Mitochondrial DNA, 2011; Early Online: 1–9

informa
healthcare

DNA barcoding discriminates freshwater fishes from southeastern Nigeria and provides river system-level phylogeographic resolution within some species

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(*Received 17 August 2010; revised 28 October 2010; accepted 28 October 2010*)

Abstract

Background and aims: Fishes are the main animal protein source for human beings and play a vital role in aquatic ecosystems and food webs. Fish identification can be challenging, especially in the tropics (due to high diversity), and this is particularly true for larval forms or fragmentary remains. DNA barcoding, which uses the 5′ region of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) as a target gene, is an efficient method for standardized species-level identification for biodiversity assessment and conservation, pending the establishment of reference sequence libraries.

Materials and methods: In this study, fishes were collected from three rivers in southeastern Nigeria, identified morphologically, and imaged digitally. DNA was extracted, PCR-amplified, and the standard barcode region was bidirectionally sequenced for 363 individuals belonging to 70 species in 38 genera. All specimen provenance data and associated sequence information were recorded in the barcode of life data systems (BOLD; www.barcodinglife.org). Analytical tools on BOLD were used to assess the performance of barcoding to identify species.

Results: Using neighbor-joining distance comparison, the average genetic distance was 60-fold higher between species than within species, as pairwise genetic distance estimates averaged 10.29% among congeners and only 0.17% among conspecifics. Despite low levels of divergence within species, we observed river system-specific haplotype partitioning within eight species (11.4% of all species).

Conclusion: Our preliminary results suggest that DNA barcoding is very effective for species identification of Nigerian freshwater fishes.

Keywords: *DNA barcoding, cytochrome* c *oxidase subunit I, mtDNA, freshwater fishes, phylogeographic structure, Nigeria*

Introduction

The inland fisheries in tropical Africa face threats both by stress from climate change and by overexploitation (Hughes et al. 1997). Species are becoming extinct and populations decline at an alarming but poorly understood rate. Many species may face extinction before they can be identified or described. This presents a problem for conservation planning and prioritization, because those species that have not been identified obviously cannot be protected effectively (Swartz et al. 2008). Caddy and Garibaldi (2000) reported that only 65.09% of worldwide fishery captures reported to the FAO for the year 1996 was identified at species level, ranging from about 90% in temperate areas to less than 40% in tropical regions. Surveys into the accuracies of species identifications have not been reported, but a significant percentage of identifications may still be erroneous (Ward et al. 2009). The limitations inherent in morphology-based identification systems and the

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ISSN 1940-1736 print/ISSN 1940-1744 online
DOI: 10.3109/19401736.2010.536537

limited pool of taxonomists paved the way for the introduction of new molecular diagnostic tools for effective species identification. Hitherto, a wide variety of protein-based and DNA-based methods have been evaluated for the molecular identification of fish species in Africa (e.g. Waters and Cambray 1997; Wishart et al. 2006; Swartz et al. 2008). These studies, however, are not comparable for the purposes of species identification because they lack standardization (e.g. different regions of the mitochondrial genome such as cytochrome *b* and 16S rDNA were used). Hebert et al. (2003) proposed a single gene sequence to discriminate the vast majority of animal species, using a 650-bp fragment of the 5′ end of the mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene as a global bioidentification sequence for animals. This technology (DNA barcoding) relies on the observation that the 'barcode' sequence divergence within species is typically much lower than the divergence exhibited between species (Hebert et al. 2003), making it an effective marker for species identification and discovery. The startling efficiency of the method may arise from selective sweeps and the intricacies of mitonuclear coadaptation, raising the profile of bioenergetics as a possible speciation mechanism (Lane 2009).

DNA barcoding has since gained global support as a rapid, accurate, cost-effective, and broadly applicable tool for species identification, particularly with respect to fishes as coordinated by the fish barcode of life (FISH-BOL; www.fishbol.org) campaign (Ward et al. 2009). Barcoding has also been adopted by the census of marine life project, a growing global conglomerate of 50 countries engaged in a 10-year initiative to assess and explain the diversity, distribution, and abundance of life in the ocean (O'Dor 2004). Although there has been criticism of both the philosophical and the practical underpinnings of DNA barcoding (e.g. Moritz and Cicero 2004; Fitzhugh 2006; Rubinoff et al. 2006; Song et al. 2008), its successful application for both species identification and discovery has been demonstrated in many studies, involving many taxonomic groups, for example birds (Hebert et al. 2004b), fish (Ward et al. 2005), fish parasites (Locke et al. 2010), bats (Clare et al. 2007), spiders (Barret and Hebert 2005), crustaceans (Costa et al. 2007), nematodes (Elsasser et al. 2009), earthworms (Chang et al. 2008), mosquitoes (Cywinska et al. 2006), and diverse arrays of *Lepidoptera* (Hebert et al. 2004a; Hajibabaei et al. 2006; Wilson 2010). In addition, DNA barcoding strategies are now being applied for other groups of organisms including plants (CBOL Plant Working Group 2009; Goa et al. 2010), macroalgae (Saunders 2005), fungi (Seifert et al. 2007; Stockinger et al. 2010), protists (Chantangsi et al. 2007), and bacteria (Sogin et al. 2006). Furthermore, DNA barcoding has gained wide application in forensic analysis to investigate cases of illegal poaching (Eaton et al. 2009), separation of species (Wilson-Wilde et al. 2010), gut content analysis in ecological studies (Smith et al. 2005; Berry et al. 2007; Clare et al. 2009), food product analysis and market substitution (Wong and Hanner 2008; Cohen et al. 2009), and Asian medicine trade regulation (Peppin et al. 2008). DNA barcoding has also been employed to validate the identity of biomaterial collections and cell lines (Lorenz et al. 2005; Cooper et al. 2007). A sufficient accumulation of DNA barcodes can also help conservation managers to identify interim priority areas for conservation efforts in the absence of species data. Currently, DNA barcode reference library records are available for more than 1 million sequences representing more than 94,000 species on the barcode of life data systems (BOLD; www.boldsystems.org; Ratnasingham and Hebert 2007), an informatics workbench aiding the acquisition, storage, analysis, and publication of DNA barcode records. Nearly, 10% of these records comprise marine and freshwater fish species (www.fishbol.org).

The *cox1* divergence and species identification success based on DNA barcodes have been previously assessed for many freshwater fish species, for example in Canada (Hubert et al. 2008), Mexico and Guatemala (Valdez-Moreno et al. 2009), and Brazil (Carvalho et al. 2010; Pereira et al. 2010). Since, to date, there is no detailed knowledge about the diversity and distribution of freshwater fish species in Nigeria, the aim of this study was to determine whether DNA barcoding can be used as an effective tool to perform unambiguous species identification of freshwater fishes in this region, with a view toward establishing a DNA barcode reference library for utilization in biodiversity assessment and conservation for the entire country.

Materials and methods

Specimen collection and documentation

We sampled 366 fish specimens, representing 70 species, 38 genera, 20 families, comprising 25% of the 285 known fish species in all Nigerian freshwater systems, as described by Olaosebikan and Raji (1998). Samples were collected from three different sites at three rivers (Afikpo, Anambra, and Ebonyi) in southeastern Nigeria between February 2008 and March 2010. The number of specimens per species collected at each site ranged from 1 to 15, with a mean of 3.5. All specimens were caught in the wild, morphologically identified *in situ* by visual inspection and taxonomically classified with standard guides (Olaosebikan and Raji 1998). Voucher specimens were imaged by digital scanning as described by Steinke et al. (2009a) and preserved in 100% ethanol at the Applied Biology Department Museum, Ebonyi State University, Ebonyi, Nigeria. Details on collection, coordinates, and dates are publicly accessible in BOLD (www.barcodinglife.org) project 'Barcoding Fishes of Eastern Nigeria'. A detailed specimen

overview is given in Table S1 in the Supplementary material.

DNA extraction

Muscle tissue (about 5 mm^3) was extracted from the left side of each fish and preserved in 95% ethanol, using tools that were treated with DNA ELIMINase (Decon Laboratories, USA) before sampling each specimen. Genomic DNA was extracted from 1 to 2 mm^3 tissue pieces, using the DNeasy Blood and Tissue kit (Qiagen, USA). The tissue pieces were incubated (and shaken at 300 rpm) overnight at 56°C in 180 µl tissue lysis buffer ATL and 20 µl proteinase K. Genomic DNA was subsequently extracted with a membrane-based approach on a Biomek FX liquid handling station (Beckman Coulter, USA) using AcroPrep 96 1.0 ml filter plates with 1.0 µm PALL glass fiber media (Ivanova et al. 2006).

PCR amplification

For this study, a 651-bp fragment of the standard *cox1* barcode region was amplified using a mammal primer cocktail (Ivanova et al. 2007), appended with M13 (Messing 1983) tails to aid in a standard sequencing protocol (Table I). Each PCR reaction mixture consisted of 6.25 µl of 10% trehalose, 2 µl ultrapure ddH_2O, 1.25 µl of 10 × PCR buffer for Platinum Taq (Invitrogen, Inc., USA), 0.625 µl of 50 mM $MgCl_2$, 0.125 µl of 10 µM primer cocktail (see Table I), 0.0625 µl of 10 mM dNTP mix, 0.06 µl Platinum Taq polymerase (Invitrogen, USA), and 2.0 µl template DNA to make a 12.5 µl total volume. PCR amplification reactions were conducted on Eppendorf Mastercycler gradient thermal cyclers (Brinkmann Instruments, USA). The thermocycling program consisted of a hot start of 94°C for 1 min; followed by five cycles of 94°C for 30 s, 50°C for 40 s, 72°C for 1 min; then 35 cycles of 94°C for 30 s, 54°C for 40 s, 72°C for 1 min; and then an extension of 72°C for 10 min and a final hold at 4°C.

DNA sequencing and sequence data analysis

PCR products were visualized on 2% agarose E-gel 96 plates (Invitrogen, USA) stained with ethidium bromide. PCR samples with a single visible band were processed further for sequencing using the BigDye Terminator v3.1 Cycle Sequencing Kit [Applied Biosystems, Inc. (ABI), USA]. Each forward or reverse cycle sequencing reaction mixture consisted of 0.25 µl BigDye (ABI), 1.875 µl of 5 × buffer (400 mM Tris–HCl, pH 9.0 and 10 mM $MgCl_2$), 5 µl of 10% trehalose, 1.0 µl primer (10 µM; M13F or M13R), 0.875 µl ultrapure ddH_2O, and 1.5 µl PCR product. The sequencing reaction thermocycling program consisted of 2 min at 96°C, followed by 30 cycles of 30 s at 96°C, 15 s at 55°C, and 4 min at 60°C, followed by a hold at 4°C. Bidirectional sequencing reactions were carried out with the M13 primers (Table I), and the fluorescent signals were recorded on an ABI 3730 DNA Analyzer. Sequences were edited using the software Sequencher 4.8. To evaluate sequence identity, we used the basic local alignment search tool algorithm (Altschul et al. 1997) to search GenBank, and the BOLD 'identification engine' to query barcode records within BOLD. Top species matches obtained from both GenBank and BOLD for each specimen were compared with the specimens' species names. Sequence divergence was calculated using the Kimura 2-parameter (K2P) model (Kimura 1980). A mid-point rooted neighbor-joining (NJ) tree of K2P distances was created to provide graphic representation of the species divergence (Saitou and Nei 1987) as implemented in the 'Sequence analysis module' of BOLD. The sequences generated in this study are deposited in GenBank (accession numbers HM882696–HM883026) (see Table S1 for details).

Results and discussion

Deep divergence between species

We applied the technique of DNA barcoding to evaluate the suitability of this approach to unequivocally

Table I. PCR and sequencing primers used in this study.

Name	Cocktail name/5′–3′ sequence	Taxonomic groups	References
	M13-tailed primers		
	Mammal cocktail: C_VF1LFt1–C_VR1LRt1 (ratio 1:1:1:3:1:1:1:3)	Mammals, reptiles, fish	Ivanova et al. (2007)
LepF1_t1	TGTAAAACGACGGCCAGTATTCAACCAATCATAAAGATATTGG		
VF1_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAAAGACATTGG		
VF1d_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCACAARGAYATYGG		
VF1i_t1	TGTAAAACGACGGCCAGTTCTCAACCAACCAIAAIGAIATIGG		
LepR1_t1	CAGGAAACAGCTATGACTAAACTTCTGGATGTCCAAAAAATCA		
VR1d_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCRAARAAYCA		
VR1_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGGCCAAAGAATCA		
VR1i_t1	CAGGAAACAGCTATGACTAGACTTCTGGGTGICCIAAIAAICA		
	Sequencing primers for M13-tailed PCR products		
M13F	TGTAAAACGACGGCCAGT		Messing (1983)
M13R	CAGGAAACAGCTATGAC		Messing (1983)

discriminate freshwater fish species from southeastern Nigeria (Africa). In this study, we obtained 363 sequences (all >500 bp) belonging to 70 species, 38 genera, and 20 families. Only three (0.8%) out of the 366 samples analyzed failed to yield a DNA barcode. No pseudogenes (short sequences with stop codons) or contaminant sequences (e.g. from bacteria) were detected by the automatic amino acid translation or contaminant screening tools when sequences were uploaded to BOLD. Sequence assemblies, electropherogram trace files, and specific primer combinations used to amplify sequenced PCR products are available for each specimen within the 'Barcoding Fishes of Southeastern Nigeria' project, which is publicly accessible in BOLD. A detailed specimen overview is given in Table S1.

A full K2P model-based NJ phenogram (Figure S1 in the supplementary material) shows the genetic distance between all specimens that generated a DNA barcode as described above. Our DNA barcoding approach separated all 70 species, with 10.29% distance among individuals of congeneric species (Table II). This result is summarized in a K2P NJ tree (Figure 1) to provide an overview of sequence divergences between all species tested in this study. For species within higher taxonomic ranks such as families, orders, and classes, pairwise genetic divergences increased to 17.20, 21.60, and 25.05%, respectively (Table II and Figure 2). Some taxa, however, showed deeper genetic divergence than others. For example, the average of within-genus divergence of the silver catfish (*Chrysichthys*) was 11.31%, considerably larger than 8.28% of the genus *Synodontis*, which is itself considerably larger than the within-*Alestes* divergence of 4.28% (Figure S1). These differences among genera probably reflect the duration and history of species divergence, although some species within genera may be older than others (Ward et al. 2005).

The analysis of the nearest neighbor distance (NND), which is the genetic distance between a species and its closest congeneric relative, revealed that while only 2.9% of the NND was lower than 1% (Table III), the divergence between conspecific individuals was lower than 1% in all cases (Figure 3a). Congeneric NND averaged 10.9% (Figure 3b), which was 64-fold higher than the mean within-species distance of 0.17% and 26-fold higher than the maximum intraspecific distance of 0.42%.

A close inspection of the K2P NJ tree (Figure 1) shows that distinction between two cichlids, *Oreochromis niloticus* and *Sarotherodon boulengeri*, might be ambiguous and may suggest shared haplotypes, because the genetic distance between both is only 0.46% (compare Figure 3(b)). Such cases of shared haplotypes or low inter-specific variation may be due to introgressive hybridization, incomplete lineage sorting, taxonomic over-splitting, recent radiation, or possibly misidentification of the original specimens (Won et al. 2005; Hubert et al. 2008; Steinke et al. 2009b). We suspect the latter, given the fact that different genera are involved and a detailed analysis into the nature of this anomaly is underway. Berra (2001) observed that the identification of cichlids might pose a taxonomic challenge with over 1000 species present in the tropics. However, the other cichlid species in this study were clearly separated (Figure 1), indicating that DNA barcodes can significantly advance our understanding of the diversity in this very important group in Nigeria.

Minimal divergence but phylogeographic variation within species

The average K2P distance estimate of intraspecies divergence in this study was 0.17% (maximum 1.08%) (see Table II), which is lower than the previous fish DNA barcoding studies, which have reported mean conspecific divergences of 0.27% (range 0–1.95%) for 1035 salmon and trout specimens from North America (Rasmussen et al. 2009), 0.30% (range 0–7.42%) for 194 Canadian fish species (Hubert et al. 2008), 0.39% (range 0–14.08%) for 207 Australian fish species (Ward et al. 2005), and 0.99% (0.19% when possible misidentifications were omitted) for 72 commercial fish species in the USA (Yancy et al. 2008). In summary, even with a significant overlap of the genetic distances at the genus level and above (see Table II), the average genetic distance among congeneric species is nearly 60-fold higher than that found within species, a ratio higher than the 12-fold and 27-fold previously reported for freshwater fishes in Mexico/Guatemala (Valdez-Moreno et al. 2009) and Canada (Hubert et al. 2008), respectively. The ratio observed in this study is also much higher than the 25-fold and 26-fold

Table II. Summary of genetic distance (according to K2P model) for increasing taxonomic levels.

Comparison	Comparisons (*n*)	Minimum (%)	Mean (%)	Maximum (%)	SE
Within species	1284	0	0.17	1.08	0.01
Genus among species	1447	1.08	10.29	34.07	0.14
Family among genera	2951	0.46	17.20	24.82	0.06
Order among families	7768	15.84	21.60	28.12	0.03
Class among orders	46,609	18.46	25.05	31.96	0.01

Note: Data are from 363 sequences, 70 species, and 38 genera.

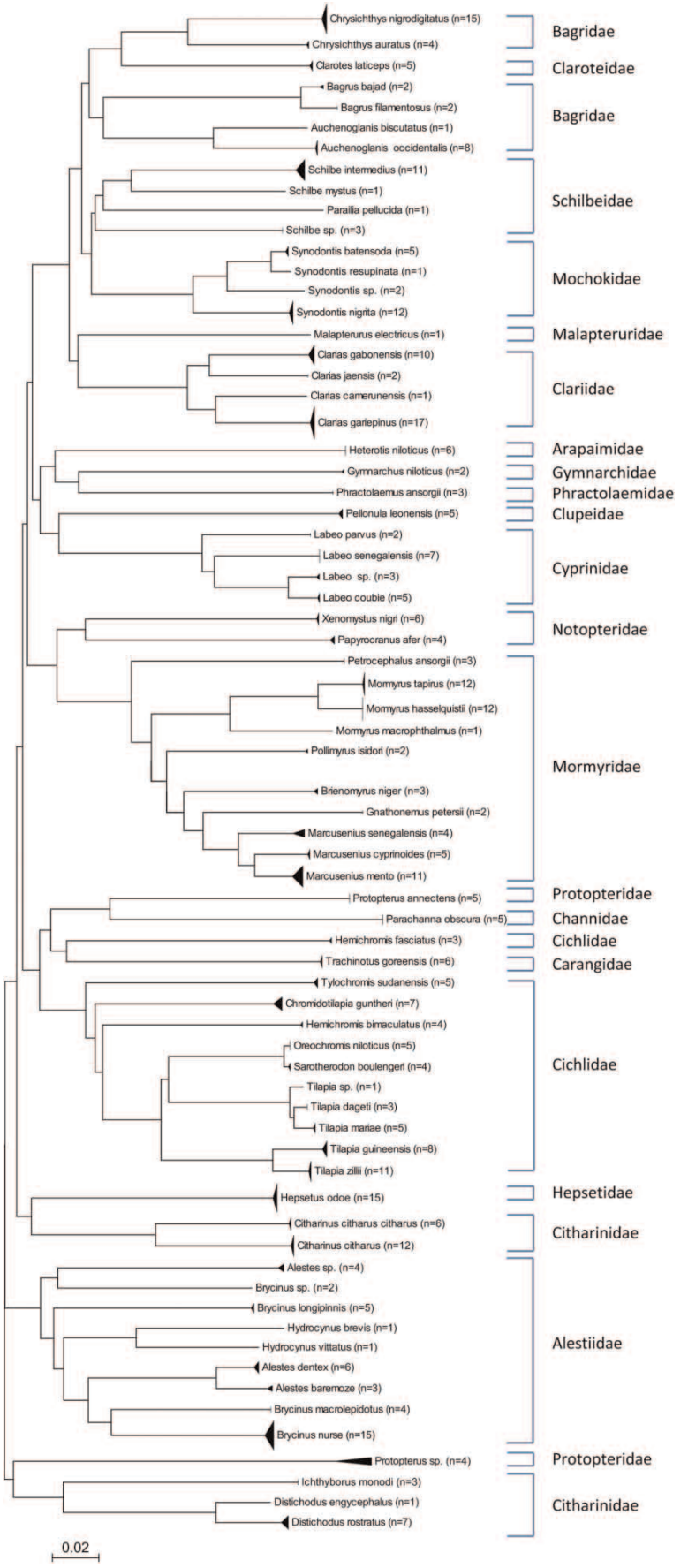

Figure 1. NJ distance tree with 70 freshwater fish species (taxonomic families outside tree) of southeastern Nigeria, using K2P model distance calculation.

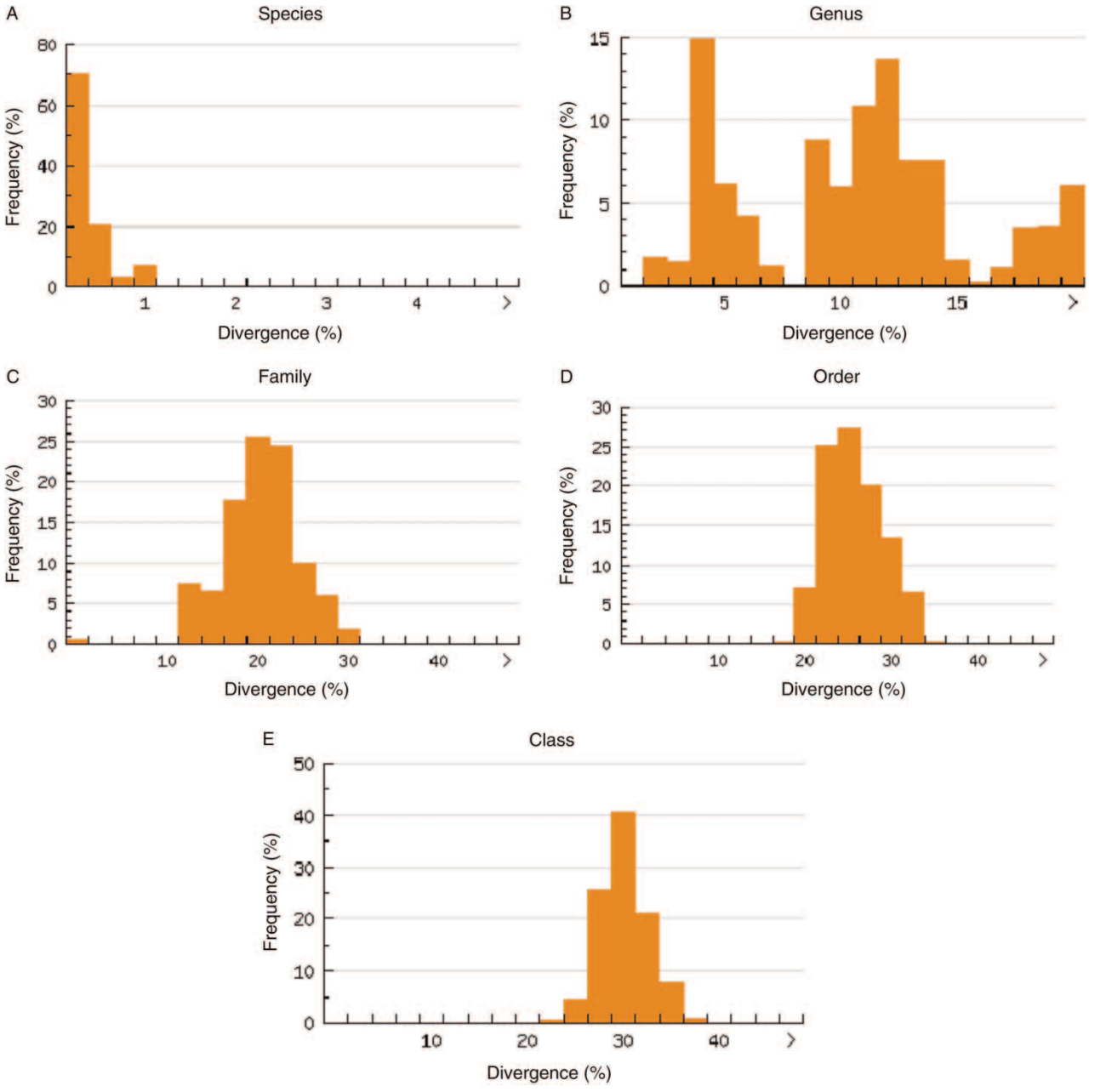

Figure 2. Genetic variability (K2P model distance of *cox1* sequences) within different taxonomic categories. Panel A, species; panel B, genus; panel C, family; panel D, order and panel E, class.

reported for marine salmon and trout (Rasmussen et al. 2009) and ornamental marine fishes (Steinke et al. 2009b), respectively.

Although the primary objective of DNA barcoding is to identify species, phylogeographic structure among *cox1* sequences within species became evident in this study. In the family *Characidae*, 15 individuals of *Brycinus nurse* were analyzed, and the full K2P NJ distance tree (Figure S1) shows two closely related (genetic distance 0.8%) but distinct clusters, with specimens BNF 178, BNF 179, BNF 181, and BNF 182 caught in Anambra river in one cluster (red color in Figure S1), while the others (BFN 102, BNF 190, BNF 191, BNF 192, BNF 194, BNF 195, BNF 196, BNF 197, BNF 198, BNF 199, and BNF 200) in the second cluster were caught in two separate but closely located rivers (Ebonyi river, blue color in Figure S1, and Afikpo river, pink color in Figure S1). A similar observation was made among the 15 specimens of *Hepsetus odoe* studied. The intraspecific genetic divergence was 0.20%, but there are two closely related distinct clusters of seven specimens from Anambra river (BNF 313, BNF 314, BNF 315, BNF 316, BNF 317, BNF 318, and BNF 319, red color in Figure S1) and eight specimens from Ebonyi river (BNF 320, BNF 321, BNF 322, BNF 323, BNF 324, BNF 325, BNF 326, and BNF 327, blue color in Figure S1). According to the river system from which the fish were captured, distinct clusters were also observed within six other species: *Marcusenius mento*,

Table III. Summary of southeastern Nigeria fish diversity and distribution of the genetic distance to the nearest neighbor; *cox1* sequences of 70 species analyzed with K2P model.

Order	Family	Species (*n*)	<0.1%	0.1–1.0%	1.0–2.7%	>2.7%
Siluriformes	Malapteruridae	1	0	0	0	1
	Mochokidae	4	0	0	2	2
	Clariidae	4	0	0	0	4
	Bagridae	6	0	0	2	4
	Schilbeidae	4	0	0	0	4
	Claroteidae	1	0	0	0	1
Osteoglossiformes	Gymnarchidae	1	0	0	0	1
	Notopteridae	2	0	0	0	2
	Mormyridae	10	0	0	0	10
	Arapaimidae	1	0	0	0	1
Perciformes	Cichlidae	11	0	2	3	6
	Carangidae	1	0	0	0	1
	Channidae	1	0	0	0	1
Cypriniformes	Cyprinidae	4	0	0	2	2
Lepidosireniformes	Protopteridae	2	0	0	0	2
Characiformes	Alestiidae	9	0	0	0	9
	Citharinidae	5	0	0	0	5
	Hepsetidae	1	0	0	0	1
Clupeiformes	Clupeidae	1	0	0	0	1
Gonorynchiformes	Phractolaemidae	1	0	0	0	1
	Total	70	0	2	9	59

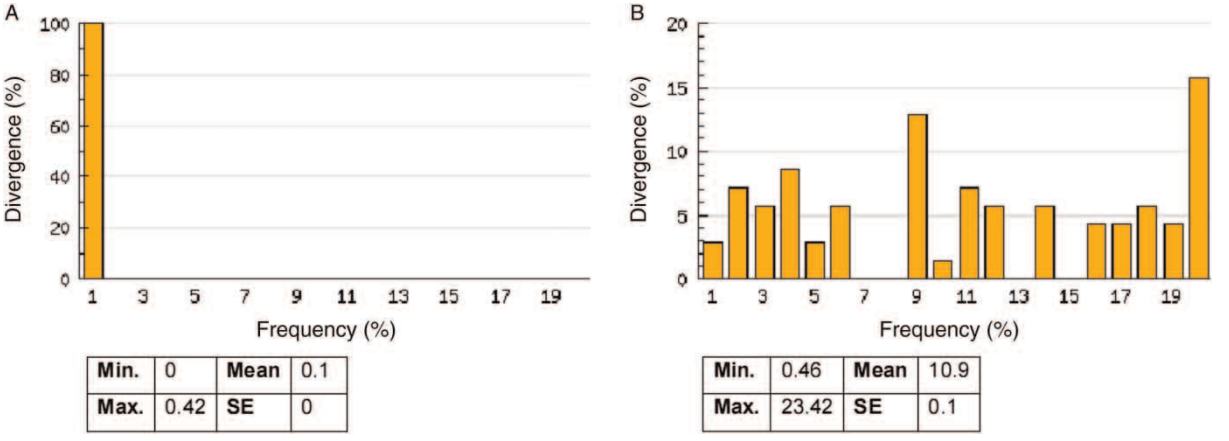

Figure 3. Distribution of the genetic variability (K2P model distance of *cox1* sequences) for the 363 individuals and 70 species analyzed. Panel A, intraspecific distance and panel B, genetic distance to the nearest neighbor. SE, standard error.

Schilbe intermedius, *Clarias gabonensis*, *Chrysichthys nigrodigitatus*, *Chromidotilapia guntheri*, and *Citharinus citharus*. These phylogeographic associations (Waters and Cambray 1997) may reflect a so-called ecosystem-dependent adaptive radiation (Ernst et al. 2003) and highlight the fact that barcoding can extend beyond just simple species identification to include phylogeographic 'source tracking' in many cases.

Conclusions

The results obtained in this study validate the efficacy of *cox1* DNA barcodes for identification of freshwater fish species and will serve as a framework for future analysis of fish population structure and other applied studies such as forensic research, market analysis, food web analysis, and identification of freshwater fish eggs, larvae, fillets, and fins. Our results will also pave the way for advanced biodiversity research in Nigeria.

Acknowledgements

The authors would like to thank Rick Turner, Jaclyn McCormick, Joao Lima, John James Wilson, and Natasha Serrao (University of Guelph) for aid in imaging, DNA analysis, and data evaluation. A travel bursary was provided to CDN from the Consortium for the Barcode of Life (CBOL) to analyze specimens at the Biodiversity Institute of Ontario and the Department of Integrative Biology at the University of Guelph. Sequencing costs were paid for by the International Barcode of Life Project, with funds provided by Genome Canada (through the Ontario Genomic Institute). The authors thank the authority

of Ebonyi State University PMB 053 Ebonyi (Nigeria) for the financial support of this research project.

Declarations of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper. SB acknowledges support from the Canadian Barcode of Life Network provided by the Natural Sciences and Engineering Research Council of Canada.

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Supplementary material available online