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Molecular phylogeny and biogeogr aphy of West Indian frogs of the genus Leptodactylus (Anura, Leptodactylidae)

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Abstract

Three endemic species of the aquatic-breeding frog genus Leptodactylus are recognized from the West Indies: Leptodactylus albilabris (Puerto Rico and the Virgin Islands), Leptodactylus dominicensis(Hispaniola), and Leptodactylus fallax (Lesser Antilles). DNA sequences were obtained from several mitochondrial genes to resolve taxonomic questions involving these species and to provide insights i nto their origin and distribution in the islands. We found low levels of sequence divergence between L. dominicensis and L. albilabris , supporting morphological evidence that the former species is a junior synonym of the latter species. Phylogenetic analysis supported previ ous species-group allocations, Wnding that L. albilabris is a member of the fuscus group and L. fallax is a member of the pentadactylusgroup. Molecular time estimates for the divergence of L. albilabris from its closest relative in South America (24–58 million years ago, Ma) and for L. fallax from its closest relative in South America (23–34 Ma) indicate that they colonized the West Indies independently by over-water dispersal in the mid-Cenozoic. The absence of detectable sequence divergence between the two extant populations of L. fallax (Dominica and Montserrat), a species used for human food and now critically endangered, suggests that one or both arose by huma n introduction from an island or islands where that species originated. The relatively minor genetic di Verentiation of populations of L. albilabris can be explained by vicariance and dispersal in the Pleistocene and Holocene, although human introduction of some populations cannot be ruled out.

Keywords: West Indies; Caribbean; Puerto Rico; Virgin Islands; Hispaniola; Lesser antilles; Biogeography; Phylogeny; Conservation; Amphib ian declines; Systematics; Taxonomy; Amphibia

1. Introduction

Frogs of the genus Leptodactylus (72 species) occur in the New World tropics and have aquatic larvae (Amphibiaweb, 2006; Duellman and Trueb, 1986). Only three species are endemic to the West Indies (Schwartz and Henderson, 1991): Leptodactylus albilabris (Puerto Rico and the Virgin Islands), Leptodactylus dominicensis (Hispaniola), and Leptodactylus fallax (Lesser Antilles). A fourth species, Leptodactylus validus, occurs in the southern Lesser Antilles (Grenada, Grenadines, and St. Vincent) and on the islands of Trinidad and Tobago, which are part of continental South America (Heyer, 1994). Its origin in the West Indies has been presumed to be Pleistocene or Holocene (Hedges, 1996; Heyer, 1994), including the possibility of recent human transport. In contrast, the terrestrial frog genus Eleutherodactylus, with direct development, has undergone a major radiation in the West Indies, with 146 endemic species known from the islands (Hedges, 2006a).

West Indian Leptodactylus are distributed in the eastern half of the West Indies, consistent with an origin from South America and the Xow of ocean currents from east to west in the Caribbean (Hedges, 1996, 2001). The Hispaniolan species, L. dominicensis (Cochran, 1923), is known from only a small area in the extreme eastern part of the island, below the Bahia de Samaná. Morphologically, it is so similar to L. albilabris of Puerto Rico and the Virgin Islands that it has been considered a junior synonym of that species

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in some taxonomic accounts (Heyer, 1978). Nonetheless, most have treated it as a distinct species since it was described (Cochran, 1941; Hedges, 2006a; Powell et al., 1996; Schwartz and Henderson, 1988, 1991; Schwartz and Thomas, 1975). Both species are relatively small (<50 mm snout-vent length, SVL), live and breed in shallow bodies of water such as flooded meadows and ditches, and probably use seismic signals in their intraspecific communication (Lewis et al., 2001; Schwartz and Henderson, 1991). The Puerto Rican species, *L. albilabris*, is widely distributed in lowland areas of the island, and occurs throughout the Puerto Rican Bank (including the Virgin Islands) and on St. Croix (Schwartz and Henderson, 1991).

Leptodactylus fallax is a large species, reaching 210 mm SVL (Daltry and Gray, 1999), and occurs now on two islands: Dominica and Montserrat. It is unusual in having maternal care that includes obligatory oophagy (Gibson and Buley, 2004). Historical records have suggested that it had a wider distribution in the past, occurring also on St. Kitts, Antigua, Guadeloupe, Martinique, and St. Lucia, although museum specimens are known only from St. Kitts (Kaiser, 1994; Lescure, 1979a,b). The disappearance of the species from those islands has been attributed to predation by the mongoose and the introduction of the predacious toad *Bufo marinus* (Kaiser, 1994). The species is also consumed by humans, and presumably this fact has had an impact on both the distribution of the species and on its current decline. In Montserrat, recent volcanic activity has affected the species range and overall health of the populations (Daltry and Gray, 1999; Gibson and Buley, 2004). Currently, the species is listed as "critically endangered" on the "Red List" of the International Union for the Conservation of Nature (IUCN, 2006).

Each of these two lineages of *Leptodactylus* is believed to have arrived to the West Indies independently from South America. Morphologically, *L. albilabris* and *L. dominicensis* belong to the *fuscus* group (Heyer, 1978) and *L. fallax* to the *pentadactylus* group (Heyer, 1979). As with most frogs, the fossil record is largely silent on the origin of these species, and therefore molecular data have been collected to offer evidence on times of divergence. Estimates of amino acid substitutions in the serum albumin protein of *Leptodactylus* have been made with an immunological technique, micro-complement fixation (Maxson and Heyer, 1988), but these data were only partly useful. Most species examined were too divergent to obtain comparable results, and some other results were inconsistent.

In the case of *L. albilabris*, one-way (antigen versus antibody) immunological distances (IDs) to two species in the *fuscus* group (*Leptodactylus fuscus* and *Leptodactylus labrosus*) were 76 and 66, respectively (Maxson and Heyer, 1988), suggesting divergence times of approximately 40–45 Ma using an albumin calibration of 1 ID = 0.6 Ma (Maxson, 1992). For *L. fallax*, the results were mixed because it exhibited very low IDs (5–11) to a species in South America (*Leptodactylus stenodema*) not considered its closest relative and similarly low IDs to *L. albilabris*, which by all other evidence is in a different species group (Maxson and Heyer, 1988). Therefore, while the immunological data supported a mid-Cenozoic to late Cenozoic origin for these lineages in the West Indies, the results were inconclusive.

To clarify these taxonomic questions surrounding the endemic West Indian species of *Leptodactylus*, and to gain insights into their origin and evolution, we have conducted DNA sequence analyses. We collected samples of each of the species and sequenced portions of three mitochondrial genes. Our analyses have helped to illuminate the evolutionary history of these frogs in the West Indies.

2. Materials and methods

The senior author collected specimens by hand at localities in Hispaniola, Puerto Rico, the US Virgin Islands, and Montserrat. These were supplemented by other available tissue samples, and by sequence data in the public databases (Genbank). Field and laboratory research was approved by the Institutional Animal Care and Use Committee of Pennsylvania State University (#17632). Tissues were removed and frozen in liquid nitrogen, or temporarily transferred to the laboratory in 75% ethanol. The remaining specimen was preserved. Tissues were maintained in the laboratory at −80 °C. The specimens sampled, localities (Fig. 1), laboratory numbers, and Genbank accession numbers. are listed in the Appendix A.

DNA was extracted from tissue samples, amplified (PCR) with primers spanning defined regions of genes, and sequenced. Two relatively slow-evolving mitochondrial genes, 12S rRNA and 16S rRNA (~1800 bp, total) were used to determine higher-level relationships of the West Indian species to other species in the genus, and for time estimation. A portion of the faster-evolving cytochrome b mitochondrial gene (804 bp) was used for examining genetic variation among individuals and populations of the species. The primers used were (listed 5-prime to 3-prime, with gene indicated in prefix of primer name): 12L29, AAAGCRTA GCACTGAAAATGCTAAGA; 12.1L4, TACACATGCA AGTYTCCGC; 12H46, GCTGCACYTTGACCTGAC GT; 12.2L4, GCTTAAAACCYAARGGAYTTGACG; 12.2H1, TCCGGTAYRCTTACCATGTTAC; 16L19, AA TACCTAACGAACTTAGCGATAGCTGGTT; 16H36, AAGCTCCAWAGGGTCTTCTCGTC; 16L37, GATTA YAAGAAAAAGAAGGAACTCGGCA; 16H37, TTAC TCCGGTCTGAACTCAGATC; CBL21, ACAGGHYT WTTCCTAGCDATACA; CBH22, GATGAYCCWGTT TCATGAAG; CBL20, GTYCAATGAATCTGAGG CG G; CBH15, ACTGGTTGDCCYCCRATYCAKGTKAG; CBL1, TCTGCYTGATGAAAYTTTGG; CBH1, GGAA TTTTRTCTGARTTSGATT; CBL2, ATRGTMGARTG AATCTGA; CBH2, GCTACRAAGACTTATCATTT. Both strands of the PCR products were sequenced using the ABI (Applied Biosystems) BigDye sequencing kit and an ABI Prism 3100 Genetic Analyser.

Sequences were aligned using BIOEDIT (Hall, 1999) and CLUSTAL (Thompson et al., 1997). Phylogenies were

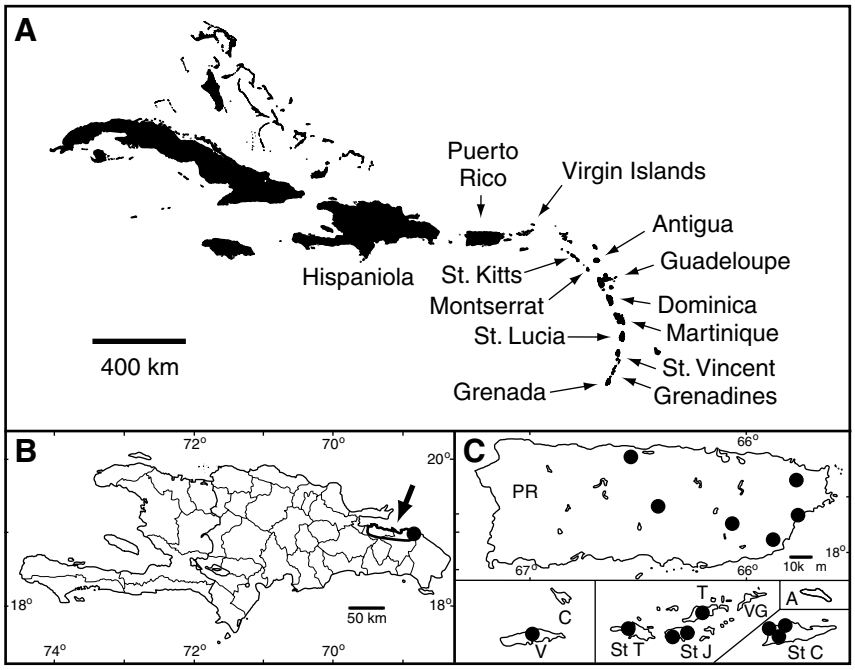

Fig. 1. Distribution of *Leptodactylus* in the West Indies. (A) Map of West Indies showing islands mentioned in the text. (B) Distribution of *L. dominicensis* in Hispaniola (solid line indicated by arrow), showing locality sampled. (C) Puerto Rico, Culebra, Vieques, and the Virgin Islands, showing localities sampled for *L. albilabris*. The species is distributed throughout Puerto and occurs on all labeled islands except Virgin Gorda. Islands abbreviated are Anegada (A), Culebra (C), Puerto Rico (PR), St. Croix (St C), St. John (St J), St. Thomas (St T), Tortola (T), Vieques (V), and Virgin Gorda (VG).

constructed with minimum evolution (ME) using MEGA 3.1 (Kumar et al., 2004), with maximum likelihood (heuristic search, GTR + gamma model) using PAUP∗ 4b10 (Swofford, 2003), and with Bayesian methods of inference using MrBayes 3.1 (Ronquist and Huelsenbeck, 2003). PAML 3.13d (Yang, 1997) (HKY85 model) was used to find the gamma parameter in the minimum-evolution analyses (Tamura-Nei model). Optimal model parameters for likelihood analyses were estimated in PAUP using MODELTEST (Posada and Crandall, 1998) and fixed before analysis. Statistical significance was evaluated with bootstrapping and Bayesian posterior probabilities.

Divergence time analyses for the rRNA dataset were conducted with the Bayesian software MULTIDIVTIME T3 (Thorne and Kishino, 2002; Yang and Yoder, 2003) and with the penalized-likelihood software r8S (Sanderson, 2003). The MULTIDIVTIME analysis used the following priors: rttm (mean of time for ingroup root), 65 Ma; rttmsd (standard deviation of time for ingroup root), 15; rtrate (mean of rate for ingroup root), 0.003; rtratesd (standard deviation of rate for ingroup root), 0.002; brownmean (mean of variance in logarithm of the rate), 0.025; brownsd (standard deviation of variance in logarithm of the rate), 0.025; bigtime (time that is greater than that of any node in the tree), 100 Ma. The prior for rttm (65 Ma) was used based on previous time estimates from molecular analysis of serum-albumin (Maxson and Heyer, 1988) showing that many interspecific divergences in *Leptodactylus* date to the early Cenozoic, although younger (45 Ma) and older (85 Ma) priors were used for comparison. Other priors chosen were based on recommendations by the authors of the software. For the r8s analysis, the gamma parameter was set using the previously determined estimate, and the smoothing factor was estimated with cross-validation. The tree used in the r8S analysis was the ML tree. Only one calibration point could be used from the serum albumin time-estimation analysis (Maxson and Heyer, 1988): the divergence of *Leptodactylus labyrinthicus* and *Leptodactylus pentadactylus*. The immunological distance (35) was a reciprocal (32 and 37), yielding a divergence time of 21 million years ago (Ma), using the calibration derived from a larger vertebrate data set (Maxson, 1992).

Sequences of the following species were obtained from Genbank (accession numbers in parentheses for 12S rRNA, 16S rRNA) and used in the analyses: *L. fuscus* (DQ283404), *Leptodactylus knudseni* (AY947882, AY947863), *L. labyrinthicus* (AY947875, AY947861), *L. pentadactylus* (AY326017), *Leptodactylus ocellatus* (AY843688). Corresponding sequences of the mitochondrial genome of the hylid frog *Hyla chinensis* (AY458593) were used for rooting the trees. Although DNA sequence analyses have shown that Leptodactylidae may be paraphyletic (Darst and Cannatella, 2004; Ruvinsky and Maxson, 1996), they also indicate that Hylidae is one of the closest lineages to the Leptodactylinae (which includes *Leptodactylus*).

3. Results and discussion

The phylogenetic analysis of the mitochondrial rRNA gene sequences (1661 aligned sites, excluding gaps) supports the species group allocation of endemic West Indian *Leptodactylus* based on morphology (Heyer, 1978, 1979):

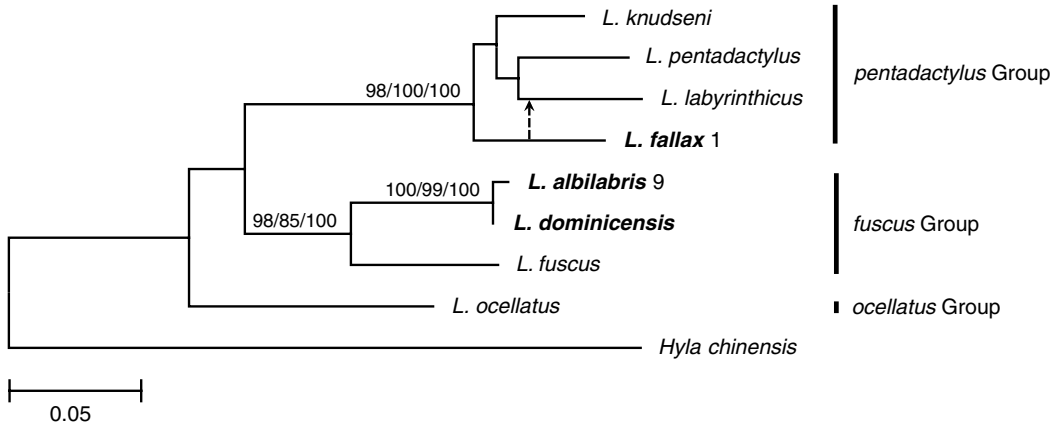

Fig. 2. Phylogenetic relationships of endemic West Indian frogs of the genus *Leptodactylus*, including several species from South America. The tree is inferred from a maximum likelihood analysis of mitochondrial DNA sequences (12S and 16S rRNA, 1661 bp) and is identical to the Bayesian tree; dashed line and arrow indicates position of *L. fallax* in the minimum evolution tree. The tree is rooted with the hylid frog *Hyla chinensis*. Confidence values are indicated at nodes (maximum likelihood bootstrap/minimum evolution bootstrap/Bayesian posterior probabilities); no values are shown for a node if all three are <95%. Numbers next to names of species are sample reference numbers (see Appendix A). Species names in bold are those from the West Indies.

L. albilabris and *L. dominicensis* cluster with a species of the *fuscus* group (*L. fuscus*), and *L. fallax* clusters with species of the *pentadactylus* group (Fig. 2). Both groupings are supported by high bootstrap confidence values. Although Bayesian posterior probabilities are also shown, that measure of nodal support is thought to represent an overestimate of statistical confidence (Simmons et al., 2004) and should be treated cautiously.

Within the *fuscus* group, the analysis also shows that *L. dominicensis* and *L. albilabris* are nearly indistinguishable at these genes. Other species in the *fuscus* group were not included, and therefore the possibility remains open that the West Indian clade may have an even closer relative on the mainland. Two South American species suggested as being closely related to *L. albilabris* based on color pattern (Heyer, 1978), *Leptodactylus amazonicus* and *Leptodactylus fragilis*, would be important to examine in the future. Within the *pentadactylus* group, *L. fallax* joins an essentially unresolved polytomy with *L. knudseni*, *L. labyrinthicus*, and *L. pentadactylus.* Again, not all members of this species group were examined, and therefore future analyses may identify a closer relative of *L. fallax*.

Sequences of the mitochondrial cytochrome b gene were collected specifically to examine genetic variation within *L. albilabris* and within *L. fallax*, because of its faster rate of evolution. The phylogenetic tree (Fig. 3) shows two results of taxonomic and biogeographic significance. The first involves the Greater Antillean species. The various samples of *L. albilabris* from throughout its range in Puerto Rico and the Virgin Islands show low levels of sequence divergence, and *L. dominicensis* is nested among them. This result does not support the recognition of *L. dominicensis* as a valid species, and therefore we agree with an earlier assessment based on morphological variation (Heyer, 1978) that *L. dominicensis* is a junior synonym of *L. albilabris.*

Among sequences of *L. albilabris*, groupings partially corresponded to geography (Figs. 1 and 3), although relationships could not be resolved statistically due to low levels of divergence and the limited number of sites. For example, the two individuals from St. John formed a cluster as did the remaining individuals from the US and British Virgin Islands. Together, those two clades joined a more inclusive group containing the sample from Vieques and one from northeastern Puerto Rico. The remaining samples from Puerto Rico, and *L. dominicensis*, were phylogenetically outside of the group just described. Additional sequences from fast-evolving genes, or from microsatellites, will be needed to draw any additional conclusions concerning the phylogeography of *L. albilabris.*

The second noteworthy aspect of the cytochrome b tree (Fig. 3) involves *L. fallax*. Sequences *L. fallax* from Dominica and Montserrat are identical, a result obtained independently by R. Thorpe, University of Wales (personal communication). This result was unexpected because the two islands have never been joined and most species of amphibians and reptiles in the Lesser Antilles are endemic to single islands or island banks (Schwartz and Henderson, 1991). Even some variation would be expected among individuals of a single population, and therefore the absence of detectable sequence divergence between the two extant populations of *L. fallax* suggests that one or both arose by human introduction. Unfortunately, it is not known where the species originated. It may have evolved on one or the other island, or a third island. The historical records indicating a more widespread distribution in the past (see above) make it more difficult to determine the island or island bank where this species originated. The reason for the introduction of this species to different islands is almost certainly related to human consumption, either by Amerindians or post-Columbian. At least reintroductions of populations to depleted areas could be accomplished without concern for mixing genetically distinct populations.

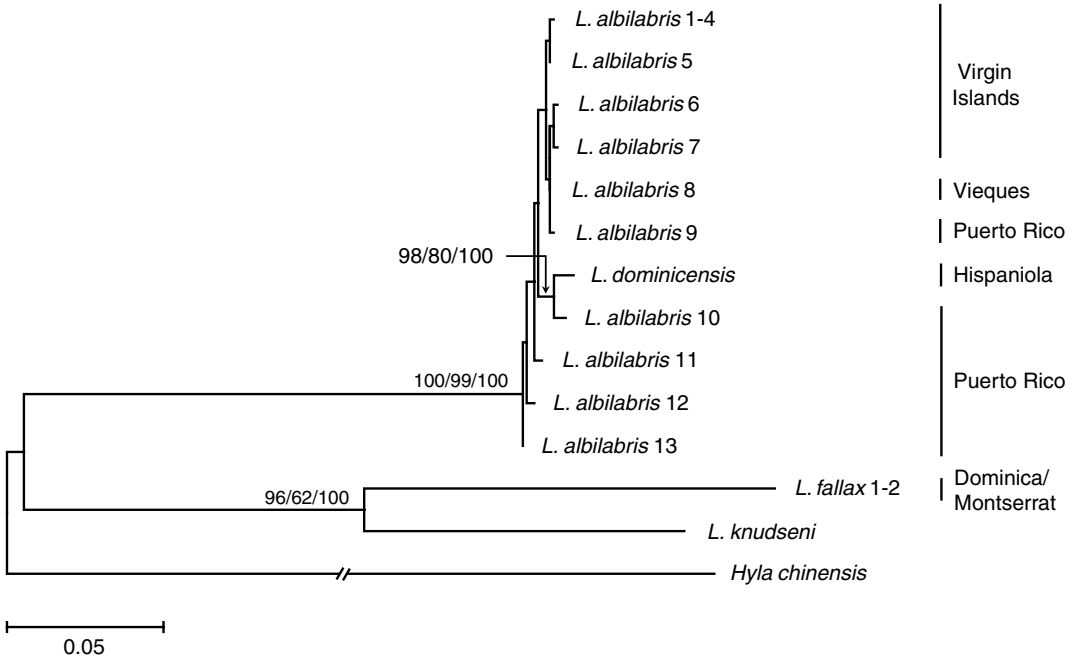

Fig. 3. Phylogenetic relationships of endemic West Indian frogs of the genus *Leptodactylus*, including one species (*L. knudseni*) from South America. The tree is inferred from a maximum likelihood analysis of mitochondrial DNA sequences (cytochrome b, 804 bp) and is identical to the Bayesian and minimum evolution trees except for relationships among populations of *L. albilabris* which are statistically unresolved.

The estimated times of divergence from the rRNA data set (Fig. 4) provide evidence bearing on the historical biogeography of West Indian *Leptodactylus*. In interpreting such evidence one must realize that the inclusion of other living species from South America, or extinct species (if they were available), could substantially reduce (but not increase) the time of origin, if those missing species were found to be closest relatives of West Indian species.

The Bayesian time estimate for the divergence of *L. fallax* from its closest relative (the South American *pentadactylus* Group clade of *L. knudseni*, *L. labyrinthicus*, and *L. pentadactylus*) was 27 (23–34) Ma and the penalized likelihood estimate was 29 Ma. The use of younger (45 Ma) and older (85 Ma) priors for rttm affected the Bayesian time estimate by only 1 million years (27–28 Ma). These dates indicate an origin by dispersal of a *pentadactylus* Group member from South America to the Lesser Antilles. As discussed above, the original island of colonization remains to be determined because of frequent introductions by humans who have used it as a food source. The data also indicate that *L. albilabris* originated by dispersal of a *fuscus* group member from South America to the Puerto Rican Bank 39 (24–58) Ma according to the Bayesian time estimation and 36 Ma according to penalized likelihood time estimation (Fig. 4). The use of younger (45 Ma) and older (85 Ma) priors for rttm affected the Bayesian time estimate by about 7% (36–42 Ma). There is no evidence that it inhabited any other island or island bank until relatively recently, when it appeared in St. Croix (not located on the Puerto Rican Bank) and in eastern Hispaniola.

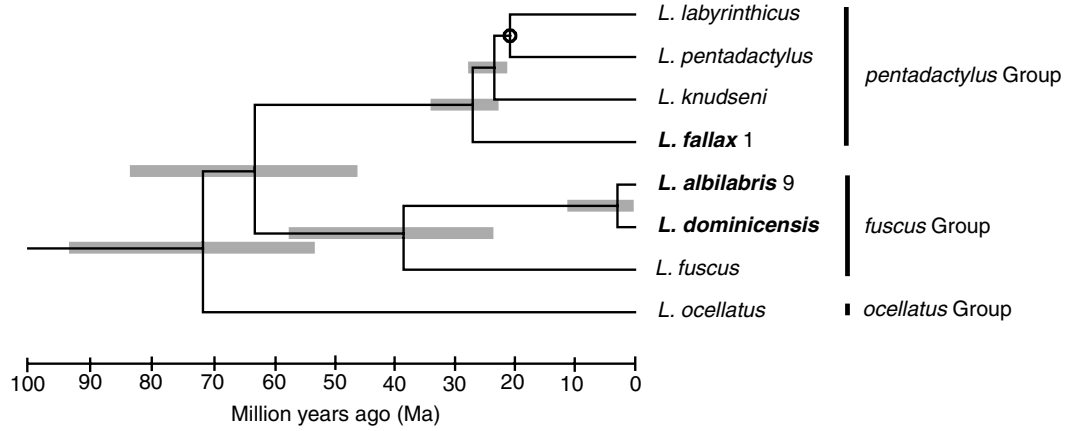

Fig. 4. A time tree of endemic West Indian frogs of the genus *Leptodactylus* (species names in bold) using a Bayesian analysis of mitochondrial 12S rRNA and 16S rRNA gene sequences. Gray bars correspond to 95% credibility intervals for the divergence time at each node. The calibration node is denoted with a filled circle.

An origin for West Indian *Leptodactylus* by proto-Antillean vicariance is rejected because their dates of origin would need to be older than approximately 65 Ma (Hedges, 2001, 2006b). Dispersal across a dry land bridge (Aves Ridge) from South America could have occurred, but geologic evidence is silent and the biological evidence argues against any dry land bridge having ever occurred (Hedges, 2001, 2006b). Moreover a land bridge would not explain the presence of *Leptodactylus* in the Lesser Antilles (never connected by land) and the absence of any ancient lineage in Hispaniola (the island presumably connected by land bridge) or Cuba.

The time estimates for the divergence of *L. albilabris* (sample No. 9) from *L. dominicensis* were 2.9 (0.3–11.4) Ma using the Bayesian method and 2.2 Ma using penalized likelihood. The use of younger (45 Ma) and older (85 Ma) priors for rttm affected the Bayesian time estimate only slightly (2.8–3.1 Ma). However, considering the wide range in the Bayesian credibility interval and the recent discovery that time estimates are often inflated for shallow divergences in trees (Ho et al., 2005), little can be inferred from these estimates. Unfortunately, there were no calibrations available to estimate divergence times with the faster-evolving cytochrome b data set separately. Rates of sequence variation in the cytochrome b gene vary widely among amphibians (Babik et al., 2004; Mulcahy and Mendelson, 2000; Tan and Wake, 1995) and therefore use of a single rate is not justified. However, given the lowland distribution of this species and the fact that most of the Puerto Rican Bank was a continuous land area during the last glaciation suggests that the populations, including the one in Hispaniola, probably diverged in the late Pleistocene or Holocene as sea levels rose. In some cases (e.g., St. Croix), dispersal may have occurred on flotsam after storms, although human introductions cannot be ruled out.

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Appendix A. Species, localities, and sequence accession numbers

In the following list, localities are provided for each sample, followed by the laboratory tissue collection number, phylogenetic tree reference number (if applicable), and Genbank sequence accession numbers (in parentheses).

Leptodactylus albilabris.—Puerto Rico: Catalina, 101755, 9 (CytB-EF091401, 12S- EF091410, 16S- EF091413). Puerto Rico: Campamento Guavate, 101774, 10 (EF091394). Puerto Rico: Playa de Humacao, 101824, 13 (EF091396). Virgin Islands: St. Croix, 0.5 km S Canebay, 266774, 1 (EF091406). Virgin Islands: St. Croix, Hams Bay, 266796, 2 (EF091403). Virgin Islands: St. Croix, Allandale, 266803, 3 (EF091405). Virgin Islands: St. Thomas, Santa Maria, 266837, 4 (EF091404). Virgin Islands: St. John, Dever's Bay, 266869, 7 (EF091399). Virgin Islands: St. John, Carolina, 266875, 6 (EF091398). Virgin Islands: Tortola, 267840, 5 (EF091402). Puerto Rico: Isla Vieques, 267841, 8 (EF091400). Puerto Rico: Manati, 267842, 11 (EF091397). Puerto Rico: Yabucoa, 267843, 12 (EF091395).

L. dominicensis.—Dominican Republic: El Seibo, Nisibon, 192453 (CytB- EF091393, 12S- EF091411, 16S- EF091414).

L. fallax.—Montserrat: St. Peter, Spring Ghut, 192787, 1 (CytB- EF091407, 12S- EF091412, 16S- EF091415). Dominica: Coulibistri, 267838, 2 (EF091408).

L. knudseni.—Brazil: Rio Madeira, Rondonia, Calama, 267844 (EF091409).

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