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Characterization of the Nigerian mudskipper species; Phylogenetic relationship, population structure and DNA barcoding using the Cytochrome oxidase 1 (CO1) gene

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Abstract
Mudskippers are a unique set of organisms being adapted to life both on land and in water. They have a worldwide distribution and are very speciose in nature, an indication of their very high adaptability. The Nigeria Mudskipper is herein being barcoded for the first time with interesting results. They form a very unique group mapping together with a very thin genetic diversity with intraspecific genetic diversity being between 0.000-0.009. The average nucleotide composition conforms to that of higher organisms (T (thiamine) 31.8, C (cytosine) 26.1, A (Adenine) 23.5 and G (guanine) 18.6. All the DNA barcodes generated have been deposited in the GenBank (Accession number: OM149397-OM149403). Our result is the first and puts paid to the controversies about what species the Nigerian mudskipper is.

Keywords: Mudskipper, DNA barcoding, population structure, phylogeny, intraspecific diversity

Introduction
The family Gobidae comprises of about 258 genera and 1,850 species with distribution in the sub tropics and tropics. Periophthalmus which happens to be one of the genera consists of 19 valid species. (Froese and Pauly 2019) [15]. One major characteristic of the group is their ability to live both on land and in water. Mudskipper species exhibit a range of adaptations to semi-terrestrialism not only within genera, but even within *morphospecies*, delineating a much more complex adaptive scenario than previously assumed (Polgar *et al*., 2017) [1]. Most mangrove ecosystems in the world are home to various species of Mudskippers. Generally they are an important member of the food chain being used as food by Man and several top carnivores. Despite the extensive documentation of the species, there are a lot of controversies about several species. Many countries in the tropics are home to a plethora of types that are poorly described. Parenti and Jaafar (2017) [17] have documented that part from the Fern *Acrostichum* aureum that live in the six biogeographical regions. Mudskippers are the only other organisms that are found in the seven disjunct divisions of the world. Many species have restricted distribution and are endemic to their proposed biogeographic regions. Cryptic species are not uncommon in the family Gobidae (Thacker 2003) [9]. Since cryptic species are morphologically similar, distinguishing them solely on morphological characteristics is nearly impossible. However, the number of Mudskipper species could well be significantly increased with further systematics sampling and associated taxonomic studies like this work seeks to do. Apart from the Atlantic mudskipper *Periophthalmus barbarus*, which is a species of mudskipper native to fresh, marine and brackish waters of the tropical Atlantic coasts of Africa, including most offshore islands, through the Indian Ocean and into the western Pacific Ocean to Guam which has been reported by various authors in Nigeria (Etim *et al*., 2002; Chukwu 2013; King and Udo 2001; Abiaobo and Udo 2017; Udoh *et al*.2013) [16, 2, 3, 14], there are also reports of species like *P. modestus, P. koelreuteric* and *P. papilio* have also been reported by authors (Elele and Aziaka, 2019; Lawson 2010; Bob-Manuel. 2011) [6, 5, 7] leading to a fairly confusing situation with the systematics as the morphological basis remains grossly unclear. The mangrove mud plains of Abonema is a rich site for various life forms including mudskippers with the attendant confusion in the nomenclature of the species. The genetic information about the species remains basic and as passed from generation to generation. Therefore, this study is aimed at identifying mudskippers of the Abonema mud planes using the CO1 mitochondrial gene as a DNA barcoding marker.

Materials and Methods

One of the molecular approaches that can be used to identify the fish species quickly and accurately is DNA barcoding using the COI mitochondrial gene. However, the research on the identification of mudskipper fish in Nigeria is sparse. No literature reporting this exists. This study is the first of such attempts. From One hundred A total of 100 samples of Periophthalmus species were collected were collected from Abonnema, Nigeria (4.73075, 6.77565) and subjected to statistical classifiers to see if there would be any subdivisions, 25 individuals were selected for DNA barcoding using the Cytochrome oxidase (CO1) gene segment.

DNA extraction and Nano pore COI barcoding.

They were transport to the laboratory using standard protocols. Approximately 50 mg of the ethanol-preserved fin samples DNA samples were treated with 1 uL of RNAse (10 mg/mL) for 30 minutes at room temperature followed by purification using bead-bead approach (Oberacker *et al*. 2019) [19]. The standard COI primers (Forward Primer: 5'-TTTCTGTTGGTGCTGATATTGCTNTCAACNAAYCAYA ARGAYATYGG-3'; Reverse Primer: 5'-ACTTGCCTGTCGCTCTATCTTCTANACYTCNGGRTGN CCRAARAAYCA-3') with partial Nanopore adapters at the 5' end (underlined) were used to amplify the fish partial COI gene fragment. Polymerase chain reaction (PCR) was performed using WizBio HS 2X Mastermix (WizBio, Korea) and the PCR reaction consists of 0.25 uM of each primer, 1 uL of gDNA and appropriate volume of distilled water and PCR master mix. The PCR profile used was an initial denaturation at 95 C for 3 minutes followed by 35 cycles of [95 °C for 15s, 45 °C for 25s, 72 °C for 30s. The PCR product was visualized on a 1% agarose gel and samples with positive band was directly used for Nanopore barcode indexing reaction to generate barcoded amplicon according to the Oxford Nanopore EXP-PBC096 protocol (Oxford Nanopore, UK). The barcoded amp icons were pooled based on band intensity and purified with 1x vol. of SPRI bead (Oberacker *et al*. 2019) [19]. Approximately 200 fmol of the pooled barcoded amp icons were used as the input for Nanopore LSK110 library preparation (Oxford Nanopore, UK) according to the manufacturer's instructions. The library was subsequently loaded into a Flongle flow cell and sequenced for 24 hours.

Data analysis for Nano pore COI barcoding

Base calling and DE multiplexing of the raw nanopore signals used Guppy v5.0.7 (super accuracy mode). The DE multiplexed fastq file were aligned to the COI gene fragment of *Periophthalmus barbarus* (GenBank: AF391339) using Minimap2 followed by consensus calling with RACON and Medaka (Vaser *et al*. 2017; Delamare-Debouteville *et al*. 2021) [12, 13]. An additional amino acid correction was performed using minibar coder (https://github.com/asrivathsan/miniBarcoder) to correct for any remaining nanopore homopolymer errors that lead to artificial frame shift mutation (Srivathsan *et al*. 2018) [10].

Seventeen individuals were successfully amplified. The final COI alignment was truncated to 657 base pairs (bp). The aligned sequences were screened for nucleotide variable sites, parsimony informative sites, the number of haplotypes (nh), and amino acid substitutions in MEGA 6.0 (Tamura *et al*., 2013) [8]. Further, the nucleotide composition was also calculated. The genetic diversity indices, namely haplotype diversity (H) and nucleotide diversity (π), were calculated in DnaSP v6 (Rozas *et al*., 2017) [18]. All haplotype sequences obtained in this study were deposited in the GenBank database (Accession number: OM149397-OM149403).

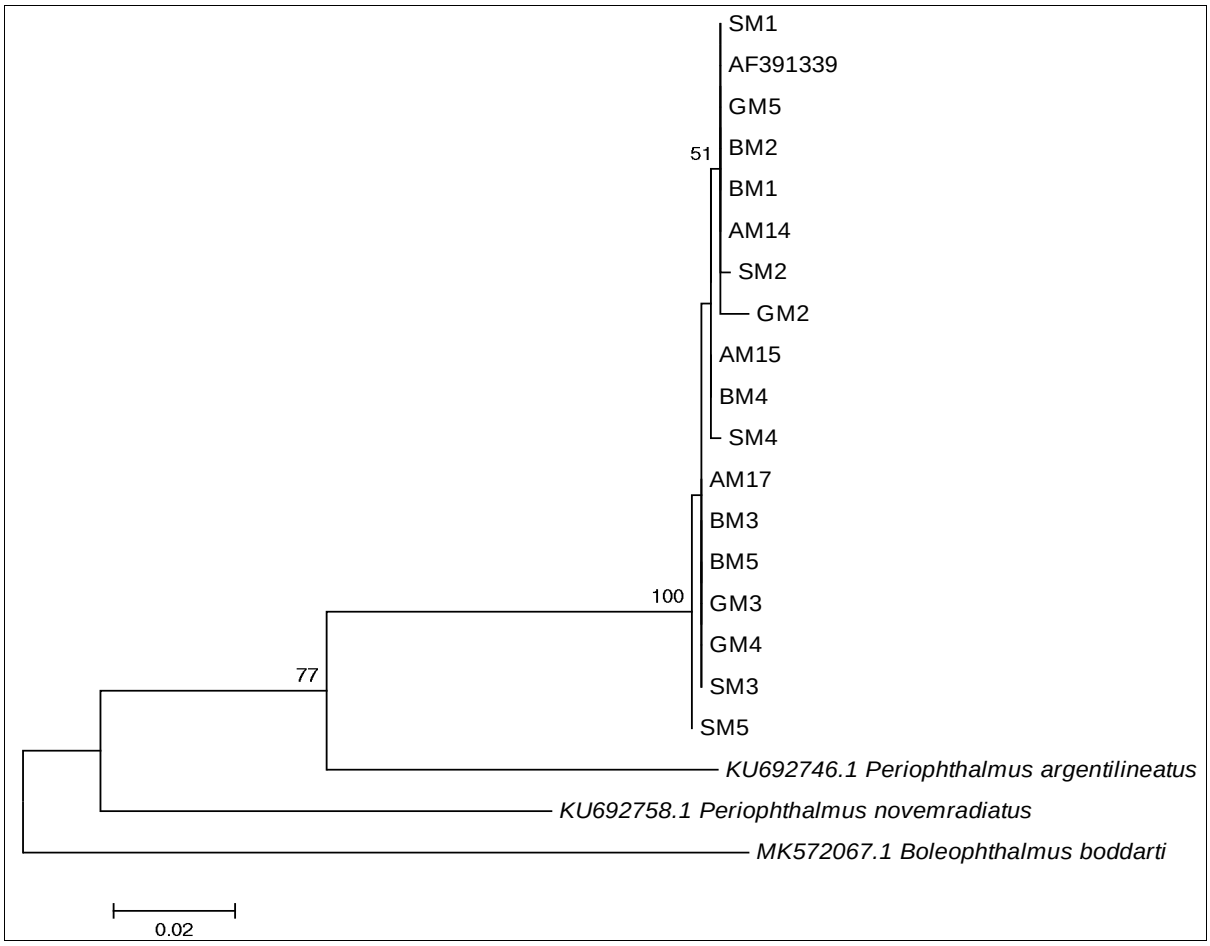
The best nucleotide substitution models with the lowest BIC (Bayesian Information Criterion) score for the dataset was the Kimura 2-parameter (K2P) model (Kimura, 1980) [11]. The phylogenetic relationships of *P. barbarus* were assessed by constructing a maximum likelihood (ML) tree in MEGA 6.0. The robustness of the statistical support for the ML tree branch was determined by 1,000 bootstrap replicates (Felsenstein, 1985) [20]. The only COI sequence of *P. barbarus* available in the GenBank database (AF391339) that was recorded from Nancy Aguilar, Nigeria (Thacker, 2003) [9] was included in the ML tree analysis. Additional CO1 mitochondrial sequences of the Silver line mudskipper (*P. argentilineatus*) (KU692746), the Pearse's mudskipper (*P. novemradiatus*) (KU692758) were obtained from the GenBank for interspecific and intraspecific analysis. Sequences of the Blue-spotted mudskipper (*Boleophthalmus boddarti*) (MK572067) were used as out group taxa. The genetic distances of the intra- and interspecies were calculated by employing K2P model in MEGA 6.0.

Results and Discussion

COI DNA barcoding of *Periophthalmus barbarus*

Final alignment of the COI sequences revealed a total of eight variable sites, resulting in a total of seven putative haplotypes. All nucleotide substitutions resulted in a silent mutation. The average nucleotide composition is T (thiamine) 31.8, C (cytosine) 26.1, A (Adenine) 23.5 and G (guanine) 18.6. The pattern of average nucleotide is persistent with the CO1 gene of higher organisms. The aligned CO1 gene sequence for Nigerian P. barbarus had 643 conserved sites and 8 variable sites of which 2 were parsimoniously informative and 6 were singletons. Our obtained sequence also has 100% similarity with five of the sequences from the only COI sequence of *P. barbarus* ((AF391339) available in the Gen Bank database. The overall haplotype and nucleotide diversity was 0.809 and 0.0046 respectively. Table 1 below also show the intraspecies genetic distances ranging from 0.000 to 0.008, while pairwise genetic distance of interspecies ranges from 0.124 to 0.203. *Periophthalmus barbarus* (the endemic Nigerian species) had inter-specific distance of between 0.197-0.201 with the *Boleophthalmus boddarti* (MK572067), while the range is from 0.124-0.194 with the *Pariophthalmus argentilineatus* (KU692746) and between 0.157-0.203 between P. barbarous and *Periophthalmus novemradiatus* (KU692758). These species (*B. boddarti*, *P. argentilineatus* and *P. novemradiatus*) are found in the Indo-Pacific, India, New Guinea and China for *B. boddarti*, while *P. argentilineatus* apart from being found in the Indo-Pacific, the southern Red Sea also extends in occurrence to South Africa and the Samoa. *Periophthalmus novemradiatusis* restricted to the Indian Ocean. All these species, like the Nigerian Periophthalmus barbarous are amphidromous.

Maximum likelihood gene tree clusters all samples obtained in this study into a single clade with 100% bootstrap support value (Figure 1).



(https://www.megasoftware.net/resources). Branches were drawn to scale and bootstrap values < 50% were not shown

Fig 1: ML gene tree of *Periophthalmus barbarus* inferred from the CO1 sequences, constructed in MEGA 6.0

These findings strongly suggest that the samples analyzed in this study belong to a single taxon despite being categorized into multiple species identity by the local community. This assertion is further supported by the fact that sequence divergence between the Nigerian specimen which is small and ranged from 0.000 to 0.009. The advanced knowledge gained from this research work is of utmost importance as it will guide future researchers who are of the assumption that there are more than one species and also because of the significance of the species as a food source vis-à-vis the effective conservation and management of mudskippers in Nigeria. This study also demonstrates the importance of genetic approach in delimiting species boundary when knowledge on the external morphology is lacking.

Table 1: Pairwise genetic distance of the intra- and interspecies of *Periophthalmus barbarus* inferred from the COI sequences

	AM14	AM15	AM17	BM1	BM2	BM3	BM4	BM5	GM2	GM3	GM4	GM5	SM1	SM2	SM3	SM4	SM5	BB	PA
AM15	0.002																		
AM17	0.003	0.002																	
BM1	0.000	0.002	0.003																
BM2	0.000	0.002	0.003	0.000															
BM3	0.003	0.002	0.000	0.003	0.003														
BM4	0.002	0.000	0.002	0.002	0.002	0.002													
BM5	0.003	0.002	0.000	0.003	0.003	0.000	0.002												
GM2	0.005	0.006	0.008	0.005	0.005	0.008	0.006	0.008											
GM3	0.003	0.002	0.000	0.003	0.003	0.000	0.002	0.000	0.008										
GM4	0.003	0.002	0.000	0.003	0.003	0.000	0.002	0.000	0.008	0.000									
GM5	0.000	0.002	0.003	0.000	0.000	0.003	0.002	0.003	0.005	0.003	0.003								
SM1	0.000	0.002	0.003	0.000	0.000	0.003	0.002	0.003	0.005	0.003	0.003	0.000							
SM2	0.002	0.003	0.005	0.002	0.002	0.005	0.003	0.005	0.006	0.005	0.005	0.002	0.002						
SM3	0.003	0.002	0.000	0.003	0.003	0.000	0.002	0.000	0.008	0.000	0.000	0.003	0.003	0.005					
SM4	0.003	0.002	0.003	0.003	0.003	0.003	0.002	0.003	0.008	0.003	0.003	0.003	0.003	0.005	0.003				
SM5	0.005	0.003	0.002	0.005	0.005	0.002	0.003	0.002	0.009	0.002	0.002	0.005	0.005	0.006	0.002	0.005			
BB	0.197	0.199	0.199	0.197	0.197	0.199	0.199	0.199	0.199	0.199	0.199	0.197	0.197	0.197	0.199	0.201	0.197		
PA	0.130	0.128	0.126	0.130	0.130	0.126	0.128	0.126	0.130	0.126	0.126	0.130	0.130	0.131	0.126	0.126	0.124	0.194	
PN	0.161	0.159	0.157	0.161	0.161	0.157	0.159	0.157	0.159	0.157	0.157	0.161	0.161	0.163	0.157	0.157	0.155	0.203	0.161

Note: BB: *Boleophthalmus boddarti* (MK572067); PA: *Periophthalmus argentilineatus* (KU692746); PN: *Periophthalmus novemradiatus* (KU692758)

Conclusion

Mudskipper can be easily misidentified due to lack of taxonomical knowledge and phenotypic plasticity. DNA barcoding is deemed appropriate and affordable for a faster and more efficient approach for an accurate species identification, as demonstrated in the current study. This study revealed that a reliable DNA barcoding reference library should be undertaken. Besides, based on very many years of traditional knowledge, the local populace had indicated that there are three different species that we sampled namely the Atlantic mudskipper (AM), the blue spotted (BM) and the silverline mudskipper (SM). At best, it is safe to assume that these are morphotypes of the same species showing diverse phenotypes. The result from the CO1 segment indicate on the contrary that they are just one species. The CO1 mitochondrial gene is confirmed here as an accurate gene for the identification of mudskippers as Parenti and Jaafar (2017) [17] had inferred from their worldwide survey of mudskippers that from the western African coast to Morocco and Angola, ust one species of mudskippers is found in this zone. This greater implication of this is that there should a review of the morphological basis of classification.

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