Research Article

Identifying Single DNA Barcode Regions of *Oreochromis niloticus* of Shahpura Lake, Bhopal and their Contribution to a Reference Library

Deepak Raghuwanshi, Rajkumar Garg*, Roshini Khandait, Parul Jagdish, Vipin Vyas

Department of Bioscience, Faculty of Life Sciences, Barkatullah University, Bhopal, Madhya Pradesh, INDIA.

Submission Date: 03-03-2024; Revision Date: 14-03-2024; Accepted Date: 14-03-2024.

ABSTRACT

Background: The present investigation was carried out on the development of a barcode and DNA sequences database of *Oreochromis niloticus* (Nile Tilapia) of Shahpura Lake, Bhopal through mitochondrial Cytochrome c Oxidase-I gene (*cox1*) for public domain uses as a reference database for identification, authentication and variation studies. **Materials and Methods:** We performed the mitochondrial genomic analyses for molecular studies of which genomic DNA was extracted from fish tissues using the standard protocol provided by Janarthanan and Vincent (2007). Then, isolated DNA was introduced to Polymerase Chain Reaction (PCR) using universal primers, after which, the electrophoresis of the PCR product was done, then, obtained the DNA bands of interest of desired molecular weight on the gel. **Results:** We generated 02 unique DNA barcodes of morphologically identified fish specimens collected from Shahpura Lake, Bhopal. Considering the ambiguous (0%), Barcode Index Number (BIN) URI (BOLD:AAC9904), Top Hit % (100%), Nearest Member of Neighbor (ANGBF54446-19), Nearest BIN URI (BOLDAET5315), Analysis of Barcode Gap as an average intra-specific (204.14) and Analysis of Cluster Sequences (RESL) (20.583717) was found significant for development of DNA barcode of *O. niloticus*. The overall mean distance among *O. niloticus* specimens (6A and 6B) was obtained as 1.82 which may be considered as good for conservation point of view. RESL in the BOLD systems has a stronger taxonomic performance than that of the Barcode Gap Analysis and thus showed better species identification, during the present investigation achieved similar results, which may be related to the species identification.

Keywords: DNA barcoding, *COX1* gene, Sanger's DNA sequencing, Barcode Index Number (BIN), Barcode Gap Analysis

Correspondence:

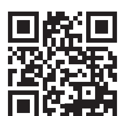
Dr. Rajkumar Garg
Associate Professor and Head, Department of Bioscience, Faculty of Life Sciences, Barkatullah University, Bhopal-462026, Madhya Pradesh, INDIA.

Email: drrkgarg136@gmail.com, rajkumar_garg@bubhopal.ac.in

INTRODUCTION

Fauna contributes more than half of the vertebrate's species of which nearly 7.7% of the world's fish variety which included 20% as vulnerable, endangered, or extinct.[1] In addition to this, fishes are also very nutritious and apart from these fishes also contribute significantly to ecological conservation.[2] However, the survival of the fishes has been negatively impacted by human activities including overfishing, sewage discharge and the construction of river dams.[3] Therefore, the molecular-based taxonomy and identification of fish fauna constitute essential requirements for the conservation of a variety of fishes.

SCAN QR CODE TO VIEW ONLINE



www.ajbls.com

DOI: 10.5530/ajbls.2024.13.28

Oreochromis niloticus (Linnaeus 1758) is a 'cichlid species' although it is an alien fish species for India, it is naturally found in the Nile River and other tropical and sub-tropical freshwater rivers, reservoirs and lakes in Africa.[4-8] *O. niloticus* is important for aquaculture and economy

and they are frequently utilized to produce intra-specific hybrids.

Characterizing the single DNA barcode-based database of this *O. niloticus* in the field of aquaculture is important because it can be used to select strains for brood stock creation and oversight that will minimize inbreeding and preserve genetic diversity.[9] The progress in taxonomy and biodiversity research has demonstrated the effectiveness of molecular methods, particularly DNA barcoding, for recognizing fish variety around the globe, especially in India.[10-14] Interestingly, DNA barcoding, which is quick and inexpensive and relies on the sequence divergence pattern of the Cytochrome c Oxidase I (*cox1*) gene, appears to be a more trustworthy method for identifying species.[15]

The primary goal of DNA barcoding is to create databases of barcode sequences, such as Fish Barcode of Life (FISH-BOL) and International Barcoding of Life (iBOL.org). The Fish Barcode of Life Project is a collaborative worldwide research effort that seeks to establish a reference library of DNA barcodes for every species of fish derived from specimen vouchers with trustworthy taxonomic assessments.[16] When finished, FISH-BOL will provide a rapid, accurate and systematic approach for molecularly identifying the aquatic fauna of the planet.

To address difficulties in fish taxonomy, morphometric analysis and DNA barcoding are being employed in tandem to identify fish. In this essence, the current investigation is aimed at identifying single DNA barcode regions of *O. niloticus* of Shahpura Lake, Bhopal and also to build a DNA sequence record for open-source usage on bold systems and NCBI, USA as a reference library for identification of mislabelled samples.

MATERIALS AND METHODS

Study site and sample collection

Shahpura Lake is a man-made pond located in one of the upscale neighbourhoods of Bhopal, the state capital of Madhya Pradesh. The lake is surrounded by urban areas and hence it is no longer used for irrigation, but it acts as recreation, a waterfront for the residents and fishery purposes. Apart from these, the lake is also having multiple inflowing drains that continue to bring wastewater to it, thus causing heavy pollution. Therefore, this study was conducted in Shahpura Lake District Bhopal, Madhya Pradesh (23.2031º N, 77.4229ºE).

The fish species under investigation, *O. niloticus* often caught by fishermen and sold in marketplaces as eating fish despite not having any protection under the Wildlife Act. As a result, no special authorization is needed to purchase this fish in India and no live specimens were used throughout the lab studies.

Since it was not possible to make repeated trips to Shahpura Lake, the samples needed for molecular analysis for DNA barcoding and sequencing examinations were obtained with the assistance of local fishermen. To save money, the muscle and liver tissues were extracted concurrently from the identical fish at each site. 2-4 *O. niloticus* specimens have been collected with 500 mg of tissue from muscles and 100 mg of tissue from the liver being taken and fish tissue has been preserved in 70% ethanol for molecular studies and then brought to the laboratory for genetic analysis. The genetic study employed standard procedures, such as genomic extraction, quantification of DNA isolated, PCR amplification, agarose gel electrophoresis and consequently Sangers sequencing of the *cox1* gene.

Extraction of Genomic DNA and quantification

Genomic DNA was isolated from the tissue by using phenol: chloroform: isoamyl (25:24:1) technique with minor modifications.[17] By using 1% agarose gel electrophoresis, the isolated genomic DNA's quantity and integrity were assessed as part of an authenticity check. To preserve the ratios of 1.8 of 260 by 280, samples were processed both using RNAase or proteinase-K and maintained the quality (proportion near 1.8 of 260 by 280) and quantity (40-60 ng/µL) of the genomic DNA. Dilution of quantified genomic DNA to a concentration of 50 ng/µL was essential for PCR-mediated amplification for the *cox1* gene.

Polymerase chain reaction of cox1gene

The COX1 gene was targeted and amplified using a set of universal primers (Table 1).[18]

Forward (FishF1: 5-TCAACCAACCACAAAGACATT GGCAC-3)

Reverse (FishR1: 5-TAGACTTCTGGGTGGCCAAA GAATCA-3)

A 96-well Thermal Cycler (Model: Veriti, Applied Biosystems, USA) was used for PCR amplification

Table 1: Universal primer set used for amplification of *cox1* gene of *O. niloticus* for species-voucher 6A and 6B.

Primer Name	Primer Sequences	GC %	Tm values	Molecular weight (g/mol)
FishF1	5'-TCAACCAACCACAAAGACATTGGCAC-3'	46.2%	60.2ºC	7886.20
FishR1	5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'	46.2%	59.8 ºC	8019.30

during the experimental work. A 25 µL reaction volume was utilized for the amplification of the *cox1* gene and it comprised 12.50 µL of using 2X PCR master mix (3B BlackBio, India), 1 µL (10 µm conc.) 9.50 µL molecular grade distilled water, 1.0 µL DNA template and each forward as well as reverse primer. Following a single cycle of initial denaturation at 95ºC for 5 min, there were 35 cycles of annealing at 72ºC for 1 min, 53ºC for 1 min and 95ºC for 30 sec. At 72ºC for 10 min and 4ºC for 10 min as the holding temperature, the last extension phase has been carried out.

Agarose gel electrophoresis

The PCR product or amplicons were separated on 1 percent gel containing 0.003% EtBr and photographed using a Transluminator (Hi-UV Duo Capture, Himedia, India) to ensure that the PCR was successfully yielded with the desired size of the amplicons or not. There was a negative control in each reaction cycle. A 1 kb DNA ladder (MBT051, Himedia, India) was utilized as a reference throughout agarose gel electrophoresis to ascertain the molecular weight associated with the specific *cox1* gene.

Analysis using DNA sequencing and bioinformatics tools

The services for Sanger's DNA sequencing of the *cox1* gene were taken from M/s. Biokart Pvt. Ltd, India. During DNA sequencing a forward primer (FishF1: 5-TCAACCAACCACAAAGACATTGGCAC-3) was used for sequencing of the forward strand. After completion of the sequencing the forward sequences were examined using ORF finder (https://www.ncbi.nlm.nih.gov), nucleotide BLAST (https://blast.ncbi.nlm.nih.gov) and Sequencing Analysis 5.2 version software (Applied Biosystems Inc., CA, USA) to exclude poor-quality reads and dropouts. All of the generated and publicly available database sequences were aligned using Clustal-X software to create a final dataset.[19] Furthermore, the dataset was produced to match the length of the sequences of DNA in the *cox1*gene of both the samples (6A and 6B) to prevent contradictory findings from tree and genetic variation analyses.

The single DNA barcode sequence of the *cox1* gene of *O. niloticus* was produced by BOLD Systems (www.boldsystem.org) which is available for reference and identification purposes. The DNA sequences of the *cox1* gene with accession numbers are available in the GenBank, NCBI, USA. The MEGA-X tool was utilized to perform sequence alignment and sequence divergence.[20] Based on K2P distances, Neighbor-Joining (NJ), closet Neighbor analysis (NN) and Barcode Index Number (BIN) URI creation, Barcode Gap Analysis as an average intra-specific, BOLD Systems and Cluster Sequence Analysis were acquired data from nucleotide composition. The BOLD data was prepared based on specimen records for which fundamentally 07 data records were taken as listed:

- Name of species;
- Voucher information;
- Collection records (collection date, name of the specimen collector);
- Identification number for the specimen;
- COI sequences of more than 600 bp;
- PCR primer details used to generate the amplicon of the *cox1* gene;
- Trace files of the sequenced gene.

The main data of the *O. niloticus* component a biphasic record in BOLD has both a 'specimen page' and a 'sequence page'. A direct connection in the project terminal allows accessibility to both sites for *O. niloticus* of the BOLD Systems (https://v4.boldsystems.org/).

RESULTS

PCR Results and DNA Sequence Analysis

In the present investigation, a total 04 individuals have been sampled from Shahpura Lake, Bhopal and universal primers set FishF1 (5-TCAACCAACCACAAAGACATTGGCAC-3) and FishR1 (5-TAGACTTCTGGGTGGCCAAA GAATCA-3) was used to amplify the targeted amplicons of the *cox1* gene. Moreover, two samples successfully sequenced were larger than 600bp and were BLAST, which showed that the sequenced samples contained the same *O. niloticus* fish species. BLAST with the NCBI databases for species-vouchers 6A and 6B, showed that ranging from 99 to 100%is identical with *O. niloticus.*

The targeted *cox1* gene was sequenced trimmed, annotated and submitted to GenBank and obtained accession numbers as OR143703 and OR143704 (Table 2) and the development of single DNA barcodes through Barcode of Life Data (BOLD) Systems USA.

Table 2: *cox1* gene sequence of *O. niloticus* submitted and available at GenBank, NCBI, USA.

Sl. No.	Species Name	Species-Voucher	Targeted gene	Accession number	Version	Sequence length
1.	*Oreochromis niloticus*	6A	*mtcox1*	OR143703	OR143703.1	654bp
2.	*Oreochromis niloticus*	6B	*mtcox1*	OR143704	OR143704.1	672bp

Table 3: Nucleotide composition of the sequenced gene of *O. niloticus*.

Sl. No.	Species Domain Data	Species-Voucher	Accession number	T(U)	C	A	G	Total
1.	*Oreochromis niloticus*	6A	OR143703	28.287	30.122	24.617	16.972	654
2.	*Oreochromis niloticus*	6B	OR143704	28.869	29.613	24.255	17.261	672
	Average	-	-	28.582	29.864	24.434	17.119	663

Estimated value, e-value, greatest coverage and bit scores were shown for *O. niloticus* authentication and varied from 99-100%. DNA fragments containing the amplified *cox1* gene produced sequences devoid of termination codons, insertions, or deletions. The observed average nucleotide frequencies were C (29.864%), T (28.582%), A (24.434%) and G (17.119%) with a total average nucleotide of 663 (Table 3).

Genetic variations at the level of nucleotide were identified at several consensus locations of the sample sequences during the sequence alignment with transitional genetic changes; nearly both fish sequences are polymorphic and varied at several places. Further, the genetic distances between the *O. niloticus* species-voucher 6A and 6B were found as 1.09 (p-dist) with an average distance of 0.3 (Table 4).

Table 4: DNA barcode database of *O. niloticus* for species-vouchers 6A and 6B developed available at BOLD Systems.

Sl. No.	DNA Barcoding Parameters	BOLD Systems Parameters for 6A and 6B
1.	Ambiguous %	0%
2.	Barcode Index Number URI	BOLD:AAC9904
3.	Top Hit %	100
4.	Max distance in BIN (p-dist) %	2.79
5.	Distance to nearest neighbor %	1.09
6.	Average distance %	0.3
7.	Barcode Compliant Number	414
8.	Member Count	1047 (776 Public)
9.	Nearest Member of neighbor	ANGBF54446-19
10.	Nearest BIN URI	BOLD: AET5315

DNA Barcoding Parameters

The present dataset observed high genetic distances for *O. niloticus* species-vouchers 6A and 6B which support species identification (Figures 1 and 2). Only 24.14 were found to be the minimal genetic distance between two individuals and their nearest congeneric cousin (Table 5) as determined by analyzing the distribution within the Nearest-Neighbor Distance (NND).

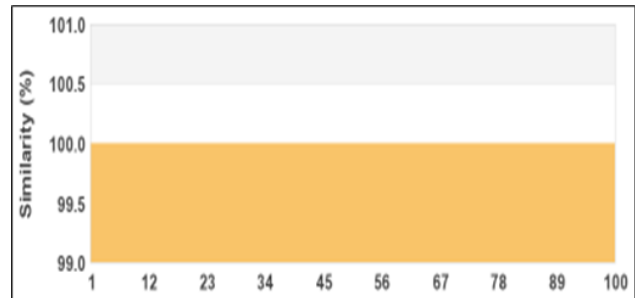


Figure 1: Similarity Scores of the top 100 matches of *O. niloticus* and BOLD Systems.

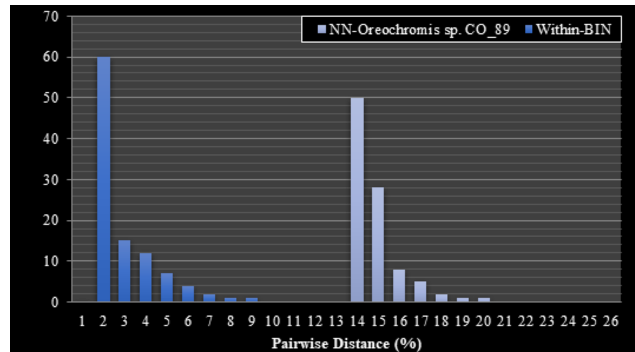


Figure 2: Distance distribution among *O. niloticus* and BIN of BOLD Systems.

O. niloticus species-voucher 6A and 6B had 100% bootstrap replications but monophyletic clustered with *O. niloticus* available on BOLD Systems with a nearest neighbor as HLALI031-23 with distance to Nearest Neighbor (NN) as 24.14 [Table 5].

Considering ambiguous (0%), Barcode Index Number (BIN) URI (BOLD:AAC9904), Top Hit % (100%), Nearest Member of Neighbor (ANGBF54446-19), Nearest BIN URI (BOLDAET5315), Barcode Gap evaluation as an average intra-specific (204.14) and

Table 5: Barcode Gap Analysis *O. niloticus* for species 6A and 6B developed and available at BOLD Systems.

Order	Family	Species	Mean Intra-Sp	Max Intra-Sp	Nearest Species	Nearest Neighbour	Distance to NN
Cichliformes	Cichlidae	*Oreochromis niloticus*	204.14	204.14	*Systomus sarana*	HLALI031-23	24.14

Cluster Sequence Analysis for species voucher 6A and 6B (20.583717) was found significant for development of DNA barcode (Table 6). Therefore, in order to verify this particular species, more barcode data from its type and topotypes had to be generated. This would aid in the creation of conservation strategies for both the invasive species and the local ecology, which are linked to the means of subsistence for the state of Madhya Pradesh ethnic people.

DISCUSSION

In recent years, DNA barcoding has become a powerful method for species identification, particularly when faced with unfamiliar organisms and limited morphological information. Utilizing a concise gene sequence, known as the DNA barcode, extracted from a standardized region of the genome, scientists can effectively identify species. The DNA barcode relies on sequencing a consistent segment of the mitochondrial cytochrome c oxidase I (*cox1*) gene, gaining recognition as a reliable approach for species identification, authentication and phylogenetic analysis. These barcodes consist of short nucleotide sequences from a specific genetic locus, designed for precise species identification. In the case of animals, the accepted barcode sequence is a 648-base pairs segments from the mitochondrial Cytochrome Oxidase subunit I (COI) gene.

DNA barcoding is used to identify species based on the idea that interspecific divergence effectively outweighs intra-specific divergence and that a threshold value, corresponding to the divergence between the closest neighbors among a group using the available barcode life database, can demarcate the biological species.[21] Hence, particularly in the fishing industry, the application of the COI gene in conjunction with DNA barcoding for species identification has been well recognized and verified.[22-24] The complete success rate in the present research indicates that DNA barcoding is effective in identifying species and this is consistent with prior publications on fish DNA Barcoding[25,26] prior investigations found success rates ranging from 90 to 95.60%.[27,28]According to a recent genetic analysis, endemism, hidden diversity, or the merging of better-defined lineages is the reasons for the increased *O. niloticus* variety of species in central India.[29] The current dataset found large genetic distances for *O. niloticus* species-vouchers 6A and 6B, supporting the identification of species [Figures 1 and 2] in line with earlier theories that K2P distances should be close to 2% for reliable species distinction in vertebrates.[30,31]The real barcode gap for species delimitation has been the subject of multiple research[32,33] but the genetic distances used to assess the gap have varied depending on how various isolates were sampled.[34] Consequently, by noting the vast genetic distance to the closest Neighbor (NN) of 24.14 and the mean intra-species (204.14) of HLALI031-23, as well as its distance from the Nearest Neighbor (NN) of 24.14 [Table 5 and Figure 3] Since the examined species is readily identifiable and barcoded, we believe that it may have strong diversity or greater hidden species diversity than nearby species [Figure 4]. Utilizing *cox1* gene sequences obtained from GenBank uploading on NCBI, each specimen (6A and 6B) of *O. niloticus* has been given a DNA barcode on the BOLD (Barcode of Life Data System) (https://v4.boldsystems.org/). This workbench has developed turn it into a tool for the DNA barcoding group by applying the workflow shown in [Figure 5].

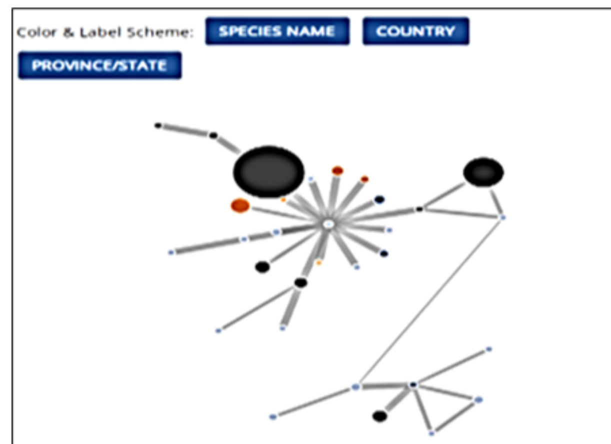


Figure 3: Haplotype network of state, species and country of *O. niloticus.*

The overall mean distance among *O. niloticus* species was obtained as 1.82 which may be considered as much as good from the conservation point of view.[35-38] However, the RESL in the BOLD systems has stronger taxonomic performance than that of the Barcode Gap Analysis and thus showed better species identification.[39] The application of mt-DNA COX1 genome sequences for DNA barcoding and genetic variation have

Table 6: Cluster Sequences (RESL) Analysis *O. niloticus* for species-vouchers 6A and 6B developed and available at BOLD Systems.

Taxon	Process ID	Sample ID	Mean	Max	Count	NN Dist
Oreochromis niloticus	HLALI021-23	6AB18	0	0	2	20.583717
Oreochromis niloticus	HLALI022-23	6BB19	0	0	2	20.583717

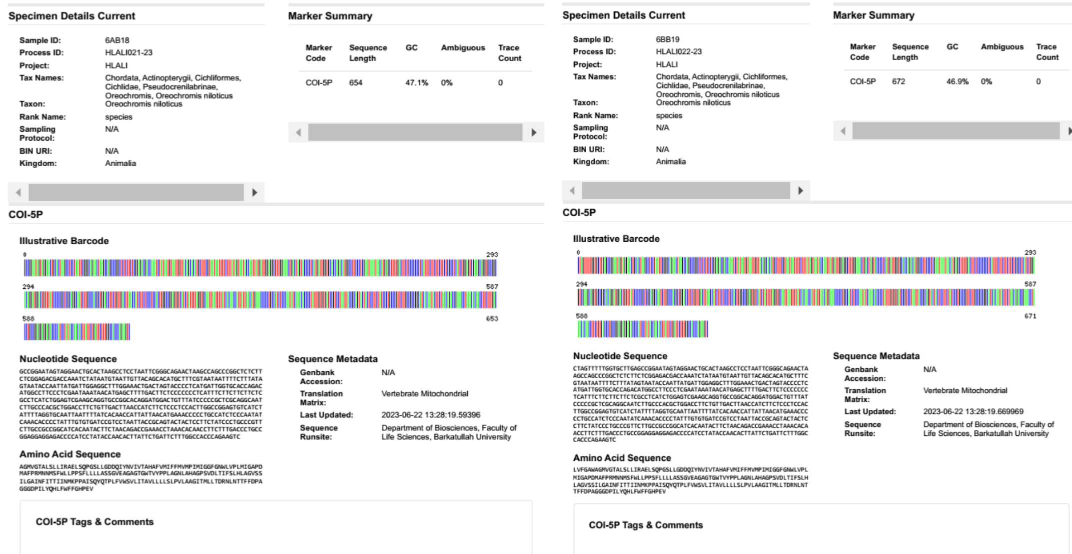


Figure 4: Developed DNA barcode database of *O. niloticus* for species-vouchers 6A and 6B on BOLD Systems.

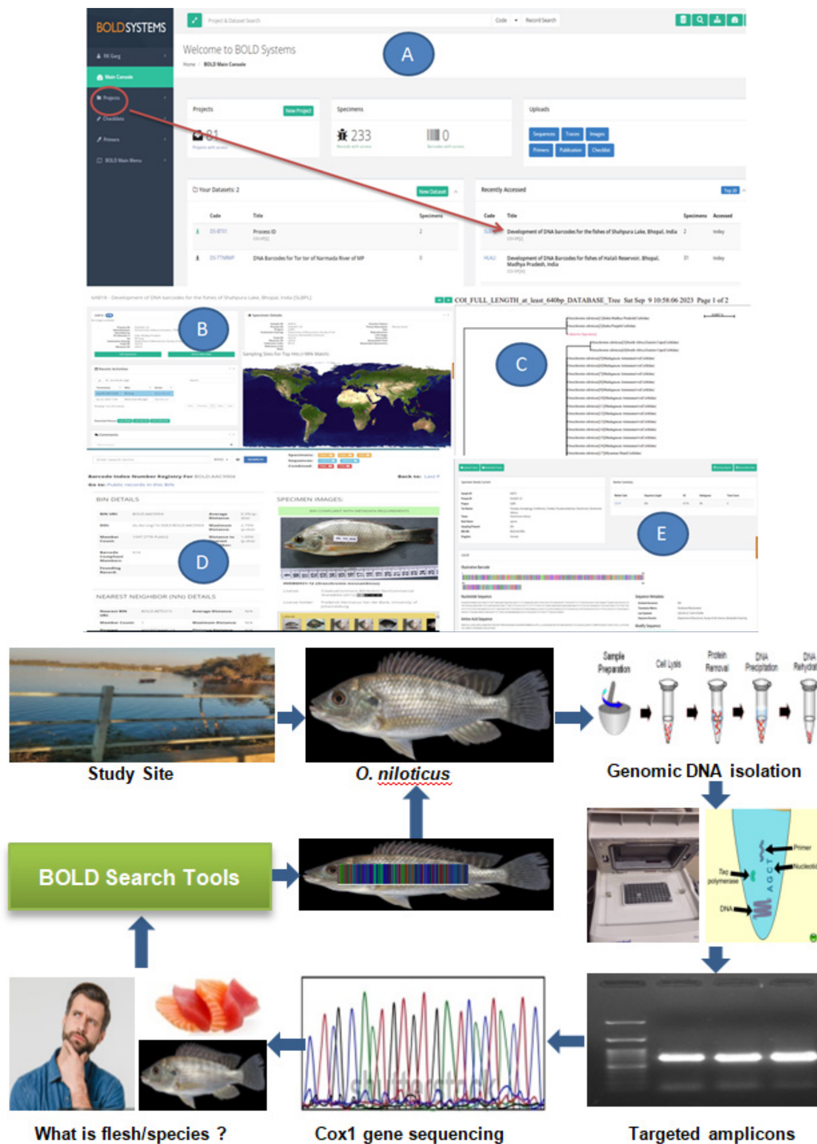


Figure 5: Complete pathway for developed DNA barcode database of Barcode of Life Data System.

demonstrated their value in phylogenetics and their capacity to identify polymorphism in populations, include fish fauna.[40] Current studies discussed towards DNA Barcode of Life project, which aims to develop a standardized, rapid and inexpensive method for identification *O. niloticus* using DNA barcodes. Studies highlighting the importance of species identification and the challenges faced by taxonomists in meeting the demand for identification and the need for a universal system. It suggests the mitochondrial *cox1* region as the ideal gene for animal species discrimination which has been achieved during investigation.

CONCLUSION

In summary Since Linnaeus's time, the conventional taxonomy has been primarily used to identify and find creatures. To identify the variety and biological species, researchers have over time presented several different notions, particularly through the use of phylogenetic theory. The worldwide database for fast and precise identification of species is strengthened by the DNA barcode database of fish with taxonomy. Furthermore, the current study contributes to the validation of the Madhya Pradesh and Central Indian ichthyo fauna checklists. All things considered, the current method of freshwater fish genetic study and barcoding would be beneficial in identifying variety, spotting invading species and developing plans for protection and sustainable management.

ACKNOWLEDGMENT

The authors wish to thank to HOD, Department of Biosciences and Dean, Faculty of Life Sciences, Barkatullah University, Bhopal for their help and encouragement during the work. The authors also wish to thank to laboratory mates for their help during this work and the local fisherman for helping us during sample collection.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

AUTHORS' CONTRIBUTIONS

DR devised the concept, developed the methodology and supervised the experiment work, **RKG, RK and PJ** collected and characterized the samples and performed the majority of experiments during the research work, **VV** has made extensive and intellectual contributions to the present work.

FUNDING SOURCE

The authors are thankful to Barkatullah University for providing us with facilities and consumables support during the research work. We are also very thankful to the co-authors who have support for financial support for primer designing, experimental kit procurements and DNA sequencing as expenses were borne by the co-authors.

ETHICAL APPROVALS

The fish *O. niloticus* under this study are not protected under the Wildlife Conservation Act and are routinely caught by professional fishermen and sold as food fish in Indian markets. No specific permission is required for obtaining these fish in India and no experimentation was conducted on live specimens in the laboratory.

ABBREVIATIONS

DNA: Deoxyribose Nucleic Acid; **PCR:** Polymerase Chain Reactions; **EtBr:** Ethidium Bromide; **NCBI:** National Center for Biotechnology Information; **BOLD:** Barcode of Life Data Systems; **COX1:** Cytochrome-C Oxidase Subunit-1; **FISH-BOL:** Fish Barcode of Life Initiative; **NJ:** Neighbor-joining; **BIN:** Barcode Index Number.

SUMMARY

Unique DNA barcode data to the mitochondrial COX1 sequences of genes and barcode life and uploaded to GenBank, NCBI, USA with a 672bp (accessions OR143703. OR143704) data with as below link:

https://www.ncbi.nlm.nih.gov/nuccore/OR143703

https://www.ncbi.nlm.nih.gov/nuccore/OR143704

The most conserved areas for *O. niloticus* are displayed in barcoded and NCBI data, which differs from neighboring species and yields more than 99% of matches with resemblance. The results of the current study indicate that high gene flow hinders genetic differentiation within populations of *O. niloticus*. The development of a barcode to distinguish between fish species is in line with an international effort to build an extensive database of *cox1* sequences associated with specific specimens.

REFERENCES

1. Froese R, Pauly D. Fishbase. World Wide Web electronic publication; 2017.
2. Oishi T. Ethno ecology and ethno medicinal use of fish among the Bakwele of south-eastern Cameroon. Revue d'ethnoécologie. 2016;10:2267-419. doi: 10.4000/ethnoecologie.2893.

3. Barman AS, Singh M, Singh SK, Saha H, Singh YJ, Laishram M, *et al.* DNA barcoding of freshwater fishes of Indo-Myanmar biodiversity hotspot. 2018;8:8579. doi: 10.1038/s41598-018-26976-3.
4. Trewavas E. Tilapiine fishes of the genera Sarotherodon, *Oreochromis* and Danakilia. British Museum Nat Hist. 1983;583. doi: 10.5962/bhl.title.123198.
5. Grammer GL, Slack WT, Peterson MS, Dugo MA. Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) establishment in temperate Mississippi, USA: multi-year survival confirmed by otolith ages. 2012;7: 367-76. doi: 10.3391/ai.2012.7.3.008.
6. Tripathi S, Gopesh A, Dwivedi AC. Framework and sustainable audit for the assessing of the Ganga River ecosystem health at Allahabad, India. Asian Journal of Environmental Science. 2017;12: 37-42. doi: 10.15740/HAS/AJES/12.1/37-42.
7. Dwivedi AC, Mayank P, Tiwari A. The River as transformed by human activities: the rise of the invader potential of *Cyprinus carpio* and *Oreochromis niloticus* from the Yamuna River, India. Journal of Earth Science and Climatic Change. 2016;7:361. doi: 10.4172/2157-7617.1000361.
8. Mayank P, Mishra N, Dwivedi A. Invasive Potential of Nile Tilapia, *Oreochromis niloticus* (Linnaeus, 1758) from the Tributary of the Ganga River, Central India. Journal of Earth and Environmental Sciences Research. 2021;3(3):1-5.
9. Ukenye EA, Megbowon I. Comparison of genetic diversity of farmed *Oreochromis niloticus* and wild unidentified tilapia (Wesafu) using microsatellite markers. Biodiversity. 2023;24(5): 2953-2957. doi: 10.13057/biodiv/d240550.
10. Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, e*t al*. Identifying Canadian freshwater fishes through DNA barcodes. PLoS ONE. 2008;3:1-8. doi: 10.1371/journal.pone.0002490.
11. April J, Mayden RL, Hanner RH, Bernatchez L. Genetic calibration of species diversity among North America’s freshwater fishes. Proc Nat Acad Sci USA. 2011;108:10602-10607. doi: 10.1073/pnas.1016437108.
12. Chakraborty M, Ghosh SK. An assessment of the DNA barcodes of Indian freshwater fishes. Gene. 2014;537:20-28. doi: 10.1016/j.gene.2013.12.047.
13. Khedkar GD, Jamdade R, Naik S, David L, Haymer D. DNA barcodes for the fishes of the Narmada, one of India’s longest rivers. PLoS One. 2014;9(7):101460. doi: 10.1371/journal.pone.0101460.
14. Chen W, Ma X, Shen Y, Mao Y, He S. The fish diversity in the upper reaches of the Salween River, Nujiang River, revealed by DNA barcoding. Sci Rep. 2015;5:17437. doi: 10.1038/srep17437.
15. Shantanu K, Kailash C, Kaomud T, Avas P, Vikas K. DNA barcoding of freshwater fishes from Brahmaputra River in Eastern Himalaya biodiversity hotspot. Mitochondrial DNA Part B. 2019;4(2):2411-9. doi: 10.1080/23802359.2019.1637290.
16. Hanner R. Proposed Standards for BARCODE Records in INSDC (BRIs); 2005. doi: 10.5479/10088/96518
17. Janarthan S, Vincent S. Practical Biotechnology, Methods and Protocols, University Press (India). 2007;13:978-81-7371-582-2.
18. Kocher TD, Thomas WK, Meyer A, Edwards SV, Pääbo S, Villablanca FX, *et al.* Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Pro. Nat. Acad. Sci. 1989;86(16):6196-6200.
19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 1997;25:4876-82. doi: 10.1093/nar/25.24.4876.
20. Tamura K, Peterson D, Peterson N, Stecher G, Nei M. MEGA5.05: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods. MolBiol and Evo. 2011;28(10):2731-9. doi: 10.1093/molbev/msr121.
21. Hebert PD, Ratnasingham S, deWard JR. Barcoding animal life: cytochrome c oxidase subunit 1 divergence among closely related species. Proc R SocLond B Biol Sci. 2003;270:96-9. doi: 10.1098/rsbl.2003.0025.
22. Kochzius M, Seidel C, Antoniou A, Botla SK, Campo D, Cariani A, *et al*. Identifying fishes through DNA barcodes and microarrays. PLoS One. 2010;5(9):12620. doi: 10.1371/journal.pone.0012620.
23. Ward R. A case study for DNA barcodes. In: Kress WJ, Erickson DL (eds) DNA barcodes: methods and protocols, methods in molecular biology. Springer, New York; 2012. doi: 10.1007/978-1-61779-591-6_21.
24. Knebelsberger T, Landi M, Neumann H, Kloppmann M, Sell AF, Campbell PD, *et al*. A reliable DNA barcode reference library for the identification of the North European shelf fish fauna. Mol Ecol Resour. 2014;14:1060-71. doi: 10.1111/1755-0998.12238.
25. Lakra WS, Singh M, Goswami M, Gopalakrishnan A, Lal KK, Mohindra V, *et al*. DNA barcoding Indian freshwater fishes. Mitochondrial DNA. 2015;27:4510-17.
26. Shen Y, Guan L, Wang D, Gan X. DNA barcoding and evaluation of genetic diversity in Cyprinidae fish in the midstream of the Yangtze River. Ecol Evol. 2016;6(9):2702-13. doi: 10.1002/ece3.2060.
27. Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, *et al*. Identifying Canadian freshwater fishes through DNA barcodes. PLoS One. 2008;3(6):2490. doi: 10.1371/journal.pone.0002490.
28. Iyiola AO, Nneji LM, Mustapha MK, Nzeh CG, Oladipo CG, Nneji IC, *et al*. DNA barcoding of economically important freshwater fish species from north-central Nigeria uncovers cryptic diversity. Ecol Evol. 2018;8:6932-51. doi: 10.1002/ece3.4210.
29. Conte-Grand C, Britz R, Dahanukar N, Raghavan R, Pethiyagoda R, Tan HH, *et al*. Barcoding snakeheads (Teleostei, Channidae) revisited: discovering greater species diversity and resolving perpetuated taxonomic confusions. PLoS One. 2017;12:e0184017. doi: 10.1371/journal.pone.0184017.
30. Johns GC, Avise JC. A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome b gene. Mol Biol Evol. 1998;15:1481-90. doi: 10.1093/oxfordjournals.molbev.a025875.
31. Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W. Ten species in one: DNA Barcoding reveals cryptic species in the neotropical skipper butterfly A straptesfulgerator. Proc Nat Acad Sci USA. 2004;101:14812-7. doi: 10.1073/pnas.0406166101.
32. Meyer CP, Paulay G. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 2005;3:422. doi: 10.1371/journal.pbio.0030422.
33. Steinke D, Zemlak TS, Hebert P. Barcoding Nemo: DNA-based identifications for the ornamental fish trade. PLoS One. 2009;4:6300. doi: 10.1371/journal.pone.0006300.
34. Meier R, Zhang G, Ali F. The use of mean instead of smallest interspecific distances exaggerates the size of the ‘barcoding gap’ and leads to misidentification. Syst Biol. 2008;57:809-13. doi: 10.1080/10635150802406343.
35. Avise JC. Molecular markers, natural history and evolution. New York: Chapman and Hall; 1994. doi: 10.2307/4088801.
36. Bickford D, Lohman DJ, Sodhi NS, Ng PKL, Meier R, Winker K, *et al*. Cryptic species as a window on diversity and conservation. Trends Ecol Evol. 2007;22:148-55. doi: 10.1016/j.tree.2006.11.004.
37. Hebert PDN, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc R Soc Lond B Biol Sci. 2003;270:313-21. doi: 10.1098/rspb.2002.2218.
38. Tyagi K, Kumar V, Singha D, Chandra K, Laskar BA, Kundu S, *et al*. DNA barcoding studies on Thrips in India: cryptic species, species complexes. Sci Rep. 2017;7:1-14. doi: 10.1038/s41598-017-05112-7.
39. Ratnasingham S, Hebert PDN. A DNA-based registry for all animals: the Barcode Index Number (BIN) System. PLoS One. 2013;8:66213. doi: 10.1371/journal.pone.0066213.
40. Garg RK, Dohre S. Evaluation of Molecular Genetic Diversity and DNA Barcoding of *Rita gogra* (Family Bagridae) from Narmada River Revealed through Mitochondrial COX1 Gene Sequencing. Asian J. Biol. L Sci. 2023;12(3):587. doi: 10.5530/ajbls.2023.12.77.

Cite this article: Raghuwanshi D, Garg R, Khandait R, Jagdish P, Vyas V. Identifying Single DNA Barcode Regions of Oreochromis niloticus of Shahpura Lake, Bhopal and their Contribution to a Reference Library. Asian J Biol Life Sci. 2024;13(1):221-8.