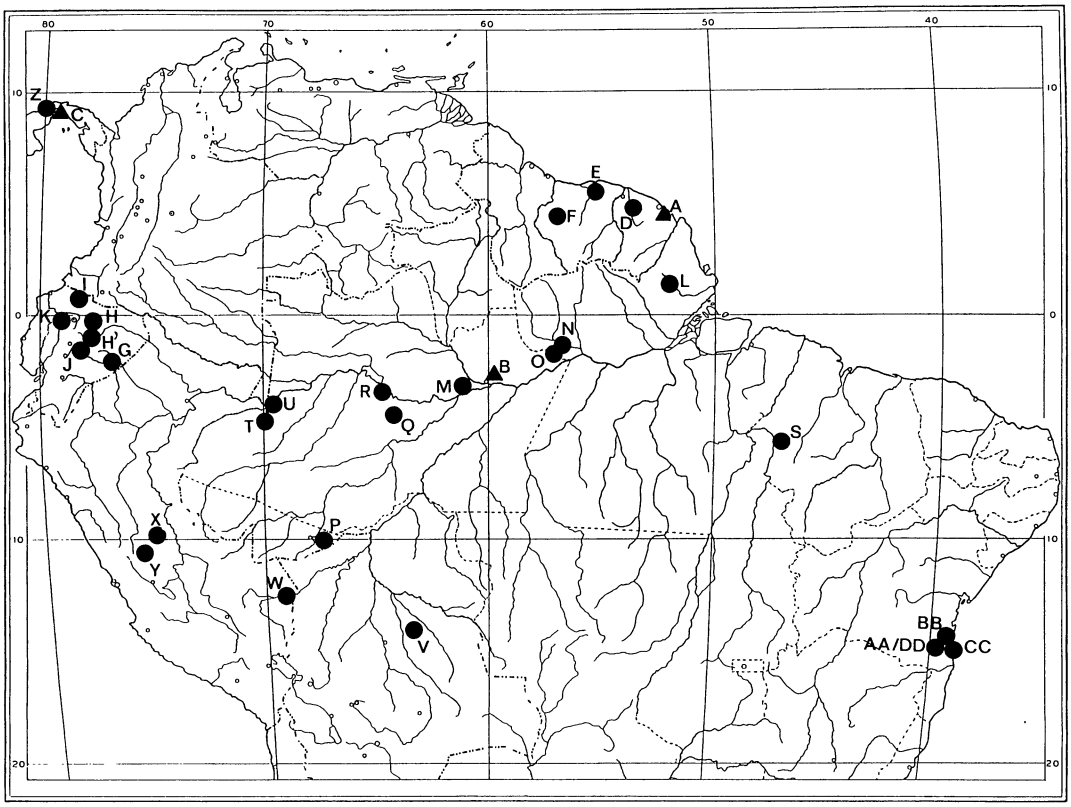

FIGURE 1. Collection localities for representatives of the *B. margaritifera* complex. Populations represented by antisera are designated with triangles; other populations are designated with circles. Letters correspond to localities described in the Appendix.

One of us (MSH) has been engaged in morphological studies of this complex since 1977 and has obtained blood samples from most specimens collected since 1980. All specimens collected by MSH have been deposited in the collection of the Nationaal Natuurhistorisch Museum (formerly the Rijksmuseum van Natuurlijke Historie, acronym RMNH), Leiden, The Netherlands. Duplicate specimens with identical data and from which blood samples also were taken have been deposited in museums in Belém, Brazil (Museu Paraense E. Goeldi), Paramaribo, Suriname (Nationaal Zoologische Collectie), and Santa Cruz de la Sierra, Bolivia (Museo de Historia Natural Noel Kempff Mercado).

Data are reported as immunological distance (ID) units; one unit of ID is approximately equal to one amino acid difference between the albumins compared (Hass & Maxson 1993, Prager & Wilson 1993). Reciprocal IDs were tested for any significant non-randomness in reciprocity (Sarich & Cronin 1976) and corrected if any such non-random deviations were observed.

The averages of raw (uncorrected) reciprocal ID values were used to construct a phylogeny using a modification (Hutchinson & Maxson 1987) of the distance Wagner method (Farris 1972); these data also were used to construct a phylogeny using the neighbor-joining algorithm of Saitou and Nei (1987). Two representative species of South American *Bufo, B. marinus* and *B. spinulosus,* were used as outgroups to the *B. margaritifera* complex. These taxa were chosen as outgroups because, while they occur in South America, they are clearly outside the *B. margaritifera* complex both morphologically and genetically (Maxson 1984, Duellman & Schulte 1992). One-way IDs to all of the *B. margaritifera* complex samples from each of the three antisera against representatives of this complex were measured to evaluate the degree of albumin divergence within the *B. margaritifera* complex and to ascertain patterns of immunological cross-reactivity.

TABLE 1. *Reciprocal immunological distance comparisons.*

a. Raw reciprocal immunological distance comparisons employing antisera from three representatives of the *B. margaritifera* complex and two representatives of other South American groups of *Bufo*. The standard deviation from reciprocity (Maxson & Wilson 1975) of these data is 6.7 percent, comparable to that seen in anuran studies (Hass & Maxson 1993). Nonrandomness correction factors, indicating the degree of deviation from perfect reciprocity (1.0) for each antiserum (Sarich & Cronin 1976), are shown.

	Antisera				
	FG	B	P	M	S
Antigens Correction factors:	1.04	0.85	0.97	0.95	1.16
B. margaritifera—Fr. Guiana (FG)	0	33	49	81	114
B. sp.—Brazil (B)	32	0	45	67	97
B. sp.—Panama (P)	40	48	0	104	106
B. marinus—(M)	72	72	96	0	33
B. spinulosus—(S)	123	130	117	36	0

b. Corrected reciprocal immunological distance comparisons. The standard deviation from reciprocity for these data is 3.8 percent.

	Antisera				
Antigens	FG	B	P	M	S
B. margaritifera—Fr. Guiana (FG)	0	30	49	78	132
B. sp.—Brazil (B)	35	0	45	64	112
B. sp.—Panama (P)	44	43	0	100	123
B. marinus—(M)	78	65	96	0	38
B. spinulosus—(S)	134	117	117	34	0

RESULTS

Antiserum titers ranged from a low of 2600 (*B.* sp.-Panama) to a high of 14,000 (*B. spinulosus*). The average titer for all five antisera was 5600, and the slopes averaged 390, relatively typical for amphibian studies (Maxson *et al.* 1979, Maxson 1984). The reciprocal ID data are presented in Table 1. Only the antisera to *B.* sp.-Brazil and *B. spinulosus* showed pronounced deviations from reciprocity (Table 1a); a corrected matrix is shown in Table 1b.

The averages of the raw reciprocal data were used to construct the tree in Figure 2. A neighbor-joining analysis of the raw data clustered the taxa from French Guiana and Panama, while a modified distance Wagner method analysis grouped the taxa from French Guiana and Brazil. However, in both cases the branch length supporting these groups is within the experimental repeatability of the technique (± 2 ID, Maxson & Maxson 1979). Therefore the relationship among the three taxa is shown as a trichotomy, although the lowest ID value is between the Brazilian and French Guianan populations. In constructing the tree, we used only comparisons with *B.* sp.-Panama to allocate branch lengths to *B. marinus* and *B. spinulosus*. If the averages of the branch lengths to the entire *B. margaritifera* complex cluster are used to allocate branch lengths to the outgroup lineages, very unequal amounts of change in albumin are found, with the length of the branch to *B. spinulosus* much larger than that to *B. marinus*. Although it clusters with *B. marinus*, both the comparisons using the antiserum to *B. spinulosus* and the reciprocal estimates indicate that there may have been a higher rate of albumin evolution along the branch leading to *B. spinulosus*. However, this rate difference was not observed in a more comprehensive study of Central and South American *Bufo* (Maxson 1984). The ID estimates for *B.* sp.-Panama to the *B. marinus*/*B. spinulosus* cluster minimize this rate difference and using them to allocate branch lengths to *B. marinus* and *B. spinulosus* results in lowering the rate difference to a factor of two.

One-way comparisons of samples from throughout the range of the *B. margaritifera* complex to the three antisera representing this complex are presented in Table 2. These comparisons estimate the amount of albumin evolution between each sample and the three reference antisera. Although phylogenetic placement of these individuals can be inferred based upon one-way comparisons (Beverley & Wilson 1982), our analysis does not include any samples without reciprocal comparisons

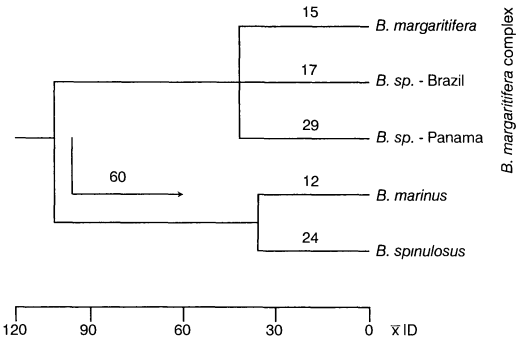

FIGURE 2. Phylogenetic tree depicting relationships of three populations representing the *B. margaritifera* complex, *B. marinus,* and *B. spinulosus* based on mean ID values from reciprocal albumin comparisons (Table 1a). The arrow indicates an unrooted branch. The goodness of fit values for the tree are: standard deviation (Fitch & Margoliash 1967) = 12.2 percent; *F* value (Prager & Wilson 1976) = 8.7 percent. These parameters suggest a reasonable fit of the data to the tree, comparable to that of other amphibian studies which average standard deviation = 13 percent and *F* value = 9 percent (Maxson 1984).

in the phylogenetic tree (Fig. 2). To place all 41 specimens (Table 2) in a phylogenetic tree, an antiserum to every population examined would be needed to determine reciprocal IDs for each pair.

If the populations in Table 2 were members of the same species, they should show an ID of 0 (± 2 due to experimental error, Maxson & Maxson 1979) to the reference antisera. For example, albumin from *B. marinus* sampled from a number of localities shows no differentiation (0 ID to antiserum against *B. marinus* from Mexico; Maxson 1984). Also, the range of ID values seen for the wide-ranging salamander, *Plethodon glutinosus,* in eastern North America is 0 to 7 (Highton *et al.* 1989). Two samples from the same population in Amazonas, Brazil (locality B in Fig. 1), illustrate variation within the experimental error of the technique. This example also demonstrates that animals sharing the same gene pool show similar patterns of ID against the reference antisera. However, the albumins from many representatives of the *B. margaritifera* complex used in this study show much more extensive immunological variation. Immunological distances range from 1 to 48 ID to the antiserum against albumin from *B. margaritifera*; 9 to 69 ID to the antiserum against albumin from *B.* sp.-Brazil; and 12 to 88 ID to the antiserum against albumin from *B.* sp.-Panama.

Table 2 also shows that albumins of some individuals placed in the *B. margaritifera* complex and previously identified as *B. "typhonius"* are more divergent from the reference antisera than albumins of other individuals recognized as distinct species. Albumins from *B. margaritifera* and *B. roqueanus* actually show less amino acid sequence divergence from the albumins of the populations represented by antisera than do those of other populations currently assigned to the *B. margaritifera* complex but unassigned to species. Apparently these very different taxa have been isolated from the reference populations longer than populations with sufficient morphological evolution to warrant recognition as a separate species. Immunological distances to representatives of some other species groups of toads in the Neotropics are shown in Table 3.

Variation within the *B. margaritifera* complex also was examined to determine if different populations might represent the same species (Table 4) or if a single locality has populations of more than one species (Table 5). Table 4 summarizes the MC'F reactions of albumins from different populations within the *B. margaritifera* complex that show similar patterns of cross-reactivity to the panel of antisera. For example, three Ecuadoran populations from Napo and Salado (localities H, H', and I) differ by an average of 2 ID to the antiserum panel. While the IDs between the representatives of these three populations are not known, the similar pattern suggests that the populations from these three localities could be members of the same species.

Similarly, toads from two localities in French Guiana, Montagne de Kaw (an antiserum locality, A) and Petit Saut (*B. margaritifera*; locality D), probably are members of the same species; this is corroborated by morphological data (Hoogmoed & Avila-Pires 1991). Animals from Maranhão, Brazil (locality S) may also belong to this taxon. It is likely that all three of these populations should be assigned to *B. margaritifera. B. margaritifera,* then, is found in the Guyanas (Hoogmoed 1989a) as well as eastern Brazil. A second species apparently occurs with *B. margaritifera* at locality D. A third taxon which overlaps this range includes toads from Amapa, Amazonas, and Pará, Brazil (L, M, and N in Fig. 1) but does not include the members of *B. margaritifera.* Additional morphological and molecular data for all these specimens should help to define species membership.

Sympatric members of the *B. margaritifera* complex that have different patterns of albumin cross-reactivity are shown in Table 5. These differences suggest at least two sympatric species inhabit French Guiana; one is *B. margaritifera,* the other

TABLE 2. *Comparisons of different populations representing the* B. margaritifera *complex to reference antisera against three members of the* B. margaritifera *complex. Localities correspond to those shown in Figure 1. The numbers correspond to specimens listed in the Appendix and are MSH numbers unless otherwise indicated (see Appendix). Specimens allocated to named species are indicated.*

Country	Locality	Specimen	Antisera			
			FG	B	P	
French Guiana	A[a]	4014–15	0	33	49	*B. margaritifera*
	A	4016	41	9	53	
	D	5402–05	30	10	43	
	D	5269	0	38	46	*B. margaritifera*
Suriname	E	CM84687	29	66	88	
	F	3397	31	68	79	
Ecuador	G	3838–43	1	44	34	
	G	3920	31	57	28	*B. roqueanus*
	H	4605	19	34	33	
	H′	4617	18	37	35	
	I	LM241	19	38	30	
	J	3746	24	48	23	
	K	3664–65, 3689	41	66	12	
Brazil	B[a]	4337–42	32	0	45	
	B	4206	30	2	43	
	L	4977	34	9	51	
	M	4411	27	10	55	
	N	5132	33	10	50	
	O	5030–33	48	14	68	
	P	5855–56	20	12	37	
	Q	5473–74	27	16	37	
	Q	5477	16	27	32	
	R	4321–22	37	29	69	
	S	5228	2	35	54	
	T	5641	20	22	31	
	U	5845	21	41	41	
	U	4247	24	49	58	
	U	5598–5600	31	39	38	
	U	4244	47	38	52	
	AA	4045	38	—[b]	38	
	BB	4079	40	69	36	
	CC	4141	34	—[b]	36	
	DD	4154	38	69	35	
Bolivia	V	5959	24	40	19	
Peru	W	KU205262–63	10	42	26	
	X	2001	27	51	19	
	X	2008	40	61	32	
	Y	SBH171039	41	51	20	
Panama	C[a]	LM2261–62	40	48	0	
	Z	LM1850	39	44	0	

[a] Serum albumins from taxa used in antiserum production.
[b] Cross-reaction was not done.

is probably one of two undescribed species recognized by Hoogmoed (1990). Similarly, specimens from Tabatinga, Brazil (locality U) that can be distinguished morphologically on the basis of iris color and body size ("larger" and "smaller") show very different cross-reactivity patterns. This molecular diversity suggests that within the *B. margaritifera* complex there are a number of sympatric, reproductively isolated populations that warrant unique species designations. Clearly, the MC'F data support Hoogmoed's (1990) observations based upon morphology that this complex includes a number of species, most with relatively small distributions which are partially overlapping or allopatric.

TABLE 3. *Albumin variation measured from reference antisera against members of the* B. margaritifera *complex to other species of Neotropical* Bufo.

Species	Specimen	Antisera FG	B	P
Unassigned to species group				
B. diptychus	LM 1767	28	45	24
B. marinus group				
B. marinus	LM 207	72	72	96
B. poeppigii	SBH171135	80	72	108
B. spinulosus group				
B. spinulosus	LM236	123	130	117
B. valliceps group				
B. luetkenii	LM333	—[a]	161	116
B. fastidiosus	LM191	—[a]	117	136

[a] Cross-reaction was not done.

DISCUSSION

Invoking the albumin clock, in which approximately 10 ID accumulate every 5.5–6 million years of lineage independence (Wilson *et al.* 1977, Maxson 1992), the ID between *B. margaritifera* and *B.* sp.-Brazil suggests that these two lineages diverged about 20 million years ago (mya). Similarly, a common ancestor of these toads would have diverged from *B.* sp.-Panama approximately 30 mya. Some taxa within the *B. margaritifera* complex may have diverged from one another as long ago as the Eocene (about 53 mya). This Eocene divergence is comparable to other early Cenozoic divergences reported among some South American anuran genera (Scanlan *et al.* 1980; Maxson & Heyer 1982, 1988). This divergence time estimate greatly predates a more recent divergence predicted by the refuge model (Haffer 1987) in which Pleistocene climatic and habitat fluctuations are proposed to have created geographically isolated refugia within which genetic differentiation of isolated populations led to allopatric speciation. While MC'F has uncovered albumin variation within several taxa and indicated the existence of cryptic species (Maha *et al.* 1983, Pinou *et al.*, in press), this is the largest amount of albumin variation ever observed within a complex of closely related species (Highton *et al.* 1989).

With the three antisera to representatives of the *B. margaritifera* complex, populations from throughout the range could be identified as different, but taxonomic groupings could not be characterized.

TABLE 4. *Different populations of toads within the* B. margaritifera *complex that demonstrate similar patterns of albumin cross-reactivity. Species designations have been given when available (see Appendix).*

Country	Locality	Specimen	Species	Antisera FG	B	P
Ecuador	H	4605		19	34	33
Ecuador	H'	4617		18	37	35
Ecuador	I	LM241		19	38	30
Brazil	Q	5473–74	*B.* sp. 2	27	16	37
Brazil	P	5855–56	*B.* sp. 7	20	12	37
Brazil	Q	5477	*B.* sp. 3	16	27	32
Brazil	T	5641	*B.* sp. 6	20	22	31
Fr. Guiana	A	4014–15	*B. margaritifera*	0	33	49
Fr. Guiana	D	5269	*B. margaritifera*	0	38	46
Brazil	S	5228		2	35	43
Brazil	L	4977	*B.* sp. 1	34	9	51
Brazil	N	5132		33	10	50
Brazil	M	4411		27	10	55
Fr. Guiana	A	4016		41	9	53
Fr. Guiana	D	5402–05		30	10	43
Suriname	E	CM84687		29	66	88
Suriname	F	3397		31	68	79
Brazil	AA	4045		38	—[a]	38
Brazil	BB	4079		40	69	36
Brazil	CC	4141		34	—[a]	36
Brazil	DD	4154		38	69	35

[a] Cross-reaction was not done.

TABLE 5. *Sympatric populations of taxa within the* B. margaritfera *complex demonstrating different patterns of albumin cross-reactivity.*

Country	Locality	Specimen	Species	Antisera		
				FG	B	P
Fr. Guiana	A	4014–15	*B. margaritifera*	0	33	49
	A	4016		41	9	53
Fr. Guiana	D	5402–05		30	10	43
	D	5269	*B. margaritifera*	0	38	46
Brazil	U	4247	*B.* sp. 5 (larger)	24	49	58
	U	5845	*B.* sp. 5 (larger)	21	41	41
	U	5598–5600	*B.* sp. 4 (smaller)	31	39	38
	U	4244	*B.* sp. 4 (smaller)	47	38	52
Brazil	Q	5473–74	*B.* sp. 2	27	16	37
	Q	5477	*B.* sp. 3	16	27	32
Peru	X	2001		27	51	19
	X	2008		40	61	32
Brazil	N	5132		33	10	50
	O	5030–33		48	14	68
Ecuador	G	3838–43		1	44	34
	G	3920	*B. roqueanus*	31	57	28

The three antisera produced appear to represent three distinct lineages within this group and the data suggest that there are more than three species involved in this group. Additional molecular data and closer examination of the morphology and biogeography of these toads (currently being studied by MSH) are needed to help better delineate the number and distribution of these species. Hoogmoed (1989a) has noted that morphologically similar specimens can be distinguished in the field based on mating call, iris color, habitat, and reproductive behavior. Chromosome morphology has not yet been studied; polyploidy may occur (Hoogmoed 1989a). More extensive work with additional antisera might help provide a definitive phylogenetic reconstruction of the taxa hidden within the *B. margaritifera* complex. However, while MC'F serves as a good molecular probe to phylogeny, the time and cost of raising antisera to a representative of each locality is impractical. A more appropriate molecular phylogeny could be recovered by an allozyme analysis or a comparison of DNA sequences.

The resolution of the number and distribution of the species contained within this complex becomes even more problematic considering the ongoing destruction of the toads' natural habitat. An estimated 15,000 km^2 of forest are cleared each year in the Brazilian Amazon (Skole & Tucker 1993). If current deforestation practices continue, even relict blocs of South American tropical forest in western Brazil and the Guyana Shield are unlikely to last beyond the middle of the next century (Myers 1988). In addition to the actual deforestation, 15 percent of the remaining forested Amazon is affected by fragmentation (Skole & Tucker 1993); this fragmentation leads to habitat isolation, and the resulting effects can adversely impact the biological diversity (Lovejoy *et al.* 1986, Simberloff 1992).

The number of species that face extinction without intervention is increasing rapidly (Heywood & Stuart 1992). Since this study has probed only a relatively small number of populations within the range of the *B. margaritifera* complex, it is likely that some cryptic species may become extinct before they can be described. The four populations from the Atlantic coastal forest of Brazil (localities AA, BB, CC, DD) appear to be representatives of the same species; however, they show a different pattern of cross-reactivity than any of the other taxa examined and thus seem to be genetically distinct. Unfortunately, they occur in an area that has been heavily deforested, reduced to only 12 percent of its original range, and the remaining forest is still being cut (Brown & Brown 1992). Moreover, McNeeley *et al.* (1990) estimated the remaining Atlantic forest cover at only 1 to 5 percent of its original range. It is clear that this habitat is highly endangered. The genetic diversity uncovered within the *B. margaritifera* complex suggests that other Amazonian species or complexes of related species could be composed of multiple, cryptic, and old lineages. However, the extent of this genetic diversity may never be known unless the tropical forest is effectively conserved.

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APPENDIX. *Locality descriptions and inventory numbers of albumin samples. The letters listed under Key correspond to collecting localities for members of the* B. margaritifera *complex on the map in Figure 1. MSH numbers are field numbers for specimens deposited in the RMNH, Leiden collection; LM numbers are specimens in the Linda Maxson frozen tissue collection, Penn State University; SBH numbers are specimens in the S. Blair Hedges frozen tissue collection, Penn State University; KU numbers are specimens cataloged at the University of Kansas; CM numbers, the Carnegie Museum of Natural History; UIMNH numbers, the University of Illinois, Urbana–Champaign. Species allocation, if known, is given. The species indicated by number are those which share morphological similarities but have not yet been described (M. Hoogmoed, pers. obs.).*

Key	Country	Locality	MSH	LM	Species
A	French Guiana	Montagne de Kaw, SE of Cayenne	4014–15	1552–3	*B. margaritifera*
A	French Guiana	Montagne de Kaw, SE of Cayenne	4016	1554	*B.* sp. 1
D	French Guiana	Petit Saut, Sinnamary River	5402–05	2813	*B.* sp. 1
D	French Guiana	Petit Saut, Sinnamary River	5269	2808	*B. margaritifera*
E	Suriname	Paramaribo	CM84687	386	
F	Suriname	Kabalebo	3397	96	
G	Ecuador	Pastaza, Shiona on Rio Conambo	3838–43	773	
G	Ecuador	Pastaza, Shiona on Rio Conambo	3920	811	*B. roqueanus*
H	Ecuador	Napo, Baeza-Lago Agrio, 3 km S of Reventador	4605	2645	
H′	Ecuador	Napo, Baeza-Tena, 5 km S of Baeza	4617	2652	
I	Ecuador	Napo, Salado	—	241	
J	Ecuador	Pastaza, Mera	3746	771	
K	Ecuador	N of Alluriquin	3664–65, 3689	769	
B	Brazil	Amazonas, Reserva Ducke, 25 km E of Manaus	4337–42	1789–94	
B	Brazil	Amazonas, Reserva Ducke, 25 km E of Manaus	4206	1769	
L	Brazil	Amapa, Serra do Navio	4977	2599	*B.* sp. 1
M	Brazil	Amazonas, 15 km NE of Manacapuru	4411	1806	
N	Brazil	Pará, between Rio Nhamundá and Rio Trombetas	5132	2636	
O	Brazil	Pará, Rio Nhamundá, Sitio Ceú Estrelado	5030–33	2629	
P	Brazil	Acre, Catuaba	5855–56	2842	*B.* sp. 7
Q	Brazil	Amazonas, Urucu	5473–74	2814	*B.* sp. 2
Q	Brazil	Amazonas, Urucu	5477	2816	*B.* sp. 3
R	Brazil	Amazonas, Tefé	4321–22	1783–84	
S	Brazil	Maranhão, Rio Paciencia, affluente del Rio Tocatins	5228	2719	
T	Brazil	Amazonas, Benjamin Constant	5641	2828	*B.* sp. 6
U	Brazil	Amazonas, Tabatinga	5845	2827	*B.* sp. 5 (larger)
U	Brazil	Amazonas, Tabatinga	4247	1780	*B.* sp. 5 (larger)
U	Brazil	Amazonas, Tabatinga	5598–5600	2823	*B.* sp. 4 (smaller)
U	Brazil	Amazonas, Tabatinga	4244	1775	*B.* sp. 4 (smaller)
V	Bolivia	Santa Cruz, Perseverancia on Rio Negro	5959	2935	
W	Peru	Cuzco, Cuzco Amazónica	KU205262–63	1844	
X	Peru	Panguana	2001	1814	
X	Peru	Panguana	2008	1818	
Y	Peru	Pasco, Oxapampa	SBH171039		
C	Panama	Gigante Ridge or Las Pavas	UIMNH95435-39	2261–62	
Z	Panama	Gigante peninsula region, S. of Barro Colorado Island	—	1850	
AA	Brazil	Bahia, 5 km E of Itabuna	4045	1624	

APPENDIX. *Continued.*

Key	Country	Locality	MSH	LM	Species
BB	Brazil	Fazenda Luzitania, Rio Almada	4079	1627	
CC	Brazil	Bahia, 10 km WNW of Ilheus	4141	1630	
DD	Brazil	Bahia, Fazenda S. Domingos	4154	1634	
	Paraguay		—	1767	*B. diptychus*
	Mexico		—	207	*B. marinus*
	Argentina	Mendoza, Mendoza	—	236	*B. spinulosus*
	Peru	Pasco, Cacazu	SBH171135		*B. poeppigii*
	Costa Rica	Liberia		333	*B. luetkenii*
	Costa Rica	Liberia		191	*B. fastidiosus*