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Hidden diversity of the widespread *Opsaridium ubangiense* (Cypriniformes: Danionidae) in Central Africa

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Abstract

Opsaridium ubangiense is a reportedly widespread species that occurs throughout the Lower Guinea ichthyofaunal province and within the Upper Congo River drainage. Recent collections throughout the region allow us to examine the genetic diversity within this species across its reported distribution. The results of a multi-locus analyses and four different species delimitation methods (GD, bPTP, ABGD, and ASAP) suggest the presence of distinct taxa within what is now considered *O. ubangiense*. The analysis also revealed potentially distinct taxa within the rivers of Equatorial Guinea and highlight some interesting patterns of diversity within the Mbini River drainage. A preliminary morphological analysis also revealed some differences among topotypic material and other studied populations. These results are discussed in the context of other recent discoveries in the region; future studies needed are also detailed.

Key words: species delimitation, biodiversity, cryptic species, headwater capture

Introduction

The Lower Guinea ichthyofaunal province of Central Africa is one of the most species-rich freshwater regions on the continent, with over 575 fishes reported to date (Stiassny *et al.* 2007). Stretching from the mouth of the Niger River in the north to the mouth of the Congo River in the south, its rivers and streams harbor extraordinary levels of diversity and endemism. In the past decade, renewed ichthyological surveys have not only revealed previously undocumented biodiversity but also led to the formal description of numerous new species (Cutler *et al.* 2019; Mipounga *et al.* 2019; Schmidt & Barrientos 2019; Schmidt *et al.* 2021; Malumbres *et al.* 2022; Valdesalici & Malumbres 2022). Yet even among the region's widespread and supposedly well-known taxa, fundamental questions remain.

One such case is the Danionidae: Chedrinae, *Opsaridium ubangiense* (Pellegrin, 1901). There are 12 valid species in the genus *Opsaridium* distributed in central and southern Africa (Fricke *et al.* 2025), but it is considered non-monophyletic. The relationship/distinction between the morphologically similar, though generally larger, genus *Raiamas* remains unresolved (Liao *et al.* 2012). *Opsaridium ubangiense*, first described from the Ubangi River basin in the Congo River drainage, has long been regarded as the only member of the genus present in the Lower Guinea province (De Weirdt 2007). Its reported broad distribution across multiple basins has never been tested, raising the possibility that this taxon may mask unrecognized diversity.

During a National Geographic Society-funded expedition (# WW-055R-17) to continental Equatorial Guinea in 2017, we collected *O. ubangiense* specimens in several different drainages. Notably, field observations indicated that there may be some morphological differences among these different populations within the species distribution range. These observations prompted us to investigate whether genetic data would support hidden structure within

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this widespread species. In this study, we assess the genetic diversity of *O. ubangiense* in Equatorial Guinea and compare it with material from other parts of Lower Guinea, including topotypic specimens from the Ubangi River basin, to evaluate whether cryptic diversity may underlie its current taxonomic treatment.

Methods

To examine the diversity of *Opsaridium ubangiense* throughout their proposed range we examined specimens and tissues from natural history collections and those recently collected in the region. *Opsaridium ubangiense* specimens and tissues from Equatorial Guinea were collected in 2017. Specimens were collected in several drainages in the country (Fig. 1), tissues samples were collected, and voucher specimens were fixed in 10% formalin and later transferred to 75% ethanol for long term storage. Additional tissues and specimens were borrowed from several natural history collections including the American Museum of Natural History (AMNH), Cornell University Museum of Vertebrates (CUMV) and the Oregon State Ichthyology Collection (OS).

Tissues were acquired from 32 specimens of *O. ubangiense* from throughout the distribution range. This included specimens from the Ogooué River drainage in Gabon, the Ubangi River drainage in the Central African Republic (type locality), upper Congo River drainage tributaries, coastal rivers in the Republic of the Congo, and from several drainages in Equatorial Guinea and Cameroon. Genomic DNA was extracted with DNeasy tissue kits (Qiagen). Two mitochondrial and two nuclear markers were amplified and sequenced for this study. Cytochome b (CYTB) was amplified with the primers Glu-2 and Pro-R1 (Hardman and Page 2003) using a touchdown protocol described in Schmidt *et al.* (2014). Cytochrome c oxidase I (CO1) was amplified using primers LCO1490 and HCO2198 (Folmer *et al.* 1994) with an initial denaturation at 94°C for 60 s then 35 cycles of 94 °C for 30 s, 42 °C for 1 min, 72 °C for 1.5 mins, and a final extension at 72 °C for 7 mins.

The nuclear gene myosin-heavy polypeptide 6-cardiac muscle-alpha (MYH6) was amplified and sequenced using primers MYH6_F507 and MYH6_R1322 with published protocols (Li *et al.*, 2007; Arroyave and Stiassny 2011). Lastly the second intron from the growth hormone (GH) was sequenced using primers 23F, 102F, and 148R designed for African Small Barbs (ASB) in the genus *Enteromius* (Schmidt *et al.* 2017). We used a nested design starting with the primers 23F and 148R and then using the resulting products in a reaction with primers 102F and 148R. The protocols for these reactions follow Schmidt *et al.* (2017). Primers 102F and 148R were then used in the sequencing reaction. Specimen metadata and GenBank accession numbers associated with the different molecular markers are in Supplemental Table 1. Additionally, CO1 data from 10 representative *O. ubangiense* specimens that were collected in Equatorial Guinea were submitted to the Barcode of Life Database (BOLDsystems.org).

In addition to the sequences produced for the study published sequences from comparative material, mostly CO1, were included in the analyses (Tang *et al.* 2010; Liao *et al.* 2012; Decru *et al.* 2016; Sungana *et al.* 2017; Nehemia and Mwakalesi 2023, 2024). The outgroup taxa *Raimas senegalensis* (Steindachner) and *R. batesii* (Boulenger) were selected based on their position in the phylogeny previously produced for the group (Liao *et al.* 2011). Each dataset was partitioned by codon position, except the GH intron, and the best model of evolution for each partition was determined by PartitionFinder2 (Lanfear *et al.* 2016). Bayesian analyses were performed in MrBayes version 3.2 (Ronquist *et al.* 2012) via the CIPRES Science Gateway (Miller *et al.* 2010). Posterior probabilities were assessed with 5 million generations, sampling trees every 100 generations. The first 25% of trees were discarded as burn-in. The datasets were run independently and then concatenated for total evidence analysis. Genetic data missing from individual specimens were treated as missing in the combined analysis. All trees were visualized in FigTree v 1.4.2 (Rambault, 2012) and annotated in CorelDraw. Four species delimitation methods were applied to the CO1 dataset. The barcoding genetic distance method (GD, Herbert *et al.* 2003), Automatic Barcode Gap Discovery (ABGD, Puillandre *et al.* 2012), assemble species by automatic partitioning (ASAP, Puillandre *et al.* 2021) and Bayesian implementation of the Poisson Tree Processes (bPTP) (Zhang *et al.* 2013). We largely followed the parameters implemented in Bragança *et al.* (2021), however we set the GD cutoff to 3% sequence divergence to be conservative in the value between operational units (Ward 2009).

A preliminary morphometric analysis of populations of *O. ubangiense* was also completed for this study. These included specimens from the Ubangi River drainage (type locality of *O. ubangiense*), several drainages in Equatorial Guinea, and a few representatives from other drainages in the region (*e.g.,*Ogooué & Congo rivers). Measurements are modified from Howes and Teugels (1989) and Stiassny *et al.* (2006) with the addition of several fin-length measurements. A Principal Components Analysis (PCA) on 18 log-transformed measurements using the covariance matrix was performed in MYSTAT and visualized in CorelDraw. Specimens included in the study are listed in the materials examined, including uncataloged material designated by field number, institutional abbreviations follow Sabaj (2020).

Results

A phylogeny inferred from partial CO1 (651 bp) from 75 taxa, including 30 sequences produced in this study, recovered *O. ubangiense* as paraphyletic. *Opsaridium ubangiense* from the type locality (Ubangi River) grouped with specimens from the Ituri and Epulu Rivers (Congo River Drainage) (Fig. 2). These populations are sister to a specimen in the Dja River (Congo River). In contrast, *O. ubangiense* populations from lower Guinea (Ntem, Mbini, Ogooué, Komo, Mbei, Kouilou, Nyanga, and smaller coastal rivers in Equatorial Guinea) clustered separately and sister to *O. zambezense* (Peters) and *O. boweni* (Fowler). These lower Guinea populations were highly divergent from Ubangi/Congo (12–13% in CO1; Supplemental table 2).

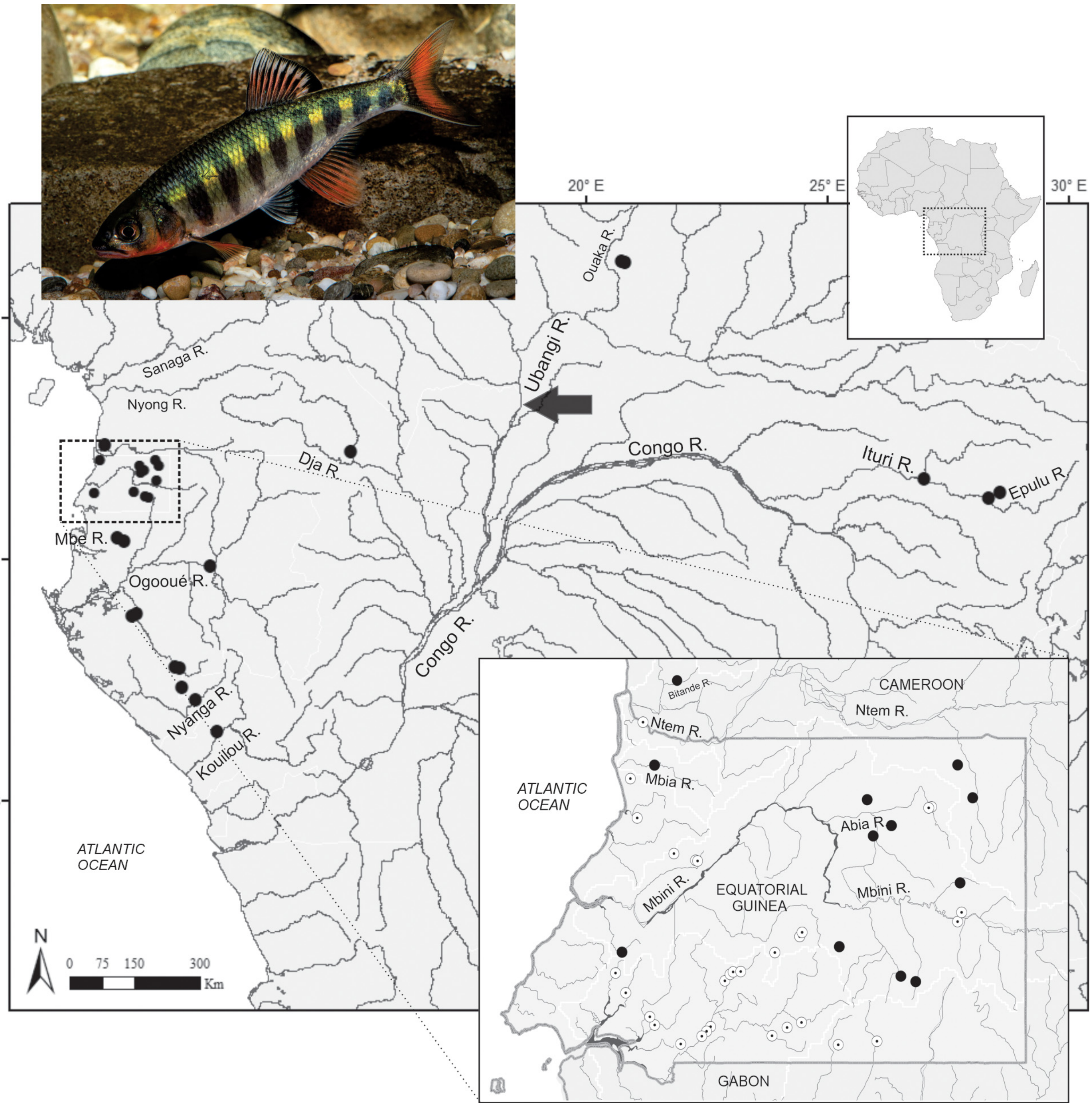

FIGURE 1. Localities of *Opsaridium ubangiense* included in the analyses. Note that the Ubangi River basin is the type locality for *O. ubangiense* (indicated by arrow). Open circles within Equatorial Guinea are collection events that didn't yield *Opsaridium ubangiense*. Inset picture of *Opsaridium ubangiense* from the Sognok River, a tributary to the Sanaga River, in Cameroon. Photograph by M.C.W. Keijman.

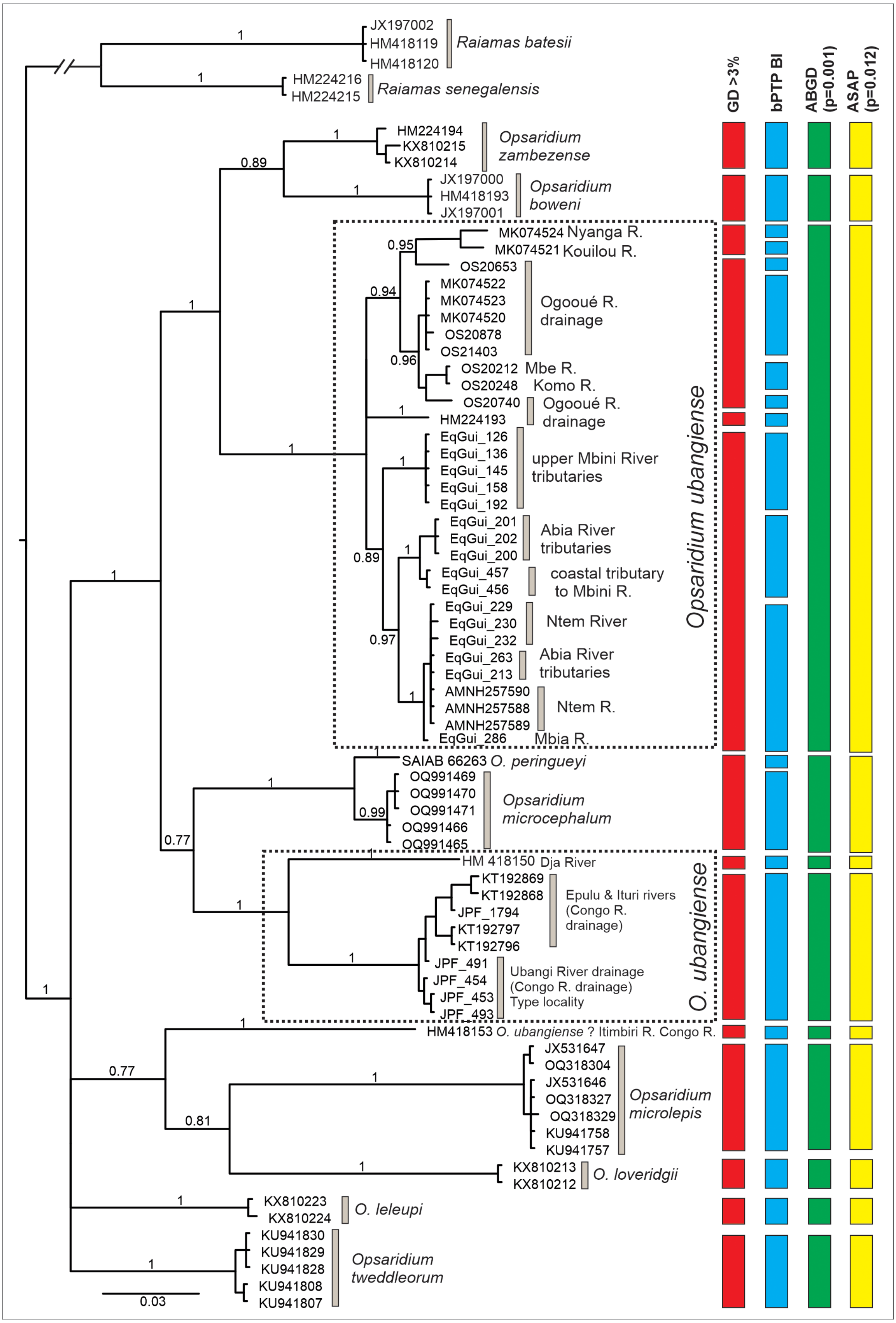

FIGURE 2. Phylogeny inferred from partial CO1 (651 bp) from 75 specimens. Branch support values are posterior probabilities from the Bayesian Inference analysis. Groups identified by different delimitation methods are shown on the left of the tree. All specimens currently considered *O. ubangiense* are enclosed in dashed box. Metadata for specimens included in this analysis can be found in supplemental table 1.

Within the Lower Guinea populations of *O. ubangiense*, further structure was evident. Populations from the Ogooué, Komo, Mbe, Louetsie, Nyanga, and Kouilou rivers formed one clade, distinct from those of the Ntem, Mbini, Abia, Mbia, and nearby coastal tributaries in Equatorial Guinea (Fig. 1). Lastly, within those rivers you see a split between populations in the upper Mbini River and those in the Ntem, Mbia, and Abia rivers and two distinct lineages within the Abia River were separated by ~ 2% at CO1. One of these lineages is closely allied to a small coastal tributary to the Mbini River (Figs. 1 & 2).

The CYTB phylogeny (1044 bp) from 25 taxa, including 20 produced in this study, showed a similar topology although less resolved (Supplemental Figure 1), again deep divergences are observed between the Ubangi River populations and those in lower Guinea (~14%) and within the lower Guinea populations. The two distinct populations in the Abia River are also recovered and have a divergence of 3% in CYTB (Supplementary Table 2).

Nuclear markers showed shallower divergence but supported the Ubangi-Lower Guinea split. The nuclear marker MYH6 (786 bp) produced from 20 specimens show divergence between the Ubangi River populations and those in Lower Guinea (1.3%, supplemental table 2) but did not resolve within Equatorial Guinea structure (Supplemental Figure 3). The GH intron 2 (140 bp) wasn't much more informative than MYH6. There was divergence (1.4-2.1%) between the Ubangi River populations and those in lower Guinea, and some variation with the lower Guinea populations but not congruent with mitochondrial groupings (Supplemental Figure 3). A concatenated data set (2621 bp) from 76 taxa) recovered a topology very similar to the CO1 phylogeny (Supplemental Figure 4) with some branches having higher support within the *O. ubangiense* complex. This analysis further reinforce separation between Ubangi/Congo and lower Guinea populations.

Species delimitation methods varied in their resolution, but all indicated multiple lineages within *O. ubangiense*. The putative number of operational units within *O. ubangiense* varied by the method implemented and ranged from 3 groups in ABGD and ASAP methods to 12 in the bPTP method (Fig. 2). The ABGD and ASAP method delimits three groups within *O. ubangiense*; the lower Guinea populations, the specimen in the Dja River, and topotypic Ubangi River and Congo River drainages. The GD method follows a similar pattern as the ABGD methods but further distinguishes the lower Guinea populations in the upper lower Guinea (*e.g.,*Mbini and Ntem Rivers), those drainages further south (*e.g.,*Ogooué and Komo rivers), and lastly the Wyanga and Niari River specimens. The bPTP method delimited 12 groups within *O. ubangiense* following patterns previously noted but further splitting the lower guinea populations (Fig. 2). Notably, the bPTP differentiates the upper Mbini River tributaries from one population in the Abia River and a coastal tributary of the Mbini River. This analysis also distinguishes the Ntem River, Mbia River, and other Abia River populations from the other populations in Equatorial Guinea. The bPTP analysis also further distinguishes two populations in the Ogooué River, the Mbe and Komo Rivers, and populations from the coastal rivers in the Republic of the Congo (Fig. 2).

Preliminary morphological analysis of 50 specimens, conducted before the genetic analyses, also revealed differences among populations (Fig. 3). A PCA of morphometric data (PC2 vs. PC3 separated Mbini River specimens and those in the Ubangi River drainage. Eye length, snout length, upper jaw length, lower jaw length, and anal-fin length, contributed most to variation observed in PC2. Caudal peduncle length and the post-orbital length of the head contributed most to the variation observed along PC3. Although ongoing analyses may refine these patterns, the results suggest morphological differentiation congruent with some of the genetic groupings.

Discussion

Our molecular analyses, and four different species delimitation methods, suggest the presence of hidden diversity within what is is currently considered *Opsaridium ubangiense* (Fig. 2). Even the most conservative species delimitation results (ABGD and ASAP) suggests three lineages within *O. ubangiense*. One being topotypic populations in the Ubangi River basin and Epulu/Ituri River in the Congo River drainage. The second from the Dja River and the third from the coastal river drainages in the Lower Guinea province (*e.g.,*Ntem, Mbini, Ogooué, Mbe, Komo, Wyanga, and Niari rivers). Other methods recover finer-scale partitions within Lower Guinea, indicating additional hidden diversity, though not always with consistent groupings. The distinction between the Dja River populations and those in the Ubangi River basin and the Epulu/Ituri River provide additional evidence for a hypothesized river capture event of a headwater stream of the Nyong by the Dja River. (Roberts 1975; Lévêque 1997, Fig. 1). A distinct population of *Distichodus notospilus* Günther was also found to occur in the Dja River (Schmidt *et al.* 2021).

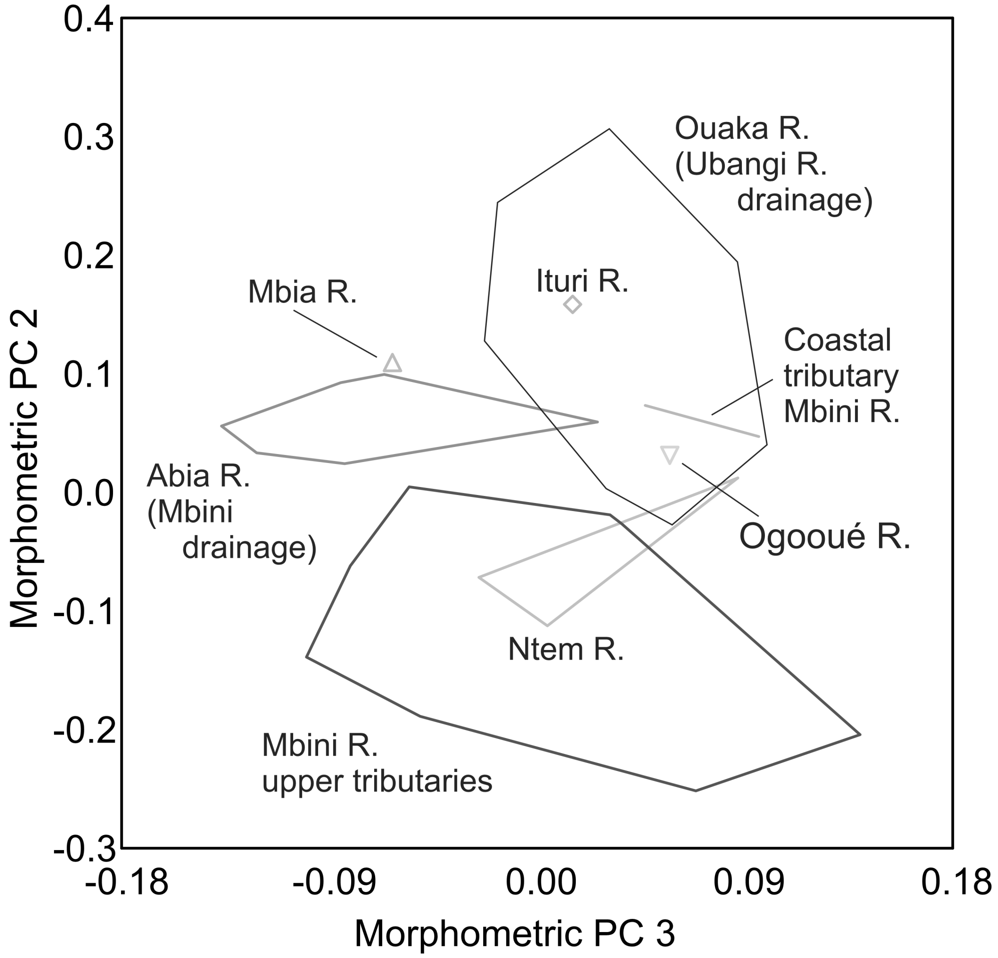

FIGURE 3. Scatter plot of PC2 vs. PC3 from principal components analysis of 18 log-transformed linear measurements from 50 specimens.

Within Equatorial Guinea, the interesting patterns of divergence highlight the biogeographic complexity of individual drainages. Within the Mbini River drainage, we observed divergences between populations in the Abia River, a northern tributary of the Mbini River, and those populations collected in southern tributaries to the Mbini River, with one population in the Abia River allied with populations from a tributary much closer to the coast. We also detected two divergent lineages within the Abia itself (2–3% divergence), suggesting the presence of more undescribed taxa. The historical drainage evolution of the Abia River may contribute to the uniqueness of the taxa found there. The elbow-shaped bend in the upper Abia River could suggest the capture of a southern tributary of the Ntem River that used to flow to the north. (Fig.1). This could explain the similarities between some Abia River populations and those in the Ntem River (Fig. 2). Similar cross-basin connections have been observed in other fishes (*e.g. Distichodus microps* Schmidt *et al.* 2021), underscoring the dynamic role of river history in shaping diversity. More taxa from these rivers need to be examined to better understand the biogeography of the fishes in the region.

A more detailed morphological assessment is critical and underway to determine which lineages merit formal recognition (*e.g.* Dja River, Ubangi/Congo River, Ogooué River, Niari, etc..). The combination of deep genetic divergence and preliminary morphological differences suggests that at least three species will be recognized within what is currently treated as *O. ubangiense*. Additional populations from Cameroon may also reveal further diversity within the group. Recent collections in coastal rivers in Cameroon discovered *Opsaridium ubangiense* populations that have some clear morphological differences (Keijman pers comm, Fig. 1).

Taken together, these findings reinforce the view that the Lower Guinea province remains a hotspot of hidden freshwater diversity. Far from being a uniform widespread species, *O. ubangiense* exemplifies how unrecognized lineages persist under a single name. Continued integrative taxonomic work in the region is likely to uncover further undescribed species, many of which may be endemics restricted to single rivers, with direct implications for conservation and management.

Material Examined

CUMV 80353: 1, Bambomo Creek on the road from Lebamba to Memba; -2.163611, 11.461667; 24 Jul 1999; Hopkins, Arnegard, and Sullivan. CUMV 79917: 2, Gabon, Ogooué River drainage, Bambomo Creek on the road from Lebamba to Memba; -2.163611, 11.461667; 24 Jul 1999; Hopkins, Arnegard, and Sullivan. CUMV 92666: 47, Central African Republic, Ubangi River drainage, Nguétépé Stream within AXMIN Ndassima mine concession area; 6.1854, 20.7395992, 23 Feb 2006, Bills, Friel, and Reid. CUMV 92767: 2, Central African Republic, Ubangi River drainage, Mbourou River at AXMIN Ndassima Camp; 6.15880001, 20.8008995; 23 Feb 2006; Bills, Friel, and Reid. CUMV 96674: 1, Democratic Replubic of the Congo, Congo River drainage, Tshopo, Small forest stream (Ruisseau Mayi Moja); 1.389723, 28.573342; 18 Jul 2010; Thumitho, Walanga, and fishermen. RCS 1716: 38, Rio Mbini drainage, Rio Chiguo on Evinayong—Aconibe Rd.; 1.41795, 10.62607; 23 Jun 2017; Schmidt, Barrientos, and others. RCS 1725: 2, Ntem River drainage, Rio Kwe; 2.0763, 11.07675; 28 Jun 2017; Schmidt, Barrientos, and others. RCS 1728: 23, Rio Mbini drainage, Tributary to Rio Binville near Nsamonkeing; 1.95036, 10.73247; 29 Jun 2017; Schmidt, Barrientos, and others. RCS 1730: 1, Rio Mbia drainage; Rio Mbia at Menzong; 2.07529, 9.92378; 30 Jun 2017; Schmidt, Barrientos, and others.

Supplementary materials

The supplementary tables and figures are available for download at 10.6084/m9.figshare.30086260.

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Supplementary Materials. The following supporting information can be downloaded at the DOI landing page of this paper.

Supplementary Table 1. Locality, catalog/field numbers, and GenBank accession numbers for specimens included in the analyses.

Supplementary Table 2. Uncorrected pairwise distance of CO1, CYTB, MYH6, and GH intron 2 among populations included in this study.

Supplementary Figure 1. Gene tree inferred from partial CYTB (1044 bp) from 25 taxa. Branch support values are posterior probabilities from the Bayesian Inference analysis. Metadata for specimens included in this analysis can be found in Supplemental Table 1.

Supplementary Figure 2. Unrooted phylogeny inferred from partial MYH6 (786 bp) from 19 specimens. Branch support values are posterior probabilities from the Bayesian Inference analysis. Metadata for specimens included in this analysis can be found in Supplemental Table 1.

Supplementary Figure 3. Unrooted phylogeny inferred from GH intron 2 (146 bp) from 23 specimens. Branch support values are posterior probabilities from the Bayesian Inference analysis. Metadata for specimens included in this analysis can be found in Supplemental Table 1.

Supplementary Figure 4. Phylogeny inferred from concatenated CO1, CYTB, MYH6, and GH (2621 bp) from 76 taxa. Branch support values are posterior probabilities from the Bayesian Inference analysis. Metadata for specimens included in this analysis can be found in Supplemental Table 1.