

DNA Barcoding of Tilapia species (Pisces: Cichlidae) from North-Eastern, Nigeria

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Abstract

The Tilapiine complexes are made up of diverse species, hence the need for proper identification. Samples of tilapia species belonging to the family of Cichlidae obtained from Upper Benue River and Lake Geriyo landing sites were used for this study. Fish species were morphologically identified *in situ* by visual inspection with the aid of field guide for taxonomic studies. DNA was extracted from fresh fish tissue and the barcode gene region cytochrome c oxidase subunit 1 (co1) was amplified using the FishF1 and FishR1 primer pair. Amplified products were visualized on 1% agarose gel electrophoresis, purified and sequenced using Genetic Analyzer (Applied Biosystems 3130 XL). Four species (*Oreochromis niloticus*, *Sarotherodon galilaeus*, *Sarotherodon galilaeus boulengeri* and *Coptodon zillii*) belonging to three genera (*Oreochromis*, *Sarotherodon* and *Coptodon*) were identified in the study area. From our sampling survey, *Oreochromis niloticus* was the most dominant species in both areas, followed by *Coptodon zillii*. *Sarotherodon* spp. was not present in Upper Benue River but present in Lake Geriyo (an ox-bow lake of Upper Benue River). A new sub-species of *Sarotherodon galilaeus* known as *Sarotherodon galilaeus boulengeri* was identified in this study from the sequencing result. This species has never been reported in any literature to be present in Nigeria, which further confirmed the efficacy of DNA barcoding in the identification of cryptic species. The phylogenetic relationship and genetic distance between generic groups obtained in this study was in concordance with the hypothesis of Trewavas that the mouth brooders *Oreochromis niloticus* and *Sarotherodon galilaeus* are more closely related to each other than the substrate brooders (*Tilapia zillii*).

1. Introduction

The distinction of species within the tilapiine genera is “notoriously difficult” [1] and most published descriptions of the species are based on their morphological characteristics which “show considerable variation and broad interspecies overlaps” [2-7]. Often, geography has played a huge role in the identification of species [1]. Over the past 3 to 5 decade, species identification has become more difficult by the extensive introduction of alien species and transfer of native from outside of their natural ranges, for example in Lake Victoria [8, 9]. This introduction has led to inter-specific hybridization, and the emergence of hybrid forms making morphological identification of species clearly impossible [10-13, 6, 14]. Incorrect description and misidentification of species and possible hybrids using the conventional methods call for better molecular techniques.

Little is known about the relationships among the tilapiine species, despite the fact that they have become an important component of African Aquaculture [1].

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DNA marker technologies have revolutionized the way aquaculture genetics research is conducted [15]. Due to the advances in the sequencing and computational technologies, DNA sequences have become the major source of new information for advance understanding of evolutionary and genetic relationships [16]. Species identification through barcoding is usually achieved by the retrieval of a short DNA sequence (the barcode) from a standard part of the genome (i.e. a specific gene region, cytochrome oxidase 1), from the specimen under investigation [16]. The barcode sequence from the unknown specimen is compared with a library reference barcode sequences derived from individuals of unknown identity (<http://www.barcodinglife.org>). Hence, this study was aimed to identify tilapia species of family cichlidae from Upper River Benue and Lake Geriyo in the northeastern part of Nigeria using DNA barcoding.

2. Materials and Methods

2.1. Study Area

The study areas used for this study are Lake Geriyo (Latitude 9.30°N and Longitude 12.40°E) and Upper River Benue fish landing site (Latitude 9.29°N and Longitude 12.47°E). DNA extraction, polymerase chain reaction and gel electrophoresis was carried out at the Chevron Biotechnology Centre Molecular Biology Laboratory, Modibbo Adama University of Technology, Yola.

2.2. Sample collection and initial identification

All specimens were caught in the wild and morphologically identified in situ by visual inspection. Taxonomic classification was done with the aid of the field guide to Nigerian freshwater fish [17] and freshwater fishes of Nigeria [18]. Fresh/frozen muscle and pectoral fin was collected from the identified fishes.

2.3. DNA Extraction

DNA extraction was carried out using Zymo Research Genomic DNA™-Tissue MiniPrep extraction kit, and the manufacturer protocol was followed. Briefly, in a clean 1.5 ml microcentrifuge tube, 25 mg of fish tissue was added. 95 µl of RNase free water, 95 µl of 2X digestion buffer and 10 µl proteinase k was added to the tube. The mixture was incubated in a water bath set at 55°C for 3 hours. RNase treatment was performed by adding 5 µl of RNase enzyme and incubated at 37°C for 15 minutes.

2.4. Polymerase Chain Reaction (PCR) Amplification

The CO1 gene located in the mitochondrial genome was amplified using the pair of primers FishF1: TCAACCAACCACAAAGACATTGGCAC and Fish R1: TAGACTTCTGGGTGGCCAAAGAATCA (Ward et. al., 2005). PCR amplification was performed with Eppendorf Mastercycler personal in 50 µl total reaction volume using OneTaq Quick Load 2X Master Mix with Standard Buffer (New England BioLabs Inc) ready-to-use kit as shown in Table 1. The PCR was carried out under the following conditions: an initial denaturation of 94°C for 30 seconds, followed by 35 cycles of 94°C for 30 seconds, 60°C for 1 minute, 68°C for 1 minute; followed by final extension of 68°C for 10 minutes and held at 20°C. The PCR products were visualized on 1% agarose gel electrophoresis stained with ethidium bromide.

Tables

PCR setup

Component	50 µl Reaction	Final Concentration
2X Mastermix	25 µl	1X
10 µM Forward Primer	1 µl	0.2 µM
10 µM Reverse Primer	1 µl	0.2 µM
Template DNA	3 µl	
Nuclease-Free Water	20 µl	

2.5. Purification of PCR Products and Sequencing

Zymo Research DNA clean and concentrator™-5 was used to purify the PCR products. In a 1.5 ml microcentrifuge tube, DNA binding buffer was added to the PCR products in a ratio of 5:1. The mixture was transferred to a Zymo-spin™ column in a collection tube. The sample was centrifuged for 30 seconds at 10,000 x g and the flow-through was discarded. To the spin column, 200 µl DNA wash buffer was added and centrifuge at 10,000 x g for 30 seconds. The wash step was repeated. DNA elution buffer was added at a volume of 15 µl directly to the column matrix and incubated at room temperature for a minute. This was transferred to a 1.5 µl microcentrifuge tube and centrifuge for 30 seconds at 10,000 x g to elute the DNA. The purified PCR products were sequenced using Genetic Analyzer (Applied Biosystems 3130 XL). Cycle

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

2.6. Sequence Analysis

The sequencing was subjected to Barcode of Life Database Identification (BOLD-ID) and BLAST algorithm from the National Centre of Biotechnology Information, Bethesda, MD, USA (<http://www.ncbi.nlm.nih.gov>) to retrieve for homologous sequences in GenBank. Sequences were edited in BioEdit [19], and aligned using ClustalX 2.1 software [20]. Phylogenetic analyses were conducted using MEGA 6 [21]. A Maximum Likelihood tree of K2P distances was created to provide a graphic pattern of divergence between the species [22]. A minimum of 1000 bootstrap replications was used for distance creation [23]. Sample identification based on sequence similarity approach was carried out using Barcode of Life Database (BOLD) (<http://www.barcodinglife.org>) and GenBank (<http://www.ncbi.nlm.nih.gov>). All sequences were submitted in GenBank under the accession number KY784669-KY784688, a sequence of *Citharinus citharus* (GenBank: HQ927822) was used as an out-group.

3. Results

3.1. PCR and Sequencing

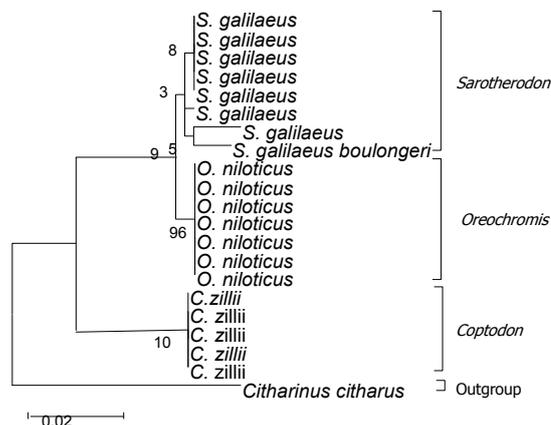
Similarities between sequences were calculated using built in algorithms for local sequence alignments. Both GenBank and BOLD-IDS databases revealed definitive identity matches in the range of 98%-100% for consensus sequences of all the species. In each case, all queried sequence returned a top hit for the morphologically identified species. One of the species morphologically identified as *Sarotherodon galilaeus* returned a hit of 98% and 100% for *Sarotherodon galilaeus boulengeri* in GenBank and BOLD-IDS respectively.

3.2. Phylogenetic Relationship and Sequence Divergence

Multiple sequence alignment was carried out using ClustalX 2.1 [20] and subsequently converted to MEGA format using MEGA 6 before constructing the Maximum Likelihood tree with a bootstrap of 1000. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. From the phylogenetic tree shown in Figure 1 the species clustered independently within their

corresponding genera (i.e *Sarotherodon*, *Oreochromis* and *Coptodon*). The *Coptodon zilli* formed a more cohesive group sharing common ancestor with a bootstrap value of 100%, *Oreochromis niloticus* were held together by a bootstrap value of 96%. The *Sarotherodon* species formed three groups within a cluster and the sequences for this genera showed more variation than the other two (*Coptodon* and *Oreochromis*). The overall mean distance of the CO1 sequences for the three generic groups is 0.081. A higher level of divergence was observed in *Sarotherodon* spp. against *Coptodon zillii* (0.150), *Oreochromis niloticus* and *Sarotherodon* spp showed the lowest level of divergence (0.010), while *Oreochromis niloticus* and *Coptodon zillii* showed a divergence of 0.145.

Figure 1: Phylogenetic tree of *cox1* sequences from *Oreochromis*, *Sarotherodon* and *Coptodon*



4. Discussion

This study has justified the use of mitochondrial DNA CO1 barcodes for identification of fish species and their relationship. All the sequences obtained showed a clear distinction of the three species studied. The result was in concordance with the work of Wu and Yang [24] where the CO1 gene was used to identify captive and wild tilapia species. Similarly, Hubert et al. [25] and Mohanty et al. [26] used the CO1 gene in distinguishing North American freshwater fishes and cultivable carps in India, respectively. In Nigeria, Nwani et al. [27] used the gene to distinguish fresh water fish species from the southern part of the country. The effectiveness of DNA barcoding technology has been validated for various animal groups and most investigated species (>94%) possess distinct barcode arrays, with low intraspecies variation and high

divergences from closely allied taxa [28, 16]. This clear sequence divergence between species, coupled with sequence conservation within species confirmed the barcode CO1 sequence as highly variable and specific.

The branch length within species is more clustered together than the branch length between species which are deeper. This confirms the findings of Meyer and Pauly [29] that branch length between species tends to be much deeper than between conspecific individuals leading to a gap in the distribution of the pairwise distance between conspecific individuals and between species that have been referred to the barcoding gap. The phylogenetic tree clearly separates the three tilapia species into three clustered groups based on their genetic distance. Ward et al. [28] in their study of Australian fish species demonstrated the efficacy of phylogenetic tree in distinguishing species by showing 14 different clusters from 14 species of flathead. Mohanty et al. [26] in their study of cultivable carp clearly distinguished different carp species into different clustered group. The result showed all individuals of a species clustered together.

The close similarities between the mouth brooders: *Sarotherodon* species and *Oreochromis niloticus* as shown in the phylogenetic tree is in concordance with the hypothesis of Trewavas [30], but in disagreement with the hypothesis of Peter and Berns [31]. Similar studies using enzyme loci showed clustering patterns according to their genera i.e. *Oreochromis*, *Sarotherodon* and *Tilapia* [32, 33]. The study of Saad et al. [34], using microsatellite DNA markers showed a clustering pattern of *Tilapia* species according to their generic group. The genetic distance between the three generic groups in this study is less than the values obtained in the study of Gu et al. [35] This could be attributed to the common source of the collection sites in this study. The efficacy of DNA barcode region (CO1), in the identification cryptic species was validated with the identification of *Sarotherodon galilaeus boulengeri*, a subspecies of *Sarotherodon galilaeus* which has never been reported in Nigeria.

5. Conclusion

Mitochondrial CO1 gene is an ideal region for fish species identification. DNA sequence from this gene will improve and aid the identification of fish species even where morphological characteristics needed for the identification of a particular species is not available as a result of predation or smaller samples that cannot be identified using morphological characteristic such as

larvae.

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