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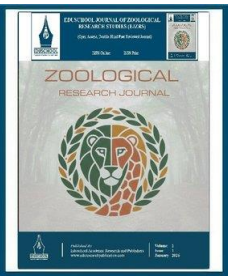

Assessment of Genetic Relationship of Freshwater Fish Species from Anuppur District, Madhya Pradesh Using Mitochondrial COI Gene Sequences

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Abstract

The present study aimed to evaluate the genetic relationships among the selected 12 freshwater fish species using the mitochondrial cytochrome C oxidase I (COI) gene sequences. The sequence data were retrieved from NCBI and analyzed the Multiple sequence alignment, Genetic distance estimation using the Kimura2-parameter model and phylogenetic tree construction using the neighbor-joining technique. The result revealed the conserved and the variable regions with in the COI gene and also showed that closely related species exhibit lower genetic distance and clustered together, especially in the cyprinidae family. while, the distantly related species form separate branches which indicates the higher genetic divergence. Some minor deviations observed in the clustering patterns maybe due to the limitation of single-gene analysis. Overall, the study demonstrates that the COI gene is an important molecular marker for the identification of species and phylogenetic analysis, with application in conservation studies and biodiversity assessment.

Keywords:- COI Gene, Multiple Sequence Alignment, Genetic Distance, Phylogenetic Relationship.

I. INTRODUCTION

Fish diversity plays an important role in the aquatic ecosystems and is crucial for the production of fisheries, nutrient metabolism and to ecological equilibrium. Especially in countries that are developing, varieties of freshwater fish play a major role in the local communities and food availability. Therefore, proper identification of the fish species and their classification are important for both successful management of aquatic diversity and the conservation of the biodiversity (Herbert et al., 2003). The fish species are traditionally classified based on their morphological appearance, structure of the fin and body colour. Although the morphological identification of fish species was the strong foundation, the variability in the phenotype, typical stages of development and environmental factors generally have an effect on it. All of these components may cause the wrong identification or classification, particularly when the species are closely related and having the similar external characteristics (Ward et al., 2005).

DNA barcoding is a modern technique for the identification of species that may precisely correspond to unrecognized species to known species while also identifying the genetic distance between the species populations possibly including unidentified species (Radulovici et al., 2010). Molecular methods have become reliable methods for the identification pf species as well as the evolutionary study to overcome these limitations because genetic markers are less likely to be affected by the environmental changes, also the DNA based techniques offer more exact information about the genetic relationships between the species. For the better understanding of patterns of evolution and divergence of species is given by the molecular methods which allows the divergence and genetic similarity (Avise et al., 2000).

Fish diversity and molecular identification have effectively used the COI gene, providing great specificity in distinguishing between the closely related species (Bhattacharjee et al., 2012). Because they make the comparison of sequence and evolutionary analysis readily accessible, the tools of the bioinformatics are very important in the molecular phylogenetic research. Whereas the phylogenetic study software like MEGA made it simple to create the evolutionary trees using the statistical models, the sequence similarity search tools like NCBI and BLAST allows quick identification of the homologous sequences (Kumar et al., 2018). By classifying the species according to their genetic similarities, phylogenetic analysis assists in the understanding of evolutionary links. The distantly related organisms have more divergent traits, indicating the evolutionary separation over the time period, while the closely related species generally cluster together and shows the lower genetic distance values (Nei and Kumar, 2000). Studying the history of evolution, overcoming the taxonomic problems and discovering the hidden species ultimately rely on these types of studies.

The present study is carried out in some freshwater habitats of Anuppur district of Madhya Pradesh, which is known for its wide range of rivers, reservoirs and inland wate bodies that are habitat to a variety of fish species. Ponds and other tiny lentic habitats are among the freshwater ecosystems that are an essential aspect of the biosphere, the rural and urban environment, and the worldwide network of metabolically active sites (Downing, 2010). Most of the previous research has been focused only on fish diversity and the morphological classification, despite the ecological importance of freshwater systems in the region of Madhya Pradesh. The molecular phylogenetic study and genetic relationship between the freshwater fish species in this area have not received much attention. A major research gap is observed by the absence of molecular data, which limits the proper identification of species, especially in situations of similar identical species or cryptic species. Therefore, the current study uses the COI gene sequences and molecular phylogenetic technique to assess the genetic relationships and the divergence among certain freshwater fish species. This work supports the sustainable fisheries management and biodiversity conservation by providing the molecular evidence for understanding the evolutionary links.

II. LITERATURE REVIEW

Fish are key markers of the health of the freshwater environment, which is home to a variety of orders of animals, plants, fungi, vertebrates and invertebrates (Karr et al., 1986). Because of the high mutation rate, maternal inheritance, and absence of the recombination the mitochondrial DNA has been used widely in the phylogenetic research among different molecular markers. For example, the mitochondrial cytochrome c Oxidase I (COI) gene has been regarded as a common barcode marker for species identification in diverse taxa (Lakra et al., 2011). Mitochondrial COI gene sequences are used to identify freshwater fish using DNA barcoding and molecular phylogenetic techniques. Evolutionary connection analysis, phylogenetic reconstruction and genetic distance estimation among certain fish species were all carried out in the study. Sequences divergence and genetic relatedness between species were assessed using phylogenetic grouping supported by bootstrap (Sambah et al., 2025). MEGA12 is a powerful bioinformatics program for phylogenetic reconstruction and molecular evolutionary study. Sequence alignment, genetic distance estimate, bootstrap analysis, and Maximum Likelihood phylogenetic analysis are all supported by the program for researching the evolutionary connections between species (Kumar et al., 2024). Fish diversity and molecular identification have effectively used the COI gene, providing great specificity in distinguishing between the closely related species (Bhattacharjee et al., 2012). Mitochondrial COI gene sequences were used for molecular phylogenetic study of ray-finned fishes. The study utilized DNA barcoding along with neighbor-joining and maximum-Likelihood phylogenetic methods to evaluate genetic relationships among fish species. Nucleotide diversity and genetic variation analysis revealed evolutionary divergence among geographically distributed fish populations and supported the effectiveness of COI- based molecular phylogeny in fish identification (Sahu et al., 2025). In order to evaluate ichthyofaunal richness and evolutionary connections among fish species, full DNA barcoding of Indian freshwater fishes was conducted utilizing mitochondrial COI gene sequences Genetic divergence analysis using K2P distance, Neighbor-Joining and Bayesian inference methods revealed hierarchical evolutionary variation among species, genera and families. The study also employed barcode gap analysis for accurate species delimitation and demonstrated the effectiveness of COI-based molecular approaches in freshwater fish identification and phylogenetic assessment (Modeel et al., 2024).

III. MATERIAL AND METHODS

Field sampling was conducted for morphological identification, whereas molecular phylogenetic analysis was carried out using publicly available COI gene sequences retrieved from the NCBI GenBank database. No wet laboratory sequencing was performed in the present study.

3.1. Study Sites

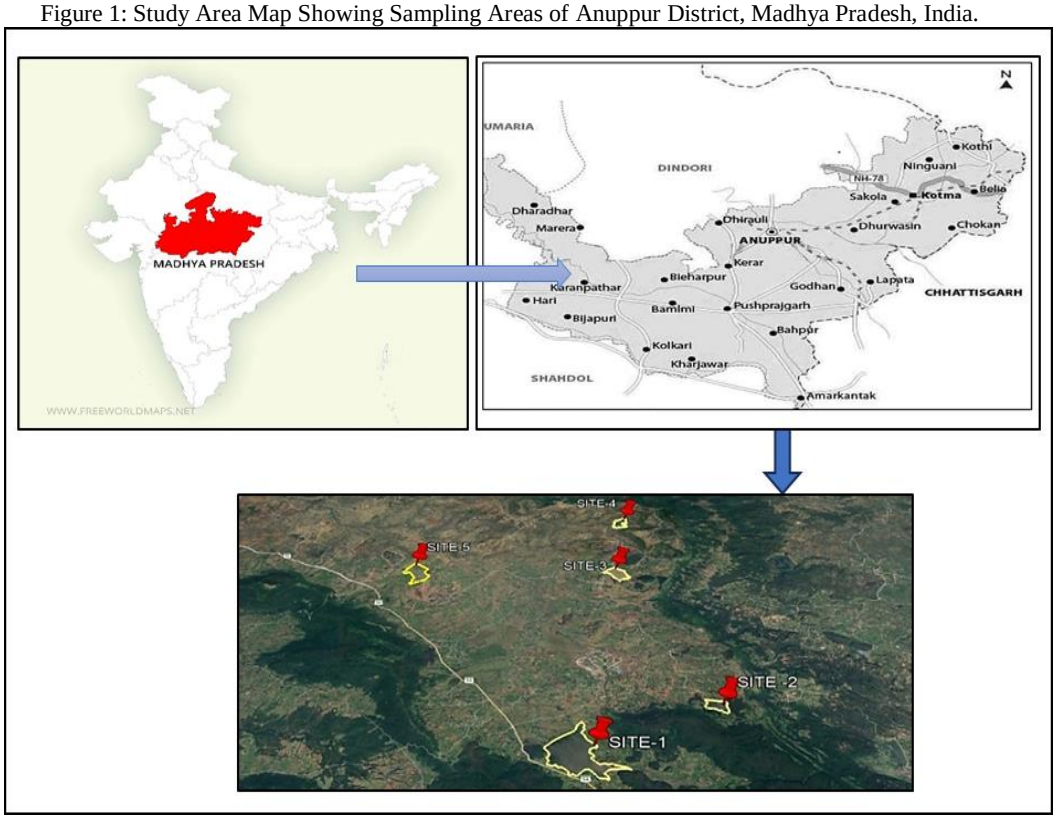
The current study was carried out in some freshwater ecosystems of Anuppur district of Madhya Pradesh, this area is known for its remarkable freshwater biodiversity and wide range of aquatic habitats. Many numbers of freshwater fish species can find suitable habitats like dams, ponds and lakes, which are generally impacted by the streams of major river systems. There were five sampling station were chosen to show the study area's ecological diversity. These consists of four reservoir system: Johila Dam, Kantur Dam, Lapti Dam, Bahpuri Dam as well as a pond called Batki which an example of lentic freshwater system with modest depth.

Localized fish populations are supported by these conditions, which may also have an impact on genetic structure and species-specific adaptations. The reservoir systems on the other hand are the bigger bodies of water, varying depths

and variations in water levels caused by human activities and seasonal influences. The species divergence and genetic diversity maybe impacted by the different levels of water flow, human activities. Seasonal changes, particularly during the monsoon period which connects between different waterbodies which facilitates gene flow in the fish communities and affect their genetic relationships.

Some of the selected study area display anthropogenic pressure, such as fishing, water extraction and habitat change which has an impact on genetic diversity. Therefore, Anuppur area provides perfect ecological surroundings for using the molecular phylogeny methods asses the genetic link among freshwater fish.

Figure 1: Study Area Map Showing Sampling Areas of Anuppur District, Madhya Pradesh, India.



Source: Google Earth Pro

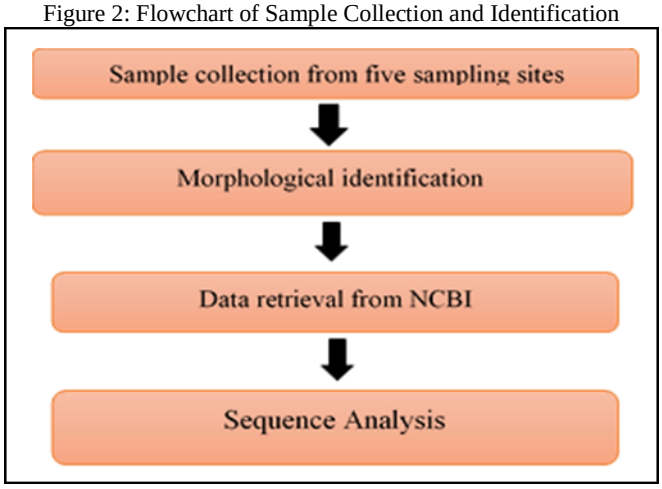
Table 1. Sampling Site Description

Site No.	Site Name	Type of Water Body	Habitat Type	Key Features
1.	Johila Dam	Reservoir	Lentic	More depth, High Anthropogenic activities
2.	Kantur Dam	Reservoir	Lentic	Sedimentation, No flow
3.	Batki Pond	Pond	Lentic	Aquatic Vegetation and Still Water
4.	Lapti Dam	Reservoir	Lentic	Moderate Depth, Seasonal fluctuation
5.	Bahpuri Dam	Reservoir	Lentic	Moderate Depth, local activities

3.2. Sample Collection and Identification

The fish samples were collected from the five selected freshwater sites in the Anuppur district of Madhya Pradesh during the period of October to march, covering the post-monsoon and winter seasons. This time period was chosen due to relatively stable climatic conditions and the fish species were more readily available. The sample collection was carried out in the morning hours between 7:00 AM to 9:00AM with the help of the local fisherman by using the cast net, gill net and standard fishing methods. Due to preserve their morphological characteristics the fish sample were handled and collected carefully. The primary identification was carried out based on the morphological characteristics such as body shape, body colour, fin structure and scale pattern (Talwar & Jhingran, 1991). Molecular verification was used to confirm the accuracy and reliability of morphological identification by comparing it with the DNA sequence data obtained from the NCBI gene bank.

Figure 2: Flowchart of Sample Collection and Identification



3.2.1. Data Retrieval

DNA sequences of the selected freshwater fish species were retrieved from the NCBI and the relevant sequences were selected and downloaded in FASTA format. Because of its strong selective capacity in species level identification and phylogenetic research, the mitochondrial cytochrome c oxidase I (COI) gene was chosen as a standard barcode marker. Only strong sequences with the complete COI region and reliable interpretations have been selected for the accuracy, and their accession numbers were recorded.

Due to limitations in molecular laboratory facilities and resources, the present study was restricted to in silico analysis using publicly available COI gene sequences retrieved from the NCBI GenBank database.

3.2.2. Selection of The Sequence and Processing

Sequences that were retrieved from the NCBI gene bank were carefully screened to ensure the consistency and quality of the data (Sayers et al., 2022). The criteria for the selection of the accurate identification of COI gene includes sequence length and sequence completeness. Sequences containing the missing regions, poor quality were trimmed from data set to get accurate result. To maintain the uniformity, the sequences which represents comparable gene regions were retained for further analysis.

3.3 Multiple Sequence Alignment

The multiple sequence alignment of the retrieved FASTA sequences was performed using ClustalW (Thompson et al., 2003). Generally Used to find the conserved and variable nucleotide regions among the chosen COI gene sequences by using the MEGA software (Kumar et al., 2018). In order to ensure the structural similarity between different sequences, the alignment was carefully checked. In order to improve the alignment quality and reduce computational mistakes, unclear and poorly aligned regions were eliminated. The next phase improved the level of accuracy of phylogenetic construction and later genetic distance estimation.

Figure 3: MEGA-12 Alignment Explorer Window

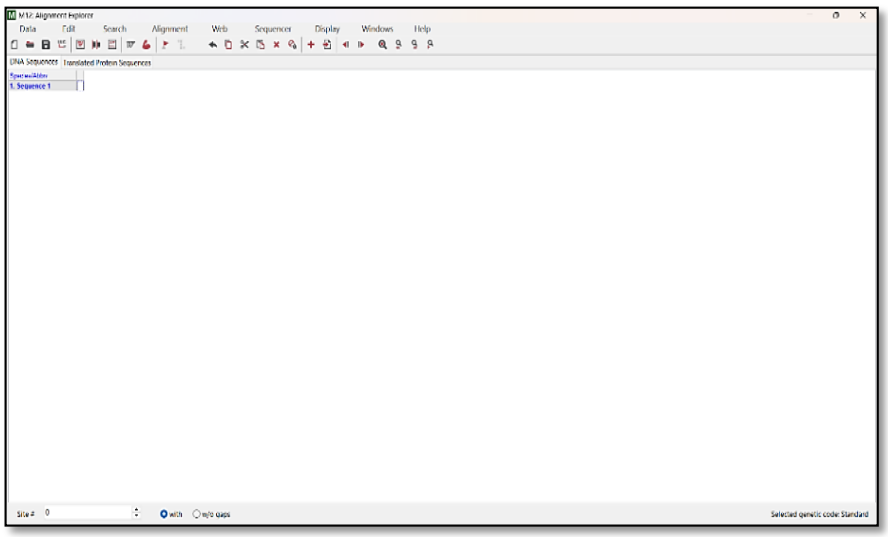


Table 2. Fish species name with their Accession Number

Serial No.	Species Name	Accession No.
1.	*Labeo rohita*	OR148140.1
2.	*Channa punctata*	JX983251.1
3.	*Catla catla*	JX983237.1
4.	*Cirrhinus mrigala*	JX983257.1
5.	*Cyprinus carpio*	JX983283.1
6.	*Labeo calbasu*	JX983339.1
7.	*Oreochromis niloticus*	MG428624.1
8.	*Pangasianodon hypophthalmus*	MG837968.1
9.	*Pethia ticto*	JX983474.1
10.	*Puntius chola*	MK599537.1
11.	*Puntius sophore*	JX983465.1
12.	*Tor putitora*	OR148221.1

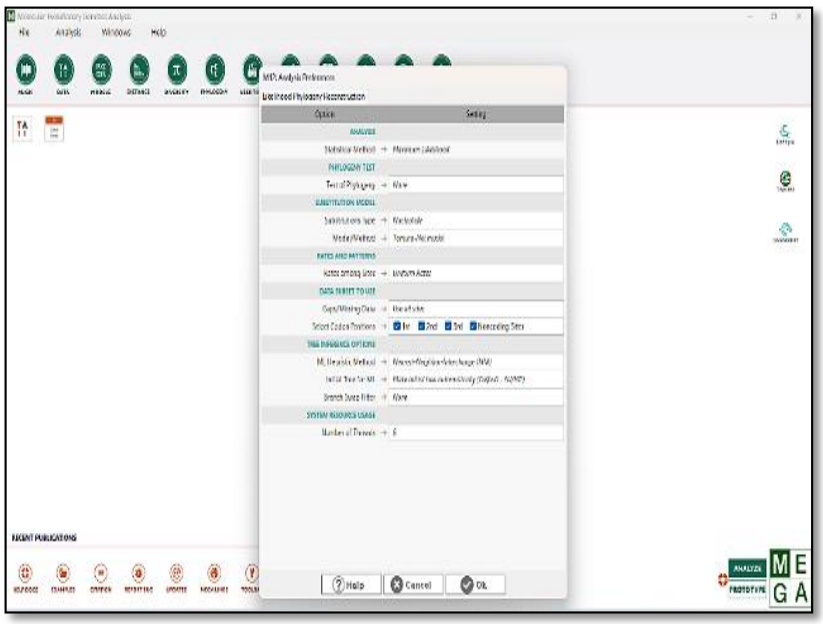
3.4 Genetic Distance Analysis

Pairwise genetic distance among the selected fish species was calculated by using the Kimura 2-parameter (K2P) model implemented in MEGA software (Kimura, 1980, Kumar et al., 2018). which is often used in the DNA barcoding studies. The higher the K2P value more the evolutionary divergence, lower the value indicates the closely related species.

3.5 Phylogenetic Analysis

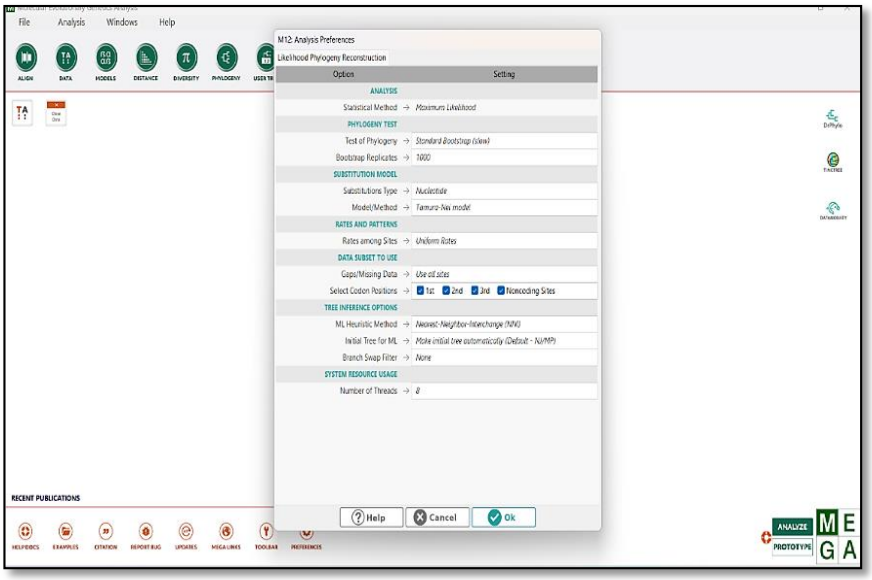
The MEGA software used to derive phylogenetic connections (Kumar et al., 2018). The phylogenetic tree was built using the neighbour-joining method technique (Saitou & Nei, 1987) and the bootstrap analysis with 1000 replicates was performed to assess the robustness of the topology.

Figure 4: MEGA Evolutionary Software for Phylogenetic Analysis



In addition, phylogenetic analysis was also performed using the Maximum likelihood (ML) method (Felsentein, 1981) in MEGA software. The Tamura-Nei model was used for the nucleotide substitution. The analysis was conducted assuming uniform rates among sites, and gaps were treated using all sites. The initial tree was automatically produced, and the ML tree was determined using the Nearest-Neighbour-Interchange (NNI) Bayesian approach with 1000 bootstrap replicates to provide a more robust and statistically reliable estimation of evolutionary relationships.

Figure 5: Maximum Likelihood Analysis Parameters



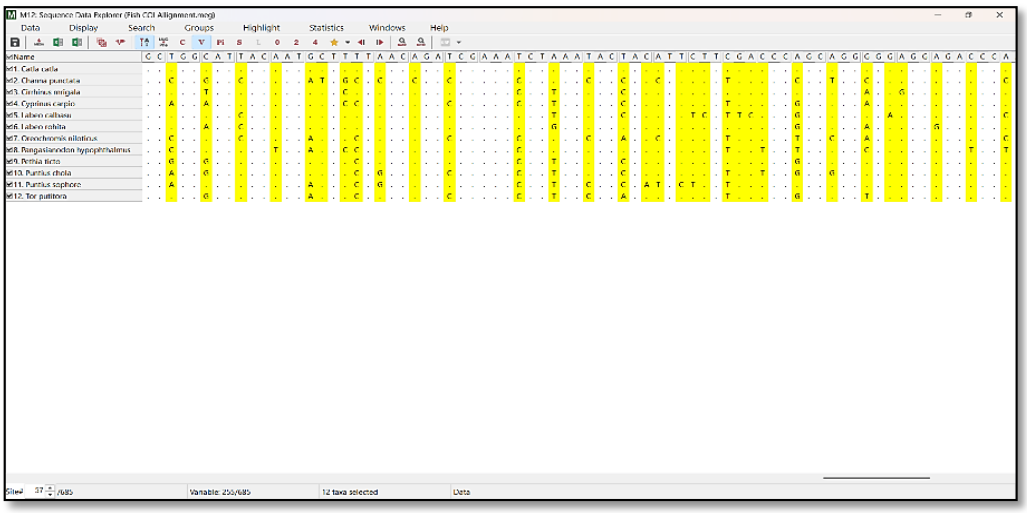
IV. RESULT

The results of the molecular analysis-based o the COI gene sequences are display through the multiple sequence alignment, Genetic distance analysis and phylogenetic analysis.

4.1. Multiple Sequence Alignment

The mitochondrial COI gene sequences of the selected freshwater fish species revealed both conserved and variable nucleotide regions and the variable regions are highlighted in yellow colour, when multiple sequence alignment was performed. All species of fish indicated a number of highly conserved sites, indicating that the COI gene region went through evolutionary conservation processes. At the same time, different distinct variation sites were identified, representing the genetic diversity between species. The distantly related species like *Oreochromis niloticus* and *Channa punctata* displayed more nucleotide variations while the closely related species like *Labeo rohita* and *Labeo calbasu* showed the less nucleotide variation.

Figure 6: Multiple Sequence Alignment of Mitochondrial COI Gene Of Selected Fish Species Showing Conserved Regions and The Variable Regions Highlighted in Yellow Colour Showing Similarity and Diversity



4.2. Genetic Distance Analysis

The pairwise distance analysis based on the Kimura 2-parameter (K2P) model, showed the divergence among the selected freshwater fishes. The genetic distance values ranged between 0.067 to 0.282 which indicates the close and distant relationship between the species. *Labeo rohita* and *Catla catla* had the lowest genetic distance that is 0.0671, indicating closer genetic similarity. Slightly higher but still low divergence (0.0830) was observed between *Labeo rohita* and *Labeo calbasu*. Similarly, relatively low divergence values were observed among other cyprinidae species. Indicating

their close relationship. The observed variations or distance may be due to the gene-specific evolutionary patterns and sequence level differences.

In contrast, higher genetic distance values were observed between *Channa punctata*, *Pangasianodon hypopthalamus* and *Oreochromis niloticus*, indicating major genetic divergence. Highest genetic distance seen between the *Puntius sophore* and *Pangasianodon hypopthalamus*. While, other species showed moderate genetic distance values, indicating intermediate evolutionary connections.

Figure 7: Pairwise Genetic Distance Among Selected Fresh Water Fish Species Using COI Gene Sequences (K2P).

M12: Pairwise Distances (Fish COI Allignment.meg)

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	1	2	3	4	5	6	7	8	9	10	11	12
1. Catla catla												
2. Channa punctata	0.2674											
3. Cirrhinus mrigala	0.1236	0.2514										
4. Cyprinus carpio	0.1412	0.2420	0.1091									
5. Labeo calbasu	0.0774	0.2714	0.1330	0.1233								
6. Labeo rohita	0.0671	0.2556	0.1188	0.1234	0.0830							
7. Oreochromis niloticus	0.2304	0.2349	0.2540	0.2438	0.2567	0.2377						
8. Pangasianodon hypophthalmus	0.2315	0.2815	0.2601	0.2611	0.2574	0.2510	0.2398					
9. Pethia ticto	0.1688	0.2789	0.1739	0.1533	0.1704	0.1676	0.2644	0.2636				
10. Puntius chola	0.1738	0.2541	0.1953	0.1494	0.1758	0.1718	0.2427	0.2541	0.0968			
11. Puntius sophore	0.1772	0.2673	0.1783	0.1532	0.1726	0.1738	0.2645	0.2829	0.1109	0.1214		
12. Tor putitora	0.1320	0.2477	0.1446	0.1327	0.1358	0.1232	0.2028	0.2560	0.1747	0.1788	0.1724	

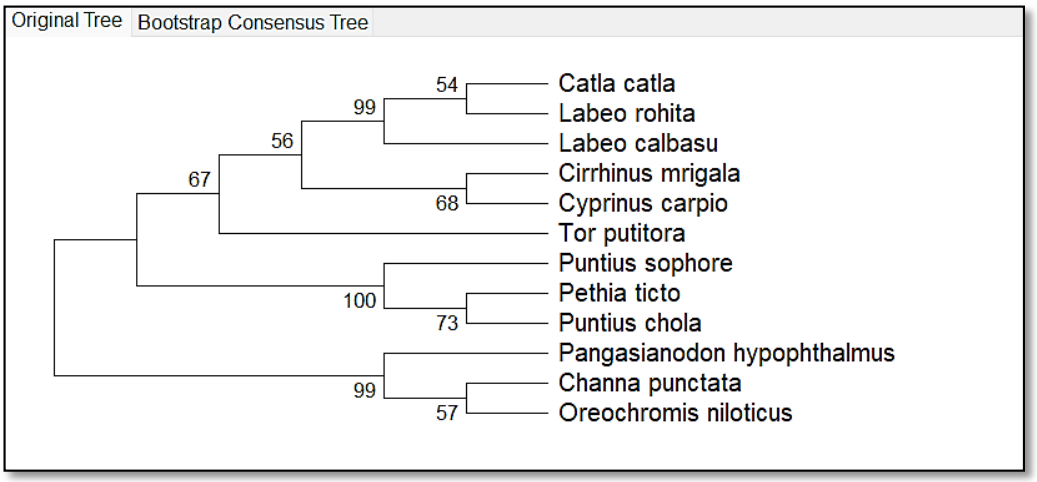
4.3. Phylogenetic Relationship Analysis

4.3.1. Neighbor-joining technique

Different clustering pattern among the selected fresh water fish species were found by phylogenetic analysis based on mitochondrial COI gene sequences by using the neighbor-joining technique of the MEGA software. Members of the cyprinidae family, such as *Catla catla*, *Labeo rohita*, *Labeo calbasu*, *Cirrhinus mrigala*, *Cyprinus carpio* and *Tor putitora* clustered closely together to create a large clade that showed a considerable genetic similarity. Within this clade close clustering and strong bootsrap support (99) between *Labeo rohita* and *Labeo calbasu*, indicating high genetic similarity. *Catla catla*, on the other hand was associated with this group but indicates relatively low bootstrap support (54), suggesting comparatively weaker support for its immediate clustering with *Labeo rohita*. *Puntius sophore*, *Pethia ticto* and *puntius chola* were found to form another well-supported sub-clade with high bootstrap support (100), indicating strong genetic affinity within these taxa. In contrast, the formation of separate branches by *Pangasianodon hypopthalamus*, *Channa punctata* and *Oreochromis niloticus* indicating significant genetic divergence and reflect their membership in different families. Among these species *Oreochromis niloticus* appeared as the most divergent lineage.

Strong support (>70) for major clade was shown by bootstrap values across the tree, with values reaching upto 99-100, while moderate support (50-70) was observed in some internal nodes, probably as a result of sequence diversity among species. Overall, the phylogenetic tree clearly demonstrates clustering consistent with taxonomic classification and validates the reliability of COI gene sequence in determining the evolutionary relationships among the selected freshwater fish species.

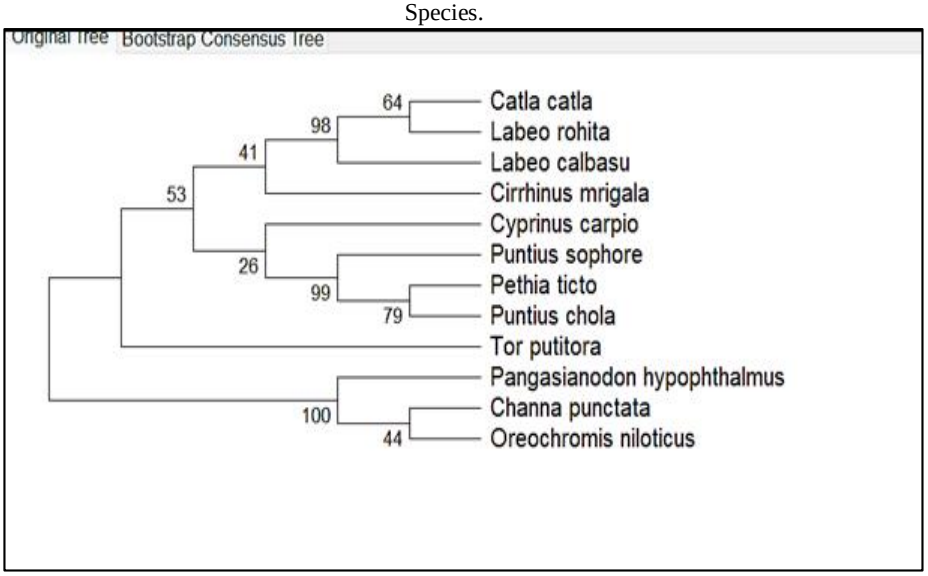
Figure 8: Construction of Neighbor-Joining Phylogenetic Tree Based on COI Gene Sequences Which Illustrates Evolutionary Relationships Among the Selected Fish Species.



4.3.2. Maximum-Likelihood method

The phylogenetic analysis using the Maximum-Likelihood (ML) method based on mitochondrial COI gene sequences revealed clear evolutionary relationships among the selected freshwater fish species. Species belonging to the family Cyprinidae, including *Catla catla*, *Labeo rohita*, *Labeo calbasu* and *Cirrhinus mrigala*, clustered closely together, indicating high genetic similarity and common ancestry. *Puntius sophore*, *Pethia ticto* and *Puntius chola* formed another closely related sub-cluster within the cyprinid group. In contrast, *Pangasianodon hypophthalmus*, *Channa punctata* and *Oreochromis niloticus* formed distinct branches, reflecting comparatively higher genetic divergence. The ML tree showed strong bootstrap support values (up to 98–100) for major clades, confirming the reliability of the clustering pattern and evolutionary relationships among the studied fish species

Figure 9: Construction of Maximum-Likelihood Phylogenetic Tree Based on COI Gene Sequences Which Illustrates Evolutionary Relationships Among the Selected Fish Species.



V. DISCUSSION

Based on the mitochondrial COI gene sequences, the present study revealed clustering patterns Largely consistent with taxonomic classification, specially within the cyprinidae family. A large clade with less genetic distances was formed by the species such as *Catla catla*, *Labeo rohita*, *Labeo calbasu*, *Cirrhinus mrigala*, *Cyprinus carpio* and *Tor putitora*, indicating close evolutionary connections. Strong clustering with high bootstrap support was formed by *Labeo rohita* and *Labeo calbasu*, however *Catla catla* comparatively showed lower support in its connection, maybe due to the gene-specific variation. Similar findings were done on supporting the effectiveness of COI gene in fish phylogenetics (Ward et al.,2005; Lakra et al., 2011). Due to their taxonomic differences, distant species such as *Channa punctata*, *Pangasianodon hypopthalamus* and *Oreochromis niloticus* displayed more divergence and formed separate branches, reflecting their taxonomic differences. In the present study, bioinformatics analysis provided deeper understanding of genetic relationships among species. Both conserved and variable regions were found in the multiple sequence alignment, which is consistent with earlier findings (Yu et al., 2022) that mitochondrial genomes contain both conserved and variable regions useful for phylogenetic analysis.

Overall, the study shows that the COI gene is a reliable molecular marker for species identification, although minor deviations may occur due to single-gene restrictions. The species were clearly grouped based on their taxonomic relationships in the Neighbor-Joining and Maximum Likelihood phylogenetic trees produced in this study. In both NJ and ML trees, species of the family Cyprinidae, such as *Catla catla*, *Labeo rohita*, *Labeo calbasu*, *Cirrhinus mrigala*, and *Cyprinus carpio*, formed a separate clade, indicating close evolutionary relatedness and genetic similarities among cyprinid fishes. Similar reports were found in a study on *Garra* and *Labeo* species utilizing COI-based phylogenetic analysis have found similar grouping tendencies among cyprinid fishes based on mitochondrial gene sequences (Barman et al., 2024; Jouladeh-Roudbar et al., 2024).The current study also showed that non-cyprinid fishes, such as *Channa punctata*, *Oreochromis niloticus*, and *Pangasianodon hypophthalmus*, inhabited distinct branches in the phylogenetic trees, showing a higher degree of genetic divergence from cyprinid fishes. Similar findings were documented by (Roy et al., 2024), who found that, according to mitochondrial genome analysis, *Mystus* species grouped apart from other freshwater fish groupings. Fish species that are closely and distantly related may be distinguished using COI-based molecular phylogeny, as demonstrated by the unique branching pattern seen in this study.

The present study was primarily based on in silico analysis using representative COI gene sequences retrieved from the NCBI GenBank database. Comprehensive wet laboratory validation and detailed barcode gap analysis could not be performed due to limited molecular laboratory resources and limited availability of multiple sequences for each species. Future studies involving larger sample size, wet laboratory sequencing and multilocus analysis may provide more comprehensive phylogenetic resolution.

VI. CONCLUSION

The present study illustrated the importance of mitochondrial COI gene sequences for the assessment of genetic relationships among freshwater fish species. Multiple sequence alignment, Genetic distance analysis, and phylogenetic relationship analysis showed the clustering patterns largely consistent with taxonomic categorization, particularly, in the cyprinidae family. Distantly linked species showed greater divergence and formed separate lineages, whereas, closely related species exhibited lower genetic distance and strong cluster formation. The minor variations observed in clustering patterns which high lights the change in genetic level. Overall, the study confirms that the COI gene is valuable molecular marker for phylogenetic analysis of different species and their identification and it may also use successfully in conservation and biodiversity assessment.

VII. Acknowledgement

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