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Kebede Beshera
Southern University and A&M College, kebede.beshera@sus.edu

Avery A. Williams Mr.
LSUE, aaavery@aol.edu

John A. Hamlin Dr.
LSUE, jhamlin@lsue.edu

Peter J. Bergeaux Mr.
pjbergeaux@yahoo.com

Christian C. Morgan Dr.
christianmorgan106@yahoo.com

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Genetic variation among populations of, and evidence of deep divergence within, the Rio Grande Chirping Frog, *Eleutherodactylus campi* (Anura: Eleutherodactylidae)

KEBEDE A. BESHERA[1,7], AVERY A. WILLIAMS[2], JOHN A. HAMLIN[3], PETER J. BERGEAUX[4], CHRISTIAN C. MORGAN[5] & AUBREY N. ARMSTRONG[6]

[1] *Department of Biological Sciences and Chemistry, Southern University and Agricultural & Mechanical College, 801 Harding Blvd, Baton Rouge, LA 70807, USA, https://orcid.org/0000-0002-5257-4693*
[2] *2032 Mohawk Dr., Lake Charles, LA 70611, aaavery@aol.com*
[3] *Division of Arts and Sciences, Louisiana State University Eunice, 2048 Johnson Hwy, Eunice, LA 70535, USA, jhamlin@lsue.edu*
[4] *1320 Merchant Road, Kaplan, LA 70548, USA, pjbergeaux@gmail.com*
[5] *288 Charlie Arceneaux Road, Rayne LA 70578, USA, christianmorgan106@yahoo.com*
[6] *1616 South Barnett Springs Street, Ruston, LA 71270, USA*
[7] *Corresponding Author kebede.beshera@sus.edu*

Abstract

Herein we report the first molecular assessment of intra-species genetic variation and interrelationships within the Rio Grande Chirping frog, *Eleutherodactylus campi*. We analyzed 548 base pairs of 16S rRNA gene for 71 ingroup individuals belonging to the genus *Eleutherodactylus* (including 42 *E. campi* sampled from 15 localities in the United States and Mexico) and four outgroup samples. By unveiling two highly divergent and geographically structured clades within *E. campi* this study provides a novel phylogenetic placement of *E. campi* populations north and south of the Rio Grande Valley as sister groups to each other. The observed level of genetic divergence between these two clades (5.8%) is, on average, comparable to or greater than the levels of divergence found between several currently valid amphibian species pairs. Estimates of Time to Most Common Ancestor (TMRCA) indicate that the phylogeographic split between the two *E. campi* clades may have occurred 7.6 MYA (i.e., late Miocene), consistent with the geologic history of southwestern North America. The study also confirms that south Texas served as the source population for populations of *E. campi* in its introduced range (i.e., Alabama, Louisiana, and Texas). Overall, this molecular study indicates that *E. campi* consists of two deeply divergent lineages corresponding to its populations north and south of Rio Grande Valley. These results suggest that the recovered lineages may represent independent species and thereby highlight the need for further research to clarify their status.

Key words: genetic divergence; haplotype; monophyletic; phylogeographic clades; 16S ribosomal RNA gene; taxonomy

Introduction

The Rio Grande Chirping Frog, *Eleutherodactylus campi* Stejneger (Fig. 1) is a small direct developing frog belonging to a primarily Neotropical family, Eleutherodactylidae. It is one of the three species of Eleutherodactylid frogs that naturally occur in the continental United States (US), the other two being *Eleutherodactylus guttilatus* Cope and *Eleutherodactylus marnockii* Cope 1878 (Lott 2019). *Eleutherodactylus campi* was originally described as a new species, *Syrrhophus campi*, based on specimens collected from Brownsville, Texas. But later it was considered as a northern subspecies of *Syrrhophus cystignathoides* that has been described as *Phyllobates cystignathoides* Cope, based on intermediate specimens collected from southeastern Mexico (Lynch 1970). Later the genus *Syrrhophus* was considered a junior synonym of the genus *Eleutherodactylus* (Hedges 1989). Subsequently, *Syrrhophus* was demoted to the subgenus rank and *E. campi* was renamed *Eleutherodactylus cystignathoides* (Hedges 1989; Heinicke *et al*. 2007). However, a more recent assessment of molecular data has raised *E. campi* from the synonymy of *E. cystignathoides* (Grünwald *et al*. 2018), although its southern range limits with *E. cystignathoides* still remain fuzzy. This decision has been recently corroborated by Hernandez-Austria *et al* (2022). However, intra-species genetic structure and interrelationships were unresolved due to the limited scope of these studies and incomplete geographic sampling of *E. campi*. For instance, no samples of *E. campi* were analyzed from the US.

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FIGURE 1. *Eleutherodactylus campi* in life, found under a palm log at the edge of a parking lot (Image by Jake M. Scott)."

The native range of *Eleutherodactylus campi* was limited to the Lower Rio Grande Valley and extended from northeastern Mexico to the southernmost tip of Texas (Powell *et al*. 2016; Lott 2019). However, in the past few decades, its geographic range has been rapidly expanding northward into Texas and eastward along the Gulf Coast into Louisiana and Alabama. Currently, the Rio Grande Chirping Frog has established populations outside the Lower Rio Grande Valley, including in several parts of Texas (McGown *et al*. 1994; Lutterschmidt & Thies 1999**;** Dixon 2013), five parishes in Louisiana with its northernmost range extending to Shreveport (Caddo Parish), and Mobile County in Alabama (Hardy 2004; Beck and Dobbs 2008; Williams *et al*. 2012; Boundy & Carr 2017; Lott 2019). Thus far no studies have examined genetic differences between *E. campi* populations from its native and introduced ranges.

To explore geographic differentiation and the nature and extent of genetic variation within *Eleutherodactylus campi*, herein we analyzed the mitochondrial 16S Ribosomal gene sequences of 42 *E. campi* sampled from 15 localities in Alabama, Louisiana, Texas (including the type locality in Cameroon County), and Nuevo Leon, northeastern Mexico. Specifically, we attempted to address the following questions: 1) Is *E. campi* a monophyletic species? 2) How many genetically differentiated populations exist? 3) When did these populations diverge? And 4) are populations from the species' native and introduced ranges genetically different?

Materials and methods

Sampling. For this study, we analyzed 16S Ribosomal gene sequences (548 bp) derived from a total of 42 *Eleutherodactylus campi* specimens from 15 localities in the US (Alabama, Louisiana, and Texas) and Northeastern Mexico. Thirty-one specimens were collected from nine localities in Louisiana. Five tissue samples of *E. campi* collected from Mobile (Alabama), two from Eastern Baton Rouge (Louisiana), and one from Cameroon County (Texas), were provided to us by Auburn University Museum of Natural History, Louisiana State University Museum of Natural Sciences, and Sternberg Museum of Natural History (Fort Hays State University), respectively. Additional three DNA sequences were retrieved from GenBank. Samples examined in this study and their GenBank accession numbers are summarized in Table 1. For *Eleutherodactylus campi*, our sampling regime included individuals collected from the native range in US and Mexico as well as its introduced range in the US.

For the 31 samples we collected, we determined the collection sites based on the presence of nocturnal calling males identified at each locality. The habitats associated with each site were similar consisting of mixed hardwoods and pines with dense thicket and briar ground cover. Upon visiting each site, we would listen for and locate calling males. Although some males were easily located from elevated substrates (e.g., atop logs, stumps, low branches, etc.), other specimens were called from concealed refuges (e.g., inside rotten logs, under bark, among dense vegetation, etc.). To entice hidden males from refugia, we played an audio recording of other calling males of this species as a lure. This method was very successful as hidden males have emerged and advanced directly toward the recording. We preserved whole frogs in 95% ethanol and extracted DNA from the muscle tissue of the hind limb of the frogs for molecular analysis.

TABLE 1. List of sampled taxa, sampling locality information, and GenBank accession numbers of sequences used in the study.

	Species	Source locality	Species group	GenBank accession #
Ingroup	*E. campi* Haplotype 1*	Nuevo Leon, Mexico	*Leprus*	MG856966
	E. campi Haplotype 2	Nuevo Leon, Mexico		MG856965
	E. campi Haplotype 3	Lake Charles, USA		MW040053
	E. campi Haplotype 4	Baton Rouge, USA		MW040054
	E. cystignathoides (Haplotype 1**)	Veracruz, Mexico		MZ203186, MZ203185
	E. cystignathoides (Haplotype 2**)	Veracruz, Mexico		MZ203181, MZ203182
	E. cystignathoides (Haplotype 3**)	Veracruz, Mexico		MZ203183, MZ203184
	E. marnockii	Travis County, Texas	*E. marnockii*	DQ283101
	E. marnockii	Travis County, Texas		EF107177
	E. marnockii	Austin, Texas		EF493642
	E. guttilatus	Mexico: San Luis Potosi		MG856994
	E. verucippes	Mexico: Tamaulipas		MG857079
	E. longipes	Mexico: Nuevo Leon	*E. longipes*	MG857006
	E. modestus	Mexico: Colima	*E. modestus*	MG857021
	E. pallidus	Mexico: Nayarit		MG857053
	E. teretistes	Mexico: Jalisco		MG857078
	E. nitidus	Mexico: Puebla, Sierra Negra	*E. nitidus*	EU186712
	E_albolabris	Mexico: Guerrero		MG856956, MG856955
	E. dilatus	Mexico: Guerrero		MG856974
	E. pipilans	Mexico: Guerrero, Carretera Tierra	*E. pipilans*	EU186711
	E. rubrimaculatus	Mexico: Chiapas		MG857057, MG857056
	E. simingtoni	Cuba: Pinar del Rio, Soroa	*E. simingtoni*	EF493643
	*E. ricordi_*	Cuba: Santiago de Cuba		EF493636
	E. planirostris	Mexico: Veracruz, Cuichapa Municipality		MF374458, MF374459
	E. inoptatus	Dominican Republic: Barahona		EF493380
	E. schwartzi	Virgin Islands: Tortola		EF493551
	E. martinicensis	Guadeloupe: Basse-Terre		EF493343
Outgroup	*Craugastor longirostris*	Ecuador: Pichincha		EF493395
	Brachycephalus didactylus	Brazil: Ilha Grande		HQ435692
	Ischnocnema parva	Brazil: Sao Paulo		EF493532
	Pristimantis orestes	Ecuador: Azuay		EF493388

*Identical DNA sequence is found in 39 *E. campi* samples obtained from 13 localities in Alabama, Louisiana, and Texas.

**Two identical DNA sequences were found in each haplotype of *E. cystignathoides.*

For phylogenetic analysis, we assembled a data set consisting of a total of seventy-five 16S rRNA gene sequences. Of these, 59 sequences (including 42 and six sequences of *Eleutherodactylus campi* and *E. cystignathoides*, respectively) were from the subgenus *Syrrhophus*. Of the 42 *Eleutherodactylus campi* sequences, 39 were generated for this study. Additional three sequences of *E. campi* were downloaded from GenBank (GenBank accession numbers JX512277, MG856965, and MG856966). To put the study in a broader phylogenetic and taxonomic context we included an additional six sequences (five species) representing the subgenera *Euhyas*, *Pelorius*, and *Schwartzius*. We also designated four species, one species from each of the genera *Brachiocephalus*, *Craugastor*, *Ischnocnema*, and *Pristimantis* as outgroup.

DNA Extraction and PCR Amplification. We extracted total genomic DNA from ethanol-preserved muscle tissue using the DNeasy tissue extraction kit (Qiagen; www.qiagen.com) following the manufacturer's instructions. Then we amplified the mitochondrial (mt) 16S ribosomal RNA (rRNA) gene (553 base pairs) using the upstream 16Sar-L (5'CGCCTGTTTATCAAAAACAT3') and downstream 16Sbr-H (5'CCGGTCTGA ACTCAGATCACGT3') primers. We performed PCR amplification of samples in 50-µl reactions using the Promega PCR Mastermix, which is a premixed ready-to-use solution containing Taq DNA Polymerase, dNTPs, $MgCl_2$, and reaction buffers at concentrations for efficient amplification of DNA templates by PCR. We purified PCR products using the QIAquick PCR Purification Kit (QIAGEN) and outsourced purified products to ACGT, INC. for Sanger sequencing. We carried out DNA sequencing reactions using BigDye terminator version 3.1, cleaned up products with magnetic beads (CleanSEQ dye terminator removal kit), and analyzed extension products using the ABI 3730 XL or 3730 Genetic Analyzer. Then we manually aligned DNA sequences employing BIOEDIT software version 5.0.9 (Hall 1999). Finally, we deposited the newly generated DNA sequences of two haplotypes (representing 39 *Eleutherodactylus campi* samples) in GenBank. The sequences were assigned accession numbers (Table 1).

Phylogenetic Analyses. We generated haplotype data for *Eleutherodactylus campi* and *E. cystignathoides* using DNASP 6.12.03 (Rozas *et al*. 2017). We determined the best performing Maximum Likelihood (ML) nucleotide substitution model for our sequence data in MEGA X (Kumar *et al*. 2018). We performed phylogenetic analyses using the Maximum Likelihood (ML) method with the Nearest Neighbor Interchange tree inference option as implemented in MEGA version X (Kumar *et al*. 2018) and Bayesian Inference (BI) employing Beast version 1.10.4 (Suchard *et al*. 2018). We ran ML analysis in MEGA X applying the GTR nucleotide-substitution model (GTR-Gamma) along with rapid bootstrapping (500 bootstrapping replicates) and ML heuristic Nearest Neighbor Interchange (NNI) tree inference option. We ran Bayesian analysis under the GTR nucleotide substitution, uncorrelated lognormal relaxed molecular clock, and Gamma rate heterogeneity, and Birth-Death Speciation models. For this analysis, we ran three independent Markov Chain Monte Carlo (MCMC) simulations for 50 million generations, sampling trees every 1000 generations. To evaluate support for resolved clades, we used Posterior Probability (PP) for Bayesian analysis and bootstrap values (BS) for ML analysis. We calculated pairwise genetic distances in MEGA X program (Kumar *et al*. 2018) using the maximum composite likelihood method. In addition, to assess relatedness among haplotypes, we generated an un-rooted TCS haplotype network employing PopART, Population Analysis with reticulate Trees version 1.7 (Leigh & Bryant 2015), which follows the statistical parsimony algorithm described in Templeton *et al*. (1992). The method calculated the maximum number of mutational steps that make parsimonious connections between haplotype sequences with 95% confidence.

Time to Most Recent Common Ancestor (TMRCA). We estimated the Time to Most Recent Common Ancestor (TMRCA) of recovered clades in Beast version 1.10.4 (Suchard *et al*. 2018) employing the same models used for our phylogenetic analysis. We used both molecular sequences and paleontological temporal constraints on sequence divergence to calibrate our phylogeny. First, we used an amphibian 16S rRNA gene sequence evolution rate of 0.16–1.98% per million years (Bittencourt-Silva *et al*. 2016). Crawford & Smith (2005) suggested that low sea levels during the Oligocene at about 30 MYA may have facilitated the dispersal of the ancestor of *Syrrhophus* to mainland north America, consistent with the fossil record (Holman 1968). Therefore, we time-scaled the node at which the subgenera *Syrrhophus* and *Euhyas* diverged from each other to 30 MYA. We ran three independent Bayesian Markov Chain Monte Carlo (MCMC) simulations each with 50 million generations, sampling trees every 1000 generations. For both phylogeny and divergence time inference, we evaluated the Effective Sample Size (ESS) values for each parameter and the stationarity of the likelihood values in Tracer v.1.6 (Rambaut *et al*. 2018). We combined outputs of the three independent simulations using LogCombiner v.2.4.2 discarding 10% of the sampled trees as burn-in and then we used these outputs to reconstruct a maximum credibility tree in TreeAnnotator v.2.4.2 (Rambaut & Drummond 2002–2018). Trees were visualized using FigTree (Rambaut 2006-2018).

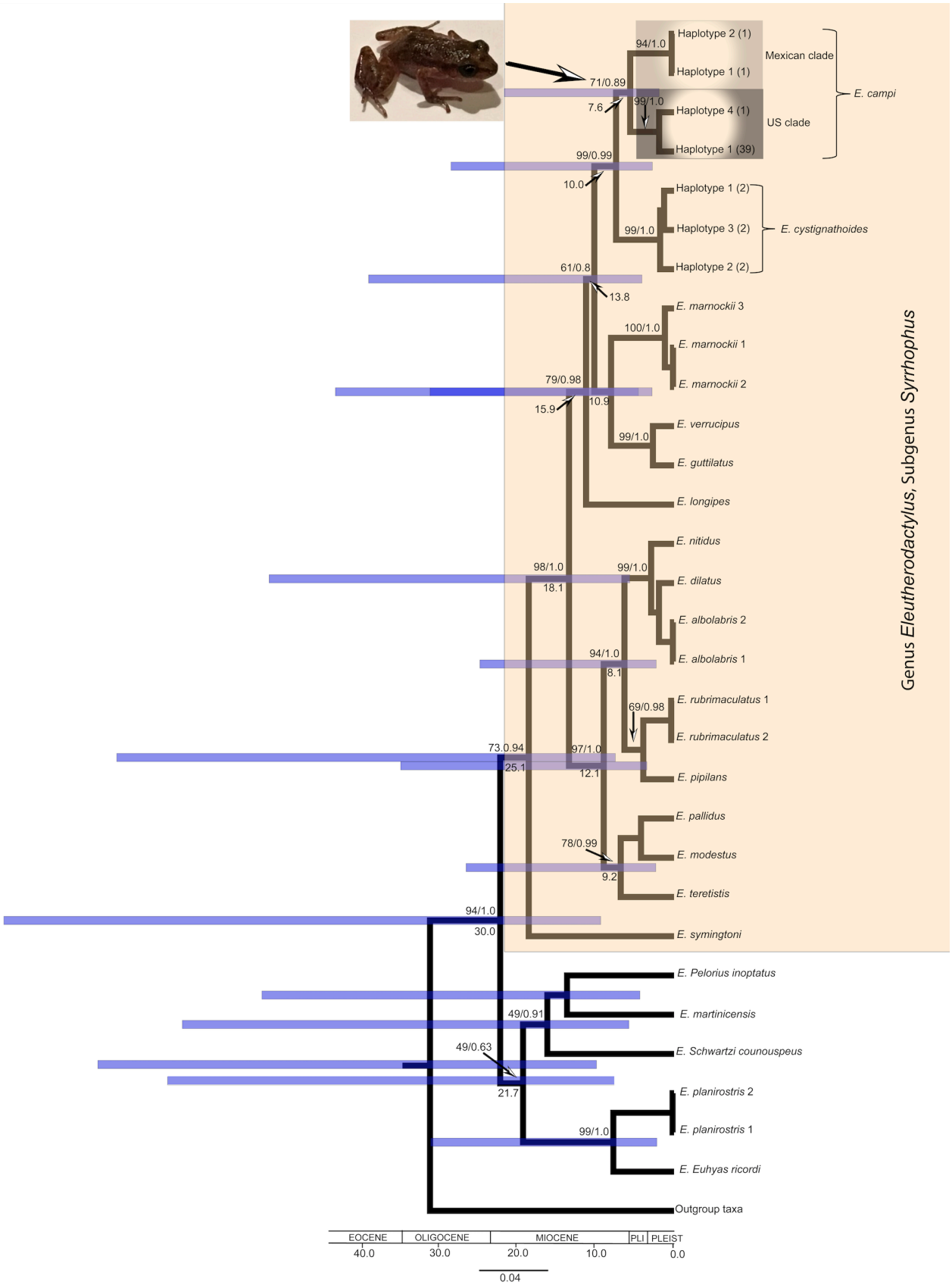

FIGURE 2. Phylogenetic tree based on Bayesian analysis of mitochondrial 16S rRNA gene sequences. Numbers above nodes are ML Bootstrap (before the slash) and Bayesian Posterior probability (after the slash) values. Numbers below branch nodes correspond to mean divergence date estimates in millions of years. Node bars indicate 95% HPD associated with divergence dates whereas the scale bars indicate time in million years.

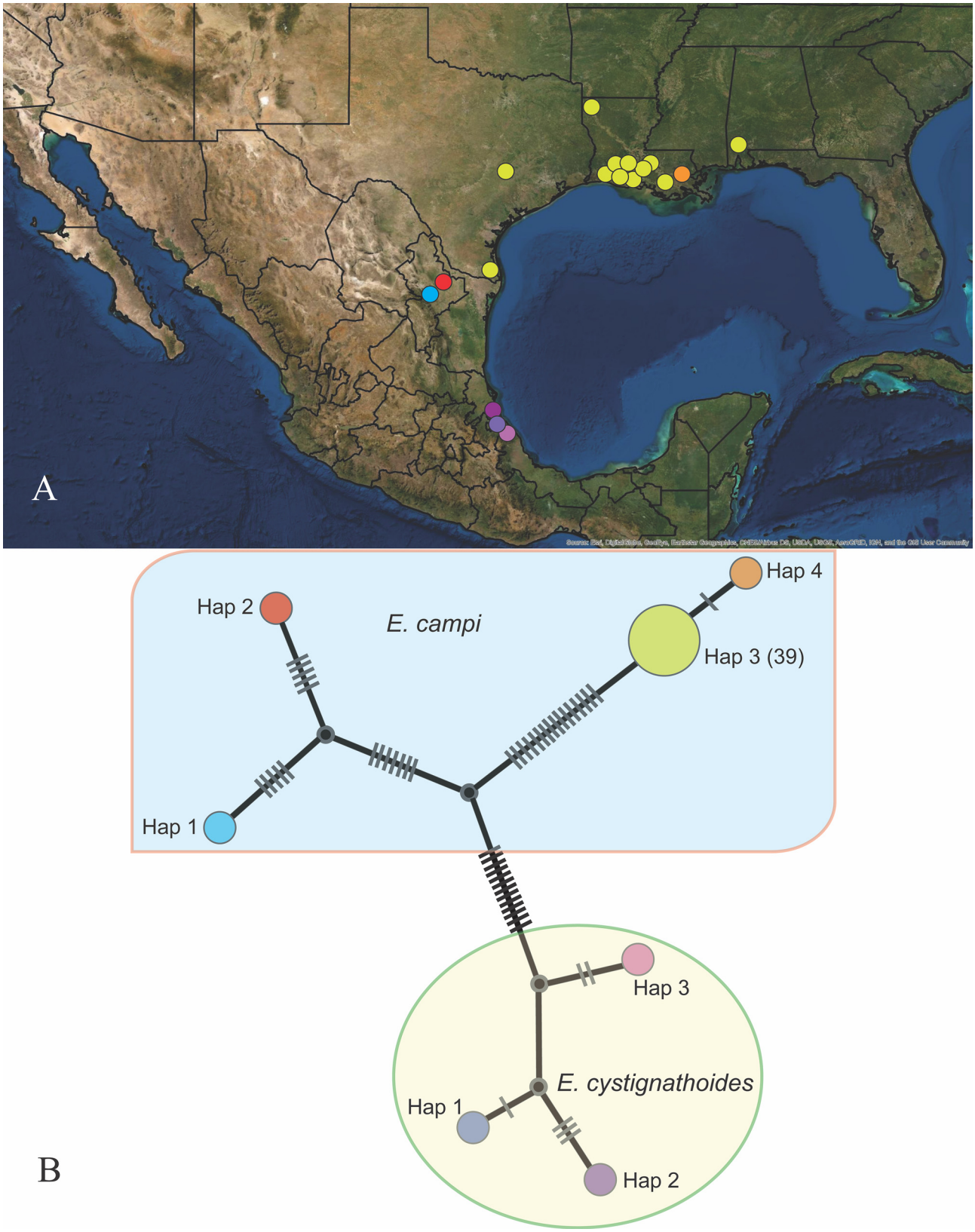

FIGURE 3. A: Sampling localities of four haplotypes (42 samples) of *Eleutherodactylus campi* and three haplotypes (six samples) of *E. cystignathoides* with some points offset for clarity. The geographic positions of sampling localities were approximated for sequences obtained from GenBank. B: Unrooted TCS network generated using PopART. Lines intersecting node lines correspond to the number of mutational steps between haplotypes and clades. The geographic origins of haplotypes are color-coded, circle sizes roughly reflect the frequency of each haplotype, and the number in parenthesis indicates the number of individuals sharing a haplotype.

Results

Genetic Diversity. The final 16S rRNA gene sequence assembled for this study (including ingroup and outgroup taxa) contained 34 sequences (representing 75 samples) with 548 nucleotide sites; Forty-two *Eleutherodactylus campi* samples are represented by 4 haplotype sequences and six *E. cystignathoides* samples are represented by three haplotype sequences. Of the 173 variable or polymorphic sites, 114 were parsimony informative sites. The overall transition/transversion bias (R) estimated under the Tamura-Nei model is 1.64. The nucleotide frequencies were A = 32.4%, T = 24.7%, C = 23.6%, and G = 19.2%. Of the 24 different substitution models evaluated in MEGA X, the nucleotide substitution model that best fit our dataset was GTR+G with a Maximum Likelihood value (*lnL*) and BIC score of -4010.571761 and 8739.797469, respectively. Among the 42 *Eleutherodactylus campi* sequences analyzed 29 variable sites and four unique haplotypes were identified. Haplotype Diversity (Hd) and nucleotide diversity were 0.14 and 0.005, respectively. Within-clade genetic divergence (0.2%) was much lower than between-clade genetic divergence (5.8%).

Phylogenetic relationships and molecular dating. The 16S rRNA gene sequence-based phylogenetic tree derived from Bayesian analysis (Fig. 2) recovered *Eleutherodactylus campi* as a monophyletic species (BS = 71%, PP = 0.91), which had a sister relationship to *E. cystignathoides* (BS = 99, PP = 0.99). The gene tree recovered two phylogroups or clades within *E. campi*; a northern clade consisting of haplotypes sampled from localities in the southern US (Alabama, Louisiana, and Texas) and a southern clade comprising haplotypes from Nuevo Leon, Mexico. Both clades were strongly supported (US clade BS=99 and PP = 1.0%; southern clade BS= 94 and PP = 1.0%). Hereafter, we refer to these northern and southern clades, as US and Mexican clades, respectively. The geographic distribution of these clades is presented in Fig. 3A. These clades showed a high level of genetic divergence (5.8% ± 0.02 SE) from each other, which indicates a high degree of cryptic diversity within *E. campi*. Similarly, the TCS haplotype network generated by statistical parsimony (Fig. 3B) recovered two connected *E. campi* networks; one of them included haplotypes of *E. campi* originating from the US, and the second contained haplotypes from Nuevo Leon, Mexico. Twenty-five mutational steps separate the two clades with no haplotypes shared between them. Genetic divergence between *E. campi* populations from the derived range of the species (i.e., Alabama, Louisiana, and Texas) and the individual from the native range (Cameroon County, Texas) is much lower (0.02%) than their divergence from Nuevo Leon (Mexico) populations (5.6-6.1%). Pairwise genetic distances (ML uncorrected distances) among the four haplotypes of *E. campi* are presented in Table 2. Divergence date estimates based on analysis of the 16S rRNA gene dataset show that *E. campi* diverged from *E. cystignathoides* around 10.0 MYA, whereas the two clades of *E campi* (US and Mexican) clades diverged from one another at 7.6 MYA (Fig. 2).

TABLE 2. Uncorrected pair-wise genetic distances ±Standard Error (SE) in the 16S rRNA gene between haplotypes of *E. campi*.

	Haplotype 1	Haplotype 2	Haplotype 3
Haplotype 1			
Haplotype 2	0.002±0.002		
Haplotype 3	0.058±0.019	0.061±0.020	
Haplotype 4	0.056±0.018	0.058±0.019	0.002±0.002

Discussion

Phylogenetic relationships. In this study, *Eleutherodactylus campi* was recovered as monophyletic with moderately high support values (BS = 71 and PP = 0.89). Hedges *et al*. (2008) defined seven species groups within the subgenus *Syrrhophus* with *E. campi* being placed within the *E.* (*syrrhophus*) *leprus* species group along with *E. leprus* and *E. rubrimaculatus* (Hedges *et al*. 2008). However, our phylogeny recovered the *E.* (*syrrhophus*) *leprus* species group as nonmonophyletic. Whereas most taxa placed by Hedges *et al*. (2008) in this species group are embedded in clade A (e.g., *E. campi*, *E. cystignathoides*, *E. marnockii*, *E. guttilatus*, *E. longipes*, and *E. verucipes*), *E. rubrimaculatus* is recovered in clade B, which includes species placed by Hedges et al (2008) in the *E. modesus*, *E. nitidus*, and *E. pipilans* species groups.

Grünwald *et al*. (2018) and Hernandez-Austria *et al.* (2022) resolved the phylogenetic relationship between *Eleutherodactylus campi* and *E. cystignathoides*. However, in both studies, intraspecific genetic structure and interrelationships were not resolved for *E. campi* due to the limited scope of these studies and incomplete geographic sampling. For instance, no samples of *E. campi* were analyzed from the US. Through increased geographic sampling the current study resolved two phylogeographic clades within *E. campi*, which provides a novel phylogenetic placement of *E campi* populations north of the Rio Grande Valley as a sister to those populations from Nuevo Leon, Mexico. The geographic range of *E. campi* in the US was once limited to a small part of Texas in the Lower Rio Grande Valley. However, during the last five decades, the species has made its way into a much wider distribution within three Gulf States, namely Alabama, Louisiana, and Texas (Lott 2019). Patterns of genetic variability indicate that there is little genetic divergence within the US clade; US populations of *E. campi* from its introduced range are not differentiated from those in its native range (southernmost Texas; sequence divergence 0. 002). In addition, 39 of 40 sequences obtained from the US including one from Cameroon County (Texas) shared one haplotype (haplotype 3). We interpret the lower genetic variability and haplotype diversity observed within *E campi* north of the Rio Grande Valley as indicative of a much more recent and currently ongoing range expansion from source populations in lower Rio Grande (Texas) northward into Texas and northeastward into Louisiana and Alabama. Because we analyzed only two haplotypes (representing two sequences) from Mexico, we cannot discuss genetic variability within the Mexican clade.

Biogeographic Implications. The results we obtained from the evaluation of genetic variation within *Eleutherodactylus campi* based on analysis of 16S rRNA gene sequences have significant biogeographic implications. Early biogeographic studies have invoked late Cenozoic and Miocene-Pliocene geological processes as well as more recent Pleistocene-Holocene Glacial-Interglacial and desertification events to explain the distribution and population structure of the fauna of southwestern North America (Morafka 1977; Riddle 1995). Our divergence date of 7.6 MYA (Fig. 2) for Mexican and US clades of *E. campi* coincides with the late Miocene-early Pliocene evolution of the Rio Grande Valley suggesting that the Rio Grande Valley may have served as a geographic barrier to gene flow between the two clades. Geological processes in the form of volcanic activity, block faulting, and tectonic uplifting during the early Cenozoic occurred at the convergence of what is now Texas, New Mexico, and Mexican borders, creating the Rio Grande Rift and a range topography (Chapin & Seager 1975; Cape *et al*. 1983; Rosenthal & Forstner 2004). The Rio Grande rift that is occupied by the Rio Grande River starts in central Colorado's Rocky Mountains and runs southward through Colorado and New Mexico into Northern Mexico. During its early formative periods (Oligocene to late Miocene, 28.0–9.0 MYA), the Rio Grande Rift was occupied by an east-flowing ancestor of the Rio Grande River. Orogenic activities of the late Miocene, specifically the uplift of the Sangre de Cristo, deflected this east-flowing system to the south (Chapin & Seager 1975). The drainage system of the Miocene-Pleistocene is believed to have been composed of several closed basins (Miller 1981), and not until late Pliocene-early Pleistocene did the Rio Grande River assume its present form. Currently, the river marks the boundary between Mexico and the U.S. The observed phylogeographic split within *E. campi* is consistent with the north-south vicariance (between northern Mexico and southern united states) of some herptile taxa (Hillis *et al*. 1983; Wake & Lynch, 1976).

Taxonomic Implications. Contra to its present status as a single species, our molecular analysis reveals the existence of two deeply differentiated and highly divergent phylogeographic clades within *Eleutherodactylus campi* suggesting that the current taxonomy of the species does not reflect the observed genetic diversity, and therefore needs to be revisited. Genetic divergence between these clades (5.8%) was high and is on average comparable to or greater than the levels of divergence commonly found between several currently valid amphibian species pairs. For example, the level of genetic divergence exhibited by the two clades is significantly higher than the proposed threshold genetic distance (3.0 %) widely used for delineating amphibian species based on 16S rRNA gene sequences (Vences *et al*. 2005; Fouquet *et al*. 2007). Similarly, some sister species surveyed in this study show comparable to, or substantially lower, genetic divergences relative to the two clades of *E. campi*: *E. nitidus* and *E. rubrimaculatus* (5.96%), *E. nitidus* and *E. pipilans* (5.75%), *E. pipilans* and *E. rubrimaculatus* (3.53%).

In their recent morphological and molecular analyses of specimens sampled from El Nacimiento, southernmost San Luis Potosi (Mexico), Hernandez-Austria *et al*. (2022) described a new species, *Eleutherodactylus potosiensis*. Based on this study Hernandez-Austria *et al*. (2022) concluded that *E. potosiensis* is similar to *E. campi* morphologically, but their molecular data recovered it as an independent lineage from *E. campi.* Because *E. potosiensis* was not included in our analysis, we could not evaluate its relationships to the two *E campi* clades recovered in this study. However, judging from the geographic origin of Mexican *E. campi* sequences analyzed

in our study (Nuevo Leon) and Hernandez-Austria *et al*. (2022; Nuevo Leon and Tamaulipas) relative to that of *E. potosiensis* (southernmost San Luis Potosi), *E. campi* sequences examined by Hernandez-Austria *et al*. (2022) are likely to belong to the Mexican clade. If this holds true, given that *E. campi* was originally described from Brownsville (Cameroon County, Texas), Mexican populations currently included within *E. campi* could potentially represent an undescribed and possibly cryptic species. Based on the recovery of several novel evolutionary lineages independent of other described species of *Syrrhophus,* Hernandez-Austria (2022) concluded that more species are awaiting to be described in eastern Mexico. An alternative, but less plausible, explanation for the observed deep genetic divergence within *E. campi* would be that the Mexican *E. campi* specimens represented by sequences we obtained from GenBank (Accession #: MG856965 and MG856966, Grünwald *et al*., 2018) may have been misidentified. Therefore, to resolve the taxonomy of *E. campi*, further comparative analyses of morphological and ecological data for populations north and south of Rio Grande Valley and clarifying the certainty of the identity of the Mexican sequences analyzed herein would be needed.

In conclusion, in this study, we present the first assessment of genetic variation and phylogenetic interrelationships within *Eleutherodactylus campi*. Our results are not consistent with the current taxonomy that hypothesizes *E. campi* as a single widespread species. Instead, they highlight the existence of two deeply differentiated and geographically structured clades (US and Mexican clades) within *E. campi* that may represent independent species. Because we analyzed samples of *E campi* populations from both its native and derived ranges, including the type locality in Cameroon County (Texas), we consider the phylogeographic patterns described herein to likely represent real patterns that exist. However, divergence at a single mtDNA gene alone is insufficient evidence to define species boundaries, but it is a cause for new hypothesis testing. Therefore, future assessment of morphological and ecological data including advertisement calls of the two populations north and south of the Rio Grande Valley will be necessary to further refine the taxonomy of *E. campi*.

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