

DNA BARCODING OF SELECTED FRESHWATER FISHES IN NIKE LAKE, ENUGU STATE NIGERIA

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ABSTRACT

Background: The continuous decline in biodiversity is a subject of concern among the scientific community. This decline is common in the fish population. Although taxonomy has been an age-long field of science, there are still undiscovered members of species and new species are waiting to be uncovered. The failure of traditional taxonomic method to address this issue has resulted in the adoption of a molecular approach-DNA barcoding. It was proposed that DNA barcoding using mitochondrion cytochrome oxidase subunit I (COI) gene has the capability to serve as a barcode for fishes. **Objectives:** The aim of this study was to use DNA barcoding in the identification of fish species in Nike Lake, Enugu State. **Materials and methods:** The DNA samples from 6 fishes collected from four Nike Lake in Enugu State respectively were isolated, amplified and sequenced. **Result:** BLAST result shows correlation between the sequence queried and the biological sequences with the NCBI database. The names of the samples, percentage ID, predicted organisms and GenBank Accession numbers were clearly identified. A total of 12 sequences (all > 500 bp) belonging to 6 species, 5 genera and 5 families and 3 orders were validated and submitted to the NCBI database. Each nucleotide peak was represented by a single color with various percentage occurrences. None of the 6 original samples analyzed corresponded with the predicted organisms from NCBI database. Pairwise sequence alignment showed different consensus positions and 1 transversion mutation in *Oreochromis aureus*. There were no deletion and nonsense codons in all the amplified sequences. **Conclusion:** This work will facilitate more research in other keys areas such as identification of mislabeled fish products, illegal trading of endangered species and effective tracking of fish biodiversity.

Keywords: DNA barcoding, Nike lake, phylogenetic trees, Pairwise DNA alignment, substitution, insertion, mutation.

1. INTRODUCTION

The continuous decline in biodiversity is a subject of concern among the scientific community. This decline is common in fish population. Still, attempts to conserve biodiversity remained uncertain because there is fragmentary information concerning the exact global birth data and the rate of loss is unknown, particularly in terms of background knowledge [1]. Although the history of taxonomy began since 250 years ago, there are still numerous undescribed members of a species. It has been reported that only 226000 of the estimated 0.7 – 1 million marine species have been described [2]. Formalized techniques need to be developed for data accession of biodiversity in order to manage up the current biodiversity crisis. Among the marine organisms, the organism with the highest form of taxonomic extremity is the fish. The term "fish" is generally an accessible description for a group of poikilothermic (cold-thoroughbred) marine invertebrates under the phylum Chordata that breathe with gills [3]. Fishes are vertebrates of great diversity in morphological appearances and numerically, accurate scientific descriptions have been noted for more than 35,000 species that contribute significantly to the number of invertebrates [4,3]. Globally, there are 86% of unidentified terrestrial species that need taxonomic identity whereas in marine and brackish territories, this number is extremely high (91% species) [5]. Freshwater is any natural liquid containing low level of dissolved solids. The term 'freshwater' excludes seawater and brackish water but does include non-salty mineral-rich waters similar as chalybeate springs [6]. Freshwater fishes are vertebrates with streamlined body and rich in different types of nutrients. They live each, or a critical part of its life in either brackish inland or brackish arms [7]. Some examples of freshwater fishes are carps, characins, cichlids, salmon, eels, shafts and sawfish, estuarine fish, e.g., archer fish and gobies; and soda pop and swab lake fish but excludes coral reef fish and fishes in the ocean and sea. Fish identification is traditionally done with the use of morphological parameters [8]. Since humans learned how to hunt fish, species were identified and given names on the ground of their simple anatomical features. The identification of fish on the ground of their morphological features is the most practical, rapid and low-cost system [7]. Besides well experienced fishermen and fish mongers, people who live by the water side would learn to identify fishes at a youthful age. This is due to knowledge and memory acquired from long-term observation or through oral tradition maintained by elders [9]. Some species like the *Silurichthys Indragiriensis* and *Wall ago attu* propel themselves forward or backward by wavelike flexure of long anal fins. Ichthyologists call these fins "strip- fins" [10], and what makes them so easy for identification is that the entire stretch of the anal fin is actuated by muscles along the body length [11,12,13]. Patterns and configuration of the cephalic-lateral system on fish bodies are occasionally used for fish identification similar as those from the *Kryptoglanis*, *Pseudorasbora* and *Caecileotri* genera [14,15,16]. A recent study describes an app that could be installed on smartphones in order to take photos of the fish and shoot them to a cloud server for processing and recognition. The answer would also arrive directly to the smart

device [17]. This method has been adjudged to be more than 50% accurate and efficient in fish identification and recognition [18]. Even with these records, morphological indices have been adjudged to be inadequate in the unambiguous identification of fish, especially the cryptic species. For this reason, scientists have adopted a DNA-based method that will eclipse the limitations with morphological parameters. DNA barcoding is a system of species identification using a short section of DNA from a specific gene or genes. The premise of DNA barcoding is that, by comparison with a reference library of similar DNA sections (also called "sequences"), an individual sequence can be used to uniquely identify an organism to species level, in the same way that a supermarket scanner uses the familiar black stripes of the Universal Product Code (UPC) barcode to identify an item in its stock against its reference database [19].

These sections of mtDNA called "barcodes" are sometimes used in an effort to identify unknown species or parts of an organism, or simply to catalog as many taxonomic groups as possible, or to compare with traditional taxonomy in an effort to determine species boundaries. Different gene regions are used to identify the different organismal groups using barcoding. Different gene regions are used to identify the different organismal groups using barcoding. The most generally used barcode region for animals and some protists is a section of the cytochrome c oxidase I (COI or COX1) gene found in mitochondrial DNA. In 2003, [20] proposed that cytochrome oxidase subunit I (COI) gene in the mitochondrial DNA has the capability to serve as a barcode for all animals. DNA barcoding serves as a golden bullet for not only species identification, but also it delimits species boundaries. With this revolution in taxonomy, the Barcode of Life (BOL) design was launched to develop generally accepted barcode system that's hinged on standard sequence of mitochondrial cytochrome oxidase I (COI) gene to identify eukaryotes and also matriculate global biodiversity at a platform [21]. In the late 2004, the Consortium for the Barcode of Life (CBOL) inaugurated this project. The goal of CBOL became to broaden a popular protocol of DNA extraction, PCR, and sequencing techniques, which is a useful resource for the formation of a worldwide DNA library.

The CBOL entered a new phase when they launched International Barcode of Life (IBOL) comprising of 26 countries that aimed to enlist eukaryotic biodiversity. In the For the first time, the IBOL concentrated substantially on a collection of maximum barcode sequences in a barcode library from all over the world to achieve the target of five million species barcodes. The IBOL also developed bioinformatics software with a huge data library [21,22]. Some other important systems include fish barcode of life (Fish BOL), Health Barcode of life (HBOL), Lepidoptera Barcode of life (LBOL), Marine Barcode of life (MarBOL), Mosquito Barcode of life (MBI), Mammalia Barcode of life crusade(MBOL), Coral Reef Barcode of life (CRBOL) and Bee barcode of life (BeeBOL) (23). Many nations of the world have come up with such projects like Norway (NorBOL), Mexico (MexBOL), Japan (JBOL) and Europe (EBOL) [22]. One or two studies have provided molecular details of the diversity and distribution of freshwater fish species in Nike Lake, however, the aim of this study is to bridge the gap and provide further details on the use of DNA barcoding in the identification of fish species in Nike Lake, Enugu State.

2. MATERIALS AND METHODS

2.1 Study Area

The capital of Enugu is Enugu. The state shares borders with Abia, Imo, Anambra, Ebonyi, Benue and Kogi States. The samples were collected from Nike Lake in Enugu East Senatorial District, Enugu state, Nigeria.

2.2 Sample Collection

A total of six (6) fish samples were collected and used for this study. Samples were collected by the help of fishermen using a standard fishing gear. Random sampling method was used for the collection of the fish samples as described Eyo and Akpati (1995) [24].

2.3 Transportation of Fish to Laboratory

The fishes were placed in a glass aquarium where they were transported to the ETF lab of Applied Biology and Biotechnology, Enugu State University of Science and Technology for sample collection.

2.4 Identification of Fish Samples by a Taxonomist

Before sample collection, a taxonomist was called to identify the fish samples using morphological parameters. The identification was done as described by Fischer [25].

2.5 Collection and Preservation of Sample

A sharp blade was used to cut the fish muscle as described early [26]. Prior to DNA extraction, the muscle samples were collected and preserved in 50 ml of DNA/RNA and were transported to Inqaba Biotech, Ibadan for further analysis.

2.6 DNA Extraction

Quick DNA Miniprep Plus kit (D4068, Zymo Research) was used for the DNA extraction as described by Zymo Research Corp. It has the advantage of working reproducibly with almost any kind of plant or animal specimen. Fish muscle

samples of 15 mg for each sample was used for DNA extraction. A clean plastic pestle was used for grinding and homogenization of the tissue in about 2 minutes. The samples were then placed in clean micro centrifuge tubes of 1.5 mL and were labeled with an identification number (1 to 6) according to the number of samples. Ninety-five (95) ul of water, 95ul of solid tissue buffer and ten (10) ul of proteinase k were added to the samples in the microcentrifuge tubes. The tubes were vortexed 10-15 seconds and then incubated at 55°C for 1-3 hours until the tissue solubilized. The tubes were centrifuged at 12,000 x g for one minute to remove the insoluble debris. The aqueous supernatants were transferred to a new microcentrifuge tube of 1.5 mL. This was followed by the addition of 400 ul of Genomic Binding Buffer and vortexing for 10-15 seconds. The mixture was transferred to a Zymo-Spin™ IIC-XLR column in a collection tube and centrifuged at ≥ 12000 x g for 1 minute. The collection tube was then discarded with the flow through. Four hundred (400)ul of DNA Pre-wash Buffer was added to the spin column in a new collection tube and centrifuged at $\geq 12,000$ x g for 1 minute. The collection tube was emptied and was immediately followed by the addition of 700ul g-DNA Wash Buffer to the spin column. It was centrifuged at $\geq 12,000$ x g for 1 minute. The collection tube was then discarded with the flow through. The spin column was transferred to a new clean microcentrifuge tube. Fifty (50) ul of DNA Elution Buffer was added to the matrix, incubated for 5 minutes at room temperature, then centrifuged at maximum speed for 1 minute to elute the DNA. The eluted DNA was stored at $\geq 20^\circ\text{C}$ in preparation for amplification. The Nanodrop Spectrophotometer was used to determine the purity and concentration of DNA samples.

2.7 Amplification of DNA by PCR

The fish target region was amplified using the OneTaq Quick Load 2X Master Mix (NEB, Catalogue No: M9486), nuclease free water, template DNA with two primers for forward and reverse reactions (FISH F1-TCAACCAACCACAAAGACATTGGCAC and FISH F2-TAGACTTCTGGGTGGCCAAAGAATCA). After thermal cycling, the amplified DNA was stored at -20°C as described by Shokrallan et al. (2010) [27].

2.8 Analyzing PCR product by gel electrophoresis

The gel-casting tray was used for the gel electrophoresis. A total of 1 g of powdered agarose gel was dissolved in 100ml of 1X Tris Acetate EDTA (TAE). It was heated until agarose was completely dissolved in the buffer and it was allowed to cool after which 4ul of SafeView Classic (gel stain) was added. It was allowed to cool for about 60°C and was poured into the tray with casting dams fit on both ends of the tray and combs in correct position and allowed to set. After the gel is set, the combs and casting dams were removed while the tray was placed in the electrophoresis tank containing the buffer of choice (TAE). The ladder and samples were carefully loaded into the wells and tank covered with its lid and connected to the negative (-ve) and positive (+ve) electrodes and power supply. The gel was kept for approximately 30 minutes 130v and was viewed using UV transilluminator as described by Lucentini et al. (2006) [28].

2.9 DNA Sequencing

The PCR products were further purified using EXOSAP method. The purified fragments were analyzed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction and every sample. The extracted fragments were sequenced in the forward and reverse directions as described by Sanger et al. (1977) [29].

2.10 Data Analysis

A total of 12 sequences were generated from the ABI 3500xl Genetic Analyzer (Applied Biosystems). Each sample was identified to the specie level using BLASTn program on the GenBank (National Center for Biotechnology Information) [30]. The sequencing results were carefully trimmed and edited using the DNA subway as described by Merchant et al. (2016) [31]. Pairwise alignment of sequences was done using MEGA 11 software [32]. The evolutionary history was inferred using the Neighbor-Joining method as described by Saitou et al. (1987) [33]. DNASTAR was used to analyze the ab1 files generated by the ABI 3500XL Genetic Analyzer.

3. RESULTS

3.1 Estimation of the Concentration and Purity of DNA Samples

The data obtained from the Nanodrop Spectrophotometer is shown in table 1. The Nanodrop was used to measure the concentration and purity of the DNA samples. The column measured light passing through the DNA at a wavelength of A280/260 nm. The ideal purity value for DNA samples at A260/280 is 1.8 and between 2.0 and 2.2 for A260/230. Scores lower than these usually indicate that there are contaminants present in the sample. The highest DNA concentration of 138.9 was observed in sample 3 (*Mormyrus kannume*) with ratios of 1.91 and 1.59 for A280/260 and A260/230 respectively. The lowest concentration of 81.3 was observed in sample 23 (*Clarias gabonensis*) with ratios of 1.86 and 0.57 respectively. Sample 4 (*Oreochromis aureus*) and 2 (*Chrysichthys nigrodigitatus*) had the highest and lowest purity ratios of 1.99 and 1.83 respectively

Table 1: Estimation of the concentration and purity of DNA samples

Name	Concentration	A260/A280	A260/230	A260	A280
Sample 1	134	1.90	2.06	2.68	1.41
Sample 2	112.9	1.83	1.72	2.26	1.23
Sample 3	138.9	1.91	1.59	2.78	1.46
Sample 4	98.3	1.99	0.13	1.97	0.99
Sample 23	81.3	1.86	0.57	1.63	0.88
Sample 29	120.1	1.83	1.77	2.40	1.31

3.2 BLAST Result from NCBI Database

Table 2 shows the BLAST result which corresponded to the similarity between the sequences queried and the biological sequences with the NCBI database. The names of the original samples, percentage identity (ID), predicted organisms from blast result and GenBank Accession numbers were clearly identified. A total of 12 sequences (all > 500 bp) belonging to 6 species, 5 genera and 5 families were and 3 orders were validated and used for the analysis. The highest percentage ID of 100% for predicted organisms were found in *Chrysichthys nigrodigitatus*, *Mormyrus kannume* and *Mormyrus tapirus* while *Clarias gabonensis* had the lowest percentage ID of 89.40%. None of the original samples analyzed corresponded with the predicted organisms from NCBI database. A total of one class (Actinopteri), 3 orders (Perciformes, Siluriformes, Osteoglossiformes and 5 families (Cichlidae, Anabantidae, Clariidae, Clariidae and Mormyridae) were detected among the analyzed samples.

Table 2: Result obtained from BLAST Analysis using COI gene marker.

Name of Sample	Percentage ID	Predicted Organism	Genbank Accession
<i>Tilapia zilli</i>	92.45%	<i>Ctenopoma ocellatum</i>	MK074177.1
<i>Ameriurusnatali</i>	100%	<i>Chrysichthys nigrodigitatus</i>	HM882776.1
<i>Clarias gariepinus</i>	100%	<i>Mormyrus kannume</i>	LC487200.1
<i>Tilapia zilli</i>	99.55%	<i>Oreochromis aureus</i>	LC487089.1
<i>Clarias batrachus</i>	89.40%	<i>Clarias gabonensis</i>	KT193141.1
<i>Mormyrus rume</i>	100%	<i>Mormyrus tapirus</i>	HM882737.1

3.2.1 Phylogenetic Tree Construction using Neighbor-Joining Method

The evolutionary history was inferred using the Neighbor-Joining method (33). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (34) and are in the units of the number of base substitutions per site. This analysis involved 6 nucleotide sequences. There were a total of 811 positions in the final dataset. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted with MEGA 11 (34). The tree showed that there were intergeneric and congeneric divergences among the organisms studied thereby proving the effectiveness of the phylogenetic tree in tracing the phylogenetic relationship of organisms. Pairwise genetic divergence increased across higher taxonomic ranks like genera, order, families and class. The genera united at a common node shows that they have a common ancestor and may have little genetic divergence. The intra- and inter-genetic distances are shown in table 3.

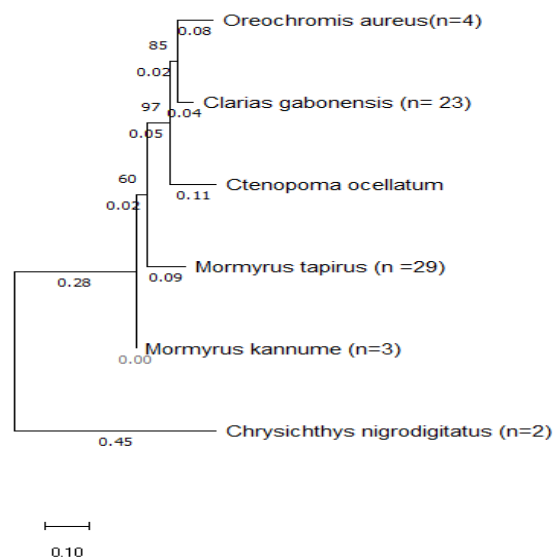


Figure 1: Phylogenetic tree of fish species from Nike Lake.

Table 3: Intra and intergenetic distances of fish samples from Nike Lake.

S/N	Name of organism	1	2	3	4	5
1	<i>Ctenopoma nigrodigitatus</i>					
2	<i>Chrysichthys nigrodigitatus</i>	0.9190				
3	<i>Mormyrus kannume</i>	0.1973	0.7407			
3	<i>Oreochromis niloticus</i>	0.2076	0.9285	0.1712		
5	<i>Clarias gabonensis</i>	0.1659	0.8728	0.1181	0.1189	
6	<i>Mormyrus tapirus</i>	0.2346	0.8537	0.0777	0.2310	0.2107

3.2.2 Electropherogram Trace Files

The electropherogram trace files obtained from the ABI 3500XL Genetic Analyzer (Applied Biosystem) were trimmed, filtered and edited using BioEdit [35,36,32]. Short sequences with stop codons known as pseudogenes or junk DNA were not detected when sequences were submitted to the NCBI database. Tables and 5 show the percentage occurrence of the four different nucleotides and the total number of base pairs for each predicted organism. In the reverse strand, the table shows that *Mormyrus tapirus* had the highest number of base pairs (672 bp) while the least number of base pairs was found in *Oreochromis aureus* (612 bp). In the forward strand, *Mormyrus tapirus* also had the highest basepairs while the least was found in *Clarias gabonensis*. The observed baseline noise was minimal and did not affect calling of the peaks. Each peak with a particular colour represents a nucleotide. Adenine (A) is represented with green colour, Cytosine (C) with blue, Guanine (G) with black and thymine with red colour.

Table 4: Table of DNA bases and their percentage occurrence (forward).

S/N	Name of organism	A	%	T	%	C	%	G	%	Total
1	<i>Ctenopoma ocellatum</i>	166	24.78%	195	29.10%	191	28.51%	118	17.61%	670
2	<i>Chrysichthys nigrodigitatus</i>	161	24.21%	186	27.97%	191	28.72%	127	19.10%	665
3	<i>Mormyrus kannume</i>	146	25.22%	145	25.04%	180	31.09%	108	18.65%	579
4	<i>Oreochromis aureus</i>	159	23.73%	189	28.21%	201	30.00%	121	18.06%	670
5	<i>Clarias gabonensis</i>	148	26.06%	158	27.82%	157	27.64%	105	18.49%	568
6	<i>Mormyrus tapirus</i>	160	23.77%	163	24.22%	213	31.65%	137	20.36%	673

Table 5: Summary of bases and their percentage occurrence (reverse).

S/N	Name of organisms	A	%	T	%	C	%	T	%	Total
1	<i>Ctenopoma ocellatum</i>	189	28.34%	168	25.19%	115	17.24%	195	29.24%	667
2	<i>Chrysichthys nigrodigitatus</i>	180	26.99%	164	24.59%	130	19.49%	193	28.94%	667
3	<i>Mormyrus kannume</i>	180	26.99%	164	24.59%	130	19.49%	193	28.94%	667
4	<i>Oreochromis aureus</i>	158	25.82%	169	27.61%	134	21.90%	151	24.67%	612
5	<i>Clarias gabonensis</i>	186	27.72%	160	23.85%	119	17.73%	206	30.70%	671
6	<i>Mormyrus tapirus</i>	157	23.36%	165	24.55%	133	19.79%	217	32.29%	672

4. DISCUSSION

Several studies published in high impact journals have proven that DNA barcoding using the Cytochrome Oxidase Subunit 1 gene (COX 1) is effective in the identification of fish species [37,38]. The detected 6 species, 5 genera, 5 families and 3 orders are lower than the studies involving 44 fish species (38) but higher than the study involving 3 species [41]. Some of the identified fish species corresponded with the ones earlier discovered by other researchers within and outside the country [40,41,42,39]. The COI sequences effectively clustered intraspecific and congeneric species at a bootstrap value of 99%. A similar result was obtained in the study involving 363 freshwater fishes from Southeastern Nigeria [42] and 194 Canadian freshwater fishes [41]. This study recorded more than 95% success rate and it corresponds with other studies on DNA barcoding of freshwater fishes [42,41,43]. The genetic distances were similar between conspecific and congeneric individuals but different at confamilial taxonomic level. This agrees with the study done by George et al. (2020) [39] where they reported computations of genetic distance between (interspecific) and within (intraspecific) species.

The genetic distances between *Ctenopoma ocellatum* and *Chrysichthys nigrodigitatus*, *Mormyrus kannume* and *Oreochromis aureus*, *Clarias gabonensis* and *Mormyrus tapirus* are 0.92, 0.17, 0.21% respectively. The lowest intrageneric distance of 0.07% was observed between *Mormyrus kannume* and *Mormyrus tapirus* while the highest intergeneric distance was observed between *Chrysichthys nigrodigitatus* and *Oreochromis aureus* (0.93%). This was also reflected in their positions on the tree.

These values fall within the range (0-7.42%) reported in the DNA barcoding of 72 commercial fish species in the USA [44] and 194 Canadian fish species [41]. Generally, the intergeneric divergences observed in the phylogenetic tree are lower than the one reported in the study of 35 fishes [45] and 44 fishes [39]. The Nanodrop Spectrophotometer showed that all the samples exceeded the purity threshold of 1.8 at a wavelength of A260/280 nm. This value suggests that the DNA samples were free of contaminants. Pairwise sequence alignment showed different consensus positions and 1transversion (substitution) mutations in *Oreochromis aureus*. There were no insertion, deletion, transversion mutations and nonsense codons in all the amplified sequence. This aligns with earlier studies reported by previous studies [46,39].

5. CONCLUSION

This work has proven that the mitochondrial cytochrome oxidase gene 1 (COI) is unambiguous and effective in discriminating species boundaries. It has generated a barcode library for fish population in new Nike Lake, and will serve as a molecular framework for identification of fish species in other freshwater sources in the Enugu state. The use of DNA barcoding in the identification of fish species appears to hold great potential for discrimination of fish species and authentication monitoring system by governments, agencies, regulatory bodies and industries to improve transparency and fair trade on domestic fish management.

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