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Biogeographic origin and radiation of Cuban *Eleutherodactylus* frogs of the *auriculatus* species group, inferred from mitochondrial and nuclear gene sequences

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ABSTRACT

We studied phylogenetic relationships of the *Eleutherodactylus auriculatus* species group to infer colonization and diversification patterns in this endemic radiation of terrestrial frogs of the genus *Eleutherodactylus* in the largest of the Greater Antilles, Cuba. An initial screening of genetic diversity based on partial sequences of the 16S rRNA gene in almost 100 individuals of all species of the group and covering the complete known geographic range of their occurrence found most species endemic to small ranges in the eastern Cuban mountains while a single species was widespread over most of Cuba. Our molecular phylogeny, based on 3731 bp of four mitochondrial and one nuclear gene, suggests that most cladogenetic events within the group occurred among clades restricted to the eastern mountains, which acted as refugia and facilitated the diversification in this group. Our results reveal two separate colonization events of Central and Western Cuba and allow inferring the timing of the subsequent diversification events that occurred between 11 and 2 Mya. Because populations previously assigned to *E. auriculatus* represent four genetically strongly divergent lineages that also differ in their advertisement calls, we propose that *E. auriculatus* as currently recognized comprises four species. The difficulties in assigning the name *auriculatus* to any of these four species, and the fact that *E. principalis* is nested within one of them, stress the need for a thorough taxonomic revision of this group.

1. Introduction

Located in the tropics of the Western hemisphere, the West Indies include more than 7000 islands, islets and keys grouped into the Bahamas, the Greater Antilles, and the Lesser Antilles (Berman, 2008; Hofmann, 2008). The West Indies have a complex geological history, sustain many endemic forms and have been considered a natural laboratory for studying evolutionary processes (Ricklefs and Bermingham, 2007). Both dispersal and vicariance might have contributed to the formation of these island biotas, and a more complex biogeographical scenario that includes elements of both models, is beginning to emerge (de Queiroz, 2005; Heaney, 2007).

In contrast to the numerous studies addressing issues related to island colonization, comparatively little attention has been given to the more recent intra-island speciation processes, and only few studies have addressed the phylogeography of terrestrial vertebrates in the West Indies (Ricklefs and Bermingham, 2007). Recent advances have shown that vicariant events associated with island fragmentation by sea-level changes play a key role in speciation or population substructuring of amphibian and reptile populations on West Indian islands (Gifford and Larson, 2008; Gifford et al., 2004; Glor et al., 2004; Velo-Antón et al., 2007). Cuba, the largest of the Greater Antilles, has a complex geological history that involves sea-level changes and tectonic movements that fragmented and rejoined the Western, Central and Eastern paleo-archipelagos (Graham, 2003; Iturralde-Vinent, 2001; Iturralde-Vinent and MacPhee, 1999; MacPhee et al., 2003). Allopatric divergences initiated during partial island submergence in the Miocene (24–6 Mya) may have played an important role in speciation during the adaptive radiation of *Anolis* lizards (Glor et al., 2004) and freshwater fishes of the genus *Girardinus* (Doadrio et al., 2009) in the Paleo-Cuban archipelago. The island constitutes thus an excellent model to test biogeographic hypotheses.

New World direct-developing frogs, Terrarana as defined by Hedges et al. (2008) comprise a radiation of 882 species grouped into four families (Brachycephalidae, Craugastoridae, Eleutherodactylidae, and Strabomantidae). Among the features

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that are likely to explain the origin of this very high number of frog species are the direct development of juveniles into adults and the production of isolated clutches, which might promote diversification and a high level of populational structuring (Dubois, 2004). Frogs of the genus *Eleutherodactylus* (Eleutherodactylidae) are grouped into five subgenera and 185 species, with a geographic distribution primarily centered in the Caribbean (Hedges et al., 2008). In Cuba, the amphibian fauna is largely dominated by these frogs, and it is noteworthy that 90% (50 species) of the native taxa are members of this genus (AmphibiaWeb, 2008; Díaz and Cádiz, 2008; Hedges et al., 2008).

The subgenus *Eleutherodactylus* is represented in Cuba by the monophyletic *Eleutherodactylus auriculatus* (seven species) and *E. varians* (five species) species groups (Hedges et al., 2008; Heinicke et al., 2007). Members of this subgenus have no communal breeding sites, occupy small home ranges, and are highly territorial (Alonso et al., 2001; Ovaska, 1992; Stewart and Rand, 1991; Townsend and Stewart, 1994; Woolbright, 1985). This combination of natural history features makes them an attractive subject for studies investigating speciation processes in amphibians. As an exclusive clade of direct-developing frogs, the *E. auriculatus* species group appears as a suitable model to study patterns and processes of diversification within Cuba. Based on a comprehensive sampling of all species and using concatenated DNA sequences of mitochondrial and nuclear genes, we reconstruct a phylogeny of the group and calculate the ages of divergences between its members. We furthermore describe the phylogeographic pattern of the widespread species *E. auriculatus,* uncover cryptic diversity, and propose a scenario for the evolutionary diversification of these frogs in Cuba.

2. Materials and methods

2.1. Sampling

Fieldwork was performed during the rainy seasons of 2005 and 2007. We visited 23 localities covering the geographic range of the *Eleutherodactylus auriculatus* species group (*E. auriculatus* (Cope 1862), *E. bartonsmithi* Schwartz, 1959, *E. eileenae* Dunn 1926, *E. glamyrus* Estrada and Hedges 1997, *E. mariposa* Hedges, Estrada and Thomas 1992, *E. principalis* Estrada and Hedges 1997, and *E. ronaldi* Schwartz 1959) (Fig. 1). Geographic coordinates and altitude of each locality visited were recorded in the field using a Garmin ETREX GPS receiver with WGS84 projection. Calling males of the species were captured by hand during the night with the aid of headlamps. Samples were obtained by toe clipping and stored in vials with 90% ethanol; some specimens were preserved as vouchers and deposited in the Zoological Collection of the Institute of Ecology and Systematics of Cuba (CZACC). Locality data and voucher specimen information is summarized in Supplementary Table S1. We allocated specimens to species based on published diagnostic features (Estrada and Hedges, 1997a,b; Hedges et al., 1992; Schwartz, 1969; Schwartz and Henderson, 1991) and aided by bioacoustic features of their advertisement calls recorded in the field. Representative calls of every species included in this study have been published in a sound guide (Alonso et al., 2007).

2.2. DNA isolation, amplification and sequencing

We used the Qiagen DNeasy Tissue Kit© to extract DNA from the tissue samples following the manufacturer's protocol. We con-

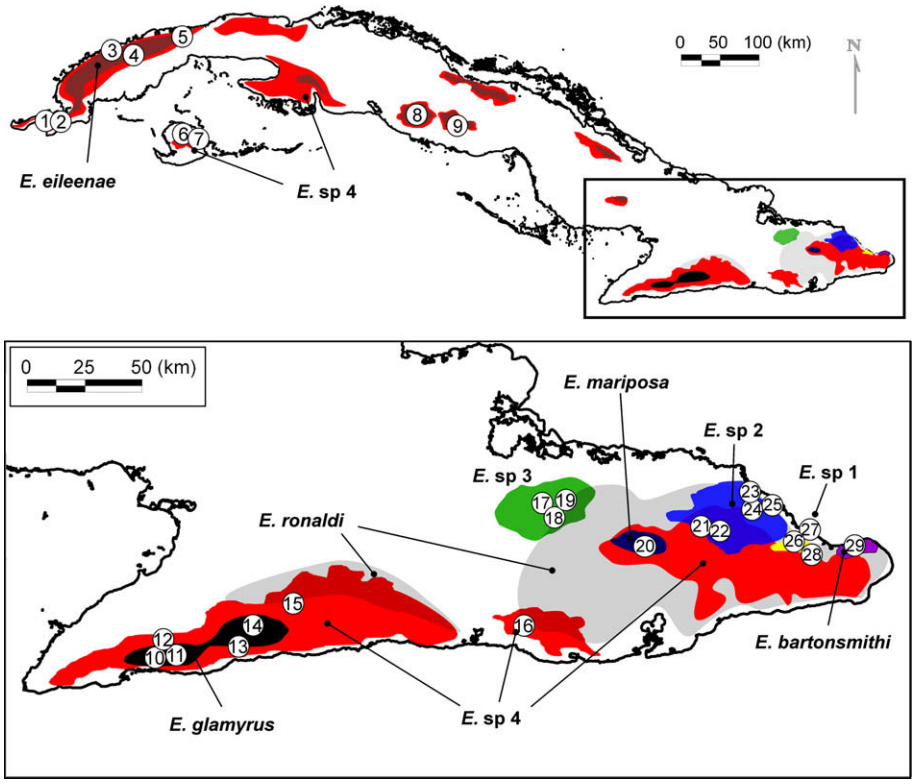

Fig. 1. Map of Cuba showing the approximate distribution of species of the *Eleutherodactylus auriculatus* species group as well as outgroup species, and localities sampled: (1) La Bajada, (2) 7 km East of La Jaula, (3) Cajálbana, (4) San Diego de los Baños, (5) Las Terrazas, (6) La Jía, (7) La Isabel, (8) Topes de Collantes, (9) Caja de Agua, (10) Pico Cuba, (11) Pico Turquino, (12) Aguada del Joaquín, (13) Peladeros, (14) La Nueve, (15) La Pimienta, (16) Gran Piedra, (17) El Palenque, (18) La China, (19) Baconal, (20) La Tagua, (21) Piedra La Vela, (22) Riito, (23) Yamanigüey, (24) Monte Iberia, (25) Bahía de Taco, (26) Yunque de Baracoa (summit), (27) Yunque de Baracoa (base), (28) 8 km South of Baracoa, (29) Yumurí. For detailed information of localities, see Supplementary Table S1.

Table 1
Primers and PCR conditions used in the present study. PCR conditions start with temperature (in °C) of each step, followed by the time in seconds between parentheses.

Gene	Primer name	Sequence (5' → 3')	Source	PCR conditions
12S rRNA	12SAL	AAA CTG GGA TTA GAT ACC CCA CTA T	modified from Kocher et al. (1989)	95(180), [94(60), 50(60), 72(60) x 40], 72(300)
12S rRNA	16SR3	TTT CAT CTT TCC CTT GCG GTA C	Vences et al. (2003)	
16S rRNA-1	16SarL	CGC CTG TTT ATC AAA AAC AT	Palumbi et al. (1991)	94(90), [94(45), 55(45), 72(90) x 33], 72(300)
16S rRNA-1	16SbrH	CCG GTC TGA ACT CAG ATC ACG T	Palumbi et al. (1991)	
16S rRNA-2	16SL3	AGC AAA GAH YWW ACC TCG TAC CTT TTG CAT	Vences et al. (2003)	95(180), [94(60), 50(60), 72(60) x 40], 72(300)
16S rRNA-2	16SAH	ATG TTT TTG ATA AAC AGG CG	Vences et al. (2003)	
cob	CBJ10933	TAT GTT CTA CCA TGA GGA CAA ATA TC	Bossuyt and Milinkovich (2000)	94(90), [94(30), 53(45), 72(90) x 35], 72(600)
cob	Cytb-c	CTA CTG GTT GTC CTC CGA TTC ATG T	Bossuyt and Milinkovich (2000)	
cox1	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	universal barcoding primers	94(90), [94(45), 50(45), 72(90) x 35], 72(300)
cox1	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	universal barcoding primers	
Rag-1	Rag1-FF2	ATG CAT CRA AAA TTC ARC AAT	Heinicke et al. (2007)	94(90), [94(45), 50(45), 72(90) x 35], 72(300)
Rag-1	Rag1-FR2	CCY CCT TTR TTG ATA KGG WCA TA	Heinicke et al. (2007)	

ducted PCR reactions targeting the gene regions to be sequenced with appropriated primers (Table 1). We performed a first screening using a partial sequence of ca. 545 bp of the 16S rRNA gene which has proven its DNA barcoding efficiency in amphibians (Fouquet et al., 2007; Vences and Wake, 2007). Based on this initial assessment of genetic variation, we selected representatives of the major clades identified (based on node support and amount of genetic divergences, see below), and for these sequenced multiple gene fragments that included partial sequences of the mitochondrial genes 12S rRNA (ca. 728 bp), 16S rRNA (the above mentioned plus another section of ca. 652 bp), Cytochrome oxidase, subunit I (cox1, ca. 675 bp), and Cytochrome b (cob; ca. 573 bp), and the nuclear recombination-activating gene-1 (Rag-1; ca. 558 bp). For primers and cycling conditions, see Table 1. PCR reactions were performed in 25 μl volume and negative and positive controls were used in each PCR reaction. Results were loaded on 1% agarose gels, stained with SybrSafe© and subjected to electrophoresis immersed in Tris–acetate–EDTA buffer (TAE). Bands of DNA were visualized on a "Gel-Doc" system (Bio-Rad©). PCR products were purified with QIAquick spin columns (Qiagen©) and sequenced using ABI (Applied Biosystems) BigDye Terminator 3.1 sequencing kit on an ABI Prism 3100 Genetic Analyzer.

We used the program BioEdit to inspect chromatograms and to edit sequences. ClustalX was employed to align sequences of non-coding genes using default parameters; the resulting alignment was later inspected by eye. Alignment of protein-coding sequences was done by eye and bases were transformed into amino acids to verify alignment and to avoid the possibility of including nuclear pseudocopies of the mitochondrial genes. Heterozygous bases in the nuclear gene Rag-1 (although uncommon) and overlapping traces in chromatograms were coded with the IUPAC symbols. All newly determined sequences were submitted to Genbank (accession numbers FJ527312-416, GQ357651-65, and GQ426489-533; for a complete list of accession numbers and voucher specimens, see Supplementary Table S1).

Lab work was conducted in the molecular laboratories of the Museo Nacional de Ciencias Naturales (Madrid, Spain), the Royal Belgium Museum of Natural History (Brussels, Belgium) and the Zoological Institute of the University of Braunschweig (Braunschweig, Germany).

2.3. Sequence data and phylogenetic analyses

In our first screening of 16S rRNA sequences of multiple specimens per population, we applied a neighbor-joining algorithm as implemented in MEGA 4.0 software (Tamura et al., 2007), with node support assessed via non-parametric bootstrap (2000 pseudoreplicates). Homologous sequence data from six species of the *E. auriculatus* group available in Genbank (accession numbers EF493344, EF493573-7, details in Supplementary Table S1) were added to the dataset for consistency. Our sampling followed Vences et al. (2005a) and Vences and Wake (2007) in considering phylogeny, genetic distances, and other characters such as the advertisement calls of specimens in the respective populations (Alonso et al., 2007; Rodríguez, 2002). We followed Vences and Wake (2007) and Vieites et al. (2009) and considered as candidate species those geographic populations sharing similar advertisement calls and diagnosed by a clade of mitochondrial haplotypes differing substantially from haplotypes of other populations (using a conservative threshold of 5% uncorrected 16S *p*-distance).

For phylogenetic reconstructions we assembled a combined dataset including sequences of the 16S, 12S, cob, cox1, and Rag-1 genes for one representative per species (including candidate species as revealed by the previous analysis) plus seven terminals representing different populations of the species with the widest distribution range. We assembled three separate data matrices, including the nuclear, mitochondrial and combined datasets. DNA sequence data were analyzed with the software ModelTest 3.7 (Posada and Crandall, 1998) to determine the substitution model, for each gene and the combined dataset. Maximum likelihood (ML) searches were conducted in PAUP* 4.0b (Swofford, 1998) using 1000 random addition replicates and TBR branch swapping, *E. eileenae* was designated the outgroup based on phylogenetic results of Hedges et al. (2008). Confidence in the resulting topology was assessed with non-parametric bootstrap (Felsenstein, 1985) with 1000 bootstrap replicates. Bayesian phylogenetic analyses were conducted with MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) and data from all sites of non-coding genes and each codon position of coding genes were treated as different partitions, with model specifications selected by the Akaike Information Criterion (AIC) in ModelTest 3.7 (Supplementary Materials, Table S2). Two Markov chain analyses were run for 10,000,000 generations and sampled every 100 generations. A majority consensus tree was obtained after discarding the first 50,000 trees as "burn-in" based on visual inspection of the log-likelihood values.

Divergence-time analyses were conducted with the Bayesian software MultiDivTime (Thorne and Kishino, 2002), which implements a relaxed molecular clock method. We assembled a dataset containing newly obtained sequences of the five genes (16S, 12S, cob, cox1, and Rag-1) for each of the Cuban species (including newly identified candidate species). Based on results of Hedges et al. (2008), we used as outgroup Hispaniolan species from the *varians* and *minutus* groups. This included three species for which homologous sequences of the 12S, 16S and Rag-1 genes were available in Genbank: *E. abboti* (12–16S: EF493540, Rag-1: EF493412), *E. lamprotes* (EF493379, EU186759) and *E. leberi* (EF493342, EF493403). Throughout the analyses we used a

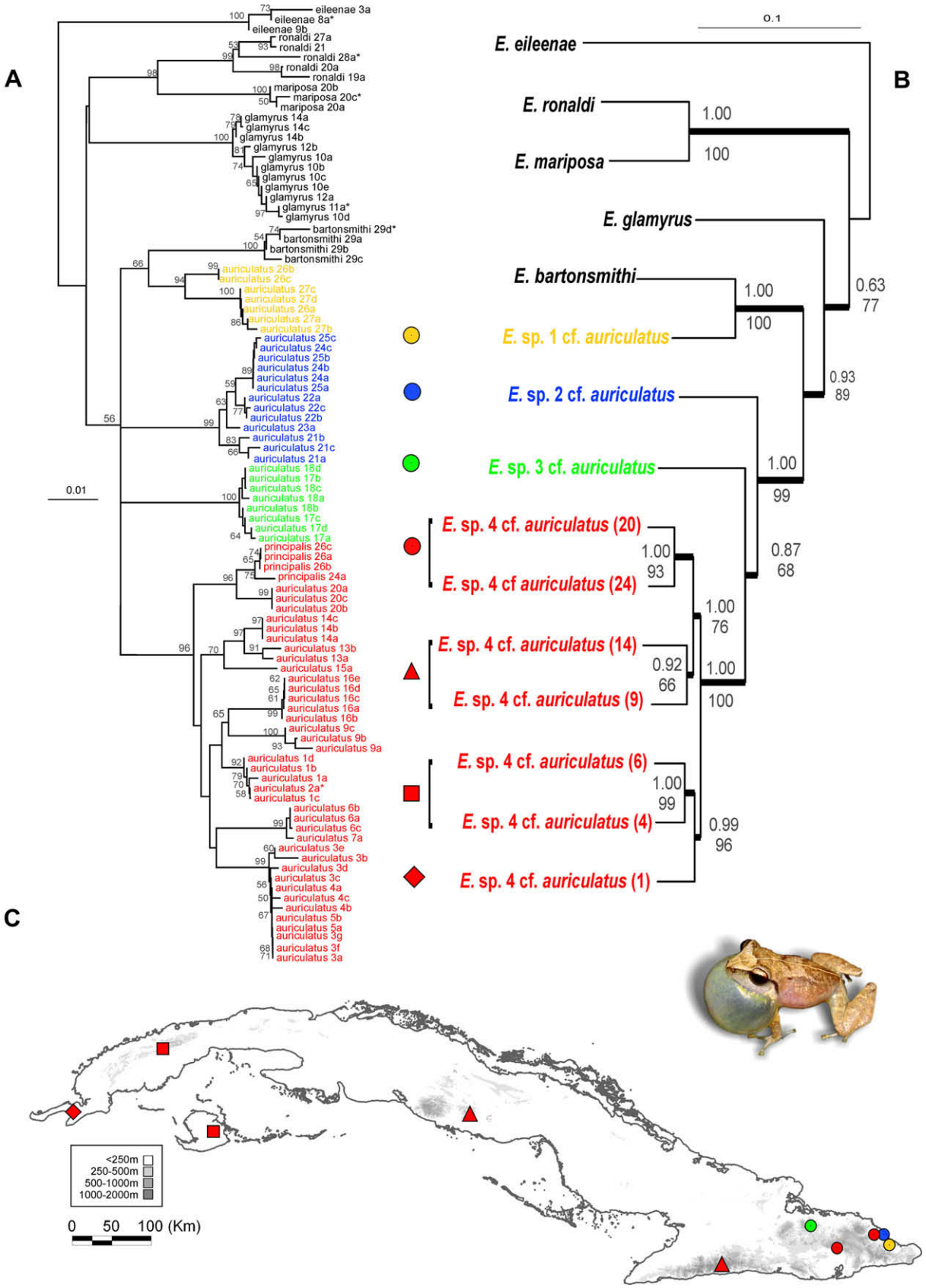

Fig. 2. Molecular phylogeny of the Cuban *E. auriculatus* species group. (A) Neighbor-joining phylogram based on uncorrected "*p*" distances among 95 partial sequences of 545 bases of the 16S rRNA gene, sample codes as in Supplementary Table S1, bootstrap support values >50 are indicated at each node. (B) Phylogeny based on a Bayesian analysis of a 3731 bp dataset of mitochondrial (12S, 16S, cob, cox) and nuclear (Rag-1) genes; Bayesian posterior probabilities (top) and Maximum likelihood bootstrap support values (bottom) are shown in each node. (C) Topographic map of Cuba showing geographic distribution of newly-discovered taxa using same colors as (A) and (B) (for *Eleutherodactylus* sp. 4, different symbols represent major clades). Refer to Supplementary Table S1 for detailed information of localities. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

topology that combines the Bayesian consensus topology obtained here for the Cuban clades rooted with the outgroups according to Hedges et al. (2008).

Data from each gene were analyzed using the BaseML program to determine the appropriate nucleotide substitution parameters under the F84 model. Subsequently the program Estbranches was used to estimate branch lengths and the variance–covariance structure of these parameter estimates for all genes. Finally we run MultiDivTime incorporating the information of the model parameters and the branch lengths of each gene. The mean time for ingroup root (rttm) was set to 16 Mya, with 14–17 Mya as lower and upper constraints, respectively, assuming that the divergence between Cuban and Hispaniolan frogs might have been associated with the split of the two Paleoislands that occurred ca. 16 Mya ago (Iturralde-Vinent, 2006; Iturralde-Vinent and MacPhee, 1999). These age estimates are in good agreement with the 12.6 ± 2.6 Mya of divergence between *E. auriculatus* and *E. abboti* estimated by Heinicke et al. (2007) using DNA sequences. Other priors were established as follows: standard deviation for ingroup root (rttmsd), 6 Mya; mean of rate for ingroup root (rtrate), 0.02; standard deviation of rate for ingroup root (rtratesd), 0.02; mean of variance in logarithm of the rate (brownmean), 0.4; standard deviation of variance in logarithm of the rate (brownsd), 0.4; time that is greater than that of any node in the tree (bigtime), 50 Mya. The rate at ingroup root was calculated by dividing the average branch lengths from root to tip (as obtained from Estbranches) by the value of rttm following recommendations of the authors. We sampled the Markov Chain every 100 generations for a total of 100,000 samples after a burn-in of 100,000. To check for consistency of results, two runs of MultiDivTime were performed using different starting seeds, and the results did not differ significantly.

3. Results

The final alignment of 16S sequences consisted of 545 characters from 95 individuals of the *auriculatus* species group. After removal of ambiguous sites or gaps there were 93 variable sites containing 114 substitutions of which 81 were parsimony informative, defining 43 haplotypes.

The inspection of the neighbor-joining (NJ) tree of uncorrected "*p*" distances obtained from the 16S dataset showed little evidence of gene flow between localities, and haplotypes from each locality generally clustered together. Clusters of haplotypes from nearby localities connected by favorable habitat provided the few exceptions (Fig. 2).

Within *auriculatus* four haplotype clades, each supported by >90% bootstrap values and differing from each other by >4.4% sequence divergence (Fig. 2A), diagnose geographic groupings of populations likely to represent species lineages (here named *Eleutherodactylus* sp. 1–4). Taking into account their distinct advertisement calls (Alonso et al., 2007) we consider these clades as candidate species, likely to represent undescribed species. Samples representing *E. principalis* from two localities in the Cuchillas del Toa mountains clustered with those of *Eleutherodactylus* sp. 4 from La Tagua. This latter candidate species appears to be distributed in most of the Cuban territory, and geographical subdivisions were evident with various levels of support (Fig. 2A). *Eleutherodactylus* sp. 3 is known only from rain forests in the Sierra de Cristal mountains, Holguín Province in the East and *E.* sp. 2 is distributed only in the Cuchillas del Toa mountains, in Holguín and Guantánamo Provinces, in densely forested areas. The most restricted form is *Eleutherodactylus* sp. 1 known only from a single mountain: El Yunque de Baracoa in Guantánamo Province at the extreme East of Cuba.

Phylogenetic reconstructions using the combined dataset (3731 bp) showed strong support for most of the clades recovered. A sister-taxon relationship was evident between *E. mariposa* and *E. ronaldi*. The clade with these two species forms the sister taxon to a clade comprising *E. glamyrus*, *E. bartonsmithi* and the four forms of *auriculatus* (*E.* sp. 1–4). Within this second clade, the first split separates *E. glamyrus* from a clade comprising the remaining species; *Eleutherodactylus* sp. 1 and *E. bartonsmithi* form the sister taxon to a clade comprising *Eleutherodactylus* sp. 2, *E.* sp. 3 and *E.* sp. 4. Within the widespread form *E.* sp. 4 a split between Western and Eastern-Central populations was strongly supported (Fig. 2B).

A separate analysis including only 3173 bp of the mitochondrial genome (not shown) differed from the above-mentioned pattern in few aspects: *E. glamyrus* clustered with *E. ronaldi* + *E. mariposa*, albeit with low support, and *Eleutherodactylus* sp. 2 and *E.* sp. 3 switched positions. The nuclear dataset (558 bp of Rag-1) showed little resolution when analyzed separately and had many polytomies in all analyses (not shown).

Divergence times estimated using a relaxed molecular clock method suggest that *Eleutherodactylus eileenae* diverged from all other members of the *E. auriculatus* group 11.5 ± 1.3 Mya, followed by a split leading to the origin of two clades (*E. mariposa* + *E. ronaldi*, and a clade comprising *E. glamyrus*, *E. bartonsmithi* and the various candidate species previously assigned to *E. auriculatus*) at 7.6 ± 1.12 Mya. Four other diversification events occurred between 3.8 and 7.1 Mya leading to the origin of the remaining clades. The diversification within populations of the widespread candidate species *Eleutherodactylus* sp. 4 started 2.1 ± 0.5 Mya with the separation of an eastern-central lineage from a western lineage; subsequently, a divergence at 1.88 ± 0.45 Mya separated populations from the Sierra Maestra and Guamuhaya from those from Nipe-Sagua-Baracoa Mountains and almost simultaneously, at 1.81 ± 0.46 Mya populations from the Isle of Youth and San Diego de los Baños diverged in Western Cuba (Fig. 3). Means, standard deviations and confidence intervals of the divergence times of each node (see Supplementary Fig. 1) are reported in Supplementary Table S3.

4. Discussion

The four major clades of frog populations previously assigned to *E. auriculatus* showed divergence levels of 4.4–5.8% from each other, which indicates a high degree of cryptic diversity. These forms also distinctly differ in advertisement call dominant frequency, call rate, and partly in general call structure (Rodríguez, unpubublished data)—some of the key features in species recognition during anuran courtships (Angulo and Reichle, 2008; Gerhardt, 1994; Schneider and Sinsch, 2007; Wells, 2007)—and therefore make it likely that all four clades represent distinct species. Within at least one of these, *Eleutherodactylus* sp. 4, there is a relevant genetic differentiation between populations as well (up to a maximum of 3.8% pairwise 16S divergences) but because this differentiation is not accompanied by relevant differences in call and morphology, it probably does not indicate the presence of additional species. Studies integrating alternative sources of information such as DNA sequences, bioacoustic and morphological data are needed to delimitate species in this group.

Due to its straightforward amplification and strong phylogenetic signal, the 16S rRNA gene has been proposed to complement the standard barcoding marker cox1 for amphibians. A preliminary threshold level of 5% uncorrected pairwise genetic divergence has been proposed to identify amphibian candidate species (Vences et al., 2005b; Vences and Wake, 2007) but assessments of Neotropical and Madagascar amphibian diversity using the 16S rRNA gene suggested that values over 3% genetic divergence may be consid-

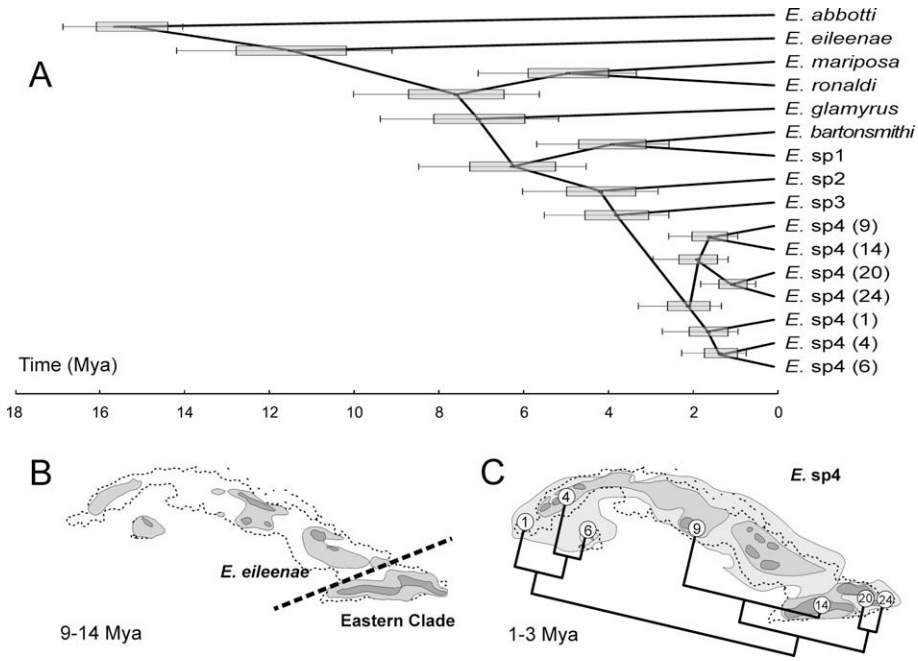

Fig. 3. Divergence times of lineages in the *E. auriculatus* species group and paleogeographic scenarios for the colonization of Western and Central Cuba. (A) Time tree obtained by a relaxed molecular clock method using sequences of the 12S, 16S and Rag-1 genes, horizontal gray boxes represent standard deviation and whiskers 95% confidence intervals. Numbers on the right indicate localities of *Eleutherodactylus* sp. 4 as illustrated in Fig. 1. (B and C) Reconstructions of island geography adapted from published paleomaps (Iturralde-Vinent, 2003; Iturralde-Vinent and MacPhee, 1999) depicting the colonization of Western and Central Cuba by *E. eileenae* and *Eleutherodactylus* sp. 4. Elevations represented in tones of grey as: Highlands, dark grey; lowlands, medium grey; and periodically flooded lowlands, light grey.

ered a reliable yardstick for the identification of undescribed species (Fouquet et al., 2007; Vieites et al., 2009; but see Padial et al. (2009a)).

The taxonomic revision of this complex of frogs is complicated by the apparent lack of clear morphological differences between the various species, which makes it difficult to decide, based on the original description by Cope (1862), which of the four candidate species is the true *E. auriculatus,* and which are the three new species. It is beyond the scope of the present paper to discuss this issue in detail, but it also needs to be taken into consideration that, apparently, *E. principalis* is nested within *E.* sp. 4, and the name *principalis* thus needs to be considered either as a synonym (if *E.* sp. 4 turns out to be the true *E. auriculatus*), or as valid name for *E.* sp. 4 (if one of the species *E.* sp. 1–3 results to be the true *auriculatus*). Furthermore, the facts that the type material of *auriculatus* is probably lost (Schwartz, 1960, Hedges, pers. comm.) and that the type locality published on the original description is only "Eastern Cuba" require designation of a neotype for *E. auriculatus*.

Special attention needs to be paid to identify new morphological diagnostic features, because in the *E. auriculatus* group, only *E. bartonsmithi*, *E. eileenae*, *E. mariposa* and *E. ronaldi* can be readily identified using morphological characters in their respective diagnoses (Dunn, 1926; Hedges et al., 1992; Schwartz, 1960). For the remaining five species, calling males can readily be identified by bioacoustics but the allocation of preserved specimens, juveniles, females or silent males is nearly impossible and at present only achievable by molecular methods. In calling anurans most of the communication takes place without visual contact between sexes, a reason that explains the higher level of variation, and hence the higher systematic value of bioacoustic features relative to morphology (Schneider and Sinsch, 2007). Thus, careful morphological studies are needed to find any diagnostic features that might have been overlooked in previous taxonomical analyses of the *E. auriculatus* group.

The addition of new sequences representing the four cryptic species and the inclusion of *E. principalis* in our data set now provide a deeper understanding of the relationships within the *auriculatus* species group. Compared with the results of Hedges et al. (2008), our tree (Fig. 2) shows a similar placement of *E. mariposa* and *E. ronaldi* as sister taxa, but *E. glamyrus* is now the sister taxon to the rest of the *auriculatus*-like species, and a sister-taxon relationship was recovered between *E. bartonsmithi* and *E.* sp. 1, which in turn are the sister taxon to a clade comprising the other three cryptic forms (*E.* sp. 2–4). These differences are likely to be the result of a much better character and taxon sampling in our study, an issue that is considered a major source of error in phylogenetic analyses (Sites and Marshall, 2003). More complete sampling often results in an improvement in our understanding of evolutionary relationships between groups of species (Padial et al., 2009b) or provides new and more detailed insight into the phylogeography of a given species (Gifford and Larson, 2008).

The topology of our tree (Fig. 2) assigns an important role to the mountains of eastern Cuba for the diversification pattern of the *E. auriculatus* group. This area harbors most of the species of the group, and most cladogenetic events have occurred between species endemic to this region and restricted to small mountain ranges. It is likely that mountains of Eastern Cuba, the largest mountain range in the country, promoted the diversification not only by tectonic uplifts but also acting as elevational refugia in periods of unsuitable climate when ecological niches got reduced geographically. Diversification in mountain refugia, either allopatric by populations remaining isolated on different mountains, or parapatric along elevational ecotones, has been proposed as the prevalent mechanism for other groups of frogs (e.g., Guarnizo et al., 2009; Wollenberg et al., 2008). Within the Antillean context, there is compelling evidence that during the Pleistocene, the alternate exposure and submergence of land, and the correlated alternation of xeric and mesic environments, would have resulted in repeated events of faunal isolation, speciation, and extinction such

that relict distributions would be superimposed one on another as a mosaic through time (Curtis et al., 2001; Pregill and Olson, 1981). Also, during this period dry forests acted as a barrier to gene flow between populations of Central American wet-forest frogs of the genus *Craugastor* (Crawford et al., 2007).

Only two species (*Eleutherodactylus eileenae* and *E.* sp. 4) have been able to colonize West and Central Cuba and the Isla de la Juventud in the latter case. Our molecular dating suggests that these two colonization events were separated by a period of 5–10 Mya (Fig. 3). Both overwater dispersal and vicariance can be invoked to explain the origin of *E. eileenae* (9–14 Mya) in Central Cuba from the Eastern ancestor of the group. Published paleogeographic reconstructions of this period depict a deep water channel (the Cauto-Nipe channel) between Eastern and Central Cuba (Fig. 3), but all water gaps might have been transitory allowing a repeated dispersal of faunal elements over land, and subsequent vicariance as the result of the reappearing water barrier (Iturralde-Vinent and MacPhee, 1999). Partial submergence of the Cuban island during the Miocene is also supported by paleontological findings (MacPhee et al., 2003) and has been proposed as a factor that may have facilitated the speciation of Cuban anoles (Glor et al., 2004).

More recently *Eleutherodactylus* sp. 4 and *E.* sp. 3 could have originated from an Eastern ancestor 2.6–5.5 Mya, and subsequently *E.* sp. 4 expanded its range and colonized Western Cuba. Also a peripatric speciation can be invoked to explain the origin of *E.* sp. 3 whose range is smaller and does not overlap with that of *E.* sp. 4. Alternatively, it remains possible that *E.* sp. 4 originated in the West and that the origin of *E.* sp. 4 and *E.* sp. 3 was caused by a differentiation event along an East–West axis rather than a split in Eastern Cuba, but this scenario implies a contraction of the range of *E.* sp. 3 to its present distribution. This seems less plausible than the first two hypotheses. More work is needed to test these or any other alternative hypothesis, bearing in mind that post-speciation range shifts can obscure patterns of speciation among closely related taxa (Losos and Glor, 2003). The population structuring of *E.* sp. 4 started later (1–3 Mya) with the split of Western populations from the Eastern and Central ones. A terrestrial range expansion model is favored by the putative existence of land connections between all Cuban paleoislands that should have occurred between 6–3 Mya (Iturralde-Vinent, 2003). However, the phylogenetic relationships between populations of *E.* sp. 4 (Figs. 2 and 3) disagree with this model of diversification because the Western and Central populations are not each other's closest relatives. Instead, we suggest that overwater dispersal from Eastern Cuba could explain the divergence of the Western populations from the Eastern-Central lineage. This later dispersal scenario that assumes pre-Pleistocene divergence and stochastic overwater dispersal events resembles the scenario proposed for the *Anolis carolinensis* group (Glor et al., 2005). At present, the data on the paleogeography of Cuba during this time period are too scarce to reach a definite conclusion on this subject. More detailed phylogeographic studies of the two widely distributed species (*E. eileenae* and *E.* sp. 4) are needed to provide additional information concerning the processes that may explain the population structuring of these taxa throughout Western and Central Cuba.

Cryptic species pose a challenge for the understanding, conservation, and sustainable use of biological diversity (Bickford et al., 2006). Through the application of molecular techniques, combined with a thorough sampling, our study uncovered the existence of several cryptic taxa in the *E. auriculatus* species group, which improved our understanding of its evolutionary history and biogeographical origin. Each of the newly-discovered cryptic taxa have much smaller distribution ranges than *E. auriculatus*; based on this knowledge new conservation priorities should be established, enforcing the preservation of forest habitats in the Eastern Mountains of Cuba. It seems plausible that also other amphibian species with a wide distribution range on Cuba contain many more cryptic species, an issue that will be the subject of future research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.08.023.

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