INTERNATIONAL JOURNAL OF APPLIED AND EXPERIMENTAL BIOLOGY
(ISSN PRINT: 2790-6523; ISSN ONLINE: 2790-6531)
2024, Vol. 3, NO. 2, 105-111
doi: 10.56612/ijaaeb.v1i1.79
https://eminentscientists.com/

Molecular marker-based species identification of carnivorous freshwater catfish (*Wallago attu*) from river Chenab, Pakistan

Parsa Riaz[1], Muhammad Naeem[1*], Shoaib Hassan[1], Ammar Danyal Naeem[1], Zara Naeem[1], Sheikh Muhammad Azam[1,2]**

[1]Institute of Zoology, Bahauddin Zakariya University Multan, Pakistan
[2]Department of Zoology, Division of Science & Technology, University of Education Lahore, Pakistan

Abstract
DNA barcoding, one of several procedures for identification of organisms, is an effective way to identify fish species. DNA-based identification is reliable and accurate to characterize an organism at different stages of its life cycle. Several reference libraries were used to link species by adding data to report species. For the sequencing of undetermined species, a customized primer was used. An unknown specimen was identified using a cytochrome c oxidase 1 primer. In this study, cytochrome c oxidase subunit 1 was used to identify *Wallago attu* at the molecular level. After sequencing, the mitochondrial cytochrome c oxidase subunit 1 gene was fragmented into 380 base pair pieces. A molecular-based identification approach, which comprised a neighbor-joining tree to estimate divergence, was used in addition to morphological identification to identify and authenticate the species under investigation. The COI barcodes were morphologically identified and matched with reference sequences of anticipated species. The GenBank databases received species reports with the entry number MT476360. The study's findings suggested that DNA barcoding is a viable and successful method for fish identification and for establishing a reliable DNA barcode reference library for these species.

ARTICLE TYPE
Research Paper (RP)

SECTION
Animal Biology (AB)

HANDLING EDITOR
Ali, M.R. (AB)

ARTICLE HISTORY
Received: 29 Jul, 2023
Accepted: 01 Jan, 2024
Published: 04 Jul, 2024

KEYWORDS
Cytochrome c oxidase;
DNA barcoding;
Fish;
Molecular identification;
Molecular markers

Introduction

In the past, fishes were used to be identified using morphological characteristics such as body shape, coloration, and meristic count (Matarese et al., 2011). In contrast to DNA barcoding, conventional morphological identification techniques exhibit reduced efficacy and precision in species identification. According to Packer et al. (2009), DNA barcoding offers a precise and effective means of identification of fish species. The reliability of species identification through DNA barcoding is contingent upon the presence of significant genetic differentiation both within and between species. For the successful identification of species, quantitative PCR had been extensively used (Yamanaka, 2016). So, PCR is the promising method for the identification of marked species. Then after PCR amplification, DNA barcoding involves DNA sequencing, which is considered efficient for accurate identification (Hofmann et al., 2016).

***CONTACT** Muhammad Naeem, dr_naeembzu@yahoo.com, Institute of Zoology, Bahauddin Zakariya University Multan, Pakistan

****CONTACT** Sheikh Muhammad Azam, sheikh.azam@ue.edu.pk, Institute of Zoology, Bahauddin Zakariya University Multan, Pakistan; Department of Zoology, Division of Science & Technology, University of Education Lahore, Pakistan

TO CITE THIS ARTICLE: Li, B., Zhang, C., Qi, M., Zheng, X., Mustafad, N.S., Ye, A., Deng, J., Liu, X., Ahmed, N., Ahanger, M.A., Zhang L. (2024). Effects of plant growth-promoting rhizobacteria on uptake and utilization of phosphorus and root architecture in apple seedlings under water limited regimes. *International Journal of Applied and Experimental Biology* 3(2): Samiah Shahid.

The K2P genetic distance is commonly employed in genetic analysis, utilizing Kimura's two-parameter model as a basis (Kimura, 1980). The K2P method offers expeditious and precise computational outcomes for numerous taxa. The p-distance model was posited as a superior alternative to the K2P model. The current investigation employed K2P and p-distance models for DNA barcode gap analysis. The phylogenetic tree describes genetic divergence and ancestral evidence (Patwardhan, 2014). The COI mitochondrial DNA marker is recognized universally for the identification of fish species (Rajeshkannan et al., 2019). In fact, sequencing of uniform proportion of cytochrome c oxidase type 1 (*COI*) gene is the basis of fish DNA barcoding (Haldar and Nath, 2020; Mu et al., 2023), and it has gained a ground as a potential tool for the authentic identification and phylogenetic analysis of species.

Wallago attu is known to be a fast-growing catfish and it has a good market value because of its high body protein content and nutritional value (Riaz and Naeem, 2024). The widespread distribution of *Wallago attu* has cast a doubt that there might be more than its one species thriving in different aquatic habitats. For example, while comparing the specimens of *Wallago attu* collected from South Asia and Southeast Asia, considerable differences in skeletal structures were recorded. Based on such findings it is naïve to expect that there could be more than one species of the genus *Wallago* (Roberts, 2014). Thus, the premier objective of the present study was to identify and authenticate fish species using DNA barcoding and compare it with that done by morphological markers. So, in the present study, the mitochondrial DNA was isolated from the tissues of freshwater catfish (*Wallago attu*), a species that occurs widely in the rivers of Pakistan, to identify fish species accurately and quickly within the genus *Wallago*.

Materials and Methods

Study Area and Sampling

Ten specimens of *Wallago attu* were obtained from the Chenab River, located near Head Muhammad Wala Bridge in Multan, Pakistan (Latitude: 30.17'32^{o}N; Longitude: 71.22'52^{o}E), through collaboration with local fishermen. The specimens were promptly preserved and brought to the Fisheries Research Laboratory, Institute of Zoology, Bahauddin Zakariya University, Multan, Pakistan.

Species Identification

Fish specimens were identified using the conventional taxonomic key (Mirza and Sandhu, 2007) and the FishBase global electronic database of fish species (Froese and Pauly, 2011), considering morphometric characteristics.

DNA extraction

DNA was isolated from the muscle sample of the fish. The size of each tissue was about 50-100 mg which was homogenized using an extraction buffer of 6-800 µL. The homogenized sample was immediately transferred into an Eppendorf tube. Proteinase K (12 µL) was added to the Eppendorf tube and mixed using a vortex. The tissue was incubated for 1 h at 37 °C and 1 h at 55 °C.

After that incubation, the solution was centrifuged at 5000 rotation per minute (rpm) for 10 min. The supernatant was isolated and to it phenol:chloroform:isoamyl alcohol with a 25:24:1 ratio was gently added with a gentle inversion for 5 min. Then the sample solution was centrifuged at 12000 rpm for 10 min. The supernatant was collected and an equal volume of chloroform:isoamyl:alcohol with a ratio of 24:1 was added to it. The treated sample was centrifuged at 12000 rpm for 10 min and collected the supernatant. Then 0.1 volume of 3M sodium acetate along with an equal volume of 100% ice-chilled ethanol, were added. The treated sample was kept at -20 °C for 1 h. The sample was properly centrifuged for 10 minutes, and DNA precipitation appeared at the base of the tube. The precipitated DNA was collected and to it an aliquot of 100 µL of 70% ethanol was added, and then centrifuged the sample at 1000 rpm for 10 min. Then the ethanol from the treated sample was discarded and the sample air-dried. An aliquot of (50 µL) of deionized water was added to the treated sample. For the purification of DNA, an aliquot of 100 µL of RNase was added and stored at -20 °C until further analysis.

PCR amplification

The *COI* gene barcoding was done successfully using the universal fish primer Fish F1 and Fish R1 (Ward et al., 2005) as shown in Table 1. The total PCR reaction was 25 µL with the composition of extracted DNA as 1 µL; primer 1.5 µL for each forward and reverse; PCR Master mix 12.5 µL (TaqNova-Red) and deionized water 10.8 µL (Table 2). The conditions for PCR thermal cycler were set as: the initial denaturation for 2 min was set at 95 °C, further with 30 complete rotations of denaturation was set for 30 s at 95 °C, annealing for 40 s at 54 °C and extension for 1 min at 72 °C. The final extension was 7 min

at 72 °C (Table 3). The quantity of the extracted DNA was checked by Nanophotometer (Implen, GmBH). DNA absorbance, including concentration and purity of the samples, was assessed by determining its absorbance at $A_{260}/_{280}$nm and $A_{260}/_{230}$nm of wavelength.

Analysis of the data

The sequences were analyzed and aligned by utilizing BioEdit version 7.0.5.3. The identity match was performed utilizing BOLD and NCBI. The Neighbor-Joining (NJ) method, as proposed by Saitou and Nei (1987) was employed to generate trees. The K2P model was utilized for this purpose, using MEGA version X software.

Table 1. Primer sequences used for PCR amplification and sequencing through *CO1* identification gene

Primer	Sequence (5'→3')	TM (°C)
Forward primer	TCAACCAACCACAAAGACATTGGAAC	64.7
Reverse primer	TAGACTTCTGGGTGGCCAAAGAATCA	66.3

TM, Melting temperature

Table 2. Components of the mixture for polymerase chain reaction

Component	Quantity (µL)
DNA Template	2.5
Master Mix (TaqNova-Red)	12.5
Forward Primer	0.1
Reverse Primer	0.1
Sterile Water	9.8

Table 3. Optimized conditions for a polymerase chain reaction in PCR thermal cycler

Cycles	Temperature (°C)	Duration
1	95	5 min
40	95	30 sec
40	55	30 sec
40	72	30 sec
1	72	7 min

Results

Morphometric-based identification

The body of the fish samples was compressed and elongated without scales. The body was straight, with a rounded abdomen. The head was large and depressed; head width was a little less than its length and equal to half of its height. The mouth was recorded as wide and its cleft extending behind the eyes; jaws were slightly long. Eyes were small. Teeth were large, numerous, cardiform in both jaws and oblique patches on either side of the vomer. Barbels were in two pairs; the longest maxillary pair reached beyond the front end of the anal fin, and the mandibular pair was as long as the snout. The dorsal fins were observed to be relatively diminutive in size, lacking in spines, and typically positioned anteriorly to the pelvic fins. The pectoral spine exhibited a moderate level of strength and possessed internal serrations of a delicate nature. The size of the pelvic fin was notably diminutive. The anal fin of considerable length was recorded to be extended beyond half of the body's length and terminated at the caudal fin base without merging with it. The caudal fin exhibited rounded lobes and a forked appearance, with the upper lobe being comparatively larger and slightly curved about the lower lobe. The body color of the specimen in question was silvery grey, which exhibited a darker hue on its dorsal side. The dorsal region might also display an olive coloration with a golden gloss, while the lateral aspect of the body appeared dull and white. Additionally, it is worth noting that a subtle orange-yellow band was frequently observed along the lateral line. The caudal and anal caudal fins exhibited a somewhat dusky appearance.

Figure 1. *Wallago attu*

DNA Extraction and PCR amplification

DNA extraction and PCR amplification were done successfully, as shown in Figures 2 and 3. PCR purified products were sent to the First BASE Lab, Malaysia, for barcode sequencing.

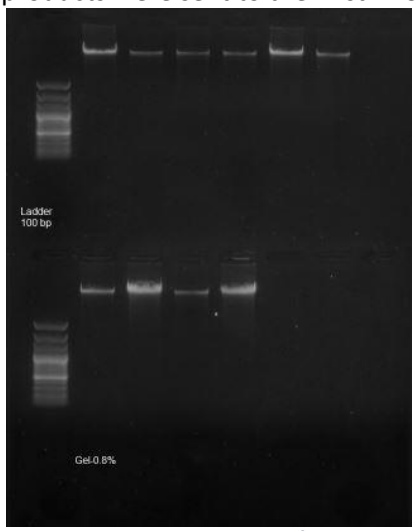

Figure 2. DNA extraction products of *Wallago attu*

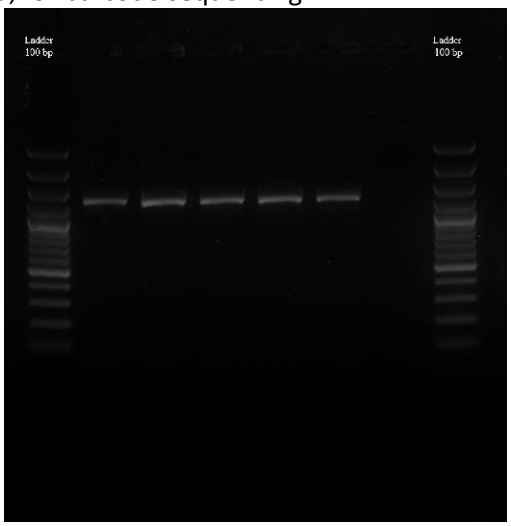

Figure 3. PCR amplified product of *Wallago attu*

Mitochondrial *COI* gene barcoding

The sequencing product was aligned using the biological sequence alignment editor. The sequence size of *Wallago attumwas* 380 base pair was as shown in Figure 4. The barcoded sequence was submitted to the GenBank Database with Accession Number MT476360 as a reference.

AACTCCCTTATTGACTTACCAGCCCCATCTAACATTTCTGCATGATGAAACTTTGGCT
CCCTCCTACTACTCTGTCTCATTATACAAATCCTAACCGGCTTATTCTTAGCCATGCA
CTACACCTCAGATATCTCAACCGCCTTCTCATCTGTAGTTCACATCTGCCGAGACGT
AAACTACGGATGACTAATCCGTAACCTGCACGCTAACGGAGCCTCTTTCTTTTTCATT
TGCATTTACCTTCACATTGGCCGGGGCCTTTATTACGGATCATACCTGTATAAAGAA
ACCTGAAACATCGGAGTTGTACTACTACTCCTAACAATAATAACCGCATTCGTAGGG
TACGTACTACCATGGGGTCAAATGTCATTCTGAGG

Figure 4. Sequence of *Wallago attu*

NCBI BLAST Identification

For the identification of unknown species, the sequence of nucleotides was BLAST on NCBI. The sequence of *Wallago attu* was found to be of 380 base pair in size, as was confirmed through gel electrophoresis by comparing it with the ladder size and when it was sequenced. The barcoded *COI* sequences of *Wallago attu* were BLAST with the National Centre for Biotechnology Information nucleotide database and examined for an accurate identity match. The BLAST analysis confirmed the 100% similarity with relevant sequences of the mitochondrial region of *Wallago attu* in the GenBank databases. The BLAST analysis revealed that *COI* gene barcoded species sequences had 100% similarity with the relevant GenBank databases of mitochondrial region sequences, so it was confirmed that the fish species under investigation was *Wallago attu* (Table 4).

Table 4. Fish specimen DNA sequence result with the GenBank confirmed the accurate identity of *Wallago attu*

Identified species GenBank accession number	Base pair maximum score	GenBank identity match	Identified species
MT476360	380	100%	*Wallago attu*

The Neighborhood-Joining method was employed to construct phylogenetic ancestry. The 100 bootstrap replicates showed the clustered taxa together. The K2P (Kimura 2-paramete) method calculated the phylogenetic ancestry genetic distances. The Neighbor-Joining hierarchy distinctly showed phylogenetic relationships among the species, with identical species clustered beneath the identical

nodes, while divergent species were clustered under discrete nodes with no exceptions. Genetic distance nucleotide divergence within species and inter-species was evaluated by the K2P method, which showed minimum distance.

Discussion

Previously, meristic features and morphometric characteristics were used for the identification and phylogenetic studies of species (Musikasinthorn, 2000). The species identification and phylogenetic studies based on morphological characteristics are still much controversial due to the complexity of morphological characteristics and evolutionary variations (Miyan et al., 2014). The utilization of DNA sequence variation in the mtDNA region of cytochrome c oxidase subunit I (*COI*) demonstrates a significant efficacy in the demarcation and recognition of animal species in a broad sense (Hebert et al., 2003), and specifically in fish (Teletchea and Fontaine, 2010). To achieve this investigation's objective, *Wallago attu* specimens were subjected to molecular analysis utilizing *COI* gene sequencing for species identification.

For identifying species, *COI* gene with 650 total base pairs was used (Lohman et al. 2009). However, in the present study, 618 bp were used. The *COI* primer was used to identify species and diversity of the population (Khaliq, 2012). The efficient identification was done on a molecular basis that differentiates species with high morphological resemblance. For taxa, *COI* barcoding is more dependable for species identification. Species taxonomic identification was inferred with DNA barcoding accurately provided by exact similarity to species in the database Genbank (Zhang and Hanner, 2012). The primary objective of the DNA barcode is record keeping for all fishes by the Fish Barcode of Life (FISH-BOL). For both intra-species and inter-species diversity, barcoding of DNA is considered to be an effective technique for species scrutiny and identity (Chakraborty and Ghosh, 2014).

The present study has effectively documented the precise identification and phylogenetic correlation of *Wallago attu* using DNA barcode data. Taxonomic confusion can arise due to imperfect recognition of characteristic morphological variability in descriptions of older species, as noted by Ndiaye et al. (2014). According to Padial et al. (2010), gathering data from both morphological and molecular sources is imperative to effectively address taxonomic confusion. In the present study, utilizing molecular data derived from *COI* gene variability has facilitated the resolution of the identification and phylogenetic relationships of *Wallago attu*. Recently, there has been a notable increase in the interest in DNA barcoding, particularly regarding fish species, as highlighted. The *COI* gene has been chosen as the standard barcode gene owing to its distinctive variation pattern across a wide range of intraspecific and interspecific species. According to Hebert et al. (2003), K2P genetic distances within populations are generally less than 1%, whereas K2P genetic distances exceeding 2% are infrequently observed in fish species across a wide range of taxa. The current investigation revealed that the genetic distance within the species *Wallago attu* based on K2P and p-distance was observed to be less than 1%.

The measurement of sequence divergence has been conducted through the utilization of the Kimura 2-parameter (K2P) model, as proposed by Kimura (1980). The NJ tree exhibited a congruent phylogenetic arrangement among the taxa, and no instances of misclassification were detected. In the majority of studies, there was a tendency to group species that were congeneric and also those that were confamilial. The *COI* sequence data exhibits a discernible phylogenetic signal, as expounded upon in the earlier work of Lakra et al. (2011). The phylogenetic tree was utilized to analyze the evolutionary relationships among species. The clustering of species under distinct nodes was observed, with different species being grouped and the same species being clustered under the same tree node. The current study's findings align with those of previous research conducted by Naeem and Hassan (2019) and Naeem et al. (2020), who utilized a molecular approach to identify *Labeo bata* and *Catla catla*, respectively, through the analysis of *COI* gene sequences and the construction of NJ trees for each fish species.

This study provides an essential validation of use of DNA barcode sequence molecular technique for fish species identification. It would be helpful for fish conservators, ecologists, and fisheries managers to understand these adaptive features and monitor the species diversity within complex ecosystems.

Conclusion

The utilization of DNA barcoding presented a viable substitute for conventional techniques in the identification of fish species. A cytochrome c oxidase I primer has been employed to identify specimens of unknown origin. The present investigation pertained to the molecular characterization of *Wallago attu* utilizing cytochrome c oxidase subunit I.

Author(s), Editor(s) and Publisher's declarations

Conflict of interest
The authors declare no conflict of interest.

Source of funding
Declared none.

Contribution of authors
Conceptualization and designing of the study: PR, MN, SH, ADN, ZN. Conduction of experiment and collection of data: PR, SMA. Analytical work: PR. Written first draft of the manuscript: PR, MN, SH, ADN, ZN, SMA. Helped to prepare figures and tables: PR, SMA. Statistical analysis of data: PR. Final draft reviewed and read by all authors.

Ethical approval
This work was approved by the Institutional Ethical Review Board/Committee (IERB/C) of University of Okara, Okara, Pakistan under approval number UO /ERC/2023/46A.

Handling of bio-hazardous materials
The authors certify that all experimental materials were handled with care during collection and experimental procedures. After completion of experiment, all materials were properly discarded to minimize any types of bio-contamination(s).

Availability of primary data and materials
As per editorial policy, experimental materials, primary data, or software codes are not submitted to the publisher. These are available with the corresponding author and/or with other author(s) as declared by the corresponding author of this manuscript.

Authors' consent
All authors contributed in designing and writing the entire review article. All contributors have critically read this manuscript and agreed for publishing in IJAaEB.

Disclaimer/Editors'/publisher's declaration
All claims/results/prototypes included in this manuscript are exclusively those of the authors and do not inevitably express those of their affiliated organizations/enterprises, or those of the publisher/journal management, and the editors/reviewers. Any product mentioned in this manuscript, or claim rendered by its manufacturer, is not certified by the publisher/Journal management. The journal management disown responsibility for any injury to organisms including humans, animals and plants or property resulting from any ideas/opinions, protocols/methods, guidelines or products included in the publication.

Declaration of Generative AI and AI-assisted technologies in the writing process
It is declared that the authors did not use any AI tools or AI-assisted services in the preparation, analysis, or creation of this manuscript submitted for publication in the International Journal of Applied and Experimental Biology (IJAaEB).

References

Chakraborty, M., Ghosh, S.K. (2014). An assessment of the DNA barcodes of Indian freshwater fishes. *Gene* 537:20-28.

Froese, R., Pauly, D. (2011). FishBase. World Wide Web Electronic publication. http://www.fishbase.org/Summary/speciesSummary.php?ID=12858&genusname=Leptonotus&speciesname=blainvilleanus&AT=Leptonotus+blainvilleanus&lang=English.

Haldar, C., Nath, S. (2020). DNA barcoding for fish species identification: current status and future prospective. *International Journal of Fauna and Biological Studies* 7(4):72-75.

Hebert, P.D.N., Cywinska, A., Ball, S.L., de Waard, J.R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Journal of Biological Sciences* 270(1512):313–321.

Hofmann, C., Schubert, G., Calvignac-Spencer, S. (2016). Aquatic biodiversity assessment for the lazy. *Journal of Molecular Ecology* 25:846–848.

Khaliq, F. (2012). "Isolation and sequencing of cytochrome oxidase c subunit 1 (COI) gene of *Tor putitora"* (Thesis). University of Sargodha, Pakistan.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16:111-120.

Lakra, W.S., Verma, M.S., Goswami, M., Lal, K.K., Mohindra,V., Punia, P., Gopalakrishnan, A., Singh, K.V., Ward, R.D., Hebert, P. (2011). DNA barcoding Indian marine fishes. *Mitochondrial DNA Part A* 27:4510-4517.

Lohman, D.J., Prawiradilaga, D.M., Meier, R. (2009). Improved COI barcoding primers for Southeast Asian perching. *Journal of Molecular Ecology Research* 9:37–40.

Matarese, A.C., Spies, I.B., Busby, M.S., Orr, J.W. (2011). Early larvae *Zesticelus profundorum* (family Cottidae) identified using DNA barcoding. *Journal of Ichthyology Research* 58:170–174.

Mirza, M.R., Sandhu, A.A. (2007). "Fishes of Punjab", Pakistan. Polymer Publications, Rahat, Market, Urdu Bazaar, Lahore.

Miyan, K., Afzal, K.M., Khan, S. (2014). Stock structure delineation using variation in otolith chemistry of snakehead, *Channa punctata* (Bloch, 1793), from three Indian rivers. *Journal of Applied Ichthyology* 30:881-886.

Mu, Y., Song, C., Yang, J., Zhang, Y., Zhang, X. (2023). Next-generation DNA barcoding for fish identification using high-throughput sequencing in Tai Lake, China. *Water* 15(4):774. http://dx.doi.org/10.3390/w15040774

Musikasinthorn, P. (2000). *Channa aurantimaculata*, a new channid fish from Assam (Brahmaputra River basin), India, with designation of a neotype for *C. amphibeus* (McClelland, 1845). *Journal of Ichthyological Research* 47(1):27-37.

Naeem, M., Hassan, S. (2019). Molecular approach for identification of *Labeo bata* based on COI gene sequence from Pakistan. *Mitochondrial DNA Part B* 4:244-246.

Naeem, Z., Masud, S., Hassan, S., Naeem, M. (2020). Molecular approach for identification of *Catla catla* using mitochondrial CO1 from Pakistan. *Mitochondrial DNA Part B*5:3018-3021.

Ndiaye, A., Hima, K., Dobigny, G., Sow, A., Dalecky, A., Ba, K., Granjon, L. (2014). Integrative taxonomy of a poorly known Sahelian rodent, *Gerbillus nancillus* (Muridae, Gerbillinae). *Zoologischer Anzeiger-A Journal of Comparative Zoology* 253:430-439.

Packer, L., Gibbs, J., Sheffield, C. Hanner, R. (2009). Barcoding methodology and applications of DNA barcoding and the mediocrity of morphology. *Molecular Ecology Resources* 9(1):42-50.

Padial, J.M., Miralles, A., De la Riva, I., Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology* 7:1-14.

Patwardhan, A., Ray, S., Roy, A. (2014) Molecular markers in phylogenetic studies: A review. *Journal of Phylogenetics & Evolutionary Biology* 2:131.

Rajeshkannan, R., Pereira, J.J., Marx, K.K., Jawahar, P., Lakshmi, K.D., Reddy, D. (2019). DNA barcoding on Cardinalfishes (Apogonidae) of Thoothukudi coast. *International Journal of Current Microbiology and Applied Sciences* 8(8):1293-1306.

Riaz, P., Naeem, M. (2024). Elemental composition of carnivorous fish *Wallago attu* (Siluridae, Siluriformes) from river Chenab. Pakistan. *Brazilian Journal of Biology* 84: https://doi.org/10.1590/1519-6984.270501

Roberts, T.R. (2014). (Wallago) Bleeker, 1851 and Wallagonia Myers, 1938 (Ostariophysi, Siluridae), distinct genera of tropical Asian catfishes, with description of Wallago maemohensis from the Miocene of Thailand. *Bulletin of the Peabody Museum of Natural History* 55(1):35-47 https://doi.org/10.3374/014.055.0103

Saitou, N., Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4(4):406–425.

Teletchea, F., Fontaine, P. (2010). Comparison of early life-stage strategies in temperate freshwater fish species: trade-offs are directed towards first feeding of larvae in spring and early summer. *Journal of Fish Biology* 77:257-278.

Ward, R.D., Zemlak, T.S., Innes, P.R., Hebert, P.D.N. (2005). DNA barcoding Australia's fish species. *Philosophical Transaction of the Royal Society B. Biological Science* 360:1847–1857.

Yamanaka, H. (2016). On site filtration of water samples for environmental DNA analysis to avoid DNA degradation during transportation. *Journal of Ecological Research* 31:963–967.

Zhang, J., Hanner, R. (2012). Molecular approach to the identification of fish in the South China Sea. *PLoS One* 7(2):e30621.