Herpetology Notes, Volume 18: 529–537 (published online on 05 July 2025)

Initial survey of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* around Bouamir Research Station, Dja Faunal Reserve, Cameroon

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Chytridiomycosis, caused by the chytrid fungus *Batrachochytrium dendrobatidis* (Bd) (Berger et al., 1998; Lips et al., 2006), is responsible for many of the recent rapid declines of amphibians worldwide (Skerratt et al., 2007; Lötters et al., 2009; Crawford et al., 2010; Farrer et al., 2011; Scheele et al., 2019). It is now considered by the International Union for Conservation of Nature as the worst infectious disease ever observed in vertebrates in terms of the number of species suffering population declines and extinctions (IUCN, 2024). The most significant losses associated with Bd have been observed in Australia, Central America, and South America, particularly in large anurans with limited ranges in humid climates. These trends are concerning given that amphibian diversity and endemism are elevated in these tropical regions. By contrast, although Afrotropical regions also harbour exceptional levels of amphibian diversity, the extent to which Bd poses a threat to this unique amphibian fauna is still unclear (Scheele et al., 2019).

Surveys for Bd in amphibian communities across the African continent (reviewed in Doherty-Bone et al., 2020; Zimkus et al., 2020; Ghose et al., 2023) indicate that Bd is widespread, but these largely opportunistic surveys have not found clear evidence that Bd infection causes large-scale mortality events like those documented in other tropical regions (e.g., Lips et al., 2006, 2008). Surveys of montane amphibian communities in Cameroon, however, found declines in some genera are associated with the apparent emergence of Bd, indicating that Bd may indeed pose a threat to at least some groups of Afrotropical amphibians (Hirschfeld et al., 2016). Herpetological surveys conducted in Africa over the past decade have significantly increased the number of confirmed Bd cases in African amphibians, and a number of studies have also screened museum specimens for Bd to elucidate the historical prevalence of the pathogen in various regions of Africa (Seimon et al., 2015; Hirschfeld et al., 2016; Hydeman et al., 2017; Nguyen et al., 2025). These field surveys were largely conducted opportunistically, often as part of more general amphibian inventories, and consequently, there is very little quantitative information regarding temporal variation in infection or community-level patterns of Bd prevalence and infection intensity for African amphibians. Furthermore, few studies of Bd in African amphibians have reported on Bd genotype variability, limiting our understanding of how Bd has spread and diversified across the continent.

Two lineages of Bd are known from Africa, BdGPL and BdCAPE (Byrne et al., 2019; Ghose et al., 2023), which differ in their global distributions and presumed virulence on local hosts. Somewhat unexpectedly, only BdCAPE has been reported from Cameroon (Byrne et al., 2019; Ghose et al., 2023) even though BdGPL is known from neighbouring countries including Gabon and the Gulf of Guinea Islands (Hydeman et al., 2017; Byrne et al., 2019). Sample sizes in these studies are still limited, however, and thus it is unclear whether BdGPL is truly absent from Cameroon or has simply

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not yet been detected and likewise, whether BdCAPE is present in these other Central African regions from which BdGPL has been reported.

We established an initial, standardized survey approach at Bouamir Research Station in the Dja Faunal Reserve, Cameroon to serve as a long-term monitoring site of Bd dynamics in a community of Central African amphibians. The Dja Reserve is one of the largest reserves in Central Africa and is situated at 600–750 m above sea level with dense humid forests. Amphibian surveys at Bouamir Research Station in the northwestern quadrant of the reserve documented at least 47 species in 10 families (Fokou et al., 2025). Most are species that are typically found in lowland Central African rainforests and species distribution projections indicate that over 100 amphibian species may be present in the vicinity of the research station (Fokou, unpub. data). With its rich amphibian community, largely intact forest habitat, and robust research infrastructure, Bouamir provides a unique opportunity for long-term monitoring of Bd dynamics in the region. Here we report the Bd prevalence, infection intensity, and genotypic results from our first sampling campaign. In addition, we provide new sequence data from Bd-positive amphibians collected in Equatorial Guinea (Marshall et al. 2023) to further document the distributions of BdCAPE and BdGPL in Central Africa.

Materials and Methods

Geographic and taxonomic sampling. We conducted amphibian surveys between 27 April and 7 May 2022 in the vicinity of Bouamir Research Station (3.1906°N, 12.8120°E, elevation 680 m). Accumulated annual rainfall in the Dja is 1600 mm with two wet seasons (short wet season between March and May, long wet season between August and November) and two dry seasons (long dry season between November and March, short dry season between June and July; Sonké, 1998). Thus, our sampling period corresponds to the middle of the short wet season.

Our sampling around Bouamir primarily included lowland forest habitat as well as some *Raphia* palm (Arecaceae) and *Uapaca* (Phyllanthaceae) swamps in lower lying areas. We established 20 100-m long transects within ~1.5 km of the research station (Figs. 1, 2): ten transects follow the course of small streams with one parallel "terrestrial" transect to each stream transect placed 50 m away from the stream. Transects were visited for three consecutive nights to enable future quantitative estimates of population size using mark-recapture approaches (Heyer et al., 1994). For each survey, researchers equipped with headlamps conducted visual encounter surveys and disturbed the leaf litter using walking sticks. All individuals encountered within 1 m on either side of the transect line were captured and placed in individual plastic bags. Data on the collection date, time, weather conditions, microhabitat, and position along the transect line were recorded. In addition, a rain gauge was placed in the centre of the research station to record rainfall and recorders (HOBO model UA-002-64, Onset Computer Corp.) were placed at the beginning and end of each transect to continuously record temperature and luminosity.

Initial amphibian species identifications were made in the field by RCB, FOR, LAS, and ABK. All individuals were photographed prior to release for further confirmation of species identification based on field guides and primary literature (Frétey et al., 2011; Channing and Rödel, 2019). For individuals that belong to genera that are challenging to identify to the species level in the field we collected a non-lethal tissue sample (toe clip) preserved in RNAlater for DNA-barcoding analyses. A parallel study documenting the amphibian fauna of Bouamir is generating a voucher specimen and DNA barcode database to serve as a reference (Fokou et al., in press). To avoid interfering with the mark-recapture study, individuals captured outside of transects were prioritized for serving as the vouchers for the reference collection. However, for a few species that are less commonly encountered (e.g., arboreal toads, genus *Nectophryne*) individuals from the transect were collected for this purpose. We collected epithelial samples from post-metamorphic individuals captured at each transect. Each individual was swabbed on the ventral surface of its abdomen, hind limbs, and feet (five strokes each side) for a total of 30 strokes with sterile fine-tip swabs (Medical Wire & Equipment Co. MW113) following the methods of Hyatt et al. (2007). Individuals of the most commonly encountered genera (*Arthroleptis*, *Leptopelis*, *Phrynobatrachus*) were marked using standard toe-clipping approaches (Heyer et al., 1994) to enable robust estimates of population size and to track the infection status of individual frogs through time. These toe clips were preserved in RNAlater for future genetic analysis. Epithelial swabs were stored in 95% EtOH and kept as cool as possible in the field and then stored at -80°C until processing. Individuals prepared as morphological voucher specimens were euthanized by immersion

in buffered MS-222, liver tissue was removed and preserved in RNAlater, and the specimen was fixed in formalin. These voucher specimens were deposited in the Herpetology Collection at the California Academy of Sciences (CAS; Appendix).

Quantitative Bd detection. Prior to DNA extraction, we placed the sample tubes in a SpeedVac until the ethanol completely evaporated. We performed DNA extractions using the PrepMan Ultra kit (Applied Biosystems). To measure Bd prevalence and infection loads, we used a 1:10 dilution of the extracted DNA samples for qPCR analysis and measured ITS copies based on synthetic standards (Pisces Molecular, Boulder, Colorado, USA) diluted from 2.6 x 10^6 to 2.6 Bd ITS rRNA gene copies. We used primers ITS-1 and 5.8s with Taqman reagents in the QuantStudio 3 system to amplify rRNA regions of Bd (Boyle et al., 2004). To validate the qPCR reaction, we also included TaqMan Exogenous Internal Positive Control reagents (IPCs); we reran samples if IPCs did not amplify.

Bd sequencing and phylogenetic analysis. We sequenced Bd-positive samples from Bouamir and from a recent survey in Equatorial Guinea (Marshall et al., 2023) using a custom genotyping assay (Byrne et al., 2017). Our samples from the Marshall et al. (2023) study include Bd-positive samples from Bioko Island (where BdGPL has previously been documented) and from mainland Equatorial Guinea, which borders Cameroon and from which Bd genotypes have not previously been reported. The genotyping assay targets 192 phylogenetically informative amplicons that are 150–200 base pairs long. To increase the sensitivity of our PCR assay, we first preamplified DNA extracts in two separate PCR reactions, each with 96 primer pairs. The preamplification master mix included: 5 µl 2x KAPA 2G Fast Multiplex Mix (Roche), 1 µl of primer pool consisting of 96 forward and reverse primers at a concentration of 520 nM each, 2.1 µl of PCR-grade water, 0.5 µl DMSO, 0.4 µl 25 mM $MgCl_2$, and 1 µl of the DNA extract. We ran the PCRs using the following

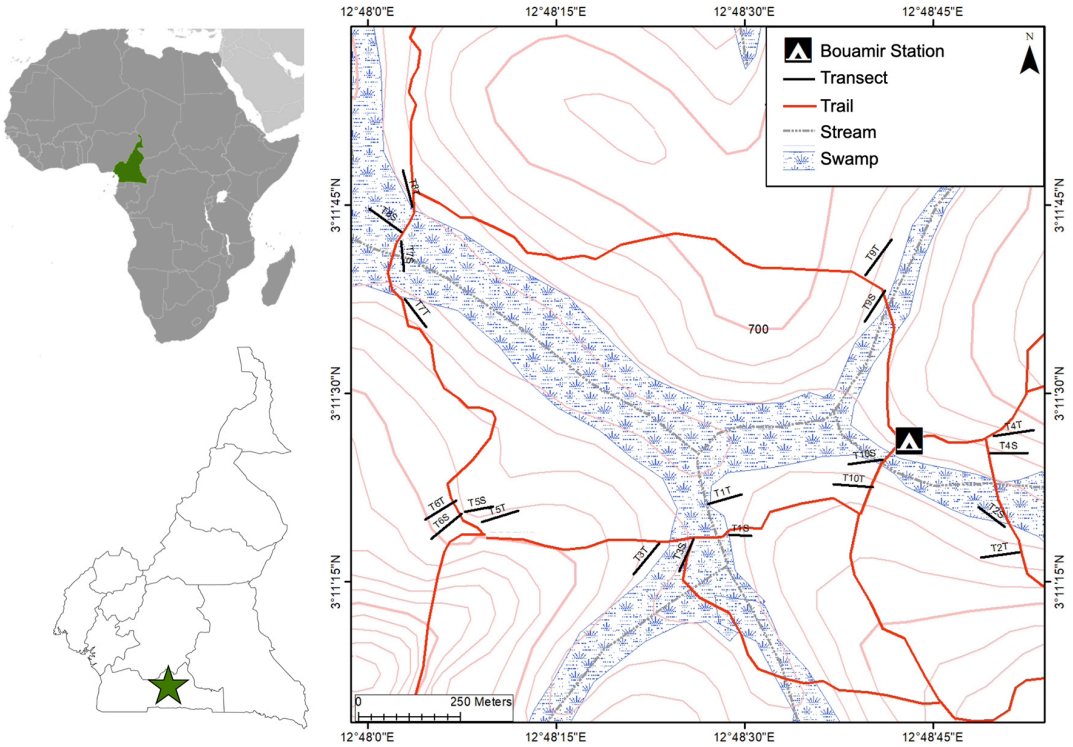

Figure 1. Map of Bouamir Research Station in the Dja Biosphere Reserve, Cameroon. The locations of the 20 transects are indicated (10 alongside streams and 10 terrestrial transects, approximately 50 m from each stream transect). The habitat map is approximate as the extent of many of the swamps and streams shift throughout the dry and wet seasons. In addition, there is a rocky stream that coincides with transects T5S and T6S in the forest at the edge of the "Petit Rocher" inselberg that does not appear on the Bouamir habitat map. Elevation from SRTM v3; Landcover digitized on SEntinel-2, 26/12/2015, ESA; Trails from GNSS tracking (Vincent Deblauwe, Congo Basin Institute). Cameroon map from vemaps.com

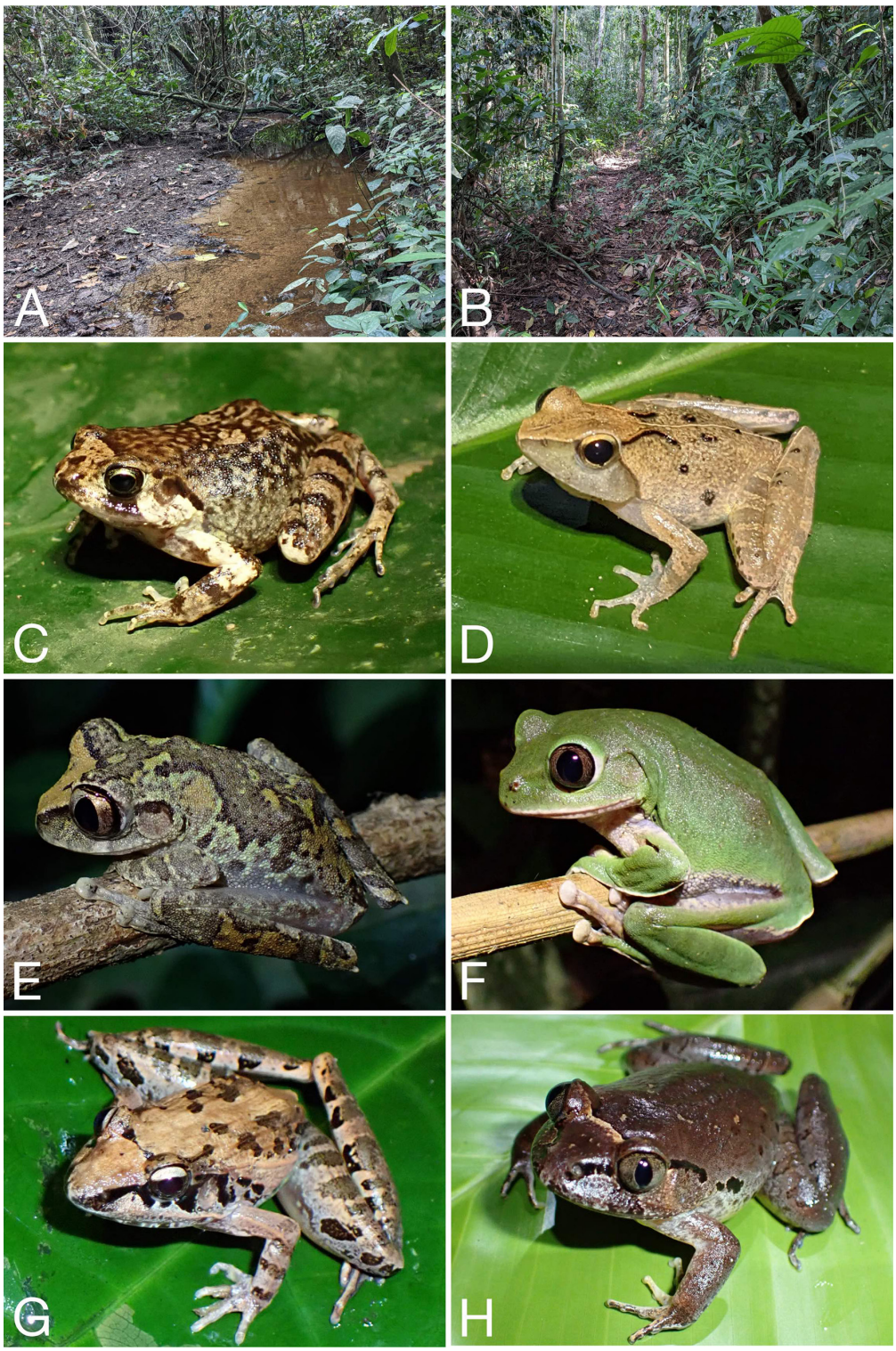

Figure 2. Photographs of the study site and representative amphibians. (A) Stream transect. (B) Terrestrial transect. (C) *Arthroleptis variabilis*. (D) *Phrynobatrachus auritus*. (E) *Leptopelis calcaratus*. (F) *Leptopelis notatus*. (G) *Ptychadena aequiplicata*. (H) *Astylosternus batesi*.

thermocycling profile: 95°C for 5 min, 20 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 90 s, then 72°C for 2 min. We combined the preamplified products from each PCR in equal volumes (5 µl from each) and cleaned the extracted DNA products using 2 µl of EXO-SAPit (Applied Biosystems) to remove primers then diluted the samples 1:5 with PCR grade water. Our cleaned, preamplified samples were then sequenced using a Fluidigm Access Array 48x48 Integrated Fluidic Circuit (Standard BioTools, South San Francisco, California, USA) at the University of Idaho IBEST Genomics Resources Core. This method uses microfluidics to amplify and barcode all 192 amplicons using 24 separate primer pools consisting of eight primer pairs. Barcoded samples were pooled and sequenced on one lane of an Illumina MiSeq using the 300-bp paired-end kit.

We pre-processed all raw sequences as described in Byrne et al. (2017). We filtered reads by selecting sequence variants that were present in at least five reads and represented at least 5% of the total number of reads for that sample/locus using dbcAmplicons (https://github.com/msettles/dbcAmplicons).We generated consensus sequences for all samples using IUPAC ambiguity codes for multiple alleles. We then used gene-tree to species-tree approach to construct a phylogeny for the target sequences that passed filtration (at least five amplicon sequences) and 33 reference samples of known Bd lineages (Byrne et al., 2019). First, we filtered our consensus sequence dataset to eliminate loci that had more than 50% missing data for the reference samples, resulting in 187 loci. Next, for each amplicon we individually aligned all sequences using the MUSCLE package (v3.4.3; Edgar, 2004) in R, checked the alignments for errors in Geneious (v2023.2.1; Kearse et al., 2012), and used the RAxML plugin (Stamatakis, 2014) in Geneious to search for the best scoring maximum likelihood tree for each locus using rapid bootstrapping (100 replicates). We then collapsed all branches in all amplicon trees with < 10 bootstrap support and used Astral-III to generate a consensus tree (Zhang et al., 2018). Astral generates an unrooted species tree given a set of unrooted gene trees and is robust to missing data (Xi et al., 2016). We collapsed nodes within the Astral consensus tree with less than 0.5 posterior probability and midpoint-rooted the resulting tree (Byrne et al., 2019).

Results

Field sampling and Bd prevalence. Our combined sampling included a total of 106 individuals representing 19 species from 11 genera in eight families. Of the frogs sampled during this initial survey, four tested positive for Bd resulting in a 3.7% global prevalence for the Bouamir community. Bd was detected in two of eight families (Arthroleptidae, Ptychadenidae) and four species (two members of the *Arthroleptis sylvaticus* complex, *Astylosternus batesi*, *Ptychadena aequiplicata*; Table 1).

Bd sequencing and phylogenetic analysis. We obtained sequence data for one of the Bd-positive samples collected at Bouamir (F1_Arth_T2S_28Apr) as well as nine Bd-positive samples from a previous survey of Bioko Island and continental Equatorial Guinea (Marshall et al., 2023). The phylogenetic analysis placed our sample from Bouamir and one sample from continental Equatorial Guinea (CAS 265225) in the BdCAPE clade (Fig. 3). The sample from Equatorial Guinea was collected in Centro Sur Province at 677 m elevation.

Table 1. Summary of amphibians detected on transects during the first field survey at Bouamir Station, Cameroon, and their Bd infection status. We list the number of positive samples along with the total sample size (Bd/*n*). Bd intensity is reported as log ITS copies.

Taxon	Bd/*n*	Intensity
Arthroleptidae		
Arthroleptis sylvaticus complex	2/45	3.510–3.870
Arthroleptis variabilis	0/14	
Astylosternus batesi	1/7	1.806
Leptopelis aubryi	0/2	
Leptopelis aubryioides	0/2	
Leptopelis boulengeri	0/9	
Leptopelis ocellatus	0/1	
Bufonidae		
Nectophryne batesii	0/1	
Hyperoliidae		
Hyperolius kuligae	0/1	
Phrynobatrachidae		
Phrynobatrachus africanus	0/7	
Phrynobatrachus auritus	0/5	
Phrynobatrachus cornutus	0/3	
Pipidae		
Hymenochirus boettgeri	0/2	
Xenopus parafraseri	0/1	
Ptychadenidae		
Ptychadena aequiplicata	1/1	6.097
Ranidae		
Amnirana albolabris	0/3	
Amnirana lepus	0/1	
Rhacophoridae		
Chiromantis rufescens	0/1	

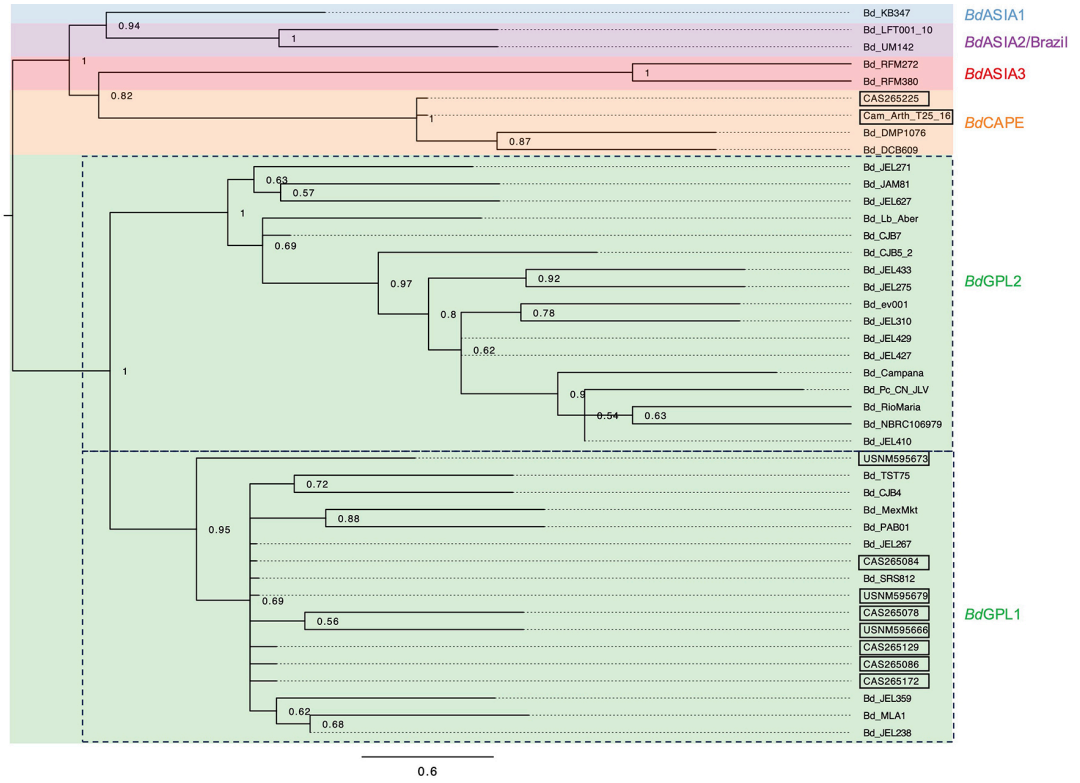

Figure 3. Consensus tree calculated from 187 amplicon trees. Samples sequenced in the present study are indicated with boxes. Nodes are labelled with posterior probability and those with a posterior probability < 0.5 have been collapsed. Major Bd lineages are labelled and coloured as in Byrne et al. (2019).

The remaining samples from Equatorial Guinea were all collected on Bioko Island and placed within the BdGPL1 clade. The sequence data for these samples is archived in the NCBI Sequence Read Archive (Bioproject PRJNA1271576).

Discussion

We report the presence of Bd in the vicinity of Bouamir station in the Dja Biosphere Reserve but did not observe any dead frogs or individuals that presented clear symptoms of chytridiomycosis. With only four Bd-positive frogs in our sample of 106 amphibians across 20 transects, our estimated prevalence is relatively low compared to other field surveys in Central African forests (e.g., Bell et al., 2011; Doherty-Bone et al., 2013; Jongsma et al., 2016; Marshall et al., 2023). Our estimates of infection intensity (based on ITS copies) cannot easily be translated into zoospore genomic equivalents without knowing the ITS copy number of the local Bd strain. However, the values we estimated likely represent moderate to high infection loads. Both Bd prevalence and infection intensity vary seasonally in other tropical amphibian communities (e.g., Longo and Burrowes, 2010; Ruggeri et al., 2018; Moura-Campos et al., 2021), but this has not been rigorously quantified for Central African amphibians (see Marshall et al., 2023). Consequently, future surveys spanning dry and wet seasons at Bouamir will provide more context for this initial characterization of Bd prevalence and infection intensity in the amphibian community.

Of the four Bd-positive frogs in the initial survey, two are direct developers (*Arthroleptis sylvaticus* species complex) and two have more aquatic life histories with a larval life stage (*Astylosternus batesi* and *Ptychadena aequiplicata*). Marshall et al. (2023) reported higher infection intensity in direct developing frogs in an amphibian community in Equatorial Guinea, and this pattern has also been documented in other tropical amphibian communities (e.g., Moura-Campos et al., 2021). Our small sample size precludes a formal test at this time but the presence of species with direct development and larval development life histories at

Bouamir will enable more robust investigation of how reproductive mode is associated with Bd prevalence, infection intensity, and risk. In addition, all four individuals were collected from transects alongside streams (T1S, T2S, and T10S) suggesting that proximity to water may be associated with transmission. Further surveys will clarify whether Bd infection at Bouamir is indeed concentrated around streams and whether this pattern is consistent throughout the year.

Identifying which strains of Bd are present in a given amphibian community is essential for understanding disease dynamics. Two strains of Bd have been documented in Central Africa: BdCAPE in Cameroon and BdGPL in Gabon and the Gulf of Guinea Islands (reviewed in Zimkus et al., 2020; Ghose et al., 2023). Both strains have the potential to cause lethal infection (Sewell et al., 2024) but their distribution across the continent, how they may compete with one another, and whether coinfection is possible remain poorly understood. Our study is consistent with previous work that exclusively reports BdCAPE in Cameroon's amphibian communities (Byrne et al., 2019; Ghose et al., 2023) and our results expand the distribution of this lineage to include continental Equatorial Guinea. This distribution is somewhat surprising given that BdGPL is reported from neighbouring Gabon on the continent and the Gulf of Guinea Islands. Furthermore, the eight Bd-positive samples of Marshall et al. (2023) from Bioko Island that we genotyped all placed in the BdGPL clade in the phylogenetic analysis, suggesting that BdCAPE may not occur on Bioko or the other islands in the Gulf of Guinea. We acknowledge that sampling is still quite limited, but this emerging pattern underscores the need to continue documenting Bd strain diversity throughout the continent.

Acknowledgements. We thank the Ministry of Forestry and Wildlife and the Ministry of Scientific Research and Innovation for research authorizations and permission to export specimens to the California Academy of Sciences for further study, the Congo Basin Institute for logistical support, local guides E. Mpomo and Ecoguard S. Bakouan for assistance in the field, our cook Gladys, and the team of porters from Somalomo. All research was conducted with approval of the California Academy of Sciences Institutional Animal Care and Use Committee protocol 2020-04-Bell. This research was funded by the U.S. National Science Foundation DEB-2003466 (to RCB) and the Academy's Lakeside Fellowship program (to AGBK, ORF, and RCB).

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Accepted by Werner Conradie

Appendix. Frog specimens collected during transects and preserved as voucher specimens at the California Academy of Sciences (CAS).

Catalog number	Species	Transect
CAS 267714	*Astylosternus batesi*	T4S
CAS 267723	*Chiromantis rufescens*	T9S
CAS 267722	*Leptopelis ocellatus*	T9S
CAS 267709	*Nectophryne batesii*	T2T