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Expansion of the known distribution and molecular systematics of *Eleutherodactylus adelus* (Anura: Eleutherodactylidae)

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Eleutherodactylus frogs constitute the highest proportion of the Cuban amphibian fauna, accounting for 52 of the 62 described species, and exhibit high levels of endemism (98%) and restricted distributions (Díaz & Cádiz 2008). The high species diversity of *Eleutherodactylus* and related genera has been hypothesized to be derived from their particular reproductive mode, with terrestrial clutches and direct development, which might promote faster speciation rates than observed in water-breeding anurans (Dubois 2004, reviewed in Vences & Wake 2007), or be influenced by a high karyological variability favouring chromosome speciation (Bogart & Hedges 1995).

One of the last discoveries in this group is *Eleutherodactylus adelus* Díaz, Cádiz & Hedges, 2003, a species described from a single locality in western Cuba (Loma del Espejo, Alturas de Pizarras del Sur, Sabanas Llanas, Pinar del Río Province). In their original description, on the basis of morphological and acoustic features, the authors hypothesized a close relationship between *E. adelus* and the widespread *E. varleyi* (Díaz et al. 2003).

More recently, Hedges et al. (2008) included *E. adelus* together with *E. varleyi* in the *E. gundlachi* species group without providing any further morphological, acoustic or molecular evidence. Other than the type series, the only additional sighting of this species to date is that of Alonso et al. (2007) who photographed and recorded a male at the type locality in July of 2006. Herein we report a new locality for the species, provide evidence of its phylogenetic position within the genus based on molecular data, and propose a hypothesis on its extent of occurrence.

Specimens of *Eleutherodactylus adelus* were collected during the day in the leaf litter of a forest. They were photographed and later euthanized by immersion in a chlorobuthanol solution, preserved in 70% ethanol, and deposited in the Zoological Collection of the Institute of Ecology and Systematics (CZACC) in Havana, Cuba. Fragments of thigh muscle were removed and preserved in 90% ethanol. Total genomic DNA was extracted using proteinase K (10mg/ml) digestion followed by a standard salt-extraction

protocol (Bruford et al. 1992). Standard polymerase chain reactions were performed in a final volume of 10 µl and using 0.3 µl each of 10 µM primer, 0.25 µl of total dNTP 10 mM, 0.08 µl of 5 u/µl GoTaq and 2.5 µl 5x GoTaq Reaction Buffer. PCR conditions comprised initial denaturation at 94°C (90 sec) and then 36 cycles of denaturation at 94°C (45 sec), primer annealing at 52°C (45 sec) and elongation at 72°C (90 sec), followed by a final extension step at 72°C (5 min). We PCR-amplified a stretch of the mitochondrial 12S rRNA and 16S rRNA genes, including the intervening tRNAVal gene, using the primers 12SAL (AAA CTG GGA TTA GAT ACC CCA CTA T) and 16SBH (CCG GTC TGA ACT CAG ATC ACG T) and sequenced three fragments using primers 12SAL, 16SL3 (ATG TTT TTG ATA AAC AGG CG), and 16SAL (CGC CTG TTT ATC AAA AAC AT) (primers from (Palumbi et al. 1991, Vences et al. 2003).

The successfully amplified products were purified using Exonuclease I and Shrimp Alkaline Phosphatase (SAP) or Antarctic Phosphatase (AP) according to the manufacturer's instructions (NEB). Purified PCR templates were sequenced on both strands using dye-labelled didesoxy terminator cycle sequencing on an ABI 3130 automated DNA sequencer. Chromatograms were checked and sequences were corrected by hand, where necessary, using Codon-Code Aligner (v. 3.5.6, Codon Code Coorporation). The newly identified sequences were submitted to GenBank (accession numbers: JF947364–JF947374).

We downloaded from GenBank partial 12S and 16S DNA sequences from the work of Hedges et al. (2008). Since BLAST searches of the newly identified sequences clearly indicated sequence similarities only with Cuban *Eleutherodactylus* species of the *E. planirostris* series, we selected a subset containing all available sequences from this species series (24 individuals, 21 species) plus representatives of all other Cuban *Eleutherodactylus* clades closely related to this series that have been sequenced so far (29 species of the *E. armstrongi, E. luteolus, E. ricordii, E. greyi, E. dimidiatus,* and *E. zugi* series). We used Muscle 3.6 (Edgar

2004) to align our new sequences against this data set. Ambiguously aligned sections were removed from the dataset using the software Gblocks 0.91b (Castresana 2000); the final alignment included 1107 characters.

We conducted maximum likelihood (ML) and Bayesian inference (BI) searches for phylogeny reconstruction. For BI, models of sequence evolution were determined by the Akaike Information Criterion (AIC) in MrModeltest (Nylander 2008). Searches were run in MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003) under default conditions (one cold and three heated chains); posterior probabilities were obtained from the 50% majority rule consensus tree. Analysis ran for 5 million generations, and every 1000th tree was sampled and 50% of them were discarded as a conservative burn-in after empirically assessing that

chains had converged and log-likelihood values had stabilized. ML analyses were carried out in Garli 0.95 (Zwickl 2006). We used default search parameters and implemented a GTR+I+G model of nucleotide evolution for the whole data matrix. Two independent searches were performed to ensure convergence. Node support was assessed by 100 bootstrap replicates.

To produce a hypothesis on the extent of the suitable habitat of the species, we used orthorectified Landsat-MSS images from the Global Land Cover Facility (available from http://www.glcf.umd.edu/data/landsat). According to the original description and our field experience, this species is a pine forest specialist, and hence we used satellite imagery and our GPS data (projected into WGS-84 UTM 17N coordinates) to identify the area covered by pine
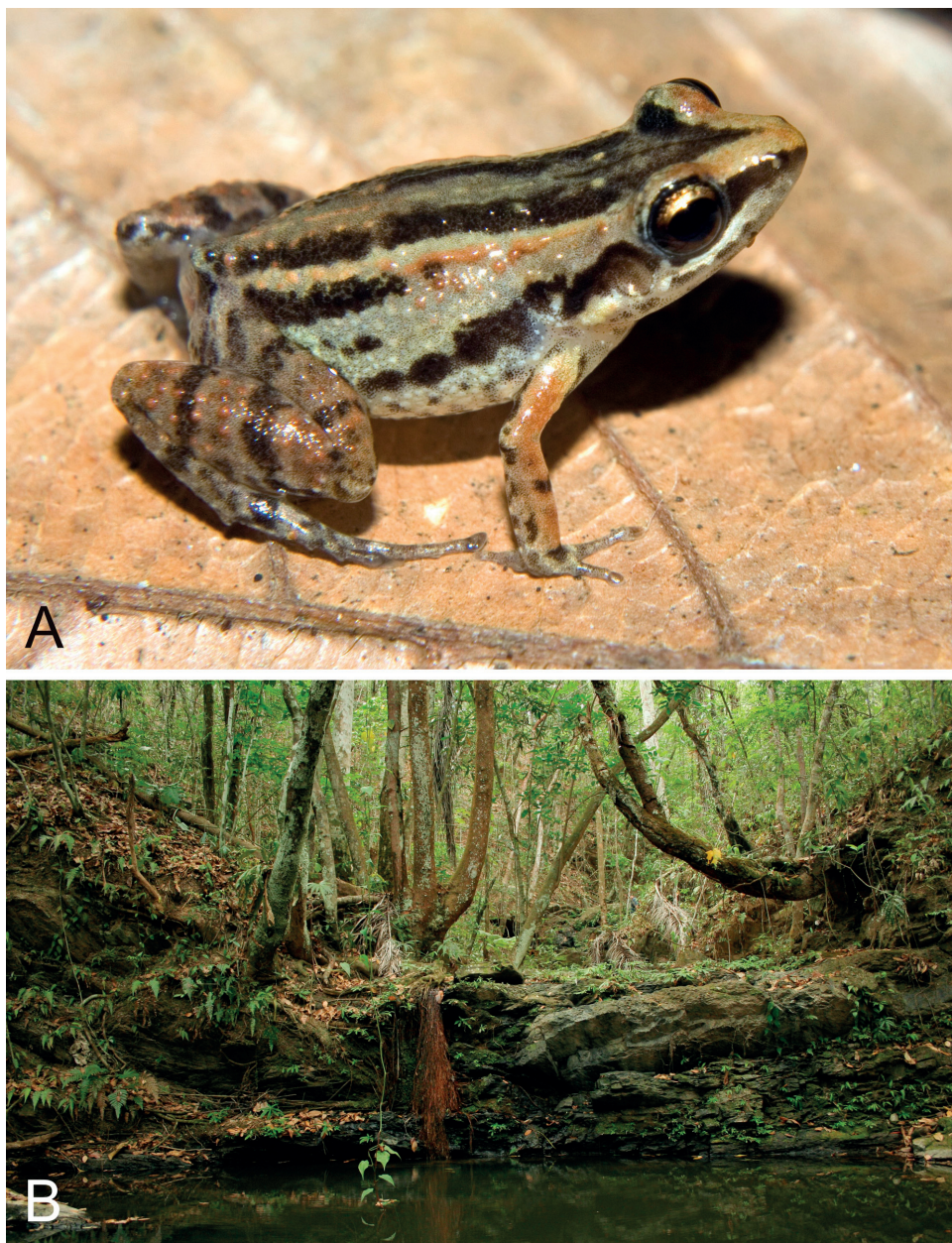

Figure 1. (A) Adult female *Eleutherodactylus adelus* (CZACC 14.3224) collected at Arroyo la Llavita, El Moncada, Pinar del Río Province, western Cuba. (B) Habitat of *E. adelus* at Arroyo La Llavita. Photographs by A. Rodríguez.
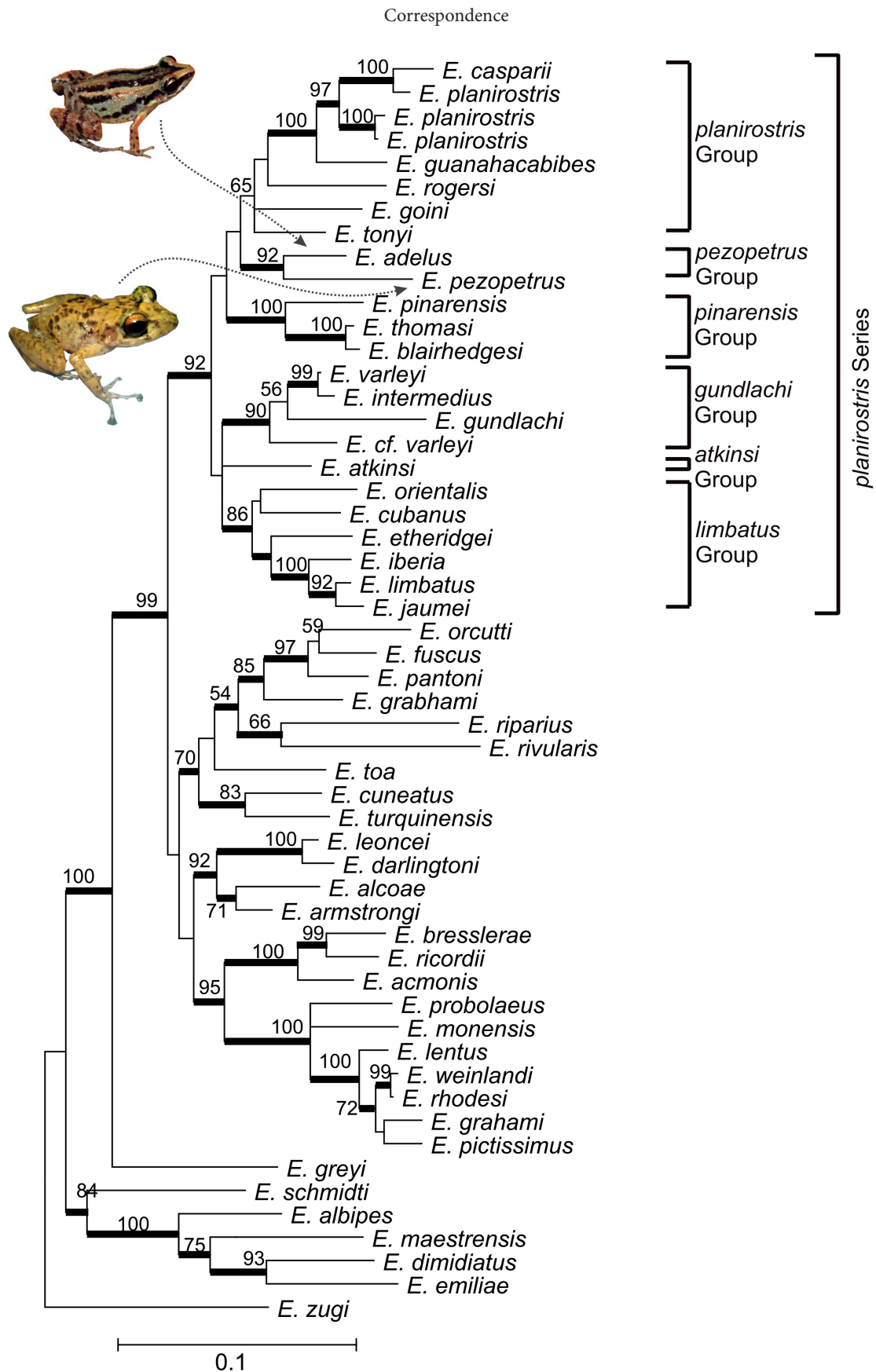
Figure 2. Phylogenetic position of *Eleutherodactylus adelus* within the *E. planirostris* species series of the family Eleutherodactylidae, as revealed by a Bayesian Inference tree obtained from 1107 base pairs of aligned mitochondrial DNA sequences of the 12S rRNA and 16S rRNA genes, including the intervening transfer RNA Valine. Nodes supported by a Bayesian posterior probability > 0.9 are represented by thick branches, numbers indicate ML bootstrap support values > 50%. Species groups within the *planirostris* species series (sensu Hedges et al. 2008) are indicated on the right. Inset pictures show *E. adelus* and its sister species *E. pezopetrus*.

forests in the region. All spatial data analyses were done in ArcGis 9.2 (ESRI, Redlands, CA).

The two newly collected specimens of *Eleutherodactylus adelus* (Fig. 1a) were found on 21 October 2010, at a locality 22.0 km east of the type locality while conducting an intensive leaf litter search during the late afternoon. The new locality is Arroyo La Llavita (83.834849° W, 22.538583° N, 272 m above sea level), El Moncada, Viñales, Pinar del Río Province, Cuba. The vegetation was dominated by pine forests with gallery forest covering the springs. The specimens were collected in the shallow leaf litter that covered a steep bank of a rivulet (Fig. 1b).

Morphologically, the specimens agreed with the original description of *E. adelus* in its distinct and probably invariant colour pattern and the presence of clearly visible continuous paravertebral folds, which are absent in the morphologically similar *E. varleyi* (see Díaz et al. 2003).

A comparison of DNA sequences of a short 12S rRNA fragment from both of the newly collected *E. adelus* specimens (CZACC 14.13224–25; field numbers: AR-1078–1079) showed high genetic divergences from all other Cuban *Eleutherodactylus* and did not indicate close affinities to *E. varleyi*, thus supporting the distinctiveness of this species. Although we did not find the species in syntopy with *E. varleyi*, we collected specimens of the latter species nearby, i.e., in the surroundings of Cueva del Indio, San Vicente, Viñales, a locality 19.5 km to the northeast. A preliminary phylogenetic analysis of the 12S rRNA sequences of three of these individuals (CZACC 14.13226–13228; field numbers AR-1045, 1080, 1081) placed them close to an *E. varleyi*

sequence from GenBank (EF493345) and thus confirmed their identification and distinctiveness from *E. adelus*.

Final phylogenetic analyses were carried out with a single *E. adelus* specimen (CZACC 14.13224) based on DNA sequences of the 12S rRNA and 16S rRNA genes. The results (Fig. 2) confirmed that *E. adelus* is a member of the *E. planirostris* series to which also *E. varleyi* belongs, but also indicated that it is not a close relative of *E. varleyi*. Instead, with a Bayesian posterior probability of 1.0 and a bootstrap support of 92%, it was placed sister to *E. pezopetrus*, a species that is restricted to eastern Cuba and the only member of the *E. pezopetrus* group (HEDGES et al. 2008). This suggests that *E. adelus* should be transferred from its previous placement in the *E. gundlachi* group to the *E. pezopetrus* group, which so far used to be monotypic. This result is remarkable given the very different morphologic and ecological features of these species: *E. adelus* is a leaf litter inhabitant of 11.44 ± 0.4 mm SVL with a striped colour pattern (Díaz et al. 2003), while *E. pezopetrus* is a cave-dwelling species of up to 50 mm SVL with a blotched colour pattern (Díaz & Cádiz 2008) (Fig. 2).

Groups above the species level in *Eleutherodactylus* and other Terrarana frogs were constructed by HEDGES et al. (2008) on the basis of their phylogenetic relationships obtained from an extensive but still incomplete sampling of known taxa. Therefore, it is to be expected that certain relationship and group membership may need to be changed as more species are incorporated into this phylogeny. This has been evident in previous studies where improved sampling in some clades has resulted in important taxonomic
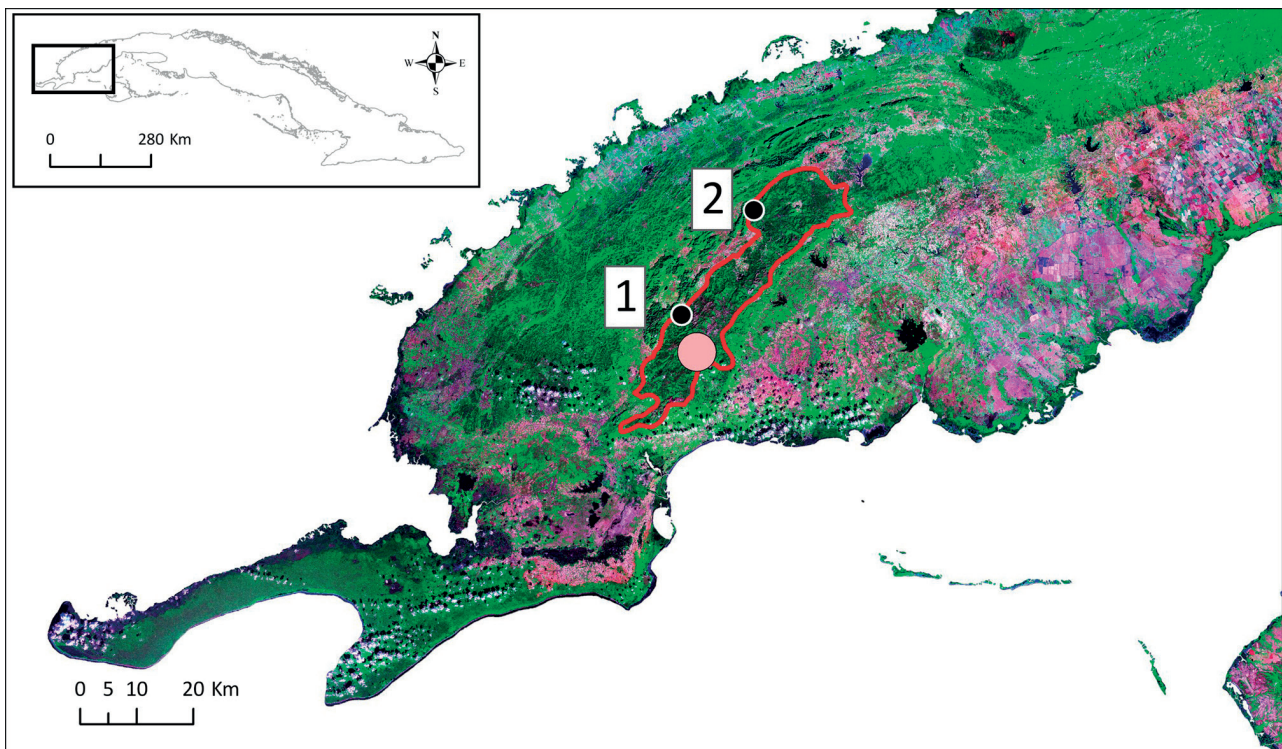

Figure 3. Updated geographic distribution of *Eleutherodactylus adelus*. The inset shows a composite of satellite pictures of western Cuba, illustrating with dots the location of the type locality "Loma del Espejo" (1) and the new locality reported herein, "Arroyo la Llavita" (2). The red line shows the possible extent of occurrence as estimated in this study. The pink circle shows the extent of occurrence of the species estimated during the Global Amphibian Assessment (STUART et al. 2004) and featured in the IUCN redlist.

re-arrangements within Terrarana (HEINICKE et al. 2009, PADIAL et al. 2009).

Until now, the extent of occurrence of *Eleutherodactylus adelus* was estimated to be 33.6 km² around the type locality (IUCN 2011). The senior author visited this locality in July 2006 and recorded its coordinates with a GPS (22.37236° N, 83.94975° W, 165 m a.s.l.), (Fig. 3). In that year, the presence of the species was confirmed through the collection of a single specimen (CZACC 14.13231), which was also photographed and had its call recorded for the sound guide of Cuban amphibians (ALONSO et al. 2007). This GPS position is 8 km to the northwest of the estimation of IUCN (Fig. 3) which was probably biased because the original description of *Eleutherodactylus adelus* did not include geographic coordinates (DÍAZ et al. 2003). The new data confirm that the distribution of this species is wider, as was originally suggested by DÍAZ et al. (2003). Tentatively, we propose as the possible extent of occurrence a polygonal area of 438 km², from the Viñales region in the east to the surroundings of Guane in the west in the hills of Alturas de Pizaras del Sur. The eastern limit was arbitrarily set in the region of Viñales where clearings and roads fragment the pine forests (Fig 3). We are aware that this species is very difficult to detect in the leaf litter and understorey of pine forests, and our knowledge about its geographic distribution is surely far from complete.

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