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Molecular Phylogeny and Biogeography of West Indian Toads (Anura: Bufonidae)

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Toads of the *Bufo peltocephalus* Group (Anura: Bufonidae) occur throughout the Greater Antilles (Cuba to the Virgin Islands), a geographic region of relatively high endemicity. Previous morphological and immunological studies suggested that the West Indian toads are a monophyletic lineage derived from Neotropical *Bufo* but were unable to clarify relationships within the group. We examined the evolutionary relationships and biogeography of this group of frogs by collecting approximately 2 kb of mitochondrial DNA sequence data from eight West Indian species and selected non-West Indian species from the New World and the Old World. Our analyses support the monophyly of native West Indian toads and a New World origin for the group. Relationships among the West Indian species are less certain, but a Cuban lineage is defined in most analyses. © 2001 Academic Press

Key Words: **Anura; Bufonidae; *Bufo peltocephalus* Group; molecular phylogeny; Greater Antilles; biogeography.**

INTRODUCTION

More than half of the species within the family Bufonidae are contained within the genus *Bufo*. The 205 species of this genus are distributed throughout most major land masses of the world, including the Americas, Eurasia, Africa and excluding the Australo–Papuan Realm and Madagascar (Duellman and Sweet, 1999). Toads of the genus *Bufo* share the presence of parotoid glands (Duellman and Trueb, 1996); in addition, most members of the group are distinctively rotund and are covered by toxin-secreting, granular skin. Typically, they are relatively poor jumpers and are ground dwellers that produce large clutches of small eggs (Graybeal, 1997). Because of their cosmopolitan distribution and variable life histories, these frogs are of interest to investigators researching evolutionary history and biogeography.

Within the Bufonidae, the *Bufo peltocephalus* Group is a distinct lineage of toads composed of 11 species that are endemic to the Greater Antilles (present on the Virgin Islands, Puerto Rico, Hispaniola, and Cuba). The West Indian toads are of interest because their distribution makes them particularly suited to the investigation of hypotheses of Caribbean biogeography. These toads are characterized by great morphological and ecological diversity and are found in a variety of habitats, ranging from xeric scrubland to localized riparian broadleaf forest (Schwartz and Henderson, 1991). For example, *B. longinasus* and *B. fluviaticus* are adapted to a riparian environment, with the former species being reported to be semiarboreal (unusual among bufonids) (Schwartz, 1972). Other taxa (e.g., *B. empusus* and *B. gundlachi*) have adaptations to prevent desiccation in semiarid habitats and spend much of the year in subterranean burrows where they use their heavily coossified heads to seal the entrances of their burrows (Barbour, 1945; Valdés de la Osa and Ruiz Garcia, 1979; Schwartz and Henderson, 1991). Because some species of native West Indian toads are fossorial and secretive in habit, many aspects of their natural history are poorly known.

Possibly as a result of different habitat restrictions, members of the *B. peltocephalus* Group vary greatly in the amount of cranial ossification (Pregill, 1981; Pramuk, 2000) and overall snout–vent length (SVL). For example, the most diminutive member of the group, *B. cataulaciceps*, has an average SVL of only 28 mm, whereas *B. fustiger* is approximately 198 mm in length (Schwartz and Henderson, 1991). The extreme variation in maximum body size and degree of cranial ossification among the West Indian toads has led some to question the monophyly of the group (Stejneger, 1905; Pregill, 1981; Graybeal and Cannatella, 1995).

Unfortunately, specimens (especially tissue samples) of some species are rare in museum collections (e.g., *Bufo fluviaticus* and *B. cataulaciceps*); consequently, a phylogeny of the West Indian toads has not been proposed since Pregill (1981) described the cranial osteology of the group and speculated about their

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evolutionary relationships. To resolve the relationships within Bufonidae, Graybeal (1997) examined regions of three mitochondrial genes (12S, 16S, and cytochrome *b*) and one region of a nuclear gene (*c*-mos) to infer a phylogeny for this nearly cosmopolitan family. Many of the clades had low node support and many of the topologies resulting from her separate parsimony, maximum-likelihood (ML), and neighbor-joining (NJ) analyses conflicted. Until now, hers was the only DNA study to include a member of the *Bufo peltocephalus* Group (*B. lemur*).

Hass *et al.* (2001) recently presented immunological distances (IDs) for the protein serum albumin in this group. Using antisera made to *Bufo guentheri* (Hispaniola), *Bufo peltocephalus* (Cuba), and *Bufo marinus* (South America), they obtained distances to those species and to *B. lemur* (Puerto Rico), *B. longinasus* (Cuba), and *B. granulosus* (South America). Distances (corrected) among West Indian species were <44, whereas IDs between West Indian and South American species ranged from 52 to 96. A low ID between *B. lemur* and *B. guentheri* of 18 suggested a close relationship compared with distances between those species and Cuban species (31–44).

Herein, we investigate the phylogenetic relationships among 8 of the 11 species of the *Bufo peltocephalus* Group and 10 non-West Indian taxa based on DNA sequence data of three mitochondrial genes. We use these data to investigate the following questions: Do the West Indian toads form a monophyletic lineage? What are the relationships within the group? What are the relationships among representative North, Central, and South American *Bufo*? What is the biogeographic origin of West Indian *Bufo*?

MATERIALS AND METHODS

DNA Protocols

Tissue samples (liver, blood, or tissue homogenate) of eight ingroup species were obtained, mostly during field collections by S. B. Hedges and colleagues (Table 1). West Indian species included *Bufo empusus, B. peltocephalus, B. fustiger, B. gundlachi, B. longinasus, B. taladai* (Cuba), *B. guentheri* (Hispaniola), and *B. lemur* (Puerto Rico and Virgin Islands). They represent all species within the *B. peltocephalus* Group, with the exception of *B. fractus* (a close relative of *B. guentheri* on Hispaniola) and two rare taxa—*B. cataulaciceps* (western Cuba) and *B. fluviaticus* (northwestern Dominican Republic)—for which we were unable to obtain tissues, despite extensive fieldwork. The six non-West Indian taxa sequenced for this study included *B. granulosus* (ranging from Panama to southeastern Brazil), *B. marinus* (Central America to northern South America), *B. luetkenii* (Central America), *B. valliceps* (southeastern United States and Central America), *B. regularis* (East and West Africa), and *B. stomaticus* (eastern Iran and Pakistan). Data for the following additional non-West Indian taxa were taken from GenBank: *B. americanus* (North America), *B. melanostictus* (southeastern Asia), *B. viridis* (Europe, Asia, and North Africa), and *B. pardalis* (South Africa). (See Table 1 for specimen numbers and locality data.) Most non-West Indian taxa were chosen based on availability of tissues and published data available on GenBank. *Bufo granulosus* was selected because it previously has been suggested to be a close relative of the West Indian species (Pregill, 1981; Graybeal, 1997).

All molecular data collection was conducted at The Pennsylvania State University. DNA was extracted with a phenol–chloroform method (Hedges *et al.*, 1991). Amplification and sequencing followed protocols described previously (Hedges *et al.*, 1991). Two regions of the mitochondrial 12S, three regions of 16S, and one region of cytochrome *b* were sequenced (Table 1). Oligonucleotide primers were used to amplify and sequence complementary strands of a 352-bp region of cytochrome *b*, approximately 800 bp of the 16S rRNA gene, and approximately 830 bp of the 12S rRNA gene, resulting in a total of 1970 bp. DNA was purified and cut on a low-melting-temperature agarose gel. After reamplification, the purified DNA was filtered with sterile water in a Millipore column filter. Cycle sequencing reactions were performed with 3′ dye-labeled dideoxynucleotide triphosphates (flourescent dye terminators) and run on a Perkin–Elmer ABI PRISM 377 DNA Sequencer.

The accuracy of the sequence data generated by the automatic sequencer was checked by comparison of ambiguous bases with the electropherogram of the complementary strand. Data from the heavy and light strands were spliced together to generate a consensus sequence (light strand) for each taxon. Preliminary alignment of the sequences was performed with the CLUSTAL option in Sequence Navigator 1.01 (Applied Biosystems). All alignments were verified by eye and corrected accordingly. Our cytochrome *b*, 12S rRNA, and 16S rRNA sequences are deposited in GenBank (Table 1) and aligned sequences are available upon request from J.B.P.

Phylogenetic Analyses

Phylogenetic analyses were performed with neighbor-joining (NJ) by MEGA (test version 2.0b2; Kumar *et al.*, 2000) and with parsimony and maximum-likelihood (ML) by PAUP* (test version 4.0b4; Swofford, 2000). Initially, data from the three genes were analyzed separately with equally weighted parsimony to detect possible areas of strongly supported incongruence and then were combined. Nodes supported in more than 95% of bootstrap replicates were considered strongly supported.

Analyses were performed with and without transitions to investigate any potential affect from transition

TABLE 1

Distribution, Catalog Number, Locality, and GenBank Accession Numbers of Species Examined

Taxon GenBank Nos.[b]	Catalog No.[a]	Locality
West Indies		
Bufo lemur AY028481, AY028494, AY028506	SBH 190657	Puerto Rico
B. guentheri AY028478, AY028491, AY028503	SBH 101227	Dominican Republic, Barahona, Cabral
B. gundlachi AY028479, AY028492, AY028504	SBH 193518	Cuba, Granma, Bartolome Maso
B. longinasus AY028480, AY028493, AY028505	SBH 266461	Cuba, Sancti Spíritus, Pico de Porterillo
B. peltocephalus AY028477, AY028490, AY028502	SBH 191380	Cuba, Santiago de Cuba, La Tabla
B. empusus AF361695, AY028489, AY028501	SBH 193517	Cuba, Granma, Bartolome Maso
B. fustiger AF361696, AF361697, AF361698	SBH 172586	Cuba, Pinar del Río, Soroa
B. taladai AY028482, AY028495, AY028507	SBH 190537	Cuba, Santiago de Cuba, La Esmajugua
North/Central/South America		
B. americanus AF160761[c], AF160779[c], AF174500[c]	Acquired from GenBank	
B. luetkenii AY028484, AY028497, AY028509	LM 205	Costa Rica, Liberia
B. valliceps AY028487, AY028499, AY028512	LM 243	USA, Texas, Austin
B. granulosus AY028483, AY028496, AY028508	LM 1493	Brazil, Rondia, Porto Velho
B. marinus AY028485, AY028498, AY028510	SBH 190696	Jamaica, St. Mary, Galina
Europe		
B. viridis AF160778[c], AF160797[c], AF174526[c]	Acquired from GenBank	
Africa		
B. regularis AY028486, AF220890[e], AY028511	LM 137	Ghana (16S Acquired from GenBank)
B. pardalis AF220850[e], AF220897[e], AF210083[d]	Acquired from GenBank	
Asia		
B. melanostictus AF249001[c], AF160793[c], AF174520[c]	Acquired from GenBank	
B. stomaticus AY028488, AY028500, AY028513	LM 237	India

[a] Collection abbreviations are as follows: SBH = S. Blair Hedges; LM = Linda Maxson.
[b] GenBank numbers are listed as follows: 12S; 16S; cytochrome *b*.
[c] Liu *et al.* (2000).
[d] B. N. Eick, E. H. H. Harley, and M. I. Cherry (1999) unpublished data.
[e] M. Cunningham (1999) unpublished data.

saturation. Sites with ambiguities or deletions (38 bp total) were removed from the analyses. The Kimura two-parameter (K2P; Kimura, 1980) model was used as the primary substitution model for maximum-likelihood and neighbor-joining analyses. For parsimony analyses, heuristic searches with tree bisection and reconnection (TBR) branch swapping and 100 addition sequence replicates per analysis were used to find the shortest tree(s). Gaps were treated as a fifth character state in parsimony analyses. For the parsimony analyses, data were analyzed with all positions and nucleotide substitution types weighted equally (Farris, 1969) and with transitions excluded.

Confidence in the phylogenetic groupings was assessed by the bootstrap (BS) method (Felsenstein, 1985), with 2000 replications for parsimony and NJ (Saitou and Nei, 1987) and 100 replications for ML analyses. Bremer support for parsimony trees (both with and without transitions) was calculated with TreeRot (Sorensen, 1996). Neighbor-joining searches

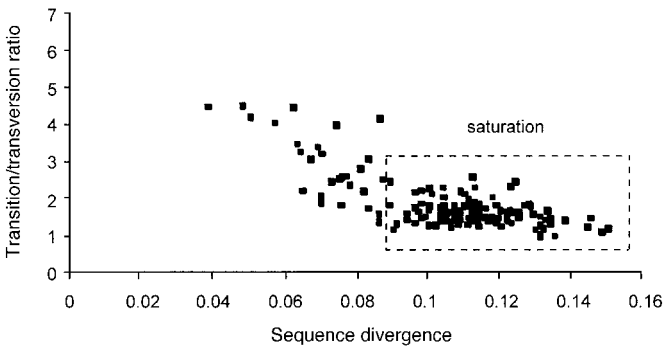

FIG. 1. Transition/transversion ratios plotted against sequence divergence (Kimura two-parameter distance). The ratio is relatively unchanged above a sequence divergence value of 0.08, indicating saturation.

using Kimura's (1980) two-parameter model were performed with MEGA.

Because sequence data were not available for all of the selected gene regions in some of the outgroup taxa, analyses were performed on two data sets: an 18-taxon data set with reduced number of sites (1269 bp) and a 10-taxon data set covering the entire region selected for sequencing (1970 bp). The former data set was used to examine the position of the West Indian toads in relation to non-West Indian taxa, with the African species to root the tree. The African species were chosen because they were identified as being the most divergent species of *Bufo* in an immunological study (Maxson, 1984). In addition, the African species possess a chromosome number (20) different from that of most *Bufo* (22), but this is likely a derived character uniting the African group (Bogart, 1967). The 1970-bp data set was used to examine relationships among West Indian taxa and was rooted with *B. granulosus* and *B. marinus*.

Because transition saturation is common in mitochondrial DNA sequence data sets (Li, 1997), and because our study includes some distantly related taxa, we were concerned about this potential bias in our results. A plot of transition/transversion ratios versus total sequence divergence (Fig. 1) indicates that transitions are saturated at distances above approximately 0.08. Also, trees that included transitions often were quite different from those that excluded transitions, especially concerning the more distantly related taxa. For these reasons, we compared trees with and without transitions but present only those trees in which transitions are excluded. Also, the results of the NJ and ML analyses were nearly identical and therefore only the NJ trees are shown. Differences between parsimony, NJ, and ML trees are discussed.

RESULTS

In the 1269-bp data set, there were 109 variable sites and 260 parsimony-informative sites. In the 1970-bp data set, there were 170 variable sites and 310 parsimony-informative sites. The transition/transversion ratio, estimated by maximum-likelihood, was 2.16. Parsimony (excluding transitions) yielded one maximum parsimony tree (MPT) for both the 1269- and the 1970-bp data sets (1269-bp data set: CI = 0.524, TL = 416; 1970-bp data set: CI = 0.615, TL = 390). Parsimony (including transitions) yielded two and one MPTs for the 1269- (CI = 0.458, TL = 1200) and 1970- (CI = 0.555, TL = 1185) bp data sets, respectively.

All trees resulting from parsimony, NJ, and ML analyses of both the 1269- and the 1970-bp data sets (with or without transitions) strongly support the monophyly of the *Bufo peltocephalus* Group (Fig. 2). The relationships among the West Indian toads are similar in the NJ and ML analyses, with each defining a Cuban clade composed of *B. empusus*, *B. fustiger*, *B. gundlachi*, *B. longinasus*, *B. peltocephalus*, and *B. taladai*. In addition, the single tree resulting from the parsimony analysis of the 1970-bp data set (excluding transitions) also supports the Cuban clade, (although with low node support [BS $<$ 50]). All analyses support a clade containing the large taxa *B. peltocephalus*, *B. fustiger*, and *B. empusus* with *B. taladai* clustering with these species in the NJ and ML analyses. The remaining two Cuban species, *B. gundlachi* and *B. longinasus*, are smaller species and cluster as a group in all analyses except the parsimony trees resulting from the 1269-bp data sets (including and excluding transitions). The results of NJ and ML analyses also are similar in that they place *B. guentheri* as closest relative to the Cuban clade, with *B. lemur* as basal among West Indian taxa when transitions are included; the NJ, ML, and parsimony analyses join those two species when transitions are excluded.

The two African species, *B. regularis* and *B. pardalis*, form a group in the NJ and ML trees. The relationships of the remaining non-West Indian taxa differ considerably between trees that include and exclude transitions. When transitions are included, the two Asian species, *B. melanostictus* and *B. stomaticus*, are separated and the North American species, *B. americanus*, is separated from the North and Central American *valliceps* Group species, *B. luetkenii* and *B. valliceps*. However, when transitions are excluded, the Asian species cluster and the North and Central American species cluster (Fig. 2).

The South American species *B. granulosus* and the Central and South American species *B. marinus* form a group in all analyses and cluster with the West Indian clade in most analyses (except NJ and ML analyses excluding transitions). In those trees, *B. granulosus* and *B. marinus* cluster with the North and Central American clade. The North and Central American species *B. luetkenii* and *B. valliceps* also are well supported as sister taxa in all analyses (BS $>$ 95).

The single-most parsimonious tree resulting from the 1269-bp data set (transitions excluded) differs in

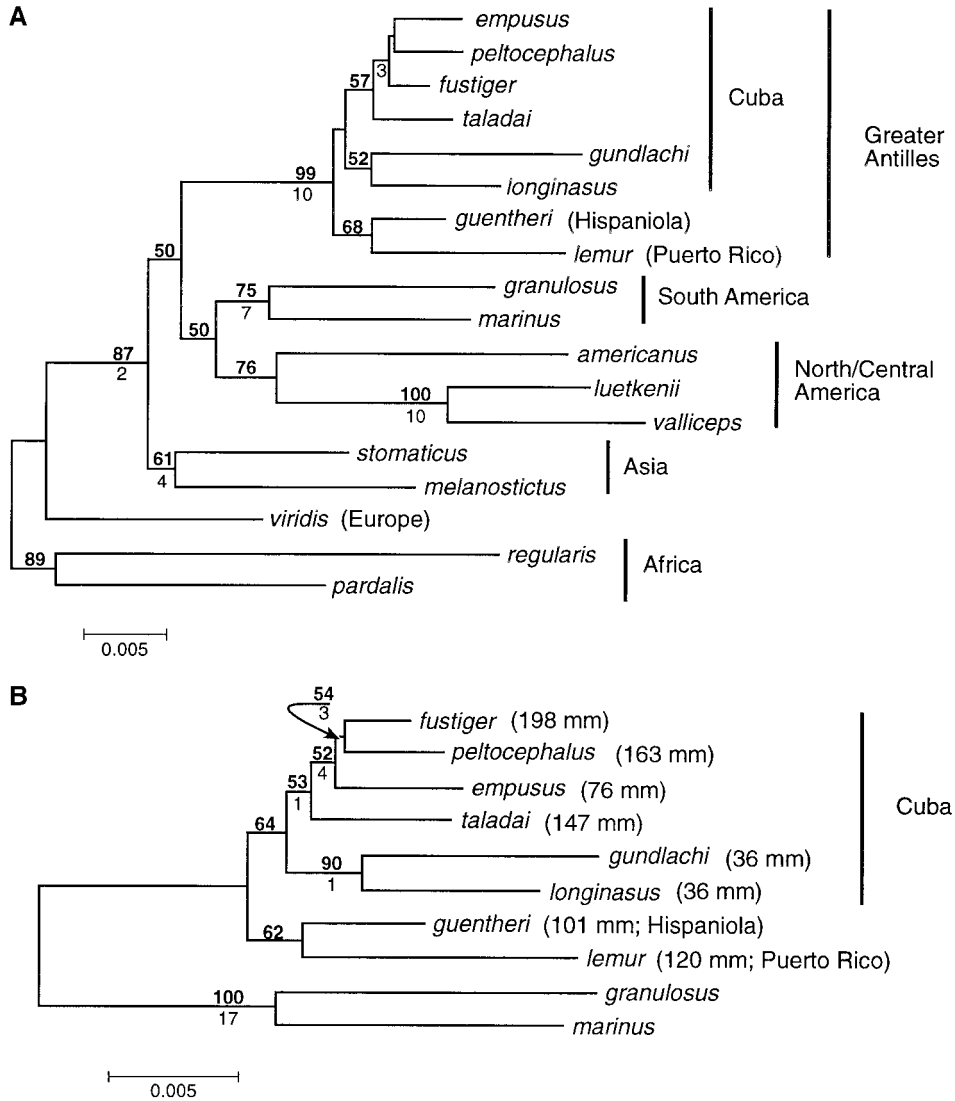

FIG. 2. Phylogenetic hypotheses of toads of the *Bufo peltocephalus* Group (Greater Antilles) and selected congeners inferred from analysis of mitochondrial DNA sequences spanning portions of the 12S rRNA, 16S rRNA, and cytochrome *b* genes. Bootstrap confidence values (50% or greater) are indicated in boldface above nodes (2000 replications); Bremer support for nodes of parsimony trees (analyses including transitions) that are common to NJ trees are presented below nodes. (A) Neighbor-joining tree of all taxa (1269 aligned sites) using the Kimura two-parameter distance (transversions only) and rooted with the African species *B. pardalis* and *B. regularis*. (B) Neighbor-joining tree resulting from an identical analysis except that incomplete sequences are omitted, allowing a larger data set (1970 aligned sites) to be analyzed. Average snout–vent lengths of ingroup taxa are presented next to species names. The tree is rooted with the South American taxa *B. granulosus* and *B. marinus*.

several respects from trees generated by the NJ and ML methods: a Cuban clade is not formed in this tree as the Cuban species *B. taladai* is basal to the clade containing *B. empusus, B. fustiger, B. peltocephalus, B. guentheri, B. lemur*, and *B. gundlachi*. In addition, *B. longinasus* is not sister to *B. gundlachi*, but is basal to all other West Indian taxa. This tree places *B. lemur* as sister to *B. guentheri* (BS = 67), with *B. gundlachi* as sister to these taxa. This clade is sister to the clade containing *B. empusus, B. fustiger, and B. peltocephalus.*

In the 1970-bp data set, the West Indian species were rooted with the South American clade because they were not missing regions of sequence and were members of the closely related American clade. In those analyses, relationships were similar to those obtained with the 1269-bp data set (Fig. 2). No relationships within the West Indian clade were supported by significant bootstrap confidence values.

DISCUSSION

Phylogeny of West Indian Toads

All analyses show strong support for the monophyly of the West Indian toads (i.e., the *Bufo peltocephalus*

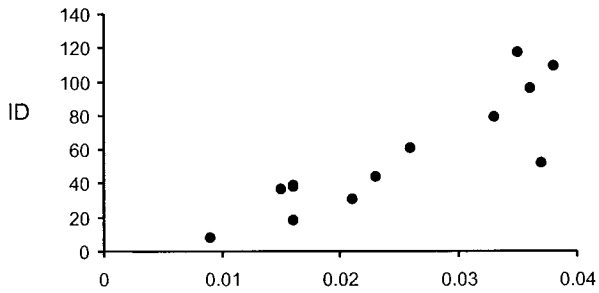

FIG. 3. Plot of immunological distances, from Hass *et al.* (2001) and Maxson (1984), versus Tamura–Nei transversion distances from this study.

Group) with bootstrap values >99%. These results concur with speculation from morphological description (Pregill, 1981) and shared, derived morphological characters for the group, including (1) presence of knob-shaped terminal phalanges and (2) shared presence of a maxillary extension (Pramuk, 1999). Unfortunately, tissues currently are unavailable for two ingroup taxa. One of these taxa, *Bufo fluviaticus*, is the most controversial species of the group. This taxon, more than any other, has led some to doubt the monophyly of these toads (Graybeal and Cannatella, 1995), because of its lack of a maxillary extension or "rostral elements" (*sensu* Pregill, 1981) shared by all members of the group. This character, however, was recently redescribed and found to be present in *B. fluviaticus* (Pramuk, 2000) as a small band of bone most easily seen in cleared-and-stained specimens. Although DNA data are unavailable for this species, the presence of this character (unique among all bufonids) in all members of the *B. peltocephalus* Group (and *Bufo goeldii* of the *B. granulosus* Group) lends support for a common evolutionary history.

In addition to the monophyly of the West Indian species, there is little else that is statistically significant in the results of the sequence analyses, suggesting that more sequence data will be needed to resolve fully the evolutionary relationships of these taxa. Nonetheless, there is considerable concordance between these results and geography, morphology, and other molecular data (e.g., Fig. 3). This agreement between independent data sets gives added support to the results and is discussed below.

Most analyses support a derived Cuban clade containing *Bufo empusus, B. fustiger, B. gundlachi, B. longinasus, B. peltocephalus,* and *B. taladai* within the West Indian toads. Within the Cuban clade, all NJ and ML trees and the parsimony analysis including transitions (1970-bp data set) contain a clade of large toads (76–198 mm snout–vent length) sharing broad, heavily ossified skulls (*B. fustiger, B. peltocephalus, B. empusus,* and *B. taladai*). The sister group relationship of *B. fustiger* and *B. peltocephalus* seen in most analyses is expected, because *B. fustiger* was once considered a subspecies of *B. peltocephalus* (Schwartz, 1960; Schwartz and Henderson, 1991). Both species are large and share similar morphologies; however, they differ in their calls and are separated by an approximately 100-km gap in distribution (Valdés de la Osa, 1989). The remaining two Cuban species, *B. gundlachi* and *B. longinasus*, are small (36 mm snout–vent length) and form a separate group (Fig. 2).

Most analyses place *Bufo guentheri* from Hispaniola as the closest relative of the Puerto Rican species *B. lemur*. This topology concurs with the previously proposed hypothesis that *B. guentheri* and *B. lemur* share a more recent common ancestor with each other than with any other species of West Indian toad (Pregill, 1981). This relationship also agrees with albumin ID data that strongly support a *B. lemur* + *B. guentheri* grouping (ID = 15 versus 30–40 for other species of West Indian toads) (Hass *et al.*, 2001). In addition, the shared features of skull shape, accuminate nasal bones, and compressed snouts of these two taxa support their presumed evolutionary alliance.

Origin of the West Indian Clade

Several authors have presented morphological (Cei, 1972; Graybeal, 1997), immunological distance (Hedges, 1996a), and molecular (Graybeal, 1997) data that suggest a close relationship of the West Indian toads to the New World *Bufo*. Based on morphological similarity, it was postulated that the *Bufo peltocephalus* Group is related to the *B. granulosus* Group (*sensu* Frost, 1985) of South America (Cei *et al.*, 1972; Graybeal, 1997). The latter group is an assemblage of three species and 11 subspecies of toads distributed throughout South America, from the Río Calobre in Panama to the eastern lowlands of Brazil and the islands of Margarita and Trinidad (Gallardo, 1965; Frost, 1985). A close relationship of the West Indian toads and the *B. granulosus* Group is supported by three previously reported shared–derived characters: (1) zygomatic ramus of the squamosal in contact with the lateral edge of the ventral ramus of the squamosal and articulating with maxilla (Cei, 1972; Pregill, 1981), (2) nasal bones comprising approximately half the margin of the orbit (Cei *et al.*, 1972), and (3) shared maxillary extension (Pramuk, 2000).

Our sequence analyses are unable to identify the closest relative of the West Indian clade, although a New World origin is suggested. It is noteworthy that the relationships of the non-West Indian species agree quite well with geography and with albumin immunological data (Maxson, 1984). The immunological data join the North American and Central American species together in a cluster that, in turn, is the closest relative of the South American species. The Asian species *B. melanostictus* and *B. stomaticus* form a cluster that is the closest relative of the American clade. Although

weakly supported by bootstrap analysis, this pattern is seen in the sequence analyses (Fig. 2).

Within the outgroup taxa, the sister group relationship of *B. luetkenii* and *B. valliceps* is expected, because both are members of the *B. valliceps* Group—an assemblage distributed from the southern United States to Costa Rica (Mendelson, 1997). The species of this group of Middle American toads are defined by several morphological characters including broad frontoparietals (Martin, 1971) and the presence of bilateral vocal slits (Mendelson, 1997).

Historical Biogeography of West Indian Toads

The results of the phylogenetic and molecular clock analyses presented here have some implications for the historical biogeography of this group. Because the Caribbean has been a tectonically active region for more than 100 million years, and the islands have moved considerably since they originated, there were opportunities for both vicariance and dispersal of the fauna in the past (Hedges, 1996b).

The fauna of the West Indies includes 1295 species of vertebrates characterized by high levels of endemism. Their taxonomic composition, relationships, and other data have been used to infer the historical biogeography of various groups. Although the geologic history of the region provides the opportunity for proto-Antillean vicariance, most groups appear to have arisen by dispersal. The evidence for this is a reduced higher-level taxonomic diversity (now and in the past), unusually large adaptive radiations of groups present, and divergence time estimates too recent (and scattered in time) to be associated with the late Mesozoic proto-Antillean vicariance. Also, most terrestrial groups have closest relatives in South America, consistent with the direction of water currents and hurricane tracks (Hedges, 1996b).

A New World origin for West Indian *Bufo* now is supported by diverse data, including morphology (Cei, 1972), albumin immunological data (Hedges *et al.*, 1992; Hass *et al.*, 2001), and nucleotide sequence data (Graybeal, 1997; this study). A close relationship with the South American *granulosus* Group (Cei, 1972) is not refuted by the molecular evidence but also is not necessarily supported by those data. There is no fossil evidence that would elucidate the timing of the origin of this group in the Antilles, so the molecular clock evidence provides the best estimate at the present time (Hedges, 1996a).

Maxson (1984) found that New World species of *Bufo* were separated from Asian and African species by ID's of 110–120 units, which corresponds to approximately 65–75 million years ago (mya) using a standard calibration (Maxson, 1992). She also found that South American species groups, such as the *granulosus* and *marinus* groups, were separated from those in North and Central America by about 70–80 ID units (~45 mya). No West Indian species was examined and no antiserum was made to *B. granulosus*. Hedges *et al.* (1992) reported an average ID of 85 (~50 mya) between two West Indian species and *B. marinus*.

More recently, Hass *et al.* (2001) reported additional data from West Indian toads. They found that *B. granulosus* had a smaller ID (52; = 31 mya) to a West Indian species (*B. peltocephalus*) than did *B. marinus* (96; = 58 mya) to another West Indian species (*B. guentheri*). However, because the sequence analyses here show a weakly supported *granulosus–marinus* grouping, it is unclear whether the immunological data for the two South American species should be combined or whether *B. granulosus* should be used by itself (assuming that it is the closest relative to the West Indian clade). We take a conservative approach here and use both time estimates, as a range of 31–58 mya, for the origin of the West Indian clade. Such time estimates would appear to be too young to be consistent with a late Cretaceous vicariant event and therefore would support an origin by over-water dispersal for this group (Hedges *et al.*, 1992; Hedges, 1996a).

The new ID data for West Indian *Bufo* (Hass *et al.*, 2001) are correlated with sequence divergence (Fig. 3; $r = 0.87$) and allow some inferences to be drawn in regard to biogeography within the West Indian clade. The average reciprocal ID (37) between *B. guentheri* and *B. peltocephalus* suggests an Early Miocene (22 mya) divergence of the Cuban clade from the Hispaniolan–Puerto Rican clade. The ID (18) between *B. guentheri* and *B. lemur* indicates a late Miocene (11 mya) divergence between these Hispaniolan and Puerto Rican species. The positions of the Greater Antilles and their extent of exposure above sea level at different times during the Cenozoic are not yet known with precision and therefore the biogeographic mechanisms involved in these events are unclear. Either vicariance or dispersal could explain them. Bufonids have been reported to raft across fresh water (Boyd, 1962) and recently, green iguanas were reported to disperse overwater from the island of Guadeloupe to Anguilla in the Lesser Antilles following a hurricane (Censky *et al.*, 1998). However, direct observation of animals floating is not necessary to infer dispersal. The presence of any native amphibian on an island that never was connected to the mainland, such as in the Lesser Antilles, is evidence that dispersal has occurred.

In summary, the sequence analyses support the monophyly of the West Indian toads (*Bufo peltocephalus* Group) and indicate a New World origin for the lineage. In addition, molecular time estimates suggest a Cenozoic divergence of the group from mainland relatives, which favors a biogeographic origin by overwater dispersal rather than vicariance.

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