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ANCIENT TEPUI SUMMITS HARBOR YOUNG RATHER THAN OLD LINEAGES OF ENDEMIC FROGS

Patricia E. Salerno,[1,2] Santiago R. Ron,[3] J. Celsa Señaris,[4] Fernando J. M. Rojas-Runjaic,[4] Brice P. Noonan,[5] and David C. Cannatella[1]

[1]*Section of Integrative Biology, University of Texas at Austin, 1 University Station, C0900, Austin, Texas 78712*

[2]*E-mail: patriciasalerno@gmail.com*

[3]*Museo de Zoología, Escuela de Biología, Pontificia Universidad Católica del Ecuador, Av. 12 de Octubre y Roca, Aptdo. 17–01-2184, Quito, Ecuador*

[4]*Museo de Historia Natural La Salle, Apartado postal 1930, Caracas 1010-A, Venezuela*

[5]*Department of Biology, The University of Mississippi, Box 1848, Mississippi 38677*

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The flattop mountains (tepuis) of South America are ancient remnants of the Precambrian Guiana Shield plateau. The tepui summits, isolated by their surrounding cliffs that can be up to 1000 m tall, are thought of as "islands in the sky," harboring relict flora and fauna that underwent vicariant speciation due to plateau fragmentation. High endemicity atop tepui summits support the idea of an ancient "Lost World" biota. However, recent work suggests that dispersal between lowlands and summits has occurred long after tepui formation indicating that tepui summits may not be as isolated from the lowlands as researchers have long suggested. Neither view of the origin of the tepui biota (i.e., ancient vicariance vs. recent dispersal) has strong empirical support owing to a lack of studies. We test diversification hypotheses of the Guiana Shield highlands by estimating divergence times of an endemic group of treefrogs, *Tepuihyla*. We find that diversification of this group does not support an ancient origin for this taxon; instead, divergence times among the highland species are 2–5 Ma. Our data indicate that most highland speciation occurred during the Pliocene. Thus, this unparalleled landscape known as "The Lost World" is inhabited, in part, not by Early Tertiary relicts but neoendemics.

KEY WORDS: **Diversification hypotheses, Guiana Shield, Hylidae, Lost World, sky islands, tepuis.**

The tepuis (flattop mountains) of northern South America are arguably the most dramatic example of sky islands on Earth. With relatively flat summit plateaus sitting atop sheer cliffs of up to 1000 m, these tabletop mountains form a discontinuous ecosystem called Pantepui (Huber 1988) and harbor numerous endemic lineages. For example, within the Pantepui, 60% of the vascular plant species and 87% of the frog species are endemics, often to a single tepui summit (Duellman 1999; Berry and Riina 2005; McDiarmid and Donnelly 2005). The tepuis are part of the Guiana Shield, which is situated between the Orinoco and Amazon Basins, accounts for 9% of the land area of South America, and shows biogeographic affinities with the Amazon and Andes.

Early reports of the unique topography and biodiversity of the Pantepui inspired Sir Arthur Conan Doyle to write of long-isolated dinosaur populations surviving to the present day in his novel "The Lost World," which inspired the namesake Lost World hypothesis (Rull 2004a; or Plateau hypothesis, Chapman 1931). This is the main biogeographic hypothesis put forward to explain the highly endemic fauna and flora of this region

(Chapman 1931; Maguire 1970; Hoogmoed 1979; McDiarmid and Donnelly 2005; Heinicke et al. 2009). This hypothesis predicts that highland species, which are often endemic to a single tepui, are relicts of formerly widespread plateau taxa and arose through vicariance following ancient fragmentation of the plateau. Thus, summit taxa are hypothesized to have been isolated for millions of years. In examining the especially high endemism levels of Pantepui frog genera, most researchers support the idea that diversification must be explained, at least partially, by long-term isolation of Plateau paleoendemics (Hoogmoed 1979; MacCulloch and Lathrop 2002; McDiarmid and Donnelly 2005; Heinicke et al. 2009). However, to date, it is unclear what fraction of the highland endemism, in frogs or other groups, is explained by the Lost World hypothesis.

Many global biodiversity hotspots are tropical mountainous areas, suggesting that highland areas promote higher rates of diversification (Orme et al. 2005). In most uplifted mountain ranges such as the Andes, the summits are the most recently exposed surfaces, and these seem to be associated with recent species divergences (Hughes and Eastwood 2006). In contrast, the tepui summits are of Precambrian origin and are thought to harbor ancient lineages (Chapman 1931; Maguire 1970; Hoogmoed 1979; McDiarmid and Donnelly 2005; Heinicke et al. 2009). The tepuis were formed about 70–90 Ma, after the Guiana Shield plateau underwent several periods of erosion and plateau uplift and fragmentation starting around 300 Ma, resulting in isolated sky islands (Briceño et al. 1990; Briceño and Schubert 1990; Gibbs and Barron 1993). Because the tepuis were formed mostly by erosion rather than uplift, the summit surfaces are geologically older than the adjacent slopes.

Many studies have suggested that vicariance alone (i.e., the Lost World hypothesis) cannot explain the current distribution of highland vascular plants (Huber 1988; Givnish et al. 1997), birds (Mayr and Phelps 1967), and ants (Jaffé et al. 1993), nor the available pollen deposits (Rull 2005; Rull and Nogué 2007). A set of alternative hypotheses states that diversification occurred more recently, after the summits were colonized by lowland species through various mechanisms such as habitat shifts, vertical (cool climate) displacement, and island hopping (Huber 1988; Mayr and Phelps 1967; Rull 2004a). The Island-Hopping hypothesis (Chapman 1931) suggests aerial dispersal among the tepuis. This hypothesis seems plausible (though not obligatory) for organisms with higher dispersal abilities (e.g., birds, vascular plants, insects; Mayr and Phelps 1967; Jaffé et al. 1993; Givnish et al. 1997; Rull and Nogué 2007) but unlikely for low-vagility organisms. The Habitat Shift hypothesis (Mayr and Phelps 1967) suggests that lowland species adapted to cooler climates, allowing colonization of the highlands (Mayr and Phelps 1967; Huber 1988; McDiarmid and Donnelly 2005). The Vertical Displacement hypothesis (Rull 2004a,c, 2005), which is not exclusive of the Habitat Shift hypothesis, suggests that cooler climates, especially during Quaternary climate oscillations, promoted downward elevational shifts of habitat and range expansion of highland species into the lowlands, thus connecting previously isolated populations. Subsequent warmer interglacials promoted upward shifts of these cold-adapted populations, isolating them on the summits. Thus, the Vertical Displacement hypothesis specifically invokes historical climate change, whereas the Habitat Shift hypothesis focuses on populations adapting to new habitats.

The Vertical Displacement hypothesis is similar to the well-known Forest Refuge hypothesis of Haffer (1969) in that both invoke glacial–interglacial climate fluctuations resulting in alternation between isolated refugia and widespread habitat types. Hypotheses of Pleistocene refugia have been extremely popular, though also highly disputed, for explaining biogeographic patterns both in temperate (Knowles 2000; Johnson and Cicero 2004; Galbreath et al. 2009) and tropical regions (Mayr and O'Hara 1986; Hooghiemstra and Van der Hammen 2004; Carnaval and Moritz 2008). The postulated Pleistocene refugia of the Guiana Shield are not restricted to tepui summits. For example, in low-elevation mountains in the eastern Guiana Shield, the effects of Pleistocene climatic fluctuation have been posited to promote extensive secondary contact among species typically found in mid-elevations (Noonan and Gaucher 2005, 2006).

Debate on the origins of the Pantepui biota thus focuses on the relative importance of recent dispersal versus ancient vicariance. Not unexpectedly, the Pantepui biota is likely to be a mosaic of remnant Guiana Shield lineages and more recent colonizers (Rull 2004b). Divergence times from molecular analyses of tepui species, and between these tepui inhabitants and their closest lowland relatives, can differentiate among these alternatives. To date, however, few studies have tested these hypotheses using phylogenetic approaches (but see Givnish et al. 1997, 2004), largely due to the great difficulty and expense of conducting fieldwork in the Pantepui ecosystem. To our knowledge, we offer the first explicit test of diversification hypotheses of a Pantepui-endemic vertebrate group by inferring the phylogenetic relationships and divergence times for species of *Tepuihyla*, which comprises seven allopatric treefrog species that occur only at mid- to high-elevations (Fig. 1).

Materials and Methods

TERMINOLOGY

The flattop mountains that make up the Pantepui ecosystem are remnants of the Guiana Shield Plateau and derived from Roraima Group sandstones (Huber 1987). The Pantepui occupies mainly southern Venezuela but also adjacent regions of northeastern Guyana, southern Suriname, and northern Brazil (Huber 1987). The concept of Pantepui that we follow is the association

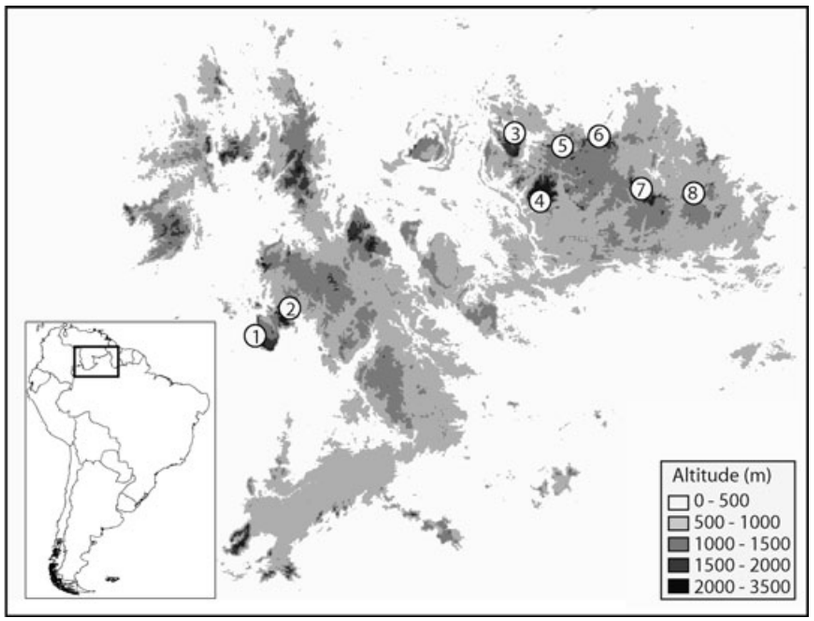

Figure 1. Map of the distribution of *Tepuihyla* within the Pantepui of the Guiana Shield: (1) *T. aecii* on Duida tepui; (2) *T. luteolabris* on Marahuaka tepui; (3) *T. edelcae* on Auyan tepui; (4) *T. edelcae* on Chimantá massif; (5) *T. rimarum* on Ptari tepui; (6) *T. rodriguezi* on Sierra de Lema; (7) *T. galani* on Guadacapiapu tepui; (8) *T. talbergae* at Kaieteur falls.

with the geological formation and not a particular elevation or floristic composition.

SAMPLES AND SEQUENCES

We sampled four of the seven recognized species of *Tepuihyla*. All *Tepuihyla* species are allopatric and inhabit montane and submontane areas. Of the seven species, four are known only from a single tepui, one (*T. edelcae*) from several adjacent tepui summits, and two (*T. rodriguezi* and *T. talbergae*) from lower elevation localities (Fig. 1). One nominal species, *T. celsae*, is not considered in our discussions. It is known only from a single locality well outside the Pantepui region. Barrio-Amorós (2004) noted that the specimens of *T. celsae*, which were not collected by the authors who described the species, have a doubtful locality and he attributed this new species and locality to mislabeling. Although the poor condition of the specimens makes identification challenging, the specimen is likely a mislabeled *T. luteolabris* from Duida tepui (C. Barrio-Amorós, pers. comm.).

Tissue samples were obtained from the Pontificia Universidad Católica del Ecuador (QCAZ), Museo de Historia Natural La Salle, Venezuela (MHNLS), and United States National Museum (USNM). See Appendix for GenBank accession numbers. The entire ingroup sample includes sequences of 86 individuals of Lophiohylini (Hylidae; Trueb 1970; Faivovich et al. 2005), of which 43 were generated by us and 43 were downloaded from GenBank. The outgroup consists of nine species of the sister clade Hylini.

Extraction and isolation of DNA, and amplification and sequencing of mitochondrial (mtDNA) and nuclear (nDNA) genes were done using standard techniques. Genomic DNA was extracted with the Viogene Blood and Tissue Genomic DNA Extraction Kit. Two different polymerase chain reaction protocols were used, one for the nDNA (the proopiomelanocortin gene POMC, approx. 460 bp) and another for the mtDNA (12S, valine tRNA, and 16S; approx. 2400 bp). The *T. aecii* tissue was from an old museum specimen, and extraction yielded low DNA concentrations, so only part of the 12S–16S could be amplified.

The mtDNA was amplified in four overlapping segments and using nine primers (MVZ59: 5′ATAGCACTGAAAAYGC TDAGATG3′ [Goebel 1999, #29], tRNA-phe: 5′GCRCTG AARATGCTGAGATGARCCC3′ [Goebel 1999, #30], 12LI: 5′AAAAAGCTTCAAACTGGGATTAGATACCCCACTAT3′ [Goebel 1999, #46], 12SM: 5′GGCAAGTCGTAACATGG TAAG3′ [Pauly et al. 2004], tRNA-val: 5′GGTGTAAGC GAGAGGCTT3′ [Goebel 1999, #73], 16SH: 5′GCTAG ACCATKATGCAAAAGGTA3′ [Goebel 1999, #76], 16SC: 5′GTRGGCCTAAAAGCAGCCAC3′ [Pauly et al. 2004], 16SA: 5′ATGTTTTTGGTAAACAGGCG3′ [Goebel 1999, #87], 16SD: 5′CTCCGGTCTGAACTCAGATCACGTAG3′ [Goebel 1999]). The nDNA was amplified in one fragment (POMCR1: GGCRT-TYTTGAAWAGAGTCATTAGWGG [Vieites et al. 2007], POMCF1: ATATGTCATGASCCAYTTYCGCTGGAA [Vieites et al. 2007]) .

The thermocycler protocol for POMC was 2 min at 94˚C followed by 12 cycles of 30 s at 94˚C, 30 s at 65˚C (–5˚C every two cycles), and 60 s at 72˚C. The thermocycler protocol for the mtDNA was 2 min at 94˚C followed by 34 cycles of 30 s at 94˚C, 30 s at 46˚C, and 60 s at 72˚C. Amplified products were purified using the Viogene Gel-M Extraction Kit. Sequencing was performed using an ABI3100 PRISM sequencer.

Contiguous sequences were assembled for 12S–16S in Sequencher 4.8 and alignments were constructed using ClustalX 2.0 with default parameters (Thompson et al. 1997) followed by manual editing in MacClade 4.08 (Maddison and Maddison 2001). Several regions in the mitochondrial dataset totaling 194 bases were judged to be unalignable and were excluded.

PHYLOGENETIC ANALYSES

The combined dataset of mtDNA and POMC included 3039 bases. All individuals were represented in the mtDNA alignment, but sequences of POMC were not available for all individuals so three analyses were performed. The first included 84 ingroup individuals plus outgroups (95 individuals total) with complete mtDNA data. To minimize missing data, the second included 46 individuals plus seven outgroup species with complete data for mtDNA and POMC (53 individuals). The third included only POMC sequences (53 individuals), so that the nDNA and mtDNA topologies could be compared. Three partitioning schemes were used in MrBayes (Ronquist and Huelsenbeck 2003): a single partition including all data; two partitions, by locus (mtDNA and POMC); and three partitions, by gene and codon position (separating third codon position for POMC). Bayes factors were used to determine the preferred partition (Kass and Raftery 1995), which was by gene and codon position. This partition was used in all subsequent analyses. The best-fitting model of evolution for each of the four partitions, GTR + G + I, was determined using MrModeltest (Nylander 2004). Maximum likelihood analyses were performed using RAxML using the model GTR + G rather than GTR + G + I (Stamatakis 2006). Node support was measured by standard nonparametric bootstrapping (1000 replicates) using RAxML, and by Bayesian posterior probabilities using MrBayes (two independent runs, 10 million generations, sampled every 1000, with burn-in of 1000 out of 10,000 samples). Convergence and stationarity of runs was assessed in Tracer (Drummond and Rambaut 2007).

We compared the results for *Tepuihyla* to another frog group, *Stefania*, with similar Pantepui distribution. *Stefania* is a Pantepui endemic clade, but only two of the species available on GenBank occur on tepui summits. We added all available GenBank data for 12S and 16S (1510 bp total) for five of the 19 described species of *Stefania* (Hylidae). The sequences included were the following: *Flectonotus fitzgeraldi* (AY819355, DQ679381), *Stefania coxi* (DQ679265, DQ679415), *Stefania ginesi* (DQ679266, DQ679417), *Stefania scalae* (DQ679267, DQ679418), *Stefania schuberti* (AY843768), *Stefania evansi* (AY843767), and *Stefania evansi* (AY819359). The sequences were aligned and analyzed using the same protocols and programs as for *Tepuihyla*.

DIVERGENCE TIME ESTIMATES

Divergence times were estimated using BEAST (Drummond and Rambaut 2007), which allows for simultaneous inference of topology and divergence times. We used two general methods of calibration to test for consistency among divergence estimates: fossils and paleobiogeography, and published substitution rates. The fossil calibrations included five hylid frogs (Smith et al. 2005). These are problematic for several reasons. First, only one pre-Holocene fossil is assigned to Lophiohylini (*Osteopilus septentrionalis* from the Pleistocene of the Bahamas; Sanchíz 1998) and none for the group under study (*Osteocephalus* and *Tepuihyla*). Second, for many frog fossils, the only recovered elements are ilia; rarely are even partially articulated frogs found, rendering identification less certain. Third, assignment of frog fossils to species or genera is often done from overall similarity rather than evidence of phylogenetic relationship from synapomorphies (Bell et al. 2010). Nonetheless, dates of occurrence of some frog fossils, assigned only on the basis of general similarity (Holman 1998, 2003) have been used to calibrate chronograms of hylid frogs (Smith et al. 2005; Lemmon et al. 2007).

We also used a paleobiogeographic calibration to the common ancestor of *Osteopilus* and *Phyllodytes auratus*, using the GAARlandia or Aves Ridge landbridge hypothesis (Iturralde-Vinent and MacPhee 1999). GAARlandia is a hypothesized Caribbean landbridge that connected the Greater Antilles to northern South America between 33 and 35 Ma. The support for this hypothesis is mixed; some authors support it as a major means of dispersal to the Caribbean islands (i.e., mammals, spiders, frogs; Dávalos 2004; Crews and Gillespie 2010; Alonso et al. 2012) whereas others strongly oppose it (Hedges 2006). However, Moen and Wiens (2009) found that the divergence of *Osteopilus* from other mainland Lophiohylini overlapped with the 33–35 Ma timeframe of this hypothesis. The prior for the GAARlandia node was drawn from a normal distribution with a mean of 34 Ma and standard deviation of 1 Ma, based on the proposed range of 33–35 Ma (Iturralde-Vinent and MacPhee 1999). Although the GAARlandia hypothesis is highly debated, we believe the inclusion of this calibration did not bias our results, since it did not change overall estimates. Furthermore, it substantially improved the computational performance of the analysis, in that good effective sample sizes (ESS > 200) were never reached (>80 M generations) without the GAARlandia calibration. With the calibrations, we analyzed the mtDNA dataset only as well as the combined mtDNA and nDNA datasets. Data partitioning was identical to that in the MrBayes analysis.

We compared chronograms obtained from fossil and paleobiogeographic calibrations to chronograms generated using two estimates of rates of evolution for anuran mitochondrial 12S and 16S genes, one from *Xenopus* (0.00249 substitutions per site per lineage per Myr, hereafter the "*Xenopus* rate"; Evans et al. 2004) and one from *Pseudacris* (0.00277 substitutions per site per lineage per Myr, the "*Pseudacris* rate"; Lemmon et al. 2007). The *Pseudacris* rate also relied in part on the hylid frog fossil calibrations (Smith et al. 2005) so it is not independent of our rate estimates. For these analyses, only the mitochondrial dataset was used because the published rates of evolution (Evans et al. 2004; Lemmon et al. 2007) were not estimated using POMC.

The BEAST analyses were run with sufficient generations (35–60 million) to yield ESS of at least 200 for all parameters. The GTR + G model was used with uncorrelated lognormal distributions of branch lengths and with no specification of a prior tree. Every 1000th generation was sampled, and the first 10% of the samples were discarded as burn-in based on examination of all parameter estimates in TRACER. Trees were summarized using TREEANNOTATOR (in the BEAST package) with the target tree type set as maximum clade credibility.

The analyses of the *Stefania* GenBank sequences were conducted similarly as in *Tepuihyla*/*Osteocephalus*. Two separate analyses were performed using the *Xenopus* and *Pseudacris* rates. Given that the *Stefania* dataset is small (compared to *Tepuihyla*), we only used BEAST (and exclude RAxML and MrBayes analyses) to estimate tree topology and divergence times.

Results

PHYLOGENETIC ANALYSES

Maximum likelihood and Bayesian analyses indicate that species of *Tepuihyla* (four out of seven recognized species; 11 individuals) form a strongly supported clade for both mitochondrial and nuclear data (1.0 Bayesian posterior probability, BPP; Figs. 2 and 3; 100% likelihood bootstrap support, BS; Fig. 3). The samples of *Osteocephalus* (11 out of 24 species) revealed that *Tepuihyla* and *Osteocephalus exophthalmus* form a strongly supported clade (100% BS, 1.0 BPP; Figs. 2 and 3) that is the sister group of all other *Osteocephalus*. Thus *Osteocephalus* as currently delimited is paraphyletic, and henceforth, we denote this by referring to "*Osteocephalus*" *exophthalmus* with quotation marks pending our resolution of this taxonomic issue, which is in progress.

We also analyzed all genera within the Lophiohylini, which includes *Osteocephalus* and *Tepuihyla*. Although the Lophiohylini was strongly supported as monophyletic, relationships among the deeper nodes of the lophiohyline clades (Fig. 3) are generally poorly supported and our topology is somewhat inconsistent with other studies (Salducci et al. 2002; Faivovich et al. 2005; Wiens et al. 2010). However, the sister-group relationship between *Tepuihyla* and *Osteocephalus* holds in all studies.

The divergences among the *T. edelcae* samples from the summits of Auyan Tepui and the Chimantá Massif (Fig. 1) are similar to the among-species divergences within *Tepuihyla*. Further, the mtDNA and nDNA phylogenies are incongruent regarding the monophyly of *T. edelcae*. The mtDNA only (Fig. 2) and the combined mtDNA and POMC (Fig. 3A) topologies both group *T. edelcae* from Chimantá as more closely related to the mid-elevation *T. rodriguezi*/*T. talbergae* than to the *T. edelcae* from Auyan indicating that *T. edelcae* is paraphyletic. However, based on nDNA only (Fig. 3B), *T. edelcae* is monophyletic, though this is poorly supported (44 BS, 0.81 BPP). The low variability in the nuclear locus does not resolve the species-level relationships within *Tepuihyla*. Thus, the apparent discrepancy between the nuclear and mitochondrial trees for *T. edelcae* might be due to mitochondrial introgression from recent hybridization between *T. edelcae* and *T. rodriguezi*/*T. talbergae* or to incomplete lineage sorting.

DIVERGENCE TIME ESTIMATES

Divergence time estimates based on calibrations from either fossils or substitution rates showed similar results (Fig. 4). Taking into account all results from different datasets and calibrations, divergences between the Pantepui-endemic *Tepuihyla* and its sister taxon were estimated to be 14.7–23.6 Ma, which is far more recent than the formation of the tepuis. Furthermore, the oldest estimates for the divergence between this clade (*Tepuihyla* + *"O." exophthalmus*) and *Osteocephalus* are still at least 20 Ma more recent than tepui formation. Divergences among *Tepuihyla* species or populations inhabiting different tepuis are also relatively recent, from 0.7 to 8.1 Ma, with several divergences overlapping the Quaternary.

The divergence times from the *Xenopus* estimate were the oldest, possibly because *Xenopus* is very distantly related. The rates found using the hylid fossil calibrations are all in close agreement, the only difference being that the estimates obtained from the fossil calibrations (compared to the *Pseudacris* estimate) had much higher variance, probably because none of the calibrations lies within the group of interest (*Tepuihyla* and *Osteocephalus*). The only calibration within Lophiohylini is the paleobiogeographic calibration of *Osteopilus*. Given this, we focus the discussion on the analysis using the *Pseudacris* estimate. Estimated divergence times among *Tepuihyla* species range from 0.7–5.3 Ma. The oldest node, the split between *T. aecii* and all other *Tepuihyla*, is estimated at 5.3 Ma (Fig. 2, node A). Notably, this split corresponds to the geographic division between the western tepui group and the eastern tepui group (Huber 1988). *Tepuihyla aecii* is from Cerro Duida in the western group, and the remaining species are from the eastern group.

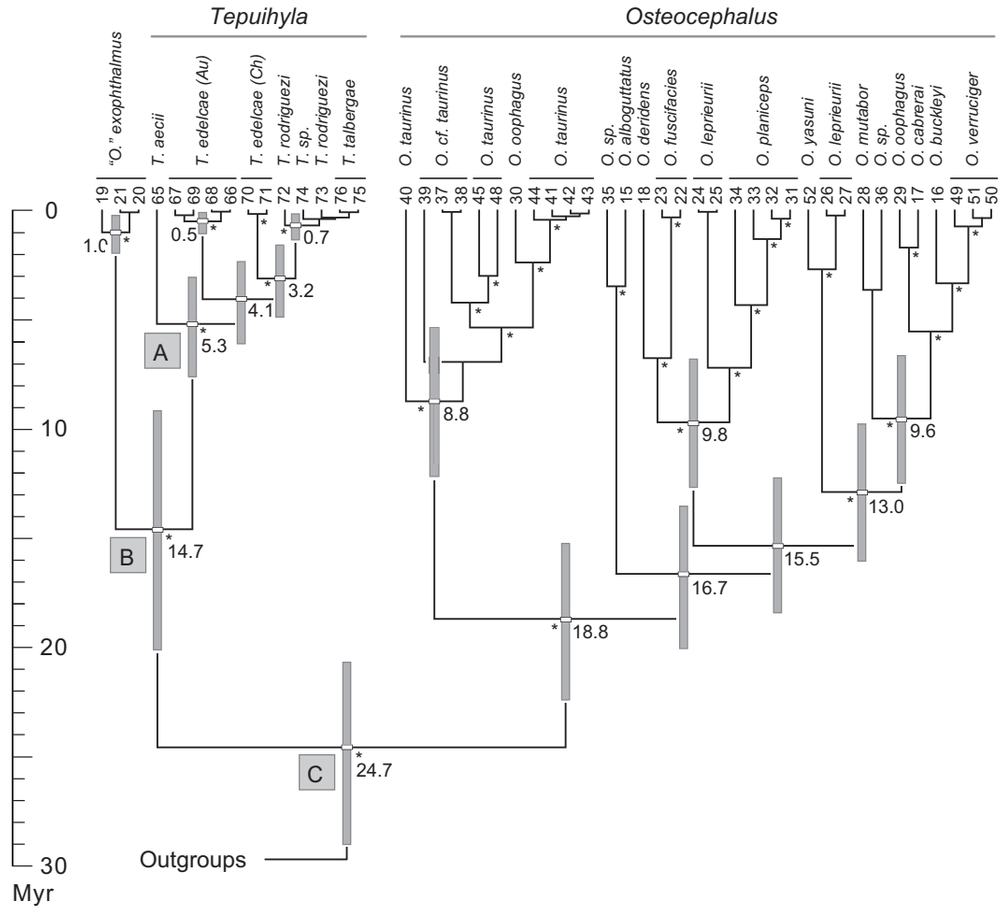

Figure 2. **Divergence time estimates and 95% confidence bars for *Tepuihyla* (node A), the sister species *"O." exophthalmus* (node B), and their lowland closest relatives, *Osteocephalus* (node C) for the mtDNA dataset. Divergence estimates of major clades are shown for outgroups. Estimates were obtained using the Lemmon et al. (2007) rate of substitution. Asterisks indicate Bayesian posterior probabilities greater than 95%. The tree and estimates were obtained from BEAST. Numbers next to terminal taxa refer to individual specimens in Appendix.**

The youngest split (0.7 Ma) is between *T. talbergae* (Kaieteur National Park, Guyana, 366 m, but known from higher elevations) and *T. rodriguezi* (Gran Sabana, Venezuela, 800–1200 m); both species inhabit the lowest elevations known for *Tepuihyla*. Not all analyses recovered this poorly supported node; in the mtDNA tree (Fig. 2) *T. rodriguezi* was paraphyletic, which suggests either incomplete lineage sorting, or simply that they are a single species. We refer to this complex hereafter as *T. talbergae*/*rodriguezi*. The divergence between *Tepuihyla* and its sister species *"O." exophthalmus* is estimated at 14.7 Ma (Fig. 2; node B), and that between *Tepuihyla* + *"O." exophthalmus* and *Osteocephalus* is estimated at 24.7 Ma (Fig. 2; node C).

The results of the *Stefania* analyses using the *Xenopus* and *Pseudacris* rates were very similar, so we only report the divergence times from the *Pseudacris* rate. The deepest divergence found is 34.4 Ma, between *S. ginesi* and all other included species (Fig. 5). The most recent divergence is estimated at 7.1 Ma, between *S. scalae* and *S. evansi*.

Discussion

MONOPHYLY AND DIVERGENCES AMONG *TEPUIHYLA* SPECIES

The low divergence estimates among *Tepuihyla* species (<5.3 Ma) indicate that this Pantepui clade did not speciate under a Lost World vicariance scenario. Even when accounting for uncertainty in rates of molecular evolution, credibility intervals, and time calibrations, it is evident that species diversity within this clade is not the product of ancient dissection of the Guiana Shield plateau. Furthermore, the origin of the Pantepui-endemic treefrogs does not predate the formation of the tepuis, which is another prediction of the Lost World hypothesis.

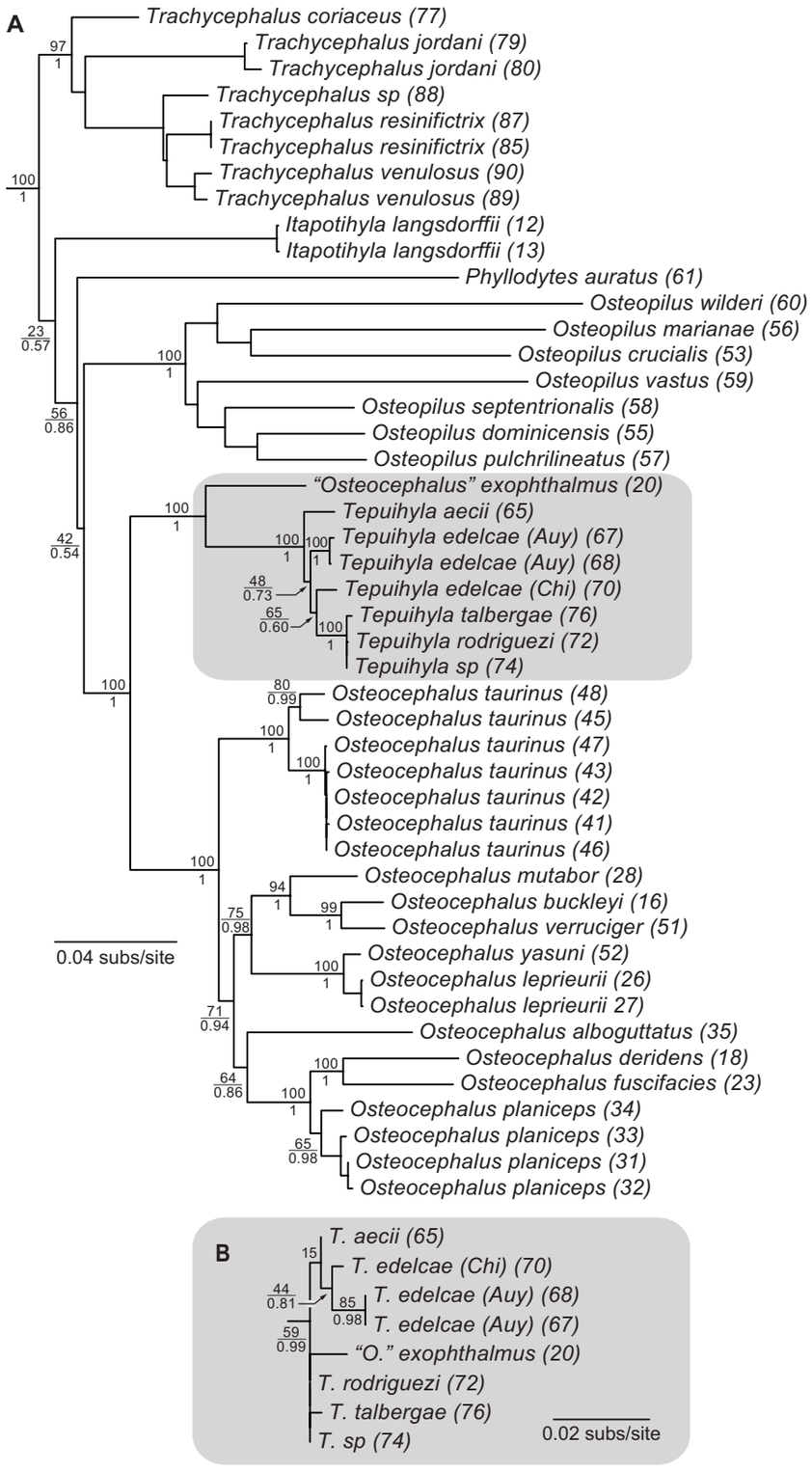

Figure 3. Maximum likelihood topology for: (A) the combined dataset of 12S, 16S, and POMC (present in all terminals), and (B) the POMC only dataset. Bootstrap values are shown above the nodes and Bayesian posterior probabilities are shown below. Support values are omitted for the outgroups for simplicity. Numbers next to terminal taxa refer to individual specimens in Appendix.

Calibration	Node 1	Node 2	Node 3	Node 4	Node 5	Node 6
Pseudacris Estimate (0.00277 subs/site/Myr) 12S–16S; 35M gen.	0.7* 0.2–1.4	3.2 1.6–4.9	4.1 2.4–6.2	5.3 3.1–7.7	14.7 9.2–20.2	24.7 20.8–29.2
Xenopus Estimate (0.00249 subs/site/Myr) 12S–16S; 35M gen.	1.1 0.3–2.2	5.0 2.5–8.1	6.4 3.5–9.9	8.1 4.5–12.4	23.6 13.4–34.1	41.5 32.9–51.0
Hylid Fossil Calibrations + GAARlandia 12S–16S; 60M gen.	0.8* 0.2–1.6	3.3 1.5–5.6	4.4 2.2–7.1	5.7 3.0–9.1	17.0 9.6–24.9	31.1 26.5–35.0
Hylid Fossil Calibrations + GAARlandia 12S–16S + POMC; 50M gen.	0.7 1.1–1.4	3.4 1.8–5.4	4.4 2.4–6.6	5.6 3.2–8.5	17.1 10.3–24.3	29.1 23.5–34.2

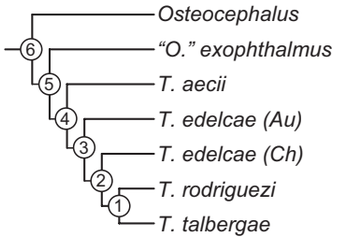

Figure 4. Divergence time estimates (top) in millions of years and 95% confidence intervals (bottom) obtained in all BEAST analyses for the main clades of the highland group, *Tepuihyla*, and its closest relatives, *"O." exophthalmus* and *Osteocephalus*. The clades are numbered in the simplified cladogram to the right. Node 1 was not always recovered, and thus we report the deepest divergence within that group and indicate the unrecovered node with *. The differences in number of generations in the left column are due to the fact that different analyses required different numbers of samples to reach stationarity. All divergence time estimates obtained with the calibrations of the outgroup fall within the 95% confidence intervals obtained with the previously estimated rates of evolution.

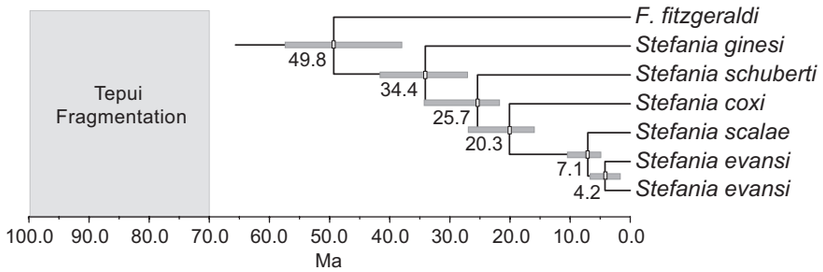

Figure 5. Divergence time estimates and 95% confidence intervals for *Stefania*. Divergence time estimates are shown in millions of years. Time of tepui fragmentation is shown as a reference. Estimates were obtained using the Lemmon et al. (2007) rate of substitution.

Previous phylogenetic analyses that included lophiohyline taxa (Faivovich et al. 2005; Wiens et al. 2010) sampled only one species of *Tepuihyla* so the monophyly of this group had never been tested. With our increased sampling (four out of seven recognized species), we found *Tepuihyla* to be monophyletic. However, *Osteocephalus* as currently delimited (Trueb 1970; Faivovich et al. 2005) is not monophyletic because "*O*." *exophthalmus* was found to be the sister taxon to *Tepuihyla*. "*Osteocephalus*" *exophthalmus* is a Pantepui-endemic known from low and middle elevations in Guyana. In contrast to this clade of Guiana shield endemics (i.e., "*O*." *exophthalmus* + *Tepuihyla*), *Osteocephalus* is primarily a lowland Amazonian clade; a few species occur in the lowlands of the Guiana Shield. Thus, the key node to understanding the divergence of the Pantepui clade is that between *Tepuihyla* + "*O*." *exophthalmus* and *Osteocephalus* (node 6, Fig. 4).

Tepuihyla + "*O*." *exophthalmus* diverged from *Osteocephalus* about 24.7 Ma (Figs. 2 and 4, node 6). This timing of separation between *Osteocephalus* and *Tepuihyla* overlaps with that of a marine incursion into the present Amazon Basin starting in the Early Miocene, 23–16 Ma (Hoorn 1993). This may explain the largely allopatric distributions of *Osteocephalus* and *Tepuihyla*.

OTHER BIOGEOGRAPHIC HYPOTHESES

Having rejected the Lost World hypothesis for *Tepuihyla*, it is now clear that the divergence times among *Tepuihyla* species require dispersal between the lowlands and the summits. All three dispersal hypotheses (Island-Hopping, Habitat Shift, and Vertical Displacement) generate predictions related to dispersal frequencies and times of divergence. The Island-Hopping hypothesis is unlikely because island hopping should be a rare event for *Tepuihyla* given its low vagility. In addition, our finding of multiple relatively recent dispersal events is inconsistent with the predictions of Island Hopping. However, given our estimates of divergence times and phylogenetic relationships, the biogeographic history of *Tepuihyla* does not definitively reject either of the remaining dispersal hypotheses.

Given the occurrence of *Tepuihyla* in mostly middle and high elevations, as well as the recent divergence times, the establishment of the current distribution probably involved elements of both the Habitat Shift and Vertical Displacement hypotheses. Because all known *Tepuihyla* species are associated with the Pantepui, we infer that the common ancestor of *Tepuihyla* was also a Pantepui inhabitant that adapted to these conditions (as the Habitat

Shift hypothesis suggests). However, the sympatry of "*O.*" *exophthalmus* with *T. rodriguezi*/*T. talbergae* throughout their limited distributions in lower Pantepui elevations of the eastern Guiana Shield suggests that the association of this clade ("*O.*" *exophthalmus* + *Tepuihyla*) with the tepuis and possible adaptation to higher elevations occurred as far back as 14.7 Ma (Fig. 2).

It is unlikely that Pleistocene climatic shifts were the only processes involved in divergences within *Tepuihyla*. The three deepest divergences within *Tepuihyla* predate the Quaternary, supporting the overlooked importance of pre-Quaternary diversification (Rull 2008). On the other hand, large confidence intervals among *Tepuihyla* divergences that overlap part of the Pleistocene, plus the small divergences between *T. edelcae* and *T. rodriguezi*/*T. talbergae* suggest that Pleistocene glacial cycles may have shaped the current distributions of these species. However, to determine the general significance of Pleistocene climate fluctuations, extensive sampling within recently diverged *Tepuihyla* species is needed to estimate recent gene flow using coalescent analyses.

COMPARISONS WITH PHYLOGENIES OF OTHER TEPUI TAXA

This analysis is the first to estimate DNA-based divergence times of a Pantepui-endemic animal taxon. Our analyses of *Tepuihyla* recover relatively recently diverged species (Plio-Pleistocene) with good species sampling (4/7 species). Although small samples of two other Pantepui frog groups have been analyzed phylogenetically (*Stefania* and *Ceuthomantis*, see below), the only other studies (to our knowledge) that explicitly treat the historical biogeography of a Pantepui clade include members of the Bromeliaceae and Rapateaceae (Givnish et al. 1997, 2000, 2004, 2011).

Bromeliaceae and Rapateaceae are families of flowering plants within the order Poales. Within the Rapataceae, the tepui-endemic crown clade Stegolepidieae (*Stegolepis*, *Amphiphyllum*, and *Epidryos*) diversified 10 Ma (Givnish et al. 2004). Dispersal, and not vicariance, is argued to be the principal correlate of divergence among these lineages (Givnish et al. 2004). Furthermore, the presence of some *Stegolepis* in lowland as well as intermediate and summit habitats corroborates recent dispersal.

Similarly, divergence times for many clades within Bromeliaceae have been estimated. The most recent common ancestor of crown-group Bromeliaceae likely inhabited the Pantepui at 19.1 Ma (Givnish et al. 2011: Fig. 7). Brocchinioideae and Lindmanioideae, the two earliest branching lineages within Bromeliaceae, are tepui endemics. The range of divergences between these clades is 8.9–19.0 Ma. The Bromeliaceae, then, does not owe its diversification (as a crown group) to the vicariant dissection of Pantepui, but more likely to late Cenozoic dispersal across a dissected landscape, a scenario similar to that observed for *Tepuihyla*.

The Pantepui endemic bromeliads in the genus *Brocchinia* are incredibly diverse in morphology and ecology (Givnish et al. 1997). This diversity could suggest that this clade is an "ancient" lineage. However, species within *Brocchinia* diverged relatively recently; the age of the crown group is at least 13.1 Ma (Givnish et al. 2011). Interestingly, *Tepuihyla* species seem to depend on water accumulation in phytotelmata of some species of *Brocchinia* (*B. hectiodes* and *B. acuminata*; Ayarzagüena et al. 1992), but no hypothesis of co-divergence of *Tepuihyla* and *Brocchinia* has been put forward.

We also examined divergence times of another Pantepui-endemic group, *Stefania*, for comparison to the *Tepuihyla* results. *Stefania*, like *Tepuihyla*, are highly dependent on the phytotelmata of pitcher plants. However, the reproductive biology of *Stefania* is quite distinct in that females are obligate dorsal egg brooders and the embryos undergo direct development (Salerno and Pauly 2012). In contrast, *Tepuihyla* have the more common reproductive mode of depositing eggs in bodies of water with the eggs hatching into a larval (tadpole) stage that undergoes metamorphosis (Ayarzagüena et al. 1992). Several authors have suggested that distributions and phylogenetic relationships in *Stefania* support a vicariant speciation scenario following the Lost World hypothesis (Hoogmoed 1979; MacCulloch and Lathrop 2002; McDiarmid and Donnelly 2005). However, as is the case for most Pantepui groups, these assessments were based on limited sampling and distributional data with relationships among individuals solely based on morphological comparisons. Our analysis of the available sequences (five out of 19 species) shows that the deepest divergence within *Stefania* is 34.4 Ma, and the youngest is 7.1 (Fig. 5). Interestingly, the youngest divergence is found between two lowland/midland species (*S. scalae* and *S. evansi*), which is the same pattern, though different timing, observed for the two *Tepuihyla* lowland/midland clades, *T. rodriguezi*/*T. talbergae*. Furthermore, these two species pairs have similar geographic distributions, which may indicate shared biogeographic histories. Even though the divergence estimates for *Stefania* clades are on average more deeply diverged than *Tepuihyla*, Stegolepidae, *Brocchinia*, and *Lindmania*, the divergences within *Stefania* indicate that this lineage radiated more recently than the dissection of the tepui summits (70–90 Ma). Thus, *Stefania* follows the general diversification pattern in *Tepuihyla* and Bromeliaceae.

Many endemic representatives of the "Lost World" are touted as "living fossils," that is, a species or group of species that was formerly speciose or widespread in time and/or space, but has suffered extinction (Brown and Lomolino 1998). However, highly endemic clades are not necessarily "living fossils." There is no evidence that the clades *Tepuihyla*, *Stefania*, *Lindmania*, *Brocchinia*, and Stegolepidae are remnants of ancient widespread lineages. These taxa are more appropriately termed neoendemics, that is, endemic taxa of relatively recent autochthonous origin (Brown and Lomolino 1998).

The frog *Ceuthomantis smaragdinus* from Mt. Kopinang, Guyana also is claimed to be a "living fossil" (Heinicke et al. 2009). In contrast to the previous examples, this species is deeply diverged from its extremely speciose sister taxon at about 60 Ma (Heinicke et al. 2009). Two other species are tentatively referred to this genus, but no sequences are available. Although this divergence time is much greater than that of the other taxa considered here, the divergence of only one species makes it difficult to determine the minimal age at which that lineage (one species) was present within the Pantepui. Thus, further work is needed to assess whether this lineage is a paleoendemic.

TEPUIS AS CURRENT PHYSICAL AND ECOLOGICAL BARRIERS

Many tepuis have sheer cliffs that have been proposed to be physical barriers to dispersal between low and high elevations, thus isolating summit taxa (Chapman 1931; Maguire 1970; Hoogmoed 1979). However, divergences among *Tepuihyla* species took place relatively recently, at least 60 Ma after the tepuis were fully formed, indicating that tepui walls are not a complete physical barrier between the summits and lowland forest even for organisms with low vagility.

Tepuihyla has an extremely fragmented distribution, but it is widespread in the Guiana Shield, occurring in three of the four Tepui provinces (Huber 1988). All species are endemic either to the summits or to mid-elevation regions of tepui remnants and are generally found in Rapateaceae- or Bromeliaceae-dominated meadows (Ayarzagüena et al. 1992). That *Tepuihyla* is absent from the lowlands between the tepuis suggests the habitat is currently unsuitable. Physiological constraints associated with increasing elevation (and thus decreasing temperature and increasing daily and yearly temperature variation) have been shown to enhance isolation among populations at different elevations, especially in ectothermic vertebrates (Navas 2006). The drastic elevational differences between tepui summits and the surrounding lowlands result in extreme differences in annual mean temperature, precipitation, soil composition, and phytogeographic regions (Steyermark 1979; Huber 2006). Drastic microclimatic and habitat differences between summits and lowlands may create a challenge for dispersal, thus restricting *Tepuihyla* to suitable mid- and high-elevation conditions. However, as stated in the Vertical Displacement hypothesis, these climatic conditions may have been different in the past, allowing for a more widespread lowland existence.

Our results indicate that the sheer escarpments of tepuis have not prevented dispersal of *Tepuihyla* species across the lowlands between the summits during the last 5.3 Ma. Furthermore, the first association of the ancestor of *Tepuihyla* + "*O*." *exophthalmus* with the Pantepui likely occurred around 14.7 Ma, which deeply postdates tepui fragmentation. Because most *Tepuihyla* divergences are during the Pliocene (5.3–2.6 Ma), our results also highlight the importance of pre-Quaternary speciation (Rull 2008). However, we cannot completely reject the effects of Pleistocene glaciation, because the timing of the most recent divergences is consistent with climatic fluctuations. Our analysis clearly demonstrates dispersal of *Tepuihyla* to and/or from the tepui summits long after their formation. Furthermore, comparisons to other taxa such as *Stefania* and Bromeliaceae seem to indicate that dispersal has occurred across widely different tepui taxa, from pollen-dispersing plants to low-vagility organisms such as frogs. Thus, even though the Lost World hypothesis is attractive in nature and has been largely popular in the literature, so far there is no empirical dataset that shows unambiguous support for it.

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Appendix

List of specimens. Specimens that were sequenced are bold and marked with an asterisk. All other specimens were obtained from GenBank. Field/museum codes are only shown for specimens sequenced herein. Field numbers correspond to Brice P. Noonan (BPN) and Patricia Salerno (PS).

			GenBank accession	
	Taxon	Field/museum code	12S–16S	POMC
1	*Acris crepitans*	–	EF566969	AY819109
2	*Aparasphenodon brunoi*	–	AY843567	–
3	*Argenteohyla siemersi*	–	AY843570	–
4	*Corythomantis greeningi*	–	AY843578	–
5	*Hyla arborea*	–	AY843601	DQ57787
6	*Hyla arenicolor*	–	EF566960	–
7	*Hyla cinerea*	–	AY680271	AY819116
8	*Hyla gratiosa*	–	AY843630	GQ374915
9	*Hyla meridionalis*	–	EF566953	GQ374915
10	*Hyla squirella*	–	AY843678	AY819120
11	*Hyla versicolor*	–	EF566953	DQ55805
12	*Itapotihyla langsdorffii*	–	AY843706	AY843706
1	***Itapotihyla langsdorffii****	USNM303287	JQ686500	JQ868470
14	*Nyctimantis rugiceps*	–	AY843781	–
15	*Osteocephalus alboguttatus*	–	DQ380347	–
16	*Osteocephalus buckleyi*	–	DQ380378	EUO34116
17	*Osteocephalus cabrerai*	–	AY843705	–
1	***Osteocephalus deridens****	QCAZ20868	JQ868501	JQ868484
1	***Osteocephalus exophthalmus****	BPN166	JQ868523	–
2	***Osteocephalus exophthalmus****	MHNLS19584	JQ868525	JQ868483
2	***Osteocephalus exophthalmus****	MHNLS19583	JQ868524	–

	Taxon	Field/museum code	GenBank accession	
			12S–16S	POMC
2	***Osteocephalus fuscifacies****	QCAZ20790	JQ868502	–
2	***Osteocephalus fuscifacies****	QCAZ20788	JQ868503	JQ868499
24	*Osteocephalus leprieurii*	–	AY843707	–
25	*Osteocephalus leprieurii*	–	AY549361	–
2	***Osteocephalus leprieurii****	MHNLS18689	JQ868505	JQ868497
2	***Osteocephalus leprieurii****	MHNLS18619	JQ868504	JQ868498
28	*Osteocephalus mutabor*	–	DQ380379	EUO34117
29	*Osteocephalus oophagus*	–	AF467267	–
30	*Osteocephalus oophagus*	–	AY843708	–
31	*Osteocephalus planiceps*	–	DQ380380	EUO43118
3	***Osteocephalus planiceps****	QCAZ19195	JQ868521	JQ868495
3	***Osteocephalus planiceps****	QCAZ20797	JQ868522	JQ868494
3	***Osteocephalus planiceps****	QCAZ18844	JQ868520	JQ868496
3	***Osteocephalus alboguttatus****	QCAZ18186	JQ868516	JQ868493
3	***Osteocephalus* sp.***	QCAZ38420	JQ868526	–
3	***Osteocephalus* cf. *taurinus****	USNM302469	JQ868514	–
3	***Osteocephalus* cf. *taurinus****	USNMFS008803	JQ868515	–
3	***Osteocephalus* cf. *taurinus****	MHNLS18325	JQ868506	–
40	*Osteocephalus taurinus*	–	AY843709	–
4	***Osteocephalus taurinus****	MHNLS18663	JQ868509	JQ868490
4	***Osteocephalus taurinus****	MHNLS15622	JQ868507	JQ868492
4	***Osteocephalus taurinus****	PS004	JQ868512	JQ868487
4	***Osteocephalus taurinus****	MHNLS19633	JQ868511	–
4	***Osteocephalus taurinus****	QCAZ18839	JQ868513	JQ868488
4	***Osteocephalus taurinus****	MHNLS17336	JQ868508	JQ868491
4	***Osteocephalus taurinus****	MHNLS18715	JQ868510	JQ868489
48	*Osteocephalus taurinus*	–	AY326041	AY819130
4	***Osteocephalus verruciger****	QCAZ13225	JQ868517	JQ868486
5	***Osteocephalus verruciger****	QCAZ17283	JQ868518	–
51	*Osteocephalus verruciger*	–	DQ380381	–
5	***Osteocephalus yasuni****	QCAZ19245	JQ868519	JQ868485
53	*Osteopilus crucialis*	–	AY843710	EUO34121
54	*Osteopilus dominicensis*	–	AY843711	–
55	*Osteopilus dominicensis*	–	AY819443	EUO34122
56	*Osteopilus marianae*	–	DQ380383	EUO34123
57	*Osteopilus pulchrilineatus*	–	AY819436	EUO34124
58	*Osteopilus septentrionalis*	–	AY843712	AY819131
59	*Osteopilus vastus*	–	AY843713	EUO34128
60	*Osteopilus wilderi*	–	DQ380385	EUO34129
61	*Phyllodytes auratus*	–	AY819383	AY819133
62	*Phyllodytes luteolus*	–	AY843721	–
63	*Phyllodytes* sp.	–	AY843722	–
64	*Pseudacris crucifer*	–	AY291103	EF988269
6	***Tepuihyla aecii****	MHNLS12013	JQ868533	JQ868478
66	*Tepuihyla edelcae*	–	AY843770	–
6	***Tepuihyla edelcae****	PS002	JQ868537	JQ868475
6	***Tepuihyla edelcae****	MHNLS16090	JQ868534	JQ868477
6	***Tepuihyla edelcae****	MHNLS05824	JQ868535	–
7	***Tepuihyla edelcae****	PS001	JQ868536	JQ868476
7	***Tepuihyla edelcae****	PS268	JQ868538	–

	Taxon	Field/museum code	GenBank accession	
			12S–16S	POMC
7	***Tepuihyla rodriguezi****	PS003	JQ868540	JQ868474
7	***Tepuihyla rodriguezi****	MHNLS19575	JQ868539	–
74	*Tepuihyla* sp.	–	DQ380389	EUO34131
7	***Tepuihyla talbergae****	BPN1101	JQ868541	–
7	***Tepuihyla talbergae****	BPN1219	JQ868542	JQ868473
77	*Trachycephalus coriaceus*	–	DQ380386	EUO34130
78	*Trachycephalus hadroceps*	–	AY843717	–
7	***Trachycephalus jordani****	QCAZ17509	JQ868527	JQ868471
80	*Trachycephalus jordani*	–	AY819395	AY819145
81	*Trachycephalus jordani*	–	AY326042	–
82	*Trachycephalus jordani*	–	AY843771	–
83	*Trachycephalus mesophaeus*	–	AY843718	–
84	*Trachycephalus nigromaculatus*	–	AY843772	–
8	***Trachycephalus resinifictrix****	QCAZ20808	JQ868528	JQ868481
86	*Trachycephalus resinifictrix*	–	AY843719	–
8	***Trachycephalus resinifictrix****	QCAZ19304	JQ868529	JQ868482
8	***Trachycephalus* sp.***	QCAZ21282	JQ868530	JQ868479
8	***Trachycephalus venulosus****	QCAZ21283	JQ868531	JQ868480
9	***Trachycephalus venulosus****	PS013	JQ868532	JQ868472
91	*Trachycephalus venulosus*	–	AY549362	–
92	*Trachycephalus venulosus*	–	AY326048	–
93	*Trachycephalus venulosus*	–	AY819382	–
94	*Trachycephalus venulosus*	–	DQ347027	–
95	*Trachycephalus venulosus*	–	AY364350	–