Molecular phylogeny of an endemic radiation of Cuban toads (Bufo: *Peltophryne*) based on mitochondrial and nuclear genes

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**ABSTRACT**

**Aim** In this study we present a molecular phylogenetic and phylogeographical analysis of *Peltophryne* (Anura: Bufonidae), an endemic genus of Antillean toads, to investigate the spatial and temporal origins of the genus, with particular focus on the eight Cuban species.

**Location** Greater Antilles, with extensive sampling of the Cuban archipelago.

**Methods** We obtained DNA sequence data from two mitochondrial genes, cytochrome *c* oxidase subunit I (COI) and ribosomal RNA (16S), for 124 toads representing all eight Cuban species, and combined this with published data from Hispaniola (one of three species) and Puerto Rico (one of one species) to establish a molecular phylogeny for *Peltophryne*. In addition, we explored the phylogeographical structure of widespread Cuban species. For a subset of 42 toads we also obtained DNA sequence data from two nuclear genes, recombination activator-1 (RAG-1) and chemokine receptor 4 (CXCR-4). We combined our molecular data with published DNA sequences from a global sample of bufonid toads to place the spatial and temporal origins of *Peltophryne* in the Caribbean within a fuller geographical and phylogenetic context.

**Results** All phylogenetic analyses supported the monophyly of West Indian toads. The ancestor of *Peltophryne* diverged from its mainland source around the Eocene–Oligocene boundary, with a subsequent radiation across the Caribbean islands taking place during the Miocene. Cuban species are monophyletic with a basal split in the early–middle Miocene that separates extant small-bodied from large-bodied species. Extensive mitochondrial DNA (mtDNA) sampling within widespread Cuban species revealed contrasting phylogeographical patterns. *Peltophryne taladai* and *P. empusa* showed deeply divergent lineages, whereas no geographical structure was observed in the widespread *P. peltocephala*.

**Main conclusions** Our timeline for *Peltophryne* diversification is consistent with a biogeographical model requiring no long-distance overwater dispersal. Although confidence intervals on divergence time estimates are wide, the stem age of *Peltophryne* coincides with the hypothesized GAARlandia landspan or archipelago, which may have connected South America briefly with the Antilles. The ages of *Peltophryne* for Puerto Rico, Hispaniola and Cuba are consistent with a recently proposed vicariance scenario for the region. Our molecular results support the recognition of all eight species in Cuba, and provide evidence of possible cryptic species.

**Keywords** Caribbean biogeography, Cuba, divergence time, GAARlandia, phylogeography, relaxed molecular clock, vicariance, West Indies, within-island diversification.

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INTRODUCTION

The West Indies have a long biotic history of colonization, radiation, speciation and extinction (Wallace, 1881; Williams, 1989; Woods & Sergile, 2001). These islands are recognized as a natural laboratory for the study of biogeography and evolution (Williams, 1969; Ricklefs & Bermingham, 2007). Recent evolutionary research has focused on adaptation, speciation within islands, and the divergence of lineages among islands (Hass et al., 2001; Losos et al., 2006; Ricklefs & Bermingham, 2007; Losos & Ricklefs, 2009). Biogeographical studies have focused on the relative importance of vicariance versus over-water dispersal in the initial establishment and subsequent partitioning of ancestral lineages among islands (Hedges, 1996a,b). Historical and adaptive processes together have created the fauna of the West Indies that we see today. This fauna includes more than 1300 native terrestrial vertebrate species, characterized by high levels of endemism, especially among amphibians and reptiles (Pregill & Crother, 1999; Hedges, 2006).

The history of the West Indies stretches back almost 100 million years, when the fault that formed the Lesser Antilles began moving from west to east between North and South America (Pindell & Kennan, 2009). During this dynamic geological history, islands have accreted and broken apart, and may have formed temporary connections with the mainland (Iturralde-Vinent & MacPhee, 1999; Graham, 2003; Pindell & Kennan, 2009). Against this geological backdrop, the relative importance of vicariance and over-water dispersal in the origins of the Caribbean fauna has been controversial (Rosen, 1985; Hedges, 1996a,b; Crother & Guyer, 1996; Roca et al., 2004; Glor et al., 2005; Hedges, 2006; Hedges et al., 2008). A third model for the origin of the Caribbean biota involves a hypothesized landspace called GAARlandia, linking South America and the West Indies during the late Eocene to early Oligocene (Iturralde-Vinent & MacPhee, 1999; MacPhee & Iturralde-Vinent, 2000, 2005; Dávalos, 2004). This landspace may have been continuous or may have been punctuated by short stretches of water (Iturralde-Vinent & MacPhee, 1999).

Molecular phylogenetic estimates of divergence times have been increasingly employed to test for correspondence between the ages of island endemics and reconstructions of past geological events (Fritsch, 2003; Hower & Hedges, 2003; Dávalos, 2004; Gifford et al., 2004; Roca et al., 2004; Glor et al., 2005; Hedges & Heinicke, 2007; Weiss & Hedges, 2007; Lavin & Beyra Matos, 2008 Doadrio et al., 2009; Crews & Gillespie, 2010; Oneal et al., 2010; Rodriguez et al., 2010). This is the approach that we adopt here for Peltophryne (Anura: Bufonidae), an endemic genus of Antillean toads. Peltophryne Fitzinger, 1843 contains 12 species endemic to the Caribbean islands (Fig. 1), which range from western Cuba to the Virgin Islands (Pregill, 1981; Schwartz & Henderson, 1991). The Cuban archipelago hosts eight endemic species: P. cataulaciceps (Schwartz, 1959), P. empusa Cope, 1862, P. fustiger (Schwartz, 1960), P. florentinoi (Moreno and Rivalta, 2007), P. gundlachi (Ruibal, 1959), P. longinasa (Stejneger, 1905), P. peltoccephala (Tschudi, 1838) and P. taladai (Schwartz, 1960). A further three species inhabit Hispaniola [P. fluviatica (Schwartz, 1972), P. fracta (Schwartz, 1972) and P. guentheri (Cochran, 1941)], and one species (P. lemur Cope, 1869) is found in Puerto Rico and on the Virgin Islands (AmphibiaWeb, 2010). A recent study of global bufonid history inferred from nuclear and mitochondrial genes suggested that the sister clade to Peltophryne is the small South American genus Rhaebo (Van Bocxlaer et al., 2010).

In this study, we follow the investigations of Peltophryne by Pramuk and colleagues (Pramuk, 2000, 2002; Pramuk et al., 2001), but expand the coverage of the genus to include all named Cuban species and phylogeographical analyses of the most widespread species. We use multi-locus DNA sequence data to gain insights into the congeneric relationships of Peltophryne and the history of its expansion in the Greater Antilles archipelago. We sought to answer the following questions regarding the biogeographical origin and diversification of these toads. (1) When did the Peltophryne ancestor diverge from its nearest mainland relative, and is this date compatible with Late Cretaceous vicariance, Eocene–Oligocene GAARlandia, or recent over-water dispersal? (2) Does the congeneric, inter-island divergence correspond temporally, spatially and phylogenetically with the geological history of the West Indies? (3) Do geographical variables explain within-island speciation in Caribbean toads? (4) Given that Cuban toads form two discrete body size categories, large and small, is body size phylogenetically conserved or is change in body size associated with speciation events? One might expect rapid change in body size under a model of adaptive radiation involving resource competition (Losos & Ricklefs, 2009) in terms of trophic niche, given that body size largely determines the prey base of generalist anurans (Wells, 2007).

Using data from two mitochondrial genes, cytochrome c oxidase subunit I (COI) and 16S ribosomal RNA (16S), and from two nuclear genes, recombination activator-1 (RAG-1) and...
chemokine receptor 4 (CXCR-4), the specific goals of this paper are to: (1) assess the taxonomic status of currently recognized species in Cuba, (2) investigate the phylogenetic relationships among all Cuban species of *Peltophryne* plus two other West Indian species, (3) estimate the temporal origins of these toads based on calibration points external to *Peltophryne*, and (4) explore the phylogeographical patterns of the more common species in the Cuban archipelago, especially *P. peltcephala*.

**MATERIALS AND METHODS**

**Sample collection**

A total of 124 individuals were used in our study (Table 1 and Appendix S1 in Supporting Information), representing all Cuban species of *Peltophryne*, including a recently described species (Moreno & Rivalta, 2007). Tissue samples were obtained from toe clips collected in the field and preserved in 90% ethanol. For most species we obtained two or more individuals per population and three or more populations per species (Table 1, Fig. 2 and Appendix S1). For *P. peltcephala*, we included 15 localities across the Cuban archipelago in order to assess phylogeographical structure in this widespread species (Fig. 2h). For the polytypic species *P. longinasa*, we obtained samples only from the western subspecies, *P. l. longinasa* and *P. l. cajalbanensis*. Despite repeated attempts, no individuals were located from the other two subspecies, namely *P. l. dunni* and *P. l. ramsdeni*. *Peltophryne l. dunni* is restricted to the Guamahaya mountain range, central Cuba (Valdés de la Osa & Ruiz, 1980), and has purportedly been impacted by the chytrid fungal pathogen *Batrachochytrium dendrobatidis* (Díaz et al., 2007; Rosenblum et al., 2010). *Peltophryne l. ramsdeni* is known only from the Guaso plateau, eastern Cuba (Valdés de la Osa & Ruiz, 1980), and has not been reported in the last three decades.

In addition to field samples, we also obtained molecular data from GenBank for a global sampling of bufonids (Appendix S2), along with previously published *Peltophryne* data (Table 1). GenBank data were obtained for the 16S, RAG-1 and CXCR-4 genes. For most non-*Peltophryne* or ’outgroup’ bufonid species, we were unable to obtain published sequences of the Folmer or ’Barcode of Life’ fragment of the COI gene (Meyer et al., 2005; Smith et al., 2008). To reduce the amount of missing data and increase phylogenetic accuracy (Campbell & Lapointe, 2009), we therefore utilized ’composite taxa’ by analysing COI sequences of different individuals of the same ’outgroup’ species or clade as a single concatenated DNA sequence (Appendix S1). We hypothesize that composite taxa may help to improve estimates of rates of molecular evolution and therefore improve divergence time estimates, as well as improving phylogenetic inference (Campbell & Lapointe, 2009). Composite taxa were never used within *Peltophryne*.

**Laboratory methods**

Total DNA was extracted using the Qiagen DNeasy blood and tissues kit (Qiagen, Valencia, CA, USA). For all samples, we amplified and sequenced portions of two mitochondrial genes, 16S and COI (Table 2). We obtained DNA sequence data from two nuclear genes, CXCR-4 and RAG-1 (Table 2), from a subset of 42 samples representing the major phylogenetic lineages revealed by the mtDNA data. Polymerase chain reaction (PCR) products were isolated on 1.5% agarose gels. The bands were then cut from the gel, digested with Gelase (Epicentre Technologies, Madison, WI, USA), and used directly in DNA sequencing reactions. Cycle sequencing reactions were performed in 10-μL reactions with BigDye sequencing kits and analysed on an ABI-3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). DNA sequences were aligned with SEQUENcher 4.2 (Gene Codes, Ann Arbor, MI, USA) and checked by eye. Sequences of COI, CXCR-4 and RAG-1 were translated to amino acids and examined for inferred pre-mature stop codons using MACCLADE 4.08 (Maddison & Maddison, 2005).

**Analytical methods**

We did not perform the incongruence length difference test, because of doubts surrounding its utility (Barker & Lutzoni,

<table>
<thead>
<tr>
<th>Species/subspecies</th>
<th>Sample size</th>
<th>Geographical provenance</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Inggroup</em> (n = 126)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Peltophryne cataulaceps</em></td>
<td>5</td>
<td>Island of Youth, Cuba (1 locality)</td>
</tr>
<tr>
<td><em>P. empusa</em></td>
<td>15</td>
<td>Mainland Cuba and Island of Youth (4 localities)</td>
</tr>
<tr>
<td><em>P. florentinoi</em></td>
<td>5</td>
<td>Mainland Cuba (Type locality only)</td>
</tr>
<tr>
<td><em>P. fusiger</em></td>
<td>15</td>
<td>Western Cuba (4 localities)</td>
</tr>
<tr>
<td><em>P. gundachi</em></td>
<td>1</td>
<td>Mainland Cuba (1 locality)</td>
</tr>
<tr>
<td><em>P. longinasa longinasa</em></td>
<td>4</td>
<td>Western Cuba (2 localities)</td>
</tr>
<tr>
<td><em>P. l. cajalbanensis</em></td>
<td>1</td>
<td>Western Cuba (Type locality)</td>
</tr>
<tr>
<td><em>P. peltcephala</em></td>
<td>63</td>
<td>Mainland Cuba, Island of Youth and Sabana-Camaguey archipelago (15 localities)</td>
</tr>
<tr>
<td><em>P. taladai</em></td>
<td>15</td>
<td>Central and Eastern Cuba (3 localities)</td>
</tr>
<tr>
<td><em>P. guevtheri</em></td>
<td>1</td>
<td>Cabral, Barahona, Dominican Republic (1 locality)</td>
</tr>
<tr>
<td><em>P. lemur</em></td>
<td>1</td>
<td>Puerto Rico (1 locality)</td>
</tr>
</tbody>
</table>

Table 1 Species, subspecies, numbers of individuals and geographical provenance (including number of localities sampled) of *Peltophryne* samples used in the molecular systematic analyses. Data for the two non-Cuban *Peltophryne* samples used obtained from GenBank (Pramuk, 2006; Pramuk et al., 2001). For detailed information on localities, see Appendix S1.
2002; Darlu & Lecointre, 2002), but instead assessed potential data incongruence in phylogenetic reconstructions by a visual inspection of single-gene phylogenies inferred from modified neighbour-joining (BIONJ; Saitou & Nei, 1987; Gascuel, 1997) and maximum parsimony (MP) methods implemented in Paup* 4.0b10 (Swofford, 2002). Preliminary BIONJ trees were based on Hasegawa–Kishino–Yano (HKY) distances (Hasegawa et al., 1985), while MP inference used heuristic searches with 100 random addition sequence replicates and tree bisection–reconnection (TBR) branch swapping.

The most appropriate models of molecular evolution for maximum likelihood (ML; Felsenstein, 1981) and Bayesian (Rannala & Yang, 1996; Yang & Rannala, 1997) phylogenetic analyses were selected using a Bayesian information criterion, as implemented in DT_MoSel (Minin et al., 2003). This method incorporates error in branch length estimation as a performance measure and tends to recommend simpler models relative to other model selection criteria (Minin et al., 2003). We applied this method to various partitions of the data, including to an unpartitioned four-gene data set, to the pair of gene fragments representing either genome (mitochondrial versus nuclear), to each of the four loci individually, and to each codon position independently in each of the three protein-coding genes (COI, CXCR-4, RAG-1).

Bayesian phylogenetic analyses employing Metropolis-coupled Markov chain Monte Carlo, also known as (MC)$^3$ (Metropolis et al., 1953; Hastings, 1970), were run using the software package MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). Each analysis consisted of paired independent runs of 5 million generations, including a burn-in period of 1 million generations. Each of the paired runs utilized four Metropolis-coupled chains with a heating parameter of $T = 0.02$, a value that gave preferred rates of chain-swapping (20–80%) compared with $T = 0.2$ (default), $T = 0.002$ or $T = 0.008$ during initial testing. Two default prior distributions were modified as follows. The gamma shape parameter was assigned a uniform (0.0001, 20.0) prior distribution. The prior for the rate multiplier was set as variable, following Marshall et al. (2006). In addition to unpartitioned Bayesian (MC)$^3$ analyses, four other data partition schemata were employed: a 2-way partition schema by genome, a 4-way partitioning by gene, a 6-way partitioning by gene with COI further partitioned by codon position, and a 10-way partitioning with each of the three protein-coding genes partitioned by codon position. The optimal partition strategy was chosen by estimating the relative Bayes factors for each partition scheme (Kass & Raftery, 1995; Castoe et al., 2005).
We calculated pairwise Kimura 2-parameter (K2P) distances between and within the two major clades of Cuban toads, among species within clades, and within each species for both genes using MEGA 4.0 (Tamura et al., 2007). To further examine the genetic variability among *Peltophryne* species within Cuba, we inferred neighbour-joining and Bayesian consensus phylogenies (as above) using just mtDNA data from Cuban *Peltophryne*. We explored the potential phylogeographical structure of the most widespread species of large Cuban toad, *P. peltcephala* (61 individuals from 13 populations), and its sister species (see below), *P. florentinoi*, by constructing a mitochondrial haplotype network based on COI and using the software *tcs* 1.21 (Clement et al., 2000), with a 95% connection limit.

We estimated divergence times of *Peltophryne* by combining our data with a global sampling of the family Bufonidae. We used a clock-free and highly parametric Bayesian MCMC approach, as implemented in *t3* (Thorinan time traveler), a modified version of the software package *multidivtime* (Thorne et al., 1998; Thorne & Kishino, 2000; Yang & Yoder, 2003). This software estimates independent rates of evolution among genes as well as among branches over a common phylogenetic history (a detailed guide to running this software can be found at http://dna.ac/ACtips_multidistribute.pdf).

To extract temporal information from the molecular data we placed restrictions on the timing of five nodes phylogenetically distant from the most recent common ancestor (MRCA) of *Peltophryne* and its closest relative, *Rheo* (see Results). We adopted the temporal framework of Roelants et al. (2007) for the Neobatrachia, and used their results to constrain two basal and one nested node within the history of Bufonidae. The time-tree of Roelants et al. (2007) utilized 15 fossil calibrations and five palaeogeographical events, and accounted for uncertainty around each point. The stem age of Bufonidae was constrained to the interval 50.9–75.5 million years ago (Ma). The oldest divergence within Bufonidae in our tree was constrained to 45.1–67.8 Ma, corresponding to *Melanophryniscus* versus *Duttaphrynus* in Roelants et al. (2007). The divergence time of two Laurasian genera (*Duttaphrynus* and *Ameitophrynus*) was constrained to the interval 18.0–31.2 Ma. Following the molecular analyses of Slade & Moritz (1998), we also constrained the MRCA of *Rhinella granulosa* and *R. marina* to >2.7 Ma, and the maximum age of two trans-Andean samples of *R. marina* to <2.7 Ma.

**RESULTS**

**Phylogeny of Cuban toads**

The combined mitochondrial DNA data (16S and COI) included 124 samples and 1183 base pairs (bp), and the combined nuclear data (CXCR-4 and RAG-1) comprised 42 samples and 1503 bp. Aligned and concatenated data for all four genes contained a total of 2683 bp (Table 3). The topologies of the mtDNA and nuclear data subsets were broadly congruent, with differences occurring in some nodes poorly supported by nuclear data. Conflicting results among inference methods are represented by polytomies in Fig. 3. Because partitioned Bayesian analyses maximally exploit the information available in the data, these results are presented and discussed in detail. The optimal partition scheme for the Bayesian analyses was the 10-way partition strategy, with a relative Bayes factor of 47.7 over the 6-way partitioning scheme (absolute Bayes factor of 572.9). All but four inferred interspecific nodes received robust statistical support, with Bayesian posterior probabilities (bpp) of 0.96–1.0 and non-parametric bootstrap (npb) support of 75–100% (Fig. 3).

All phylogenetic trees supported the monophyly of the West Indies toads included in this analysis (*P. lemur* from Puerto Rico, *P. guentheri* from Hispaniola and all Cuban species) relative to mainland taxa. Within *Peltophryne*, the Cuban species also made up a monophyletic group in all analyses (Fig. 3). Within Cuba, we recovered a well-supported clade containing the three smaller species of <36 mm snout–vent length, with *P. guadallachi* as the sister to the clade *P. catulaciceps* plus *P. longinasa* (Fig. 3). Average pairwise genetic distances within this clade were 2.1 and 8.3% for 16S and COI, respectively (Table 4). Subspecies of *P. longinasa* (*P. l. longinasa* and *P. l. cajabanensis*) were clearly differentiated, with average pairwise genetic distances of 1.7% for 16S and 3.1% for COI. Within populations of *P. l. longinasa*, pairwise distances were 0.4% for 16S and 0.5% for COI.

Maximum likelihood reconstructions based on the complete dataset suggested that large-bodied species formed a mono-

### Table 2 Primers used in this study and polymerase chain reaction (PCR) conditions specific to each gene. Each primer pair was used for PCR and sequencing.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5'–3')</th>
<th>Source</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>16Sar-L</td>
<td>ACGCCTGGTTATCAAAAAACAT</td>
<td>Kessing et al. (2004)</td>
<td>Q-Taq 0.05 U µL⁻¹, [MgCl₂] = 2.25 mm; Annealing: 0:45 s at 54 °C</td>
</tr>
<tr>
<td>16Sbr-H</td>
<td>CGGCTCTGAACCTGATACGCT</td>
<td>Meyer et al. (2005)</td>
<td>Q-Taq 0.05 U µL⁻¹; Annealing: 1:30 min at 50 °C</td>
</tr>
<tr>
<td>BOL-dgHCO</td>
<td>TAAACTTCAAGGGTGACAAARAYCA</td>
<td>Chiari et al. (2004)</td>
<td>AmpliTaq 0.05 U µL⁻¹, [MgCl₂] = 3.0 mm; Annealing: 0:45 s at 55 °C</td>
</tr>
<tr>
<td>BOL-dgLCO</td>
<td>GGTCAAACTAAGAAAGAYTGGG</td>
<td>Biju &amp; Bossuyt (2003)</td>
<td>AmpliTaq 0.05 U µL⁻¹, [MgCl₂] = 2.50 mm; Annealing: 0:45 s at 56 °C</td>
</tr>
<tr>
<td>RAG-1 MartFl1</td>
<td>CCTCGACCGTAYCAYAARATGTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAG-1 AmpR1</td>
<td>AAATCGCATGCATTTCACATRTCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR-4-C</td>
<td>GTCAATGAGGTCTAYCARAAGAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCR-4-F</td>
<td>TGAATTTGGCCCTACAGGAAGC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
phylogenetic group with relationships [P. taladai (P. empusa (P. fustiger, (P. florentinoi (P. peltocephala)))). The only interspecific node that received high statistical support, however, was P. florentinoi + P. peltocephala (Fig. 3). When the analysis was limited to only those samples with all four genes, the clade (P. empusa, P. fustiger, P. florentinoi, P. peltocephala) also received significant support (Fig. 4). Mean pairwise genetic distances among all large-bodied toads were 0.9 and 5.5% at 16S and COI, respectively (Table 4).

Peltophryne taladai individuals were split into two deeply divergent and well-supported clades (Fig. 3). The first included all haplotypes found near the Duaba river (DUABA; see Appendix S1 for locality details) and the second was subdivided further into two branches, with haplotypes from the highlands of La Tagua (TAG) in eastern Cuba and Jarico (JAR) in central Cuba, despite the fact that DUABA and TAG are geographically proximal localities relative to JAR (Fig. 2). Intraspecific pairwise genetic distances for P. taladai from DUABA versus TAG–JAR were 1.9% for 16S and 6.7% for COI, whereas TAG versus JAR showed 0.1 and 0.6%, respectively.

Within P. empusa, toads from the Island of Youth (IJLos–Ind) were reciprocally monophyletic with respect to their relatives from the main island of Cuba (Figs 3 & 4). The average genetic distances between the island and Cuban mainland for 16S and COI were 0.8 and 2.1%, respectively, with significant phylogeographical structure (high bpp) among haplotypes from the main island despite pairwise genetic distances below 1% for both genes. Peltophryne fustiger, whose distribution is limited to western Cuba, showed no obvious spatial genetic structure (Fig. 3), and the average genetic distances among populations of this species were below 1% for both mitochondrial genes.

### Phylogeography of Peltophryne peltocephala

Samples of P. peltocephala obtained from throughout its range in the Cuban archipelago (61 individuals from 13 localities) showed low levels of sequence divergence and little statistical support for phylogenetic relationships among haplotypes (Fig. 3). We recovered a widespread COI haplotype at all 12 sampling localities in mainland Cuba and the Sabana-Camagüey archipelago (Fig. 5), suggesting scant geographical structuring of genetic variation. The Island of Youth samples, however, were monophyletic relative to mainland P. peltocephala samples (Fig. 3) and separated by three mutational steps at COI (Fig. 5). Island of Youth samples showed an average pairwise distance for 16S and COI of 0.4 and 0.6%, respectively, from mainland conspecifics (Table 4).

### Peltophryne in the global bufonid phylogeny: divergence time estimates

Combining our data with a global sampling of bufonids, we inferred a sister relationship between Rhaebo and Peltophryne, with suggestive but non-significant support (bpp = 0.92; Fig. 4). Old World bufonids formed a single well-supported clade nested within New World toads. Our estimated divergence times suggested that the ancestor of Peltophryne diverged from its mainland relative around the Eocene–Oligocene boundary (mean stem age 33.1 Ma; Fig. 4). An apparent expansion across the Caribbean islands took place 13.0–28.3 Ma (the mean crown age of Peltophryne was 19.7 Ma), with the split among Hispaniolan and Cuban ancestral forms estimated at c. 16.2 Ma (95% credible interval 10.3–24.3 Ma). The major diversification of Peltophryne within Cuba began in

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**Table 3** Total ingroup (Peltophryne) DNA sequence data analysed by partition, length in base pairs (bp), percentage and number of variable and parsimony-informative sites, and evolutionary model chosen for each partition by the decision theory method (DT_MonoSel). The total number of samples was 126, and gapped sites were excluded. Mitochondrial (mt) DNA datasets (16S and COI) contained 124 samples. Nuclear (nuc) DNA datasets (CXCR-4 and RAG-1) contained 42 samples.

<table>
<thead>
<tr>
<th>Partition</th>
<th>Included bp</th>
<th>Percentage variable sites (no. of sites)</th>
<th>Percentage parsimony-informative sites (no. of sites)</th>
<th>Model chosen by DT_MonoSel</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 genes</td>
<td>2683</td>
<td>14.6 (391)</td>
<td>10.7 (288)</td>
<td>HKY+I+Γ</td>
</tr>
<tr>
<td>2 mt genes</td>
<td>1183</td>
<td>22.1 (262)</td>
<td>18.9 (224)</td>
<td>HKY+I+Γ</td>
</tr>
<tr>
<td>2 nuc genes</td>
<td>1503</td>
<td>8.1 (122)</td>
<td>4.1 (61)</td>
<td>HKY+I+Γ</td>
</tr>
<tr>
<td>16S</td>
<td>543</td>
<td>14.4 (78)</td>
<td>10.1 (55)</td>
<td>HKY+I</td>
</tr>
<tr>
<td>COI</td>
<td>648</td>
<td>28.9 (187)</td>
<td>26.4 (171)</td>
<td>TrN+Γ</td>
</tr>
<tr>
<td>COIpos1</td>
<td>216</td>
<td>10.6 (23)</td>
<td>8.8 (19)</td>
<td>TrN+Γ</td>
</tr>
<tr>
<td>COIpos2</td>
<td>216</td>
<td>0.9 (2)</td>
<td>0.0 (0)</td>
<td>F81</td>
</tr>
<tr>
<td>COIpos3</td>
<td>216</td>
<td>75.0 (162)</td>
<td>70.4 (152)</td>
<td>TrN+Γ</td>
</tr>
<tr>
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<td>714</td>
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<td>3.9 (28)</td>
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</tr>
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<td>CXCR-4pos2</td>
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<td>F81</td>
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<tr>
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the Miocene, 12.7 (7.95–19.3) Ma (Fig. 4). *Peltophryne taladai* diverged from other members of the clade of large-bodied toads 10.2 (5.96–16.3) Ma, which was followed by a split separating *P. empusa* from a sister clade comprising the remaining three large Cuban species (3.45–12.2 Ma). After the split that gave rise to *P. fastiger*, the most recent speciation event among Cuban toads separated *P. florentinoi* from *P. peltcephala* (Fig. 4). The crown age of the small Cuban *Peltophryne* dates to 9.90 (5.66–15.6) Ma, with *P. cataulaciceps* and *P. longinasa* diverging 6.16 (2.66–11.0) Ma.

**DISCUSSION**

**Peltophryne in a global phylogeny of Bufonidae**

Our multi-genic molecular phylogenetic analyses confirm the monophyly of Old World toads nested within New World toads (Van Bocxlaer et al., 2009, 2010), as well as the monophyly of *Peltophryne* toads and the monophyly of Cuban species within *Peltophryne*. We hypothesize that the mainland Neotropical genus *Rhaebo* is the sister lineage to *Peltophryne* (Fig. 4). Our estimated stem age of *Peltophryne* precludes the possibility that this taxon represents a Cretaceous Caribbean lineage (Fig. 4). Although we find that this Antillean lineage of toads is younger than previously thought (Pramuk et al., 2008), our estimated stem age of *Peltophryne* is similar to that of all Old World toad genera combined (Fig. 4). Our analyses suggest that the *Peltophryne* ancestor colonized the Caribbean archipelago in the early Oligocene. These results are consonant with, but independent of, the findings of Van Bocxlaer et al. (2010), who used the geological hypotheses of Iturralde-Vinent & MacPhee (1999) as a temporal calibration point.

**Phylogeny and biogeography of *Peltophryne* in the Caribbean**

Contrasting hypotheses of vicariance versus over-water dispersal have been proposed to explain the origin of the endemic flora and fauna of the West Indies from mainland ancestors.
Bayesian consensus phylogeny inferred from only those *Peltophryne* samples with all four gene sequences, combined with a global sampling of bufonids. Branch lengths represent divergence times as estimated using *multidivtime* and assuming the temporal framework for the ages of extant amphibians provided by Roelants et al. (2007). Mean and 95% credible intervals (CI) around divergence times in the history of *Peltophryne* are indicated in the table within the figure. Topology is based on the 10-way partitioned Bayesian phylogenetic analysis of four genes (COI, 16S, RAG-1 and CXCR-4). Open circles indicate nodes supported by 0.96–1.0 marginal posterior probability. Support for four nodes of interest with < 0.95 posterior probability is indicated by the corresponding probability. The open star indicates a well-supported clade containing all Old World bufonid samples. The five black squares indicate nodes with *a priori* divergence time constraints. Horizontal grey bars indicate bounded constraints for the root node (50.9–75.5 Ma), the most recent common ancestor (MRCA) of *Melanophryniscus* and *Duttaphrynus* (45.1–67.8 Ma), and the MRCA of *Duttaphrynus* and *Amietophrynus* (18.0–31.2 Ma). The left-pointing grey triangle indicates the minimum age constraint on the MRCA of *Rhinella granulosa* and *R. marina* (> 2.7 Ma), and the right-pointing grey triangle indicates the maximum age of two trans-Andean samples of *R. marina* (< 2.7 Ma), according to Slade & Moritz (1998).

**Figure 4** Bayesian consensus phylogeny inferred from only those *Peltophryne* samples with all four gene sequences, combined with a global sampling of bufonids. Branch lengths represent divergence times as estimated using *multidivtime* and assuming the temporal framework for the ages of extant amphibians provided by Roelants et al. (2007). Mean and 95% credible intervals (CI) around divergence times in the history of *Peltophryne* are indicated in the table within the figure. Topology is based on the 10-way partitioned Bayesian phylogenetic analysis of four genes (COI, 16S, RAG-1 and CXCR-4). Open circles indicate nodes supported by 0.96–1.0 marginal posterior probability. Support for four nodes of interest with < 0.95 posterior probability is indicated by the corresponding probability. The open star indicates a well-supported clade containing all Old World bufonid samples. The five black squares indicate nodes with *a priori* divergence time constraints. Horizontal grey bars indicate bounded constraints for the root node (50.9–75.5 Ma), the most recent common ancestor (MRCA) of *Melanophryniscus* and *Duttaphrynus* (45.1–67.8 Ma), and the MRCA of *Duttaphrynus* and *Amietophrynus* (18.0–31.2 Ma). The left-pointing grey triangle indicates the minimum age constraint on the MRCA of *Rhinella granulosa* and *R. marina* (> 2.7 Ma), and the right-pointing grey triangle indicates the maximum age of two trans-Andean samples of *R. marina* (< 2.7 Ma), according to Slade & Moritz (1998).
Historically, the emphasis has been on vicariance explanations (Rosen, 1985; Crother & Guyer, 1996), but our phylogenetic and divergence time analyses of *Peltophryne* preclude a vicariant origin via the break-up of a hypothetical Late Cretaceous Proto-Antillean land bridge (Hedges, 1989a). Our findings are consistent with recent molecular dating analyses suggesting that the Caribbean vertebrate fauna post-dates the end-Cretaceous bolide impact (e.g. Heinicke et al., 2007). The arrival of the ancestral *Peltophryne* to the Antilles 33 Ma could have occurred by over-water dispersal (Hedges et al., 1992; Hedges, 1996a,b, 2006), and indeed recent studies suggest that the importance of oceanic dispersal has been underestimated (de Queiroz, 2005; Heaney, 2007). Although amphibians are often regarded as poor dispersers across marine barriers (Darwin, 1859, p. 393; Vitt & Caldwell, 2009), recent molecular genetic evidence has revealed cases of probable transmarine dispersal by frogs (Hedges et al., 1992; Bossuyt & Milinkovitch, 2001; Vences et al., 2003; Heinicke et al., 2007). Amphibians could cross open water on floating vegetation (Boyd, 1962), and given enough time even unlikely events such as rafting may become probable.

The estimated time of divergence of *Peltophryne* from its mainland sister, *Rhaebo*, is consistent with a third model for the origin of the Antillean biota: the hypothesized GAARlandia connection between northern South America and the nascent Caribbean islands (Iturralde-Vinent & MacPhee, 1999; Iturralde-Vinent, 2006). This short-lived landspan is thought to have developed 35–33 Ma and is now evidenced by the submarine Aves Ridge (Iturralde-Vinent & MacPhee, 1999; Iturralde-Vinent, 2006). The temporal match between the proposed age of GAARlandia (Iturralde-Vinent, 2006) and our divergence estimates (Fig. 4) implies that the ancestral *Peltophryne* could have colonized the current Caribbean islands from South America during the Eocene or Oligocene epoch. As the Aves Ridge continued its eastward displacement (Pindell & Kennan, 2009), *Peltophryne* presumably diverged from *Rhaebo* owing to the subsequent disappearance of sub-areal GAARlandia (MacPhee & Iturralde-Vinent, 2000; Iturralde-Vinent & Gahagan, 2002). Finally, our estimated divergence times allow us to reject Plio-Pleistocene over-water dispersal as a possible explanation for the origin of these toads (Fig. 4).

A roughly 3 million year biotic exchange from northern South America into GAARlandia has also been invoked to understand the origin of other terrestrial elements of the West Indian biota, including mammals such as megalonychid sloths (MacPhee & Iturralde-Vinent, 2000; White & MacPhee, 2001; Dávalos, 2004), hystricognath rodents (Woods et al., 2001; MacPhee et al., 2003; Dávalos, 2004), bats (Dávalos, 2004) and primates (Horovitz & MacPhee, 1999; Dávalos, 2004). Some frogs (Crawford & Smith, 2005; Moen & Wiens, 2009), fishes (Hulsey et al., 2011), plants (Fritsch, 2003; van Ee et al., 2008) and spiders (Binford et al., 2008) may have also dispersed through GAARlandia. In a recent study similar to the present work, Crews & Gillespie (2010) tested the GAARlandia

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**Figure 5** Parsimony haplotype network based on 61 cytochrome c oxidase subunit I gene fragments of *Peltophryne peltocephala* and *P. florentinoi*. Each unique haplotype is designated with a unique letter and contains the sample codes as in Fig. 3 and Appendix S1. Mainland localities such as YAR and VEL contained haplotypes from across the network, suggesting scant geographical structure within the extensive intraspecific sampling of *P. peltocephala*. 
framework using a dispersal-limited genus of spiders, Selenops (Selenopidae), and also demonstrated compatibility between the age of the hypothetical GAARlandia landspan and the estimated divergence time between South American and Caribbean lineages.

The consonance between node dates on the Peltophryne tree and independently derived dates of geological activity in the Greater Antilles extends to the divergence of toad lineages found on Puerto Rico, Hispaniola and Cuba. Based on palaeogeographical evidence, the marine inundation of the Mona Passage that isolated Puerto Rico from Hispaniola is hypothesized to have occurred 30–20 Ma (MacPhee et al., 2003). The Peltophryne phylogeny presented here establishes the separation of P. lemur on Puerto Rico from Hispaniola + Cuba Peltophryne at 19.7 (13.0–28.3) Ma. The subsequent separation of Hispaniola from Cuba at 14–17 Ma (Iturralde-Vinent, 2006) represents a good match to the estimated divergence time of 16.2 (10.3–24.3) Ma for the MRCA of Hispaniolan versus Cuban Peltophryne species (Fig. 4, node C and Fig. 6). These island–island vicariant events form the second component of the Iturralde-Vinent & MacPhee (1999) landspan–vicariance model, with GAARlandia as the first component.

Phylogeny and biogeography of Peltophryne within Cuba

The morphological diversification of Cuban toads appears to have taken place quickly and early in their phylogenetic history, with the formation of two major clades, small versus large Peltophryne (cf. Pramuk et al., 2001; Pramuk, 2002), although statistical support for the monophyly of large toads is weak (Fig. 3; Node E in Fig. 4). Most anurans are dietary generalists, with prey size largely determined by body size (Wells, 2007; Moen et al., 2009). Given that recent speciation is not associated with obvious changes in body size, divergence among these toads does not appear to have involved adaptation in terms of tropic niche, although studies of diet in Peltophryne are few (e.g. Sampedro et al., 1982). Whether adaptations in other morphological, ecological or behavioural traits may have contributed to the radiation of these toads requires further study.

The subsequent radiation within large- and small-bodied clades across the emerged Cuban archipelago appears to have started in the Miocene, during which time Cuba underwent fragmentation owing to geological processes and changing sea level. These processes may have contributed to speciation (and extinction) in Peltophryne, although the geographical patterns are not clear from the phylogeny. Such geological dynamism of Greater Antillean islands is thought to have contributed to the diversification of other amphibians and reptiles, such as Cuban anoline lizards (Glor et al., 2004), geckos of the genus Tarentola from Cuba (Weiss & Hedges, 2007), Hispaniolan teiid lizards of the genus Ameiva (Gifford et al., 2004; Gifford & Larson, 2008), and some eleutherodactyline frogs from Jamaica, Hispaniola and Cuba (Hedges, 1989b; Heinicke et al., 2007; Rodriguez et al., 2010). Within Cuba, we focused attention on the P. fustiger, P. peltocephala plus P. florentinoi clade, three closely related species with intriguing geographical distributions. Peltophryne fustiger and P. peltocephala geographically replace one another (Fig. 2) and were previously regarded as one species (Schwartz, 1960; Schwartz & Henderson, 1991). Peltophryne fustiger is separated from P. peltocephala by an approximately 100-km gap in distribution (Valdés de la Osa, 1988). The phylogenetic results support the recognition of these two species plus P. florentinoi (Schwartz & Thomas, 1960).
Origin and diversification of Cuban toads

Peltophryne fustiger is particularly abundant in geologically old elevations of western Cuba, such as Sierra de los Organos and Sierra del Rosario, which remained land-positive during Pleistocene sea-level oscillations. Although the phylogenetic placement of P. fustiger is not statistically well supported, the optimal reconstructions (cf. Figs 3 & 4) are consistent with a scenario involving the vicariant separation of a western Cuban lineage from the ancestor of P. peltcephala + P. florentinoi 5.77 Ma (2.55–10.4 Ma). During this period, temporary water gaps formed between western and central Cuba (Iturralde-Vinent, 2003). Western Cuba achieved dry land contact with central Cuba after the disappearance of the Havana–Matanzas Channel in the Miocene (Iturralde-Vinent & MacPhee, 1999).

Peltophryne florentinoi forms the sister species to P. peltcephala with strong support (Figs 3 & 4). Peltophryne florentinoi is restricted to the Zapata peninsula, a region that emerged for the last time during the late Pleistocene and early Holocene (20,000–8000 years ago). Given the recent split of P. florentinoi from P. peltcephala (Figs 4 & 5), their relative geographical distributions (Fig. 2) and the palaeogeographical history of the Cuban archipelago (Iturralde-Vinent, 2003), P. florentinoi may have formed by peripheral isolation sometime during the Pleistocene, as has been suggested for some species of fishes, lizards and amphibians (Lynch, 1989; Friesen & Anderson, 1997; Johnson & Cicero, 2002; Robalo et al., 2008). The habitat of P. florentinoi is quite distinct from that of P. peltcephala, and recent work shows that P. florentinoi is distinctive in terms of calling and oviposition sites (Alonso et al., 2007; Moreno & Rivalta, 2007; Díaz & Cádiz, 2008), advertisement call (Hernández et al., 2010) and larval morphology (Díaz & Cádiz, 2008). Given the young age of the MRCA of these two species, it would be useful to investigate the role that ecological speciation might have played in the origins of P. florentinoi (Schluter, 2001).

The wide geographical distribution of individual mtDNA haplotypes of P. peltcephala across the main island of Cuba suggests recent and widespread gene flow within P. peltcephala (Avise, 2000), including expansion into formerly inundated areas (Figs 2 & 5). In contrast, the isolated haplotypes from the Island of Youth (haplotype T) may indicate a Pleistocene or Holocene separation from mainland Cuba. Iturralde-Vinent (2006) suggested that the present-day Island of Youth (Isla de Pines) and Cuba were connected between 125,000 and 8000 years ago, providing a route for animal migration between these lands. The population of P. peltcephala from the Island of Youth is distinctive in terms of coloration (Schwartz, 1960), morphology (Valdés de la Osa, 1988) and allozymes (Rivalta et al., 2009). Resolution of the evolutionary and taxonomic status of this population awaits further systematic work, for which we recommend the inclusion of the more rapidly evolving molecular markers, such as microsatellites.

Like P. peltcephala, P. empusa is widespread across Cuba, reaching the Sabana–Camaquiey (northern) and Los Canarreos (southern) archipelagos (Fig. 2), but P. empusa shows considerably more phylogeographical structuring among sampling localities than does P. peltcephala. Peltophryne empusa inhabits lowlands and seasonally flooded areas with a marked rainy season, and thus lowland habitat use is broadly similar to that of P. peltcephala. Given that lowland sites were probably affected by sea-level oscillations and climate fluctuations, we posit that differences in dispersal capacity, rather than ecological preferences, may better explain the greater phylogeographical structuring in P. empusa relative to P. peltcephala.

Although our phylogeographical sampling of other Cuban toads was less extensive, we observed deeply divergent lineages also within P. taladai in all phylogenetic analysis (Figs 3 & 4). The relatively old divergence time between these populations (3.07–11.1 Ma) suggests that isolation might have been caused by the Pliocene sea-level highstand roughly 4–5 Ma, with inundation of the lowlands separating these localities. The appreciable genetic distance within P. taladai between localities DUABA and TAG-JAR suggests the existence of cryptic or ‘candidate’ species (Vences & Wake, 2007; Vieites et al., 2009; Padial et al., 2010). A formal evaluation of the specific status of populations of P. taladai, however, should involve an integrative approach combining morphology, behaviour and genetics (Padial et al., 2009).

The position of the specimen of P. longinasa (GenBank accession number AY028493) is an unexpected result in our divergence time-tree (Fig. 4). This sample corresponds to specimen SBH 266461 collected in Pico de Potrerillo, Sancti Spiritus, Cuba. We have not been able to study the specimen, but, given its collection locality, we previously assumed that it represented the central subspecies, P. longinasa dunni. This specimen’s sequence rendered P. longinasa polyphyletic and raises the question of whether it could represent an additional species of Peltophryne. Valdés de la Osa & Ruiz (1980), in their systematic considerations of Peltophryne (Bufo) longinanus, described a new subspecies (P. l. cajalbunensis) and noted some differences from P. l. dunni, including: the absence of well-defined dorsal markings, a complex combination of dorsal colours, and labial bars lacking. Lateral bands are fragmented in P. l. dunni, and the pectoral bands are wider, more diffuse and do not touch the jaw. Furthermore, P. l. dunni is more spotted ventrally, more webbed and has paler feet. Valdés de la Osa & Ruiz (1980) noted a significant ecological difference between P. l. longinasa and P. l. dunni in their modes of oviposition: P. l. longinasa females deposit their eggs in submerged compacted clumps, while the eggs of P. l. dunni are deposited in double strings. Other noticeable differences have been observed in tadpole coloration between western forms and P. l. dunni (Valdés de la Osa & Ruiz, 1980; Schwartz & Henderson, 1991; Díaz & Cádiz, 2008). One explanation for the unusual haplotype could be that the distinctive form ascribed to P. longinasa is in fact a ninth species of Cuban Peltophryne. We expect that future work will clarify the taxonomic status of the currently recognized subspecies of P. longinasa, and resolve the identity of P. longinasa (GenBank number AY02843).
Implications and future directions

In this contribution we have examined the evolutionary patterns of one endemic genus of toad from the West Indies in the context of the long and complex Caribbean geological history. Our phylogenetic and phylogeographical results set the stage for further testing of island biogeographical models. Because we found no evidence that toads dispersed out of Cuba once it was isolated, however, island biogeographical models that assume a non-zero colonization rate (Heaney, 2000; Whittaker et al., 2010) may be of limited utility in understanding the evolution of Peltophryne. Various models predict that species diversity within islands should be correlated with island size and age (Arrhenius, 1921; MacArthur & Wilson, 1967; Whittaker et al., 2008, 2010). Such a pattern could be explained by large islands providing more opportunities for vicariance, leading to stochastic divergence, or by large islands providing a wider variety of habitats, which may promote deterministic divergence through local adaptation (Losos & Schluter, 2000). Peltophryne appears to be a good example of the former hypothesis, given the many opportunities for vicariant processes in the complex geological history of the Cuban archipelago. Given the historical inundations of the lowlands, we may expect the highlands to host relatively older lineages, a prediction made by a dynamic model of biogeography of volcanic islands (Whittaker et al., 2008, 2010). Within Cuban Peltophryne, we note that the most basal lineage among the small-bodied toads (P. dunnii, GenBank accession number AY02843) and the most basal lineage among the large-bodied toads (P. taladai) are both montane lineages, consistent with this prediction. However, nested within small- and large-bodied toads are two other typically montane species (P. longinasa and P. fustiger), making it difficult to estimate ancestral areas with confidence.

The diversification of toads within islands may provide a useful testing ground for theories explaining radiations following an initial colonization event (Losos & Ricklefs, 2009). Superficially, Cuban toads appear remarkably similar in their natural histories, with body size being the only morphological trait of obvious ecological relevance; however, body size has changed little across speciation events since the initial diversification of Peltophryne (Fig. 3). Thus, before we ask questions about allopatric adaptation versus character displacement in sympathy (e.g. Grant & Grant, 2008), we need to identify what traits, if any, facilitate the coexistence of Cuban toads. The Island of Youth in western Cuba (locality IJG in Fig. 2h) hosts four species, none of which form sister pairs (Fig. 3), and this site may provide clues regarding mechanisms of coexistence. Island radiations may also be driven by sexual selection (Mendelson & Shaw, 2005), and in Cuban toads a potential mechanism may be the evolution of female preferences for male calls (Ryan & Rand, 1993; Boul et al., 2007). In contrast to earlier divergences, the recent divergence between P. florentinoi and P. peltoccephala may have involved either adaptation or sexual selection, and this is a subject currently under investigation.

CONCLUSIONS

Our study provides a biogeographical context for future evolutionary studies of West Indian toads. We find that the phylogenetic history and divergence times of Peltophryne support the GAARlandia palaeogeographical model (Iturralde-Vinent & MacPhee, 1999; Iturralde-Vinent, 2006). The inferred monophyly of Cuban Peltophryne, and of the genus as a whole, indicates an important role for island–island vicariance in the isolation of these lineages following dispersal from South America (Iturralde-Vinent & MacPhee, 1999). A more complete understanding of the evolution of Peltophryne in the West Indies requires sampling of the other two known species from Hispaniola (P. fluviatica and P. fracta) and of P. lemur from the Virgin Islands.

Our extensive sampling of Cuban Peltophryne establishes a phylogenetic picture consistent with current taxonomy. Nonetheless, our results point to opportunities for additional systematic study and suggest that the count of toad species in Cuba may increase as a result. We require a more extensive survey of the widely distributed species, further use of multilocus markers and samples of the subspecies of P. longinasa. Moreover, an integrative approach combining all sources of evidence (morphological, acoustic and molecular) will permit an improved evaluation of Cuban toad diversity that will enrich our understanding of divergence and speciation of Peltophryne.

Finally, our phylogenetic analyses suggest that ecomorphological diversity among Cuban toads arose quickly and has been phylogenetically conserved. While historical processes such as island–island vicariance and marine incursions (Iturralde-Vinent, 2006) may explain the basal divergences within Peltophryne, the lack of concordant phylogeographical structure among co-distributed toad species suggests that recent lineage diversification may have been more strongly influenced by autecological differences among species than by common palaeogeographical factors.

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REFERENCES


Rivalta, V., Berovides, V., Rodríguez-Shettino, L. & Chamizo, A. (2009) Variabilidad genética de tres especies cubanas del...
dynamic theory of oceanic island biogeography. *Journal of
dynamic theory of oceanic island biogeography: extending
the MacArthur–Wilson theory to accommodate the rise and
fall of volcanic islands. *The theory of island biogeography
Princeton University Press, Princeton, NJ.
Williams, E.E. (1969) The ecology of colonization as seen in
the zoogeography of anoline lizards on small islands. *The
Quarterly Review of Biology*, 44, 345.
Williams, E.E. (1989) Old problems and new opportunities in
West Indian biogeography. *Biogeography of the West Indies:
Sandhill Crane Press, Gainesville, FL.
Indies: patterns and perspectives*. CRC Press, Boca Raton, FL.
patterns and radiations of West Indian rodents. *Biogeogra-
phy of the West Indies: patterns and perspectives* (ed. by C. A.
Woods and F.E. Sergile), pp. 335–353. CRC Press, Boca
Raton, FL.
Yang, Z. & Rannala, B. (1997) Bayesian phylogenetic inference
using DNA sequences: a Markov chain Monte Carlo meth-
Yang, Z. & Yoder, A.D. (2003) Comparison of likelihood and
Bayesian methods for estimating divergence times using
multiple gene loci and calibration points, with application to
a radiation of cute-looking mouse lemur species. *Systematic
Biology*, 52, 705–716.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the
online version of this article:

**Appendix S1** Sampling information and GenBank accession
numbers for original data.

**Appendix S2** GenBank accession numbers for previously
published data used in phylogenetic and divergence time
analyses.

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