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Diversity and distribution of reed frogs (*Hyperolius* spp.) on Bioko Island, Equatorial Guinea

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Reed frogs (*Hyperolius* spp.) are the most species-rich genus in the family Hyperoliidae, a group of frogs endemic to sub-Saharan Africa and the Gulf of Guinea islands. Three species are endemic to oceanic islands in the archipelago, but the diversity and distribution of reed frogs on Bioko – a land-bridge island in the archipelago – remains unclear. Two species, *H. tuberculatus* and *H. ocellatus ocellatus*, were formally documented on Bioko in the colonial era, however, two additional reed frog species have been detected in recent surveys. In addition, observations of heterospecific amplexus suggest one of the unidentified species and *H. o. ocellatus* may be hybridising on the island. In this study, we examined mitochondrial DNA (mtDNA), nuclear DNA (nuDNA), morphological and acoustic data to confirm the tentative identifications of the reed frogs and determine if there is any evidence of hybridisation. Our data confirm that the two unidentified species are *H. endjami* and *H. fusciventris*, and that Bioko *H. endjami* have unique genetic diversity not shared with the mainland population. Additionally, our mitochondrial (16S) and nuclear DNA (CMYC) haplotype networks suggest that *H. o. ocellatus* and *H. endjami* are not hybridising on Bioko. Finally, we report colour pattern variation in *H. endjami* and *H. o. ocellatus* that is unique from populations on the continent, and describe male advertisement calls of Bioko *H. endjami* and *H. o. ocellatus*. Our study provides a first look at the diversity and distribution of reed frogs on Bioko Island and sets the stage for future studies of the ecology, evolution, and conservation of this island community.

Keywords: hybridisation, polymorphism, mitochondrial and nuclear DNA, morphology, land-bridge island

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Ranas de caña (*Hyperolius* spp.) son el género más rico en especies de la familia Hyperoliidae, un grupo de ranas endémicas del África subsahariana y las islas del Golfo de Guinea. Tres especies son endémicas de las islas oceánicas del archipiélago, pero la diversidad y distribución de las ranas de caña en Bioko, una isla puente terrestre en el archipiélago, sigue sin estar clara. Dos especies, *H. tuberculatus* y *H. ocellatus ocellatus*, fueron documentadas formalmente en Bioko en la época colonial; sin embargo, se han detectado dos especies adicionales de ranas de caña en estudios recientes. Además, las observaciones de amplexo heteroespecífico sugieren que una de las especies no identificadas y *H. o. ocellatus* puede estar hibridando en la isla. En este estudio, examinamos el ADN mitocondrial (mtDNA), el ADN nuclear (nuDNA), los datos morfológicos y acústicos para confirmar las identificaciones tentativas de las ranas de caña y determinar si hay alguna evidencia de hibridación. Nuestros datos confirman que las dos especies no identificadas son *H. endjami* y *H. fusciventris*, y que Bioko *H. endjami* tiene una diversidad genética única que no comparte con la población continental. Además, nuestras redes de haplotipos de ADN mitocondrial (16S) y nuclear (CMYC) sugieren que *H. o. ocellatus* y *H. endjami* no hibridan en Bioko. Finalmente, reportamos la variación del patrón de color en *H. endjami* y *H. o. ocellatus* que es único de las poblaciones del continente, y describe los cantos masculinos de Bioko *H. endjami* y *H. o. ocellatus*. Nuestro estudio proporciona un primer vistazo a la diversidad y distribución de las ranas de caña en la isla de Bioko y sienta las bases para futuros estudios sobre la ecología, la evolución y la conservación de esta comunidad isleña.

INTRODUCTION

Biodiversity in the Gulf of Guinea archipelago reflects the region's geologic and geographic history (Jones, 1994; Melo et al., 2022a). The archipelago sits along the western terminus of the Cameroon volcanic line, a 1,600 km chain of Cenozoic volcanoes that stretches from the Gulf of Guinea to the interior of Central Africa (Marzoli et al., 2000). During the Miocene, an event of crustal uplift formed four volcanic islands (Bioko, São Tomé, Príncipe and Annobón) that comprise the archipelago today (Meyers & Rosendahl, 1991). Bioko, a land-bridge island, is separated from continental Africa by <35 km of shallow sea (Deruelle et al., 1991; Bell et al., 2017). Cycles of rising and retreating sea resulted in periods of isolation and connectivity between Bioko and continental Africa with the last period of connectivity taking place during the Last Glacial Maximum (~21 kya) when sea levels lowered enough to connect Bioko to continental Africa via a broad land bridge (Jones, 1994; Bell et al., 2017). By contrast, São

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Tomé, Príncipe and Annobón are oceanic islands and have never been connected to continental Africa (Ceríaco et al., 2020). Bioko comprises three stratovolcanoes that formed 1–3 mya and, relative to the oceanic islands which formed 5–30 mya, is young in age (Jones, 1994). The combination of oceanic and land-bridge islands in the Gulf of Guinea archipelago provides an excellent framework to compare patterns of species diversity and endemism between islands that differ in geological history.

Biological patterns of species diversity and endemism on the islands indicate that periods of connectivity likely facilitated the dispersal of biodiversity from continental Africa to Bioko (Estrela, 1994; Juste & Ibañez, 1994; Melo et al., 2011; Ceríaco et al., 2016; Bell et al., 2017; Loiseau et al., 2019). Consequently, Bioko exhibits high species richness, but low endemism, reflecting its proximity and short periods of isolation from the continent (Jones, 1994). Conversely, the oceanic islands are less species-rich but have high endemism as a result of millions of years of geographic isolation from the mainland. For example, only two bird species are endemic to Bioko while São Tomé and Príncipe host seventeen and eight single-island endemic birds, respectively (Jones, 1994; Melo et al., 2022b). Intermittent periods of isolation and connectivity may contribute to population-level diversification on Bioko, particularly for species that are poor dispersers across saltwater barriers, such as amphibians. For instance, there is evidence of moderate genetic divergence between mainland and Bioko populations of some reed frogs (*Hyperolius o. ocellatus*, *H. tuberculatus*; Bell et al., 2017), African tree toads (genus *Nectophryne*; Liedtke et al., 2021), and leaf-folding frogs (*Afrixalus paradorsalis*; Charles et al., 2018). However, for larger-bodied foam nesting frogs (*Chiromantis rufescens*; Leaché et al., 2019), torrent frogs (genus *Petropedetes*; Barej et al., 2014), and giant tree frogs (*Leptopelis rufus*; Jaynes et al., 2022), genetic divergence between mainland and island populations is seemingly less pronounced. Investigating the genetic history of island populations relative to their mainland counterparts can reveal how amphibians are responding to periods of geographic separation during glacial cycles and whether these cycles are generating unique diversity on Bioko.

Reed frogs (*Hyperolius* spp.) are a diverse group of frogs endemic to Africa and widely distributed across the sub-Saharan region (Channing & Rödel, 2019). They are the most species-rich genus of frogs in the family Hyperoliidae, totaling 144 species (Frost, 2023). A number of *Hyperolius* species have been the subject of taxonomic discussion and reclassification, in part due to the reliance of colour patterns as taxonomic characters, which can be highly variable (i.e. polymorphism) within species (Channing, 2022). Sexual dichromatism (differences in colouration between the sexes) further contributes to the challenge of species identification in *Hyperolius* frogs. In some groups of closely related species, differences in female colouration are more pronounced than those in males (Portik et al., 2019), while the males of different species are often similar looking; this can make it difficult to identify species in the field without finding both sexes or hearing the male advertisement calls. Identifying *Hyperolius* frogs is particularly challenging on the African continent where there can be five or more species at a single breeding site (Drewes & Vindum, 1994). Species identification can still be challenging in less diverse communities, however, due to high regional variation in colour pattern (Channing, 2022). Consequently, an integrative approach that accounts for morphological, acoustic and genetic variation is recommended for making reliable species identifications in this genus.

Two reed frog species, *H. tuberculatus* (Mertens, 1965; Sánchez Vialas et al., 2020) and *H. ocellatus* (Günther, 1858), were formally documented on Bioko in the colonial era. On the mainland, these two species have a distribution stretching from Nigeria and Cameroon (*H. o. ocellatus* and *H. tuberculatus*) to the interior of Central Africa (*H. tuberculatus* and *H. o. purpurescens*). Morphological and genetic studies of *H. ocellatus* indicate that populations on Bioko Island correspond to the subspecies *H. o. ocellatus* (Laurent, 1943; Perret, 1975; Bell et al., 2017). Two additional reed frog species were detected on Bioko in surveys led by the California Academy of Sciences in 1998, but the identification of these species remains tentative (both were identified in the field as *H.* cf. *ocellatus*). Moreover, we observed one of the unidentified species and *H. o. ocellatus* in heterospecific amplexus at a breeding site where they co-occur. There is evidence of hybridisation in reed frogs on the nearby island of São Tomé (Bell et al., 2015; Gilbert & Bell, 2018; Bell & Irian, 2019) which raises the question of whether reed frogs may also be hybridising on Bioko. In this study, we use multiple lines of evidence to clarify the identification of the two unidentified reed frogs on Bioko and assess whether one of the unidentified species and *H. o. ocellatus* may be hybridising. We describe the species' distributions on the island, estimate genetic diversity, characterise male advertisement calls and report colour pattern variations unique from populations on the mainland.

MATERIALS & METHODS

Field sampling

We conducted amphibian surveys on Bioko Island over five field seasons: 27 September–18 October 1998, 19 August–12 September 2011, 1 January–22 January 2013, 29 August–17 September 2013, 11 April–30 April 2019. Our sampling spanned a range of habitats including lowland forest, agricultural fields, montane forest and Schefflera forest/Mossy forest, spanning elevations from sea level to >1900 m. Access to much of the northernmost volcano (Pico Basile) is restricted, thus the majority of our sampling effort was based in the southern half of the island (Bioko Sur Province; Fig. 1). Animals were euthanised via immersion in MS-222, tissue samples (liver) were preserved in 95% ethanol or RNAlater, and voucher specimens were deposited at the California Academy of Sciences (CAS), Cornell University Museum of Vertebrates (CUMV), and U.S. National Museum of Natural History (USNM). Voucher specimen information is included in Table S1, see Supplementary Material.

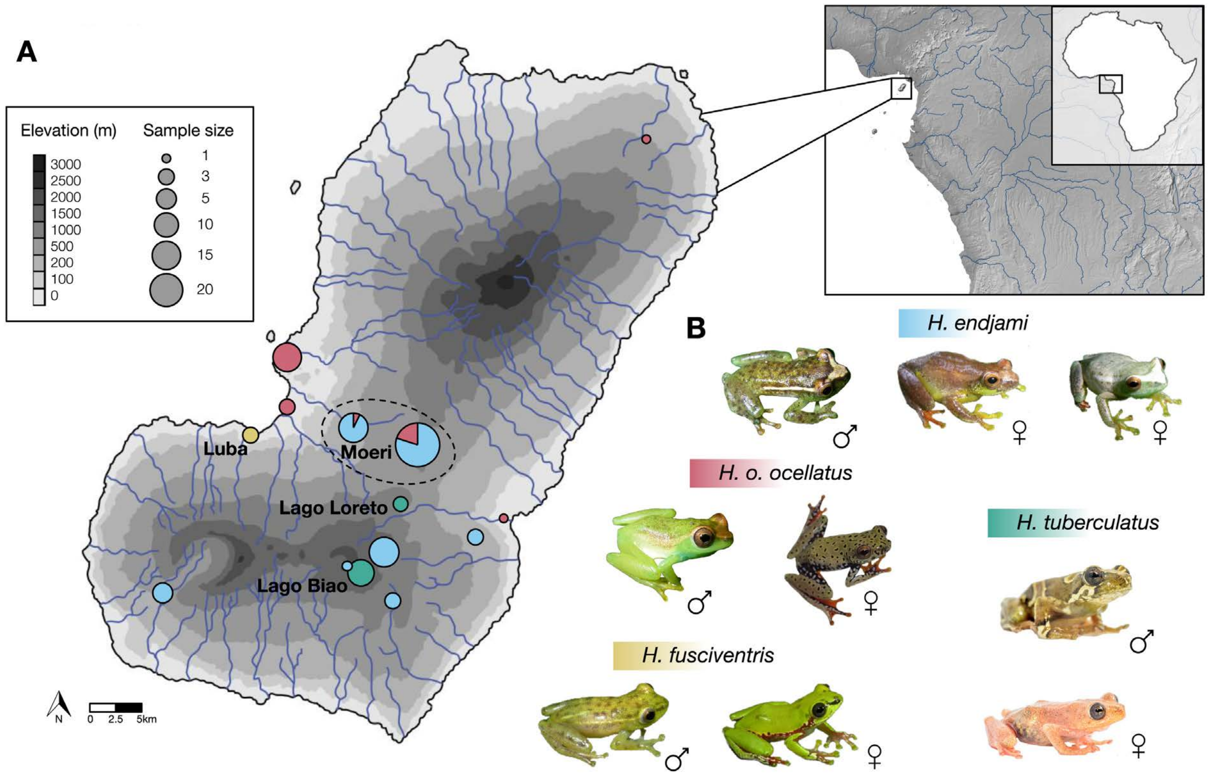

Figure 1. **A.** Sampling distribution of *Hyperolius endjami*, *Hyperolius ocellatus ocellatus*, *Hyperolius fusciventris* and *Hyperolius tuberculatus* on Bioko Island. Sampling localities are scaled according to sample size and coloured according to the proportion of each species present at the locality. The two crater lakes (Lago Biao and Lago Loreto), port town of Luba, and *H. endjami* and *H. o. ocellatus* zone of sympatry (Moeri) are indicated. **B.** Representative photographs of males and females of each species, all of which exhibit sexual dichromatism. Two colour morphs of female *H. endjami* are shown. Photos of *Hyperolius fusciventris* are of individuals in Cameroon because those from Bioko were not photographed prior to preservation.

Genetic data collection

We extracted genomic DNA from 102 tissue samples using the Qiagen DNEasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA). We amplified the 16S mitochondrial and CMYC nuclear genes using polymerase chain reaction (PCR) and the respective primers (16S: 16SA and 16SB (Palumbi et al., 1991), CMYC: CMYC-1U and CMYC-ex2dR (Crawford, 2003; Wiens et al., 2005)). Each PCR reaction was carried out in a volume of 18 µl containing: 1 µl template DNA, 0.18 µl DreamTaq DNA polymerase, 2.4 µl dNTPs, 0.75 µl of each primer, 2 µl 10X DreamTaq Buffer, and 10.92 µl molecular grade water. Amplifications were carried out with initial denaturation for 5 min at 94 °C, followed by 35 cycles consisting of 60s denaturation at 94 °C, 60s annealing at 48 °C, 60s extension at 72 °C and a final extension at 72 °C for 5 min. PCR products were visualised using gel electrophoresis, purified using ExoSAP-IT (USB Corp., Cleveland, OH) and sequenced using a BigDye Terminator Cycle Sequencing Kit v3.1 (Applied Biosystems, Foster City, CA, USA) on an ABI Automated 3730x1 Genetic Analyser (Applied Biosystems). All sequences were aligned and edited using Geneious Prime v2021.1 (https://www.geneious.com). Several of the Bioko *H. tuberculatus* and *H. o. ocellatus* specimens had previously been sequenced for a Central African phylogeography study by the authors (Bell et al., 2017) and these sequences were included in our Bioko Island datasets (Table S1).

Species identification and population genetic structure

To assess the identifications of *Hyperolius* sp. 1 and *Hyperolius* sp. 2 from Bioko, we used a recent phylogeny of Hyperoliidae as a reference. Portik et al. (2019) estimated species relationships with >1000 sequence capture nuDNA loci and included 16S because it is a commonly used barcoding gene for African amphibians. We generated a phylogeny based on this 16S dataset with all *Hyperolius* taxa included in the Portik et al. (2019) study (n = 90), one representative sequence of each haplotype for each of the unidentified Bioko reed frog taxa, and *Afrixalus brachycnemis* (also from Portik et al., 2019) as an outgroup. We aligned the sequences with MAFFT using the automatic algorithm selection option (--auto) (Katoh et al., 2002; Katoh & Standley, 2013). To perform maximum likelihood phylogenetic analyses, we used IQ-TREE v1.6.12 on the IQ-TREE server with the built-in model selection tool ModelFinderPlus and 1000 ultrafast bootstraps (Nguyen et al., 2015; Trifinopoulos et al., 2016; Kalyaanamoorthy et al., 2017; Hoang et al., 2018). We calculated the percent sequence divergence between the closest reference samples to the Bioko reed frogs in Geneious. Finally, we examined the physical specimens to confirm the presence of diagnostic characters in species descriptions and field guides.

To estimate genetic diversity and divergence of Bioko reed frogs relative to their continental counterparts, we downloaded GenBank sequences of 16S for the species, which included samples from Cameroon, Gabon, Ghana and Nigeria (Table S1). For each species, we generated alignments in Geneious and constructed haplotype networks using TCS v1.21 (Clement et al., 2000) and PopART v1.7 (Leigh & Bryant, 2015).

Preliminary investigations of potential hybridisation

We sequenced one mtDNA (16S) and one nuDNA gene (CMYC) for *H. o. ocellatus* and the sympatric *Hyperolius* sp. 1 as a preliminary investigation of hybridisation. In particular, we wanted to assess whether any individual frogs exhibited mismatched mtDNA-nuDNA haplotypes (e.g. an individual exhibiting the mtDNA haplotype of species 1 and the nuDNA haplotype of species 2), or heterozygosity in the nuDNA gene indicative of mixed ancestry (e.g. one phased haplotype attributable to each of the parental species). Our sampling included 113 individuals from Bioko Island with 51 collected from Moeri (3.467317 N, 8.6411 E), the site at which heterospecific amplexus was observed.

Advertisement call analysis

We collected acoustic recordings for *Hyperolius* sp. 1 and *H. o. ocellatus* during the 2011 field season. *Hyperolius* sp. 2 was detected in 1998 and despite searching near the 1998 locality in subsequent field seasons, no additional individuals have been seen or recorded. Calls were recorded in situ at breeding sites using a Marantz PMD-661 recorder paired with a Sennheiser ME-67 microphone (2011) at an approximate distance of 0.5–2.0 m from the males. For each recording, we measured the ambient air temperature, described the male calling site and behavioral context, and prepared the males as voucher specimens (Table S1). The recordings were archived in the Macaulay Library at the Cornell Lab of Ornithology (Table S1).

We digitised recordings at a resolution of 24-bit 96 000 Hz sampling rate and analysed them in Raven Pro v1.4 (Cornell Lab of Ornithology) with a Fast Fourier transformation window of 512 points, a brightness of 70 points and a contrast of 70 points. The total pulse groups, average dominant frequency (Hz), and average pulses were recorded for each call as these metrics capture much of the variation in *Hyperolius* calls (Amiet & Goutte, 2017). We compared our Bioko recordings to those obtained by Amiet & Goutte (2017) in Cameroon. Oscillograms and spectrograms were made in R using the Seewave package v2.1.8 (Sueur et al., 2008).

Comparisons of colour pattern variation

Most specimens collected in 2011, 2013 and 2019 were photographed immediately following euthanasia to provide a record of colour and pattern, as colouration typically fades in museum specimens over time. We compared colour pattern variation in male *Hyperolius* sp. 1 (n = 38), *Hyperolius* sp. 2 (n = 3) and *H. o. ocellatus* (n = 32) to comprehensive descriptions and photos of each species (Amiet, 2012).

RESULTS

Hyperolius diversity and distribution on Bioko Island

We found that Bioko *Hyperolius* sp. 1 and *Hyperolius* sp. 2 formed strongly supported clades with *H. endjami* and *H. fusciventris* respectively in the Hyperoliidae phylogeny (Fig. S1, see Supplementary Material). Percent sequence

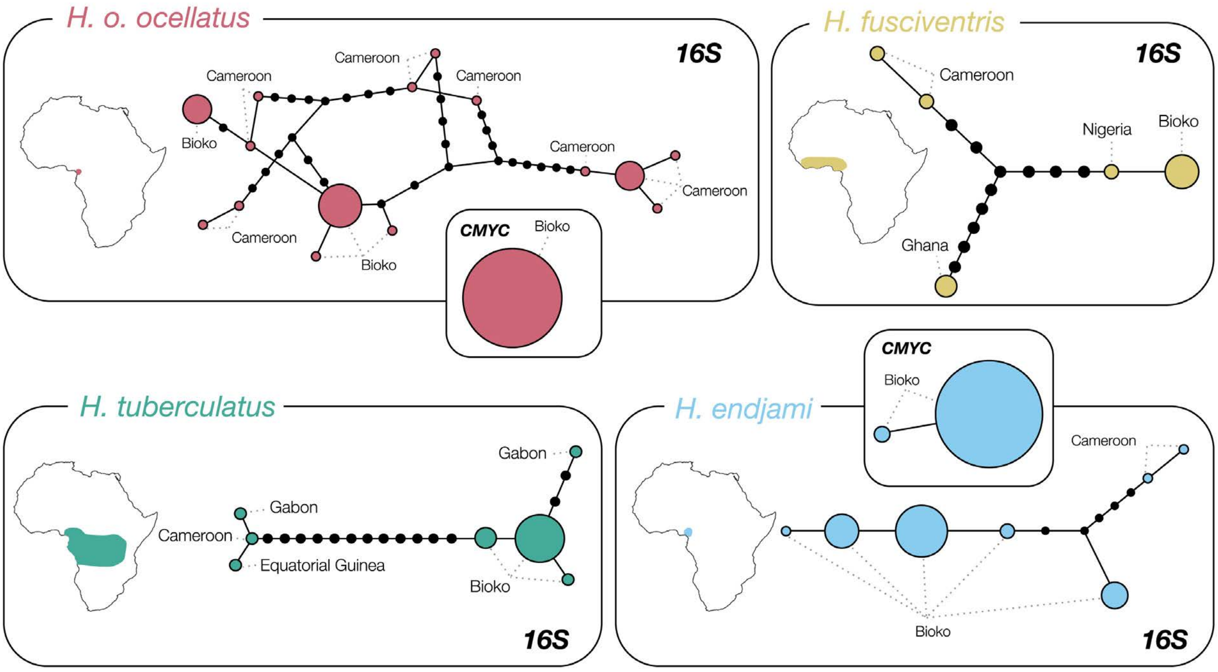

Figure 2. Mitochondrial (16S) and nuclear (CMYC) haplotype networks for *Hyperolius ocellatus ocellatus*, *Hyperolius endjami*, *Hyperolius tuberculatus* and *Hyperolius fusciventris* and distribution of species on mainland Africa. Haplotype networks are scaled according to sample size and coloured according to species. No mtDNA or nuDNA haplotypes were shared between *H. endjami* and *H. o. ocellatus*. Bioko and reference (Cameroon, Ghana, Equatorial Guinea, Gabon, and Nigeria) specimens are indicated.

divergence was low (0.0–0.4% and 0.7% for *H. endjami* and *H. fusciventris*, respectively) and the morphological features of the specimens fit these species descriptions; thus, we are confident in these species identifications. Consequently, the island *Hyperolius* community includes *H. endjami*, *H. o. ocellatus*, *H. fusciventris* and *H. tuberculatus* (Fig. 1). We found *H. endjami* at sites ranging from approximately 300–1950 m elevation, *H. o. ocellatus* from sea level to ~550 m, and a narrow zone of sympatry between the species around 450 m. This area of sympatry is where individuals in heterospecific amplexus were observed by the authors. By contrast, *H. tuberculatus* were only found in habitats surrounding Lago Biao and Lago Loretto, two crater lakes on the southern half of the island. Finally, only three *H. fusciventris* were found, and all were collected in 1998 from a single locality in the port town of Luba (3.4613 N, 8.5524 E). Several subspecies of *H. fusciventris* are recognised on the basis of differences in colouration (*H. f. burtoni*, *H. f. fusciventris*, *H. f. lamtoensis*), however, with only preserved male specimens we were not able to attribute the Bioko samples to a particular subspecies.

Genetic diversity in Bioko reed frogs and divergence from continental populations

We found moderate genetic diversity at 16S for Bioko *H. endjami* with five haplotypes, none of which are shared with individuals from Cameroon (Fig. 2). By contrast, the three individuals of *H. fusciventris* shared one 16S haplotype (Fig. 2). Of the available reference sequences for *H. fusciventris* from West and Central Africa, the Bioko individuals appear most similar to populations in Nigeria. We recovered four 16S haplotypes for Bioko *H. o. ocellatus* that clustered in two groups, both with variation distinct from that in Cameroon. We found three 16S haplotypes for Bioko *H. tuberculatus*, all three of which clustered with samples collected in Gabon but with unique diversity. There was little to no genetic diversity at CMYC for Bioko

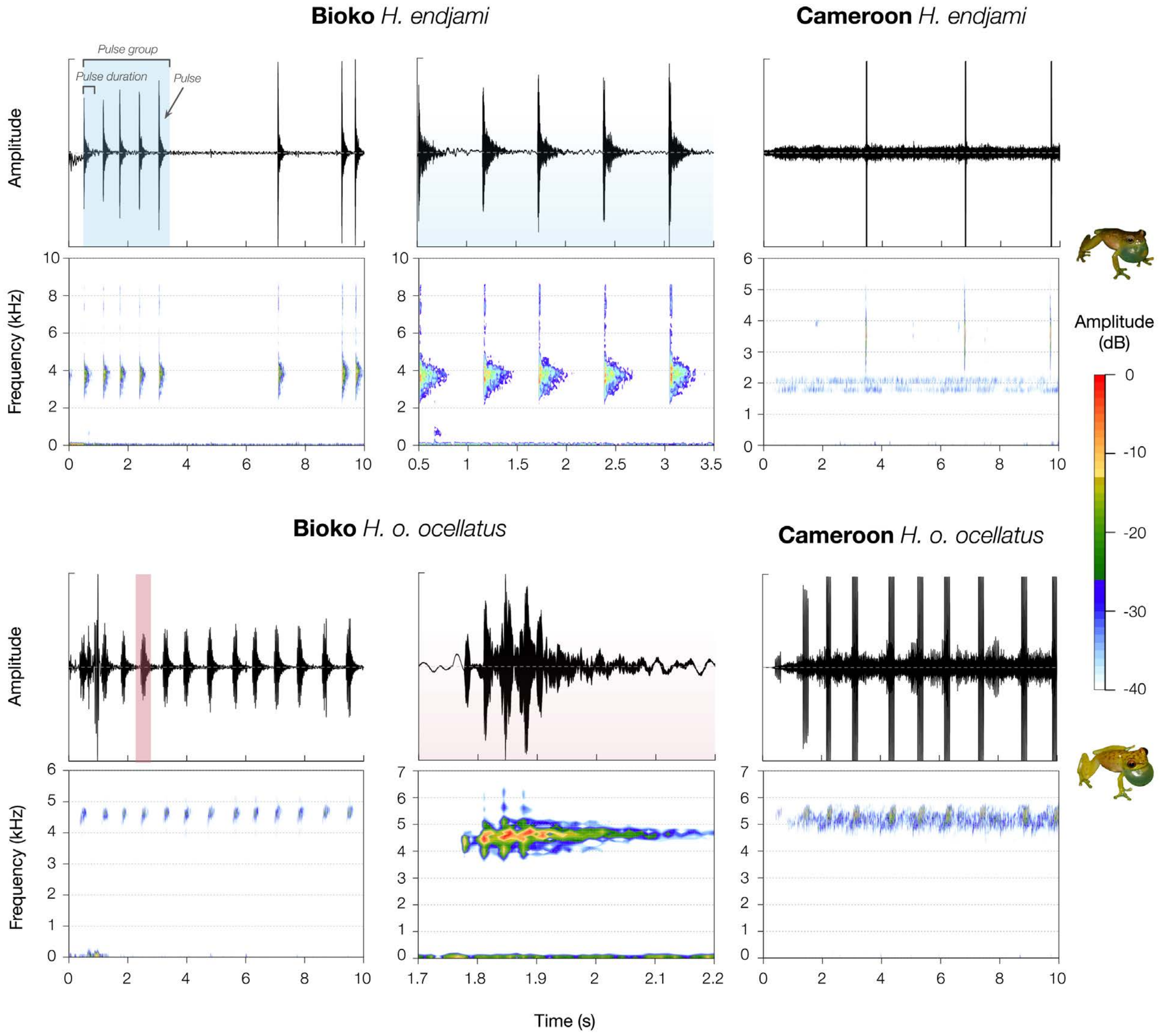

Figure 3. Oscillograms and spectrograms of advertisement calls of *Hyperolius endjami* and *Hyperolius ocellatus ocellatus* from Bioko Island (this study) and Cameroon (Amiet & Goutte, 2017). Amplitude and frequency of calls is shown over a duration of ten seconds and over a duration of a single pulse group for each species' call. Each vertical line represents a single pulse. The pulse group is defined as a set of pulses. The pulse duration is the duration of the pulse. The middle panels show a zoomed-in view of the advertisement calls of Bioko *H. endjami* and Bioko *H. o. ocellatus*.

Table 1. Advertisement calls of *Hyperolius endjami* and *Hyperolius ocellatus ocellatus* from Bioko Island and Cameroon. Snout-vent length (SVL) and ambient temperature are reported for the recordings generated in the present study.

Species	Location	Voucher	SVL (mm)	Temperature (°C)	Recording	Total Pulse Groups	Avg Dominant Freq (Hz)	Avg Pulses (range)
H. endjami	Bioko	CUMV 15162	23.5	18.3	ML 173579	18	3510	2.7 (1 – 6)
H. endjami	Bioko	CUMV 15201	20.7	20.1	ML 173581	4	3795	5.3 (1 – 10)
H. endjami	Cameroon	N/A	--	--	2.9, Amiet & Goutte 2017	9	3732	1.0 (1)
H. endjami	Cameroon	N/A	--	--	2.10, Amiet & Goutte 2017	9	3531	1.1 (1 – 2)
H. o. ocellatus	Bioko	CUMV 15217	22.0	19.4	ML 173582	37	4505	4.4 (3 – 7)
H. o. ocellatus	Cameroon	N/A	--	--	2.37, Amiet & Goutte 2017	15	4272	2.5 (1 – 3)
H. o. ocellatus	Cameroon	N/A	--	--	2.38, Amiet & Goutte 2017	28	5349	5.0 (4 – 5)

H. endjami and Bioko *H. o ocellatus* (Fig. 2). We recovered one haplotype for Bioko *H. ocellatus* and two haplotypes for Bioko *H. endjami*; none of the CMYC haplotypes were shared.

No evidence of hybridisation between *H. endjami* and *H. o. ocellatus*

In 2011 and 2019 we collected ten amplectant pairs at Moeri. Our updated identifications indicate that we collected two pairs of *H. endjami*, seven pairs of *H. o. ocellatus* and one heterospecific pair. Mitochondrial and nuclear haplotypes showed no individuals with admixed genetic ancestry (Fig. 2, Table S1). Thus, there is no evidence of hybridisation between *H. endjami* and *H. o. ocellatus* despite multiple observations of heterospecific amplexus.

Male advertisement calls

Advertisement calls for *H. endjami* and *H. o. ocellatus* differ in call dominant frequency and pulses per call (Fig. 3, Table 1): *H. o. ocellatus* has a higher average dominant frequency and typically produces more pulses per call than *H. endjami*. Although there are slight genetic differences between *H. endjami* from Bioko and Cameroon, male advertisement calls for both localities are similar in amplitude and share a distinct 'clicking' timbre (Fig. 3). Likewise, advertisement calls of *H. o. ocellatus* from Bioko and Cameroon are similar with males in both populations producing high frequency trills (Fig. 3).

Colour pattern variation in Bioko *H. endjami* and *H. o. ocellatus*

Recorded variants of male *H. endjami* from Cameroon exhibit yellow to light-green mid-dorsal pigmentation and dorsolateral spots. The degree of connectivity of dorsolateral spots and pigmentation give way to a diversity of variants (Figs. 86 & 87 in Amiet (2012)). Male *H. fusciventris* display a range of colour patterns including solid green or green with white dorsolateral lines, with various degrees of dark speckling (Fig. 135 in Amiet (2012)). Male *H. o. ocellatus*, however, are described as possessing a distinctive colour pattern: white dorsolateral lines that join at the snout and form a triangle. Morphological variants of *H. o. ocellatus* differ in occelation and colour but all retain the above diagnostic colour pattern (Fig. 138 in Amiet (2012)). The three male *H. fusciventris* from Bioko had colour patterns consistent with previous descriptions of the species from Cameroon; however, we observed colour pattern variation in male *H. endjami* and *H. o. ocellatus* from Bioko that have not previously been described for these species (Amiet, 2012).

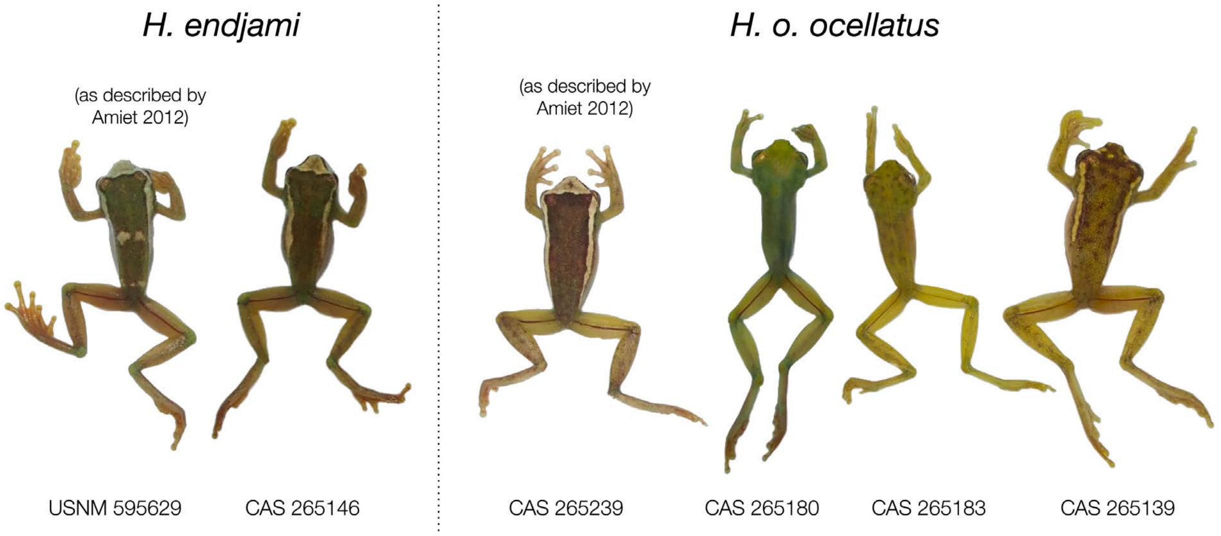

Figure 4. Newly described colour pattern variations of male *Hyperolius endjami* (CAS 265146) and *Hyperolius ocellatus ocellatus* (CAS 265180, CAS 265183, and CAS 265138 from left to right) compared to colour patterns described by Amiet (2012; *H. endjami*: USNM 59629, *H. o. ocellatus*: CAS 265239).

Three *H. endjami* specimens (CAS 265146, CAS 265245, CAS 265246) exhibited white dorsolateral stripes that join at the snout, forming a triangle. This colour pattern is typically diagnostic for male *H. o. ocellatus* and confounded some of our initial field identifications. By contrast, we observed three colour patterns in *H. o. ocellatus* that lacked the diagnostic white triangle on the snout: ocellated with no white dorsolateral stripes (CAS 265180, CAS 265141, CAS 265240), ocellated with white dorsolateral stripes that do not join at the snout (CAS 265183), and solid green with no white dorsolateral stripes (CAS 265138, CAS 265139, CAS 265140; Fig. 4).

DISCUSSION

Our integrative datasets confirm the presence of a robust population of *H. endjami* on Bioko Island. Many field identifications have confused *H. endjami* with *H. o. ocellatus*, likely due to the shared colour patterns we report in males of the two species. Thus, our study provides a significant range expansion for *H. endjami*, which was described in 1980 (Amiet) and is known from only a few localities in Cameroon. Specimens and other records from Bioko previously attributed to *H. ocellatus* (e.g. MNCN specimens examined in Sánchez-Vialas et al., 2020) should be reassessed in light of this new information. Our modest mtDNA dataset indicates that the Bioko population of *H. endjami* harbours unique genetic diversity not shared with Cameroon. A similar pattern has been observed in Bioko populations of other hyperoliid frogs including *Afrixalus paradorsalis* (Charles et al., 2018), *H. o. ocellatus* (Bell et al., 2017), and *H. tuberculatus* (Bell et al., 2017). Divergence time estimates for Bioko populations of these species indicate they likely became isolated from their continental counterparts during recent Pleistocene glacial cycles (Bell et al., 2017; Charles et al., 2018). The amount of divergence we observed between Bioko and Cameroon populations of *H. endjami* is similar to that in these other taxa, suggesting the populations have been isolated for a comparable period of time. Quantifying population genomic and phenotypic divergence between *H. endjami* on Bioko and in Cameroon would enable more robust, model-based investigations of the demographic history of the island population.

Despite extensive amphibian surveys on the southern half of Bioko Island, *H. fusciventris* are only known on the island from three males collected in the port town of Luba. Subsequent searches at this locality and in adjacent areas indicate there is suitable habitat for amphibians, including breeding choruses of *H. o. ocellatus*, but no other *H. fusciventris* have been found. On continental Africa, this species ranges from Sierra Leone to south-western Cameroon with four subspecies that differ in female (phase F) colouration (Schiøtz, 1999; Amiet, 2012). Unfortunately, only males were sampled, therefore the Bioko frogs could not be attributed to a particular subspecies based on distinctive colouration; however, the sequences were most similar to continental populations from Nigeria, which is within the distribution of *H. f. burtoni* (Boulenger, 1883). A larger genetic dataset and more sampling across the species range could help clarify whether *H. fusciventris* is native to Bioko or was introduced, and further surveys in lowland habitats across Bioko are needed to determine whether they are still extant on the island.

Although *H. endjami* and *H. o. ocellatus* occur at the same breeding sites and can be found in heterospecific amplexus, no evidence was found of successful hybridisation. Hybridisation of *Hyperolius* frogs occurs on the nearby island of São Tomé. In localities where *H. thomensis* and *H. molleri* are sympatric, Bell et al. (2015) documented extensive genetic admixture between species. However, the São Tomé species are closely related, unlike *H. endjami* and *H. o. ocellatus* (Portik et al., 2019). The call of *H. endjami* is rather distinct from *H. o. ocellatus*: *H. endjami* produces lower frequency clicks, while *H. o. ocellatus* produces higher frequency trills. The pulse rates, which some female frogs use to distinguish between males of their own species and closely related sympatric species (Lemmon, 2009), also differ between *H. endjami* and *H. o. ocellatus*. Despite these differences, the two species were observed in heterospecific amplexus, indicating that advertisement call is an incomplete prezygotic behavioural barrier. Further prezygotic barriers that come into play during courtship, such as species-specific gular gland compounds in reed frogs in particular (Starnberger et al., 2013), and postzygotic barriers like hybrid sterility (Richards & Schiøtz, 1977) may prevent successful hybridisation between sympatric species.

Reed frogs are notorious for their sexual dichromatism, and extensive intraspecific and interspecific variation in colouration (Schiøtz, 1999). On Bioko, all the colour pattern variations for *H. endjami* and *H. o. ocellatus* detailed by Amiet (2012) were observed, as well as new colour patterns for both species that have not been documented in mainland populations. Polymorphism is common in the family Hyperoliidae, with species such as those in the *H. viridiflavus*, *H. marmoratus* and *H. parallelus* complexes exhibiting extreme diversity in dorsal colour patterns (Channing, 2002). The function of colour polymorphism and sexual dichromatism in *Hyperolius* is still unclear, but may play a role in species and mate recognition (Portik et al., 2018; 2019). Our findings further suggest that colouration is variable in *H. endjami* and *H. o. ocellatus* much like other *Hyperolius* species, though surprisingly this novel variation is in the male colouration, and more work is needed to understand the genetic basis and ecological consequences of these differences.

In summary, we confirmed the presence of *H. endjami* and *H. fusciventris* on Bioko Island and recovered unique genetic diversity that is not shared with mainland populations. We described male advertisement calls of *H. endjami* and *H. o. ocellatus* on Bioko, and found that they are not hybridising on Bioko despite observations of heterospecific amplexus. Finally, we reported new dorsal colour pattern variations in male *H. endjami* and *H. o. ocellatus*, further documenting polymorphism in these two species. Unfortunately, our dataset was limited to frogs collected from the southern half of Bioko Island due to restricted access in the northern region (particularly for nocturnal surveys). Future studies sampling the northern half of Bioko would provide a more complete account of the distribution of *Hyperolius* frogs on the island and a better understanding of how reed frog species are partitioning geographic and ecological space. For instance, although *H. tuberculatus* are a widespread lowland forest species in their continental distribution (Channing & Rödel, 2019), to date they have only been documented in the vicinity of the two, high elevation crater lakes on the southern half of Bioko Island and it is unclear whether they are present at higher elevations on the northern peak (Pico Basile). Likewise, *H. o. ocellatus* seem to occur at lower elevations and *H. endjami* at higher elevations on the southern half of the island with a narrow range of sympatry, and it is unclear whether this pattern is replicated on the northern peak. Future surveys of *Hyperolius* on Bioko will benefit from careful attention to advertisement calls and/or genetic data to differentiate between male *H. o. ocellatus* and *H. endjami* given the challenges in identifying them based on morphology alone. Our study sets the stage for future studies of the ecology, evolution and conservation of this island community.

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Author contributions

R.C.B. designed the project; R.C.B., P.J.M., L.A.S., and C.I. collected field samples and took photographs; R.C.B. and J.V.N. performed the lab work; R.C.B. and J.V.N. analysed the data; J.V.N. produced the figures; J.V.N led the writing and all authors contributed to paper revision.

Ethical Statement

All research was conducted with IACUC approval (Cornell University 1999-0010, Drexel University 18748, Smithsonian Institution National Museum of Natural History 2016-09, California Academy of Sciences 2017-02).

DATA ACCESSIBILITY

GenBank accession numbers (16S: PP431295 - PP431384, CMYC: PP431075 - PP431154), sampling localities, voucher specimen information, and Macaulay Library accession numbers for audio recordings are available in Table S1.

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