



Unearthing the names of burrowing frogs: The taxonomic status of the Chacoan populations of *Leptodactylus fuscus* (Schneider, 1799) (Anura: Leptodactylidae)

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Abstract

The widely distributed Neotropical frog *Leptodactylus fuscus* has long been suspected to represent a species complex. Among its numerous synonyms is *Leptodactylus gualambensis* Gallardo, 1964, described based on specimens from the western South American Gran Chaco and regarded as a typical Chacoan species. This species was synonymized 40 years ago and has not been reassessed since. In this study, we evaluate the taxonomic validity of the Chacoan populations of *L. fuscus* potentially assignable to *L. gualambensis*, based on molecular phylogenetic analyses, lineage delimitation, bioacoustics, and morphology using extensive sampling from the South American Gran Chaco and adjacent regions. Our phylogenetic analyses revealed three clades: One distributed across northern South America and Central America, including topotypes of *L. fuscus*; a second primarily occurring in the South American Gran Chaco, including topotypes of *L. gualambensis*; and a third found in parts of Paraguay, Argentina, and Brazil. This structure is corroborated by lineage delimitation analysis, genetic differentiation, gene flow estimations, and by differences in advertisement call duration and frequency, as well as head and limb proportions. Based on these results, we support the revalidation of *L. gualambensis*, redefine the geographic distribution of *L. fuscus* sensu stricto, and discuss the status of other available synonyms. In addition, we discuss variation in certain morphological traits of *L. gualambensis* that may be associated with its broad geographic distribution, which encompasses both dry, highly seasonal environments and humid, relatively homogeneous habitats.

Keywords

Dry Chaco, endemic species, Humid Chaco, *Leptodactylus gualambensis*

Introduction

The frog genus *Leptodactylus* Fitzinger, 1826 comprises 83 species distributed across southern North America, Central America, including the West Indies, and South America (Frost 2024). Currently, the genus is divided into four species groups, among which the *Leptodactylus fuscus* group is the most diverse, encompassing 35 formally named species (Silva et al. 2020; Carvalho et al. 2021). Members of the *L. fuscus* group are adapted to terrestrial environments and display distinctive reproductive traits, including oviposition in underground chambers constructed by males (Heyer 1969, 1978).

Some species of the *L. fuscus* group have wide geographic distributions across climatically contrasting ecoregions, including semiarid environments. One such widely distributed species is *Leptodactylus fuscus* (Schneider, 1799), a common frog found from Panama to northern Argentina, inhabiting open formations east of the Andes to the Atlantic coast (de Sá et al. 2014). The original types of *Rana fusca* Schneider, 1799 are lost (Heyer 1968). To address this issue, Heyer (1968) designated the specimen MNHN 680 from Suriname, northern South America, as neotype. *Leptodactylus fuscus* has repeatedly been referred to as a species complex by several authors (e.g., Maxson and Heyer 1988; Wynn and Heyer 2001; Heyer and Reid 2003; Camargo et al. 2006; Lucas et al. 2008) and has an extensive list of synonyms (see de Sá et al. 2014), most of which have a defined type locality, although with varying degrees of precision.

Among the synonyms of *L. fuscus* is *Leptodactylus gualambensis* Gallardo, 1964, originally described from Urundel, Salta Province, Argentina, in the western part of the South American Gran Chaco, specifically in the transition between the ecoregions of Dry Chaco and Yungas. This is the only name among the synonyms associated with the Chaco region. In the description of *L. gualambensis*, besides specimens from the type locality, Gallardo (1964) analyzed specimens from other Chaco localities of Argentina and Paraguay (including the Dry Chaco and Humid Chaco ecoregions), and from the Pantanal ecoregion (specifically from the states of Mato Grosso and Mato Grosso do Sul, Brazil). The author considered the species a typical inhabitant of the Chaco, naming the taxon in reference to an old name for the Chaco region, “Gran Chaco Gualamba”. Gallardo mentioned that *L. gualambensis* was commonly confused with *Leptodactylus gracilis* (Duméril & Bibron, 1840), but that was more similar to *L. fuscus* (as *Leptodactylus sibilatrix* [Wied, 1824]), distinguished from the latter by the length of the legs, shape of the head, and dorsal color pattern of the body.

Just a year after the description of *L. gualambensis*, Barrio (1965) compared advertisement calls from Mato Grosso (municipalities of Nobres and Santo Antonio de Leverger), the northern distribution of *L. gualambensis* according to Gallardo (1964), and advertisement calls from the Argentine provinces of Chaco and Corrientes, which Barrio considered as *L. fuscus*. Based on the strict

similarity between spectrograms, Barrio questioned the validity of *L. gualambensis*; however, its formal synonymy with *L. fuscus* was proposed by Heyer (1978). In a comprehensive revision of the *L. fuscus* species group, this latter author compared specimens identified by Gallardo (see Heyer 1978) as *L. gualambensis* and others from Chacoan populations with specimens of *L. fuscus* from non-Chacoan populations. Although discriminant analyses using morphometric measurements showed some level of differentiation of the Chacoan populations, Heyer (1978) considered it within the variation range of *L. fuscus*.

As mentioned previously, several authors have found evidence supporting the idea that *L. fuscus* could represent more than one species. Heyer and Diment (1974) found some cytogenetic differences among specimens from Embarcación, Salta Province, Argentina (in the western Dry Chaco ecoregion) and São José do Rio Preto, São Paulo State, Brazil (in the Cerrado ecoregion). Later, Wynn and Heyer (2001) included samples from western Dry Chaco (Embarcación and Joaquín V. González, Salta Province, Argentina) in a wide sampling, specifically to test the existence of more than one species under *L. fuscus*. Using 24 allozyme loci, they found three genetic units: northern South America (Trinidad and Tobago, the Brazilian state of Roraima, and French Guiana), Panama, and southern South America (Argentina, Bolivia, and other parts of Brazil). Although the two Chaco localities were clustered together in all analyses, there was no clear differentiation between them and the samples from other localities. Based on these results, Heyer and Reid (2003) assessed the correlation between geographic variation in advertisement calls and genetic differentiation. They included calls from only one Chaco locality, Embarcación, and although some geographical differentiation was found, it was not associated with the genetic structure found by Wynn and Heyer (2001). Also testing the hypothesis of a species complex, Camargo et al. (2006) identified three main clades without geographic overlap based on the sampling of Wynn and Heyer (2001) but using sequences of mitochondrial genes. These clades correspond to Central America and northern South America (north of the Amazonas River), including Panama, Trinidad and Tobago; western South America (populations from Argentina and Bolivia); and populations from the eastern, southwestern, and southern parts of the species range (see Camargo et al. 2006). Additionally, Lucas et al. (2008) found some differences in reproductive ecology among populations of the different genetic groups identified by Camargo et al. (2006). However, despite showing genetic structure and other evidence supporting that *L. fuscus* is a species complex, no taxonomic rearrangement was suggested in any of the mentioned studies.

In the phylogenetic assessment of the genus *Leptodactylus* by de Sá et al. (2014), part of the sampling of Wynn and Heyer (2001) was included, recovering a polyphyletic *L. fuscus*. The two Chaco samples and two non-Chaco samples from Bolivia clustered together as sister to a clade that includes all samples of *Leptodactylus mystaceus* (Spix, 1824). However, Schneider et al.

(2019), while focusing on the taxonomy of *Leptodactylus mystacinus* (Burmeister, 1861), included part of the *L. fuscus* samples from de Sá et al. (2014) (excluding certain sequences due to low quality or high levels of missing data), recovering all *L. fuscus*, including those from Chaco populations, in a well-supported clade. These last two studies, not focused on the taxonomy of *L. fuscus*, employed essentially the same sequences of *L. fuscus*, which included only two from Chaco localities, and a few others from localities across Brazil, Bolivia, and Guyana.

In the present work, we assessed the taxonomic validity of the Chacoan populations of *L. fuscus* that could be assignable to *L. gualambensis* Gallardo, 1964. To achieve this, we improved both the sampling in Chaco localities and adjacent areas (i.e., potential *L. gualambensis*) and the sampling of several other localities to cover the known range of *L. fuscus*, including its type locality. Although Gallardo (1964) provides a detailed description based on a broad and widely geographic sampling, the diagnosis was focused only on comparing *L. gualambensis* with *L. gracilis* and *L. fuscus* (as *L. sibilatrix*). Taking this into account, we also provide an expanded diagnosis of *L. gualambensis* in the context of the entire genus *Leptodactylus*. The other names under the synonymy of *L. fuscus* are beyond the scope of the current work; however, we discuss them in the context of our resulting phylogenetic topology. We found that the clarification of the taxonomic status of *L. gualambensis* is an important step to better understand and describe the diversity within the *L. fuscus* species complex.

Materials and methods

Species concept

We follow the unified species concept, considering a species as an independent evolutionary lineage with consistent diagnostic traits (e.g., morphological, behavioral, genetic; de Queiroz 2005, 2007). Species delimitation based solely on genetic data is likely inadequate (Carstens et al. 2013). To address this, taxonomic decisions were made based on a combination of information sources, including mitochondrial, morphological, and acoustic data, which represent independent lines of evidence.

Molecular phylogenetic analyses

To assess the taxonomic status of Chaco populations of *L. fuscus* that could be assignable to *L. gualambensis* and its phylogenetic position within *Leptodactylus*, we included representatives of all species groups recognized for the genus by de Sá et al. (2014), with special emphasis on the *L. fuscus* species group. Considering the broad distribution of *L. fuscus* and its recognition as a species complex, we included sequences of specimens from several localities covering the geographic distribution of the

species, including the type locality. Given our aim of testing the taxonomic validity of the Chaco populations of *L. fuscus*, we focused efforts on sampling from the South American Gran Chaco and its surroundings (including samples from the *L. gualambensis* type locality), following the geographic distribution of the species suggested by Gallardo (1964) (Fig. 1; File S1). Representation of other taxa from the *L. fuscus* group included samples from *Leptodactylus aepitya* Schneider et al., 2019, *Leptodactylus avivoca* Carvalho et al., 2021, *Leptodactylus barrioi* Silva et al., 2020, *Leptodactylus bufonius* Boulenger, 1894, *Leptodactylus camaquara* Sazima & Bokermann, 1978, *Leptodactylus cunicularius* Sazima & Bokermann, 1978, *Leptodactylus cupreus* Caramaschi, Feio & São Pedro, 2008, *Leptodactylus didymus* Heyer, García-López & Cardoso, 1996, *Leptodactylus elenae* Heyer, 1978, *Leptodactylus fragilis* (Brocchi, 1877), *Leptodactylus furnarius* Sazima & Bokermann, 1978, *L. gracilis*, *Leptodactylus jolyi* Sazima & Bokermann, 1978, *Leptodactylus kilombo* Silva et al., 2020, *Leptodactylus labrosus* Jiménez de la Espada, 1875, *Leptodactylus laticeps* Boulenger, 1918, *Leptodactylus latinasus* Jiménez de la Espada, 1875, *Leptodactylus longirostris* Boulenger, 1882, *Leptodactylus marambaiae* Izecksohn, 1976, *Leptodactylus mystaceus* (Spix, 1824), *L. mystacinus*, *Leptodactylus notoaktites* Heyer, 1978, *Leptodactylus oreomantis* Carvalho, Leite & Pezzuti, 2013, *Leptodactylus plaumanni* Ahl, 1936, *Leptodactylus poecilochilus* (Cope, 1862), *Leptodactylus syphax* Bokermann, 1969, *Leptodactylus tapiti* Sazima & Bokermann, 1978, *Leptodactylus troglodytes* Lutz, 1926, *Leptodactylus ventrimaculatus* Boulenger, 1902, and *Leptodactylus watu* Silva et al., 2020. From the other groups of *Leptodactylus* we included: *Leptodactylus insularum* Barbour, 1906 and *Leptodactylus latrans* (Steffen, 1815) from the *Leptodactylus latrans* group; *Leptodactylus colombiensis* Heyer, 1994 and *Leptodactylus podicipinus* (Cope, 1862) from the *Leptodactylus melanonotus* group; *Leptodactylus labyrinthicus* (Spix, 1824) and *Leptodactylus pentadactylus* (Laurenti, 1768) from the *Leptodactylus pentadactylus* group. External to *Leptodactylus* we included samples of *Adenomera thomei* (Almeida & Angulo, 2006) and *Lithodytes lineatus* (Schneider, 1799) (Leptodactylinae); *Physalaemus cuvieri* Fitzinger, 1826 and *Engystomops freibergeri* (Donoso-Barros, 1969) (Leiuperinae), *Paratelmatobius poecilogaster* Giaretta & Castanho, 1990, *Crossodactylodes septentrionalis* Teixeira et al., 2013, and *Scythrophrys sawayae* (Cochran, 1953) (Paratelmatobiinae), and to root the trees, *Hyalinobatrachium fleischmanni* (Boettger, 1893).

We extracted total genomic DNA from samples conserved in 100% ethanol (muscle or liver) using the DNeasy extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. We used gene fragments of the 16S ribosomal RNA gene (16S; up to 580 base pairs [bp] per terminal) and of the cytochrome c oxidase subunit I (COI; up to 597 bp per terminal), two markers commonly used in taxonomic approaches (Vences et al. 2005) and proposed as barcoding fragments (see Lyra et al. 2017). For 16S we used the primers 16S-AR

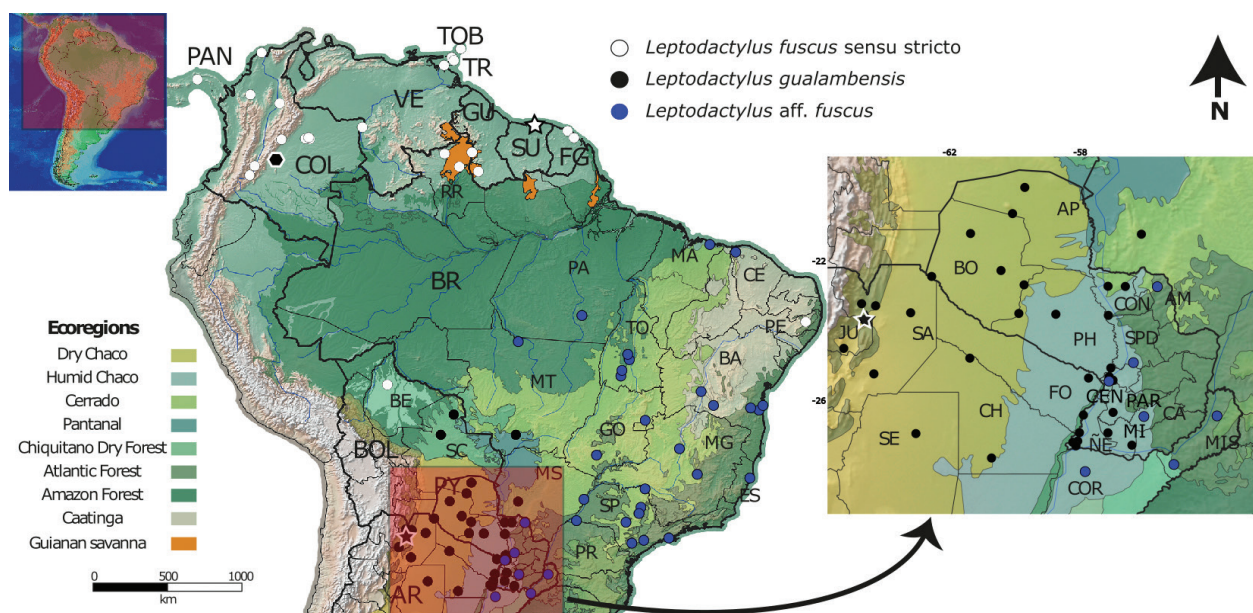


Figure 1. Sample sites for molecular analyses. *Leptodactylus fuscus sensu stricto*, *Leptodactylus gualambensis*, and *Leptodactylus aff. fuscus* correspond to mitochondrial clades (see Fig. 2). White star indicates *Leptodactylus fuscus* type locality; black star indicates *Leptodactylus gualambensis* type locality; black hexagon indicates *Leptodactylus raniformis* type locality. Relevant localities are indicated with the following abbreviations. Countries: AR (Argentina), BOL (Bolivia), BR (Brazil), COL (Colombia), FG (French Guiana), GU (Guyana), PAN (Panamá), PY (Paraguay), SU (Suriname), TOB (Tobago), TR (Trinidad), VE (Venezuela). Provinces of Argentina: CH (Chaco), COR (Corrientes), FO (Formosa), JU (Jujuy), MIS (Misiones), SA (Salta), SE (Santiago del Estero). Departments of Bolivia: BE (Beni), SC (Santa Cruz). States of Brazil: BA (Bahia), CE (Ceará), ES (Espírito Santo), GO (Goiás), MA (Maranhão), MT (Mato Grosso), MS (Mato Grosso do Sul), MG (Minas Gerais), PA (Pará), PR (Paraná), PE (Pernambuco), SP (São Paulo), TO (Tocantins), RR (Roraima). Departments of Paraguay: AP (Alto Paraguay), AM (Amambay), BO (Boquerón), CA (Caazapá), CEN (Central), CON (Concepción), MI (Misiones), NE (Neembucú), PAR (Paraguari), PH (Presidente Hayes), SPD (San Pedro).

(CGCCTGTTTATCAAAAACAT; Palumbi et al. 1991) and 16S-Wlk2 (GACCTGGATTACTCCGGTCTGA; Wilkinson et al. 1996), and for COI, AnF1 (ACHAAY-CAYAAAGAYATYGG; Lyra et al. 2017) and AnR1 (CCGGTCTGAACTCAGATCACGT, Lyra et al. 2017). For fragment amplifications through polymerase chain reactions (PCR) we used a commercial kit (Master Mix, Thermo Fisher; Waltham, MA, USA). For the 16S fragment, the protocol included an initial denaturation step of 3 min at 94°C; followed by 35 cycles consisting in 30 s at 94°C for denaturation, 30 s at 50°C for annealing, and 90 s at 72°C for extension; the final step consisted in 7 min at 72°C for extension. For the COI fragment, the protocol consisted in an initial denaturation step of 3 min at 95°C; followed by ten cycles consisting in 20 s at 95°C for denaturation, 20 s at 50°C (increasing +0.5°C in each subsequent cycle) for annealing, 50 s at 60°C for extension; the next step consisted in 25 cycles including 15 s at 95°C for denaturation, 20 s at 50°C for annealing, 50 s at 60°C for extension; the final step consisted in 5 min at 60°C for extension (Lyra et al. 2017). We sent PCR products for purification and sequencing to Macrogen Inc. (Seoul, South Korea). We checked chromatograms and edited the sequences in CodonCode Aligner 3.5.4 (Codon Code Corporation). See File S1 for GenBank accession numbers.

We aligned both 16S and COI sequences with MAFFT (Katoh and Toh 2008). The 16S sequences were aligned

with the Q-INS-i strategy (which considers RNA secondary structure) and the COI sequences with the G-INS-i strategy (which considers global homology). All other parameters were set as default. Sequence files were merged with SequenceMatrix (Vaidya et al. 2011).

To reconstruct phylogenetic trees, we used Bayesian inference (BI) and maximum parsimony (MP) as criteria. While parsimony allows the usage of gaps as a fifth state of character (i.e., it considers the value of indels in the evolutionary process; Giribet and Wheeler 1999; Goloboff and Pol 2005), the Bayesian approach permits the implementation of distinct evolutionary models for distinct DNA fragments, acknowledging that these fragments may be subject to different evolutionary pressures (Ronquist and Deans 2010). To construct trees under BI, we first used PartitionFinder v2.1.1 (Lanfear et al. 2016) to infer the best partition scheme and the evolutionary model that best fits each partition. Branch lengths were treated as linked, and the analysis considered only the models employed by MrBayes 3.2 (Ronquist et al. 2012). We analyzed 16S and codon positions of COI as distinct partitions. The best models were selected under the corrected Akaike information criterion (AICc) using the “greedy” heuristic search algorithm and PhyML software (Guindon et al. 2010). We performed the BI analysis in MrBayes v3.2, implementing the inferred models of nucleotide substitution on two independent runs, each one with four chains, sampling every 3,000 generations for

30 million generations and discarding the first 2,500 trees as burn-in. We verified convergence with TRACER v1.6 (Rambaut et al. 2014), and by examining the standard deviation of split frequencies between independent runs (<0.01), Estimated Sample Size (values >100), and Potential Scale Reduction Factor (values ~ 1). Node support was estimated as posterior probability (PP).

For MP analyses, we used T.N.T. Willi Hennig Society Edition (Goloboff et al. 2003, 2008; Goloboff and Catalano 2016) in the command line version. Searches were conducted with new technology search level 5, finding the best score 100 times. Gaps were treated as fifth state. To infer branch support, we calculated bootstrap (BT) as absolute frequencies (Farris et al. 1996) using new technology search under level 2 and hitting the best score 5 times, totaling 1,000 replicates. These parameters (level of search and best score hit number) were chosen based on achieving the best length in preliminary analyses. BI and MP trees were edited with FigTree (Rambaut 2014). We considered MP bootstrap (BT) values $\geq 70\%$ and BI posterior probabilities (PP) above 0.95 to indicate strongly supported clades (Hillis and Bull 1993; Alfaro et al. 2003; Erixon et al. 2003).

We calculated uncorrected p distance (proportion of nucleotide sites at which two compared sequences are different) for both gene fragments, within and between clades containing *L. fuscus* specimens using MEGA 7 (Kumar et al. 2016), with the “pair-wise deletion” option of gaps/missing data treatment.

Lineage delimitation, genetic differentiation and gene flow

To define independent evolving lineages, we used the multispecies coalescent model (Rannala and Yang 2003; Edwards 2009; Yang and Rannala 2014) implemented in BPP v4.6.2 (Yang and Rannala 2010). We applied the analysis A11 that estimates the number of populations and topology of trees simultaneously. For this analysis we used a matrix that contained mitochondrial sequences of all *L. fuscus* specimens, and a guide tree based on the lineages found in phylogenetic analyses. We estimate priors for population size (θ) and divergence time (τ) with Minimalist BPP (<https://brannala.github.io/bpps>). We ran two replicates with the two rjMCMC algorithms (0 and 1) 100,000 generations, with 8,000 generations discarded as burn-in, with automatic finetune.

Based on the BPP delimitation, we estimated genetic differentiation (F_{ST}) and gene flow (Nm, derived from F_{ST}) between lineages, analyzing each gene fragment separately using DnaSP 5.10.01 (Librado and Rozas 2009). Following Wright (1965) we considered values of F_{ST} between 0 and 0.05 as indicative of no genetic differentiation, between 0.05 and 0.15 as indicative of limited genetic differentiation, between 0.15 and 0.25 as indicative of moderate genetic differentiation, and larger than 0.25 as indicative of substantial genetic differentiation. Regarding Nm, values <1 indicate that the differentiation between populations is due to genetic drift (i.e., no gene

flow), >1 indicates high gene flow and low genetic differentiation, and >4 indicates nearly complete gene flow and very low differentiation (Grant and Bowen 1998).

We also estimated gene flow using the Bayesian algorithm implemented in Migrate-n 5.0.4 (Beerli and Felsenstein 1999, 2001). Both mtDNA fragments were treated as a single dataset, and migration was estimated in both directions among all lineages (i.e., a full migration model). Priors for the mutation-scaled population size (θ) were uniform, ranging from 0 to 0.1, and for the mutation-scaled migration rate (M), from 0 to 3,000. Following a burn-in of 50,000 steps, 50,000 steps were recorded every 500 steps, resulting in a total of 25,000,000 genealogies sampled. Three heated chains were run with temperatures of 1.0, 1.5, and 3.0, and 1,000,000. The number of migrants per generation (Nm) was calculated using the formula: $Nm_{(ij)} = \theta_j * M_{(ij)}$.

The datasets used in phylogenetic analyses, lineage delimitation, genetic differentiation, and gene flow are available at https://github.com/franbrusquetti/chacoan_leptodactylus_taxonomy.

Morphological analyses

As in our molecular phylogenetic analyses, we placed special emphasis on the South American Gran Chaco and its surrounding areas, including topotypes of *L. gualambensis* (213 specimens). We also included specimens of *L. fuscus* from various localities, including the type locality of the species (55 specimens; see File S2). We compared specimens assignable to *L. gualambensis* with all the other *Leptodactylus* species. For species for which it was not possible to study specimens, we based comparisons on the pertinent literature, including original species descriptions and taxonomic revisions. Comparisons with *L. fuscus* sensu stricto were restricted to specimens from Suriname (type locality) and surrounding areas, in concordance with our phylogenetic results. Specimens initially identified as *L. fuscus*, which, based on the phylogenetic results, were not considered as *L. fuscus* sensu stricto, were also compared, but as *L. aff. fuscus* (407 specimens).

We based the comparisons between species in structures frequently used in taxonomic studies in terms of presence/absence, shape, position, size relative to other structures, and color pattern, following the terminology of Duellman (1970), Cei (1980), Heyer (1978), Heyer et al. (1990), and de Sá et al. (2014). We took the following measurements based on Duellman (1970): Snout–vent length (SVL), from the tip of the snout to the vent; head length (HL), from the tip of the snout to the posterior edge of the jaw articulation; head width (HW), measured at the level of the jaw articulation; tympanum diameter (TD), measured horizontally; eye diameter (ED), measured horizontally; interorbital distance (IOD), distance between the interior margins of the eye bulges; eye–nostril distance (EN), from distal corner of the orbit to proximal margin of external nostril; internostril distance (IND), between the interior margins of the nostrils; eye–snout distance (ESD),

from the tip of the snout to the distal corner of the eye; forearm length (FAL), from elbow to the proximal margin of the thenar tubercle; thigh length (THL), from vent to the knee; shank length (SHL), from knee to heel; and tarsus length (TAL), from the tibiotarsal articulation to the proximal margin of the internal tarsal tubercle. We measured specimens with digital calipers, to the nearest 0.1 mm under a dissecting microscope. To avoid ontogenetic morphological variation, we measured only adult specimens. Adult males were determined by visual examination of secondary sexual characters (i.e., presence of vocal sac and vocal slits). Adult females were determined by the presence of ovarian follicles (when possible, i.e., abdominal cavity opened) coupled with the absence of vocal sac and vocal slits. Alternatively, we considered specimens with over 40 mm SVL as adults, since all confirmed adults were larger than this. Final datasets for morphometric analyses consisted in 11 males and 10 females of *L. fuscus* sensu stricto, 49 males and 33 females of *L. gualambensis*, and 33 males and 33 females of *L. aff. fuscus*.

To assess differentiation between the clades, we performed a Principal Component Analysis (PCA) and Canonical Discriminant Analysis (CDA). PCA was applied as an unsupervised method to explore patterns of multivariate variation, while CDA aimed to maximize discrimination between a priori defined groups based on mitochondrial clades. CDA plots included individual scores, group centroids, and spider diagrams connecting individuals to their respective group centroids. To reduce dimensional redundancy, pairwise Spearman correlation coefficients were calculated, and variables showing high collinearity ($\rho > 0.75$) were excluded. Additionally, variables with very low contributions to the first canonical axes in preliminary CDA analyses were also removed, as they did not contribute meaningfully to group discrimination. We also determine differences of each measurement between the three clades. We first tested each variable for normality with the Shapiro-Wilk test (Shapiro and Wilk 1965). As not all variables had a normal distribution, we used the non-parametric Kruskal-Wallis test (Kruskal and Wallis 1952). For each variable with significant difference, we applied the Dunn test (Dunn 1961) with Bonferroni correction, as a post-hoc test to detect between-clade differences. All morphometric analyses were conducted with the MorphoTools2 package (Šlenker et al. 2022) in R (R Core Team 2023) separately for males and females.

Advertisement call analysis

We analyzed advertisement call records from specimens identified as *L. fuscus* from its type locality (Suriname), from the type locality of *L. gualambensis* (Urundel, Argentina) and from other localities through the known distribution of the species (File S3). The records were digitized with 16-bit resolution and 44,100 Hz sampling rate using the software Raven Pro 1.6 (Cornell Ornithology Lab). We produced audio-spectrograms and oscillograms, and measured the selected variables with Fast Fourier Transformation, using a window of 256, and contrast and

brightness of 50%. We estimated call duration (in seconds), dominant frequency (as the peak frequency function of the software, in Hz), 5% frequency (in Hz), 95% frequency (in Hz), 90% bandwidth (difference between 95% and 5% frequency, in Hz), low frequency (in Hz), high frequency (in Hz), and the difference between high and low frequencies (delta frequency, in Hz). The terminology and the “call centered” approach were applied following Köhler et al. (2017). Figures were generated with the R package seewave 2.2.3 (Sueur et al. 2008).

To assess call differentiation between the clades, we first tested each variable for normality with the Shapiro-Wilk test. As no variable had a normal distribution, we used the non-parametric Kruskal-Wallis test to determine differences of each bioacoustics variable between the three clades. We applied the Dunn test as a post-hoc test for the variables with significant difference to detect between-clade differences. The mentioned tests were conducted with the MorphoTools2 package in R.

Results

Molecular phylogenetic analyses

The best fit model for 16S and for COI codon 1 was GTR (General time reversible) + I (proportion of invariable sites) + G (gamma distribution), for COI codon 2 was F81 + I, and for COI codon 3 was GTR + G. Overall, both BI (Figs 2, S1) and MP (Fig. S2) analyses resulted in very similar topologies. The “*Leptodactylus fuscus* species complex” is recovered as monophyletic and well supported in both analyses (PP 1.0, BT 96%) and structured in three main clades, of which two were well supported and one poorly supported. The well supported clades correspond to two main geographic areas: Central America + northern South America (*L. fuscus* clade 1; PP 0.97, BT 79%) and southwestern South America (southeastern Bolivia, southwestern Brazil, western Paraguay, northern and northwestern Argentina; *L. fuscus* clade 2; PP 1.0, BT 84%). The unsupported clade contains the rest of the samples, which are from eastern Paraguay, northeastern Argentina, and northern, central and southeastern Brazil (*L. fuscus* clade 3; PP 0.86, BT < 50%). The relationships among the three clades were not well supported.

Regarding the *L. fuscus* clade 1, two samples, geographically distant from all the other samples of the clade, were clustered together, nested within in MP analysis, and as sisters of the rest of the clade in BI. One of the samples is from Beni department, northern Bolivia (MJ 1357) and the other is from Pernambuco state, northeastern Brazil (USNM 284551). Besides the above-mentioned samples, the *L. fuscus* clade 1 grouped all samples from Panama, Trinidad, Tobago, Venezuela, Guyana, French Guiana, Colombia, Roraima State in Brazil, and Suriname, type locality of *L. fuscus*. The *L. fuscus* clade 2 grouped all samples from Dry Chaco, western Humid Chaco, northern Pantanal, southern Cerrado, and Chiquitano dry for-

est, including samples from the *L. gualambensis* type locality (Urundel, Salta Province, Argentina). The *L. fuscus* clade 3 includes all samples from southern State of Bahia, Brazil (southern Caatinga and Bahia coastal forest), Cerrado and transition with southern Caatinga and Upper Parana Atlantic Forest.

The uncorrected pairwise genetic distances (p distances) between clades in both gene fragments were similar, with means around 3% in 16S and 10% in COI. Within the clades, we found larger internal genetic distances in the *L. fuscus* clade 3, with values up to 3.3% in 16S and 7.5% in COI (Table 1).

Lineage delimitation

Lineage delimitation using BPP recovered the same three lineages identified in the phylogenetic analyses. All runs, two replicates using algorithms 0 and 1, recovered the *L. fuscus* lineages with posterior probabilities of 1.0. The same tree topology as in the phylogenetic analyses was also consistently recovered (Table 2).

Genetic differentiation (F_{ST}) among all populations showed similar values for both gene fragments, ranging from 0.55 to 0.68, indicating substantial genetic differentiation. Nm values based on F_{ST} were <1 for all lineage comparisons, suggesting that differentiation is mainly driven by genetic drift (i.e., no gene flow). Migrate-n results support low immigration rates, with mean Nm values among populations ranging from 0.47 to 0.59 individuals per generation (Table 3).

Morphological analysis

We identified some character states that distinguished specimens attributable to the different clades, corresponding to shape-related characters, such as snout shape in dorsal view, which is rounded to nearly rounded in individuals of the *L. fuscus* clade 2, but sub-elliptical to subovoid in the *L. fuscus* clade 1 (Fig. 3).

Regarding morphometric analyses, the CDA revealed clear morphological separation among genetic groups in both sexes. In males, the first canonical axis (Can1) ex-

Table 1. Genetic distances between and within each of the *Leptodactylus fuscus* main clades. Minimum, maximum, and mean (in parenthesis). Values in percentage (%).

COI	<i>L. fuscus</i> clade 1	<i>L. fuscus</i> clade 2	<i>L. fuscus</i> clade 3
<i>L. fuscus</i> clade 1	0–6.1 (3.7)	—	—
<i>L. fuscus</i> clade 2	9.5–12 (11)	0–4.9 (1.6)	—
<i>L. fuscus</i> clade 3	7.7–12.6 (10.4)	6.9–10.8 (9.2)	0–7.5 (3.2)
16S	<i>L. fuscus</i> clade 1	<i>L. fuscus</i> clade 2	<i>L. fuscus</i> clade 3
<i>L. fuscus</i> clade 1	0–2.4 (1.1)	—	—
<i>L. fuscus</i> clade 2	1.8–4.6 (3.1)	0–2 (0.5)	—
<i>L. fuscus</i> clade 3	1.7–5.3 (3.3)	1.3–4.0 (2.6)	0–3.3 (1.4)

Table 2. Summary of BPP analysis results. ‘rj’ indicates the rjMCMC algorithm used, ‘ θ ’ and ‘ τ ’ are the inverse-gamma priors IG (α , β) for theta (population size) and tau (divergence time) parameters, ‘rep’ indicates the replicates performed, ‘PPt’ is the posterior probability for the best models of species tree and ‘PPd’ for the best models of lineage delimitation, ‘N’ is the number of species delimited.

rj	θ	τ	rep	PPt	Best model	PPd	N
0	3, 0.039	3, 0.25	1	0.81	(A,(B,C))	1.00	3
0	3, 0.039	3, 0.25	2	0.82	(A,(B,C))	1.00	3
1	3, 0.039	3, 0.25	1	0.78	(A,(B,C))	1.00	3
1	3, 0.039	3, 0.25	2	0.79	(A,(B,C))	1.00	3

Table 3. Estimates of genetic differentiation (F_{ST}), gene flow (Nm, based on F_{ST}), and immigrants per generation (Nm) between the *Leptodactylus fuscus* lineages: Population 1 (*L. fuscus* clade 1), Population 2 (*L. fuscus* clade 2), Population 3 (*L. fuscus* clade 3).

	Nm (Migrate-n)			COI		16S	
	mean	2.5%	97.5%	F_{ST}	Nm (F_{ST})	F_{ST}	Nm (F_{ST})
Pop. 2–1	0.52	0	4.4	0.63	0.29	0.68	0.24
Pop. 3–1	0.58	0	4.4	0.55	0.41	0.6	0.33
Pop. 1–2	0.47	0	4.13	—	—	—	—
Pop. 3–2	0.53	0	4.34	0.64	0.27	0.58	0.36
Pop. 1–3	0.53	0	5.92	—	—	—	—
Pop. 2–3	0.59	0	6.08	—	—	—	—

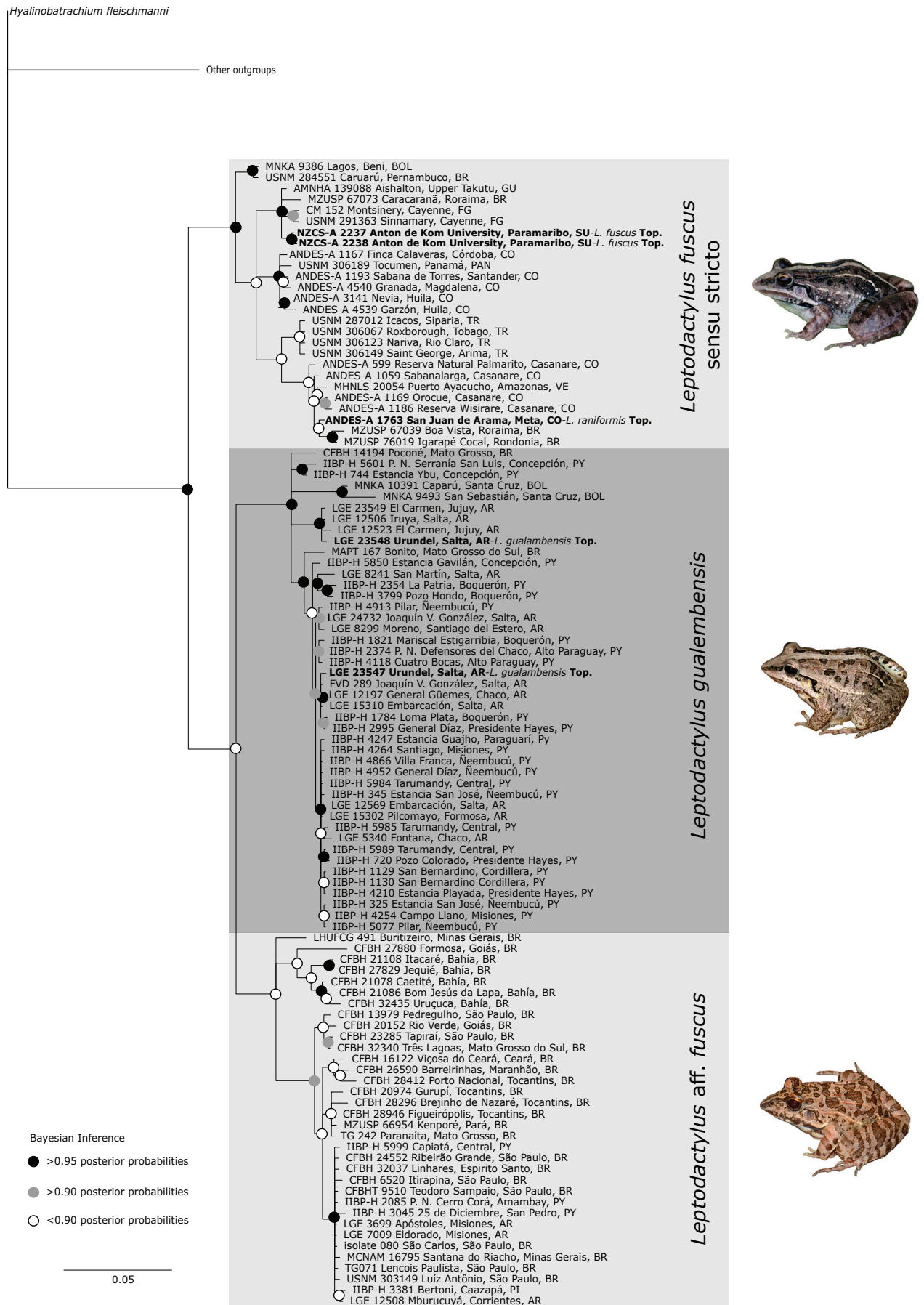


Figure 2. Bayesian inference tree. The 50% majority-rule consensus tree of concatenated mitochondrial fragments (16S ribosomal RNA [16S] and cytochrome *c* oxidase subunit 1 [CO1]). Node dots indicate different ranges of support values of BI posterior probabilities. Country abbreviations: AR (Argentina), BOL (Bolivia), BR (Brazil), Colombia (CO), French Guiana (FG), Guyana (GU), PAN (Panamá), PY (Paraguay), SU (Suriname), TR (Trinidad and Tobago), VE (Venezuela).

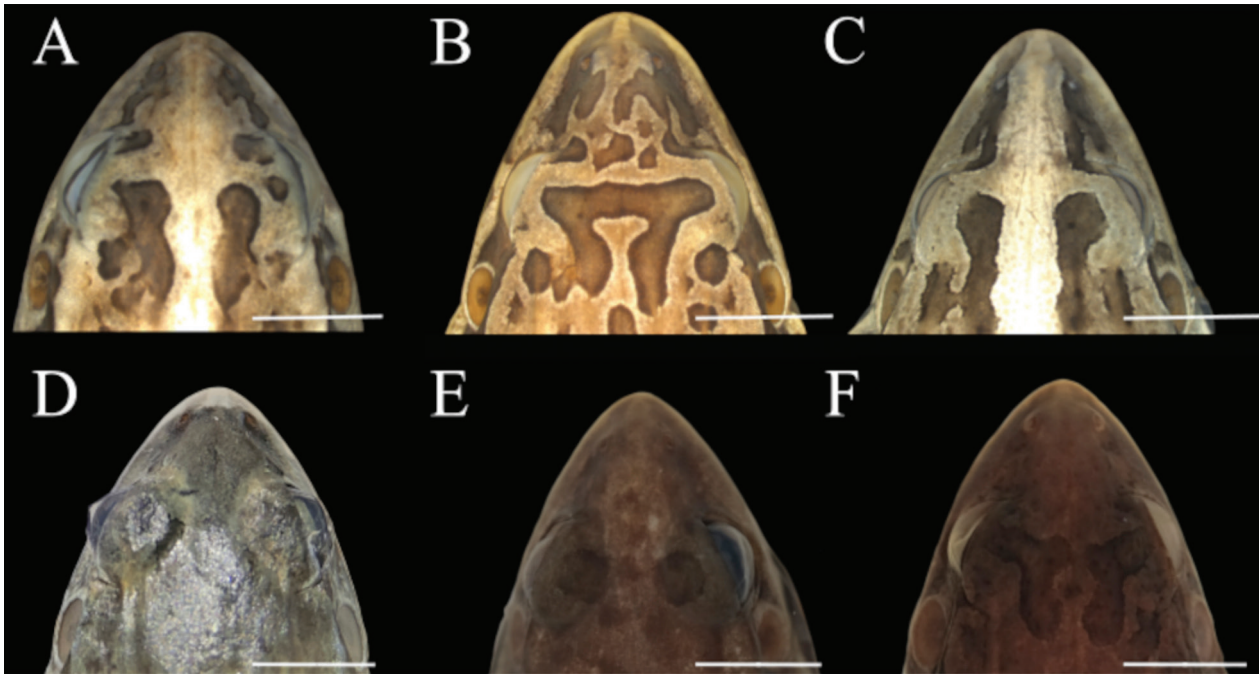


Figure 3. Dorsal view of the head of *Leptodactylus gualambensis* (*L. fuscus* clade 2; **A, B, C**) and *L. fuscus* sensu stricto (*L. fuscus* clade 1; **D, E, F**). **A** LGE 23548, Urundel, Salta, Argentina, male, SVL 44.19 mm (topotype); **B** IIBP-H 720, Pozo Colorado, Presidente Hayes, Paraguay, male, SVL 42.4 mm; **C** IIBP-H 5984, Tarumandy, Central, Paraguay, male SVL 42.33 mm; **D** NZCS-A 2339, Anton de Kom University, Paramaribo, Suriname, male, SVL 38.81 mm; **E** MZUSP 65632, Ilha de Maraca, Roraima, Brazil, male, SVL 39.70 mm; **F** MZUSP 58252, Kourou, French Guiana, male, SVL 41.98 mm. Scale bar = 5 mm.

plained 69.2% of the total variation and was primarily associated with HL (0.81), SVL (−0.68), and IND (0.44), while the second axis (Can2, 30.8%) was most influenced by SHL (−0.94), IOD (0.56), and ESD (−0.72) (Fig. 4A, B; Table 4). In females, Can1 explained 72.8% of the variation and was driven by HL (−0.75) and IOD (0.37), while Can2 (27.2%) was influenced by SHL (−0.78), ED (−0.58), and TD (−0.50) (Fig. 4C, D; Table 4). The PCA supported these general patterns, although group overlap was greater. In males, PC1 and PC2 together explained 54.7% of the variance, with HL (−0.39), ESD (−0.39), SHL (−0.37), and EN (−0.32) loading most heavily on PC1 (Fig. S3A, B; Table S1A). In females, PC1 and PC2 explained 51.0% of the variance, with HL (−0.44), EN (−0.46), and SHL (−0.37) as the dominant contributors to PC1 (Fig. S3C, D; Table S3B). In Kruskal-Wallis and Dunn tests we found statistically significant differences in some morphometric proportions among the members of the different clades. For example, members of the *L. fuscus* clade 2 tend to have a proportionally shorter and wider head than members of the *L. fuscus* clade 1 ($p < 0.01$), and proportionally shorter shanks than members of the *L. fuscus* clade 3 ($p < 0.01$; Fig. 5; Tables 5, 6).

Advertisement call analysis

The advertisement call corresponding to the three clades is structurally the same. It consists of a short whistle-like note with an increasing frequency throughout most of the call, except at the end, where it shows a slight drop. The call also has harmonic structure with up to three addition-

al harmonic bands, being the first one the most energetic (Fig. 6). In the Kruskal-Wallis test we found statistically significant differences between the three clades in all the bioacoustics variables (Tables 7, 8). Based on the post-hoc test (Dunn-test) the *L. fuscus* clade 2 had advertisement calls with shorter duration than the others, as well as higher delta frequency. *Leptodactylus fuscus* clade 3 had lower frequency and longer duration than the others (Table 8).

From the morphometric and bioacoustics variables that significantly differentiated the clades, we proposed as diagnostic characters only those with null or low overlap values. These include head length, leg length, advertisement call duration, and advertisement call frequency (Figs 5, 7).

Taxonomic conclusions

Based on the nesting position of the topotypes of *L. fuscus* (NZCS-A 2237–8 from Surinam) within the *L. fuscus* clade 1, we consider all samples of that clade as *L. fuscus* sensu stricto, restricting the distribution of the species to the northern South America and Central America, from northern Bolivia to Panama (Fig. 1). Similarly, all topotypes of *L. gualambensis* (LGE 23547–9 from Urundel, Salta, Argentina) were nested within the *L. fuscus* clade 2. Based on these results, together with morphological and bioacoustic evidence, we propose the revalidation of *Leptodactylus gualambensis* Gallardo, 1964, restricting its distribution to the Dry Chaco, its transition with the Yungas, the Humid Chaco, the Pantanal, and the south-

Table 4. Canonical structure coefficients for the first two axes of the canonical discriminant analysis (CDA) performed on the *Leptodactylus fuscus* clades based on morphometric variables. SVL = snout–vent length; HL = head length; HW = head width; TD = tympanum diameter; ED = eye diameter; IOD = interorbital distance; EN = eye–nostril distance; IND = internostril distance; ESD = eye–snout distance; FAL = forearm length; THL = thigh length; SHL = shank length. Loadings above ± 0.4 in bold indicate variables with the strongest contributions to the canonical axes. Missing values (–) indicate variables excluded from the respective model.

Variable	Can1 (♂)	Can2 (♂)	Can1 (♀)	Can2 (♀)
SVL	–0.676	0.176	0.322	–0.135
HL	0.806	–0.222	–0.752	–0.025
HW	—	—	0.333	0.179
TD	0.299	–0.179	0.019	–0.503
ED	–0.203	–0.069	–0.188	–0.576
IOD	–0.211	0.564	0.374	0.097
EN	0.139	–0.253	–0.198	–0.471
IND	0.441	–0.270	—	—
ESD	0.065	–0.715	—	—
FAL	—	—	–0.144	0.335
THL	–0.041	–0.566	–0.058	–0.423
SHL	0.162	–0.941	–0.353	–0.777

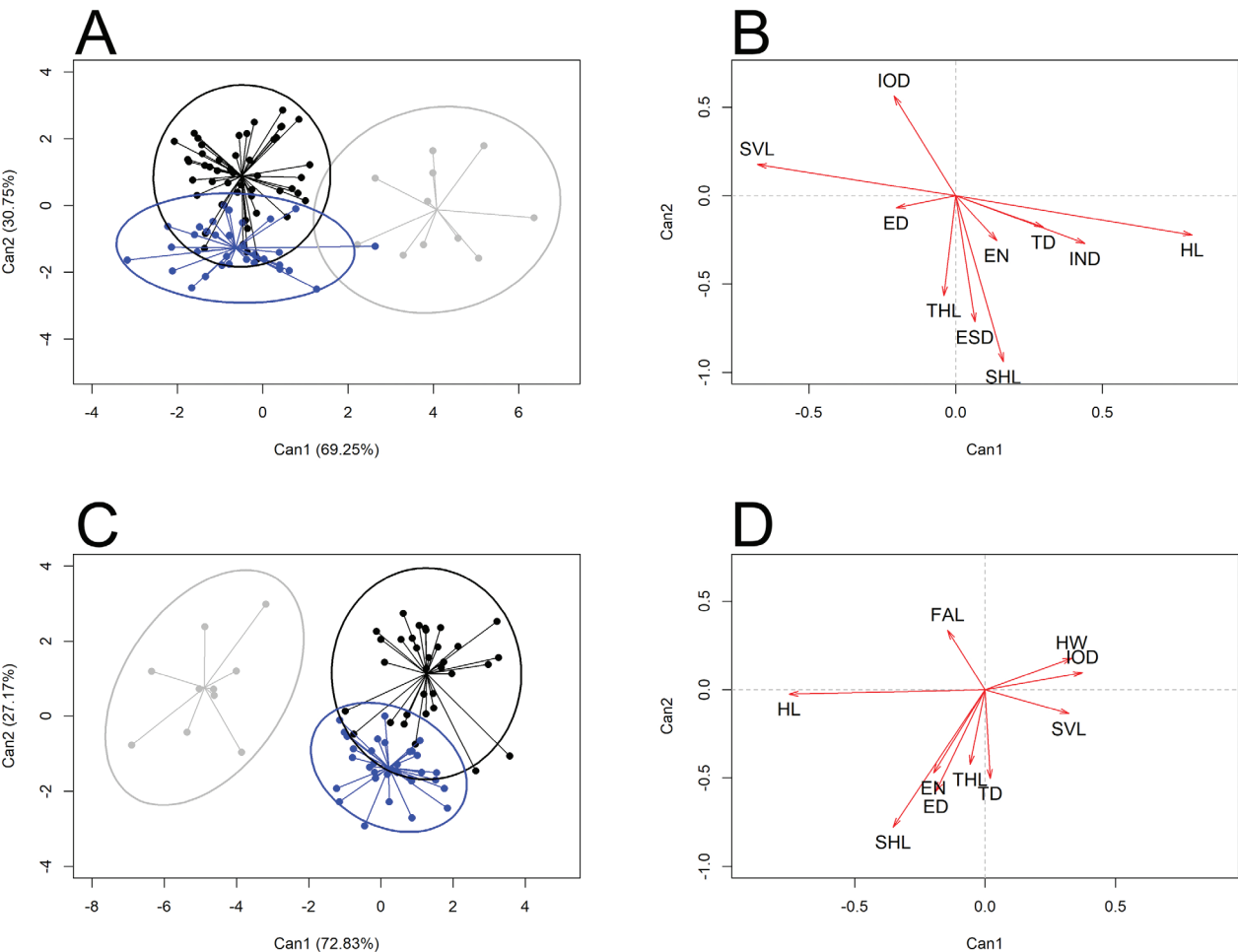


Figure 4. Canonical Discriminant Analysis (CDA) based on morphometric measurements. **A** scatterplots of individual male specimens, with points connected to the centroid of their assigned group **B** relative contribution of each morphometric variable to the first two canonical functions for males **C** scatterplots of individual female specimens, with points connected to the centroid of their assigned group **D** relative contribution of each morphometric variable to the first two canonical functions for females. SVL = snout–vent length; HL = head length; HW = head width; TD = tympanum diameter; ED = eye diameter; IOD = interorbital distance; EN = eye–nostril distance; IND = internostril distance; ESD = eye–snout distance; FAL = forearm length; THL = thigh length; SHL = shank length. Colors indicate mitochondrial *Leptodactylus fuscus* clades: gray = *L. fuscus* clade 1, black = *L. fuscus* clade 2, blue = *L. fuscus* clade 3.

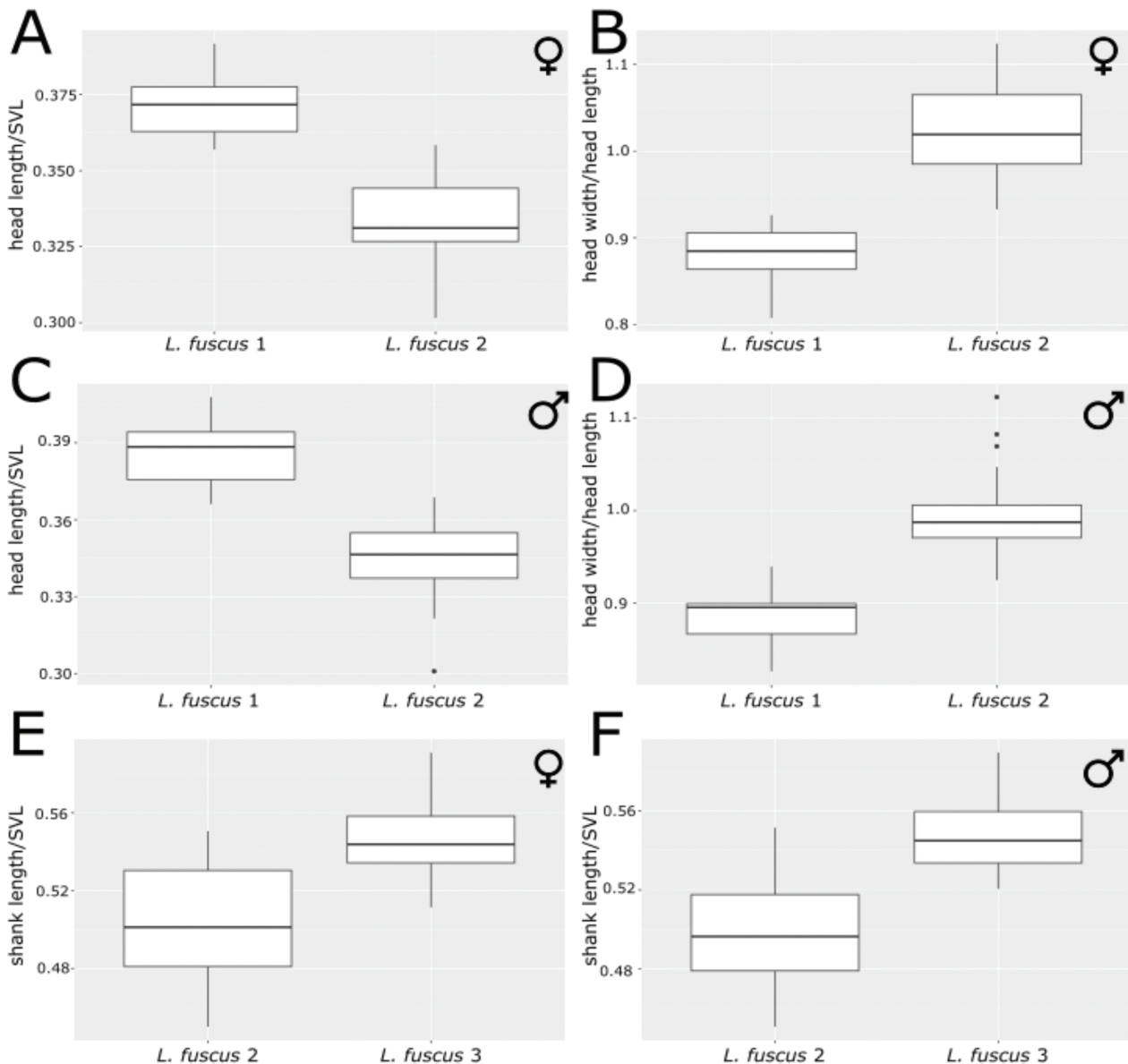


Figure 5. Morphometric variables that significantly differentiate the *Leptodactylus fuscus* clades based on Kruskal-Wallis and Dunn tests. **A** ratio of head length to snout vent length (SVL) between females of the *L. fuscus* clade 1 and *L. fuscus* clade 2; **B** ratio of head width to head length between females of the *L. fuscus* clade 1 and *L. fuscus* clade 2; **C** ratio of head length to SVL between males of the *L. fuscus* clade 1 and *L. fuscus* clade 2; **D** ratio of head width to head length between males of the *L. fuscus* clade 1 and *L. fuscus* clade 2; **E** ratio of shank length to SVL between females of the *L. fuscus* clade 2 and *L. fuscus* clade 3; **F** ratio of shank length to SVL between males of the *L. fuscus* clade 2 and *L. fuscus* clade 3. Boxplots denote median, first and third quartiles, and outliers.

ern Cerrado (Fig. 1). For the *L. fuscus* clade 3, which includes all the remaining samples, from eastern Paraguay and northeastern Argentina to northern Brazil (south of the Amazonas River), we maintain it as *Leptodactylus* aff. *fuscus* due to its weakly supported relationships and high internal genetic distances. We expect that a deeper sampling could lead to the detection of more than one independent lineage in this clade, which may correspond to some of the other names available under *L. fuscus* synonyms (see Discussion).

Re-diagnosis of *Leptodactylus gualambensis*

Leptodactylus gualambensis is distinguishable from all other species in the genus by the following combina-

tion of characters: (1) absence of toe webbing and fringes; (2) absence of chest and thumb spines in males; (3) median SVL in males (42.92–47.87 mm) and females (45.61–50.1 mm); (4) presence of lateral, dorso-lateral and dorsal dermal folds; (5) absence of longitudinal dermal folds on the dorsal surface of hind limbs; (6) absence of white tubercles on the sole of the feet; (7) rounded to nearly rounded snout; (8) proportionally short head (HL/SVL); (9) head almost as wide as long to wider than long (HW/HL); (10) proportionally short legs (SHL/SVL); (11) advertisement call with duration of 0.125–0.315 s ($\bar{x}$ = 0.1772 s); (12) low frequency of 567.901–888.889 Hz ($\bar{x}$ = 740.902 Hz); (13) delta frequency of 1679.739–2469.136 Hz ($\bar{x}$ = 2174.728 Hz); (14) high frequency of 2389.732–3283.951 Hz ($\bar{x}$ = 2915.630 Hz); (15) 95%

Table 5. Morphometric measurements of adult males and females by clade. Mean, standard deviation, minimum and maximum (in parenthesis). SVL = snout vent length.

	Males			Females		
	<i>L. fuscus 1</i> (n = 11)	<i>L. fuscus 2</i> (n = 49)	<i>L. fuscus 3</i> (n = 33)	<i>L. fuscus 1</i> (n = 10)	<i>L. fuscus 2</i> (n = 33)	<i>L. fuscus 3</i> (n = 33)
SVL	38.49 ± 2.26 (35.58–42.27)	43.32 ± 2.12 (38.06–48.79)	42.76 ± 2.19 (38–47.25)	41.85 ± 1.72 (40.03–44.14)	44.01 ± 2.13 (40.95–49.24)	44.17 ± 2.79 (40.11–49.43)
Head length/SVL	0.39 ± 0.01 (0.37–0.41)	0.34 ± 0.01 (0.3–0.37)	0.35 ± 0.01 (0.31–0.38)	0.37 ± 0.01 (0.36–0.39)	0.33 ± 0.01 (0.3–0.36)	0.34 ± 0.01 (0.31–0.37)
Head width/SVL	0.34 ± 0.02 (0.31–0.37)	0.34 ± 0.01 (0.32–0.37)	0.34 ± 0.01 (0.31–0.38)	0.33 ± 0.02 (0.29–0.35)	0.34 ± 0.01 (0.32–0.37)	0.33 ± 0.01 (0.31–0.36)
Head width/head length	0.89 ± 0.03 (0.83–0.94)	0.99 ± 0.04 (0.92–1.12)	0.98 ± 0.04 (0.9–1.09)	0.88 ± 0.03 (0.81–0.93)	1.02 ± 0.05 (0.93–1.12)	0.98 ± 0.04 (0.89–1.05)
Tympanum diameter/SVL	0.08 ± 0.01 (0.07–0.09)	0.08 ± 0 (0.07–0.09)	0.08 ± 0 (0.07–0.09)	0.07 ± 0.01 (0.06–0.08)	0.07 ± 0 (0.06–0.08)	0.08 ± 0 (0.07–0.08)
Eye-diameter/SVL	0.11 ± 0.01 (0.1–0.13)	0.12 ± 0.01 (0.1–0.13)	0.12 ± 0.01 (0.1–0.13)	0.11 ± 0.01 (0.09–0.12)	0.1 ± 0.01 (0.09–0.11)	0.11 ± 0.01 (0.1–0.12)
Interorbital distance/SVL	0.07 ± 0.01 (0.06–0.07)	0.07 ± 0.01 (0.06–0.09)	0.07 ± 0.01 (0.05–0.08)	0.07 ± 0.01 (0.05–0.07)	0.07 ± 0.01 (0.06–0.09)	0.07 ± 0.01 (0.06–0.08)
Eye-nostril distance/SVL	0.09 ± 0.01 (0.08–0.1)	0.09 ± 0.01 (0.07–0.1)	0.09 ± 0.01 (0.08–0.1)	0.09 ± 0.01 (0.08–0.1)	0.09 ± 0.01 (0.08–0.1)	0.09 ± 0 (0.08–0.1)
Internostril distance/SVL	0.07 ± 0.01 (0.07–0.08)	0.07 ± 0 (0.06–0.08)	0.07 ± 0 (0.06–0.08)	0.07 ± 0.01 (0.05–0.07)	0.07 ± 0 (0.06–0.07)	0.07 ± 0 (0.06–0.07)
Eye-snout distance/SVL	0.17 ± 0.01 (0.15–0.18)	0.16 ± 0.01 (0.14–0.18)	0.17 ± 0.01 (0.16–0.19)	0.17 ± 0.01 (0.15–0.18)	0.16 ± 0.01 (0.15–0.18)	0.17 ± 0.01 (0.15–0.19)
Forearm length/SVL	0.2 ± 0.01 (0.16–0.21)	0.19 ± 0.02 (0.16–0.23)	0.19 ± 0.01 (0.16–0.21)	0.19 ± 0.02 (0.17–0.22)	0.19 ± 0.01 (0.17–0.21)	0.18 ± 0.01 (0.16–0.2)
Thigh length/SVL	0.47 ± 0.04 (0.4–0.54)	0.46 ± 0.02 (0.4–0.51)	0.48 ± 0.02 (0.44–0.53)	0.46 ± 0.04 (0.38–0.51)	0.46 ± 0.02 (0.41–0.5)	0.48 ± 0.03 (0.43–0.53)
Shank length/SVL	0.54 ± 0.03 (0.48–0.58)	0.5 ± 0.03 (0.45–0.55)	0.55 ± 0.02 (0.52–0.59)	0.54 ± 0.02 (0.51–0.56)	0.5 ± 0.03 (0.45–0.55)	0.55 ± 0.02 (0.51–0.59)
Tarsal length/SVL	0.27 ± 0.03 (0.22–0.31)	0.27 ± 0.02 (0.24–0.31)	0.28 ± 0.01 (0.26–0.31)	0.28 ± 0.01 (0.26–0.29)	0.26 ± 0.01 (0.23–0.29)	0.28 ± 0.01 (0.26–0.3)
internostril distance/head width	0.21 ± 0.01 (0.2–0.23)	0.19 ± 0.01 (0.17–0.22)	0.2 ± 0.01 (0.18–0.24)	0.2 ± 0.02 (0.16–0.24)	0.19 ± 0.01 (0.17–0.22)	0.2 ± 0.01 (0.17–0.22)
Internostril distance/head length	0.19 ± 0.01 (0.17–0.21)	0.19 ± 0.01 (0.17–0.22)	0.19 ± 0.01 (0.17–0.23)	0.18 ± 0.02 (0.14–0.19)	0.2 ± 0.01 (0.18–0.21)	0.19 ± 0.01 (0.17–0.22)
Tarsal length/thigh length	0.59 ± 0.05 (0.53–0.68)	0.58 ± 0.03 (0.51–0.68)	0.59 ± 0.03 (0.54–0.66)	0.6 ± 0.06 (0.54–0.72)	0.57 ± 0.03 (0.54–0.63)	0.59 ± 0.03 (0.53–0.65)
Tarsal length/shank length	0.52 ± 0.03 (0.45–0.56)	0.54 ± 0.02 (0.49–0.57)	0.52 ± 0.02 (0.47–0.56)	0.51 ± 0.01 (0.48–0.53)	0.52 ± 0.02 (0.47–0.57)	0.51 ± 0.02 (0.48–0.56)
Shank length/thigh length	1.15 ± 0.06 (1.07–1.25)	1.09 ± 0.04 (1–1.23)	1.14 ± 0.05 (1.03–1.25)	1.17 ± 0.11 (1.07–1.41)	1.1 ± 0.05 (1.04–1.19)	1.15 ± 0.05 (1.06–1.25)
Eye-nostril distance/eye-snout distance	0.54 ± 0.03 (0.49–0.57)	0.54 ± 0.04 (0.48–0.66)	0.52 ± 0.03 (0.47–0.62)	0.55 ± 0.04 (0.48–0.62)	0.53 ± 0.02 (0.5–0.58)	0.54 ± 0.02 (0.5–0.59)
Eye-snout distance/head length	0.44 ± 0.02 (0.4–0.47)	0.47 ± 0.02 (0.42–0.51)	0.49 ± 0.02 (0.46–0.54)	0.45 ± 0.03 (0.4–0.51)	0.49 ± 0.01 (0.45–0.52)	0.5 ± 0.01 (0.47–0.54)
Eye-snout distance/head width	0.49 ± 0.02 (0.44–0.52)	0.47 ± 0.03 (0.41–0.53)	0.5 ± 0.02 (0.46–0.54)	0.51 ± 0.05 (0.46–0.6)	0.48 ± 0.03 (0.43–0.56)	0.51 ± 0.03 (0.47–0.61)
Eye-nostril distance/head length	0.23 ± 0.01 (0.21–0.26)	0.25 ± 0.02 (0.23–0.29)	0.26 ± 0.02 (0.23–0.3)	0.24 ± 0.01 (0.22–0.26)	0.26 ± 0.01 (0.24–0.28)	0.27 ± 0.01 (0.25–0.29)

frequency of 2156.250–3100.781 Hz ($\bar{x}$ = 2745.950 Hz); (16) dominant frequency of 1781.250–2928.516 Hz ($\bar{x}$ = 2462.907 Hz).

Comparisons with other *Leptodactylus* species

Leptodactylus gualambensis differs from the species of the *L. latrans* group by the absence of toe fringes and absence of thumb spines in adult males (both present in all the species of the *L. latrans* group; de Sá et al. 2014), and

by the smaller male SVL (42.92–47.87 mm in *L. gualambensis* and 48.7–126.3 mm species of the *L. latrans* group combined, except *L. silvanimbus* with 35.8–55.0 mm; de Sá et al. 2014; Magalhães et al. 2020) and female SVL (45.61–50.1 mm in *L. gualambensis* and 55.9–115.9 mm species of the *L. latrans* group combined, except *L. silvanimbus* with 35.9–48 mm; de Sá et al. 2014; Magalhães et al. 2020).

From the species of the *L. melanonotus* group, *L. gualambensis* differs by the absence of toe fringes (present in all species of the *L. melanonotus* group; de Sá et al.

Table 6 – part 1. Statistical tests used to determine significant differences for each morphometric variable between *Leptodactylus fuscus* clades. Kruskal-Wallis non-parametric test: H, Kruskal-Wallis statistics; df, degree of freedom; p, probability.

Kruskal-Wallis test					
	Females			Males	
Variable	H	df	p	Chi-squared	p
Eye-diameter/SVL	19.161	2	<0.01	3.737	>0.01
Eye-nostril distance/eye-snout distance	4.825	2	>0.01	6.412	>0.01
Eye-nostril distance/head length	27.571	2	<0.01	12.159	<0.01
Eye-nostril distance/SVL	11.105	2	<0.01	3.413	>0.01
Eye-snout distance/head length	17.810	2	<0.01	37.635	<0.01
Eye-snout distance/head width	11.337	2	<0.01	21.110	<0.01
Eye-snout distance/SVL	6.242	2	>0.01	24.190	<0.01
Forearm length/SVL	4.953	2	>0.01	3.157	>0.01
Head length/SVL	27.501	2	<0.01	29.889	<0.01
Head width/head length	32.348	2	<0.01	27.899	<0.01
Head width/SVL	7.646	2	>0.01	0.653	>0.01
Internostril distance/head length	13.361	2	<0.01	4.824	>0.01
Internostril distance/head width	4.268	2	>0.01	15.418	<0.01
Internostril distance/SVL	0.612	2	>0.01	12.575	<0.01
Interorbital distance/SVL	7.646	2	>0.01	18.210	<0.01
Shank length/SVL	34.976	2	<0.01	44.694	<0.01
Shank length/thigh length	14.523	2	<0.01	19.075	<0.01
SVL	7.530	2	>0.01	22.064	<0.01
Tarsal length/shank length	5.843	2	>0.01	16.196	<0.01
Tarsal length/SVL	21.975	2	<0.01	16.039	<0.01
Tarsal length/thigh length	3.227	2	>0.01	0.190	>0.01
Tympanum diameter/SVL	10.628	2	<0.01	4.323	>0.01
Thigh length/SVL	8.237	2	>0.01	17.127	<0.01

Table 6 – part 2. Post-hoc Dunn-test to examine pairwise comparisons for each morphometric variable with significance differences in Kruskal-Wallis test: Z, Dunn-test statistic; p, probability, with Bonferroni correction. Significant differences ($p < 0.01$) are in bold.

	<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 2			<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 3			<i>L. fuscus</i> 2 vs <i>L. fuscus</i> 3		
Females									
Variable	z	p	adjusted p	z	p	adjusted p	z	p	adjusted p
Eye diameter/SVL	-1.376	>0.01	>0.01	1.607	>0.01	>0.01	4.375	<0.01	<0.01
Eye-nostril distance/head length	3.196	<0.01	<0.01	5.124	<0.01	<0.01	2.826	<0.01	<0.01
Eye-nostril distance/SVL	-1.735	>0.01	>0.01	0.492	>0.01	>0.01	3.266	<0.01	<0.01
Eye-snout distance/head length	3.068	<0.01	<0.01	4.212	<0.01	<0.01	1.677	>0.01	>0.01
Eye-snout distance/head width	-1.800	>0.01	>0.01	0.442	>0.01	>0.01	3.288	<0.01	<0.01
Head length/SVL	-5.244	<0.01	<0.01	-4.001	<0.01	<0.01	1.822	>0.01	>0.01
Head width/head length	5.602	<0.01	<0.01	3.671	<0.01	<0.01	-2.831	<0.01	<0.01
Internostril distance/head length	3.651	<0.01	<0.01	2.906	<0.01	<0.01	-1.092	>0.01	>0.01
Shank length/SVL	-3.101	<0.01	<0.01	0.848	>0.01	>0.01	5.791	<0.01	<0.01
Shank length/thigh length	-2.360	>0.01	>0.01	0.106	>0.01	>0.01	3.617	<0.01	<0.01
Tarsal length/SVL	-2.190	>0.01	>0.01	0.976	>0.01	>0.01	4.643	<0.01	<0.01
Tympanum diameter/SVL	-0.199	>0.01	>0.01	1.933	>0.01	>0.01	3.127	<0.01	<0.01
	<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 2			<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 3			<i>L. fuscus</i> 2 vs <i>L. fuscus</i> 3		
Males									
Variable	z	p	adjusted p	z	p	adjusted p	z	p	adjusted p
Eye-nostril distance/head length	3.151	<0.01	<0.01	3.395	<0.01	<0.01	0.581	>0.01	>0.01
Eye-snout distance/head length	3.090	<0.01	<0.01	5.717	<0.01	<0.01	4.260	<0.01	<0.01
Eye-snout distance/head width	-2.318	>0.01	>0.01	0.654	>0.01	>0.01	4.447	<0.01	<0.01
Eye-snout distance/SVL	-2.063	>0.01	>0.01	1.164	>0.01	>0.01	4.857	<0.01	<0.01
Head length/SVL	-5.467	<0.01	<0.01	-4.298	<0.01	<0.01	1.454	>0.01	>0.01
Head width/head length	5.241	<0.01	<0.01	4.511	<0.01	<0.01	-0.790	>0.01	>0.01
Internostril distance/head width	-3.875	<0.01	<0.01	-2.637	<0.01	>0.01	1.663	>0.01	>0.01
Internostril distance/SVL	-3.373	<0.01	<0.01	-1.960	>0.01	>0.01	1.967	>0.01	>0.01

	<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 2			<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 3			<i>L. fuscus</i> 2 vs <i>L. fuscus</i> 3		
Interorbital distance/SVL	2.898	<0.01	<0.01	0.319	>0.01	>0.01	-3.801	<0.01	<0.01
Shank length/SVL	-3.287	<0.01	<0.01	1.051	>0.01	>0.01	6.495	<0.01	<0.01
Shank length/thigh length	-3.085	<0.01	<0.01	-0.490	>0.01	>0.01	3.813	<0.01	<0.01
SVL	4.691	<0.01	<0.01	3.821	<0.01	<0.01	-1.042	>0.01	>0.01
Tarsal length/shank length	2.528	>0.01	>0.01	0.029	>0.01	>0.01	-3.700	<0.01	<0.01
Tarsal length/SVL	-1.604	>0.01	>0.01	1.028	>0.01	>0.01	3.967	<0.01	<0.01
Thigh length/SVL	-0.540	>0.01	>0.01	2.131	>0.01	>0.01	4.095	<0.01	<0.01

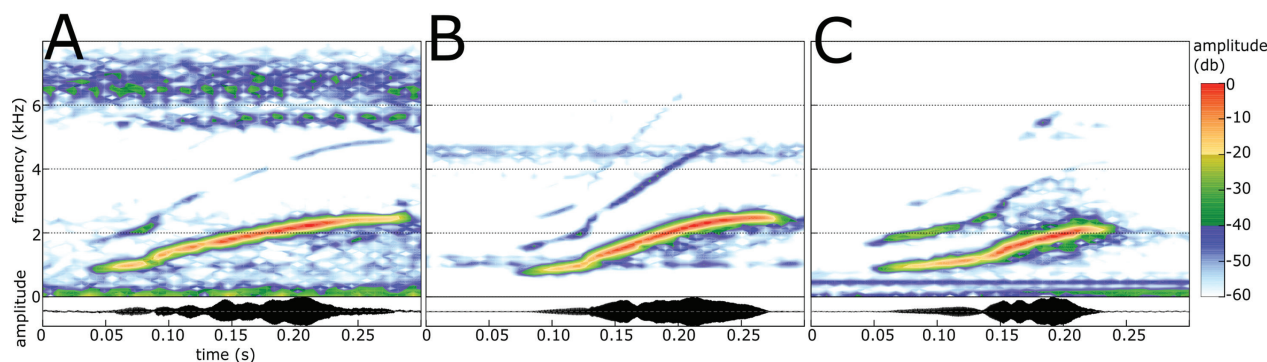


Figure 6. Advertisement call of *Leptodactylus fuscus* corresponding to the three main clades recovered in this study. **A** *L. fuscus* clade 1 (IIBP-F 028; Surinam, Paramaribo, Anton de Kom University, type locality of *Leptodactylus fuscus*) **B** *L. fuscus* clade 2 (LGE-B 762; Argentina, Salta, Urundel, type locality of *Leptodactylus gualambensis*) **C** *L. fuscus* clade 3 (IIBP-F 006; Paraguay, Amambay, Estancia Kai Rague).

2014) and absence of thumb spines on adult males (except *L. discodactylus* and *L. pustulatus*, present in all the species of the *L. melanonotus* group; de Sá et al. 2014).

From the species of the *L. pentadactylus* group differs by the absence of thumb spines in males (present in the species of the *L. pentadactylus* group, except *L. stenoderma*) and by the smaller male SVL (42.92–47.87 mm in *L. gualambensis* and 50.9–195 mm species in the *L. pentadactylus* group combined, except *L. lithonaetes* with 45.3–71.4 mm; de Sá et al. 2014) and female SVL (45.61–50.1 mm in *L. gualambensis* and 52.5–174.2 mm in species of the *L. pentadactylus* group combined; de Sá et al. 2014).

Within the *L. fuscus* group, the SVL of *L. gualambensis* (42.92–47.87 mm in males, 45.61–50.1 mm in females) differentiates it from *L. aepyta* (46.8–61.41 mm in males, 51.67–66.21 mm in females; Schneider et al. 2019), *L. cupreus* (48.5–57.0 mm in males, 55.7–57.9 mm in females; Cassini et al. 2013), *L. laticeps* (88.10–109.7 mm in males, 88–117 mm in females; de Sá et al. 2014), and *L. syphax* (55.59–83.4 mm in males, 70.5–89.8 mm in females; de Sá et al. 2014) by smaller SVL in males and females (with some overlap with *L. aepyta* males). Furthermore, it differs from *L. laticeps* and *L. syphax* by the absence of spines on the thumbs and chest (present in *L. laticeps* and *L. syphax*).

By its larger SVL (42.92–47.87 mm in males, 45.61–50.1 mm in females), *L. gualambensis* differs from *L. caatingae* (32.1–36.9 mm in males, 36.2–39.1 mm in females; de Sá et al. 2014), *L. camaquara* (30.7–33.7 mm in males, 31.8–38.3 mm in females; de Sá et al. 2014), *L. fragilis* (27–43 mm in males, 30.1–43.6 mm in females; de Sá et al. 2014), *L. kilombo* (37.6–41.3 mm in

males, 41.8 mm female; Silva et al. 2020), *L. latinasus* (27.46–29.70 mm in males, 27.29–31.66 mm in females), *L. marambaiae* (35.8–39.3 mm in males; 40–41.3 mm in females; de Sá et al. 2014), *L. oreomantis* (28.1–33.8 mm in males, 33.2–38.3 mm in females; de Sá et al. 2014), *L. tapiti* (29.8–33.4 mm in males; 35.8–41.4 mm in females; de Sá et al. 2014).

By having a pair of dorsal folds, *L. gualambensis* is distinguished from *L. albilabris*, *L. aepyta*, *L. barrioi*, *L. bufonius*, *L. caatingae*, *L. cupreus*, *L. didymus*, *L. elenae*, *L. fragilis*, *L. kilombo*, *L. labrosus*, *L. laticeps*, *L. latinasus*, *L. mystaceus*, *L. mystacinus*, *L. notoaktites*, *L. poecilochilus*, *L. spixi*, *L. syphax*, *L. troglodytes*, *L. ventrimaculatus*, *L. watu* (in *L. notoaktites*, *L. poecilochilus*, and *L. spixi* present only in specimens with light mid-dorsal stripe, absent in the rest; Heyer et al. 1996; Kwet and Di Bernardo 1999; de Sá et al. 2014; Schneider et al. 2019; Silva et al. 2020). By having a pair of well-defined and continuous dorsolateral folds, *L. gualambensis* differs from *L. bufonius* (usually absent or with weak dorsolateral folds), *L. caatingae* (dorsolateral folds absent or interrupted), *L. syphax* (dorsolateral folds absent), and *L. troglodytes* (usually absent dorsolateral folds) (de Sá et al. 2014). By having a pair of lateral folds, *L. gualambensis* differs from *L. caatingae*, *L. cupreus*, *L. laticeps*, *L. mystaceus*, and *L. ventrimaculatus* (absent lateral folds; Heyer et al. 1996; Cassini et al. 2013; de Sá et al. 2014). By the absence of longitudinal dermal folds on the dorsal surface of hind limbs, *L. gualambensis* is distinguished from *L. gracilis*, *L. jolyi*, *L. marambaiae*, *L. plaumanni*, and *L. tapiti* (with longitudinal dermal folds on dorsal surface of hind limbs; Kwet and Di Bernardo 1999; Giaretta and Cos-

Table 7. Bioacoustic variables by clade. Mean, standard deviation, minimum and maximum (in parenthesis). Advertisement call frequencies are shown in Hertz (Hz) and duration in seconds (s).

Variable	<i>L. fuscus</i> clade 1	<i>L. fuscus</i> clade 2	<i>L. fuscus</i> clade 3
Dominant frequency	2418.927 ± 10.165 (1968.75–2531.25)	2462.907 ± 7.936 (1781.25–2928.52)	1966.699 ± 11.491 (1636.523–2325.586)
Low frequency	880.383 ± 4.520 (768.215–977.728)	740.902 ± 2.274 (567.901–888.889)	750.880 ± 3.329 (657.675–882.247)
5% frequency	1584.906 ± 3.211 (1406.25–1593.75)	1580.930 ± 9.353 (1031.25–2153.32)	1231.879 ± 10.648 (947.461–1550.391)
High frequency	2917.207 ± 6.063 (2758.589–3037.94)	2915.631 ± 7.154 (2389.732–3283.951)	2357.758 ± 11.954 (2005.106–2712.472)
95% frequency	2724.057 ± 10.651 (2437.5–2906.25)	2745.951 ± 6.550 (2156.25–3100.781)	2158.255 ± 12.709 (1808.789–2497.852)
Delta frequency	2036.823 ± 5.222 (1885.618–2164.969)	2174.728 ± 6.268 (1679.739–2469.136)	1606.878 ± 12.216 (1170.982–2007.609)
Delta frequency (90%)	1139.151 ± 10.347 (937.5–1312.5)	1165.021 ± 7.232 (750–1593.75)	926.376 ± 8.040 (602.93–1205.859)
Call duration	0.228 ± 0.002 (0.1819–0.2655)	0.177 ± 0.001 (0.1251–0.3147)	0.247 ± 0.004 (0.1521–0.347)

Table 8 – part 1. Statistical tests used to determine significant differences for each bioacoustic variable between *Leptodactylus fuscus* clades. Kruskal-Wallis non-parametric test: H, Kruskal-Wallis statistic; df, degree of freedom; p, probability.

Kruskal-Wallis test			
Variable	H	df	p
Dominant frequency	411.016	2	<0.01
Low frequency	250.722	2	<0.01
5% frequency	373.387	2	<0.01
High frequency	429.433	2	<0.01
95% frequency	455.287	2	<0.01
Delta frequency	505.854	2	<0.01
Delta frequency (90%)	200.298	2	<0.01
Call duration	329.122	2	<0.01

Table 8 – part 2. Post-hoc Dunn-test to examine pairwise comparisons for each bioacoustic variable with significance differences in Kruskal-Wallis test: Z, Dunn-test statistic; p, probability, with Bonferroni correction. Significant differences (p<0.01) are in bold.

Variable	<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 2			<i>L. fuscus</i> 1 vs <i>L. fuscus</i> 3			<i>L. fuscus</i> 2 vs <i>L. fuscus</i> 3		
	z	p	adjusted p	z	p	adjusted p	z	p	adjusted p
Dominant frequency	2.020	>0.01	>0.01	–12.132	<0.01	<0.01	20.178	<0.01	<0.01
Low frequency	–15.795	<0.01	<0.01	–12.397	<0.01	<0.01	–2.003	>0.01	>0.01
5% frequency	–3.372	<0.01	<0.01	–15.335	<0.01	<0.01	17.999	<0.01	<0.01
High frequency	0.108	>0.01	>0.01	–13.900	<0.01	<0.01	20.300	<0.01	<0.01
95% frequency	1.415	>0.01	>0.01	–13.333	<0.01	<0.01	21.156	<0.01	<0.01
Delta frequency	8.254	<0.01	<0.01	–8.039	<0.01	<0.01	22.132	<0.01	<0.01
Delta frequency (90%)	1.665	>0.01	>0.01	–8.262	<0.01	<0.01	14.108	<0.01	<0.01
Call duration	–12.890	<0.01	<0.01	–0.886	>0.01	>0.01	–15.040	<0.01	<0.01

ta 2007; de Sá et al. 2014). By the absence of evident white tubercles on the sole of the foot, *L. gualambensis* differs from *L. caatingae*, *L. didymus*, *L. elenae*, *L. latinasus*, *L. mystaceus*, *L. spixi* (all with prominent white tubercles on the sole of the foot; de Sá et al. 2014; Heyer 1978, 1983; Heyer et al. 1996). By having relatively short legs (thigh 45–55% of SVL), *L. gualambensis* differs from *L. furnarius* (thigh 53–66% of SVL; de Sá et al. 2014), and from *L. longirostris* by the brown color in the hidden parts of the inguinal and axillar regions (yellow in *L. longirostris*; Lima et al. 2012).

From specimens of *L. fuscus sensu stricto* (*L. fuscus* clade 1) *L. gualambensis* is distinguished by having rounded to nearly rounded snout (sub-elliptical to sub-ovoid snout in *L. fuscus*; Fig. 3), shorter head (males head length 30–37% of SVL, $\bar{x}$ =34%, in *L. gualambensis* and 37–41%, $\bar{x}$ =39%, in *L. fuscus*; females head length 30–36% of SVL, $\bar{x}$ =33%, in *L. gualambensis* and 36–39%, $\bar{x}$ =37%, in *L. fuscus*), and head almost as wide as long to wider than long (males head width 92–112% of head length, $\bar{x}$ =99%, in *L. gualambensis* and 83–94%, $\bar{x}$ =89%, in *L. fuscus*; females head width 93–112% of head length,

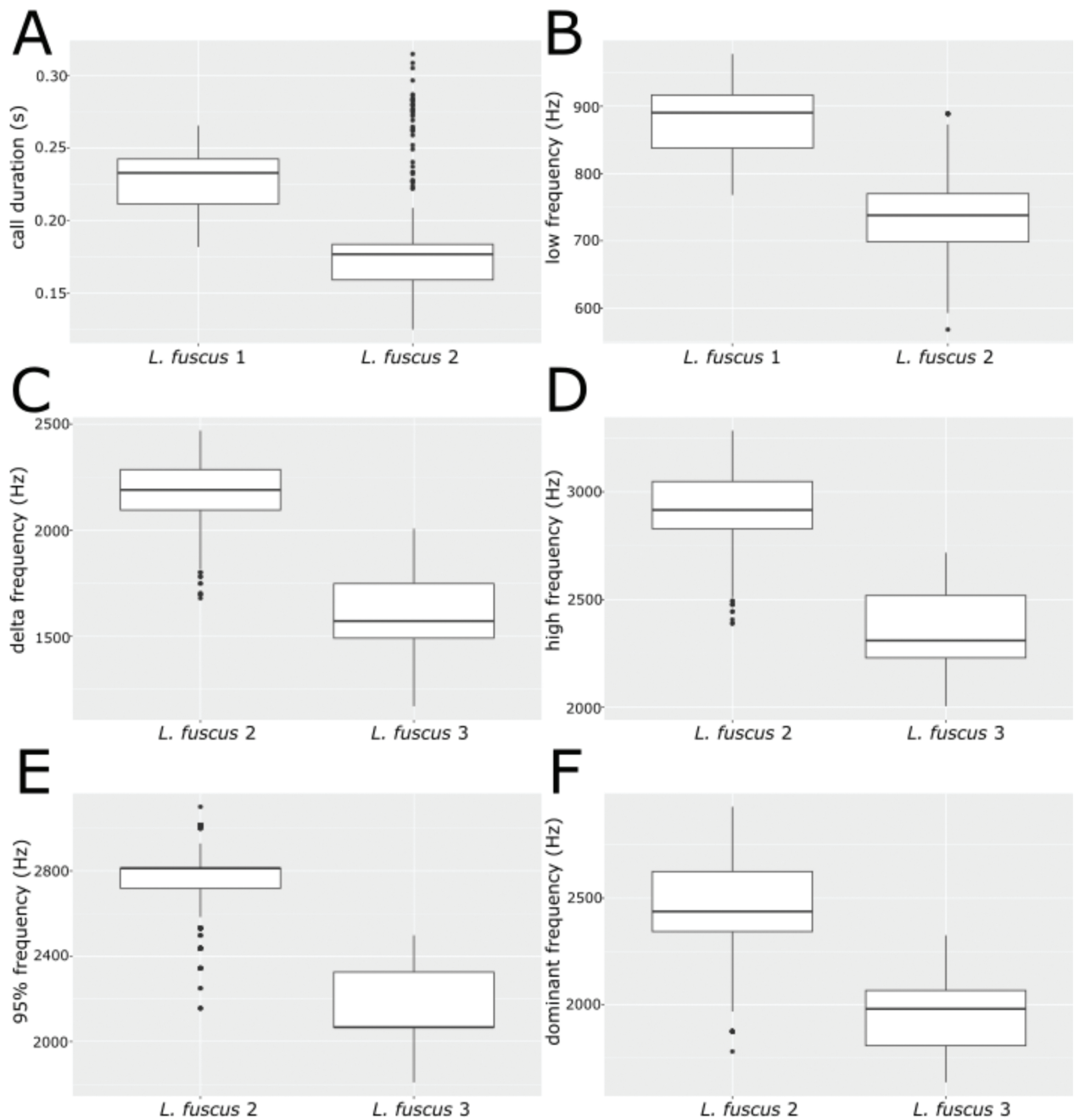


Figure 7. Bioacoustic variables that significantly differentiate the *Leptodactylus fuscus* clades based on Kruskal-Wallis and Dunn tests. **A** Call duration between advertisement calls of males of the *L. fuscus* clade 1 and *L. fuscus* clade 2; **B** low frequency between advertisement calls of males of the *L. fuscus* clade 1 and *L. fuscus* clade 2; **C** delta frequency between advertisement calls of males of the *L. fuscus* clade 2 and *L. fuscus* clade 3; **D** high frequency between advertisement calls of males of the *L. fuscus* clade 2 and *L. fuscus* clade 3; **E** 95% frequency between advertisement calls of males of the *L. fuscus* clade 2 and *L. fuscus* clade 3; **F** dominant frequency between advertisement calls of males of the *L. fuscus* clade 2 and *L. fuscus* clade 3. Boxplots denote median, first and third quartiles, and outliers. S = seconds, Hz = Hertz.

$\bar{x}$ =102%, in *L. gualambensis* and 81–93%, $\bar{x}$ =88%, in *L. fuscus*; Fig. 5; Table 5). *Leptodactylus gualambensis* is also distinguished from *L. fuscus* by shorter call duration (0.125–0.315 s, $\bar{x}$ =0.177 s, in *L. gualambensis* and 0.182–0.265 s, $\bar{x}$ =0.228 s, in *L. fuscus*), and lower low frequency (567.901–888.889 Hz, $\bar{x}$ =740.902 Hz, in *L. gualambensis* and 768.215–977.728 Hz, $\bar{x}$ =880.383 Hz, in *L. fuscus*; Fig. 7; Table 7).

From specimens of *L. aff. fuscus* (*L. fuscus* clade 3), *L. gualambensis* is distinguished by having proportional-

ly shorter shank (male shank 45–55% of SVL, $\bar{x}$ =50%, in *L. gualambensis* and 52–59%, $\bar{x}$ =55%, in *L. aff. fuscus*; female shank 45–55% of SVL, $\bar{x}$ =50%, in *L. gualambensis* and 51–59%, $\bar{x}$ =55%, in *L. aff. fuscus*; Fig. 5; Table 5; see discussion about hind limbs length variation). *Leptodactylus gualambensis* is also distinguished from *L. aff. fuscus* by the higher delta frequency (1679.739–2469.136 Hz, $\bar{x}$ =2174.728 Hz, in *L. gualambensis* and 1170.982–2007.609 Hz, $\bar{x}$ =1606.877 Hz, in *L. aff. fuscus*), higher high frequency (2389.732–3283.951 Hz, $\bar{x}$ =2915.630

Hz, in *L. gualambensis* and 2005.106–2717.472 Hz, $\bar{x}$ =2357.757 Hz, in *L. aff. fuscus*), higher 95% frequency (2156.25–3100.781 Hz, $\bar{x}$ =2745.950 Hz, in *L. gualambensis* and 1808.789–2497.852 Hz, $\bar{x}$ =2158.255 Hz, in *L. aff. fuscus*), and higher dominant frequency (1781.25–2928.516 Hz, $\bar{x}$ =2462.907 Hz, in *L. gualambensis* and 1636.523–2325.586 Hz, $\bar{x}$ =1966.699 Hz, in *L. aff. fuscus*; Fig. 7; Table 7).

Variation

Variation in body measurements and proportions in males and females are shown in Table 2. We found great variation in dorsal color pattern with respect to the presence of a mid-dorsal stripe from tip of the snout to the vent (in males, 37.03% of 107 specimens present mid-dorsal stripe; in females, 38.04% of 103 specimens present mid-dorsal stripe). Considering both groups together, specimens with and without mid-dorsal stripe, the dorsal color pattern variation is related to the distribution and density of brown blotches. Some specimens present brown blotches only on the dorsolateral region and from the scapula to the vent; others, also from the scapular region to the vent, but occupying dorsal and dorsolateral region of the body; and others show higher density of brown blotches, covering from the interocular region to the vent, in dorsal and dorsolateral regions. In the dorsal view of the head, some specimens present only a diffuse and mostly straight brown stripe on the canthus rostralis (in males, 51.6% of 67 specimens; in females 39.2% of 63 specimens), while others show some brown blotches on the snout and a well-marked and ornamented stripe on the canthus rostralis. A pair of interocular blotches are present (in males, 48.4% of 67 specimens; in females 54.9% of 63 specimens) or absent (Figs 3, 8).

Geographic distribution

Leptodactylus gualambensis is primarily distributed in the Dry Chaco, where it is found widely in western Paraguay and northern Argentina (Fig. 1). Type locality (Urundel, Salta) and records from surrounding localities (Iruya, Salta and El Carmen, Jujuy) are in transition areas between the Dry Chaco and Yungas. The species is also known from the Humid Chaco, as well as from some localities in the southern Cerrado, northern Pantanal and southern Chiquitano Dry Forest.

Discussion

We assessed the taxonomy of the Chaco populations of *L. fuscus* using molecular data, external morphology, and bioacoustic characters of the advertisement call. In BI and MP analyses, *L. fuscus* is composed of three major clades. The phylogenetic topology and lineage delimitation are further supported by morphological and acoustic evidence, leading to the recognition of *L. fuscus* (*L. fuscus* clade 1) for populations from northern South America

and Central America, and the resurrection of *L. gualambensis* for the Chaco populations (*L. fuscus* clade 2), and leaving the populations belonging to the *L. fuscus* clade 3 as the undetermined taxon *L. aff. fuscus*.

In both phylogenetic analyses we found that genetic structure follows geographic distribution of samples, except for those from northern Bolivia (Beni, Los Lagos, MJ 1357) and northeastern Brazil (Pernambuco, USNM 284551), which clustered on the *L. fuscus* clade 1 (Central America and northern South America, *L. fuscus sensu stricto*). We could not dismiss the possibility that this result could be a consequence of issues in the available sequences of the specimens mentioned. Some sequences of *Leptodactylus* species included in previous studies (e.g., Camargo et al. 2006; de Sá et al. 2014), were excluded from subsequent ones due to doubtful identities and poor sequence quality (see Schneider et al. 2019; Silva et al. 2020). In this case, we lacked access to sequences of other gene fragments of the same specimens or samples from other specimens from the same localities to assess this possibility. Alternatively, this could also be due to sampling bias, as there are large geographic gaps between both the sample from northern Bolivia, the sample from northeastern Brazil, and the remaining samples of *L. fuscus sensu stricto* (*L. fuscus* clade 1). The position of the Pernambuco sample is also incongruent with the findings of Wynn and Heyer (2001), who used allozymes, and Camargo et al. (2006), who used mtDNA. The differences in the resolution power of the markers used in each study are evident: Both recovered a northern clade, but in Wynn and Heyer (2001), samples from Brazil, Bolivia, and Argentina were clustered together, whereas in Camargo et al. (2006), these samples formed two distinct clades. In the latter study, the Pernambuco sample clustered with specimens from Palos Blancos (La Paz, Bolivia) and Embarcación (Salta, Argentina), the latter locality being close to the type locality of *L. gualambensis*. These sequences were not included here following Schneider et al. (2019); however, we included additional sequences from Embarcación. In our analyses, the Embarcación samples clustered with the other Chacoan samples, as discussed later. We do not rule out other phenomena, such as incomplete lineage sorting; however, additional samples and markers are needed to formally test this hypothesis. Moreover, considering the availability of names for the region, populations from northeastern Brazil should be further assessed.

Nonetheless, phylogenetic clustering of the sample from northern Bolivia within the *L. fuscus* clade 1 is not totally unexpected; some species of birds, snakes, plants and other open-area frog species were known to inhabit both sides of the Amazon (Mittermeier et al. 2010; Lima-Rezende et al. 2019, 2022; Ritter et al. 2021; Wüster et al. 2005; Quijada-Mascareñas et al. 2007; de Oliveira Buzatti et al. 2018; Resende-Moreira et al. 2019; Brusquetti et al. 2023; Caballero-Gini et al. 2024). Various trans-Amazonian connection routes (by Atlantic coast, by central Amazon forest, by the base of the Andes and by the Madeira River basin, see Ribeiro et al. 2016), as well the historical dynamics of the fluvial system in central Amazonia (see Pupim et al. 2019) have been proposed

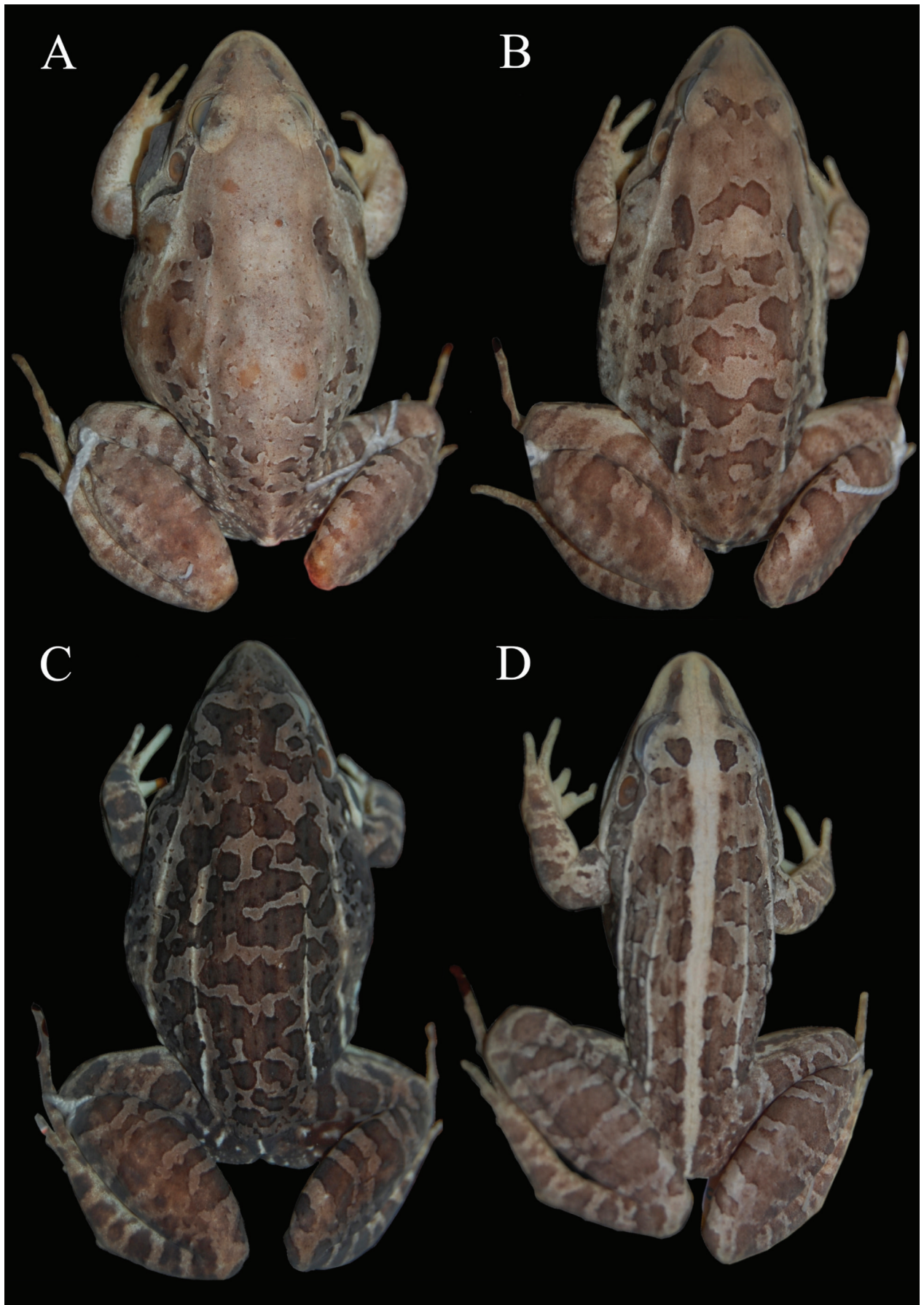


Figure 8. Intraspecific variation of dorsal color pattern of *Leptodactylus gualambensis*. **A** IIBP-H 1792, Chaco Boef farm, Boquerón, Paraguay, male, SVL 46.53 mm; **B** IIBP-H 1824, Chaco Boef farm, Boquerón, Paraguay, male, SVL 45.99 mm; **C** IIBP-H 5601, Parque Nacional Serranía San Luis, Concepción, Paraguay, male, SVL 43.64 mm; **D** IIBP-H 1129, San Bernardino, Cordillera, Paraguay, male, SVL 46.79 mm.

to explain relatively recent genetic connections between populations on both sides of the Amazon. However, to robustly test potential routes of connection or any other hypothesis related to riverine barriers, adequate sampling design and markers must be implemented (Naka and Pil 2020).

Considering the doubtful sequences issue, we had special interest in the sequences of specimens from Chaco localities included in Camargo et al. (2006). These authors included sequences of specimens from two neighboring localities (FML 4788 from Embarcación and FML 4789 from Joaquín V. González, Salta, Argentina) that in phylogenetic Bayesian analyses did not cluster together (see Camargo et al. 2006). In that study, the sample from Embarcación grouped with samples from northern Bolivia, and the sample from Joaquín V. González grouped with samples from São Paulo State (Brazil) and northeastern Brazil. To better assess this unexpected topology, we included sequences of new specimens from Embarcación (LGE 12569 and LGE 15310) and Joaquín V. González (FVD 288 and FVD 289), which in both analyses (MP and BI) grouped together with all samples from Dry Chaco, western Humid Chaco, Pantanal, and Chiquitano Dry Forest, thus corresponding to the geographic distribution of *L. gualambensis* as suggested by Gallardo (1964). Based on our results, we suspect that the sequences from these two specimens used by Camargo et al. (2006) may be affected by contamination or sample mixing and should be reconsidered in future analyses.

Hind limb length variation and the other diagnostic characters of Gallardo (1964)

Gallardo (1964) compared *L. gualambensis* with *L. fuscus* (as *L. sibilatrix*) based on specimens from localities of the Argentinian provinces of Corrientes and Misiones, the Brazilian states of São Paulo and Rio Grande do Sul, and northern Uruguay, which, judging by the geographic distribution, could be attributed to *L. aff. fuscus* (*L. fuscus* clade 3). As diagnostic characters Gallardo mentioned that *L. gualambensis* has shorter legs (especially thighs and shanks); a more concave loreal region, with a major inclination to the outside; interocular spots situated further back; and with all tibial transversal stripes of equal width, and the middle one notably wider in what he considered *L. fuscus*. Although with some overlap, we also found that *L. gualambensis* differs from specimens of the clade *L. aff. fuscus* by leg length, but in this case mainly in the relative length of the shank. We found that the position of the interocular spots and the tibial color pattern were highly variable in our sample and, therefore, not useful as diagnostic characters.

As mentioned previously, the ratios related to shank length (SH/SVL) partially overlap among *L. gualambensis* and *L. aff. fuscus* specimens. Notably, most *L. gualambensis* specimens with overlapping values (SH/SVL >0.54, 15% of 49 male specimens; >0.51, 33% of 33 female specimens) are from the eastern distribution of

the species, in the Humid Chaco and Cerrado, particularly from localities near transition areas between these ecoregions and the Atlantic Forest. In contrast, virtually all specimens (except for one female, IIBP-H 3634) from the western Humid Chaco and Dry Chaco have relatively short legs (SH/SVL <0.54, 85% of 49 male specimens; <0.51, 67% of 33 female specimens).

We suggest that the variation in leg length, which seems to reflect the geographical distribution of the specimens, could be associated with the exposure to different climatic conditions. Different environments may exert local selective pressure, resulting in phenotypic differentiation for species with a wide geographical range and populations facing diverse climatic conditions (Nali et al. 2023).

One possibility is that the variation in the hind limb length is related to burrowing behavior. In semiarid environments, a strategy used by terrestrial frogs to reduce the risk of desiccation during prolonged periods of drought is to bury themselves in self-made underground burrows (Carvalho et al. 2010). Heyer (1978) suggested that in species of the *L. fuscus* group the burrowing behavior is exclusive to males and used only for breeding purposes; burrows are used as incubation chambers. However, in the Caatinga, a semiarid ecoregion in northeastern Brazil, an individual of *L. fuscus* was found at a depth over 100 cm depth, aestivating in a skin cocoon (Varjão and Ribeiro 2018). A similar strategy may be employed by individuals of *L. gualambensis* to survive in drier parts of their distribution and this behavior could be reflected in the hind limb length, as in other species of head-first burrowers (e.g., Myobatrachidae, Vidal-García et al. 2014). Furthermore, although no data exists regarding digging behavior in the species, based on descriptions of burrow construction in other species of the *L. fuscus* group, an active function of hind limbs could be expected. Males of *L. fuscus* sensu stricto construct burrows by pressing their snouts against the ground, powered by their hind limbs (Martins 1988). Similar burrowing behavior was reported for *L. bufonius*, although a specific function of the hind limbs was not mentioned (Philibosian et al. 1974). We suggest that to reduce the risks of desiccation, individuals from populations inhabiting areas with more pronounced seasonal climate, such as the Dry Chaco, are forced to rely on fossorial behavior more frequently than individuals inhabiting areas with more homogeneous climatic conditions, such as those in the Humid Chaco and Cerrado. Based on the idea that hind limbs play an active role in digging behavior in *L. gualambensis*, and that shorter legs are more efficient in assisting digging in head-first burrowers, shorter leg lengths could be related to an increased fossoriality in populations inhabiting drier areas.

Another possible explanation for the intraspecific variation in *L. gualambensis* hind limb length is also related to the different climatic conditions faced by populations of the species, specifically with the developmental effects influenced by the duration of larval periods. Some species from arid or semi-arid environments exhibit a positive correlation between the larval period and hind limb length, potentially resulting in intraspecific morpho-

logical variation (Gómez-Mestre and Buchholz 2006). A suggested explanation of this phenomenon is that during pre-metamorphosis, a short larval period determines a short elongation phase of the hind limbs (Gómez-Mestre and Buchholz 2006). The larval period is influenced by diverse factors (see Székely et al. 2017), for pond-breeding species inhabiting semi-arid environments one of the main factors is the duration of temporary ponds. Some level of plasticity in larval development time in response to pond duration is an important adaptation to survive in an environment where breeding sites availability is a highly unpredictable resource (Newman 1992).

Leptodactylus gualambensis deposits eggs in a foam nest inside the underground nuptial chamber, constructed at the water's edge (e.g., temporary ponds). During rains, the tadpoles are washed out of the nest and develop as free larvae in the ponds (Fabrezi 2011, as *L. fuscus*). Differences in the availability and duration of superficial water throughout its geographic distribution are marked. In the Humid Chaco and Cerrado, where higher rain regimes and clayish soils prevail, permanent or semi-permanent superficial waters are very common. In contrast, a large portion of the Dry Chaco, characterized by less rain and sandy soils, offers mainly temporary ponds that may last only a few days. This could accelerate the metamorphosis and reduce the duration of the elongation phase of the hind limbs. We suggest that in individuals from populations in the drier portions of the species' distribution, where the reproduction occurs mainly in temporary ponds of short duration, comparatively shorter legs could result from a shorter elongation phase.

Other variation in morphological characters could also be related to inhabiting different environments in this species. *Rhinella granulosa*, with a similar geographic distribution pattern that includes contrasting environments, shows differences in ventral skin width and vascularization. Individuals from more xeric environments show thinner and more vascular ventral skin, a combination of traits more effective for water uptake (Navas et al. 2004). *Leptodactylus gualambensis*, with a geographic distribution including dry and highly seasonal environments as well as humid and relatively homogeneous environments, is a promising model for studying morphological adaptations associated with surviving in semiarid environments at intraspecific level and for better understanding the history of the Chaco anurofauna.

Other available names within the "*Leptodactylus fuscus* complex"

Although our main goal was to assess the taxonomic validity of *L. gualambensis*, the available names for different populations within *L. fuscus* deserve some discussion. As mentioned above, several synonyms have been attributed to *L. fuscus*, and throughout these years, with the goal of clarifying the taxonomic situation of the *L. fuscus* species complex, samples from type localities or localities attributable to some of these names have been included in different studies (e.g., Wynn and Heyer

2001; Heyer and Reid 2003; Camargo et al. 2006) but never discussed.

Before addressing the other names, we need to clarify an issue detected in the type series of *L. gualambensis*. Gallardo (1964) designated the specimen MACN 9752 as the holotype and included a drawing of a head in dorsal view in page 47, and a photograph of the complete body of an unlabeled specimen in the Plate II. In both figures the legend refers to the specimen represented as the holotype, with the following sentence: *Leptodactylus gualambensis* **nov. sp.**, MACN 9752 (typus). By the position of the legs is possible to note that the photographed specimen is the one currently labeled MACN 9753 (Fig. 9). To determine whether this was an error in the photograph or caption of Plate II in Gallardo (1964), or an error made when labeling the holotype, we examined the species description. However, it appears that only a few parts of the description are based solely on the holotype (e.g., measurements), while others, such as the reference to the medio-dorsal stripe being "frequently present", suggest an observation based on a group of specimens. Based on new measurements of the specimens MACN 9752 (SVL 43.76 mm) and MACN 9753 (SVL 45.53 mm), along with the total body length reported by Gallardo (1964) as 47 mm, it is evident that the specimen designated as holotype in the original description of *L. gualambensis* corresponds to the one currently labeled as MACN 9753, reinforcing the idea of a mistake when labeling the specimens. Given that both the photograph and the measurements indicate that the current MACN 9753 is the actual holotype described, despite the original description attributing the number MACN 9752 to it, we recommend switching the current labels to assign MACN 9752 to the correct specimen.

Leptodactylus fuscus clade 1 (*L. fuscus* *sensu stricto*)

Regarding the oldest names, such as *R. fusca* and *Rana typhonia* Daudin, 1802, we do not have new information, and the synonymy with *L. fuscus* is currently well accepted (see Heyer 1968, 1978; de Sá et al. 2014). We relate those names to the *L. fuscus* clade 1 because both share Suriname as their type locality (Heyer 1978). On the other hand, the synonymy of *Rana virginica* Laurenti, 1768 with *L. fuscus* is controversial. Both Heyer (1968) and Dubois and Ohler (2009) considered that the figure in Seba (1734: pl. 75, fig. 4), type of the species, represents a specimen of the genus *Lithobates* Fitzinger, 1843 (as *Rana pipiens* Schreber, 1782 in Heyer 1968) because of its webbed feet. However, in de Sá et al. (2014), this information was ignored, and *R. virginica* was included in the list of synonyms of *L. fuscus* without any comment. Furthermore, besides the doubts about the correspondence between Seba's (1734) figure and *L. fuscus*, the type locality of *R. virginica* was not designated (de Sá et al. 2014). Considering the mentioned evidence, we suggest the removal of the name *Rana virginica* Laurenti, 1768 from the synonymy of *Leptodactylus fuscus* and

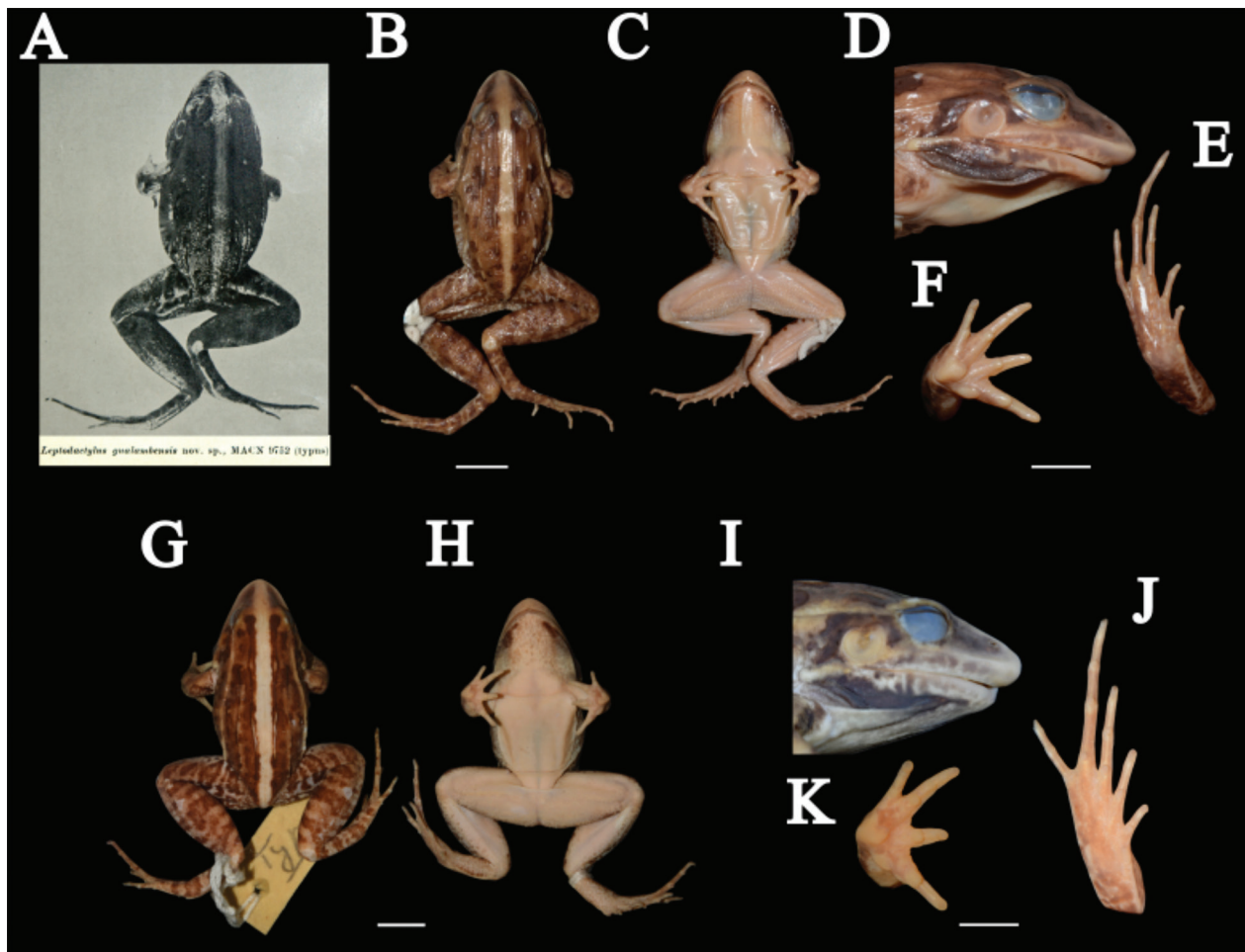


Figure 9. Part of the type series of *Leptodactylus gualambensis*. **A** capture of the photograph of the holotype of *Leptodactylus gualambensis* (MACN 9752) published in the original description of the species (Gallardo, 1964); **B** dorsal view of the body; **C** ventral view of the body; **D** lateral view of the head; **E** ventral view of the feet; **F** ventral view of the hand of the specimen currently labeled as MACN 9753; **G** dorsal view of the body; **H** ventral view of the body; **I** lateral view of the head; **J** ventral view of the feet; **K** ventral view of the hand of the specimen currently labeled as MACN 9752. Scale bars: 10 mm (B, C, G, H); 5 mm (D, E, F, I, J, K).

designate it as a nomen dubium until new evidence clarifies its application.

Another name that could be considered in the *L. fuscus* clade 1 is *Cystignathus schomburgkii* Troschel, 1848 (type locality Guyana). Boulenger (1882) synonymized this species with *Leptodactylus typhonius* (Daudin, 1802). Heyer (1978), following some traits from the original description, such as the species being closest to *C. gracilis* and having a uniform brown dorsal color, suggested that *L. fuscus*, *L. longirostris* (based on the *C. gracilis* similarity), and northern populations of *L. mystaceus* (based on dorsal color pattern), are the only known species from Guyana that could correspond to Troschel's description.

In the absence of type material (considered as lost), Heyer (1978) tentatively treated the name *C. schomburgkii* Troschel, 1848 as a *L. fuscus* synonym. Recently, the syntypes were found in the Museum für Naturkunde, Berlin, Germany. They were both originally labelled ZMB 3337, but one of them was renumbered as ZMB 75292 in November 2010 (Frank Tillack pers. comm.). The locality, according to the original catalog entry and jar label, is "Guiana", both collected by Robert Herman Schomburgk.

We had access to pictures of both specimens (Fig. 10). One of them shows serious skin and muscle damage on the left side of the dorsal surface (ZMB 3337).

Both specimens lack toe fringes, restricting their taxonomic identity to the species of the *L. fuscus* and *L. pentadactylus* groups (all species of the *L. latrans* group and *L. melanonotus* group have toe fringes; de Sá et al. 2014). Furthermore, both are adult males (evident vocal sac; Fig. 10A, B) and lack chest and thumb spines, which restricts them to the *L. fuscus* group (all species in the *L. pentadactylus* group have chest and/or thumb spines; de Sá et al. 2014). According to Cole et al. (2013), distribution maps provided by de Sá et al. (2014), and previous reports by Heyer (1978), only three species of the *L. fuscus* group are present in Guyana; *L. fuscus*, *L. longirostris*, and *L. mystaceus*.

Both syntypes of *C. schomburgkii*, in general, appear faded; however, the dorsal dermal folds are still evident (Fig. 10C, D). These folds are present only in *L. fuscus* and in individuals of *L. longirostris* with median dorsal stripe (Heyer 1978; de Sá et al. 2014; Silva et al. 2020). Another distinguishing character between *L. fuscus* and

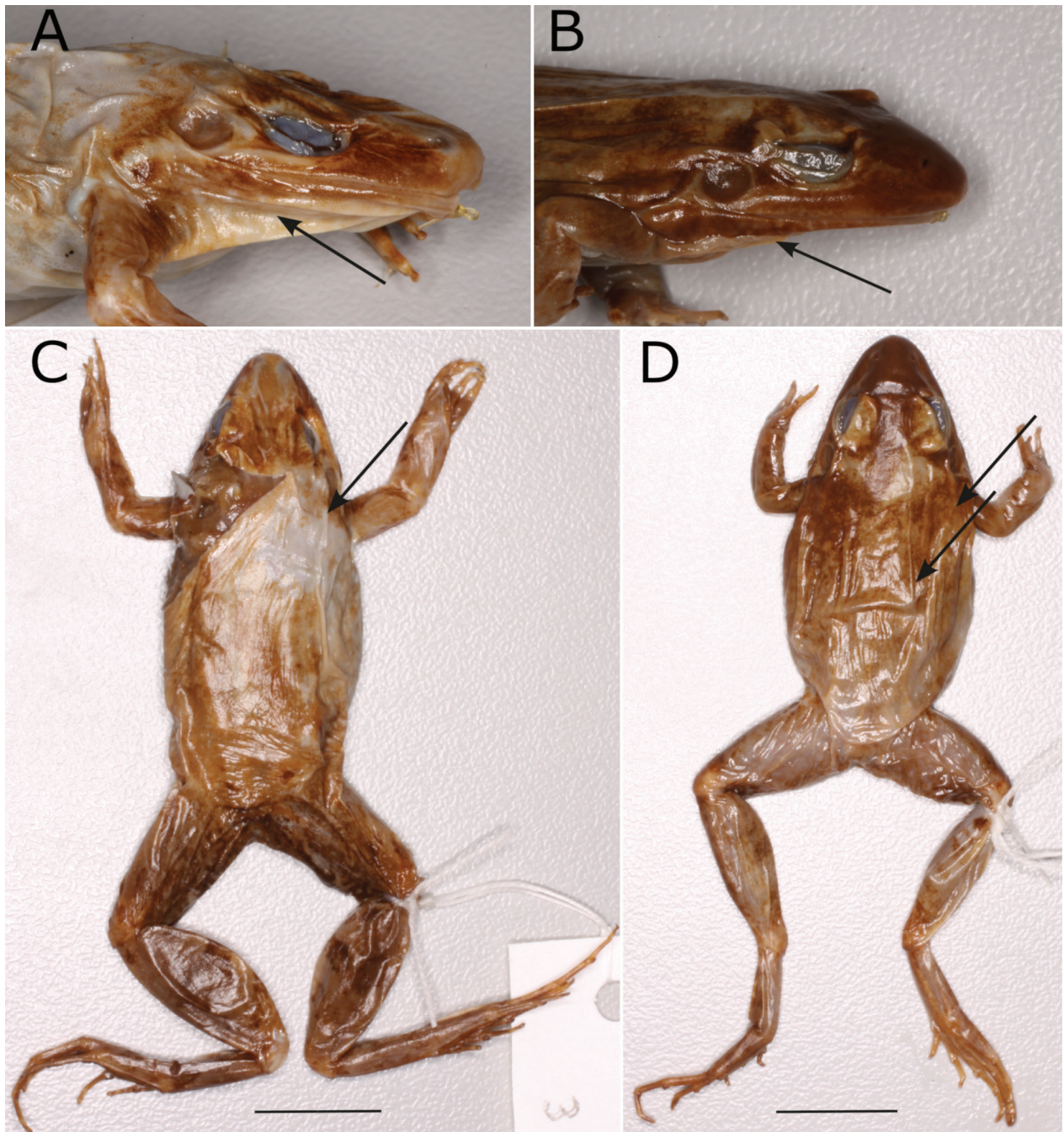


Figure 10. Syntypes of *Cystignathus schomburgkii*. **A** Lateral view of the head of the specimen ZMB 3337; **B** lateral view of the head of the specimen ZMB 75292; **C** dorsal view of the specimen ZMB 3337; **D** dorsal view of the specimen ZMB 75292. Black arrows denote vocal sacs (A, B) and dorsal dermal folds (C, D). Scale bars = 10 mm. Photos by Frank Tillack.

L. longirostris compared to *L. mystaceus* is the shape of the fingertips: *L. fuscus* and *L. longirostris* have narrow and thin fingertips, whereas *L. mystaceus* has globular fingertips (Heyer 1978; Toledo et al. 2005; de Sá et al. 2014; Silva et al. 2020; PDPP pers. observation).

Furthermore, in the original description of *C. schomburgkii*, Troschel (1848) mentioned that the vocal sac is very notable when inflated, forming a bubble-like extension on each side of the head. In *L. fuscus* and *L. longirostris* the vocal sac is double and lateral, while in *L. mystaceus* it is subgular and not expanding laterally (Heyer 1978; Toledo et al. 2005; de Sá et al. 2014). Both syntypes exhibit a double, lateral vocal sac (Fig. 10A, B),

dorsal folds, and narrow, thin fingertips (Fig. 10C, D). Those traits align with *L. fuscus* and *L. longirostris*, distinguishing them from *L. mystaceus*.

By discarding *L. mystaceus* from the list of potential synonyms with *C. schomburgkii*, two possibilities remain: *L. fuscus* and *L. longirostris*. According to Heyer (1978) and de Sá et al. (2014) there are no clear morphological characteristics that differentiate those species, as the intraspecific variation of both overlaps. Our examination of specimens of both species collected in Guyana also revealed no morphological differences (see Material Examined). This presents a challenge in resolving the taxonomic identity of *C. schomburgkii*, rendering its

synonymization with *L. fuscus* or *L. longirostris* inconclusive.

In light of this uncertainty, we follow the ICZN (1999) recommendation on the stability of nomenclature, maintaining the name *Cystignathus schomburgkii* Troschel, 1848, as a junior synonym of *Leptodactylus fuscus* (Schneider, 1799), as designated by Heyer (1978). However, it would be important to study the population genetics of *L. fuscus* and *L. longirostris* in Guyana. Given their sympatry and morphological similarities, the possibility of a complex interaction between them, including potential hybridization, cannot be ruled out.

Besides *L. gualambensis*, another *L. fuscus* synonym with a well-defined type locality is *Leptodactylus raniformis* Werner, 1899. In the phylogenetic analyses, we included a sample from the region of the type locality (previously included by Guarnizo et al. 2015; from Meta, Colombia, Andes-A 1763). Heyer (1978) mentioned that the holotype of *L. raniformis* (ZFMK 28484, adult male, Fig. S4), although with indistinct dorsolateral folds, presents a combination of traits characteristic of *L. fuscus*, such as the dorsal spots pattern, and tarsal and foot surfaces smooth with light pigmented spots. Based on the conclusion of Heyer (1978) and on our resulting topology (Fig. 2), with all samples from Colombia grouped together with the samples from Suriname (type locality of *L. fuscus*) in the *L. fuscus* clade 1 (i.e., *L. fuscus* sensu stricto), we corroborate that *L. raniformis* is a junior synonym of *L. fuscus*.

***Leptodactylus fuscus* clade 3**

Two names are available for populations of *L. fuscus* from Brazil: *Rana pachypus* var. 2 Spix, 1824 from Pará State and *Rana sibilatrix* Wied, 1824 from the coast of southern Bahia state. The only character of *R. pachypus* var. 2 mentioned by Spix (1824) that could be attributable to *L. fuscus* is the presence of six longitudinal folds. However, the synonymy was suggested by Peters (1872), who according to Hoogmoed and Gruber (1983) was the only one who examined the type-specimen, currently lost. We were not able to include any sample that could be assigned to *R. pachypus* var. 2 in our analyses. Vanzolini (1981), based on Spix and Martius' itinerary, restricted the possible type locality to the surroundings of the city of Belém (State of Pará). Our nearest sampled locality is Barreirinhas, Maranhão, which is 650 km E of Belém (Fig. 1). In view of the type specimen being lost and the lack of samples attributable to *R. pachypus* var. 2, we suggest maintaining the synonymy as proposed by Peters (1872).

Regarding *R. sibilatrix*, the short morphological description, comments about its vocalization, and the figure (see Wied 1824), correspond with a specimen that could be part of the *L. fuscus* species complex. Additionally, Heyer (1978) pointed out that *L. fuscus* is the only species with the characteristics described on the coast of southern Bahia. Heyer (1978) also mentioned some uncertainties about the correspondence between the only available syntype (AMNH A-485) and the figure pub-

lished in Wied (1824). Vanzolini and Myers (2015), based on a direct comparison of the syntype and Wied's figure, agree with Heyer (1978) that it is not the specimen drawn. This idea is reinforced by size differences between the syntype AMNH A-485 (SVL of about 36 mm, estimated from the photograph in Vanzolini and Myers 2015) and the size mentioned by Wied (~ 42.8 mm, "an inch plus seven lines"), although without a reference to a specific specimen in Wied (1824). Vanzolini and Myers (2015) recommended that, if necessary, either the only existing syntype (AMNH A-485) or the lost specimen drawn in Wied (1824) could be designated as lectotype.

We refrain from suggesting any taxonomic decisions regarding *R. sibilatrix* given the lack of support for the *L. fuscus* clade 3, and our sampling missing any locality at least near its type locality. Müller (1927) restricted the type locality of *R. sibilatrix* to the first locality mentioned by Wied (1824), Peruípe River, Nova Viçosa (Peruhybe bei Villa Viçoz). The other localities mentioned by Wied are all proximal to Nova Viçosa; Caravelas to the north and Mucurí to the south, both also on the coast of southern Bahia. Our sequences from the closest localities to these mentioned are from Linhares, Espírito Santo (CFBH 32037), 195 km S of the type locality, and from Itacaré (CFBH 21108) and Uruçuca (CFBH 32435), Bahia, 400 and 350 km N from Nova Viçosa, respectively.

The geographic distances between our samples and the type locality are not the only challenges in associating any of the samples with this name. The samples mentioned were grouped into different sub-clades, which, although not well-supported, show relatively high genetic distance among them (16S p distance from 1.4 to 3.3%). Within sub-clades, genetic distances among some localities, such as Buritizeiro, Minas Gerais state, Brazil (LHUF CG 491) and those from Bahia state, Brazil (CFBH 21078, CFBH 21086, and CFBH 21108), were also relatively high (16S p distance from 1.6 to 2.3%).

As mentioned above, our main goal was to test the taxonomic validity of *L. gualambensis*, focusing our efforts on sampling the Chaco and surroundings areas. With our current sampling, we are not able to clarify the taxonomic status of all populations of *L. fuscus* and the still available names; however, we believe this study significantly reduces the problem. With the recognition of *L. gualambensis* as a valid species for Chaco populations and the restriction of *L. fuscus* sensu stricto (and its synonyms) to the populations of northern South America and Central America, only two names remain unresolved: *Rana sibilatrix* and *Rana pachypus* var. 2.

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Supplementary Material 1

Figures S1–S4

Authors: Brusquetti F, Bueno-Villafañe D, Caballero-Gini A, Pinheiro PDP, Netto F, Burgos-Gallardo F, Fernández Ríos D, Bernabé Cardozo E, Baldo D (2025)

Data type: .pdf

Explanation notes: **Figure S1.** Bayesian inference tree. — **Figure S2.** Maximum parsimony tree. — **Figure S3.** Principal Component Analysis (PCA) based on morphometric measurements. — **Figure S4.** Holotype of *Leptodactylus raniformis* (ZFMK 28484).

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Link: <https://doi.org/10.3897/vz.74.e159878.suppl1>

Supplementary Material 2

Table S1

Authors: Brusquetti F, Bueno-Villafañe D, Caballero-Gini A, Pinheiro PDP, Netto F, Burgos-Gallardo F, Fernández Ríos D, Bernabé Cardozo E, Baldo D (2025)

Data type: .pdf

Explanation notes: Loadings (eigenvectors) and variance explained by the first four principal components (PC1–PC4) obtained from the principal component analysis (PCA) of morphometric variables.

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Link: <https://doi.org/10.3897/vz.74.e159878.suppl2>

Supplementary Material 3

Files S1–S3

Authors: Brusquetti F, Bueno-Villafañe D, Caballero-Gini A, Pinheiro PDP, Netto F, Burgos-Gallardo F, Fernández Ríos D, Bernabé Cardozo E, Baldo D (2025)

Data type: .zip

Explanation notes: **File S1.** Geographical data and GenBank accession numbers of the sequences of 16S (16S ribosomal RNA gene) and COI (cytochrome *c* oxidase subunit I) used in phylogenetics analyses and genetic distance calculations [.xlsx file]. — **File S2.** Specimens examined for morphological analyses and species comparisons with their respective localities [.doc file]. — **File S3.** Advertisement call records analyzed [.xlsx file].

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Link: <https://doi.org/10.3897/vz.74.e159878.suppl3>