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Molecular validation of Cichlids from Zobe Reservoir, Dutsin-Ma, Katsina State, Nigeria using Cytochrome Oxidase (COI) Genes

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Abstract

The species of cichlids are easily misidentified due to their morphological similarities. This study, therefore, was designed to discriminate the cichlid species inhabiting Zobe reservoir using a molecular approach. Samples of different cichlid species were collected from the reservoir between August and November 2022 and identified using their morphological features following the field guide to Nigerian freshwater fish. Fin clips of two samples from each of the species were fixed in 100% ethanol and the DNA was extracted. The mitochondrial cytochrome oxidase (CO1) gene area was amplified (using the FishF1 and FishR1 primer pair), purified and sequenced to reveal the identity of the species. Morphologically, the result revealed the presence of four species, namely: *Oreochromis niloticus, Oreochromis mossambicus, Sarotherodon galilaeus, and Coptodon zilli*. The genetic identity of the samples agreed with the earlier attempt made at morphological discrimination except for *O. mossambicus* which matches the partial and complete genome of *Oreochromis aureus, hence,* solving misidentification. The phylogenetic tree of the CO1 genes constructed using MEGA 11 software reveals the species were grouped independently into the three different genera of the Cichlidae family (i.e., the *Coptodon*, *Oreochromis* and *Sarotherodon*). This study affirms the need for molecular confirmation of morphologically identified cichlids in Nigeria.

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Keywords: Cichlids, DNA Barcoding, Identification, Phylogeny, Polymerase Chain Reaction.

Introduction

Fishes are among the most varied vertebrate species in the world, hence, identifying them using rapid and accurate means is essential (Sachithanandam and Mohan, 2020). Historical techniques of recognizing, classifying and naming fishes are mostly based on visual morphology (Okomoda *et al*., 2021). While morphological characteristics are a simpler and time-saving method for taxonomy, it can be challenging to recognize fishes at different stages of development

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relying solely on the morphological features (Neira *et al*., 2015)., More so, the identification of adult specimens of fish with missing morphological taxonomic features or keys also presents serious challenges even for the most experienced taxonomists (Gyamfua *et al*., 2019). In addition, there are certain species with a few physical differences, hence, making them nearly identical (Vivek *et al*., 2014). One such group of fish are those generally called Tilapia which are made up of a variety of very similar species (Sogbesan *et al*., 2017). According to Olufeagba *et al*. (2015), most of the available information on these species is based on morpho-meristic features that reveal extensive differences and broad interspecific overlaps. Therefore, identification of these species in their preserved forms using taxonomic keys may lead to wrong conclusions (Vivek *et al*., 2014).

Tilapia is one of the groups of freshwater fish considered invasive as it has taken over numerous rivers and lakes across the world, especially in Africa (Gu *et al*., 2014; Hu, 2015; Okomoda *et al.,* 2021). This attribute alone makes it particularly difficult to distinguish between the species within the tilapiine genus (Laith *et al*., 2020). It is therefore imperative to engage methods which can guarantee accurate identification during taxonomic studies of the Tilapia species. Using a straightforward, standardised genetic tag, DNA barcoding is a contemporary taxonomy tool that aspires to document all life at the molecular level (Vivek *et al*., 2014). Using a specific gene or genes, DNA barcoding is a methodological approach that expedites fish identification, taxonomy, phylogeny, and the mapping of the fish's biogeographic distribution (Hosam *et al*., 2021). DNA barcoding is created on the idea that a target specimen's uniform mitochondrial genome can be sequenced to produce an unknown barcode, which can then be compared to a database of known barcodes to determine the species identity (Sachithanandam and Mohan, 2020). Fish species identification is best accomplished using the mitochondrial DNA (mtDNA) (Sogbesan *et al*., 2017). According to George *et al*. (2019) and Changizi *et al*. (2013), stock structure in a range of fish species has been well studied using analysis of the mtDNA markers to produce phylogenetic trees.

Phylogenetic trees not only describe the similarities and distinctions between species but also aid scientists in understanding how species have developed (Lam *et al*., 2010; Bbole *et al*., 2018). If the visual traits required for the identification of a species are not accessible, the DNA sequence from the mtDNA has been proven handy as an effective method of fish identification (Victor *et al*., 2009; Sogbesan *et al*., 2017). Also, monomorphic fish species have been successfully identified, distinguished, and distributed biogeographically using a pool of DNA barcode sequence data (Bhattacharjee *et al*., 2012). In Nigeria, several researchers have used DNA barcoding in validating Cichlid species, these include, Olefegaba *et al*. (2015) in Kainji Lake, Nigeria, Sogbesan *et al*. (2017) in North-eastern Nigeria and Okomoda *et al.* (2021) in Makurdi, Nigeria. Therefore, to forecast future stocks and set fishing quotas appropriately for fisheries research, accurate species identification is crucial in ichthyological surveys of important freshwater ecosystems (Vivek *et al*., 2014). Zobe Reservoir is an important freshwater resource in the Dutsin-Ma Local Government Area of Katsina State (Sadauki *et al*., 2022). Although it was originally built for irrigation, water supply, and electricity generation, the reservoir is richly blessed with fish species (Salele *et al*., 2023). The fish species in the family tilapia are arguably the most caught and sold in the reservoir (Nababa *et al*., 2022: Ahmad *et al.,* 2014). This study is therefore designed to molecularly identify the tilapia species of the Zobe Reservoir for effective management of the fisheries resources.

Materials and Methods

Study Area (Zobe reservoir) and Tilapia Sample Collection

The sample collection for this study was carried out in the Zobe reservoir located in Dutsin-Ma LGA of Katsina State. The reservoir is an earth-fill structure that was constructed in 1983. The geographical coordinates are 12°23'18" N (latitude) and 7°28'29" E (longitude) (Figure 1). The dam is 48 meters high, 360 meters wide, and 2,750 meters long. Due to its impoundment from two significant rivers namely Karaduwa and Gada,

the reservoir has a storage capacity of 179 Mca (Dasuki *et al*., 2014, Salele *et al*., 2023).

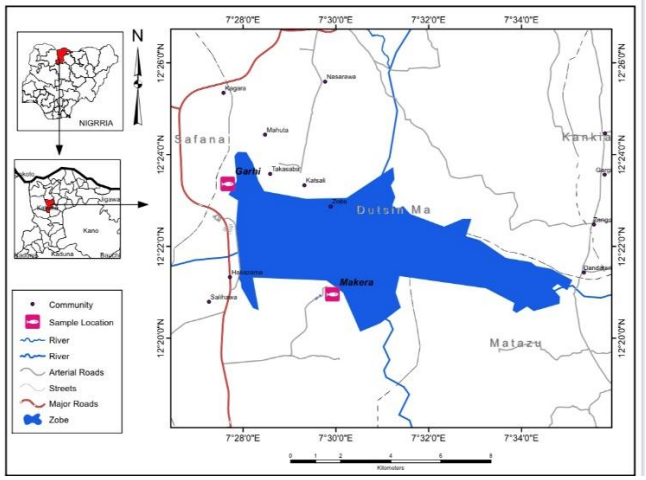

Figure 1: Map of Zobe Reservoir (Source: Salele *et al*., 2023)

Collection of fish was done from (2) two landing sites (Makera and Garhi) of the Zobe reservoir. One hundred (100) Tilapia fish species were randomly sampled and brought in an ice box to the biology lab of Federal University Dutsin-ma. Using the field guide to Nigerian freshwater fish by Olaosebikan and Raji (2013), the specimens were morphologically identified. The identified fish were divided into their distinct species and the fins clip of two samples of each species were preserved in 100% ethanol in different 200ml plastic containers.

Molecular Extraction and Sequencing

DNA extraction of the preserved samples was carried out at the National Institute for Trypanosomiasis Research (NITR), Kaduna, Nigeria using Zymo Research Genomic DNA™-Tissue Mini Prep extraction kit following the procedures by the manufacturer. The extracted DNA quality and quantity were then affirmed using a thermo-scientific nanodrop 2000c spectrometer. The polymerase chain reaction (PCR) was used to amplify the cytochrome C oxidase subunit 1 (CO1) gene using the test primers sequences of FishF1 and FishR1 as stated below.

FishF1(Forward):
TCAACCAACCACAAAGACATTGGCAC

FishR1(Reverse):
TAGACTTCTGGGTGGCCAAAGAATCA (Ward *et al*., 2005)

The 650-bp fragment of the standard CO1 barcode was amplified using a reaction consisting of the following (Table 1).

Table 1: PCR set up concentration

S/N	Reagents	Quantity
1	Polymerase Chain Reactior buffer	1 µl
2	Magnesium Chloride	0.4 µl
3	Forward primer	0.5 µl
4	Reverse primer	0.5 µl
5	Dimethyl sulfoxide	0.8 µl
6	Deoxynucleoside triphosphates	0.8 µl
7	Taq 5u/ul	0.1 µl
8	DNA fragment	6 ng/µl
9	Distilled water	2.9 µl
	Total volume	**10 µl**

PCR amplification reactions were conducted on MyGene Series Peltier Thermal cycler Model MG96G (Long Gene Scientific Instruments, company limited, USA). The thermocycling program consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 60°C for 1 minute and extension at 72 °C for 30 sec, and lastly a final extension at 72 °C for 10 min and a final hold at 10°C for the amplification of CO1 gene (Abubakar *et al*., 2018).

The PCR products were examined for the existence of amplified DNA bands using gel electrophoresis on one per cent (1%) agarose gel prepared by dissolving one gram of agarose powder into 100ml 1X TAE buffer. The approach made use of a Biolab 50 bp gene ladder. The PCR products were sequenced at Inqaba Biotec West Africa Ltd (IBWA) in Ibadan, Nigeria using the

Genetic Analyzer (Applied Biosystems 3130 XL). Cycle sequencing was carried out using the Big Dye Terminator 3.1 Cycle Sequencing Kit.

DNA Data Analysis

MEGA 11 was used to visualize, modify, align, and calculate the percentage nucleotide of the sequences. The gene sequence was submitted to the BLAST algorithm from the National Center of Biotechnology Information, Bethesda, Maryland, USA, and Barcode of Life Database Identification (BOLD-ID) to find comparable sequences in the GenBank. Based on maximal BLAST scores and sequence matches between 96% and 100% coverage, the species identity was validated. Utilising MEGA 11 software, molecular phylogenetic analyses were carried out. Kimura 2 Parameter distances (K2P) (Kimura, 1980) were used to generate a Maximum Likelihood tree and distance matrix. For distance calculation, a minimum of 1000 bootstrap replications were used. There were 9 nucleotide sequences in this analysis. Codon positions 1st+2nd+3rd+Noncoding were included. The Maximum Parsimony approach was used to automatically generate the initial tree(s) for the heuristic search.

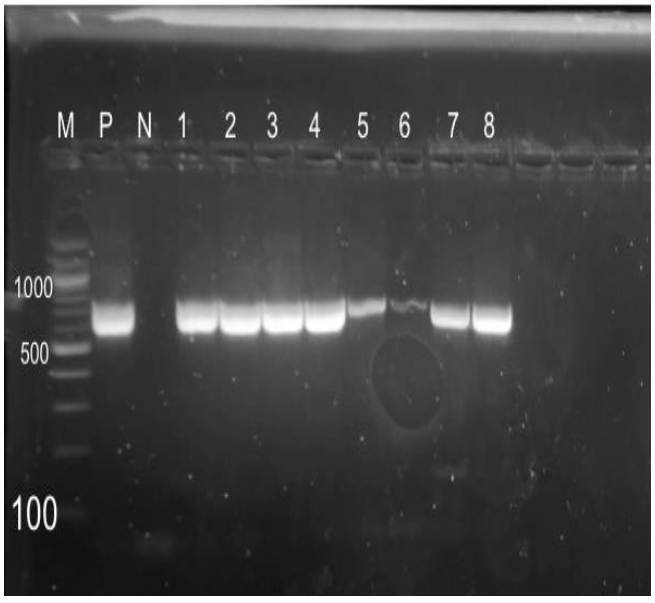

Figure 2: The size of the amplified PCR products for the samples

Results

Four Cichlids species; *Oreochromis niloticus*, *Oreochromis mossambicus*, *Sarotherodon galilaeus* and *Coptodon zilli*i were identified morphologically. The samples' amplified PCR products exceeded the 500 bp threshold specified by the Barcode of Life Database (BOLD) for CO1 gene-based species identification (Figure 2). For the CO1 gene, Table 1 displays the percentage nucleotide compositions of the sampled fish species. The average nucleotide content of the Cytochrome oxidase (1) gene in tilapia species from the Zobe reservoir is adenine (24.4%), thymine (27.7%), guanine (17.6%), and cytosine (30.2%) in average 651 base pair (Table 2).

Sequence Similarity Approach

Table (3) shows the sequence identity carried out using BLAST and BOLD IDs. The two databases showed absolute identity matches ranging between 98%-100% for three species except *O. mossambicus.* Our finding showed that morphologically identified *O. mossambicus* matches 100% with *O. aureus* partial and complete genome in BOLD and 99.5% in BLAST.

Keys: M-Molecular marker, P-Positive control N-Positive control 1-8-Sample

Table 2: Average Nucleotide Compositions of CO1 gene for the Studied Fish Species

Species code	Nucleotides				Total Base pair
	A (%)	T (%)	G (%)	C (%)	
A1	24.0	28.2	17.1	30.7	649
A2	24.2	27.5	17.9	30.4	661
B1	23.1	27.7	17.4	31.9	593
B2	24.8	27.7	17.3	30.3	654
C1	24.7	27.7	17.5	30.1	664
C2	23.6	28.5	18.0	29.9	673
D1	24.2	26.4	19.1	30.3	656
D2	26.7	27.9	17.0	28.4	659
Average	24.4	27.7	17.6	30.2	651

Keys: A- Adenine; T-Thymine, G-Guanine, C- Cytosine. A1-A2 *Oreochromis niloticus,* B1-B2 *Oreochromis aureus, C1-C2 Sarotherodon galilaeus and D1-D2Coptodon zillii.*

Table 3: Identification using BOLD Identification System (BOLD-IDS) and Basic Local Alignment Search Tool (BLAST) GenBank

Species morphologically identified	BLAST GenBank species match	Similarity (%)	BOLD-IDS species match	Similarity (%)
O. niloticus	*O. niloticus*	100	*O. niloticus*	99.9
O. niloticus	*O. niloticus*	99.8	*O. niloticus*	100
O. mossambicus	*O. aureus*	99.5	*O. aureus*	99.5
O. mossambicus	*O. aureus*	99.5	*O. aureus*	100
S. galilaeus	*S. galilaeus*	99.8	*S. galilaeus*	99.3
S. galilaeus	*S. galilaeus*	99.6	*S. galilaeus*	99.2
C. zillii	*C. zillii*	99.8	*C. Zilli*	99.8
C. zillii	*C. zillii*	99.8	*C. Zilli*	99.9

Phylogenetic Relationship

The corresponding taxa's percentage of clustered trees is displayed next to the branches. The final dataset contained 584 locations altogether. The species in the current study were clustered independently within the relevant genera according to the evolutionary relationships of taxa indicated in Figure (3). A varied sequence of *Citharinus citharus* (Out-group) with the accession number HF548669 was added from GenBank to the sequence of the sample fish to identify the phylogenetic tree's origin. The ideal tree is displayed, with a branch length sum of 0.620. A gamma distribution with a shape parameter of 2 was used to model the rate variance between sites.

The average separation between sequences is 0.620 as displayed in Table (4). Within the genus Oreochromis, the average separation is 0.0034. *O. niloticus* is 0.0 within species and 0.0034 between *O. niloticus* and *O. aureus*. The highest mean distance within the group belongs to the Coptodon genus, with a mean distance of 0.026, while Sarotherodon has a mean distance within the group of 0.0052. These figures demonstrated that in the sequence, Oreochromis and Sarotherodon species displayed the highest degree of resemblance, followed by Coptodon species, which demonstrated the greatest degree of variance. The longest mean distance is 0.450 between the genera Oreochromis and Coptodon, and the shortest one is 0.086 between the genera Sarotherodon and Coptodon. The mean distance between the genus Oreochromis and the genus Sarotherodon is 0.034. These figures indicated that from the sequence obtained, the genus Oreochromis and genus Sarotherodon had a higher sequence similarity, followed by the genus Sarotherodon and Coptodon while genus Oreochromis and Coptodon are further apart in terms of their nucleotide composition.

Discussion

The objective of this study was to identify the Cichlids in the Zobe Reservoir with a higher degree of certainty, using a standardized and verified DNA-based technique. DNA barcoding improves the chances for species-level identifications on a worldwide scale (Landi *et al*., 2014). The obtained nucleotide distribution pattern is consistent with the pattern described for fish (Abubakar *et al*., 2018). Guanine (G) frequency is of the lowest percentage while Cytosine (C) is the highest. This study disagrees with that of George *et al*. (2019), who reported that Cytosine has the lowest average nucleotide frequency. Additionally, it demonstrates that Tilapia species had larger cytosine and thymine compositions while having lower guanine compositions, it was found that the nucleotide compositions of *O. niloticus, O. aureus*, and *S. galilaeus* were remarkably comparable. This finding is consistent with that of Hannes *et al*. (2021), who showed that cichlids have low nucleotide diversity within species, with 92% of the populations they assessed having less variety than the median value for nucleotide diversity in other vertebrates. The use of the CO1 gene and DNA barcoding for species identification, particularly in fisheries, has been widely accepted

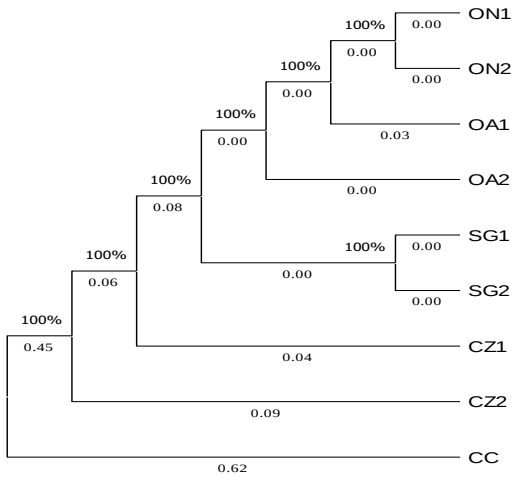

Figure 3: Phylogenetic tree of Cichlids species from Zobe reservoir using the Maximum Likelihood method and Kimura 2-parameter model with 1000 bootstrap replications for distance creation. The initial tree for the heuristic search was obtained automatically by the Maximum Parsimony method. **Keys**; ON-*Oreochromis niloticus* OA-*Oreochromis aureus* SG-*Sarotherodon galilaeus* CZ-*Coptodon zillii* CC-*Citharinus citarus* (outgroup)

Table 4: Overall Mean Distance based on the maximum likelihood tree

Species	Within group	Between-group
Oreochromis niloticus	0.0	0.0
Oreochromis niloticus	0.0	
Oreochromis aureus	0.026	0.026
Oreochromis aureus	0.004	
Sarotherodon galilaeus	0.008	0.038
Sarotherodon galilaeus	0.005	
Coptodon zillii	0.145	0.189
Coptodon zillii	0.262	
Citharinus citarus	2.734	2.734

and verified (Kochzius *et al*., 2010; Ward 2012; Knebelsberger *et al*. 2014; George *et al*. 2019). From the blasting results obtained, all the sequences match with their morphologically identified species, with the exception of *O. mossambicus* which matches 100% with the partial and full genome of *O. aureus* in BOLD and 99.5% in BLAST. The four (4) morphologically identified species were consistent with previous attempts at visual identification reported for the cichlid species in the water body, (Nababa *et al*., 2022: Ahmad *et al*., 2014). Our exploitation of genetic barcoding has revealed for the first time the presence of *O. aureus* rather than *O. mossambicus* in the reservoir. This study supports the idea put forth by Landi *et al*. (2014) that DNA barcoding can be used to correct instances of morphological misclassification. This study is also in agreement with Lakra *et al.* (2016) who reported the potential utility of the CO1 gene in barcoding the fish, as DNA barcodes generated from 72 species of freshwater fish species in India discriminated congeneric species without any confusion. The findings of this study also agree with Sogbesan *et al*. (2017) who identified a cryptic species initially grouped under *Sarotherodon galilaeus* to *be Sarotherodon galilaeus* boulengeri in the upper Benue River in the northeastern part of Nigeria. More so, Iyiola *et al*. (2018) detected mismatched names assigned to 27 species from North-central Nigeria using DNA barcoding.

The phylogenetic tree portrays a relationship between sets of species and represents a model of evolution. The current forms of species retain many of their ancestral features, some of which gradually change to help these species adjust to their environment (Geetika *et al*., 2018; Lam *et al*., 2010). Phylogenetics also relies on information extracted from genetic material such as deoxyribonucleic acid (DNA), ribonucleic acid (RNA) or protein sequences (Bbole *et al.*, 2018). In this study, the evolutionary relationships in the phylogenetic tree were clustered separately within their matching genera. Three distinct genera of the Cichlidae family belonging to Oreochromis, Sarotherodon, and Coptodon were identified. The study indicated that from the sequence obtained, the

genus Oreochromis and Sarotherodon have a higher sequence similarity, followed by the genus Sarotherodon and Coptodon while genus Oreochromis and Coptodon are further apart in terms of their nucleotide composition.

A high level of similarity between sequences usually indicates a remarkable physical and functional resemblance which are related closely in a phylogenetic tree. To obtain accurate information about the similarities that exist between species, a comparison should be made with a set of sequences already stored in a database (Vinga, 2014). However, the results from this study are in agreement with the findings of Iyiola *et al.* (2018) and Popoola *et al.* (2022) in the phylogeny of Cichlids from North-central and Jos, Plateau State (Nigeria) respectively. The close likeliness between the mouth-brooding Tilapia species; Genus Oreochromis and Sarotherodon as shown in the study tallies with the assumption of Sogbesan *et al.* (2017). Fish identification, taxonomy, phylogeny, and biogeographic distribution mapping are all accelerated by DNA barcoding. In general, genetic barcodes are useful for identifying undiscovered fish species, differentiating between overlapping species, or comparing with conventional morphological taxonomy to establish species boundaries (Hosam *et al*., 2021). From the findings of this study, DNA barcoding using mitochondrial CO1 for the identification of fish species especially Cichlids that have high levels of similarity in morphological features is more efficient.

The BLAST results and phylogenetic three revealed a clearer speciation of Cichlid species in Zobe reservoir using DNA barcoding. The observations from this study concur with those made by Sogbesan *et al*. (2017), Iyiola *et al*. (2018) and Popoola *et al*. (2022). Their studies on the phylogeny of cichlids revealed the close resemblances between the genera Sarotherodon and Oreochromis. According to the study's findings, DNA barcoding utilizing mitochondrial CO1 is more effective than typical morphomeristic traits for identifying fish species, especially for Cichlids, which have a high degree of morphological resemblance.

Conclusion

The CO1 gene correctly distinguished the samples to the species level and also fixed the issue of the misidentification between *O. mossambicus* and *O. aureus* that is discovered for the first time in Zobe reservoir. Phylogenetic tree clustered the species independently within three distinct genera of the Cichlidae family belonging to Oreochromis, Sarotherodon and Coptodon. DNA barcoding instead of morphomeristic characteristics was found to be more effective for identifying Cichlids from the Zobe reservoir.

Conflict of Interest

The authors declare no conflict of interest

Ethical Approval

The research was carried out in line with the ethical approval obtained from the ethics committee under the Directorate of Research and Development of Federal University Dutsin-Ma, Katsina

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