

Molecular characterization of *Malapterurus minjiriya* Sagua, 1987 and phylogenetic relationships within the genus *Malapterurus* (Siluriformes, Malapteruridae) from Nigerian inland water bodies

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Abstract

Molecular (mitochondrial cytochrome C oxidase subunit 1– COI) analysis was performed to characterize the poorly known *Malapterurus minjiriya* from Nigerian inland water bodies. Integrative taxonomy, involving morphological and molecular data, confirms the identity of *M. minjiriya*. Matrilineal genealogy reveals a sister relationship of *M. minjiriya* with *Malapterurus electricus* and *Malapterurus microstoma*. The genetic analysis further shows evidence of population divergence within *M. electricus* and *Malapterurus beninensis*. The findings of the study highlight the importance of the integration of DNA barcoding in biodiversity documentation of freshwater fish species in Nigeria.

KEYWORDS

conservation, DNA barcoding, freshwater fish, morphometrics, Nigeria

The African electric fishes in the family Malapteruridae are an economically important group of freshwater fishes in Nigeria (Bake *et al.*, 2016). They are characterized by the presence of a well-developed electrogenic organ, three pairs of barbels, a swim bladder elongating to posterior chambers and the lack of dorsal fins (Olaosebikan & Raji, 2013; Sagua, 1979). Endemic to tropical African rivers (Leveque *et al.*, 1991; Norris, 2002), malapterurids form an essential fishery resource as benthopelagic fish catch (Belbenoit *et al.*, 1979; Garba & Arome, 2006; Norris, 2002). In Nigeria, it is also used in ethnomedicine for the treatment of brain disorders, infertility and stroke (Sowunmi, 2007).

Previous studies have documented the presence of four species of malapterurids from the Nigerian freshwater ecosystem (Bake *et al.*, 2016; Olaosebikan & Raji, 2013): *Malapterurus electricus* Gmelin 1789, *Malapterurus microstoma* Poll & Gosse 1969, *Malapterurus beninensis* Murray 1855 and *Malapterurus minjiriya* Sagua 1987 (Bake *et al.*, 2016; Idodo-Umeh, 2003; Moller, 1995; Olaosebikan & Raji, 2013; Sagua, 1987; Teugels, 1996). These species differ remarkably in their patterns of geographic distribution in Nigeria (Bake *et al.*, 2016). For instance, whereas *M. electricus* is widely distributed across inland water bodies of Nigeria (Olaosebikan & Raji, 2013), *M. minjiriya* is present in the Niger River and Lake Kainji (Sagua, 1987). On the contrary, *M. beninensis* is found in Benin rivers (Olaosebikan & Raji, 2013), whereas

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M. microstoma occurs in the Zaire River basin (Bake *et al.*, 2016). In Nigeria, *M. minjiriya* occurs sympatrically with *M. electricus* (Sagua, 1987).

The systematics of *M. minjiriya* is a matter of continuing debate, mainly because of the similarities in their external morphology and internal anatomy with other *Malapterurus* species (Norris, 2002; Sagua, 1987). The difficulty in the species-level assignment of *M. minjiriya* could be attributed to the reliance on the use of morphological characters in species identification. Sagua (1987) reported that body spotting and pattern of skin blotches could be used to differentiate species of *Malapterurus*. Nonetheless, the author opined that these characters alone are not a reliable criterion for species delineation as individuals of different species tend to have a similar pattern of body spotting and skin blotches (Sagua, 1987). Recently, Iyiola *et al.* (2018) reported an unidentified *Malapterurus* species from north-central Nigeria. It is generally unknown if this species could be identified as any of the known *Malapterurus* species in Nigeria or an entirely new species.

Correct identification of morphologically similar species is essential for fisheries management and biodiversity documentation (Amanda & Luciane, 2010). Recent studies on Nigerian freshwater fishes showed that genetic data, in combination with morphological data, could aid accurate and unambiguous identification of the fish species (Nneji *et al.*, 2020; Oladipo *et al.*, 2020; Uden *et al.*, 2020). The application of molecular tools, involving mitochondrial cytochrome c oxidase subunit I (COI), has contributed significantly to fish systematics (Iyiola *et al.*, 2018; Nwani *et al.*, 2011; Oladipo *et al.*, 2020). This method, commonly referred to as DNA barcoding, has been successfully used to resolve species-level placement, especially in cases where conventional methods may result in ambiguities (Abdullah & Rehbein, 2017; Hubert *et al.*, 2008). Currently, DNA barcoding has been advocated as a useful tool for the species identification of various life stages and identification of hidden diversity (Iyiola *et al.*, 2018; Nneji *et al.*, 2020; Ward *et al.*, 2005). Further, the mitochondrial COI gene has been used in elucidating phylogenetic relationships between and within fish species (Iyiola *et al.*, 2018; Nneji *et al.*, 2020; Uden *et al.*, 2020).

To date, there is no information on the DNA barcodes of *M. minjiriya*. Such data are essential for the rapid and accurate identification of *M. minjiriya*. To address this, the authors of this study amplified the mitochondrial COI gene sequence of *M. minjiriya* from Nigeria. Further, they analysed the newly and previously published COI genes of *Malapterurus* species to resolve the identity of the unidentified *Malapterurus* species described by Iyiola *et al.* (2018). Using the DNA barcodes, the authors attempted to investigate the phylogenetic relationship among *Malapterurus* species. The field data provided evidence of the range extension of *M. minjiriya* in Nigeria.

The care and use of experimental animals complied with Nigerian animal welfare laws, guidelines and policies as approved by the University Ethical Review Committee of the University of Ilorin, Ilorin, Nigeria, and the permit reference number UERC/ASN/2017/991. Between May and October 2018, individuals of *M. minjiriya* ($n = 5$) and closely related *M. electricus* ($n = 14$) were collected from the lower Niger River in Jebba and Kpateji in Kwara State, and River Kaduna in Kaduna State, Nigeria. Field surveys were conducted in the morning

with the aid of local fishermen. Fishes were collected using gillnets and hook-and-line. Captured individuals were packed in a box containing ice blocks and transported to the Zoology Laboratory of the Department of Bioscience and Biotechnology, Kwara State University, Malete, Nigeria.

Preliminary identification of specimens was conducted with the aid of published literature (Norris, 2002; Olaosebikan & Raji, 2013; Sagua, 1987). The morphometric and meristic counts were done following Sagua (1987). The morphometrics of the voucher specimens was documented using a digital calliper with a precision of 0.01 mm. Fourteen linear measurements were taken as follows: head length, body height, snout length, snout width, pre-anal distance, pre-pelvic distance, anal length, pelvic length, pectoral length, depth of adipose fin, caudal peduncle length, head depth, eye diameter and mouth width. Measurements were taken to the percentage standard length and head length. Further, the meristic characters (gill rakers, anal-fin ray, caudal-fin ray, pectoral-fin ray and pelvic-fin ray) were examined and counted visually.

For the molecular-based identification, a caudal-fin tissue was taken from the voucher specimens of *M. minjiriya* and *M. electricus*. Tissue samples were preserved in 95% ethyl alcohol and stored at 20°C for further use. The voucher specimens (SOO201901 and SOO201902) were fixed with 10% formalin, preserved in 75% ethanol and were deposited in the Zoology Unit, Department of Biosciences and Biotechnology, Kwara State University, Nigeria. DNA extraction of the ethanol-preserved tissue was performed following the phenol-chloroform extraction procedure (Sambrook & Russell, 2001). The laboratory protocols for the PCR amplification and sequencing followed the method described by Iyiola *et al.* (2018). The primers used for the amplification were designed by Ward *et al.* (2005): FishF1–5'TCAA CCAACCACAAAGACATTGGCAC3' and FishR1–5'TAGACTTCTGG GTGGCCAAAGAATCA3'. The purified PCR products were directly sequenced in both forward and reverse directions using an automated DNA sequencer. The nucleotide sequences were viewed using SeqManTMII (DNASTAR Lasergene 7). An alignment of 651 bp of COI sequences was built with ClustalW with default parameters in MEGA 6.0 (Tamura *et al.*, 2013). The aligned sequences were translated into amino acids to check for premature stop codons and to confirm that the open reading frame was maintained in the protein-coding locus.

To confirm the identity of the amplified sequences, all sequences were compared with COI barcode sequences in the GenBank using "BLAST" (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), and on the Barcode of Life Database (BOLD) using the "Identification Request" function (https://www.boldsystems.org/index.php/IDS_IdentificationRequest). The authors searched the GenBank and BOLD databases for the partial sequences of the COI-5' barcode region of other *Malapterurus* species. In this study, only sequences from the BOLD database were used because it provides information such as collection data, specimen depository and identifier. For a more comprehensive inference, two other species of *Malapterurus* known to occur in west-central Africa – *Malapterurus melanocheir* Norris, 2002 and *Malapterurus monsembeensis* Roberts 2000 – were included. In sum, a total of 22 COI

sequences were downloaded from BOLD as follows: *M. melanochir* ($n = 1$), *M. monsembeensis* ($n = 5$), *M. electricus* ($n = 5$), *M. beninensis* ($n = 8$), *M. microstoma* ($n = 1$) and *M. minjiriya* ($n = 1$). Two species were selected as out-group taxa: *Gymnarchus niloticus* Cuvier, 1829 (MG824592) and *Labeo parvus* Boulenger, 1902 (MG824605), based on a previous study (Iyiola *et al.*, 2020).

Prior to this, the best-fit nucleotide substitution model of the *COI* data set was selected as GTR + I + G using the AIC implemented in MrModel Test version 3.7 (Posada and Buckley 2004). Phylogenetic analysis was performed using a maximum likelihood (ML) as inferred in RAXML version 7.2.8 (Stamatakis, 2006). The analysis used a gamma distribution evolutionary model with 100 random addition replicates and per partition branch lengths. The branch support for the optimal likelihood tree was estimated using 1000 bootstrap replications. The phylogenetic tree was constructed in the CIPRES Science Gateway version 3.3 web service (Miller *et al.*, 2010). The resulting phylogenetic tree was viewed using Fig Tree v1.4.2 (Rambaut, 2012). The intra- and interspecific genetic divergences were estimated using the uncorrected *p* distances as implemented in MEGA 6.0 (Tamura *et al.*, 2013).

The results showed that the morphological characters of *M. minjiriya* were consistent with previous descriptions by Sagua (1987) (Supporting Information Table TABLE S1). The voucher specimen was morphologically identified as *M. minjiriya* by the presence of a relatively narrower head, mouth and snout, maxillary and outer mandibular barbels of almost equal length and short adipose fin that is posteriorly sloped (Figure 1). *M. minjiriya* can be distinguished from the sympatric *M. electricus* by the pattern of body spotting and the intensity and distribution of the skin blotches (Figure 1).

The field survey discovered, for the first time, the presence of *M. minjiriya* in River Kaduna ($10^{\circ} 48' 52.6''$ N, $07^{\circ} 40' 15''$ E). Previously, its distribution in Nigeria was thought to span across the Niger River and Lake Kainji (Sagua, 1987). The authors' observation, therefore, strengthens the importance of an extensive survey of freshwater bodies in Nigeria to document the fish species richness, distribution and diversity patterns.

The BLAST search for the *COI* sequence of the newly collected *M. minjiriya* showed a 100% DNA sequence similarity with *Malapterurus* sp. LMN-2018 (MG824612) recorded by Iyiola *et al.* (2018). The Neighbour Joining tree constructed via the BOLD database and the ML tree analysis (Figure 2) showed that the newly

collected *M. minjiriya* clustered genetically in a clade consisting of *Malapterurus* sp. (MG824612) from Nigeria. This was strongly supported by a bootstrap support of 100% in the ML phylogram. This finding confirms the identity of *Malapterurus* sp. LMN-2018 (MG824612) collected by Iyiola *et al.* (2018) as *M. minjiriya*. The mean genetic distance of the partial sequence of the *COI* gene between species *M. minjiriya* and other species of *Malapterurus* included in the present study ranged from 7.0% to 11.80%. The lowest level of interspecific distance was detected between *M. minjiriya* and *M. melanochir* (7.00%), whereas the highest pairwise distance occurred between *M. minjiriya* and *M. beninensis* (11.80%). This result aided unambiguous identification and delineation of *M. minjiriya*. This study shows that the production of DNA barcodes and extensive morphological evaluation is an essential contribution to the rapid identification of *Malapterurus* species from Nigeria. All the newly amplified sequences were deposited in the GenBank (MT890459-MT890460).

The phylogeny generated as part of this study was not only informative to confirmation of the identity of *M. minjiriya* but also had implications for phylogenetic relationships within the genus *Malapterurus*. The ML analysis revealed that *M. minjiriya* formed a sister group relationship with *M. microstoma* and *M. electricus* (Figure 2). This study revealed the possibility of population divergence within *M. electricus* and *M. beninensis*. Divergences among populations of *M. electricus* and *M. beninensis* were evident, as shown in the matrilineal phylogram (Figure 2). Further, this study indicates that *M. electricus* and *M. beninensis* had higher intraspecific divergences compared to other *Malapterurus* species included in this current study. The finding on population divergence was consistent with previous studies (e.g., Benzaquem *et al.*, 2015; Iyiola *et al.*, 2018; Mat Jaafar *et al.*, 2012; Mohammed *et al.*, 2016; Nneji *et al.*, 2020; van der Bank *et al.*, 2012) that showed the effectiveness of DNA barcoding in uncovering cryptic lineage diversities in fishes. Increasing sample size and geographic sampling range may discover more cryptic diversity within *M. electricus* and *M. beninensis*. This will aid the investigation of processes governing population divergence within these species. Also, a careful examination of possible morphological variations and inclusion of more genetic analyses (including multiple mitochondrial and nuclear genetic markers) would aid in testing the hypothesis on the population divergence.

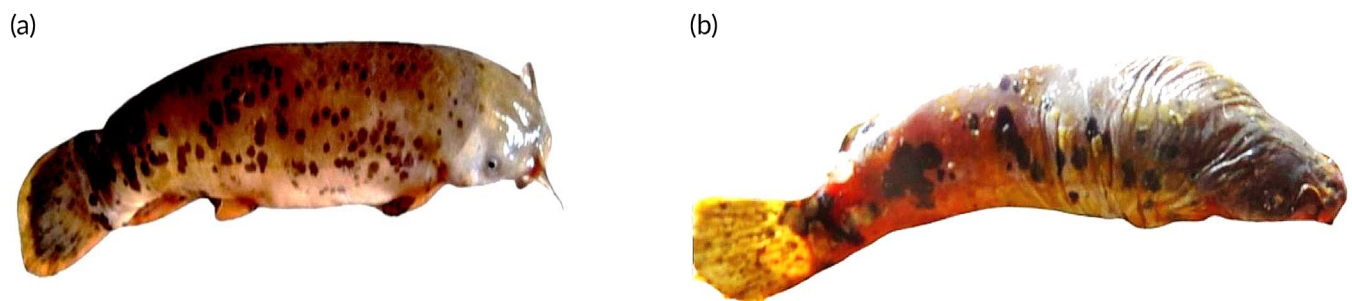


FIGURE 1 (a) *Malapterurus minjiriya* and (B) *Malapterurus electricus* from River Niger in Jebba, Kwara State, Nigeria

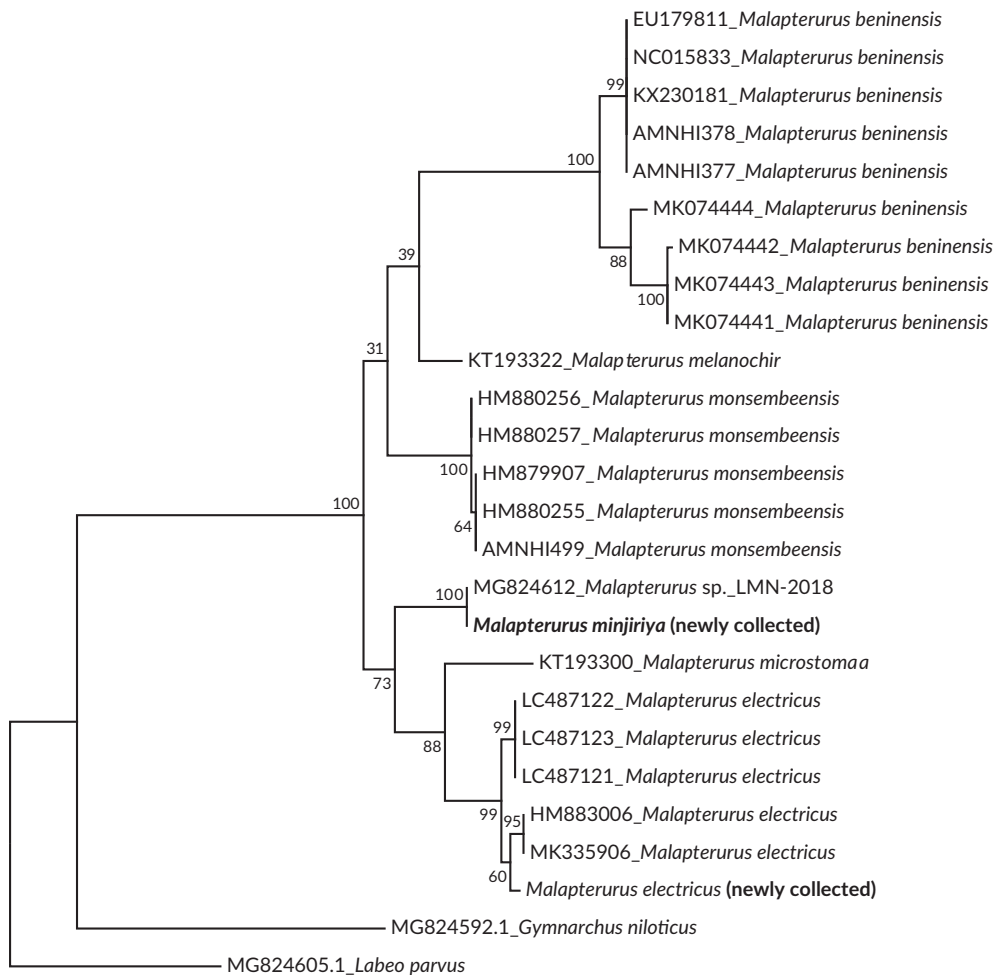


FIGURE 2 Phylogenetic tree inferred from maximum likelihood analysis of *Malapterurus* species based on mtDNA COI data set. Values above branches are the bootstrap supports. Individuals written in bold letters were newly sequenced, whereas others were downloaded from the Barcode of Life Database

In conclusion, this study enabled the generation of DNA barcode of *M. minjiriya* for quick and reliable identification. Also, this study confirms the identity of previously unidentified *Malapterurus* species described by Iyiola *et al.* (2018) as *M. minjiriya*. This study shows that the pattern and distribution of body spotting and blotches can be used to morphologically distinguish *M. minjiriya* and *M. electricus*. Fieldwork discovered a new distribution record of *M. minjiriya* in Nigeria, thus expanding the geographic range for this species in Nigeria. Therefore, the authors recommend more exhaustive sampling of freshwater fishes in Nigeria from broad geographical areas to get a clear insight into the species richness and diversity patterns in Nigeria.

Presently, there is an increasing threat to fishery resources in Nigeria due to human activities such as overfishing and pollution (Oladiipo *et al.*, 2020). Given this, there is a need for improved conservation and management of the fishery resources. The best sustainable measures for the protection of fish species in Nigeria may be the implementation of fisheries regulations such as the prohibition of unsafe fishing practices such as the use of chemicals for fishing, the prohibition of fishing in the breeding grounds and proper management guidelines for mesh sizes for fishing. Also, conservation outreach among the fishermen and involvement of local communities in developing long-term plan for the conservation of fish are vital in achieving sustainable protection of aquatic resources in Nigeria.

Finally, the authors recommend further study investigating evolutionary processes governing the population divergence observed in *M. electricus* and *M. beninensis*. Such a study would involve extensive sampling across the species' range and inclusion of multiple data sets such as morphological, ecological and genetic data. The genetic analyses will demonstrate if these species have several management and conservation units, and if each lineage should be regarded separately in determining long-term conservation strategies.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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