Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 427-445
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)
Received: 20 August 2024, Accepted: 25 September 2024
DOI: https://doi.org/10.33282/rr.vx9i2.25

MOLECULAR PHYLOGENETIC STUDY BASED UPON MITOCHONDRIAL GENOME OF FRESHWATER FISHES OF PAKISTAN

Mubashar Hussain Malik[1], Syeda Rida Zahra Naqvi[2], Rabia Ishaq[3,7], Iffat Fatima[4], Ayesha Sajjad[1], Anisa Iftikhar[5], Samara Irfan[1], Iqra Asif[*3,6]

[1]Department of Zoology, The University of Lahore, Sargodha Campus, Pakistan
[2]College of Fisheries and Life Science, Shanghai Ocean University, 201306N Shanghai, China
[3]Department of Zoology University of Sargodha, Pakistan
[4]Department of Chemistry, University of Sargodha. Pakistan
[5]Department of Biology, Clarkson University, USA
[6]Department of Biological Sciences, Superior University Lahore Pakistan
[7]Government Associate college 75/SB Sargodha

Abstract

This study presents a phylogenetic analysis of six freshwater fish species from Pakistan, utilizing molecular markers from the 12S and 16S rRNA mitochondrial genes. We conducted PCR amplification and direct sequencing of gene fragments from *Catla catla*, *Rita rita*, *Mystus seenghala*, *Labeo calbasu*, and *Bagarius bagarius*. This analysis is the first molecular investigation of freshwater fish in Pakistan, offering insights into their evolutionary relationships. Results indicated that Labeo rohita belongs to the genus Labeo, with genetic similarities of 91.4% and 88% observed between *Rita rita* and *Bagarius bagarius*, respectively. Furthermore, *Mystus seenghala* and *Catla catla* showed similarities of 91.2% and 92.28% with Mystus refescens and Labeo catla. This research enhances our understanding of the taxonomy and diversification of these species in the region.

Keywords: Molecular Marker, 12s and 16s, Fresh water fishes, mitochondrial genome

INTRODUCTION

Pakistan is endowed with diverse water resources, including rivers, lakes, and oceans, with the Indus River system being the largest. This country features a rich variety of fish fauna, influenced by its transitional zoogeographical zones among the Palearctic, Ethiopian, and Oriental regions (Mirza, 1994).

Various studies have documented the freshwater fish diversity in Pakistan, identifying 183 species across 11 orders, 26 families, and 83 genera (Mirza, 2003; Khan et al., 2011). Estimates suggest approximately 193 freshwater species, with 16 commercially significant for aquaculture (Rafique et al., 2012; Peter, 1999).

Thirty freshwater fish species in Pakistan are of high economic importance (Khan et al., 2012). However, fish populations are declining due to habitat destruction, urbanization, and pollution (Pervaiz, 2011; Limburg, 2011; Brown et al., 2005). Increased agricultural runoff and pollution further threaten fish diversity (Khan et al., 2012). Previous studies have focused on fish species diversity but often overlook conservation status and population dynamics (Rafique et al., 2012). Overfishing for commercial and nutritional purposes is significantly impacting native fish habitats, particularly ornamental species. The aquaculture industry is emerging, with the Anabantoidei suborder being particularly important in this context (Degani, 2001).

Phylogenetic studies of fish utilize molecular, biochemical, geographical, and morphological parameters (Liu et al., 2006; Feulner et al., 2006). The Anabantoidei suborder, part of the Perciformes order, is characterized by air-breathing chambers above the gills and comprises about 80 species across 16 genera, though its systematic classification remains debated (Britz, 1994; Vierke, 1988; Sattar et al., 2024).

Vierke (1988) categorized labyrinth fishes into four families: Anabantidae, Belontiidae, Osphronemidae, and Helostomatidae, with the Anabantidae family showing diverse isozyme frameworks (Veit et al., 1990; Rizwan et al., 2024).

Species such as Trichogaster leerii and Trichogaster trichopterus are said to exhibit extreme diversity of morphology and distributional ranges. Isoenzyme assays and DNA tickling comprising of Growth Hormone (GH) sequences are necessary for phylogenetic work and especially so in the diminutive Anabantoidei suborder (Degani, 2004; Roos, 2005). While Cytochrome and 12S genes remain favorites in evolutionary genetic studies, Reliable Restriction Fragment Length Analysis performed on mt DNA has been efficient in species discrimination as well

MATERIALS AND METHODS

Sample Collection

18 samples of six fresh water fish species *Labeo rohita*, *Rita rita*, *Mystus refescens*, *Catla catla*, *Labeo calbasu* and *Bagarius bagarius* were collected from different three different places of Pakistan

Table 1: Collection of fish samples

Sr. No	Fish Name	Quantity	Place	Date
1	*Labeo rohita*	03	Khushab, Sahiwal	21/07/2019
2	*Rita rita*	03	Shorkot, Sahiwal	02/08/2019
3	*Bagarius bagarius*	03	Khushab, Shorkot	05/11/2019
4	*Labeo calbasu*	03	Sahiwal, Khushab	06/11/2019
5	*Mystus refescens*	03	Sahiwal Jehlum	03/12/2019
6	*Catla catla*	03	Shorkot, Sahiwal	08/12/2019

Preservation

All the samples were preserved in 100% ethanol in the molecular lab of university of Lahore Sargodha campus.

DNA Extraction Method

DNA Extraction

Fish samples were taken and fish meat of *Labeo rohita*, *Rita rita*, *Mystus refescens*, *Catla catla*, *Labeo calbasu* and *Bagarius bagarius* was taken using surgical blades. DNA extraction was carried out by following Russel and Sambrook method. Fish meat was minced and put into the eppendrof tube.330µl lysis solution, 40 µl SDS (Sigma Aldrich, Germany), 30 µl proteinase K (Biomatik Corporation, Canada) were used. The eppendrof tubes were labeled and then put in the water bath on a floater for overnight at a temperature of 56℃

Purification of DNA

As the second day begins, the tubes were drawn out from the water bath and then all the samples were centrifuged for 8 minutes at the speed of 1200rpm. Two layers were formed in each tube and from all the tubes the upper lysate was transferred to another tube to prevent contamination. Equal volume of buffered phenol was added to each tube and shaked with hands the tubes became milky. The tubes were again subjected to centrifuge for 8 minutes at speed of 1200rpm. The lysate was transferred to new tubes and chloroform and isomyl (Merk KGoA,Germany) were added in 24:1 ratio and centrifuged at 10,000rpm for 6 minutes.

Precipitation of DNA

Double volume of ice-chilled ethanol was added to supernatant. The tubes were kept overnight at -20℃. The tubes were again centrifuged for 8 minutes at 12000rpm. White pallot was obtained at the bottom of the tubes.

Washing

70% ethanol was added in the eppendrof tubes and centrifuged for 5 minutes at 6000rpm. The ethanol was discarded carefully and the tubes were open to dry.

Addition of PCR water

After washing the Eppendorf tubes 40µl PCR water was added in each fish sample for the elution of DNA.

Agarose Gel Electrophoresis

The molecules of the DNA were isolated based on their size and accused thickness of the assistance of Agarose gel electrophoresis.

Gel Preparation

1g Agarose (Electro, England) was weighted in an electronic weight machine and mixed in a 100ml TBE buffer

Gel Casting

The comb was fixed in a gel casting tray for the preparation of wells. TBE buffer with ethidium bromide was added into the gel casting try slowly to avoid the formation of bubbles. The gel was solidified after sometime and the comb was removed which created wells in gel. Gel tank filled with 0.5X TBE buffer and caster tray was shifted to the gel tank.

The scotch tape was pasted on the working table for the mixing of DNA with Bromophenol blue. It is blue in color and it glow in gel documentation system. It enhances the density of DNA so the samples were loaded well in the wells.

3µl labeled sample was mixed with the 3µl of Bromophenol blue on the tape and with the help of micropipette the mixer was loaded in the wells. The gel was run to segregate high molecular weight bands on electrophoresis apparatus for 15 minutes at 100 volts. As the DNA is negatively charged molecule so it moves from negative to positive electrode when the switch is on .the molecules of DNA were separated on the basis of their molecular weight.

Gel Documentation

The gel with loaded DNA of fish samples were shifted in the gel documentation system under the ultraviolet light. The DNA was seen and the picture was saved.

Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 427-445
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)

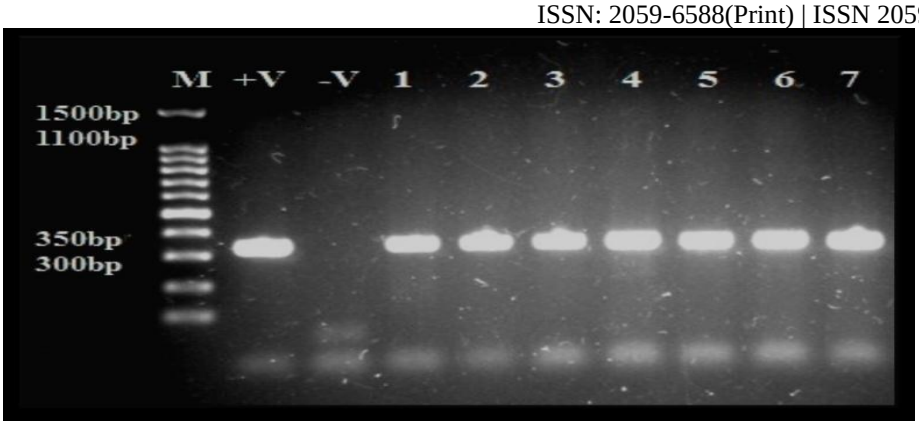

Figure 1: Gel results of fish samples.

Selection of Primers

The primer used for the PCR were selected from the literature

Table 2: primers for PCR

Sr.No	Primer name	Sequence
1	MarVer3F	AGACGAGAAGACCCTRTG
2	MarVer3F	GGATTGCGCTGTTATCCC

Polymerase Chain Reaction

The two strands of sample DNA were separated and form single strand of DNA at the temperature of 94℃. The selected primers started binding with the template and at the end of each primer, DNA polymerase enzyme added nucleotide bases by primers. The primers were extended by template and new double stranded DNA molecules were produced. The cycles were repeated many times to get the desired number of genes.

PCR Master Mix

The PCR master mix contain 2.5µl MgCl2 ,PCR buffer 2.5µl, forward primer 1.25µl, reverse primer 1.25µl, dNTPs 0.6µl, Taq polymerase 0.5µl and DNA template 2µl. The PCR water was added to make the total volume equal to 25µl.

Table 3: Master Mix for PCR

Substance	Unit Quantity	Quantity for number of samples
MgCl2	2.5µl	2.5×2=5 µl
PCR buffer	2.5µl	2.5×2=5 µl
Forward primer	1.25µl	1.25×2=2.5 µl
Reverse primer	1.25µl	1.25×2=2.5 µl
dNTPs	0.6µl	0.6 ×2=1.2 µl
Taq polymerase	0.5 µl	0.5×2=1 µl
DNA template	2 µl	2×2=4 µl

PCR Amplification Process

The course of amplification comprised of three stages. Introductory denaturation was done for 5 minutes at 94℃. 30 cycles were repeated at a similar temperature for one minute at 72℃ for 2 minutes. Annealing process was completed at the optimized annealing temperature of every primer. Extension at 72℃ for 5 minutes keeping temperature of 4℃.

Analysis of PCR Products

Gel Electrophoreses

The PCR amplified products were subjected to gel electrophoreses. This time a ladder was run on the gel to compare the data. The sequence was visualized on the gel documented apparatus and the picture was saved. The ladder length use in this process was 1000picomole.

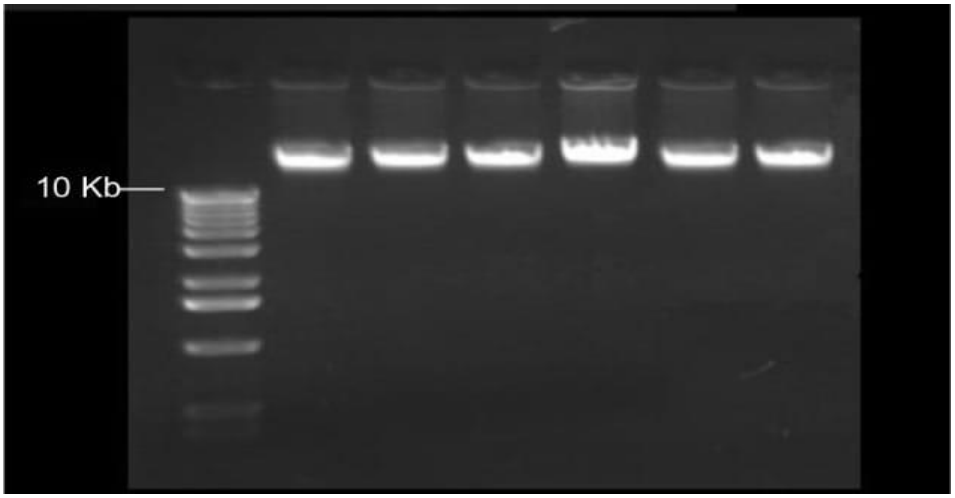

Figure 2: Visualizing DNA after PCR.

Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 427-445
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)

Gene Sequencing

The samples were sent to CAMB Lahore for gene sequencing.

Annealing

The process of annealing was done by using the software MEGA X and Bio-edit.

RESULTS

DNA Extraction and Quantification

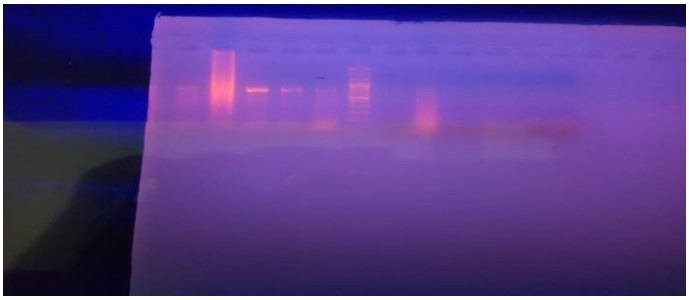

Figure 3: DNA Extraction and Quantification

The DNA of genome obtained from 15 samples of fishes and visualized and confirmed by Gel-electrophoresis. The whole DNA of genome obtained from meat of the fishes. The DNA was extracted by using Russel and Sambrook protocol with little modification. The standard optimized through cautious optimization of proteinase K concentration, standard Phenol: Chloroform extraction techniques. The results of genomic DNA extraction of PCR were visualized and recorded.

The genomic data of other related members was received by the Gene Bank for more genetic evolutionary analysis. Source and the coming number of the specimens are given in the following table 4.1

Table 4.1 showing specimen names, Accession number and source of related species.

Species Name	Accession Number	Source
C.carpio	MK088487.1	GeneBank
Ctenopharyngodon idella	MG827396.1	GeneBank
Danio rerio	KM244705.1	GeneBank
Hypophthalmichthys molitrix	Mk180232.1	GeneBank
Labeo bata	AP011198.1	GeneBank
Labeo bogut	AP013338.1	GeneBank

Labeo fimbriatus	KP025676.1	GeneBank
Laeo gonius	KT001152.1	GeneBank
Catla catla	KY419138.1	GeneBank
Cirrhinus cirrhosus	AP012150.1	GeneBank
Bangana tungling	KF752481.1	GeneBank
Labeo pangusia	AP013340.1	GeneBank
Labeo angra	AP011329.1	GeneBank
Labeo catla	MN830933.1	GeneBank
Samilabeo notabilis	NC045916.1	GeneBank
Bangana decora	KM999224.1	GeneBank
Cirrhinus moliterola	KF160921.1	GeneBank

To evaluate the correct phylogenetic data of our fish samples many related fish species were studied ant their data was taken from NCBI and BLAST results helped in the identification of species. The complete mitochondrial genome of closely related species was collected from Nucleotide blast and the data was saved in FASTA formate so that it can easily be used for the construction of phylogenetic tree on the basis of maximum likelihood method.

Construction of Phylogenetic Tree with Mega Software

To construct the phylogenetic tree from the MAGA X software the DNA sequences were saved and name (fish mega file) was given the software also demanded the title of the file so the title of (fish tree) was given the phylogenetic tree was constructed by using the option phylogeny in the software.. The software automatically reconstruct the phylogenetic tree by likelihood method (386) and also the detail of closely related species are also given with the phylogenetic tree.

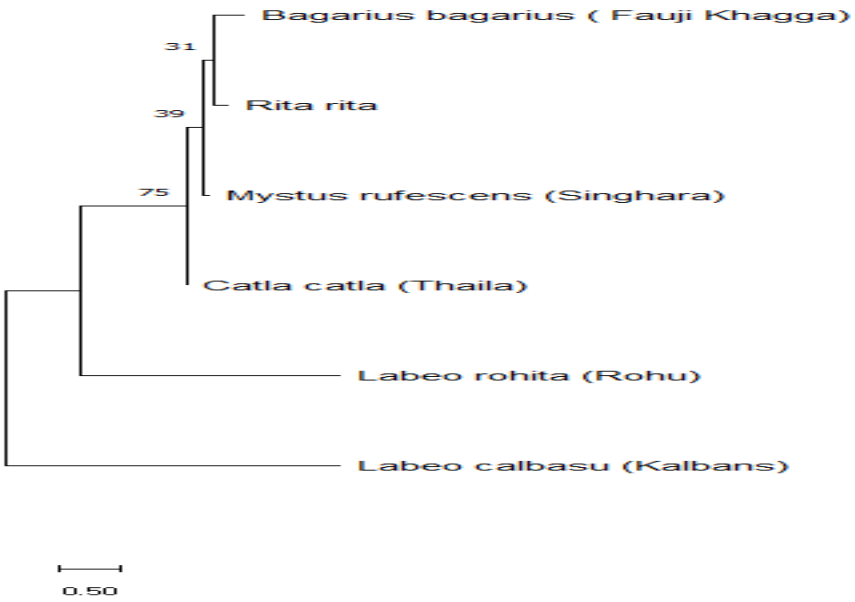

Figure 4: Evolutionary relationships of taxa (Neighbor-Joining)

The evolutionary history was inferred using the Neighbor-Joining method (saitu et al, 1987). The optimal tree is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (saitou et al, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al, 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 386 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al, 2021)

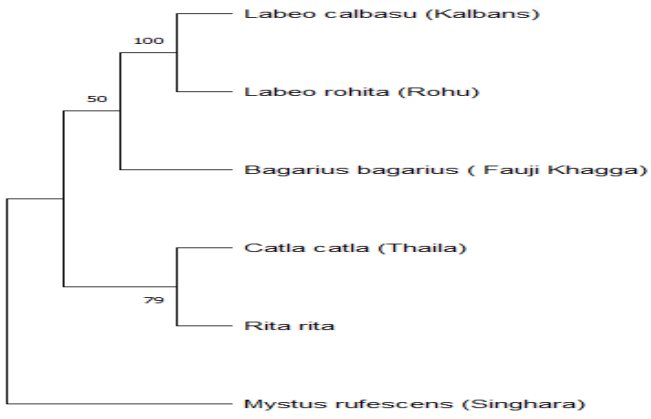

Figure 5: Maximum Parsimony analysis of taxa

The evolutionary history was inferred using the Maximum Parsimony method. The most parsimonious tree with length = 520 is shown. The consistency index is 0.905769 (0.768868), the retention index is 0.588235 (0.588235), and the composite index is 0.532805 (0.452275) for all sites

and parsimony-informative sites (in parentheses). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein,1985). The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (pg. 126 in ref. (Nei et al,2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). This analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd. There were a total of 386 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al, 2021

Morphological Characteristics of Fish Samples

Labeo rohita (Hamilton, 1822)

FISH BASE

COMMON NAME	**Dum-bra, Rohu**
Occurrence	Native
Salinity	Freshwater
Distribution	Pakistan, India, Bangladesh

Maximum weight, size and age

The maximum weight of the fish is 45kg, maximum size of the fish is 200cm reported is and the maximum age of the fish is 10 years.

Mating behavior and Life Cycle

It spawns where flood waters spreads in middle reaches. It also mates in the reservoirs. (Khan et al, 1975)

Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 427-445
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)

***Labeo calbasu* (Hamilton, 1822)**

FISH BASE

Common name	**No common name**
Occurrence	Native
Salinity	Freshwater
Distribution	Sindh, Punjab, KPK

Maximum size of the fish

The Fishes Maximum size is 90cm. The mature size of fish is 33cm.

Life cycle of fish

The adults feed on the plankton and live in the rivers and ponds. The fish always feed generously on the slow-moving water. (Talwar et al, 1991)

Rita rita (Hamilton, 1822)

FISH BASE

Common name	**No common name**
Occurrence	Native
Salinity	Freshwater
Distribution	Sindh, Punjab, Baluchistan

Distribution

It is distributed among Pakistan, Myanmar, Afghanistan, Nepal and Bangladesh.

Biology

It lives in the muddy to clear water of rivers and estuaries. It is a very good type of food fish and it feed on shrimps, insects, mollusks and fish (Talwar et al, 1991).

Saperata seenghala (skyes, 1839)

FISH BASE

Common name	**Singhara**
Occurrence	Native
Salinity	Freshwater
Distribution	Found in river Indus

Distribution

It is found in Nepal, Afghanistan, Bangladesh and Pakistan. It is also reported from Thailand, Greece and China (Talwar et al, 1991)

Size of Fish

The total length of the male unsexed is approximately 150cm but common length of the male unsexed is round about40 cm

Biology

It is found in ditches, rivers, canal and other fresh water areas. It is a carnivorous fish which mostly feed on insects and small fishes. It breeds before the start of the monsoons.

Catla catla **(**Hamilton, 1822; Talwar et al, 1991)

FISH BASE

Common name	**Thaila, Boassa, Plakaraho, Taylee**
Occurrence	Native
Salinity	Freshwater
Distribution	Punjab, Sindh, Baluchistan (Native), Azad and Jammu and Kashmir (introduced). KPK (introduced).

Size and Weight of Fish

The maximum size of the fish is approximately 182cm unsexed male and the weight of the fish is near to 38.6kg

Remittances Review
September 2024,
Volume: 9, No: S 4, pp. 427-445
ISSN: 2059-6588(Print) | ISSN 2059-6596(Online)

Biology

The adult fish occur in the lakes, rivers and ponds. It is omnivorous fish and at the juvenile stage it feed on aquatic and terrestrial insects. It also feed on detritus and phytoplanktons. (Menon, 1999)

***Bagarius bagarius* (Hamilton,1822)**

FISH BASE

Common name	**No name**
Occurrence	Native
Salinity	Freshwater
Distribution	Punjab and Sindh

Size and Weight of Fish

The size of the fish is 39cm (Rahman, 2005). The weight of the fish is 215kg (Shafi et al, 2001)

Biology

It lives in the rocky pools of the medium size rivers. It feeds on small insects, small fishes and shrimps. It is an important food fish but the meat of the fish spoils quickly and causes illness. (Talwar et al, 1991).

DISCUSSION AND CONCLUSION

Comparative phylogenetic study of closely related organisms has been successfully executed by using genetic sequence data by many scientists. Through the utilization of genetic sequence data, numerous scientists have effectively conducted comparative phylogenetic studies on closely related organisms. This achievement has been made feasible by the abundance of vast genomic

datasets stored in biological databases, allowing for in-depth exploration and analysis of evolutionary relationships among species. The wealth of genetic information available in these databases has opened up new avenues for researchers to unravel the intricacies of evolutionary history and genetic relatedness across diverse taxa, leading to groundbreaking discoveries and insights into the interconnectedness of life forms on our planet. This might be possible due to availability of large genomic data deposited to biological data basis (Zimer et al, 2012).

Comparative genomics also helps biologists to identify similarities and differences among different organisms. On the basis of these similarities and differences we can construct phylogenetic trees. A phylogenetic tree is a graphical representation to study relationship between different groups of organisms. Phylogenetic trees can be constructed by using partial as well as complete genome (Irwin et al, 1991; Bilal[a,b], 2021). Among mitochondrial genes cytochrome oxidase 1 and ribosomal gene are highly conserved, thus they can be valuable tool for comparative genomic analysis and construction of phylogenetic tree. Comparative genomics plays a pivotal role in enabling biologists to discern and analyze the similarities and distinctions existing among a diverse array of organisms. By scrutinizing these shared traits and variations, researchers can meticulously craft phylogenetic trees, which serve as intricate graphical depictions illustrating the evolutionary relationships between various groups of organisms (Torriani et al, 2009). These phylogenetic trees are instrumental in shedding light on the intricate web of connections that link different species across the tree of life (Basharat et al., 2024). The construction of such trees can be facilitated through the utilization of both partial and complete genomic data, offering a comprehensive perspective on the evolutionary history and genetic relatedness of organisms. Notably, within mitochondrial genes, the cytochrome oxidase 1 and ribosomal genes stand out for their high degree of conservation, rendering them invaluable tools for conducting in-depth comparative genomic analyses and constructing robust phylogenetic trees that delineate the evolutionary pathways and relationships among different taxa (Hsieh et al 2001).

Also, the morphological differentiations do not necessarily translate into molecular speciation. In addition, although many informative bio geographic studies have been conducted many times, ambiguities about classification are not always resolved. Moreover, it is essential to recognize that morphological variations observed among organisms do not always directly correspond to distinct molecular speciation events. While the field of biogeography has seen numerous informative studies conducted over the years, it is important to acknowledge that these

investigations may not always conclusively resolve uncertainties surrounding species classification and evolutionary relationships. Despite the wealth of data and insights gathered from biogeographic research, there remain persistent ambiguities and complexities that continue to challenge our understanding of the intricate processes shaping biodiversity and species diversification across different ecological contexts and evolutionary time scales (Mayden et al, 2009; Jawad et al., 2023).

The 12S and 16S genes, prominent components of the mitochondrial genome, hold significant importance in the realm of phylogenetic research and homology analysis, particularly within the diverse world of fish species. These genes serve as valuable molecular markers that offer insights into the evolutionary relationships and genetic similarities among different fish taxa, providing researchers with a deeper understanding of the intricate patterns of divergence and shared ancestry within aquatic ecosystems. By harnessing the genetic information encoded within the 12S and 16S genes, scientists can unravel the complex evolutionary history of fish species, elucidating the processes of speciation, adaptation, and diversification that have shaped the remarkable diversity of fishes across various habitats and geographical regions. Through the utilization of these mitochondrial genes, researchers are equipped with powerful tools to explore the evolutionary dynamics and phylogenetic patterns that underpin the rich tapestry of fish biodiversity, paving the way for new discoveries and insights into the fascinating world of aquatic life (Dimmick et al, 2002; Shoaib et al., 2022)

In the present study six freshwater fish species were studied 3 samples of each species were taken and analyzed for morphological and molecular identification. All the work was done in the molecular lab of the university of Lahore Sargodha campus. DNA was extracted from all the 18 samples and were successfully analyzed through agarose gel electrophoresis. 16s gene was successfully amplified and sequenced for each sample successfully.

After amplification the raw sequence were analyzed by using bio edit software and subsequently were identified using online BLAST tool. *Labeo rohita* and the specimens showed 98 to 99% similarity with *L.rohita* in the data base. *L.rohita* was both morphologically and molecularly identified and confirmed that the fish in river Jehlum of Pakistan belongs to genus Labeo and species rohita.

Whereas some of the specimens were identified as novel from Pakistan. Sequenced data of *Rita rita* and *Bagarius bagarius* showed 94.14% and 88% similarity to both *Rita rita* and *Bagarius* respectively. However, *Saperata seenghala* (singhara) and *Catla catla* (Thaila) showed 91.2% and

94.28% similarity to *Mystus rufescens* and Labeo catla respectively. This showed and confirmed that the singhara fish inhibiting in the river Jehlum belongs to genus Mystus where the Thaila fish which inhibit in Jehlum belongs to genus labeo. It was also concluded that 16s is an efficient marker for the identification of the fish at molecular level.

The fishes inhibiting the fresh water of Pakistan are identified morphologically but in the present study we used a molecular method to determine the species and the research conclude that species were identified morphologically by the biologist are the same according to molecular analysis with respect to 12S and 16S. So, we can say that earlier which fishes are same on the basis of morphology they are also same on the basis of molecular data. So, it is suggested to reevaluate the freshwater species inhabiting Pakistan both morphologically and molecularly to evaluate their exact taxonomic status.

REFERENCES

Basharat, M., Bilal, A., Rizwan, M., Asif, I., Shahin, F., & Hussain, M. (2024). Identification Of Fish Diversity, Distribution, And Fauna At Head Qadirabad, Marala And Khankis, Chenab River, Punjab, Pakistan. Journal of Survey in Fisheries Sciences, 75-81.

Bej, D., Sahoo, L., Das, S. P., Swain, S., Jayasankar, P., & Das, P. (2013). Complete mitochondrial genome sequence of Cirrhinus mrigala (Hamilton, 1822). Mitochondrial DNA, 24(2), 91-93.

Betancur-R, R., Wiley, E. O., Arratia, G., Acero, A., Bailly, N., Miya, M., ... & Orti, G. (2017). Phylogenetic classification of bony fishes. BMC evolutionary biology, 17(1), 162.

Bibi S, Fiaz Khan M. (2019). Phylogenetic association of Schizothorax esocinus with other Schizothoracinae fishes based on protein coding genes. Mitochondrial DNA Part B, 4(1), 352–354.

Bilal, A. (2021). Impacts of depression on pregnancy: A review. Occup Med Health Aff, 9(2).

Bilal, A. (2021). Rabies is a zoonotic disease: a literature review. Occup. Med. Health Aff, 9(2).

Briolay J, Galtier N, Brito RM, Bouvet Y. Molecular Phylogeny of Cyprinidae Inferred from cytochrome b DNA Sequences. Molecular Phylogenetics and Evolution, 9(1), 100-108.

Britz, R. (1994). Ontogenetic features of Luciocephalus (Perciformes, Anabantoidei) with a revised hypothesis of anabantoid interrelationships. Zoological Journal of the Linnean Society, 112, 491-508. doi:10.1111/j.1096-3642.1994.tb00333.x

Brown WM, George M, Wilson AC. (1979). Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci USA, 76(4), 1967–1971.

Cairney M, Taggart BJ, Hoyheim B. (2000). Characterization of microsatellite and minisatellite loci in Atlantic salmon (Salmo salar L.) and cross-species amplification in other salmonids. Molecular Ecology, 9, 2175–2178.

CAMP. (1998). Conservation Assessment and Management Plan for freshwater fishes of India. Zoo outreach organization and NBFGR, Lucknow, p. 156.

Casavas I, Doulman DJ, Petr TO, Padro J, Debas L. (1996). Cambodia-rehabilitation and development needs of the fishery sector. FAO, Rome.

Chaturvedi A, Mohindra V, Singh RK, Lal KK, Punia P, Bhaskar R, Mandal A, Narain L, Lakra WS. (2011). Population genetic structure and phylogeography of cyprinid fish, Labeo dero (Hamilton, 1822) inferred from allozyme and microsatellite DNA marker analysis. Molecular Biology Reports, 38, 3513–3529.

Chauhan T, Rajiv K. (2010). Molecular markers and their applications in fisheries and aquaculture. Advances in Bioscience and Biotechnology, 1, 281–291.

Comi G, Iacumin L, Rantsiou K, Cantoni C, Cocolin L. (2005). Molecular methods for the differentiation of species used in production of cod-fish can detect commercial frauds. Food Control, 16, 37–42.

Degani, G. (2001). Blue gourami (Trichogaster trichopterus) model for labyrinth fish. Laser Pages Publishing, Jerusalem, Israel.

Degani, G. (2004). Application of RAPD and cytochrome b sequences to the study of genetic variations in F1 and F2 generations of two different strains of guppy (Poecilia reticulata, Peter 1854). Aquaculture Research, 35, 807-815. doi:10.1111/j.1365-2109.2004.01071.x

Degani, G., & Veit, M. (1990). Electrophoretic variations of isozyme systems in the muscle and liver of Anabantidae fish. Israeli Journal of Aquaculture, 42, 67-76.

Desai, V. R. (2003). Synopsis of biological data on the Tor mahseer Tor tor, (Hamilton, 1822). FAO Fisheries Synopsis, Rome, 36, 158.

DeWoody, J.A., Walker, D., & Avise, J.C. (2000). Genetic parentage in large half-sib clutches: Theoretical estimates and empirical appraisals. Genetics, 154, 1907-1912.

Dimmick, W. W., & Edds, D. R. (2002). Evolutionary genetics of the endemic schizorathicine (Cypriniformes: Cyprinidae) fishes of Lake Rara, Nepal. Biochemical Systematics and Ecology, 30(10), 919-929.

Dores, R. M., Rubin, D. A., & Quinn, T. W. (1996). Is it possible to construct phylogenetic trees using polypeptide hormone sequences? General and Comparative Endocrinology, 103, 1-12. doi:10.1006/gcen.1996.0088

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution, 39, 783-791.

Feulner, P. G., Kirschbaum, F., Schugardt, C., Ketmaier, V., & Tiedemann, R. (2006). Electrophysiological and molecular genetic evidence for sympatrically occurring cryptic species in African weakly electric fishes (Teleostei: Mormyridae: Campylomormyrus). Molecular Phylogenetics and Evolution, 39, 198-208. doi:10.1016/j.ympev.2005.09.008

Forselius, S. (1957). Studies of anabantid fishes. Parts I, II, III. Zoologiska Bidrag Fran Uppsala, 32, 593-597.

Jawad, M., Bilal, A., Khan, S., Rizwan, M., & Arshad, M. (2023). Prevalence and awareness survey of tuberculosis in the suspected population of Bajaur Agency in Fata, Pakistan: Prevalence and awareness survey of tuberculosis. Pakistan Journal of Health Sciences, 56-61.

Rizwan, M., Mushtaq, M., Bilal, A., Nawaz, T., Riaz, K., Hussain, M., ... & Basharat, M. (2024). Trace Out the Improvement Level and Awareness of Polycyctic Ovary Syndrome (Pcos) among General People and Educational Institute of Developed and Developing Countries. Journal of Bioresource Management, 11(3), 5.

Sattar, R. Z., Bilal, A., Bashir, S., Iftikhar, A., & Yaqoob, I. (2024). Embryotoxicity of fluconazole on developing chick embryos. The Journal of Basic and Applied Zoology, 85(1), 8.

Shoaib, M., Bilal, A., Mehmood, R., Naveed, N., Alfahad, M., & Rashid, U. (2022). Effect of Methionine on Growth Performance and Microflora in Common Carp. Journal of MAR Case Reports, 5(1).

Takagi, A. P., Ishikawa, S., Nao, T., Song, S. L., Hort, S., Thammavong, K., Saphakdy, B., Phomsouvanhm, A., Nishida, M., & Kurokura, H. (2010). Genetic differentiation and distribution routes of the bronze featherback Notopterus notopterus (Osteoglossiformes: Notopteridae) in Indochina. Biological Journal of the Linnean Society, 101, 575–582.

Talwar, K. P., & Jhingran, A. G. (1991). Inland fishes of India and adjacent countries. Oxford & IBH.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA), 101, 11030–11035.

Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution. https://doi.org/10.1093/molbev/msab120

Taverne, L. (1975). A propos de Tselfatia Arambourg, C., 1943 (Cénomanien du Maroc), de Protobrama (Cénomanien du Liban) et du rapport éventuel des Tselfatioïdes fossiles (Crétacé) et des Téléostéens du super-ordre des Ostéoglossomorphes. Annales de la Société Royale Zoologique de Belgique, 105(1–2), 53–72.

The formation of tetraploid stocks of red crucian carp × common carp hybrids as an effect of interspecific hybridization. Aquaculture, 192(2-4), 171–186.

Torriani, S. F., Penselin, D., Knogge, W., Felder, M., Taudien, S., Platzer, M., ... & Brunner, P. C. (2014). Comparative analysis of mitochondrial genomes from closely related Rhynchosporium species reveals extensive intron invasion. Fungal Genetics and Biology, 62, 34-42.

Vences, M., Thomas, M., Bonett, R. M., & Vieites, D. R. (2005a). Deciphering amphibian diversity through DNA barcoding: Chances and challenges. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 360, 1859–1868.

Vences, M., Thomas, M., Van Der Meijden, A., Chiari, Y., & Vieites, D. R. (2005b). Comparative performance of the 16S rRNA gene in DNA barcoding of amphibians. Frontiers in Zoology, 2(5), 1–12.

Vierke, J. (1988). Betta, gouramis and other anabantid labyrinth fishes of the world. TFH Publications.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. (2005). DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences, 360, 1847–1857.

Wilson, E. O. (2003). The encyclopedia of life. Trends in Ecology & Evolution, 18, 77–80.