



New record of the introduced species *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae) in the state of Veracruz, Mexico

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Numerous direct developing species of the genus *Eleutherodactylus* native to the Caribbean Islands have been introduced outside its natural range by human activities. The greenhouse frog, *Eleutherodactylus planirostris* is native to Cuba and the Bahamas and has been introduced to many parts of the world. Here, we report the rediscovery of *E. planirostris* in the Mexican Gulf. The species was not reported in the region since 1974. Molecular identification of the species was possible by comparing 16S and COI sequences with samples from the type locality, five introduced populations and 20 other *Eleutherodactylus* species. The species was also verified by morphological characters. By means of phylogenetic reconstruction we propose that its introduction in Veracruz is independent to the Mexican Caribbean event. This is the first record of the species in a small rural region from Veracruz, and thus a comprehensive evaluation of the distribution of the species in Mexico is needed.

Key words: Introduced species, DNA barcoding, Mexico, *Eleutherodactylus*

Introduced species have been considered among the main threats for the preservation of biodiversity worldwide (Bellard et al., 2016). Among the amphibians, several Antillean *Eleutherodactylus* species have been introduced to new areas mainly through the trade of ornamental plants (Kraus et al., 1999; Kaiser et al., 2002). For example, *Eleutherodactylus coqui* is listed as one of the world's worst invasive alien species by IUCN (Lowe et al., 2000), and *Eleutherodactylus johnstonei* has been widely introduced, currently present in the Caribbean countries and recently recorded in Brazil (Kaiser, 1997; Ernst et al., 2011; Melo et al., 2014).

The greenhouse frog (*Eleutherodactylus planirostris*) has been introduced to many regions throughout the

world such as continental and island USA (including Hawaii), Hong Kong, Guam, Philippines, Jamaica, Honduras, Panama and Suriname (McCrane et al., 2008; Crawford et al., 2011; Heinicke et al., 2011; Olson et al., 2012; McCranie & Valdés-Orellana 2014; Lee et al., 2016). In Mexico, it was reported on one occasion for the state of Veracruz, 43 years ago (Schwartz, 1974) at the port of Veracruz (Flores-Villela & McCoy, 1993), and recently in the Yucatan Peninsula (Cedeño-Vázquez et al., 2014; García-Balderas et al., 2016; Pavón-Vázquez et al., 2016; Gómez-Salazar & Cedeño-Vázquez, 2017; Ortiz-Medina et al., 2017). Molecular data from specimens collected in the Mexican Caribbean showed they are closely related to populations from Philippines and Panama (Cedeño-Vázquez et al., 2014).

As shown by Crawford et al. (2011), "the identification of invasive species in new localities may be difficult, especially when local knowledge and comparative material of the invader may be limited". The identification of introduced Caribbean *Eleutherodactylus* species is a challenge, considering their huge diversity (191 species to date) and low morphological variation. In this case, DNA sequencing provided characters to assist in species identification. Here, we report the rediscovery of *E. planirostris* in the Mexican Gulf, and we discuss the application of molecular tools in the detection of introduced species.

During field surveys performed on Ejido La Laja (Cuichapa Municipality) in Veracruz, Mexico (18°45.17' N, 96°47.13' W, 423 m. Figure 1A), we recorded an *Eleutherodactylus* population in the leaf litter and under trunks in a house garden. The morphology of the individuals did not coincide with any of the species known for the region. The individuals were found in the same site by visual and acoustic records in April, September and November 2016. Six individuals were captured and voucher specimens were deposited in the Colección Nacional de Anfibios y Reptiles (CNAR), Instituto de

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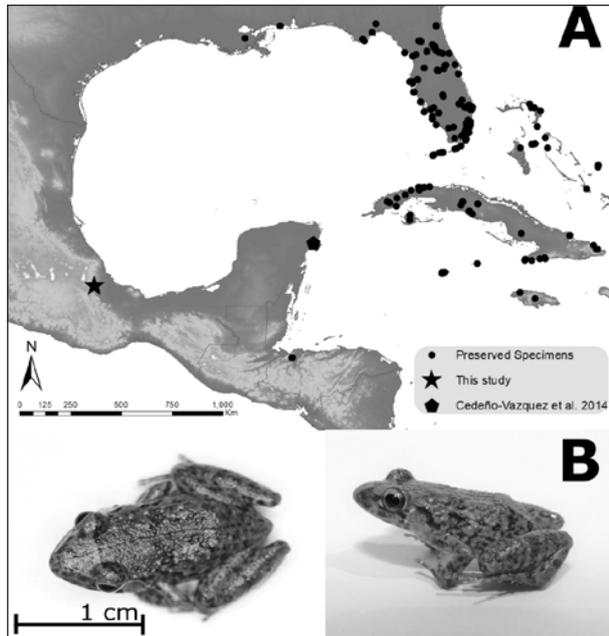


Figure 1. (A) Map with localities recorded for *E. planirostris* near the Gulf of Mexico. The record of Schwartz (1974) from Veracruz, Mexico has no locality details so it was omitted in the map. **(B)** Specimen of *E. planirostris* (IBH-31562) captured in Ejido La Laja, Municipality Cuichapa, Veracruz, México.

Biología, UNAM. Liver and muscle tissue were collected for two individuals (IBH-31562-63) and stored in RNA-later™ Tissue Storage Reagent (Ambion).

We extracted DNA using the modified protocol of phenol-chloroform (Sambrook & Russell, 2006). For molecular identification, we amplified the mitochondrial genes COI and 16S. PCR amplifications were performed using the primers and procedures detailed in Mendoza et al. (2012) for 16S and Hebert et al. (2004) for COI. We included sequences from GenBank and BOLD databases for 21 species of *Eleutherodactylus* with available information for COI gene and/or species recorded for Mexico (*E. cystignathoides*, *E. marnockii*, *E. nitidus*, *E. pipilans* and *E. planirostris*). Those species are distributed in the states of Veracruz, Queretaro, Guerrero, Oaxaca and Chiapas (Flores-Villela & McCoy, 1993; Lemos-Espinal & Smith, 2007). The sequences obtained were aligned with Geneious using default settings and verified visually. We calculated pairwise distances using the Kimura 2 Parameter model in MEGA7 (Kumar et al., 2016) and performed a Bayesian phylogenetic analysis using the program MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003; Ronquist et al., 2012). The models of nucleotide substitution were defined following Crawford et al. (2010): one data partitions scheme for 16S (model GTR+I+ Γ) and three data partitions for codon positions 1 through 3 in COI (models GTR+I, GTR+ Γ , and GTR+I+ Γ , respectively). Rates of evolution were allowed to vary across partitions using a rate multiplier. We ran two independent analyses for 20 million generations, each sampling trees and parameter values every 1000 generations. Burn-in was set to 25% and thus the first 5 million generations were discarded. A sequence of *Diasporus quitiddus* (AJC-1789) recovered from GenBank

was used as outgroup. Considering that available COI and 16S sequences for *Eleutherodactylus* belong to different sets of species, we generated two analyses (one per gene). Sequences obtained were deposited in the Genbank repository (accession numbers MF374458-MF374461).

The BLAST of both genes matched with *E. planirostris* sequences, with a 99% identity, 0% gaps and an e-value of 0.0. Online BOLD identification generated a match with *E. planirostris* for COI sequences with 99.84% identity. Bayesian trees for both genes corroborate this result, placing the sequences obtained in the *E. planirostris* clade (Figure 2). The K2P estimated distances show a low divergence of our two sequences to all other *E. planirostris* sequences (0.000-0.016 for 16S and 0.002-0.017 for COI, minimum interspecific distance was 0.045 for 16S and 0.040 for COI). COI tree shows a closer relationship between our samples and those from Cuba than to samples than to samples reported by Cedeño-Vázquez et al. (2014) in the Mexican Caribbean (not available for 16S). To confirm the molecular results, we verified the morphological characters of the species, which coincided with the species description (snout-vent length less than 34 mm, finger discs slightly expanded and absence of interdigital webbing in toes; Schwartz, 1974; Köhler, 2010). *Eleutherodactylus planirostris* is native to the Caribbean islands including Bahamas, Cayman Islands Caicos Islands and Cuba (Lee et al., 2016). This species appears to be a generalist, occupying a diversity of habitats including mesic and xeric broadleaf forest as well as secondary forests, shrub land, agricultural fields, near fishponds, urban gardens and parks, and near human settlements (Lee et al., 2016; García-Balderas et al., 2016; Gómez-Salazar & Cedeño-Vázquez, 2017; Ortiz-Medina et al., 2017).

New records of *E. planirostris* have been achieved by help of molecular tools. The species was first reported in Panama (Crawford et al., 2011) and Hong Kong (Lee et al., 2016) through DNA barcoding. In Honduras, molecular data from three specimens showed them to be genetically identical to the Florida-western Cuba populations (McCrane et al., 2014). Cedeño-Vázquez et al. (2014) identified it on the Mexican Caribbean through molecular and morphological information. In the Mexican Gulf, there are no more records of the species after the record of Schwartz (1974) despite being a region constantly studied. It is possible that this previous record was a non-successful introduction. Here, we report the presence of a population 1 000 km west of the recent observation (Cedeño-Vasquez et al., 2014, Figure 1) and 83 km from the record by Schwartz (1974), and we may infer by the Bayesian tree that both populations are coming from independent introduction events. The invasion in Mexican Caribbean in the Yucatan Peninsula may be related to the same dispersal event that occurred for Philippines and Panama, while the Veracruz samples are more related to Cuban populations. Thus, *E. planirostris* is being introduced into Mexico from different source populations at different times.

In general, the introduced species of *Eleutherodactylus* have been reported in big cities or in localities with high commercial trade. *Eleutherodactylus planirostris* has not been recorded for the Veracruz state in the last 43 years. Thus, the presence of *E. planirostris* in Cuichapa is an

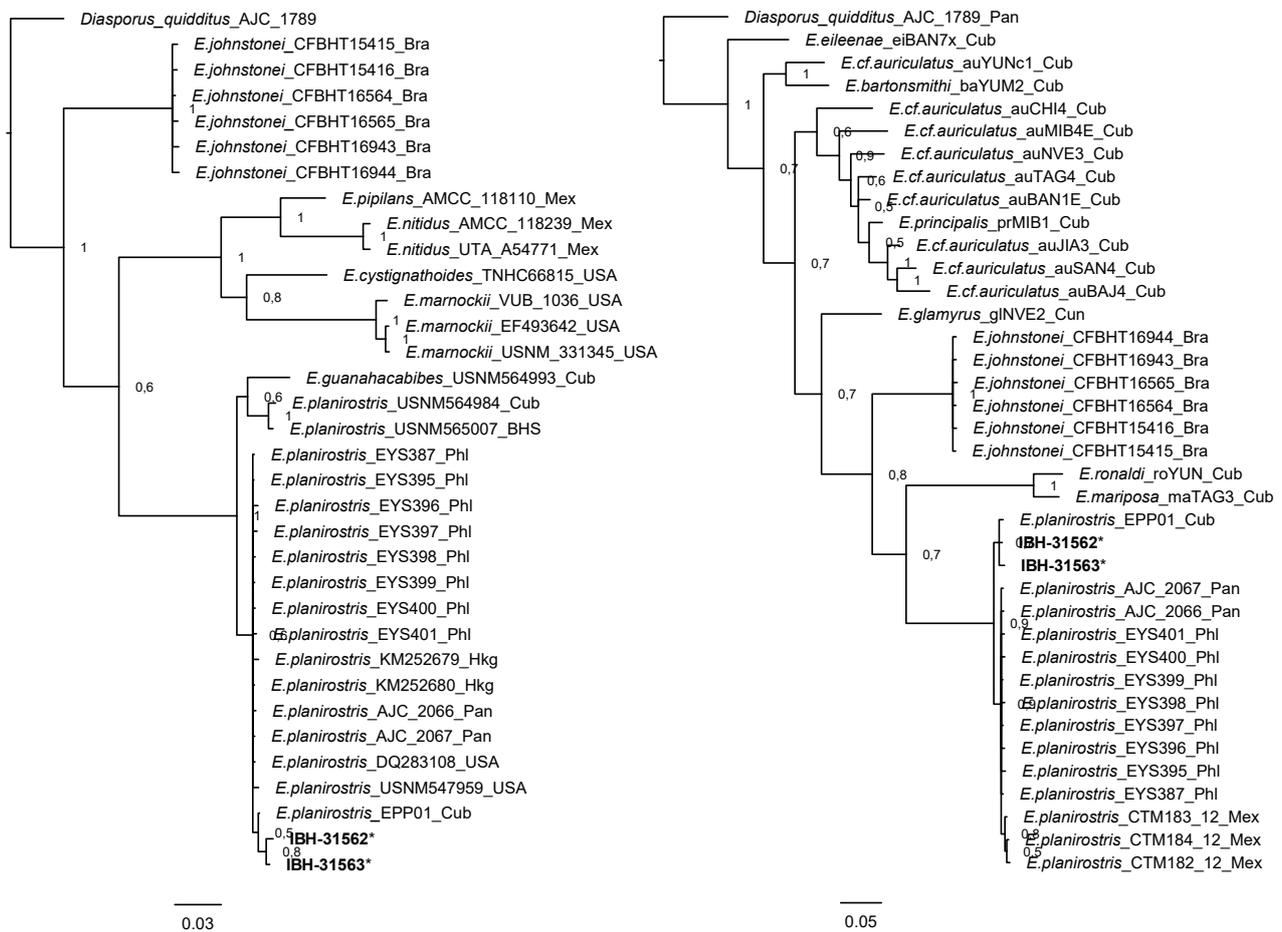


Figure 2. Phylogenetic position of two samples of *E. planirostris* from Veracruz, Mexico in a phylogenetic tree of this species as inferred from Bayesian Analyses (16S rRNA left and COI right). The samples with code IBH (in bold) were obtained for this study, while the other sequences were obtained from GenBank. Country of origin is by the corresponding ISO 3166-1 3-letter country code.

important record for expansion of this invasive species in Mexico. Cuichapa is a small rural municipality with 12,375 inhabitants according to the Instituto Nacional de Estadística y Geografía (INEGI) census (INEGI, 2009). The presence of the species in small rural regions (likely by ornamental plant trade) implies an extensive route of dispersal likely with multiple halfway localities where the species can also establish itself.

When introduced species become established, they feed, compete for food, transform and destroy the habitat, and carry transmissible diseases and parasites, capable of exterminating whole populations of native species (Williamson, 1996). Kraus et al. (1999) and Kraus & Campbell (2002) suggest that invasive populations of *E. planirostris* in Hawaii may be a serious threat to native arthropods, generating a new predation pressure primarily for insects and spiders. In Hawaii, specimens of *E. planirostris* with a population density of 12,522 frogs/ha were found to primarily consume leaf-litter invertebrates and were estimated to consume up to 129,000 invertebrates ha⁻¹ night⁻¹ (Olson & Beard, 2012). The only population survey of this species in Mexico recorded a density of 20.3 individuals/km in Playa del Carmen, Quintana Roo (Gómez-Salazar & Cedeño-Vasquez, 2017), thus demonstrating that the species was effectively established in the sampled area. The reappearance of the species in the Mexican Gulf

implies that an evaluation of the population status and the threats for biodiversity in Mexico is urgently needed, including population density, encounter frequency, spread to nearby localities, and arthropod species consumed.

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