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Phylogenetic Analyses of Cyprinid Species from the Rokel River Basin of Sierra Leone, West Africa: Taxonomic, Biogeographic, and Conservation Implications

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Abstract: The Rokel River (RR) basin is one of the most neglected ichthyofaunal basins, despite the potential for undetected diversity and high levels of endemism. Data on the molecular phylogeny of freshwater fish from this river are rare. Morphological features alone are inadequate for precise species identification. Here, a phylogenetic analysis performed based on the mtDNA *Cytb* gene for eleven cyprinid fish from the RR basin recovered eleven distinct lineages. The same was also observed for two of our species delineation analyses, of which four are identical to six morphospecies, one is of taxonomic uncertainty, and the rest are currently unrecognized. The disjunct distribution found here in some cyprinid species from the RR basin and their sister species suggests that this river had a past complex historical inter-basin connection exchange with the nearby river basins of the Zaire and lower Guinean ecoregions. The unrecognized diversity observed from cyprinid species of this area may have significant implications for the conservation of biodiversity.

Keywords: fish faunal exchange; unrecognized diversity; *Cytb*; disjunct distribution; taxonomic uncertainty

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1. Introduction

The Rokel River (RR) is positioned in the northern portion of Sierra Leone with an elevation between 950 m in the northeastern highlands and less than 7 m in the western coastal lowlands [1,2]. It has its source from the Fouta Djallon Highlands in the upper Guinean ecoregion. This river is a hotspot of fish diversity and endemism [1,3,4], as it harbors a total of about 72 freshwater fish, 17 of which are endemic to this river [1,3]. The high rate of endemism would have been facilitated by vicariant events that allowed geodispersal and successive divergence from ancestral populations [5–9]. The ancient connections of many of these smaller coastal rivers or streams in the Guinean ecoregions remain unclear, however recent studies from neighboring countries are beginning to offer some insight into the evolution of these drainages [9–12].

Fish species' identification from the RR basin, like all other freshwater systems in Sierra Leone, is poorly explored. The available checklist of freshwater fish of this river basin and the entire country is based on literature records [1,3]. Furthermore, existing morphological and molecular data were combined to decipher unappreciated diversity, which has contributed to the discovery of new species and the re-identification of formerly described species [10–12]. Phylogenetic analyses of freshwater fish of this area and the entire country have not been carried out.

Previously, molecular work from neighboring basins has shown that many currently identified widespread species in the Guinean ecoregions consist of individuals from genetically divergent lineages [9,13–15]. It has been shown that the morphological approach alone

is insufficient to uncover species of the cyprinid genus *Enteromius* [13,14]. An integrative taxonomy approach is essential to understand fish species diversity and endemism of poorly sampled rivers of the upper Guinean ecoregion, particularly the RR basin [10–16].

Considering this knowledge gap, there is a critical need for a better understanding of freshwater fish of the RR basin. Documentation of species diversity is vital for fish conservation in this river and could also help in the sustainable management of freshwater systems in the country. The current conservation strategy plan for the upper Guinean Rain Forest focuses on the terrestrial environment, including birds, plants, amphibians, and chimpanzees, with little or no attention on its aquatic systems, particularly on fish species [17]. Certainly, documentation of unknown diversity such as in this study is a key to achieve conservation goals [17].

The present view of diversification within the freshwater system of the RR basin or upper Guinea ecoregion is mainly centered on faunal records [1,4]. These faunal records were a compounding of diverse collections from checklists of individual drainages in the area [3,4] and the Checklist of the Freshwater Fish of Africa [3]. Despite these checklists demonstrating large progress in the knowledge of freshwater fish taxonomy of the Rokel River basin or the upper Guinea ecoregion, they no longer sufficiently define the diversity and distribution of species within the RR basin or the entire country. From the late 1990s to the present, 72 species of fish have been recognized across this area [1,3,4].

The Cyprinidae, with 13 currently recognized species, is the second dominant component of the fish fauna of the RR basin, accounting for 18.1% of the total number of species in this river. Among them, there are two regional endemic species: *Leptocypris guineensis,* Daget 1962 and *Prolabeo batesi,* Norman 1932. The remaining species are widely regarded as "amphi-Guinean", occupying freshwater systems in the lower and upper Guinean ecoregion or even more broadly throughout West Africa [1,3]. However, the taxonomic status of some currently recognized morphology-based species, particularly widespread species, of the upper Guinea ecoregion needs to be scrutinized using an integrated taxonomy approach.

This study represents the first step in this process, being the first molecular assessment of the RR basin to determine the level of genetic distinctiveness of these populations to ascertain if impending concerns with their existing taxonomic status can be identified.

2. Material and Methods

2.1. Study Area

The Rokel River basin drains the uplands of northern Sierra Leone at altitudes of 300–400 m, where the coastal plain borders the Guinea Plateau, and drains about 300 km into the Atlantic through the estuarine Sierra Leone River (Figure 1). Specimens were caught from different portions within the RR basin (Figure 1) by monofilament gill nets with mesh sizes of 10–35 mm, seine, cast net, and fish traps. Samples were preserved in 95% ethanol and other specimens were fixed in 10% formalin and stored in 10%, 50%, to 70% ethanol for prolonged storage. Preliminary species' identification was based on morphology. Samples were assigned to five genera: *Enteromius* spp., *Labeo* spp., *Labeobarbus* spp., *Raiamas* spp., and *Prolabeo batesi*.

All collected specimens are deposited in the Museum of Aquatic Organism at the Institute of Hydrobiology (IHB), Chinese Academy of Sciences, Wuhan city, Hubei Province. The "cf." was used in this study to describe species whose identity was uncertain. The "aff" was used in this study to describe species whose identity was uncertain, and it was classified as inquirenda to indicate the need for further taxonomic review to confirm its taxonomic validity, following the definition of [18].

Total DNA was extracted from ethanol-stored fin tissue using a TIANamp Genomic DNA Kit (Tiangen Biotech, Beijing, China). A single fragment of the mitochondrial cytochrome b (*Cytb*) gene was utilized for phylogenetic analysis. This gene was amplified by a PCR reaction, using universal fish DNA primers LA-CYTB (Yang)/HA-CYTB (Yang), following published protocols [19,20]. All sequences generated from this study were deposited in GenBank.

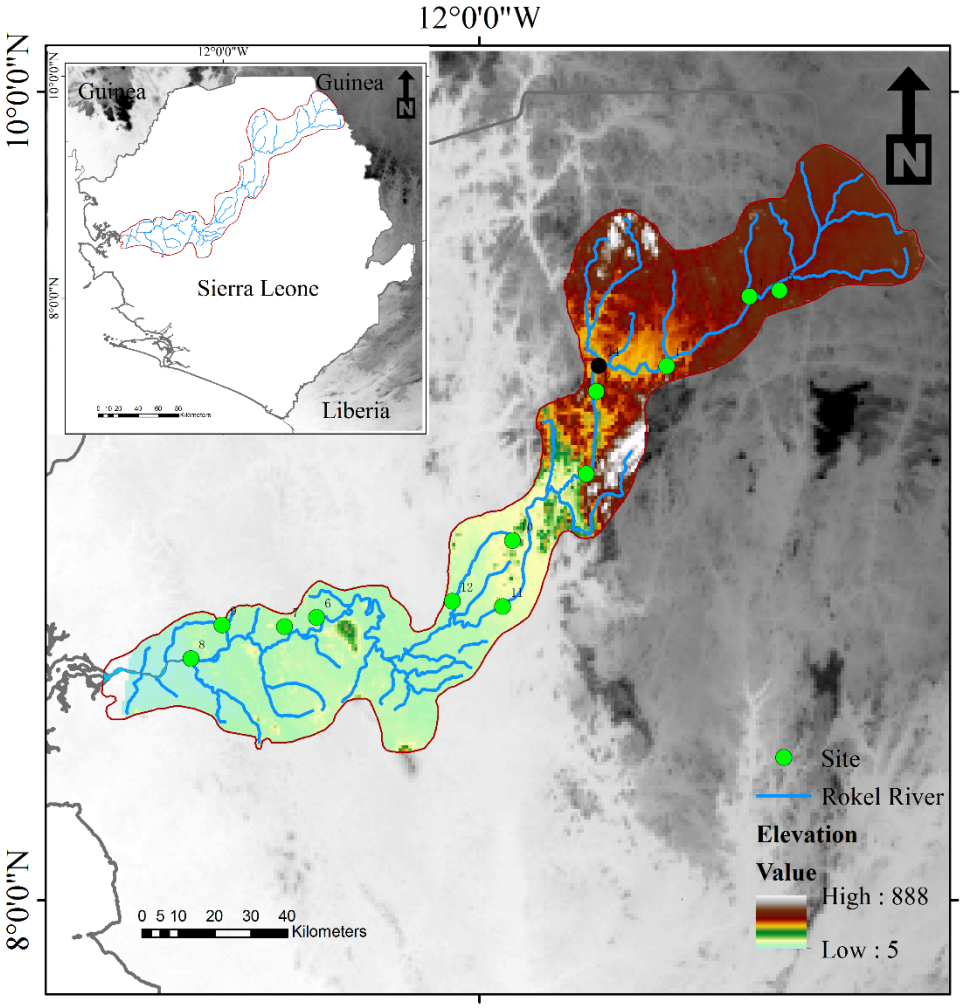

Figure 1. Distributions of sampled specimens of fish species from the Rokel River basin. The green dots show the sampling site, and the black dot is the position of the Bumbuna hydro-dam.

2.2. Data Analysis

The DNA analysis included published cytochrome b (*Cytb*) data from other cyprinids in the area [9,20] and newly amplified *Cytb* gene sequences markers. Sequences were cleaned, aligned, and trimmed to equal lengths utilizing the following programs: MAFFT [21] and AliView [22]. Amino acid sequences were inspected to ascertain that there were no stop codons present. Datasets were examined for redundancy and saturation using DAMBE v7.0.35 [23]. Individual haplotypes were determined by DnaSP v5 [24].

The suitable models of sequence evolution for each dataset were selected under Akaike's Information Criterion (AIC) [25]. RAxML v8.2 [26] was utilized for Maximum Likelihood (ML) analysis, with model substitution rates from Modeltest applied [27]. M_RB_{AYES} 3.1.1 [28] was utilized for Bayesian Inference (BI) analysis with the best-fit model for each partition selected by Partionfinder2 in PhyloSuite [21]. Minimum and maximum pairwise differences were estimated in MEGA 7 [29] using the Kimura 2-parameter model, and partial deletion of missing data (90% site coverage cut-off). Species, sampling locality, voucher numbers, and GenBank accession numbers used for phylogenetic analysis are listed in Table 1.

Table 1. Sequences used for this study and published congeneric sequences from West Africa or Africa ecoregions.

Species	Specimens ID	Countries	Locality/River	GenBank No.	Year Collected
Enteromius anema	AUF5493	Guinea	Bafing	MF135226.1	Hayes and Armbruster 2017
Enteromius anema	AUF5494	Guinea	Bafing	MF135225.1	
Enteromius ablabes	Unknown	Ivory Coast	Agnebi	AF180835.1	Tsigenopoulos et al., 1999
Enteromius macrops	AUF 5524	Guinea	Forécariah	MF135212.1	
Enteromius macrops	Gui048	Guinea	Forécariah	MF135201.1	
Enteromius macrops	Gui0237	Guinea	Forécariah	MF135200.1	
Enteromius macrops	AUF5481	Guinea	Safa-Khoure	MF135210.1	
Enteromius macrops		Guinea	Koumba	MF135203.1	
Enteromius macrops		Guinea	Koumba	MF135202.1	
Enteromius macrops	AUF5454	Guinea	Tinkisso	MF135204.1	
Enteromius macrops		Guinea	Tinkisso	MF135205.1	
Enteromius macrops		Guinea	Kolenté	MF135208.1	
Enteromius macrops	AUF5476	Guinea	Kolenté	MF135206.1	
Enteromius macrops		Guinea	Kolenté	MF135209.1	
Enteromius macrops		Guinea	Kolenté	MF135207.1	
Enteromius macrops		Guinea	Konkouré	AF180832.1	
Enteromius macrops		Guinea	Doulou	MF135211.1	
Enteromius camptacanthus	Unknown	Ghana	Lake Volta	KF791270.1	
Enteromius anema	Unknown	Nilo-Sudan	Blue Nile	KP712159.1	
Enteromius guildi	AUF5505	Guinea	Zie	MF135218.1	
Enteromius cadenati	Unknown	Sierra Leone	Taia/Jong	AF180834.1	
Enteromius cadenati	AUF5364	Guinea	Dimmah	MF135224.1	
Enteromius liberiensis	AUF5483	Guinea	Safa-Khoure	MF135213.1	
Enteromius bigornei	MNCN: 46CK	Guinea	Kaba, Kouloundela	AY004752.1	Machordom and Doadrio 2001
Enteromius aspilus	Unknown	Guinea	Konkouré	KF791275.1	Schmidt et al., 2019
Enteromius foutensis	Gui0858	Guinea	Little Scarcies	MK329230.1	
Enteromius foutensis	Gui0146	Guinea	Little Scarcies	MK329231.1	
Enteromius foutensis	Gui0167	Guinea	Little Scarcies	MF135220.1	
Enteromius foutensis	Gui0168	Guinea	Little Scarcies	MF135219.1	
Enteromius foutensis	Gui3435	Guinea	Konkouré	MK329229.1	
Enteromius foutensis	Gui3494	Guinea	Konkouré	MK329228.1	
Enteromius foutensis	Gui0145	Guinea	Konkouré	MK329227.1	
Enteromius foutensis	Gui_0206	Guinea	Konkouré	MK329233.1	
Enteromius foutensis	Gui0146	Guinea	Konkouré	MK329226.1	
Enteromius foutensis	Gui 0146	Guinea	Konkouré	MK329225.1	
Enteromius foutensis	Gui_0204	Guinea	Konkouré	MK329232.1	
Enteromius foutensis	Gui 0167	Guinea	Konkouré	MK329224.1	
Enteromius foutensis	Gui 0018	Senegal	Gambie/Senegali	MK329241.1	
Enteromius foutensis	Gui 0133	Senegal	Gambie/Senegali	MK329240.1	
Enteromius cf. *guildi*	AUF5443	Guinea	Bafing	MF135223.1	Hayes and Armbruster 2017
Enteromius ablabes	AUF5431	Guinea	Bafing	MF135227.1	
Enteromius ablabes	AUF5441	Guinea	Bafing	MF135228.1	
Enteromius trispilos	AUF5496	Guinea	Mia	MF135193.1	Hayes and Armbruster 2017
Enteromius trispilos	AUF5498	Guinea	Mia	MF135194	
Enteromius anema	AUF5493	Guinea	Mia	MF135225.1	
Enteromius anema	AUF5494	Guinea	Mia	MF135226.1	
Enteromius huguenyi	AUF5589	Guinea	Masseni	MF135214.1	

Table 1. *Cont.*

Species	Specimens ID	Countries	Locality/River	GenBank No.	Year Collected
Enteromius punctitaeniatus	AUF5610	Guinea	Mafou	MF135199.1	
Enteromius profundus	DNG_PROF2_2	Kenya	Kisumu	MH484558.1	Ndeda, Mateos, and Hurtado 2018
Enteromius profundus	DNG_PROF4_4	Kenya	Kisumu	MH484556.1	
Enteromius callipterus	Unknown	Gabon	Loa -Loa	AP009313.1	Saitoh 2006
Enteromius callipterus	CBM-ZF-11498	Gabon	Loa -Loa	KP712230.1	
Labeobarbus sacratus	MNCN 4CK	Guinea	Tangala	AF287445.1	Tsigenopoulos, Naran, and Berrebi 1999
Enteromius tiekoroi	UAIC14166.05	Sierra Leone	Mao	KP659410.1	Yang et al., 2015
Labeobarbus sacratus		Guinea	Tangala	AF180868.1	Tsigenopoulos, Naran, and Berrebi 1999
Labeobarbus sacratus				AF287445.1	
Labeobarbus wurtzi	MNCN 92CK	Guinea	Kouloundela	AF287448.1	
Labeobarbus wurtzi	MNCN 91CK	Guinea	Kaba,	AF180864.1	
Labeo forskalii	CU 94562	Ethiopia	Alwero	JX074287.1	Yang and Mayden 2012
Labeo forskalii	UAIC14744.4	Ethiopia	Alwero	FJ196833.1	Beshera and Phillip 2019
Labeobarbus cyclorhynchus	CBM ZF 11452			AP011359.1	Miya 2009
Labeo forskalii	AAU:0512009	Ethiopia	Alwero	FJ196831.1	Tang, Getahun, and Liu 2009
Labeo lukulae		DRC	Lukula	JX097084.1	Hirt 2012
Labeo parvus	BMNH:2006.3.7.1	Benin	bei Malauville	JX074292.1	Yang and Mayden 2012
Labeo parvus	CBM: ZF: 12695	Ethiopia	Alwero	AP013339.1	Beshera and Phillip 2019
Labeo parvus		Ethiopia	Baro	JX074285.1	Yang and Mayden 2012
Labeo parvus		Ethiopia	Baro	JX074286.1	
Raiamas senegalensis		Benin	Iguidi	AP010780.1	Saitoh et al., 2008
Raiamas senegalensis				HM224332.1	
Labeo horie		Ethiopia	Alwero	JX074288.1	
Labeo nasus		DRC	Congo Basin	AP013333.1	Miya 2013
Labeo lineatus				AP012154.1	
Labeo altivelis				AP013322.1	Miya 2011
Labeo coubie		Nigeria	Niger Basin	AP012149.1	
Labeo coubie				JX074261.1	
Labeo altivelis				JX074228.1	
Raiamas steindachneri		N/A		AP012113.1	
Raiamas cf. *steindachneri*	IHB29666	Sierra Leone	Rokel/Seli/upper	MW660585	This study
Raiamas aff. *scarciensis*	IHB29555	Sierra Leone	Rokel/lower	MW660586	This study
Labeo aff. *coubie*	IHB29688	Sierra Leone	Rokel/Seli/upper	MW660599	This study
Labeo aff. *parvus*	IHB29699	Sierra Leone	Rokel/Seli/upper	MW660600	This study

Table 1. *Cont.*

Species	Specimens ID	Countries	Locality/River	GenBank No.	Year Collected
Labeo aff. *parvus*	IHB29799	Sierra Leone	Rokel/Seli/upper	MW660601	This study
Labeobarbus wurtzi	IHB29999	Sierra Leone	Rokel/Seli/lower	MW660597	This study
Labeobarbus wurtzi	IHB29999B	Sierra Leone	Rokel/Seli/lower	MW660597B	This study
Labeobarbus sacratus	IHB29898	Sierra Leone	Rokel/Seli/upper	MW660598	This study
Enteromius aff. *foutensis*	IHB29317	Sierra Leone	Rokel/Seli/upper	MW660590	This study
Enteromius aff. *foutensis*	IHB29318	Sierra Leone	Rokel/Seli/upper	MW660591	This study
Enteromius aff. *foutensis*	IHB29319	Sierra Leone	Rokel/Seli/upper	MW660592	This study
Enteromius aff. *foutensis*	IHB29320	Sierra Leone	Rokel/Seli/upper	MW660593	This study
Enteromius aff. *foutensis*	IHB29444	Sierra Leone	Rokel/Seli/upper	MW660594	This study
Enteromius aff. *liberiensis*	IHB29241	Sierra Leone	Rokel/Seli/upper	MW660587	This study
Enteromius aff. *liberiensis*	IHB29362	Sierra Leone	Rokel/Seli/upper	MW660589	This study
Enteromius aff. *liberiensis*	IHB29242	Sierra Leone	Rokel/Seli/upper	MW660588	This study
Enteromius aff. *macrops*	IHB29355	Sierra Leone	Rokel/Seli/upper	MW660595	This study
Enteromius aff. *macrops*	IHB29610	Sierra Leone	Rokel/Seli/upper	MW660596	This study
Enteromius aff. *ablabes*	IHB29544	Sierra Leone	Rokel/Seli/upper	MZ013921	This study
Enteromius aff. *ablabes*	IHB29545	Sierra Leone	Rokel/Seli/upper	MZ013922	This study
Prolabeo batesi	IHB29377	Sierra Leone	Rokel/lower	MZ013919	This study
Prolabeo batesi	IHB29399	Sierra Leone	Rokel/lower	MZ013920	This study
Paracanthocobitis zonalternans		Asia		MK608087.1	Slechtova and Dvorak 2019
Paracanthocobitis mockenziei				MK608121.1	

2.3. Phylogenetic Analysis

An alignment of partial Cytb (1052 bp) from 101 specimens was included in the analysis. This included 22 specimens from *Enteromius*, 3 specimens of *Labeo*, 3 specimens of *Labeobarbus*, 2 specimens from *Raiamas*, and 2 specimens of *Prolabeo* from the RR basin, and published congeneric species from West Africa or Africa.

Phylogenetic relationships among morphospecies between groups were inferred using IQ-Tree 2.1.2 [21]. The analysis for each group had two replicate searches, six million generations, with four Markov chains. Trees were sampled every 1000 generations to obtain 10,000 sampled trees. We discarded 25% of the sampled trees as burn-in, and the remainder were used to estimate the consensus tree and Bayesian posterior probabilities (PP). The average standard deviation of split frequencies and the potential scale reduction factor were estimated. The values depicted are a posteriori probability for BI/ML. Posteriori probability was based on nodal support for BI/ML trees. Model-corrected genetic distances between unique lineages recognized for each genus were estimated using MEGA7 [29]. To discover the probable taxonomic individuality of the genetic lineages that will be unveiled from the RR basin, sequences of topotypes (i.e., published sequences in the upper Guinea Province of West/West-Central Africa of formerly described species) were included in the analysis. Mean distances within and between species were computed for two of the

better-performing versions of each species delimitation method described below: ASAP (recursive partitioning) and bPTP (maximum likelihood).

2.4. Species Delineation

The ASAP analysis using the online server (https://bioinfo.mnhn.fr/abi/public/asap (accessed on 15 December 2021)) was performed to divide the group into hypothetical species based on the genetic distance, which can be observed whenever the divergence among populations that belonged to the same species is smaller than the divergence among populations from different species. The coalescent clustering-based method (bPTP) was performed using the online server (https://species.h-its.org/ (accessed on 15 December 2021)) and the Bayesian Inference trees from MrBayes 3.1.1 [28]. We ran bPTP analyses for 500,000 MCMC generations, with a thinning of 500 and a burn-in of 0.1. Convergence of the MCMC chain was assessed as recommended by Zhang et al. [21,27]. Outgroups were pruned before conducting bPTP analyses to avoid bias that may arise if some of the outgroup taxa were too distantly related to the ingroup taxa. The phylogenetic trees were visualized and edited in FigTree v.1.4.3 (Institute for Evolutionary Biology | Centre for Infection, Immunity & Evolution Ashworth Laboratories, University of Edinburgh, Edinburgh, EH9 3FL, UK), Adobe Illustrator CS6, and Adobe Photoshop CS6 (Adobe Systems Inc., San Jose, CA, USA).

3. Results

Highly supported phylogenies inferred by both BI and ML analysis discovered 11 distinct populations within cyprinids from the RR (Figures 2 and 3).

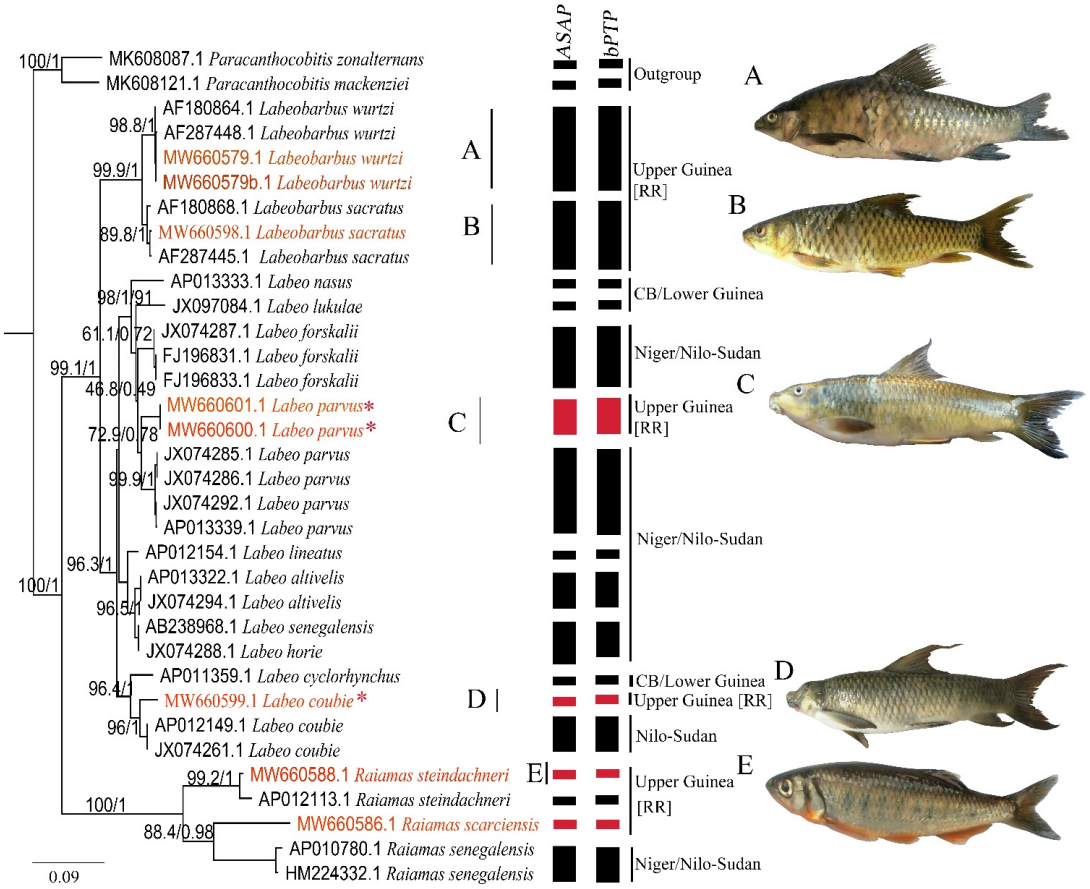

Figure 2. Phylogeny of species of *Labeo*, *Labeobarbus*, and *Raiamas* inferred from partial cytochrome b. Branch support for each node is shown from Bayesian Inference (Lineages A–E). The right vertical bars indicate partitions and final MOTUs from ASAP and bPTP. Note: Species marked with asterisks are considered ambiguous or possible misidentification. Species in red are those from the RR basin.

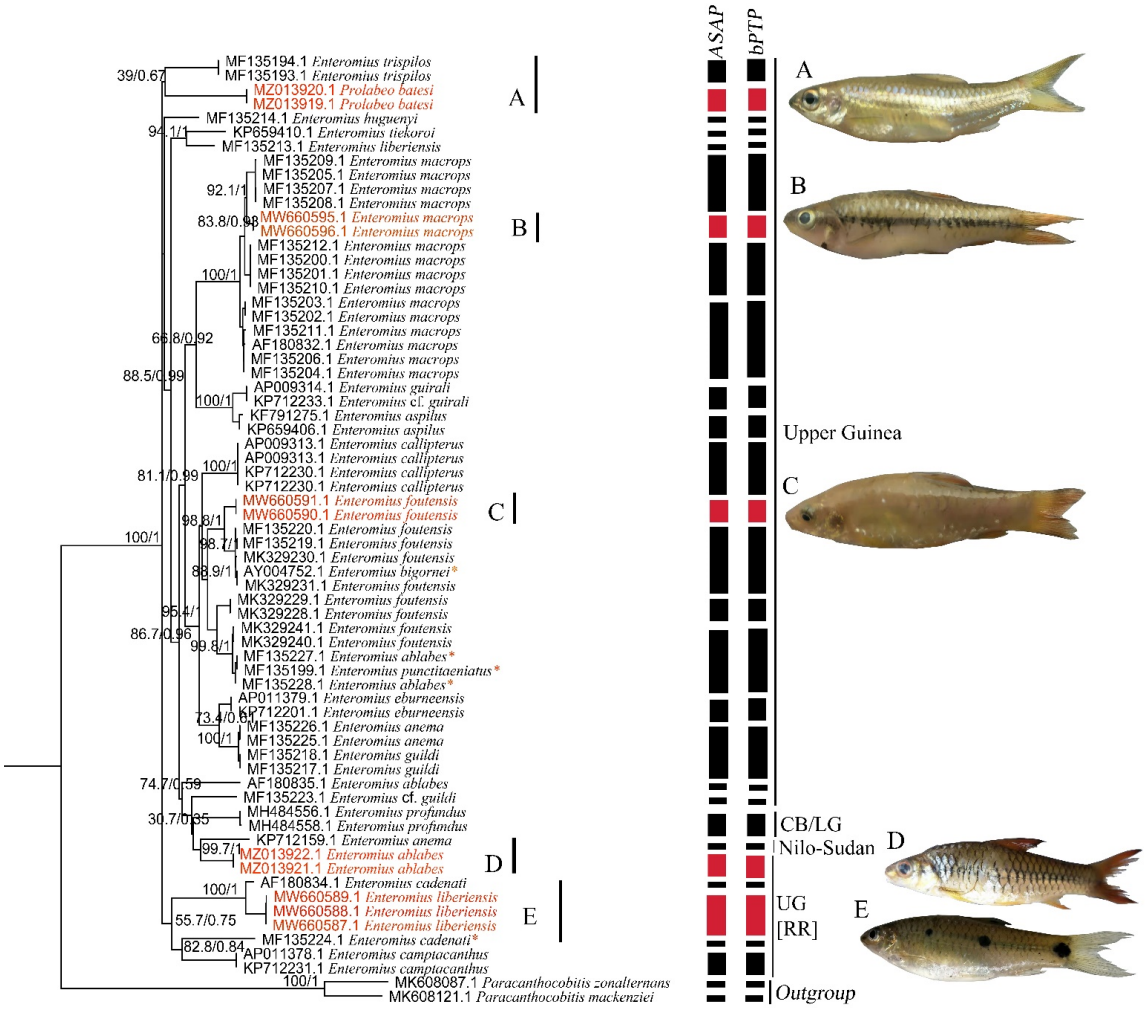

Figure 3. Phylogeny of species of *Enteromius* and *Prolabeo* inferred from partial cytochrome b. Branch support for each node is shown from Bayesian Inference (Lineages A–E). The right vertical bars indicate partitions and final MOTUs from ASAP and bPTP. Note: Species marked with asterisks are considered ambiguous or possible misidentification. Species in red are those from the RR basin.

Phylogenetic analysis of 8 unique haplotypes of *Labeo* (3), *Labeobarbus* (3), and *Raiamas* (2) and 29 published sequences of the same gene from closely allied West and West-Central African species of these 3 genera yielded identical tree topology from ML and BI methods, with most branches receiving strong support. ASAP and bPTP analyses detected six putative species for cyprinids of the RR basin (Figure 3).

Samples identified as *Labeobarbus*, *Labeo*, and *Raiamas* species from the RR basin of Sierra Leone were clustered within four clades (A–E) in the resulting molecular phylogenetic tree (Figure 3). Two species delineation analyses recognized six putative species: *L. coubie*, *L. parvus*, *L. wurtzi*, *L. sacratus*, *R. steindachneri*, and *R. scarciensis*.

Specimens from the RR basin described here as *L. wurtzi* clustered within Clade A, where it grouped with published sequences (AF287445 and AF180868) from the species; in addition, sequences from *L. sacratus* (Clade B) of the RR basin also clustered with published sequences (AF287448 and AF180864) of the species from Guinea (type locality). The genetic divergence between these two species was 5.4% and their intraspecific genetic divergence was 0.6% and 1.3%, respectively (Table 2).

Table 2. Mitochondrial *Cytb* genetic distances between lineages and species of the *Labeo* spp., *Labeobarbus* spp., and *Raiamas* spp., from the RR basin, Sierra Leone, West Africa.

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12
1	*Labeobarbus wurtzi*												
2	*L. sacratus*	0.054											
3	*Labeo forskallii*	0.182	0.157										
4	*L* aff. *parvus*	0.180	0.159	0.080									
5	*L. parvus*	0.177	0.157	0.072	0.071								
6	*L. cyclorhynchus*	0.146	0.147	0.135	0.135	0.113							
7	*L. longipinnis*	0.147	0.154	0.142	0.141	0.137	0.098						
8	*L.* aff. *coubie*	0.161	0.168	0.135	0.143	0.130	0.097	0.068					
9	*L. coubie*	0.147	0.148	0.121	0.131	0.118	0.082	0.065	0.057				
10	*R.* aff. *steindachneri*	0.283	0.268	0.233	0.245	0.238	0.246	0.260	0.257	0.251			
11	*R. steindachneri*	0.275	0.269	0.236	0.245	0.241	0.253	0.262	0.259	0.258	0.037		
12	*R. scarciensis*	0.305	0.289	0.274	0.263	0.276	0.294	0.283	0.297	0.290	0.218	0.233	
13	*R. senegalensis*	0.292	0.284	0.268	0.259	0.263	0.249	0.281	0.260	0.260	0.216	0.229	0.204

Labeo aff. *parvus* of the RR basin was clustered within Clade C, where it formed a strongly supported lineage, being a sister to *L. parvus* of the Nile River basin (type locality), and their genetic distance was 7.1%. Their interspecific genetic distance was 8.0% with *L. parvus* from this river basin.

Sequences of *Labeo* aff. *coubie* were clustered within Clade D, which was a sister to *L. coubie*, a widespread species of West-Central and East Africa (GenBank number: AP012149.1; JX074261.1). Their genetic distance was 5.7%. The paired species nested with *L. cyclorhynchus* of the Ogooué River basin (type locality). The genetic distance of *L.* aff. *coubie* was 9.7% with *L. cyclorhynchus* (Table 2).

Sequences of *Raiamas* from the RR basin were nested within Clade E. The sequence from the species under the name of *R.* cf. *steindachneri* was clustered with *R. scarciensis* from the same river basin as the GenBank-retrieved sequence (AP012113.1) of *R. steindachneri*, without precise sampling location. The genetic divergence between species *Raiamas* from the RR basin was 21.8% (Table 2).

The data matrix of the *Cytb* gene for 11 haplotypes from species of *Enteromius* (9) and *Prolabeo* (2) from the RR and 56 published sequences from closely allied species of *Enteromius* from West and West-Central Africa (including East Africa) were subjected to phylogenetic analyses, with *Paracanthocobitis zonalternans* and *P. mockenziei* from Asia used as the outgroup (Figure 3). Both ML and BI methods yielded identical tree topology, with most branches receiving strong support, and both ASAP and bPTP species delineation analyses recognized five putative species for cyprinids of the RR basin.

Species of *Prolabeo* and *Enteromius* from the RR basin were clustered within five clades (A–E) in the BI and ML trees (Figure 3). The putative species was represented in Clade A by samples of *P. batesi* from the RR basin of Sierra Leone (type locality). Its sister species was *E. trispilos* from the Mia River at Bourata Village (type locality). Their genetic divergence was 16.5% (Table 3).

Among four putative species delineated within clade B, one was represented by samples from *E.* aff. *macrops* of the RR basin. Its sister species was *E. macrops* s.str., from type locality: Kolenté or Tinkisso River. The genetic distance between these paired species was 2.4%. These two populations had an interspecific genetic distance of 2.7% and 3.5% with the other two *E.* aff. *macrops* 1 and 2 (Figure 3) putative species formed, respectively, by GenBank-retrieved sequences (MF135212, MF135200, MF135205, MF135210) and (MF135202, MF135202, MF135211, MF135206, MF135204, AF180832) from samples of Forécariah, Koumba, and Tinkisso River basins, and those from the Konkouré and Doulou River basins (Guinea; type localities).

Table 3. Mitochondrial *Cytb* genetic distances between lineages and species of the Enteromius Species groups from the RR basin, Sierra Leone, West Africa.

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1	*Prolabeo batesi*																						
2	*Enteromius trispilos*	0.155																					
3	*Enteromius cadenati*	0.161	0.157																				
4	*Enteromius* aff. *cadenati*	0.183	0.147	0.165																			
5	*Enteromius* aff. *liberiensis* RR	0.178	0.154	0.182	0.046																		
6	*Enteromius liberiensis*	0.149	0.118	0.159	0.129	0.133																	
7	*Enteromius tiekoroi*	0.161	0.132	0.171	0.145	0.160	0.090																
8	*Enteromius macrops s.str.*	0.156	0.147	0.166	0.173	0.179	0.130	0.134															
9	*Enteromius* aff. *macrops1*	0.150	0.137	0.162	0.158	0.166	0.119	0.132	0.035														
10	*Enteromius* aff. *macrops2*	0.148	0.143	0.159	0.157	0.162	0.120	0.138	0.027	0.027													
11	*Enteromius* aff. *macrops* RR	0.148	0.145	0.169	0.169	0.175	0.121	0.134	0.027	0.029	0.024												
12	*Enteromius anema*	0.150	0.134	0.170	0.145	0.163	0.131	0.139	0.132	0.128	0.136	0.139											
13	*Enteromius guildi*	0.155	0.138	0.171	0.150	0.169	0.135	0.139	0.135	0.129	0.142	0.142	0.006										
14	*Enteromius* aff. *foutensis* RR	0.153	0.148	0.169	0.139	0.156	0.132	0.123	0.126	0.124	0.123	0.123	0.101	0.104									
15	*Enteromius foutensis s.str.*	0.151	0.144	0.167	0.137	0.141	0.128	0.134	0.121	0.118	0.117	0.122	0.105	0.109	0.039								
16	*Enteromius* aff. *foutensis1*	0.162	0.148	0.167	0.143	0.154	0.133	0.126	0.122	0.115	0.117	0.124	0.087	0.089	0.068	0.068							
17	*Enteromius* aff. *foutensis2*	0.155	0.149	0.166	0.157	0.164	0.137	0.125	0.120	0.118	0.117	0.121	0.087	0.089	0.083	0.080	0.051						
18	*Enteromius ablabes*	0.160	0.151	0.163	0.161	0.165	0.137	0.125	0.121	0.120	0.120	0.124	0.094	0.097	0.087	0.080	0.052	0.009					
19	*Enteromius punctitaeniatus*	0.162	0.153	0.165	0.162	0.165	0.137	0.126	0.122	0.121	0.122	0.124	0.096	0.098	0.087	0.081	0.054	0.011	0.001				
20	*Enteromius* aff. *ablabes1*	0.144	0.137	0.134	0.164	0.161	0.134	0.145	0.129	0.128	0.123	0.129	0.137	0.140	0.118	0.125	0.118	0.112	0.112	0.113			
21	*Enteromius profundus*	0.147	0.139	0.162	0.170	0.190	0.131	0.134	0.135	0.131	0.129	0.133	0.127	0.127	0.128	0.128	0.130	0.118	0.125	0.127	0.138		
22	*Enteromius* aff. *anema*	0.147	0.149	0.167	0.151	0.168	0.128	0.132	0.139	0.124	0.135	0.135	0.133	0.134	0.116	0.128	0.120	0.108	0.113	0.115	0.126	0.116	
23	*Enteromius* aff. *ablabes* RR	0.142	0.137	0.156	0.133	0.139	0.106	0.120	0.135	0.130	0.137	0.139	0.118	0.120	0.118	0.113	0.116	0.106	0.104	0.105	0.119	0.101	0.098

Samples of *E. foutensis* from the RR basin represented a putative species (*E.* aff. *foutensis*), one of four putative species delineated within Clade C. Its sister species was formed by samples of *E. foutensis* s.str., and also one sample formerly identified as *E. bigornei*, from the Little Scarcies River basin (type locality). The interspecific genetic distance between *E. foutensis* s.str. and *E.* aff. *foutensis* was 3.9%. The paired species were sisters to the paired species (*E.* aff. *foutensis* of the Konkouré River basin and the Gambie/Senegal River basins), with which *E.* aff. *foutensis* of the RR basin had an interspecific genetic distance of 5.1% and 6.8%, respectively. Nested within *E.* aff. *foutensis* of the Gambie/Senegal River basins were GenBank-retrieved sequences formerly misidentified as *E. punctitaeniatus* (MF135199) and *E. ablabes* (MF135227 and MF135228). Their interspecific genetic distance ranged from 0.01% to 0.09% (Table 3).

Only two putative species were delineated within Clade D. One was formed by samples from *E.* aff. *ablabes* of the RR basin. It was sister to the other represented by the sample from *E. anema* s.str. (type locality: Nile basin). The paired species clustered with *E. profundus* from Lake Victoria into a lineage, being sister to *E.* cf. *guildi* from the Bafing River (at Sokotoro, East of Timbo). The basal placement of Clade D was occupied by *E. ablabes* s.str. from the Agnebi River of the Ivory Coast (type locality). The interspecific genetic distances of *E.* aff. *ablabes* in the RR basin were 9.8% with *E. anema*, 10.1% with *E. profundus*, 14.4% with *E.* cf. *guildi*, and 11.9% with *E. ablabes* s.str. Samples from *E.* aff. *liberiensis* of the RR basin represented a putative species of Clade E (Figure 3), where it stood out as the sister species of *E. cadenati*, endemic to the Pampana/Jong or Taia River basin of Sierra Leone, with their interspecific genetic distance of 4.6%. The true *E. liberiensis* of the Safa-Khoure River was sister to *E. tiekoroi* from Kolenté and Little Scarcies basins. *Enteromius liberiensis* had a distinct genetic distance of 13.3% with *E.* aff. *liberiensis*.

4. Discussion

4.1. Misidentification and Unrecognized Diversity

This study exhibits, to large extent, the misidentification of the currently recognized cyprinids and thus the existence of unappreciated diversity of the RR basin. This river harbors ten species of four genera within the Cyprinidae: *Enteromius* (four), *Labeo* (two), *Labeobarbus* (two), and *Raiamas* (two) [1,3,4]. Our samples of cyprinid fish collected from the RR basin were initially recognized as 11 morphospecies. While *E. leonensis* eluded capture, two other newly recorded species of the river were caught: *Enteromius foutensis* and *Prolabeo batesi*. The *Cytb* gene-based phylogenetic analyses utilizing BI and ML approaches recovered 11 distinct lineages, and species delineation analyses also recognized them as 11 putative species (Figures 2 and 3). The identification of four species (*Labeobarbus wurtzi*, *L. sacratus*, *Prolabeo batesi*, and *Raiamas scarciensis*) is confirmed. *Raiamas steindachneri* is tentatively considered as of taxonomic uncertainty. However, two species of *Labeo* from the RR basin are demonstrated to be currently misidentified, and so are four species of *Enteromius*.

Raiamas steindachneri and *Raiamas scarciensis*, typical species of the upper Guinean ecoregion, occur in the coastal rivers of Guinea, Liberia, and Sierra Leone. The species herein recognized from the RR basin formed two distinct lineages that were delineated as a putative species (Figure 3). These species had a distinct genetic distance of 23.3%, greater than the threshold value (2%), which is the cut-off value commonly utilized to denote intraspecific variation [29,30].

Two currently recognized species of *L. coubie* and *L. parvus* from the RR basin are misidentified. Samples initially referred to as each of both formed a distinct lineage, which was distantly allied to the lineage made up of its topotypical samples in the molecular phylogenetic tree (Figure 2). There is a distinct genetic distance between *L.* aff. *coubie* (RR basin) and *L. coubie* s.str. (6.8%) and *L.* aff. *parvus* (RR basin) and *L. parvus* s.str. (7.1%). Perhaps these two species, i.e., *L.* aff. *coubie* and *L.* aff. *parvus*, represent species of *Labeo curriei*, Fowler 1919 and *L. obscurus*, Pellegrin 1908, originally described from this

area [4]. The taxonomy of these species is not clear, and more specimens and sampling areas are required.

The type locality of *E. macrops* is the Little Scarcies River basin of Sierra Leone. Samples initially identified as this species from the RR basin formed a distinct lineage that was delineated as a putative species (*E.* aff. *macrops*) (Figure 3). Its genetic distance with *E. macrops* s.str. was 2.7%, slightly greater than the 2% threshold (Table 3). Hence, more specimens and the use of the integrative method are essential to determine its taxonomic status. The same holds for the samples initially identified as *E. foutensis* from the river. These samples represent a distinct species (*E.* aff. *foutensis*) due to its monophyletic nature recovered in the *Cytb* gene-based tree (Figure 3) and its significant genetic distance with *E. foutensis* s.str. (3.9%) from the Little Scarcies of Sierra Leone (Table 3).

Samples initially identified as either *E. ablabes* or *E. liberiensis* from the RR basin formed a distinct lineage distantly allied to the lineage made up of its topotypical samples, and two distinct lineages were delineated as two putative species: *E.* aff. *ablabes* and *E.* aff. *liberiensis* (Figure 3). There was a significant genetic distance between *E.* aff. *ablabes* of the RR basin and *E. ablabes* of the Agnebi River basin (type locality) of the Ivory Coast (10.1%), and between *E.* aff. *liberiensis* and *E. liberiensis* from the Safa-Khoure River basin (type locality) of Guinea (13.3%). The genetic distance of *E.* aff. *ablabes* and *E.* aff. *liberiensis*, respectively, with its sister species *E.* aff. *anema* from the Blue Nile River basin and *E. cadenati* from the Pampana/Jong River basin of Sierra Leone, was 9.8% and 4.6%. Based on these molecular data, it can be concluded that *Enteromius* aff. *ablabes* and *Enteromius* aff. *liberiensis* of the RR basin belong to two unnamed species. This study also highlights a need to put the current morphology-based species of *Enteromius* under molecular scrutiny, as indicated in the previous investigation. Type specimens of two new species, *E. alberti* and *E. mimus,* were previously considered conspecific, respectively, with *E. perince* and *E. stigmatopygus* [31]. Decru et al. [13] also reported deep divergence among four morphology-based species of *Enteromius* from the northeastern part of the Congo River basin, and their reported genetic divergence was greater than 5% and even up to 20% between lineages of morphologically similar specimens, clearly surpassing the 2% threshold. Taxonomic revisions of fish in the upper Guinean ecoregion suggest the likely discovery of new species in the Fouta Djallon Highlands. Integrative analyses applied to the African mountain catfish (*Amphilius* spp.) of Fouta Djallon Mountain resulted in the discovery of at least nine new species of *Amphilius* and small barbs [10–12]. Only 11 morphospecies of cyprinid fish from the RR basin were investigated here in a molecular phylogenetic context, but seven of them were shown to be misidentified or of taxonomic uncertainty. If this level of scrutiny is extrapolated to all morphospecies collected from the RR basin, it is likely that new species and even more endemic species may be discovered.

4.2. Genetic Placement of Prolabeo Batesi

Despite the mouth structure and body shape, *Prolabeo batesi* is considered a monotypic genus (i.e., distinct from *Enteromius* and *Labeo*) based on morphological characteristics [1,3,4]. Several characters placed this genus close to *L. wurtzi* [3,4]. This genus has never been compared with large taxa in a molecular phylogenetic-based approach. This is the first molecular analysis of the phylogenetic relationship of *Prolabeo* with others. It is revealed that *Prolabeo* was not recovered as a distinct lineage; instead, it was resolved as the sister species of *Enteromius trispilos* (see Figure 3). According to Paugy et al. [4], *E. trispilos* differs from all other three-spotted barbs by its elongated body, a character very similar to the genus *Prolabeo.* We therefore suggested that *E. trispilos* should be assigned to the genus *Prolabeo*.

4.3. Biogeographic Implication

Biogeographic and phylogenetic relationships of cyprinids from the RR basin with their sister species suggest that the RR had complicated historical inter-basin connections

with other nearby river basins of the upper Guinean and Nilo-Sudan ecoregions, or even with the Congo basin and Lower Guinean ecoregion [8,32].

The past connection of the RR basin with some nearby river basins of the upper Guinean ecoregion is suggested by several paired species. The sister pair *E.* aff. *macrops* and *E. macrops* have an allopatric distribution in the RR basin and the Forécariah basin, and the same pattern is also repeated for the sister pair *E.* aff. *foutensis* of the RR basin and *E. foutensis* of the Little Scarcies River basin. The paired species *E.* aff. *liberiensis* and *E. cadenati* exhibit a disjunct distribution in the RR basin and the Pampana/Jong River basin (Figure 3). All these paired species are usually found in the upper reaches of rivers [10]. It is suggested here that headwater capture may have been the cause of inter-basin transfer, in which one stream cuts through the watershed separating the two basins, allowing the stream that clears the cut to get the stream from the second basin. Fish and other biota from the captured stream are thereby introduced into the stream that makes the capture, establishing new subdivided populations. The watershed of the stream that makes the capture is increased in the area only by the part of the captured watershed above the cut [7–9]. These events possibly occurred during interglacial phases when the forests would have expanded as the weather became wetter. This climatic undulation and the concomitant expansion/contraction of forest cover were probably the main factors leading to the diversity and geodispersal patterns of the biota seen today [5,8,32,33].

In the BI and ML trees (Figure 3), *E.* aff. *ablabes* from the upper reaches of the RR basin had a closer relationship with *E. anema* from the upper Niger River basin of the Nilo-Sudan ecoregion. This allopatric distribution is also displayed by the two sister pairs *L.* aff. *parvus* and *L.* aff. *coubie* from the RR basin and *L. parvus* and *L. coubie* from the upper Niger River basin of the Nilo-Sudan ecoregion. The tectonic events during the Miocene period modified the hydrographic system, wherein populations from the Nilo-Sudan were separated by the uplift of the Fouta Djallon. Hence, this event can be a plausible explanation for the vicarious forms of their ancestral population [34]. The later formation of the watershed between them was the key driving force for their differentiation into distinct species to form a repeated disjunct distribution. More research on inter- and intra-specific relationships between different taxa occurring in neighboring rivers or ecoregions could help in unraveling the complex biogeographic patterns of this area.

4.4. Endemism and Conservation Implications

The revelation of unrecognized diversity of cyprinid fish from the RR basin in this study suggests important implications for the conservation of biodiversity. Inaccurate taxonomy on purely morphological and/or molecular grounds only leads to an underestimation of species richness and endemism, which can misdirect conservation efforts [13,16,34]. However, our DNA molecular approach used in this study is not enough to identify species for conservation efforts, and the team is working on a morphological approach. An integrative taxonomic revision of cyprinids from the RR basin in the future is likely to confirm that four putative species (*L.* aff. *parvus*, *L.* aff. *coubie*, *E.* aff. *ablabes,* and *E.* aff. *macrops*) delineated here are endemic to the upper Guinean ecoregion/Sierra Leone or RR basin, and thus of particular conservation concern. Unfortunately, these putative species are currently misidentified as four amphi-Guinean or Pan-African species (*L. parvus*, *L. coubie*, *E. ablabes,* and *E. macrops*), which are so far assessed as Least Concern (LC) [35]. Subsequently, freshwater fish species from this area are not listed as target species for conservation planning and priority [17], probably due to the underestimated level of endemism and the overestimated level of widely distributed populations. This ecoregion is presently not listed among the priority freshwater Key Biodiversity Areas within the upper Guinean Biodiversity Hotspot [17]. Unveiling this undetected diversity of the RR basin fish might warrant reconsideration for conservation priorities.

Two species of *Enteromius* (*E. foutensis* and *E. liberiensis*), endemic to the upper Guinean ecoregion, are currently listed as Endangered (EN) [16,35]. Both species are conventionally defined based on morphological characteristics alone, a cryptica species complex. *E. fouten-*

sis consists of at least three putative species [10], and *E. foutensis* and *E. liberiensis* are shown here to contain four and two putative species, respectively. These results permit an updated assessment of the conservation status of *E.* aff. *foutensis* and *E.* aff. *liberiensis* of the RR basin. The two putative species have a narrower distribution than previously identified morphospecies. The extent to which both are under threat is more severe than presently supposed. The same is the case for the other four putative species (*L.* aff. *parvus*, *L.* aff. *coubie*, *E.* aff. *ablabes*, and *E.* aff. *macrops*) of the RR basin. This unappreciated diversity merits consideration in conservation planning.

Considering that, six putative cyprinid species delineated here from the RR basin remain unnamed, efforts to catalog and assess the fish diversity of this river need to be prioritized as the first step for conservation of fish species diversity. The high level of endemism observed today is mainly attributed to the isolation of the RR basin from neighboring river basins in Sierra Leone or the upper Guinean ecoregion and its geographic position [1,2]. This river also holds a wealth of mineral resources and high hydropower potential. Fish species of the RR basin, particularly the upper reaches upstream of the Bumbuna dam, are susceptible to natural factors and anthropogenic disturbances. The most common threats observed in this area are uncontrolled timber logging, gold-mining activities, the expansion of the Bumbuna dam, and uncontrolled fishing activities [1]. These activities all bring about habitat degradation triggered by deforestation and pollution. These modifications are deleterious to native species, including the six putative species delineated herein. Thus, these putative species may go extinct before they are officially described. Species identification is essential for species-based conservation and management [34,36,37]. It is an urgent need to gain a better understanding of the biodiversity of the RR basin and beyond to prioritize biodiversity conservation, particularly that under threat from ongoing deforestation.

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Institutional Review Board Statement: Specimens utilized for this study were sampled in accordance with the Chinese Laboratory Animal Welfare and Ethics animal welfare laws (GB/T 35892–2018).

Data Availability Statement: The nucleotide sequence data that support the findings of this study are openly accessible in GenBank of NCBI at https://www.ncbi.nlm.nih.gov/websub/?form=history&tool=genbank under the accession no. MW660579-601; MZ013919-22.

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Abbreviations

The following abbreviations were used in this manuscript:

RR	Rokel River basin
IHB-CAS	Institute of Hydrobiology, Chinese Academy of Sciences
ASAP	Assemble Species by Automatic Partitioning
bPTP	Poisson Tree Processes

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