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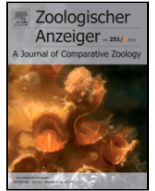
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## Research paper

# Filling the gaps: The mitogenomes of Afrotropical egg-guarding frogs based on historical type material and a re-assessment of the nomenclatural status of *Alexteroon* Perret, 1988 (Hyperoliidae)

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## ABSTRACT

The African reedfrog taxon *Alexteroon* consists of only three described species with rather restricted geographical ranges. Although the assignment to a distinct genus is supported by multiple evidence, its position within the larger African hyperoliid radiation has been disputed. Available molecular data are scarce and the geographic records are few and scattered. The partially formalin fixed type series were previously not accessible to molecular analyses. This changed only very recently with the advancement of Next Generation Sequencing and ancient DNA techniques. Here we provide a reassessment of the current distribution and identity of all known species in this taxon based on (a) historical and new records, (b) morphological reanalyses of the type material and newly collected specimens from Angola and Gabon, and (c) newly established, nearly complete mitochondrial genome data from historical type and modern non-type material. We also present a molecular phylogeny (five mitochondrial loci 12S, 16S, ND1, ND2, COI, Cytb) for 78 sequences from 75 different species of *Hyperolius* retrieved from GenBank and 14 newly established *Alexteroon* sequences. We demonstrate that *Alexteroon* is more widely distributed than previously thought with records from northern Angola representing major southern range extensions. Results of the quantitative morphometric analyses show that the group has a rather conserved general body plan. Therefore qualitative phenotypic features observable in live specimens appear to be more useful for ad hoc species delimitation. We found *Alexteroon* to be nested within *Hyperolius*, corroborating previous findings. However, the combination of molecular data and consistent differences observed in morphology and ecology provide strong support for the distinctiveness of this evolutionary lineage within *Hyperolius sensu lato*. We therefore treat *Alexteroon* as a subgenus of *Hyperolius* and argue that the large and diverse genus *Hyperolius* is in need of revision that may result in new generic arrangements.

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## 1. Introduction

Reedfrogs of the family Hyperoliidae Laurent, 1943 constitute a major portion of the diverse frog radiation known as Gastrechmia Cope, 1867 [Afrobatrachia Frost et al., 2006] (Arthroleptidae Mivart 1869, Brevicipitidae Bonaparte 1850, Hemisotidae Cope 1867, Hyperoliidae). More than half of the 228 currently recognized species in this family belong to the genus *Hyper-*

*olius* Rapp, 1842, while the remaining genera are remarkably species poor or even monotypic (Frost 2020).

One such example is the small group of peculiar afrotropical egg-guarding hyperoliids of the genus *Alexteroon* Perret, 1988. Only three species, *A. obstetricans* (Ahl 1931), *A. hypsiphonus* Amiet, 2000, and *A. jynx* Amiet, 2000, have been attributed to this genus based on morphological and etho-ecological characters. They occupy rather restricted geographic ranges spanning from lowland to mid-altitude rainforests in Southern Cameroon and Equatorial Guinea to Gabon and the western Republic of the Congo (Amiet 2012; Dewynter et al. 2018; Dewynter & Frétey 2019; Channing & Rödel 2019; Sánchez-Vialas et al. 2020). However, recent new records from northwestern Angola suggest that the ranges of *A. obstetricans* and *A. hyp-*

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*siphonus* are in fact larger than previously assumed (Baptista et al. 2019; Ernst et al. 2020).

Molecular data for *Alexteroon* are scarce and the taxon is entirely missing in most of the recently published large scale hyperoliid phylogenies and supraspecific treatments (Rödel et al. 2009; Portik & Blackburn 2016; Portik et al. 2019). By 6 November 2020 GenBank lists only nine entries for *Alexteroon*, two of which are of mitochondrial origin (DQ283171, Gabon, 554bp of 16S; DQ283344, Cameroon, 1490bp of 16S) and restricted to *A. obstetricans*, so far the only species included in molecular phylogenies (Pyron & Wiens 2011; Dubois et al. 2021; Hime et al. 2021). This general lack of data is at least partly due to the fact that these species are rather secretive and difficult to find in the field. This also partly explains the limited number of individuals deposited in collections throughout the world. Moreover, the available type material was originally fixed and/or preserved in formalin and therefore previously not accessible for extended molecular analyses. However, a molecular analysis of *Alexteroon* material is urgently needed to address some of the persisting controversies and ambiguities pertaining to the status of the taxon with respect to its position within the larger hyperoliid family tree (Portik et al. 2019). As with other underappreciated and neglected hyperoliid taxa (e.g. the monotypic genera *Arlequinus* Perret 1988, *Callixalus* Laurent 1950, *Chrysobatrachus* Laurent 1951), a reassessment of *Alexteroon* and the integration of extended molecular data into available family level phylogenies and large amphibian supertrees would also open new avenues for a better understanding of evolutionary patterns in these lineages (compare also Conrardie et al. 2020; Nečas et al., 2021).

Despite the unresolved situation with respect to the taxonomy of *Hyperolius*, *Alexteroon* deserves a special mention in context of reproductive diversity of *Gastrechmia* (Portik & Blackburn 2016) and of parental care in anuran amphibians in general (Furness & Capellini 2019; Vági et al. 2019) as it is the only hyperoliid displaying egg-guarding behaviour (Amiet 1991), perhaps only with the exception of the East African *Hyperolius spinigularis* Stevens, 1971, for which anecdotal observations of a similar reproductive behaviour are available (Stevens 1971; Wells 2007). This prompted Perret (1988) to assign frogs previously described under the generic name *Hyperolius* to the newly created genus *Alexteroon* (type species: *Hyperolius obstetricans*, Ahl 1931). The generic name makes specific reference to this peculiar reproductive behaviour (Greek: ἀλέξειν = aléxein = to protect or defend; ὠόν = oón = egg) and was purposefully chosen to indicate the distinction from the species rich reedfrog genus *Hyperolius sensu lato*. In addition to this prominent behavioural trait, several morphological characters, mainly observed in males, were used to distinguish *Alexteroon* from *Hyperolius* (summarized in Amiet 2000, 2012). These included: **a.** absence of a gular flap in males, in which the gular gland(s) is/are covered by a whitish-transparent tegumentum of the throat (not yellow as in many reproductively active *Hyperolius* spp.) **b.** indistinct vocal sac that is not discernible from surrounding tissue in resting position **c.** small but visible tympanum. Tadpoles of *Alexteroon* differ from those of members of the genus *Hyperolius* by being very slender (adaptation to living in the interstitial habitats of fast running creeks, comparable to tadpoles of the neotropical family Centrolenidae Taylor 1951 that occupy a similar niche) and having two rows of supra-buccal keratodonts, rather than one as in *Hyperolius*. These larval features already led Amiet (1974) to suggest the placement of *H. obstetricans* in a separate genus that is distinct from *Hyperolius*. On grounds of large scale molecular analyses that found *Alexteroon* to be nested within the larger *Hyperolius* radiation and despite the seemingly coherent differences in morphology and behaviour, the validity of the genus has been questioned in order to maintain a monophyletic genus *Hyperolius* (Pyron & Wiens 2011). However, a conclusive taxonomic solution that would dismantle the paraphyletic *Hyperolius* was not provided. While several sources still treated *Alexteroon* as a valid taxonomic entity distinct from *Hyperolius* (Frost 2020; Channing & Rödel 2019), others recently proposed and subsequently adopted the synonymy with *Hyperolius* (Dubois et al., 2021; Frost 2021).

With the advancement of molecular methods (e.g., Next Generation Sequencing and ancient DNA studies) and recently intensified field survey activities in previously inaccessible localities, such as the forests of northern Angola,

the limitations that have previously hampered a thorough reassessment of *Alexteroon* can now be overcome and existing knowledge gaps can be filled.

Here we provide: **1.** a thorough reassessment of the current distribution and identity of all known species within *Alexteroon*; **2.** a morphological reanalysis of the type material and newly collected specimens from Angola and Gabon; **3.** nearly complete mitochondrial genomes for all three *Alexteroon* species from historical type and modern non-type material and **4.** a reassessment of the phylogenetic position of *Alexteroon* with respect to the larger *Hyperolius* radiation. We critically discuss current gaps in observed distribution patterns in the genus *Alexteroon* and its phylogenetic position with respect to an assumed paraphyly (*sensu* Hörandl & Stuessy 2010) of the large genus *Hyperolius*.

## 2. Material and methods

### 2.1. Specimens studied

The specimens under investigation included the type material of *A. hysiphonus*, *A. jynx*, and *A. obstetricans* (neotype designated by Perret [1966]) and additional corresponding specimens from Amiet's original collection all deposited in the Museum National d'Histoire Naturelle Paris (MNHN) and the Museum d'Histoire Naturelle de la Ville de Genève (MHNG), as well as additional vouchers from more recent collections deposited in the Zoologisches Forschungsinstitut und Museum Alexander Koenig (ZFMK), the Senckenberg Naturhistorische Sammlungen Dresden (MTD), the Coleção Herpetológica do Lubango of the Instituto Superior de Ciências da Educação da Huíla (CHL/ISCED), Lubango, Angola, and material collected by L. Chirio and M. Dewynter deposited in the MNHN. For full voucher and tissue list see Table 1 & S1.

### 2.2. Morphological comparison

While the qualitative morphological and behavioural characters originally used to distinguish the three species (Amiet 2000, 2012) are useful in the identification of live specimens, many of Amiet's proposed characters cannot fully be assessed in preserved material and it is unclear, whether or not quantitative morphometric data can be used to discern particular species under preservation. We therefore established morphometric data for the type series and additional corresponding specimens from Amiet's collection, more recently collected *A. obstetricans* and *A. jynx* from Cameroon, *A. obstetricans* (one) and *A. hysiphonus* (five) from Gabon, as well as specimens of *A. hysiphonus* (three) and *A. obstetricans* (two) from the newly discovered Angolan population (Table S1) to test whether a statistically significant separation of respective "morphotaxa" that can be assigned to either the defining type material or genetically identified specimens is possible. Morphometric data include the following 12 parameters: HW, head width; HL, head length; IO, interorbital distance; IND, internasal distance; ED, eye diameter; TYD, tympanum diameter; FeL, femur length; TiL, tibiofibula length; FL, Foot length; LA, body length from foreleg angle to hind leg angle; LatFringA, maximum width of lateral fringes on arm; LatFringL, maximum width of lateral fringes on leg. Measurements were taken with a dial calliper ( $\pm 0.1$  mm) or a measuring ocular in a dissecting microscope ( $\pm 0.01$  mm, Leica M125).

We quantified morphological differences using a metric multidimensional scaling approach (PCoA = Principal coordinate analysis) with a posteriori validation of identified groupings using a similarity profile permutation test (SIMPROF, Clarke et al., 2008). All statistical analyses were performed using the packages vegan (Oksan et al. 2012) and clustsig (Whitaker & Christman 2010) in the R statistical software, version 3.4.4 (R Development Core Team 2018). To account for allometric relations in measured characters and for covariation among characters, measurements were **a.** corrected for body-size (snout-vent length = SVL) and **b.** tested for collinearity using Variance Inflation Factors (VIF) with a conservative threshold of 0.5 as exclusion criterion prior to analysis.

**Table 1**  
Genetically investigated specimens and tissue vouchers.

Collection/field number	Species	Status	Country	Locality	Data
MHNG 2685.1/NA	<i>hypsiophonus</i>	Paratype	Cameroon	Ekotolo	mt-genome
MTD 48924/RE_ANF76	<i>hypsiophonus</i>	Voucher	Angola <sup>a</sup>	Serra do Pingano, Uíge	mt-genome
MNHN-RA-2021.0010/AF 4216	<i>hypsiophonus</i>	Voucher	Gabon	Zone RAMSAR de Birougou	mt-genome
MNHN-RA-2021.0006/AF 4289	<i>hypsiophonus</i>	Voucher	Gabon	Kinguele Aval	16S
MNHN-RA-2021.0007/AF 4720	<i>hypsiophonus</i>	Voucher	Gabon	Pont du Komo	16S
MNHN-RA-2021.0008/AF 4721	<i>hypsiophonus</i>	Voucher	Gabon	Pont du Komo	16S
MNHN-RA-2021.0009/AF 4722	<i>hypsiophonus</i>	Voucher	Gabon	Pont du Komo	16S
MNHN 1999.5561/NA	<i>jynx</i>	Holotype	Cameroon	Betenga	mt-genome
ZFMK 89431/NA	<i>jynx</i>	Voucher	Cameroon	Rumpi Hills	mt-genome
MNHN-RA-2021.0011/AF 4215	<i>obstetricans</i>	Voucher	Gabon	Zone RAMSAR de Birougou	mt-genome
CHL 927/NB 927	<i>obstetricans</i>	Voucher	Angola <sup>b</sup>	Serra da Canda	16S
CHL 928/NB 928	<i>obstetricans</i>	Voucher	Angola <sup>b</sup>	Serra da Canda	mt-genome
MHNG 995.48/NA	<i>obstetricans</i>	Neotype	Cameroon	Foulassi	–

<sup>a</sup> first country record, Ernst et al. (2020).

<sup>b</sup> first country record (this study).

### 2.3. Wet lab techniques

Molecular work was divided into two batches. Five recently collected (< 20 yrs) ethanol preserved specimens were analysed in the main molecular laboratory at MTD. DNA was extracted with the innuPREP DNA Mini Kit (Analytik Jena AG), sheared down to approximately 150bp using a Covaris M220 ultrasonicator (Covaris Ltd), cleaned with the MinElute PCR Purification Kit (Qiagen N.V.), and converted into single-indexed double-stranded Illumina sequencing libraries following Meyer & Kircher (2010) with modifications by Fortes & Paijmans (2015). DNA concentration and quality were assessed using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Inc.) and a 4200 TapeStation system (Agilent Technologies, Inc.). Three historical specimens, originally formalin-preserved, were processed in the clean room facility at MTD according to established guidelines (Fulton & Shapiro 2019). DNA was extracted from muscle tissue using the DNeasy Blood and Tissue Kit (Qiagen N.V.), and converted into single-indexed single-stranded Illumina sequencing libraries following Gansauge & Meyer (2013) with modifications by Korlević et al. (2015), including the removal of uracil residues. To enrich all libraries for mtDNA, two rounds of in-solution hybridisation capture with self-made baits from PCR products of *A. hypsiophonus* (MNHN-RA-2021.0010) and *A. obstetricans* (MNHN-RA-2021.0011) were performed following Horn (2012) and Maricic et al. (2010, see supplementary information and Table S6 for preparation of bait molecules). Sequencing was performed in-house on an Illumina MiSeq platform (Illumina, Inc.), generating 75-bp-paired-end reads. 16S sequences were produced using Sanger sequencing for five additional

modern samples (MNHN-RA-2021.0006, MNHN-RA-2021.0007-9, CHL 928) in the labs of NB and AF. DNA extraction was achieved using the Wizard® Genomic DNA Purification Kit (Promega; Madison, WI, USA) following the manufacturer's protocols. Amplification of the targeted locus followed a standard PCR protocol described by Salducci et al., (2005).

### 2.4. Bioinformatics

Assembly of mt-genome sequences involved adapter trimming with Skewer v0.2.22 (Jiang et al. 2014), as well as read merging (minimum length 30 bp), quality filtering (minimum Q-score 20) and duplicate removal with BBmap-suite 37.24 (<https://sourceforge.net/projects/bbmap/>) (Bushnell et al. 2017). Resulting readpools were subjected to a two-step baiting and iterative mapping approach in MITObim with an allowed mismatch value of 2 (Hahn et al. 2013) (see supplement information for further details). Resulting scaffolds were visualised and checked for coverage (minimum per-site coverage: 3-fold) and assembly artefacts in Tablet 1.16.09.06 (Milne et al. 2013). If assembled contigs were largely discontinuous, each fragment was submitted to a nucleotide BLAST search to check for its genuineness (Madden 2002). Discovered contaminations were removed. Sequence length distribution of mapped reads was calculated with a customised awk command and Microsoft Excel. Assembly details of *Alexteroon* material is summarised in Table S2. The temporal authenticity of the mapped historical reads was tested with mapDamage 2.0 (Jónsson et al. 2013), which accounts for nucleotide misincorporations due to DNA degradation (see Suppl. Info). Annotation of assembled mt-DNA was performed with MITOS2 (Bernt et al. 2013). See Table S3 for sequence accession numbers issued by the European Nucleotide Archive (ENA) for *Alexteroon*.

### 2.5. Alignment and phylogenetic analyses

Apart from the newly generated molecular data of *Alexteroon*, sequences of five mitochondrial loci used for phylogenetic analyses were downloaded from GenBank (Table S3). The concatenated final alignment of 5421 bp contained 422 bp of 12S, 545 bp of 16S, 941 bp of ND1, 1032 bp of ND2, 1356 bp of COI, and 1125 bp of Cytb. Aligning each locus was achieved with ClustalW (Thompson et al. 1994) as implemented in BioEdit (Hall 1999) and by subsequent manually proofreading. Coding regions were checked with MEGAX (Kumar et al. 2018) for the correct reading frame and the presence of internal stop codons. The ingroup is comprised of 78 sequences of *Hyperolius* representing 75 species or subspecies, plus 14 *Alexteroon*. Three additional species of Hyperoliidae and two species of Arthroleptidae represent the outgroup, with *Leptopelis vermiculatus* (Boulenger 1909) as the root.

Phylogenetic reconstruction involved Maximum Likelihood (ML) and Bayesian Inference (BI) approaches using RAXML 8.0.0 (Stamatakis 2014) and MrBayes 3.2.6 (Ronquist et al. 2012), with Tracer 1.7.1 (Rambaut et al. 2018) being used for the assessment of calculation parameters of the latter. The best evolutionary models and partitioning schemes were determined with PartitionFinder2 (Lanfear et al. 2016) applying the greedy search scheme and the Bayesian Information Criterion. The resulting partitioning schemes (ML & BI) and substitution models (BI only) are shown in Table S4. For ML, five independent searches were carried out using the GTR + G substitution model (as recommended by Stamatakis 2016: 59f), different starting conditions, and the rapid bootstrap option. Subsequently, 1000 non-parametric thorough bootstrap replicates were calculated and the values plotted against the best tree. For BI, four parallel runs (each with eight chains) were performed with 4 million generations (burn-in 0.25; print frequency 1000; sample frequency 1000). Uncorrected *p*-distances were calculated for the 16S dataset (79 sequences of 544 bp aligned length) with MEGAX using uniform rates, transitions and transversions, and the pairwise deletion option (Table S5). For a more convenient presentation of the phylogenetic results, three or more samples forming a monophyletic entity in the ML analysis are tentatively referred to as a clade, regardless of their respective statistical support values.

### 3. Results

#### 3.1. Distribution and new records

Historically, members of the genus *Alexteroon* were considered to have very restricted distribution ranges, confined to Cameroon, Gabon, Equatorial Guinea, and the southern Republic of the Congo (compare Channing & Rödel 2019 and Fig. 1). Since the description of the three taxa, the originally described ranges were only slightly extended. *Alexteroon jynx* remains known from only very few localities near the original type locality on the eastern slopes of Rumpi Hills, southwestern Cameroon. To our knowledge, the most recent record corresponds to the specimen ZFMK 89431, collected in 2009 that is part of our genetic analyses. Because of this extremely small range it is listed as Critically Endangered under IUCN-Red List criteria (IUCN 2020). *Alexteroon hypsiphonus* has previously been recorded from the aforementioned countries and a recent treatment by Dewynter et al. (2018) provided new records of this species, as well as for *A. obstetricans* from Gabon. Very recently *A. hypsiphonus* was also discovered in the last remaining rainforest fragments of Uíge province, northwestern Angola (see account in Ernst et al. 2020), which represented an unexpected and significant range extension of about 500 km to the southeast. In a recent survey led by PVP, a new country record was added for *A. obstetricans*. Two specimens, a pair in amplexus, were collected on October 18th 2017 in Serra da Canda, Zaire province, northern Angola (S06° 11' 38"; E014° 40' 46"; 950 m a.s.l.), on top of a leaf, about 2 m above water. Serra da Canda represents the northernmost outlier of the Angolan escarpment. The recorded locality was situated in a patch of rainforest located above a second order clear-water stream of about 4 m width, and tributary of Mbridge River. The riparian forests in that area are comparatively undisturbed but canopy height did not considerably exceed 12–15 m. A summary of the

known ranges including new locality records from Angola is depicted in Fig. 1.

#### 3.2. Morphological identification of preserved material

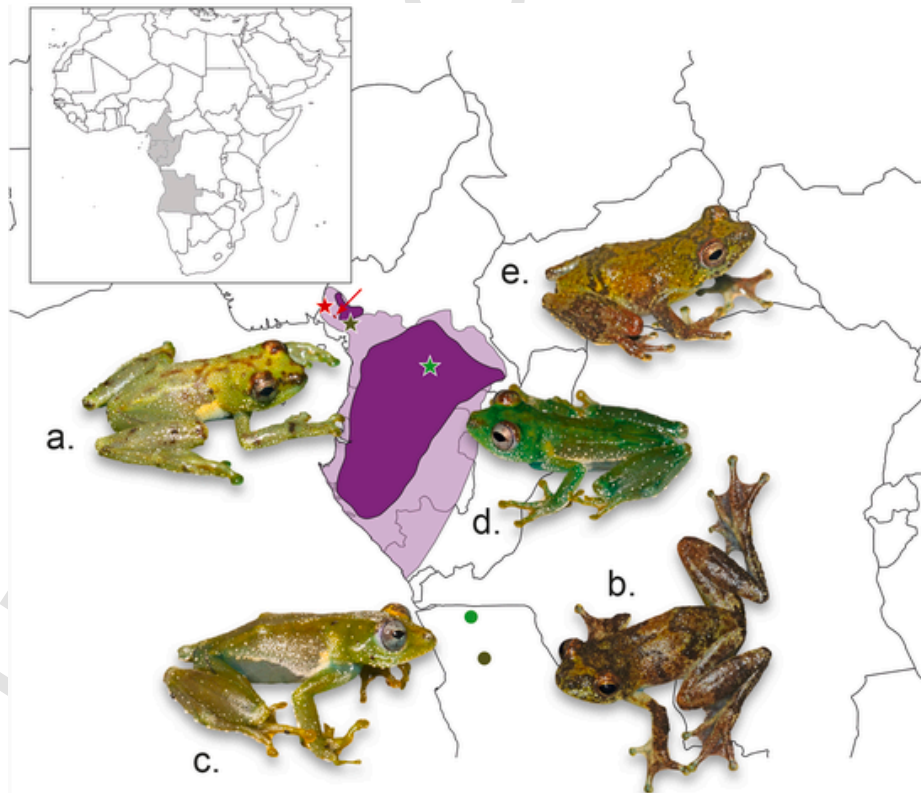
While it is comparatively unproblematic to distinguish *Alexteroon hypsiphonus* from *A. jynx* and *A. obstetricans* based on dorsal patterns and gular pigmentation, differentiating the latter two based on these characters remains difficult. Moreover, results of the quantitative statistical analyses of the 12 selected standard morphometric characters indicate that the main body shape and proportions are rather conserved in the entire genus. All specimens overlapped largely in the shared multivariate PCoA morphospace and fell into only two SIMPROF-validated clusters ( $p < 0.001$ , at 10,000 simulation runs) showing only little substructure (Fig. S5).

The general phenotypes of all examined specimens largely correspond with the original descriptions and those summarized in Amiet (2012). However, we observed a fundamental deviation in life gular coloration in males of *A. hypsiphonus* and *A. obstetricans* from Angola. Contrary to the descriptions provided in Amiet (2000; 2012), in which he stated that the gular coloration is always white or transparent-whitish, distinguishing males of the genus *Alexteroon* from males of many *Hyperolius* species, we found the gular coloration of reproductively active and/or calling males of *A. hypsiphonus* and *A. obstetricans* to be bright yellow (Fig. 2), thus rendering this character less useful for distinction between the two genera.

#### 3.3. Molecular approach

##### 3.3.1. Sequencing success

We assembled almost complete mitochondrial genomes of approximately 15 kb for all five modern samples and two out of three historical type



**Fig. 1.** Map showing known distribution range of *Alexteroon* spp. and newly recorded Angolan localities. *Alexteroon obstetricans* (dark purple polygon), *Alexteroon hypsiphonus* (light purple polygon), *Alexteroon jynx* (red polygon indicated by red arrow). Angolan records of *Alexteroon obstetricans* (light green dot) and *Alexteroon hypsiphonus* (olive dot). Bright green star, neo-type locality of *Alexteroon obstetricans* at Foulassi, Cameroon; olive star, type locality of *Alexteroon hypsiphonus* at Ekomtolo, Cameroon; red star, type locality of *Alexteroon jynx* at Betenge, Cameroon. Live pictures: a. *Alexteroon jynx* ZFMK 89431, Cameroon; b. *Alexteroon hypsiphonus* MTD 49697, Angola; c. *Alexteroon obstetricans* CHL 928 Angola. d. *Alexteroon obstetricans*, Gabon; e. *Alexteroon hypsiphonus*, Gabon. Spatial data derived from IUCN Red List of Threatened Species. Version 2017.3 (<http://www.iucnredlist.org>), accessed 17 September 2020, and this study. Photos credits: a (AS), b (RE), c (PVP), d-e (MD).





Fig. 2. Ventral coloration of live *Alexteroon* spp. males from Angola. a. *Alexteroon obstetricans* CHL 928 from Serra da Canda, Zaire province, b. *Alexteroon hypsiphonus* MTD 48923 from Serra do Pingano, Uíge province (right).

mens processed at MTD, reaching from tRNA-Phe to Cytb (Table 2). Only the assembled sequence data of the neotype of *A. obstetricans* (MNHNG 995.48) was of such poor quality that it had to be removed from subsequent analyses. However, the few fragments positively identified as endogenous DNA (nine fragments with a coverage between 3- and 16-fold and covering 380bp) were concordant with the sequence data assembled from the two modern *A. obstetricans*. Although, the amplified fragments for bait library construction theoretically covered the entire mt-genome, a ring closure, indicating a complete mitochondrial genome, could not be achieved.

### 3.4. Mitochondrial genome arrangement of *Alexteroon*

The observed arrangement of the assembled 13 coding genes, 2 rRNAs and 19 tRNAs of *Alexteroon* spp. largely corresponds to that of a typical vertebrate mt-genome, e.g., *Homo sapiens* Linnaeus, 1758 (Table 2). The only difference being a shifted tRNA-Ala and a presumably shifted (or absent) tRNA-Leu2, tRNA-Thr, tRNA-Pro, and CR. The published complete mitochondrial genome of *Hyperolius marmoratus* Rapp, 1842 shares the shifted position of

Table 2

Comparison of mitochondrial genome arrangement between *Homo sapiens*, *Hyperolius marmoratus* and *Alexteroon* spp. Whereas the mt-genomes of the first two taxa are complete, *Alexteroon* spp. is incomplete, hence, the presence of two tRNAs (trnT & trnP) and the control region (CR) is not specified (n.s.).

<b>Typical vertebrate mt-genome, e.g., <i>Homo sapiens</i></b>	trnF	12S	trnV	16S	trnL <sub>1</sub>	ND1	trnI	-trnQ	trnM		ND2	trnW
<b><i>Alexteroon</i> spp.</b>	trnF	12S	trnV	16S	trnL <sub>1</sub>	ND1	trnI	-trnQ	trnM		ND2	trnW
<b>AB777218 <i>Hyperolius marmoratus</i></b>	trnF	12S	trnV	16S	trnL <sub>1</sub>	ND1	trnI	-trnQ	trnM	ND2-CR2-trnM	ND2	trnW

<b>Typical vertebrate</b>	-trnA	-trnN		-trnC	-trnY	CO1	-trnS <sub>1</sub>	trnD	CO2	trnK	atp8	atp6
<b><i>Alexteroon</i> spp.</b>		-trnN	-trnA	-trnC	-trnY	CO1	-trnS <sub>1</sub>	trnD	CO2	trnK	atp8	atp6
<b>AB777218</b>		-trnN	-trnA	-trnC	-trnY	CO1	-trnS <sub>1</sub>	trnD	CO2	trnK	atp8	atp6

<b>Typical vertebrate</b>	CO3	trnG	ND3	trnR	ND4L	ND4	trnH	trnS <sub>2</sub>	trnL <sub>2</sub>	ND5	-ND6	-trnE
<b><i>Alexteroon</i> spp.</b>	CO3	trnG	ND3	trnR	ND4L	ND4	trnH	trnS <sub>2</sub>		ND5	-ND6	-trnE
<b>AB777218</b>	CO3	trnG	ND3	trnR	ND4L	ND4	trnH	trnS <sub>2</sub>		ND5	-ND6	-trnE

<b>Typical vertebrate</b>	Cytb	trnT	-trnP	CR								
<b><i>Alexteroon</i> spp.</b>	Cytb	n.s.	n.s.	n.s.								
<b>AB777218</b>	Cytb			CR1	-trnP	NC	trnT	trnL <sub>2</sub>	NC			

tRNA-Ala and has tRNA-Leu2 located after its first control region (CR1), a region not recovered from *Alexteroon* spp. (see supplementary information). In contrast to *H. marmoratus*, *Alexteroon* spp. do not exhibit the duplicated and presumably non-functional ND2-CR2-tRNA-Met complex as well as two long non-coding fragments (NC).

### 3.5. Phylogenetic placement of *Alexteroon* and uncorrected *p*-distances

The obtained topologies from ML and BI retrieve *Alexteroon* as a strongly supported clade deeply nested within *Hyperolius* (Fig. 3 and Fig. S6). The same applies to *Hyperolius koehleri* Mertens, 1940, a species previously placed in the monotypic genus *Chlorolius* by Perret (1988) and subsequently

treated as subgenus by Amiet (2012). These taxa together with all remaining *Hyperolius* form a strongly supported monophylum thereafter referred to as *Hyperolius sensu lato*. The subdivision within *Hyperolius s. l.* lacks good statistical support for most clades. Considering the ML tree, only the clade containing *Alexteroon* (clade E), and clades A, C, and D are well supported, the latter being sister to *Alexteroon* although with a low bootstrap value. Clades F and G show reasonable support values. Considering BI, *Hyperolius s. l.* is characterised by three reasonably (F, 0.91) to well (A, E, 1.0) supported clades and a large polytomy, from which several smaller clades originate. The comparatively lower support for clade F is also reflected in the retrieved composition that comprises the morphologically well-defined *nasutus*-group (Amiet 2005) and taxa otherwise not considered to be closely related to the former. Topology



**Fig. 3.** Maximum Likelihood phylogeny based on 5421 aligned positions of mtDNA (12S: 422bp, 16S: 545bp, ND1: 941bp, ND2: 1,032bp, COI: 1,356bp, Cytb: 1,125bp). For convenience, support values (bootstrap/posterior probabilities) are only depicted above a certain threshold ( $\geq 70/\geq 0.70$ ). Asterisks indicate maximum support under both tree-building approaches. Bold branches indicate congruence with Bayesian Inference analysis. Colours of the bar on the right side indicating the clades correspond to Table S5. Scale bar indicates 0.2 substitutions per nucleotide position. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

within *Alexteroon* in ML and BI sees *A. hypsiphonus* and *A. jynx* as strongly supported sister taxa, compared to a basal *A. obstetricans*. The seven samples of *A. hypsiphonus* fall into two subgroups, one of them exclusively comprising the five samples from Gabon, the other consisting of a sample from Angola and Cameroon, respectively.

Uncorrected genetic *p*-distances using 16S data for *Hyperolius sensu lato* (Table S5) show a minimum divergence between *Alexteroon* and *Hyperolius*, at 6.8% (*A. hypsiphonus* MTD 48924 & MHNG 2658.1 and *A. obstetricans* DQ283171 compared to *Hyperolius montanus* [Angel 1924]). Within *Alexteroon*, the individual species have a maximum intraspecific distance of 1.5% (*A. hypsiphonus*, *n* = 7), 0.4% (*A. jynx*, *n* = 2), and 1.5% (*A. obstetricans*, *n* = 5). The minimum interspecific distance is 2.3% between *A. jynx* and *A. hypsiphonus*, 4.2% between *A. obstetricans* and *A. hypsiphonus*, and 4.8% between *A. jynx* and *A. obstetricans*.

#### 4. Discussion

Molecular phylogenetics has become a standard tool for generating evolutionary hypotheses in taxonomy and systematics (Whelan et al. 2001). Historically, it has been good scientific practice in alpha-taxonomic treatments and extended supraspecific phylogenetic analyses to include name-bearing specimens in differential diagnoses. This material is defined as the ultimate reference-point for describing variation within and significant deviation from a distinct taxonomic entity, in animals usually a species, genus, or family. Ideally, any taxonomic analysis should be based on different kinds of character or trait sets including morphology, bioacoustics, life-history data and nucleotide sequences that can be used to describe particular taxonomic entities in an integrative framework (Padiál et al. 2010; Vences 2020). Name-bearing types should then be allocated to the groups identified within the scope of these integrative analytical frameworks, and only those, for which no taxon names are available, are to be recognised as new taxa (Ohler & Dubois 1999; Evans et al. 2019).

However, a major drawback of this nomenclatural procedure is the fact that many historical type specimens are unavailable to molecular analyses because they suffer from suboptimal preservation, for example due to the widely used fixation in formalin, the use of denatured alcohol or simply unfavourable storing conditions that result in the fragmentation and deterioration of DNA. As a result, historical type material is often neglected in modern molecular analyses, unless relevant species were only recently described and fresh material is available (but see Rancilhac et al. 2020; Scherz et al. 2020). The situation is in fact aggravated in taxa that have not frequently been collected and, for which fresh, ideally topotypic, material for extended analyses is simply not accessible, as is the case in *Alexteroon* spp.

Taxon-Omics, as a recently proposed discipline that integrates traditional taxonomy and genomic/transcriptomic technology (Amann et al. 2014; Raupach et al. 2016) can help to overcome these issues and here, we provide an example demonstrating that lab procedures developed for historical and subfossil material (see Material and methods) can provide a loophole for making these data accessible (e.g., Kehlmaier et al. 2019a, b; Lord et al. 2018; Mohandesan et al. 2017; Oswald et al. 2019; Stelbrink et al. 2019; Rancilhac et al. 2020; Scherz et al. 2020; Woods et al. 2020). While the divergence of mitochondrial DNA should not be used as the sole justification for species distinctiveness, the newly established data significantly aid in the identification of our three target species and in distinguishing between them, especially when comparing preserved material. Results of our quantitative morphometric analysis indicated that the three known species in the genus *Alexteroon* exhibit highly conserved general body plans and external morphology, rendering a quantitative distinction between species based on preserved material alone difficult, particularly when comparing the two superficially similar patterned yet genetically well separated *A. obstetricans* and *A. jynx*. Meanwhile, phenotypic features that can only be observed in live specimens, such as the gular coloration observed in reproductively active males of *A. hypsiphonus* and *A. obstetricans* or marked differences in coloration between *A. hypsiphonus* on one hand and *A. obstetricans* and *A. jynx* on the other hand appear to be more useful for ad hoc species delimitation (compare Amat et al. 2013;

Glaw et al. 2018 for similar findings in mantellid frogs). These observations call for detailed morphological studies, as well as for photo-documentations of live specimens that should be a standard feature of amuran species descriptions. This is likewise true for bioacoustic data, which were not included in our study since multiple recordings required for quantitative statistical analyses were not available. However, published calls of all three species (Amiet & Goutte 2017) can readily be distinguished and a call recording of an individual of the Angolan population of *A. hypsiphonus* (MTD 49697; available at <https://sonotheque.mnhn.fr>) matches acoustic properties of the previously published call of this species. *Alexteroon* may be a good example for the validity of the recently proposed cyber- or e-taxonomy concept that seeks to integrate synthesize, and analyse all available evidence that is informative at the taxonomic level(s) being studied, including extended electronic phenotypic data such as call recordings and colour images of live specimens (Wheeler & Valdecasas 2010). In our case, the genomic data generated using both historic type and recently collected material significantly helped in corroborating the distinction between the three previously described species particularly between the rarely collected and data deficient *A. jynx* and the remaining two species. Moreover, the data showed that the new Angolan records can safely be assigned to the nominal taxa *A. hypsiphonus* and *A. obstetricans* and hence represent major range extensions. This reinforces the perception that *Alexteroon* is not endemic to the Cameroonian Province of the Intertropical Montane region as previously defined by Poynton (1999) and it emphasizes the fact that the actual ranges of many sub-Saharan African amphibian species are still poorly understood (Ernst et al. 2014, 2015). It also highlights the pivotal role of the northern Angolan forest remnants in shaping and ultimately understanding the biogeography of the entire West Central African realm (Ernst et al. 2020).

Our phylogenetic analyses of the larger hyperoliid radiation corroborate previous studies that found *Alexteroon* to be nested within *Hyperolius* (Pyron & Wiens 2011; Dubois et al., 2021) and thus supports the synonymy with *Hyperolius* that was recently proposed by Dubois et al. (2021). But at the same time our analysis provides strong support for the distinctness of this evolutionary lineage within *Hyperolius s. l.*, which is in line with the observed consistent differences in morphology, ecology and bioacoustics. The well supported phylogenetic pattern within *Hyperolius s. l.* with *Alexteroon* representing one of at least eight divergent clades indicates that the large and diverse genus *Hyperolius* is in need of revision. As the taxonomic status of several lineages within *Hyperolius* are not resolved and because the clade herein recognized as *Alexteroon* is monophyletic and can be diagnosed by morphological and ethno-ecological characters, we treat *Alexteroon* as a subgenus of *Hyperolius* containing the three taxa *Hyperolius (Alexteroon) hypsiphonus*, *Hyperolius (Alexteroon) obstetricans*, and *Hyperolius (Alexteroon) jynx* until the data required for such a revision are available and the taxonomic position of *Alexteroon* can conclusively be resolved. Evidence is accumulating that several branches within the larger *Hyperolius* tree require a re-assessment (Nečas et al., 2021) and recently published research that shows the genus *Kassinula* Laurent, 1940 to be nested within the genus *Afrixalus* Laurent, 1944, despite readily observed morphological differences (Conradie et al. 2020) points in a similar direction in another species rich hyperoliid group. Splitting the genus *Hyperolius* into smaller monophyletic taxonomic entities to depict the natural groupings more accurately therefore warrants serious consideration. However, such a task is beyond the scope of this contribution and requires a thorough, truly integrative effort that includes a re-assessment of all morphological, molecular, bioacoustics and ecological traits available. The subgenus *Alexteroon* appears to be an ideal starting point to address this problem. With nearly complete mitochondrial genome data and species validations based on type material now being available, we hope to stimulate further studies addressing these issues and advocate research in this direction.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jcz.2021.06.002>.

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