



New evidence for distinctiveness of the island-endemic Príncipe giant tree frog (Arthroleptidae: *Leptopelis palmatus*)

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The Príncipe giant tree frog *Leptopelis palmatus* is endemic to the small oceanic island of Príncipe in the Gulf of Guinea. For several decades, this charismatic but poorly known species was confused with another large tree frog species from continental Africa, *L. rufus*. Phylogenetic relationships within the African genus *Leptopelis* are poorly understood and consequently the evolutionary history of *L. palmatus* and its affinity to *L. rufus* remain unclear. In this study, we combined mitochondrial DNA (mtDNA), morphological, and acoustic data for *L. palmatus* and *L. rufus* to assess different axes of divergence between the species. Our mtDNA gene tree for the genus *Leptopelis* indicated that *L. palmatus* is not closely related to *L. rufus* or other large species of *Leptopelis*. Additionally, we found low mtDNA diversity in *L. palmatus* across its range on Príncipe. We found significant morphological differences between females of *L. rufus* and *L. palmatus*, but not between males. We characterised the advertisement call of *L. palmatus* for the first time, which is markedly distinct from *L. rufus*. Finally, we summarised our observations of *L. palmatus* habitats and additional notes on phenotypic variation and behaviour. Our study reinforces the distinctiveness of *L. palmatus* and provides information important for the conservation of this endangered species.

A rã gigante de Príncipe, *Leptopelis palmatus*, é endêmica da pequena ilha oceânica de Príncipe no Golfo da Guiné. Por várias décadas, esta espécie carismática mas pouco conhecida foi confundida com outra espécie grande de rã da África continental, *L. rufus*. As relações filogenéticas dentro do gênero africano *Leptopelis* são mal compreendidas e, conseqüentemente, a história evolutiva de *L. palmatus* e sua afinidade com *L. rufus* permanecem obscuras. Neste estudo, combinamos dados de DNA mitocondrial (mtDNA), morfológicos e acústicos de *L. palmatus* e *L. rufus* para avaliar diferentes eixos de divergência entre as espécies. Nossa árvore de genes de mtDNA para o gênero *Leptopelis* indicou que *L. palmatus* não está proximalmente relacionada a *L. rufus* ou outras espécies grandes de *Leptopelis*, e encontramos baixa diversidade de mtDNA em *L. palmatus* em toda a sua distribuição em Príncipe. Encontramos diferenças morfológicas significativas entre as fêmeas de *L. rufus* e *L. palmatus*, mas não entre os machos. Caracterizamos o canto reprodutor de *L. palmatus* pela primeira vez, que é marcadamente distinto do de *L. rufus*. Finalmente, resumimos nossas observações dos habitats de *L. palmatus* e notas adicionais sobre variação fenotípica e comportamento. Nosso estudo fornece informações importantes para a conservação dessa espécie ameaçada de extinção.

Keywords: bioacoustic, morphology, endemic, mitochondrial DNA, island, São Tomé and Príncipe

INTRODUCTION

The Príncipe giant tree frog *Leptopelis palmatus* (Peters, 1868) is endemic to the small (142 km²) oceanic island of Príncipe in the Gulf of Guinea. For several decades, this charismatic but poorly known species was confused with another large tree frog species from continental Africa, *L. rufus* Reichenow 1874 (Anderson, 1909; Parker, 1936; de Witte, 1941; Perret, 1962). Phylogenetic relationships within the African genus *Leptopelis* are poorly understood and consequently the evolutionary history of *L. palmatus* and its affinity to *L. rufus* remain unclear. Previous authors have hypothesised that *L. palmatus* is closely related to *L.*

rufus and other large-bodied species in West and Central Africa (*L. macrotis*, *L. millsoni*) based on a combination of mtDNA and morphological data (e.g., Idris, 2004). A more recent study estimating diversification across Afrobratrachia, which is composed of the families Arthroleptidae, Brevicipitidae, Hemisotidae, and Hyperoliidae, does not support this relationship (Portik et al., 2019); however, relationships among *Leptopelis* were not the primary focus of that study and the molecular data matrix was a combination of mtDNA and very sparse nuDNA loci. Here we include more comprehensive sampling of *Leptopelis* diversity (55 % of currently recognised species) and use a complete mtDNA data matrix with the sole aim of identifying the

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closest continental relatives of the island endemic. We also generate mtDNA sequence data from *L. palmatus* collected across its elevational and geographic range on Príncipe to assess genetic diversity and phylogeographic structure within the species.

Throughout nearly a century of taxonomic confusion between *L. palmatus* and *L. rufus*, *L. palmatus* was reported from several countries including Cameroon, Equatorial Guinea (including Bioko Island), Gabon, and Nigeria (Boulenger, 1882; Mocquard, 1902; Boulenger, 1906; Nieden, 1910; Ahl, 1931; Schiøtz, 1963; Mertens, 1965). After comparing a large series of male and female *L. rufus* with the sole female holotype of *L. palmatus* available for study, Perret resurrected *L. rufus* and clarified that *L. palmatus* was an insular species (Perret, 1973). Perret also remarked that *L. rufus* and *L. palmatus* differed in tympanum size and several additional morphological features, concluding that despite decades of taxonomic confusion the two species may not even be closely related (Perret, 1973). Loumont later collected a series of eight *L. palmatus* females, confirmed the results of Perret's morphological study and reported a snout-vent length of up to 110 mm, which remains the largest size record for the entire genus (Loumont, 1992; Channing & Rödel, 2019). Male specimens of *L. palmatus* were finally collected and formally described following an expedition to Príncipe in 2002 (Drewes & Stoelting, 2004). We have since collected additional male and female specimens of *L. palmatus*, providing the opportunity to compare large series of both sexes of *L. palmatus* and *L. rufus* to assess phenotypic variation within and between the species.

Very little is known about the natural history of *Leptopelis palmatus*, an endangered island endemic (IUCN SSC Amphibian Specialist Group, 2020). Although males lack vocal sacs (Drewes & Stoelting, 2004), they do produce advertisement calls and here we report the first recording and analysis of their call, which we compare to that of *L. rufus*. We also summarise our observations of *L. palmatus* habitats and additional notes on phenotypic variation and behaviour. In summary, our study combines mitochondrial DNA (mtDNA), morphological, and acoustic data for *L. palmatus* and *L. rufus* to assess different axes of divergence between the species. We couple our findings of distinctness of *L. palmatus* with additional notes on phenotypic variation and behaviour to provide important information for the conservation of this endangered species.

METHODS

Field sampling

We conducted six herpetological expeditions to Príncipe Island between 2001 and 2016, during which we searched for *Leptopelis palmatus*. Tissue samples (including liver or muscle) were preserved in 95 % ethanol or RNAlater and voucher specimens were fixed in formalin. The specimens were deposited at the California Academy of Sciences (CAS) and the U.S. National Museum of Natural History (USNM).

Phylogenetic and Population Genetic Analyses

We obtained 16S mtDNA sequences for 30 species in the genus *Leptopelis* (55 % of the currently recognised species) and two confamilial outgroups (*Arthroleptis* and *Cardioglossa*; Portik & Blackburn, 2015) from GenBank, only selecting sequences with associated voucher specimens (Table S1). We aligned the sequences with MAFFT using the automatic algorithm selection option (--auto) (Katoh et al., 2002; Katoh & Standley, 2013) and selected the HKY + I + G substitution model based on BIC in jModelTest (Darriba et al., 2012). To perform Bayesian phylogenetic analyses, we used BEAST v1.8 (Drummond et al., 2012) with a birth-death tree prior (Stadler, 2009), and a lognormal relaxed molecular clock. Two independent analyses were run for 10 million generations each with sampling every 1,000 generations, producing a total of 20,000 trees. We assessed convergence and the effective sample size of parameter estimates using Tracer v1.7 (Rambaut et al., 2018) and repeated simulations without sequence data to test the influence of priors on posterior distributions. We discarded a burn-in of 10 % prior to generating a maximum clade credibility tree from the remaining 18,000 trees.

To assess mtDNA structure across the entire known geographic and elevational range of *L. palmatus*, we extracted DNA from 35 tissue samples using a DNeasy Blood & Tissue kit (Qiagen Inc., Valencia, CA, USA), and polymerase chain reaction (PCR)-amplified and cycle sequenced a portion of the 16s mitochondrial gene using the primers 16SA and 16SB (Palumbi et al., 1991). Each reaction contained the following components: 1 µL of template DNA, 6.05 µL H₂O, 1.75 µL 10X Buffer, 0.3 µL of each primer, 0.25 µL dNTPs, 0.1 µL of Bovine serum albumin (BSA), 0.1 µL of MgCl, and 5 µL of goTAQ DNA Polymerase (Promega, Madison, WI, USA). We used a thermocycler protocol for amplification beginning with denaturation for 1 min 30 seconds at 94 °C, followed by 33 cycles, which consisted of 45s denaturation at 94 °C, 45s annealing at 55 °C, and 1 min 30s extension at 72 °C, followed by the final extension occurring at 72 °C for 5 min. We purified PCR products using ExoSAP-IT (USB Corp., Cleveland, OH, USA) and carried out sequencing using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI automated 3730xl Genetic Analyzer (Applied Biosystems). All sequences were edited using Geneious v.R8.0.04 (Biomatters Ltd.) and are available on GenBank (see Supplementary Materials). Due to the limited genetic diversity in our dataset, we estimated a mtDNA haplotype network using the TCS algorithm (Clement et al., 2000) implemented in PopArt (Leigh & Bryant, 2015).

Morphological data collection and analysis

To assess the maximum reported female body size of *L. palmatus* relative to other species included in the phylogeny of the genus we obtained measurements from the literature (Table S1) and plotted them alongside the phylogeny. To compare variation in diagnostic traits between *L. rufus* and *L. palmatus* (Perret, 1973; Schiøtz, 1999), we took morphological measurements of adult

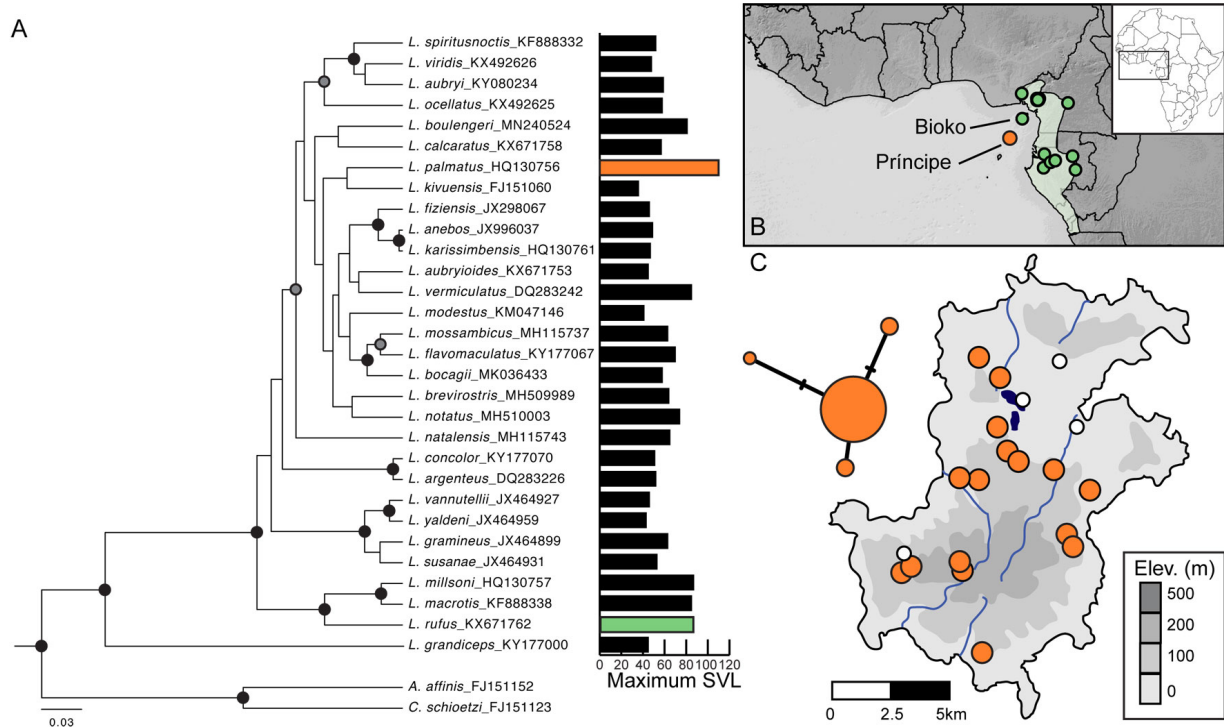


Figure 1. (a) 16S mtDNA gene tree of 30 currently recognised species in the genus *Leptopelis* with maximum female (the larger sex) snout-vent length (SVL) in millimetres. Black circles on nodes indicate > 0.95 posterior probability, grey circles indicate > 0.85 posterior probability. All other nodes are poorly supported. (b) Geographic sampling of *L. rufus* and *L. palmatus* examined in this study. (c) Sampling map and mtDNA haplotype network for *L. palmatus* on Principe Island. Open circles indicate additional localities with vouchered *L. palmatus* specimens that are currently housed in the Museu Nacional de História Natural e da Ciência, Universidade de Lisboa, Portugal (Ceríaco, 2016; Ceríaco & Marques, 2018).

frogs to the nearest 0.1 mm using Mitutoyo Absolute Digimatic Callipers. The following measurements were taken by KEJ and RCB: snout-vent length (SVL); eye diameter, measured as the ocular aperture (EYE); maximum horizontal diameter of left tympanum, measured to outer margin of tympanic rim (TMP); and maximum diameter of disc toepad, measured from the fourth digit of the left hindfoot (DSC). The ratios TMP:EYE and DSC:TMP have been proposed as useful diagnostic comparisons, but without accounting for potential sexual variation (Schiøtz, 1999). Sex was determined by a) snout-vent length, b) the presence (or absence) of pectoral glands in preserved specimens, c) the presence of eggs, or d) field notes indicating calling behaviour. A total of 109 adult specimens were measured (39 *L. palmatus* and 70 *L. rufus*). Male and female measurements were analysed separately to account for sexual size dimorphism. We omitted 10 samples that could not be identified to sex, resulting in a dataset of 58 male (19 *L. palmatus* and 39 *L. rufus*) and 41 female specimens (17 *L. palmatus* and 24 *L. rufus*). To account for allometry across individuals, we corrected TMP measurements (Thorpe, 1975; Thorpe, 1983a; Thorpe, 1983b; Turan, 1999) using the allometric equation: $X_{adj} = X - \beta(SVL - SVL_{mean})$. In this equation, X_{adj} is the adjusted value of the morphometric variable measurement, X is the original value taken from the dial callipers, β is the coefficient of the linear regression of X against SVL in the dataset, SVL is the snout-vent length of the individual, and SVL_{mean} is the overall mean snout-vent length in the dataset. To quantify divergence in

SVL, TMP, TMP:EYE, and DSC:TMP, we fit an ANOVA for each set of traits with measurements grouped by species and used a Tukey Honest Significant Differences test to calculate adjusted P values for group mean comparisons. All statistical analyses were performed in R v 4.0.2 (R Core Team 2020), and data were visualised using the ggplot2 package (Wickham, 2016).

Bioacoustic data collection and analysis

We collected an acoustic recording of a male *L. palmatus* on November 28, 2016 (25.9 °C) in situ at a presumed breeding site. The call was recorded using a Roland R-26 portable recorder paired with a Sennheiser ME-66 microphone at an approximate distance of 2 m from the male. We described the calling site and behavioural context, and prepared the male as a voucher specimen for genetic and morphological analysis (USNM 591754). The recording was archived in the Macaulay Library at the Cornell Lab of Ornithology (ML 206529). We analysed advertisement calls of *L. rufus* that were recorded in Cameroon (Amiet & Goutte, 2017). Audio spectrograms and oscillograms were made using Raven Pro 1.4 (Cornell Lab of Ornithology), and analysed with a Fast Fourier Transformation window of 512 points, a brightness of 70 points, and a contrast of 70 points following Gilbert and Bell (2018). The following parameters were measured for each call because they capture the primary axes of variation in *Leptopelis* advertisement calls (Amiet & Goutte, 2017): pulses per call, pulse duration, peak frequency (Hz), frequency range (Hz), and total number of notes recorded.

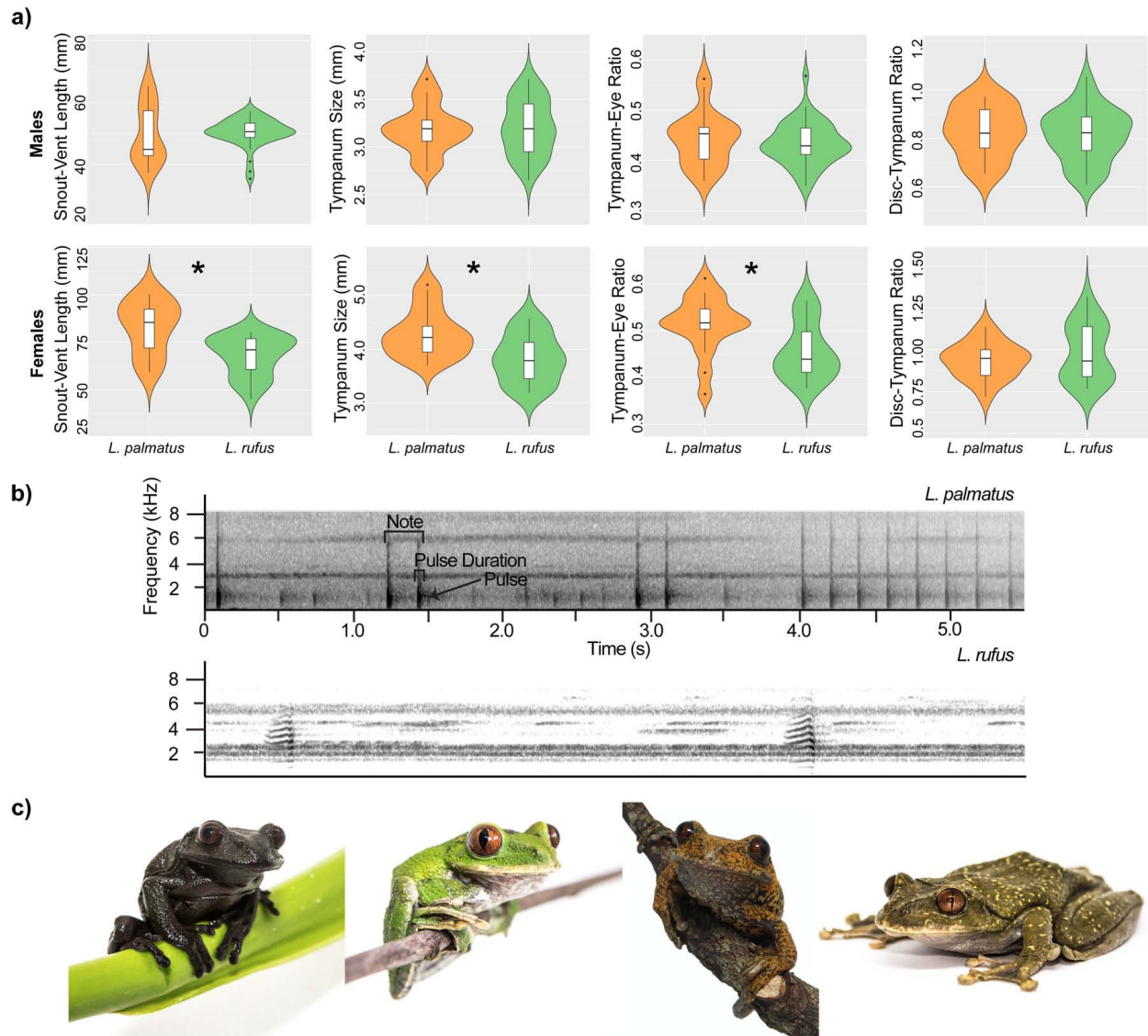


Figure 2. (a) Violin plots of adjusted Snout-Vent Length (SVL), Tympanum size (TMP), Tympanum-Eye ratio (TMP:EYE), and Disc-Tympanum ratio (DSC:TMP) in *L. palmatus* and *L. rufus*. Comparisons significant at an adjusted $P < 0.05$ with a Tukey Honest Significant Difference test are indicated with an asterisk. (b) Call parameter definitions and representative audio spectrograms of *L. palmatus* and *L. rufus* male advertisement calls. (c) colour variation in *L. palmatus* (from left to right: CAS 258958, CAS 258910, USNM 591753, USNM 591758) Photos A. Stanbridge and R. Bell.

RESULTS

Phylogenetic relationships within *Leptopelis* and genetic variation within *L. palmatus*

The 16S mtDNA gene tree of species-level relationships within the genus *Leptopelis* indicated strong support for the monophyly of some species groups including the large-bodied West and Central African species *L. rufus*, *L. macrotis*, and *L. millsoni*, and the Ethiopian radiation *L. vannutellii*, *L. yaldeni*, *L. gramineus* and *L. susanae* (Fig. 1A). By contrast, the phylogenetic placement of most species including *L. palmatus* was poorly supported in the present dataset. However, our results indicate that *L. palmatus* does not appear to be closely related to *L. rufus* or to other, larger-bodied species in the genus. We found very low diversity at 16S across the range of *L. palmatus* on Príncipe Island with one dominant haplotype, three minor haplotypes, and no pattern of phylogeographic structure (Fig. 1C).

Morphological divergence between *L. rufus* and *L. palmatus*

We found that morphological differences between *L. rufus* and *L. palmatus* were distinct in females versus males. SVL, adjusted TMP size, and TMP:EYE ratios were all significantly different between female specimens of *L. palmatus* and *L. rufus*, but not male specimens (Fig. 2). Overall, *L. palmatus* females exhibited larger body sizes and had larger TMP than *L. rufus* ($P < 0.05$; Fig. 2A). Likewise, the ratio of TMP:EYE was typically $> \frac{1}{2}$ in female *L. palmatus* and $< \frac{1}{2}$ in female *L. rufus* ($P < 0.05$; Fig. 2A). DSC:TMP ratios were not significantly different between *L. palmatus* and *L. rufus* in either males or females, and none of the remaining measurements were significantly different between males of the two species (Fig. 2A).

Table 1. Summary of call analysis for each species. Abbreviations are as follows: APF (Average Peak Frequency), APD (Average Pulse Duration)

Species	Location	Notes	Pulses	APF (Hz)	APD (s)	Recording Source
<i>L. palmatus</i>	Príncipe, trail from São Joaquim village to Pico Príncipe	14	1–8	1170 (937–1406)	0.04 (0.03–0.05)	This study (ML 206529)
<i>L. rufus</i> 1	Cameroon, Ototomo	6	1	2857 (2756–3359)	0.24 (0.22–0.26)	Amiet & Goute, 2017
<i>L. rufus</i> 2	Cameroon, Kala Afomo	7	1	3248 (2842–3962)	0.23 (0.23–0.24)	Amiet & Goute, 2017

Calling sites, advertisement call, and colour variation in *L. palmatus*

We observed calling males on two occasions in 2015 (6 October 2015 and 7 October 2015) but were unable to obtain recordings. The first site was along the road between Gaspar and Sundi where we observed a calling male perched 2 m above ground on a vine at 1958h (CAS 258910). A gravid female was found nearby several minutes later (CAS 258911). The second site was along a small stream flowing through secondary forest in the vicinity of Nova Estrela where we found two calling males at 4 m (CAS 258916) and 2.5 m (CAS 258917) above ground at approximately 1800h. On 28 November 2016 we observed several calling males along the trail from São Joaquim village to Pico Príncipe, in a tributary of the Ribeira Banzú. Males were perched 2–3 m above ground on palm fronds, tree branches, and tree trunks in the vicinity of a medium stream (3 m across) and above a swampy ditch. We recorded one male (USNM 591754) at 1805h, which was perched approximately 2 m above the ground on a palm frond. The advertisement call consisted of a simple note with a dominant frequency of 1170 Hz, average pulse duration of 0.04 seconds, and a range of pulses from 1–8 (Fig. 2B, Table 1). In the period we recorded, several calling bouts progressed from notes with one pulse to two pulses to a series of eight pulses (Fig. 2; Table 1). The waveform and structure of the *L. palmatus* call is quite different from that of *L. rufus*, which has a much higher dominant frequency (2800+ Hz), longer average pulse duration (0.23 seconds), single-pulse notes, and numerous harmonics (Fig. 2B; Table 1). We documented extensive dorsal colour pattern variation in both male and female *L. palmatus* including dark green/black with or without spots, bright green, and bright yellow with dark mottling (Fig. 2C; Table S2).

DISCUSSION

Phylogenetic relationships within the genus *Leptopelis* are poorly understood; however, relationships among some species groups are strongly supported in our mtDNA gene tree and are consistent with phenotypically and/or geographically cohesive groups (Portillo & Greenbaum, 2014; Reyes-Velasco et al., 2018). Although the phylogenetic placement of *L. palmatus* remains unclear, our inference does not support a close affinity to *L. rufus*, as foreshadowed by Perret's morphological comparisons (Perret, 1973). More complete taxonomic

sampling and a larger genetic dataset will be necessary to establish a robust phylogenetic inference for the genus *Leptopelis* and to inform the biogeographic history of *L. palmatus*. Our results indicate very low genetic diversity in *L. palmatus* and no evidence of phylogeographic structure across its range. Two other amphibian species are also endemic to Príncipe Island: the puddle frog *Phrynobatrachus dispar* (Peters, 1870) and the reed frog *Hyperolius drewesi* Bell 2016. Both are very abundant and based on our surveys, their distributions span even more of the geographic and elevational range of the island than does *L. palmatus* (Loumont, 1992; Drewes & Stoelting, 2004; Uyeda et al., 2007; Bell, 2016). Population genetic studies for both *P. dispar* and *H. drewesi* found very low genetic diversity and no patterns of genetic structure based on mtDNA-only (*P. dispar*, Uyeda et al., 2007) or a combination of mtDNA and genome-wide SNPs (*H. drewesi*, Bell et al., 2015). Consequently, there do not appear to be geographic barriers to gene flow across the ranges of the endemic amphibians of Príncipe.

Cryptic morphological variation has eluded species relationships in the genus *Leptopelis*, including nearly a century of taxonomic confusion between *L. palmatus* and *L. rufus*. Our morphological assessments of overall body size and tympanum measurements indicate significant differences between *L. palmatus* and *L. rufus* in females but not in males. In particular, the ratio of tympanum size to eye size, which has been proposed as a diagnostic character among large-bodied *Leptopelis* (*L. macrotis*, *L. millsoni*, *L. palmatus*, *L. rufus*; Schiøtz, 1999) is adequate for differentiating among *L. rufus* and *L. palmatus* females, but not males. Although the ratio of disc to tympanum width has also been proposed as diagnostic (Schiøtz, 1999), toe disc dimensions may change with preservation and be a less reliable feature to measure than tympanum or eye diameter. Sexual dimorphism in tympanum size is known in other anurans and may have functional consequences for acoustic signalling and sensitivity (Fox, 1995). Consequently, estimating acoustic sensitivity in male and female *L. palmatus* relative to male and female *L. rufus* would be an interesting extension of the pattern we identified here. Our results support that exceptional body size in *L. palmatus* is due to females reaching very large sizes whereas male body size appears to be within the range of male *L. rufus*. Despite large adult body sizes, post-metamorphic individuals of *L. palmatus* are quite small (10–11 mm; Drewes & Stoelting, 2004) and it is unknown how long individuals take to reach

reproductive maturity or how old very large females are. It is unclear what selective pressures (or lack thereof) on Príncipe have resulted in the apparent island gigantism of this species.

The advertisement call of *L. palmatus* is rather quiet and our observations thus far indicate that males form small breeding aggregations. The call of *L. palmatus* is quite distinct from that of *L. rufus*, further suggesting that these species are not closely related. We observed the other two island endemic anurans *P. dispar* and *H. drewesi* at breeding sites with *L. palmatus*. The calls of the three species are different in waveform and frequency with *P. dispar* producing long trills at higher frequencies (4500–5000 Hz; Loumont, 1992), *H. drewesi* producing single to multi pulsed notes at intermediate frequencies (2520–3020 Hz; Gilbert & Bell, 2018), and *L. palmatus* producing single to multi pulsed notes at lower frequencies (937–1406 Hz; this study). Consequently, there is very little acoustic overlap between the species and passive acoustic recording devices could be an effective strategy to gather more extensive data on geographic occupancy and seasonal activity in the three species (e.g., Sugai et al., 2019).

Previous authors have reported extensive dorsal colour pattern variation in *L. palmatus* ranging from dark green/black with or without small white spots to light brown and bright green (Manaças, 1958; Loumont, 1992; Drewes & Stoelting, 2004). We have observed all of these colour variants as well as vibrant yellow coloration with dark mottling. From our observations to date, it is not clear if colour variation differs systematically between the sexes or across ontogeny. Colour and pattern variation is known in other species of *Leptopelis* in which there are green phases and brown phases that may vary across ontogeny in some species (e.g., *L. bocagii*, *L. boulengeri*, *L. cynnamomeus*, *L. mossambicus*, *L. natalensis*, *L. notatus*, *L. occidentalis*, *L. ragazzii*, *L. susanae*, *L. vermiculatus*, *L. uluguruensis*, *L. yaldeni*; Schiøtz, 1999; Amiet, 2012). The genetic basis and ecological relevance of this colour pattern variation have not yet been characterised and coloration in *L. palmatus* seems to be among the most variable of all species in the genus.

Extensive surveys indicate the Príncipe giant tree frog, *L. palmatus*, is primarily found in forested habitats from sea level to over 600 m elevation (Loumont, 1992; Drewes & Stoelting, 2004; this study). Consistent with previous survey efforts, we found both males and females perched one meter or higher off the ground on branches or leaves, especially near small, flowing streams at night (Loumont, 1992; Drewes & Stoelting, 2004). Previous reports indicated that large females may be encountered on or near the ground (Drewes & Stoelting, 2004); correspondingly, we encountered one large female (CAS 258958 SVL = 100.0 mm) on the ground near a waterfall at 1245h, a second large female (USNM 591753 SVL = 76.6 mm) on a thin branch approximately 20 cm above ground in the forest at 1735h, a third large female (CAS 261010 SVL = 97.7 mm) on a tree root approximately 10 cm above ground near a wide, muddy stream at 1817h, and a fourth large female (USNM 591798 SVL = 90.5

mm) in a puddle in the road at 1943h. Unfortunately, the reproductive biology of *L. palmatus* is entirely unknown and we have been unsuccessful in locating eggs/larvae or observing amplexus in this species whereas we have observed egg deposition sites and collected larvae of the other two anuran species on Príncipe (RCD, RCB, per. obs.). Documenting this species' reproductive mode and specific breeding habitat will be critical to understanding the habitats *L. palmatus* relies upon throughout its lifecycle.

Our study confirms the distinctiveness of *L. palmatus* relative to other large-bodied tree frogs in the genus. Our mtDNA gene tree for the genus *Leptopelis* indicated that continental species of large tree frogs (*L. rufus*, *L. macrotis*, *L. millsoni*) form a distinct clade, and that *L. palmatus* is not closely related to this species group, reinforcing that gigantism has evolved multiple times in the genus. We found low mtDNA diversity and high colour variation within *L. palmatus*, suggesting no mtDNA genetic structure associated with phenotypic variation across the island, although more studies are needed to characterise the genetic basis and ecological relevance of colour variation. Morphological differences between *L. palmatus* and *L. rufus* are most apparent in females, which could be in part why taxonomic confusion persisted for decades. Finally, we demonstrated that the advertisement call of *L. palmatus* is markedly distinct from *L. rufus* and further supports *L. palmatus* being distinct from *L. rufus*. Future studies that describe the reproductive strategy of *L. palmatus* will provide essential information to guide conservation of this unique and endangered species.

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