



DNA barcoding identifies a third invasive species of *Eleutherodactylus* (Anura: Eleutherodactylidae) in Panama City, Panama

ANDREW J. CRAWFORD^{1,2,3}, ROBERTO ALONSO⁴, CÉSAR A. JARAMILLO A.^{2,3,5},
SAMUEL SUCRE⁶ & ROBERTO IBÁÑEZ D.^{2,3,6}

¹Departamento de Ciencias Biológicas, Universidad de los Andes, AA 4976, Bogotá, Colombia. E-mail: andrew@dna.ac

²Círculo Herpetológico de Panamá, Apartado 0824-00122, Panamá, Republic of Panama

³Smithsonian Tropical Research Institute, Apartado 0843-03092, Balboa, Ancón, Republic of Panama.

E-mail: jaramilc@si.edu, ibanezr@si.edu ⁴Museo Felipe Poey, Facultad de Biología, Universidad de La Habana, Cuba. E-mail: ralonso@fbio.uh.cu ⁵Universidad de Panamá, Facultad de Medicina, Departamento de Histología y Neuroanatomía Humana, Panamá, Republic of Panamá ⁶Escuela de Biología, Departamento de Zoología, Universidad de Panamá, Panamá, Republic of Panamá. E-mail: sam@samfrogs.com ⁷Corresponding author. E-mail: andrew@dna.ac

Eleutherodactylus planirostris Cope 1862, the greenhouse frog, is native to the Bahamas, Cayman Islands and Cuba, with introduced populations in the southeastern United States, Jamaica, Honduras, Mexico, Grenada, Caicos Islands, the Miskito Cays of Nicaragua, and on the Pacific islands of Hawaii and Guam (Heinicke *et al.* 2011; Somma 2011). This species was not previously recorded in the Republic of Panama, although two other invasive *Eleutherodactylus* species, *E. antillensis* Reinhardt & Lütken 1863 1862 and *E. johnstonei* Barbour 1914, have established populations within Panama City (Jaramillo *et al.* 2010). Based on standardized mitochondrial gene fragments, we report for the first time the presence of *E. planirostris* in Panama.

Eleutherodactylus planirostris was first heard calling in 2007 and specimens were collected by Norbert Kunert and AJC at Cerro Ancón (71 meters elevation, 08.95674 N, -079.55164 W [datum WGS84]), Corregimiento Ancón, Distrito Panamá, Provincia de Panamá on 07 May 2008, at approximately 20:00 hours. Three specimens were collected initially: Gravid female, SVL= 22.3 mm (Museo de Vertebrados de la Universidad de Panamá specimen number MVUP 2042 [collector number AJC 2066]; adult male, SVL=17.5 mm (AJC 2067); adult female, SVL=22.5 mm Círculo Herpetológico de Panamá specimen number CH 7778. Images of MVUP 2042 can be seen online at <http://biogeodb.stri.si.edu/bioinformatics/dfm/metas/search?stxt=AJC+2066>. Subsequently, we observed this species in additional neighborhoods within Panama City, including Costa del Este, Dorado Lakes and Balboa in 2009, and in Altos del Chase and Fuentes del Fresno in 2010. These frogs were found primarily associated with houses and gardens, but occasionally in grassy or forested areas within roughly 100 m of human habitations.

We employed DNA barcoding as a third source of standardized data for species identification. We sequenced two mitochondrial DNA barcode markers for amphibians, the 5' end of the cytochrome oxidase I (COI) gene and a fragment of the ribosomal 16S gene, using published primers and protocols (Vences *et al.* 2005; Smith *et al.* 2008; Crawford *et al.* 2010). GenBank accession numbers for each gene (COI, 16S) for each Panamanian specimen are as follows: MVUP 2042 (JF769001, JF769004) and AJC 2067 (JF769000, JF769003). We also obtained sequence data from one *E. planirostris* from Havana, Cuba, deposited in the Museum of Natural History "Felipe Poey", Havana, with specimen number MFP.11512 (JF769002, JF769005). Gene sequences and metadata were also deposited at Barcode of Life Data Systems (Ratnasingham & Hebert 2007) under project code "BSINV". Species identification utilized character-based phylogenetic inference and genetic distances (Goldstein & DeSalle 2011), as well as qualitative observations of morphology and advertisement call.

We compared the 16S DNA data with 16 closely related sequences (Frost *et al.* 2006; Heinicke *et al.* 2007) from GenBank (Fig. 1). Note, specimen USNM 564984 is currently identified as *P. casparii* in GenBank EF493599, but was re-identified as *P. planirostris* in Heinicke *et al.* (2011). Excluding gapped sites, the alignment contained 518 base pairs (bp), of which 57 were parsimony-informative and 37 were singletons. Phylogenetic analysis of 16S data followed protocols in Crawford *et al.* (2010). Parsimony inference resulted in 4 shortest trees of 148 steps (not shown), with support measured by 2,000 bootstrap pseudoreplicates. A maximum likelihood-based tree (-Ln score = 1520.37670) is shown in Fig 1.

Panamanian and US samples were genetically identical at the 16S gene fragment, containing 556 bp. The US–Panamanian haplotype differed by a 1–base T indel, a 4–base poly–C indel and a base substitution from the 559 bp Cuban sample from Havana. The *E. planirostris* samples from Topes de Collantes in central Cuba and the Bahamas differed by a single base substitution. *Eleutherodactylus planirostris* from Central Cuba versus Havana, however, showed 1.35% (6 mutational steps) divergence. Samples from Havana, Florida and Panama form a well-supported clade relative to conspecifics from central Cuba (Topes de Collantes) and the Bahamas (Fig. 1).

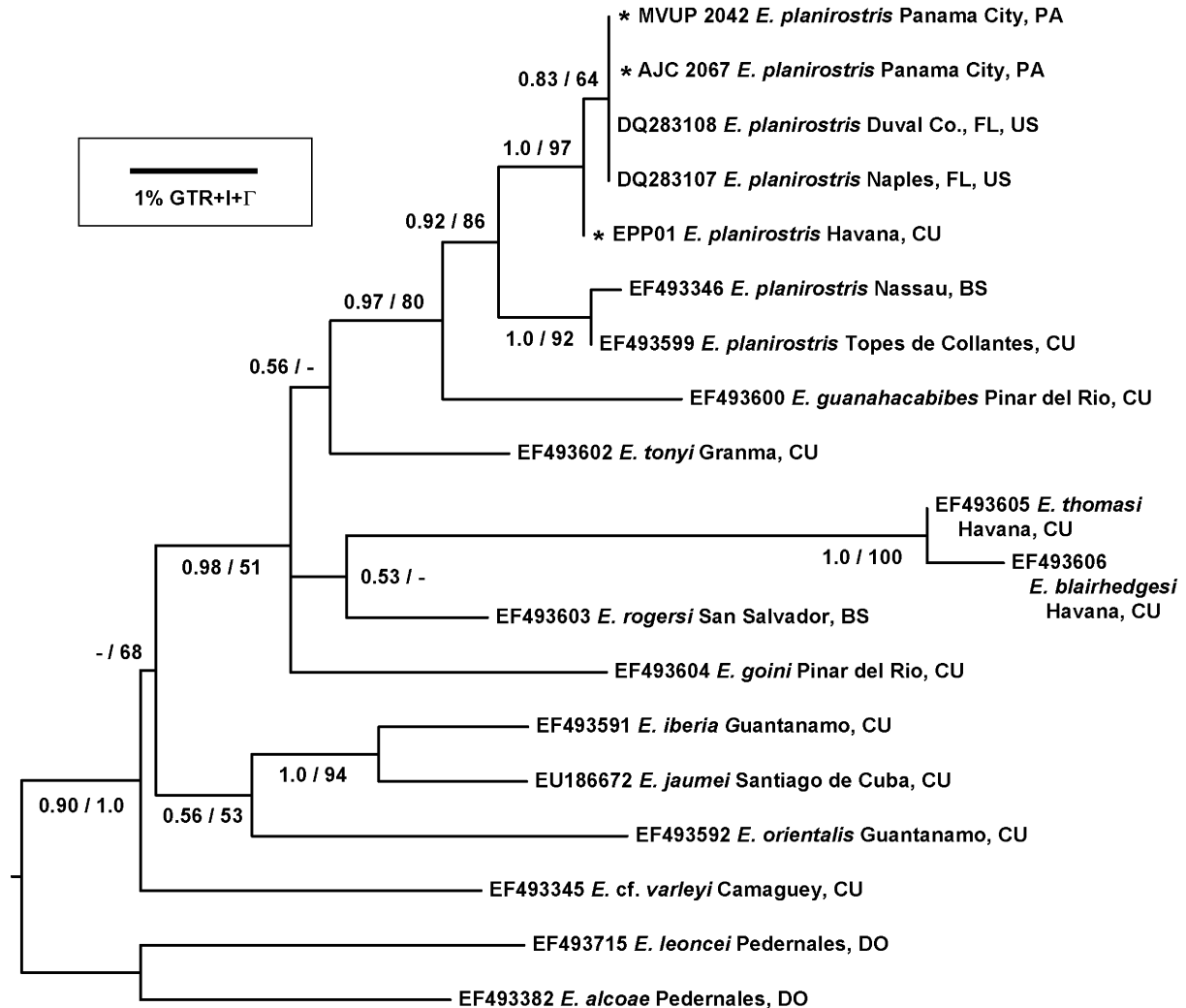


FIGURE 1. Maximum likelihood phylogeny of *Eleutherodactylus* species based on a 518 base pair fragment of the 16S gene and assuming the general time-reversible model of molecular evolution with 2 rate heterogeneity parameters (GTR+I+Γ). Tree rooted based on preliminary analyses of 42 GenBank samples and on Heinicke *et al.* (2007). Scale bar indicates an inferred branch length of 0.01 (1%). Statistical support for each node is indicated by its Bayesian marginal posterior probability followed by its parsimony bootstrap percentage, where a dash (-) indicates a probability <0.5 or a bootstrap score <50%. The three samples marked with an asterisk (*) were obtained for this study, while the other 16 sequences were obtained from GenBank (sample name starts with its GenBank accession number). Country of origin is by the corresponding ISO 3166-1 2-letter country code. Additional sample information is provided in text and in GenBank.

Our 16S molecular phylogeny agrees with that of Heinicke *et al.* (2011), based on the *cyt b* gene, in grouping samples of *E. planirostris* from Havana and Florida (western lineage) relative to central Cuba and the Bahamas (eastern lineage). Our data place the *E. planirostris* samples from Panama in the western lineage. Given our current sampling, however, we cannot pinpoint the origin of Panamanian *E. planirostris*, although Florida is a likely candidate source given the sequence identity.

Identification of invasive species may be difficult, especially when encountered for the first time in a new country, as local knowledge and comparative material of the invader may be limited. DNA sequencing provides ready access to standardized characters to aid in species identification. Comparative data for the COI barcode are still uncommon among amphibians (Smith *et al.* 2008), but we expect greater taxonomic and geographic sampling for all amphibians under a globally expanding DNA barcoding campaign. Regardless of the gene, mitochondrial DNA sequence data aid in quickly identifying taxonomic problems, while an integrative approach to taxonomy may best solve such problems (Padial *et al.* 2010).

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