Genetic characterization of minor carp (*Labeo gonius*) from Indian rivers revealed through mitochondrial ATPase 6/8 and D-loop region analysis: implications for conservation and management

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India is the habitat of many freshwater fish species that make a significant contribution to endemism. However, the native species population is declining at a very fast rate due to pollution, habitat loss or degradation, new species introduction, hydrologic alterations, and overfishing. The minor carp, *Labeo gonius*, is one of the important fish species in the Indian river systems and holds enormous potential in culture fisheries. Therefore, the precise status of population structure is a prerequisite for the management of the species. In the present study, we assessed the potential of mtDNA ATPase 6/8 and D-loop regions as markers to determine the genetic diversity and phylogeography of *L. gonius* from Indian rivers. We have selected five major Indian rivers for investigation which could represent geographically distant populations of the studied fish. A total of 210 ATPase 6/8 sequences (842 bp) and 203 D-loop region sequences (683 bp) of *L. gonius* from the five major Indian rivers were submitted to NCBI. Results revealed that ATPase 6/8 has 17 haplotypes and 70 polymorphic sites with an overall haplotype diversity of 0.560 ± 0.036 and nucleotide diversity of 0.00347 ± 0.00. Furthermore, D-loop analysis revealed 58 haplotypes, 67 polymorphic sites, 0.954 ± 0.006 overall haplotype diversity, and 0.01914 ± 0.00 nucleotide diversity. The hierarchical AMOVA analysis indicated that a high proportion of the genetic variation (76.46% and 70.22% for the D-loop and ATPase 6/8 regions, respectively) was attributed to among-population variation under gene pool-1 (considering all the stocks from Ganga, Brahmaputra, Teesta, Narmada, and Mahanadi as a single pool). Furthermore, in gene pool-2, samples were divided into two groups: group 1 (Himalayan rivers)—Ganga, Brahmaputra,

and Teesta; and group 2 (peninsular rivers)—Narmada and Mahanadi. Genetic variations in the D-loop and ATPase 6/8 regions were found among populations within groups at 70.65% and 53.48%, respectively. Haplotype network analysis revealed that there were significant differences in distributions and frequencies. The observed unique haplotypes might be useful for differentiating the stocks. In brief, the present study suggested that the D-loop and ATPase 6/8 regions are potential markers and represent a significant contribution to the genetic characterization of the minor carp *L. gonius* from major Indian rivers. Moreover, the *L. gonius* population along the east coast especially in the Himalayan rivers (Ganga, Brahmaputra, and Teesta river basin) is found to be genetically more diverse. The results would provide a scientific basis for the assessment of fisheries resources and management of this important species.

Introduction

India is endowed with a vast network of rivers and tributaries, which play crucial roles in inland fisheries, agriculture, hydroelectricity, transportation, and livelihoods for a vast number of people. Riverine resources provide essential ecological services that contribute to overall health, supporting biodiversity and productivity of the environment. Some of the major rivers in India include the Ganges, Brahmaputra, Teesta, Mahanadi, and Narmada. The river Ganga/Ganges is the largest river in India and the fifth longest in the world and has high cultural and religious value (Das et al., 2023). The river Ganga originates from the western Himalayas "Gaumukh" and discharges into the Bay of Bengal. The Brahmaputra is another major river that originated from the Himalayas, which flows through three countries, viz., China, India, and Bangladesh. The river Teesta is a tributary of the Brahmaputra; historically, the orientation of the Teesta River has shifted several times, and it has intermittently flowed into both of the major rivers (Ganga and Brahmaputra) (Richards et al., 2021; Chaubey et al., 2023). The river Mahanadi is a major rainfed peninsular river in East Central India, and it is very vulnerable to climate change and seasonal variability (Jin et al., 2018; Gunjan et al., 2020). The Narmada is another important peninsular river that rises on the Amarkantak Plateau in Madhya Pradesh and discharges into the Arabian Sea, making it the fifth biggest river in India. India is also home to many freshwater fishes contributing a high degree of endemism to the world's biological resources (Mogalekar et al., 2017). However, the riverine ecosystem of India is suffering from intense human intervention resulting in habitat loss/degradation, pollution, and hydrologic alterations, and as a result, many freshwater fish species are at risk and are declining

(Sarkar et al., 2012; Das et al., 2023). Moreover, with the global climate change scenario, the diversity and populations of many species will have to adapt or will go extinct (Peñuelas et al., 2013; Scheffers et al., 2016).

Genetic diversity is important for the long-term survival of species and, thus, is of high conservation concern (Funk et al., 2019). In this context, there is an urgent need to understand the genetic variation of freshwater fish species for the conservation of genetic resources (Weitemier et al., 2021). Genomics technologies have aided population characterization using species-specific markers, and an appropriate marker must be selected to determine genetic diversity in a population. Molecular genetic markers have become valuable tools in population genetics, conservation biology, and evolutionary studies. Among the different available DNA markers, mitochondrial DNA (mtDNA) markers have been widely and effectively used for the assessment of population genetic analysis, genetic diversity, gene flow, population structure, phylogeography, evolutionary studies, and stock characterization (Habib et al., 2012; Sati et al., 2015). The mtDNA markers are generally variable within populations and widely used to determine the variation at interspecific and intraspecific levels. This has been attributed to its maternal inheritance, lack of recombination, high rate of evolution, and practical advantages such as simpler operation, universal primer, and quick and comparable results (Satoh et al., 2016; Forcina et al., 2021). Moreover, mtDNA markers are cost-effective and easy to develop in comparison to microsatellite and single nucleotide polymorphism (SNP) markers (Zimmerman et al., 2020). Several mtDNA regions such as the control region (D-loop), ATPase 6/8, COI, Cyt b, and 16S RNA have been employed for population genetic characterization of many fish species (Swain et al., 2014;

Fang et al., 2022; He et al., 2022). Among these markers, the D-loop is a non-coding region and exhibits generally higher genetic variations relative to coding regions. The D-loop control region is the marker of choice and has important value for investigating intraspecific genetic differentiation (Mandal et al., 2012; Sahoo et al., 2020; Zhang et al., 2020), and it has been the most widely used mtDNA marker in many phylogenetic genetic studies in fish (Xie et al., 2006; Paixão et al., 2018; Jiang et al., 2019; Gao et al., 2020; Sahoo et al., 2020). In addition, another important mitochondrial region ATPase 6/8 also mutates at a faster rate compared with the nuclear genes and is a potential marker analyzed for both phylogeny and phylogeography; hence, it is very useful in genetic diversity, population genetics, and evolution studies (Divya et al., 2015; Singh et al., 2016; Balasubramaniam et al., 2022). The ATPase 6/8 gene has been used successfully in many fish species (Behera et al., 2015, 2018a, 2018b; Mandal et al., 2012; Paixão et al., 2018).

The minor carp fish species *Labeo gonius* (Hamilton, 1822), commonly known as "ghonia" or "kuria labeo" (belonging to the Order Cypriniformes, Family Cyprinidae), is widely distributed in Indian rivers. The genus "*Labeo*" represents the dominant group in cyprinids comprising 103 fish species around the world. Though freshwater aquaculture is dominated by major carps in India, the minor carp *L. gonius* holds enormous potential as a new candidate species for diversification in aquaculture, due to its higher consumer preference, better taste, and high market value (Mohanta et al., 2008; Jayasankar, 2018). In many areas throughout the country, *L. gonius* trades for more money than Indian major carps (IMCs). The fish can be cultured for short periods (two crops in a year), along with IMCs in composite fish culture and integrated fish culture systems, and can be grown in cages or pens in open water bodies. As an herbivorous bottom feeder, the fish can be substituted for *Cirrhinus mrigala* in a grow-out carp culture system without reducing productivity, giving farmers the option to diversify their culture techniques (Das et al., 2010). Similar to rohu, *L. gonius* is a high-fecund fish between 245,000 (800–900 g fish) and 540,000 (1.5–1.6 kg fish). It breeds naturally in riverine environments during the monsoon season; however, captive breeding can be induced using pituitary gland extract and synthetic hormones. Since *L. gonius* possesses culture potential, introducing the species into the culture system along with the IMCs would help in the diversification of culture practices. Earlier, the species was listed under the Lower risk-Near threatened (LR-nt) category (CAMP 1998: IUCN 2010); however, after management efforts from farmers, policymakers, and researchers, this fish now falls under the Least-concern (LC) category (Mogalekar et al., 2017). To facilitate *in-situ* conservation as well as aquaculture prospects, active research is being conducted on the artificial propagation, captive breeding, and seed production of this species (Behera et al., 2009). For the implementation of any effective conservation and management programs, it is desirable to obtain the genetic background of this species. There are also efforts to increase the production of the species through genetic improvement programs; however, it requires proper evaluation of stocks in terms of measuring genetic variability within and among populations. Nevertheless, very limited research has been done on genomics, genetic diversity, and population structure for the genetic management of this species.

Therefore, in the present study, the genetic diversity and structure of the important minor carp species (*L. gonius*) were assessed by analyzing the two mtDNA markers, D-loop region and ATPase 6/8 gene, after collecting the individuals from five major Indian rivers (namely, Ganga, Brahmaputra, Teesta, Mahanadi, and Narmada). These results are particularly important given the paucity of evidence available on the genetic diversity and structure of *L. gonius*. The knowledge gained from this study will provide future work having conservation management and marker-assisted selection implications for the studied fish.

Materials and methods

Study area and sampling

The *L. gonius* fish specimens (juvenile as well as maturing and mature) were collected from the five designated major Indian rivers (Ganga, Brahmaputra, Teesta, Mahanadi, and Narmada) situated in different geographical areas in India with multiple samplings from each location (Figure 1; Supplementary Table 1). *Labeo gonius* is identified with the taxonomic keys of Talwar and Jhingran (1991) and Rahman (2005). Fifty milligrams of tissue and fin samples were obtained from each specimen and stored in 95% ethanol until genomic DNA extraction.
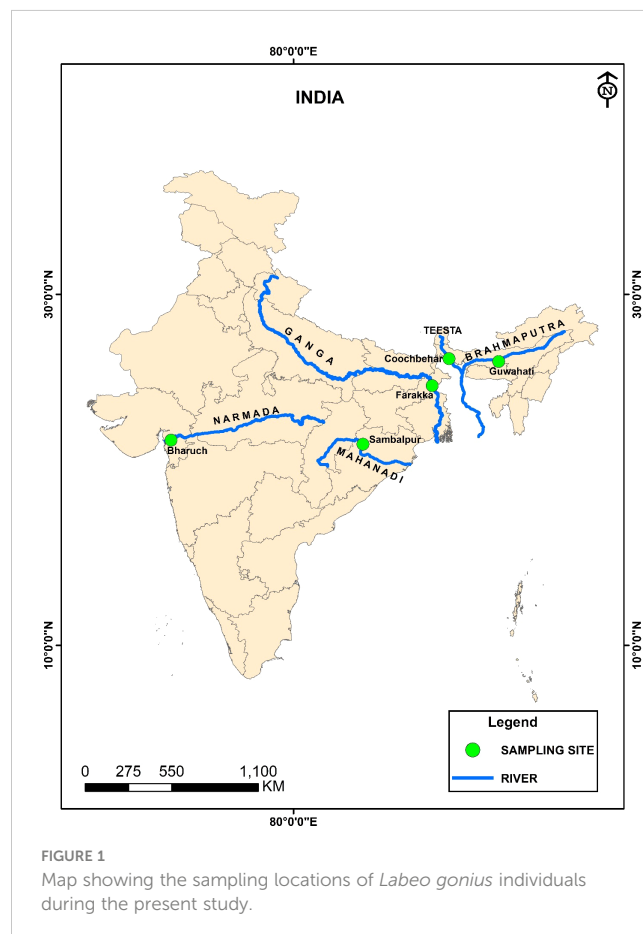

FIGURE 1
Map showing the sampling locations of *Labeo gonius* individuals during the present study.

Genomic DNA extraction and amplification of the mitochondrial ATPase 6/8 and D-loop region

Total genomic DNA was isolated from each tissue and fin samples/individuals using the phenol–chloroform method with slight modifications. The isolated genomic DNA from each sample was resuspended in TE buffer (10 mM of Tris–HCl, 0.1 mM of EDTA, pH 8.0), and its quality and concentration were estimated using a UV spectrophotometer. Then, the isolated genomic DNA was diluted to 25 ng/ml and stored at −20°C until use for further PCR analysis. Individuals/samples were amplified through PCR for the mitochondrial ATPase 6/8 gene (using primer forward: 5′ AAAGCRTYRGCCTTTTAAGC3′ and reverse: 5′GTTAGT GGTCAKGGGCTTGGRTC3 ′) and D-loop gene (using primer forward: 5′CATCGGTCTTGTAATCCGAAGAT3 ′ and reverse: 5′ GTTTCGGGGTTTGACAAGGATA3′) (Sivasundar et al., 2001). PCR amplification was carried out in 25 ml of reaction mixture, containing PCR reaction buffer (10 mM of Tris–HCl, pH 9.0, 50 mM of KCl, and 0.01% gelatine), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each primer, 1.5 U of Taq polymerase, and 100 ng of template DNA. PCR was performed using the thermal cycler Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) with following the procedure: initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 30 s and at 56°C annealing temperature for 1 min, elongation at 72°C for 2 min, with a final elongation at 72°C for 5 min. The PCR-amplified products were checked through 1.8% agarose gel electrophoresis and were purified using the QIAquick PCR purification kit (Qiagen, UK).

Data analysis

The PCR-amplified products of the two mitochondrial genes—ATPase 6/8 and D-loop—from each sample/individual of the five rivers were sequenced bidirectionally using an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA, USA). A total of 210 ATPase 6/8 sequences and 203 D-loop control region sequences from $L. gonius$ samples taken from the five main rivers in India have been submitted to NCBI (Supplementary Table 2). The sequences were aligned with ClustalW, and evolutionary analysis was conducted in MEGA11 software (Felsenstein, 1985; Tamura et al., 2021). A phylogenetic tree was inferred by using the maximum likelihood method based on the general time reversible model in the MEGA11 software with 1,000 bootstrap replicates in the phylogeny test to represent the consensus tree. DnaSP 6.1 (Rozas et al., 2017) was used to determine the molecular indices such as the number of polymorphic sites, haplotype diversity (H_d), nucleotide diversity (π), and pairwise F_{ST} within and between the populations to assess the population structure of $L. gonius$. To determine the population genetic structure within and between populations, a hierarchical analysis of molecular variance (AMOVA) was performed for $L. gonius$ populations based on the mtDNA ATPase and D-loop control region using Arlequin v3.5.1.3 (Excoffier and Lischer, 2010). The pairwise population comparison (F_{ST}), Tajima's D,

and mismatch distributions were estimated using Arlequin v3.5.1. The sum of squared deviation (SSD) and Harpending's raggedness index (Hri) were used to test the mismatch distribution under the population expansion model (Harpending, 1994). The phylogeographic haplotype network was constructed using NETWORK 4.612 (Bandelt et al., 1999).

Results

Sequence composition and variation

ATPase region

The ATPase genes of $L. gonius$ (842 bp) from the five major rivers of India were analyzed, and the 210 total sequences were submitted to NCBI with gene accession numbers KT005344–KT005384 for Ganga River; KU686247–KU686276, KU686297–KU686302, KU686360–KU686363, and KX244995–KX244997 for the river Brahmaputra; KT001131–KT001151, KU686232–KU686246, KU686294–KU686295, and KX244994 for the river Teesta; KU686222–KU686231, KU686303–KU686333, and KX244992–KX244993 for the river Mahanadi; and KU686277–KU686293, KU686296, and KU686334–KU686359 for the river Narmada (Supplementary Table 2). The average nucleotide frequencies of the mtDNA ATPase region for all the 210 samples of $L. gonius$ were adenine (A): 31.80%, thymine (T): 26.08%, guanine (G): 12.50%, and cytosine (C): 29.62%, and the region was rich in A+T content (57.88%). There were 16 parsimony informative sites (site positions: 54, 185, 197, 209, 219, 410, 515, 602, 609, 650, 653, 734, 803, 821, 824, and 825) and 54 singleton variable sites.

D-loop region

The D-loop region of $L. gonius$ (683 bp) from the five major rivers of India was analyzed, and the 203 total sequences were submitted to NCBI with gene accession numbers KP676818–KP676860 for the river Ganga; KP676861–KP676883 and KY488396–KY488411 for the river Teesta; KX943249–KX943289 for the river Mahanadi; KY488354–KY488395 for the river Brahmaputra; and KY646046–KY646048 and KY488412–KY488449 for the river Narmada (Supplementary Table 2). The average nucleotide frequencies of mtDNA for the D-loop region in all the 203 samples of $L. gonius$ were adenine (A): 31.80%, thymine (T/U): 26.08%, guanine (G): 12.50%, and cytosine (C): 29.62%, and the region was rich in A+T content (57.88%). There were 6 singleton variable sites (site positions: 104, 231, 287, 321, 430, 575), 69 polymorphic sites, and 64 parsimony informative sites.

Genetic diversity and genetic differentiation

ATPase region

The genetic variability indices based on polymorphic site, haplotype diversity (H_d), and nucleotide diversity (π) are presented in Table 1. Overall, the sequence analysis of the

ATPase 6/8 region of *L. gonius* revealed 70 polymorphic sites, with the highest polymorphic sites observed in the river Mahanadi (58). Based on the nucleotide variations, a total of 17 haplotypes were identified among the five rivers, with Brahmaputra having the highest number of haplotypes (8), followed by Mahanadi (6). The haplotype diversity (H_d) from all the major river sampling stations ranged from 0.095 ± 0.095 (Ganga) to 0.594 ± 0.072 (Mahanadi), whereas nucleotide diversity (π) was relatively low for all the studied populations ranging between 0.000117 ± 0.00 (Ganga) and 0.00407 ± 0.00285 (Mahanadi). The overall average number of nucleotide differences (K) for the ATPase 6/8 gene was found to be 2.918, with the river Mahanadi having the highest number and Ganga having the lowest number (Table 1). Hierarchical AMOVA analysis was carried out for the *L. gonius* ATPase 6/8 region among and within populations based on the two principal clusters (Table 2). The populations were analyzed as gene pool-1 (considering all the stocks from Ganga, Brahmaputra, Teesta, Narmada, and Mahanadi as a single pool) and gene pool-2 [where samples were grouped into two groups: group 1 (Himalayan rivers)—Ganga, Brahmaputra, and Teesta; and group 2 (peninsular rivers)—Narmada and Mahanadi]. This analysis provided insights into the proportion of genetic variation attributable to within-population (F_{ST}), within-group (F_{SC}), and among-group (F_{CT}) differences. The F_{ST} values were significant for the ATPase 6/8 region in both comparisons, with the highest value (F_{ST}: 0.724; $p = 0.000$) observed in gene pool-2 in comparison to gene pool-1 (F_{ST}: 0.702; $p = 0.000$). Under gene pool-1, the percentage of variation among populations (70.22%) was greater than within populations (29.78%), whereas in gene pool-2, a maximum percentage of variation was observed among populations within groups (53.48%). These results indicate that

the genetic divergence both between populations and groups was significant. In the pairwise comparison of the genetic differentiation indices (F_{ST}), among all the five Indian major rivers, the highest genetic differentiation was exhibited between groups from Ganga and Narmada ($F_{ST} = 0.917$) followed by groups from Teesta and Narmada ($F_{ST} = 0.896$) and the lowest between groups from Brahmaputra and Teesta (0.035) followed by Ganga and Brahmaputra (0.048), respectively. The results were in agreement with the fact that there was greater genetic differentiation among populations than within populations (Table 3). The *p*-distance evaluation depicts that the groups between Narmada and Mahanadi recorded the highest value. Estimates of genetic differentiation between all five river populations of *L. gonius* using the sudden expansion model (τ, θ_0, θ_1, SSD, and RI) and execution of neutrality are given in Table 4. For both Tajima's *D* (Tajima, 1989) and Fu's F_s statistics (Fu, 1997), Mahanadi, Brahmaputra, and Teesta river populations exhibited moderate to high negative values. Moreover, the Narmada population showed the lowest non-significant Tajima's *D* and F_s values. However, the Ganga population depicted non-significant positive Tajima's *D* and F_s values.

D-loop region

The genetic variability indices based on polymorphic site, haplotype diversity (H_d), and nucleotide diversity (π) are presented in Table 1. Overall, the sequence analysis of the mitochondrial D-loop region of *L. gonius* revealed 67 polymorphic sites, with the highest polymorphic sites observed in the Ganga River (26) followed by Brahmaputra (23) and Teesta (21). Altogether, based on nucleotide variations, 58 haplotypes were identified, while the Ganga River had the highest number of

TABLE 1 Genetic diversity of *Labeo gonius* from the major rivers of India estimated using the mtDNA ATPase and D-loop control regions.

Sampling sites	N	S	N_h	H_d	(π)	K
ATPase 6/8 region						
Ganga	41	1	2	0.095 ± 0.095	0.00011 ± 0.000	0.095
Brahmaputra	43	8	8	0.564 ± 0.085	0.00089 ± 0.000	0.749
Teesta	39	2	3	0.413 ± 0.081	0.00052 ± 0.434	0.434
Mahanadi	43	58	6	0.594 ± 0.072	0.00407 ± 0.003	3.428
Narmada	44	3	3	0.542 ± 0.050	0.00125 ± 0.000	1.049
Overall	210	70	17	0.560 ± 0.036	0.00347 ± 0.000	2.918
D-loop region						
Ganga	43	26	24	0.965 ± 0.0002	0.00739 ± 0.004	5.049
Brahmaputra	42	23	16	0.899 ± 0.0008	0.00642 ± 0.001	4.385
Teesta	39	21	11	0.752 ± 0.0024	0.00887 ± 0.005	6.057
Mahanadi	41	12	9	0.688 ± 0.071	0.00249 ± 0.000	1.700
Narmada	38	11	4	0.573 ± 0.059	0.00621 ± 0.0007	4.240
Overall	203	67	58	0.954 ± 0.006	0.01914 ± 0.001	13.075

N, number of samples; S, number of polymorphic sites; N_h, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; K, average number of nucleotide differences.

TABLE 2 Results of the hierarchical analysis of molecular variance (AMOVA) of populations of *Labeo gonius* based on the mtDNA ATPase and D-loop control regions.

	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation indices	p-value
ATPase 6/8 region							
Gene pool-1	Among populations	4	235.047	1.386	70.22	F_{ST}: 0.702	0.000
	Within populations	205	120.448	0.588	29.78		
Gene pool-2	Among groups	1	90.255	0.404 Va	18.96	F_{SC}: 0.660	0.000
	Among populations within the group	3	144.792	1.140 Vb	53.48	F_{ST}: 0.724	
	Within populations	205	120.448	0.587 Vc	27.56	F_{CT}: 0.189	
D-loop region							
Gene pool-1	Among populations	4	899.031	6.253	76.46	F_{ST}: 0.765	0.000
	Within populations	175	336.986	1.926	23.54		
Gene pool-2	Among groups	1	273.779	0.534 Va	6.38	F_{SC}: 0.755	0.000
	Among populations within the group	3	625.252	5.921 Vb	70.65	F_{ST}: 0.770	
	Within populations	175	336.986	1.926 Vc	22.98	F_{CT}: 0.064	

Gene pool-1: considering all the stocks of Ganga, Brahmaputra, Teesta, Narmada, and Mahanadi as a single pool; gene pool-2: group 1 (Himalayan rivers): Ganga, Brahmaputra, and Teesta; and group 2 (peninsular rivers): Narmada and Mahanadi.

haplotypes with 24 haplotypes, followed by the rivers Brahmaputra with 16 haplotypes and Teesta with 11 haplotypes, respectively. The haplotype diversity (H_d) ranged from 0.573 ± 0.059 to 0.965 ± 0.00015, with the Ganga River population having the highest diversity and the Narmada river population having the lowest diversity. Furthermore, the overall haplotype diversity was 0.954 ± 0.006. Nucleotide diversity from all the major rivers ranged from 0.00249 ± 0.000 to 0.00887 ± 0.005, with the river Teesta having the highest diversity and the river Mahanadi having the lowest diversity. Moreover, an overall nucleotide diversity (π)

was observed (0.01914 ± 0.001). The overall average number of nucleotide differences (K) was found to be 13.075, with the river Mahanadi having the lowest number and the Teesta River having the highest (Table 1). AMOVA analysis of hierarchical gene diversity for the D-loop region of *L. gonius* was also carried out among and within populations based on the two principal clusters: gene pool-1 and gene pool-2 (Table 2). The F_{ST} values were significant for the ATPase 6/8 region in both comparisons, with the highest value (F_{ST}: 0.770; $p = 0.000$) observed in gene pool-2 in comparison to gene pool-1 (F_{ST}: 0.765; $p = 0.000$). Under gene

TABLE 3 Pairwise difference F_{ST} value (below) and p-distance value (above) diagonals of *Labeo gonius* from the major rivers of India (significant at $p < 0.05$).

	Narmada	Ganga	Brahmaputra	Teesta	Mahanadi
ATPase 6/8 region					
Narmada	–	0.008	0.009	0.009	0.011
Ganga	0.917	–	0.0053	0.0004	0.002
Brahmaputra	0.879	0.048	–	0.0007	0.003
Teesta	0.896	0.135	0.035	–	0.003
Mahanadi	0.746	0.075	0.068	0.083	–
D-loop region					
Narmada	–	0.047	0.047	0.049	0.048
Ganga	0.845	–	0.008	0.009	0.007
Brahmaputra	0.859	0.175	–	0.009	0.007
Teesta	0.877	0.404	0.319	–	0.008
Mahanadi	0.907	0.255	0.319	0.601	–

"-" denote no value.

TABLE 4   Parameters of the sudden expansion model and execution of neutrality of *Labeo gonius* sampled from the major rivers of India.

Statistics	Ganga	Brahmaputra	Teesta	Mahanadi	Narmada
ATPase 6/8 region					
τ	3.000	0.816	0.547	1.484	2.578
θ_0	0.000	0.000	0.000	0.000	0.000
θ_1	0.110	99999	99999	2.1255	1.639
SSD	0.000 (0.24)	0.003 (0.55)	0.009 (0.13)	0.01809 (0.34)	0.098 (0.13)
RI	0.665 (0.80)	0.089 (0.43)	0.176 (0.27)	0.093 (0.73)	0.385 (0.08)
Tajma's D value	1.112 (0.85)	−0.155 (0.44)	−0.841 (0.21)	−1.673 (0.03)	0.000
Fu's F_s	2.121 (0.85)	−0.053 (0.37)	−0.709 (0.09)	−4.550 (0.00)	0.000
D-loop region					
τ	6.255	6.019	8.951	2.914	0.000
θ_0	0.87	0.004	0.000	0.000	0.000
θ_1	17.681	12.129	1.663	3.017	99999
SSD	0.016 (0.16)	0.019 (0.16)	0.084 (0.25)	0.027 (0.33)	0.483 (0.00)
RI	0.025 (0.48)	0.035 (0.29)	0.177 (0.32)	0.105 (0.38)	0.36 (0.93)
Tajma's D value	−0.515 (0.36)	−0.438 (0.33)	−1.101 (0.12)	−1.207 (0.12)	1.899 (0.98)
Fu's F_s	−9.354 (0.001)	−3.316 (0.09)	−0.844 (0.69)	−2.159 (0.14)	6.959 (0.99)

τ, time in number of generations; θ_0, pre-expansion population size; θ_1, post-expansion population size; SSD, sum of squared deviations; RI, raggedness index.

pool-1, the percentage of variation among populations (76.46%) was greater than within populations (23.54%), whereas in gene pool-2, a maximum percentage of variation was observed among populations within groups (70.65%). These results indicate that there is genetic divergence in both between populations and groups. In the pairwise comparison of the genetic differentiation indexes (F_{ST}), the highest genetic differentiation was observed between Narmada and Mahanadi (F_{ST} = 0.907), followed by Narmada and Teesta (F_{ST} = 0.877) and Narmada and Brahmaputra (F_{ST} = 0.859), respectively, and the lowest genetic differentiation was observed between Ganga and Brahmaputra (F_{ST} = 0.175) (Table 3). Estimates of genetic differentiation between all five river populations of the *L. gonius* D-loop region using the sudden expansion model (τ, θ_0, θ_1, SSD, and RI) and execution of neutrality are given in Table 4. Tajima's D and F_s values for the *L. gonius* D-loop region were found to be negative in all the river sample populations except the Narmada population.

Evolutionary and phylogeographic relationships

ATPase region

The phylogenetic tree showed that haplotypes were clustered into four principal clusters (Figure 2A; Supplementary Table 3). Haplotypes H1–H3 belonging to the river Narmada were grouped into one principal clade and in another principal clade subdivided into two more subgroups with a mixture of haplotypes belonging to all the studied rivers including the most common haplotype H5

except from the river Narmada. However, haplotype H17 (Mahanadi) was observed to be clustered with two other *L. rohita* haplotypes. The haplotype median-joining network from sequences of the ATPase region is presented in Figure 3A. The haplotype network revealed that there were significant differences in distributions and frequencies and H5 was the most common and extensively distributed haplotype observed in the studied rivers. The most common haplotype H5 represents individuals from the rivers Ganga, Brahmaputra, Teesta, and Mahanadi, except from the river Narmada. The next common haplotypes are H4 and H6, which were present in the rivers Brahmaputra and Teesta. Some haplotypes were observed to be exclusive or river-specific, such as H1–H3 haplotypes found in the river Narmada, H7 in the river Ganga, H8–H12 in the river Brahmaputra, and H13–H17 in the river Mahanadi (Figure 3A; Supplementary Table 3).

D-loop region

The phylogenetic tree showed that haplotypes were clearly clustered into two principal branches. One principal clade was again subdivided into three subgroups, wherein one group is exclusive to the other two branches containing H55–H58 from the river Narmada; the second subgroup has haplotypes from Ganga, Brahmaputra, and Teesta; and the third subgroup has haplotypes from Brahmaputra, Ganga, and Teesta and only sharing one haplotype from Mahanadi. Another principal clade was subdivided into three more subgroups, where one subgroup has haplotypes from Brahmaputra and Teesta, the second subgroup has haplotypes only from Mahanadi, and the third subgroup has haplotypes from Ganga and Brahmaputra and one haplotype from
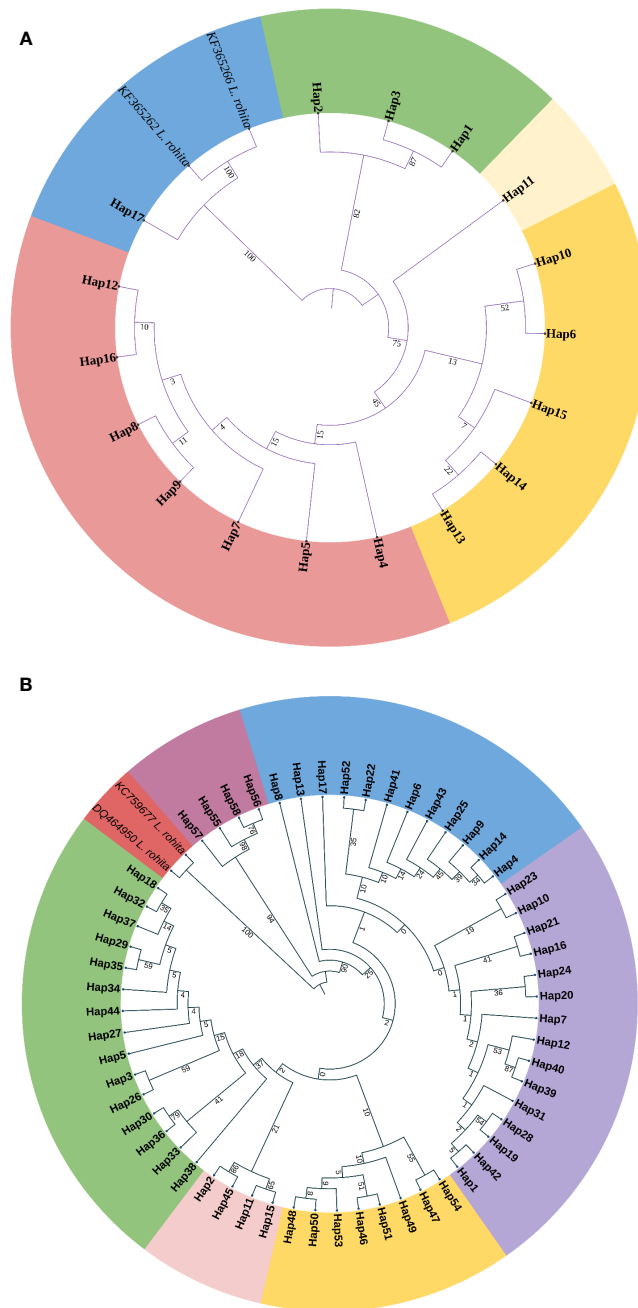
FIGURE 2
Phylogenetic tree of *Labeo gonius* by taking representatives of haplotypes of the **(A)** ATPase and **(B)** D-loop control regions. The sympatric species *Labeo rohita* is the outgroup here. The evolutionary history was inferred using the maximum likelihood method, and evolutionary distances were computed using the general time reversible method in MEGA11 software. The number at the nodes indicates bootstrap values based on 1,000 replications. The circular phylogenetic tree was better visualized using the iTOL software (https://itol.embl.de/), and different colors represent the clusters.

Teesta. In addition, *L. rohita* haplotypes clustered together separately (Figure 2B; Supplementary Table 3).

The haplotype median-joining network from sequences of the D-loop region is presented in Figure 3B. The haplotype network showed that haplotypes were observed to be exclusive or river-specific: H1–H16 in the river Brahmaputra, H17–H38 in the river Ganga, H39–H45 in the river Teesta, H46–H54 in the river Mahanadi, and H55–H58 in the river Narmada (Figure 3B, Supplementary Table 3).

Discussion

The maintenance of genetic diversity is crucial for both an individual's probability of survival and a population's capacity for evolution (Sahoo et al., 2019). The population structure analysis and understanding of fish species genetic diversity can provide us with vital information and a scientific basis for developing conservation and management strategies for fish populations (Pallavi et al., 2012; Jayasankar, 2018). The goal of the present study was to generate
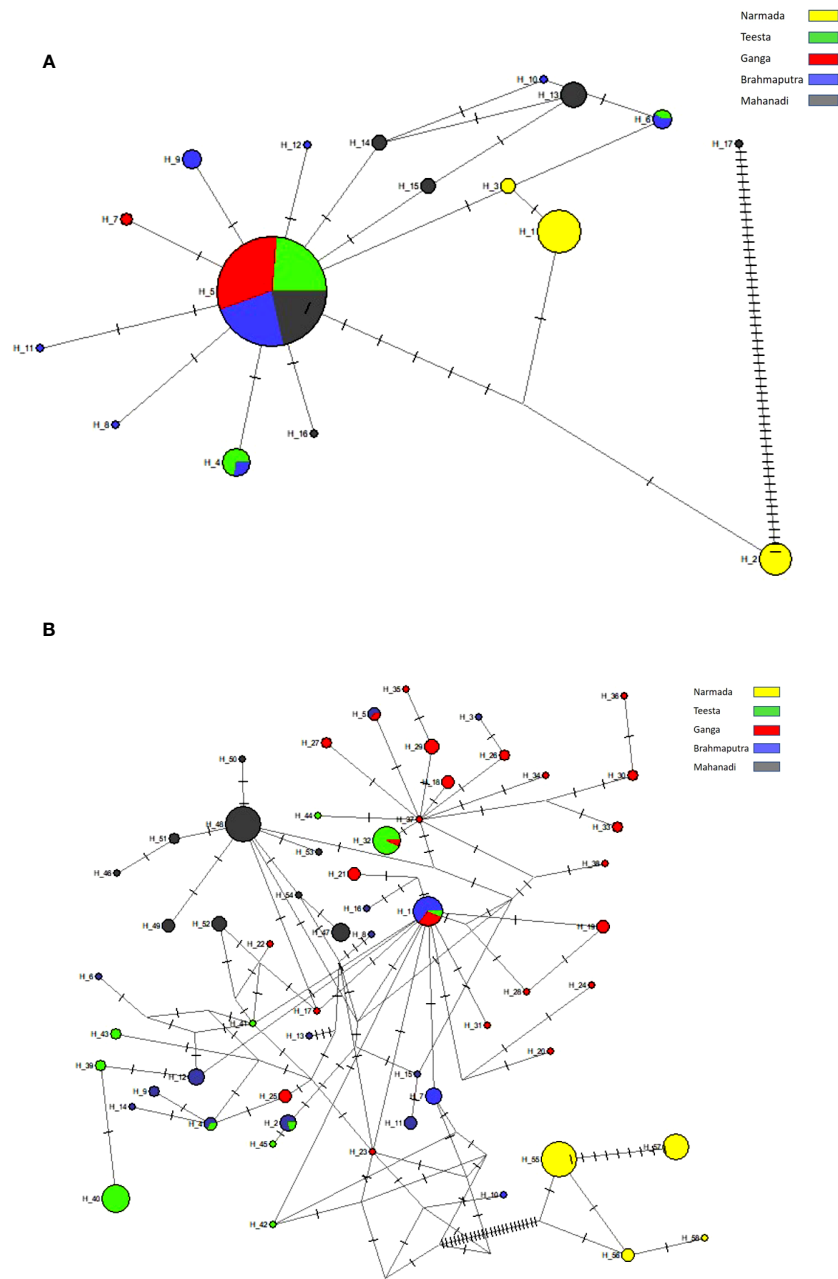
FIGURE 3
The median-joining network of *Labeo gonius,* based on haplotypes of the mtDNA **(A)** ATPase region and **(B)** D-loop region. Each circle represents a haplotype and circle size is proportional to the haplotype frequency. The colors indicate locality according to the legend, and hatch marks represent the number of mutations by which haplotypes differ.

information on genetic diversity, population structure, and evolutionary relationships of *L. gonius* within and among populations from the selected five major rivers of India based on the mitochondrial D-loop and ATPase 6/8 analysis (Paixão et al., 2018; Sahoo et al., 2020; Balasubramaniam et al., 2022). Since mitochondrial genes have a high rate of evolution, it allows for distinguishing between haplotypes in small sample sizes and threatened species (Habib et al., 2012; Das et al., 2024). The observed nucleotide frequencies of both the mtDNA D-loop and ATPase 6/8 regions were rich in A+T content (57.88%), which is in accordance with many other cyprinids and *Labeo* sp., e.g., *L.*

fimbriatus (Swain et al., 2014), *L. calbasu* (Singh et al., 2016), *L. rohita* (Luhariya et al., 2012), and *Chitala chitala* (Mandal et al., 2012). The low percentage of GC content and the least representation of nucleotide G (12.50%) were reported in other fish species as well (Swain et al., 2014; Sahoo et al., 2019), and these might be due to anti-G bias, which is characteristic for the mitochondrial genome (Kathirvelpandian et al., 2014; Balasubramaniam et al., 2022). In the present study, the D-loop region generated 58 haplotypes, with the Ganga River population having the highest number of haplotypes (24) followed by the river Brahmaputra (16), whereas the ATPase 6/8 region generated 17

haplotypes, with the Brahmaputra River population having the most haplotypes (8). Furthermore, haplotype (H_d) and nucleotide diversity (π) were considered indicators of within-population genetic variation and higher genetic variation in the group which depicts higher genetic diversity (Zhao et al., 2006; Zhang et al., 2017; Zhang et al., 2020; Liu, 2017; Zhang et al., 2017; Fang et al., 2022). In the current study, an overall haplotype diversity of 0.954 was observed as revealed by the D-loop analysis with the highest haplotype diversity in the river Ganga (0.965), followed by the river Brahmaputra, and the lowest diversity was found in the river Narmada. In the ATPase 6/8 region, the highest haplotype diversity was observed in the river Mahanadi followed by Brahmaputra. Overall, moderate to high haplotype diversity was observed for all river populations (Zhang et al., 2017; Zhang et al., 2020; Das et al., 2024). Due to a high number of haplotypes, haplotype diversity, and polymorphic sites, the Ganga River population could be considered a highly divergent population. Nevertheless, low nucleotide diversity was detected in all of the studied river populations except for the river Mahanadi population. In parallel, the characteristic higher haplotype diversity rather than nucleotide diversity was also observed in other freshwater fish species (Luhariya et al., 2012; Mandal et al., 2012; Gupta et al., 2013; Dutta et al., 2020). Genetic diversity can be influenced by many factors such as anthropogenic activity and habitation. Low nucleotide diversity in a species shows the characteristic of rapid population expansion after a period of small effective population size. This suggests that rapid population growth increased the accumulation of mutations where expansion time was enough to accumulate haplotype diversity, but the expansion time was insufficient to increase nucleotide diversity (Behera et al., 2018a; Behera et al., 2018b; Sahoo et al., 2020; Das et al., 2024). The D-loop region has more haplotypes and nucleotide diversity than the ATPase region, and this could be explained by the fact that the D-loop region has a high mutational rate and is an evolving region than the ATPase region (Guo et al., 2014). The combined mitochondrial region analysis has proven to be very useful in delineating genetic diversity and differentiation in many fish species (Sharma et al., 2019; Sahoo et al., 2020).

In a relatively rapid evolving genome within a population, haplotype diversity will generally reach 1.0 as many individuals will tend to have unique haplotypes (Freeland et al., 2010). Furthermore, it needs special mention that the populations along the east coast, especially the Ganga, Brahmaputra, and Mahanadi river basin populations, are genetically more diverse than the other river populations as evidenced by the high haplotype and nucleotide diversity. The Ganga and Brahmaputra river populations might be carrying the original founder population of the parental population, and the Mahanadi population resides in habitats that are less exploited and less impacted by destructive human activities. This may potentially account for the higher genetic diversity of the Ganga–Brahmaputra and Mahanadi river basin populations. This also indicates that other river populations might have experienced a population bottleneck followed by a rapid population growth (Bowen et al., 2001; Meng et al., 2015). The mismatch distribution and neutral test analysis helps us to investigate demographic history (Guo et al., 2014; Lau et al., 2018). Tajima's D value of low significance indicates that a population is deviating from the neutral expectations, and negative F_s values signify deviation from neutrality, which may be due to the effect of population expansion and/or selection. In the present study, Tajima's D and Fu's F_s values were found to be negative in the studied river populations; however, these were only significant in the Mahanadi population (for ATPase 6/8) and Ganga population (Fu's F_s value, for D-loop). Negative and insignificant Fu's F_s and Tajima's D values indicate the presence of more rare nucleotide variants and haplotypes than what would be predicted from neutrality. Altogether, the high values of haplotype diversity, the low values of nucleotide diversity, and the negative values of Tajima's D and Fu's F_s imply a recent population expansion following a bottleneck or small effective population size.

Several evolutionary forces, which either act as locus-specific (selection and mutations) or at the genome-wide level (genetic drift and gene flow), influence the variation patterns of genomes and populations. The hierarchical AMOVA analysis for the D-loop region indicated that a high proportion of genetic variation was attributed to differences by among-population variation, i.e., 76.46% under gene pool-1 (considering all the stocks from Ganga, Brahmaputra, Teesta, Narmada, and Mahanadi as a single pool), and among populations within groups, 70.65% under gene pool-2 [where samples were grouped into two groups: group 1 (Himalayan rivers)—Ganga, Brahmaputra, and Teesta; and group 2 (peninsular rivers)—Narmada and Mahanadi]. Similar results were obtained in the ATPase 6/8 region analysis, where 70.22% of the variation among populations was found under gene pool-1 and 53.48% of the variation was found among populations within groups in gene pool-2 (Habib et al., 2012; Balasubramaniam et al., 2022). It has been reported that non-migratory fish species have higher variation among the populations and low variation within populations (Vrijenhoek, 1998). In the present study, within- and among-population variation is similar to that reported for non-migratory and freshwater fishes such as *L. fimbriatus*, *C. catla*, and *Clarias magur* (Swain et al., 2014; Behera et al., 2018b; Sahoo et al., 2020). Significant genetic differentiation (F_{ST}) was observed for the D-loop and ATPase 6/8 regions in both comparisons, with the highest F_{ST} value observed in gene pool-2 in comparison to gene pool-1, suggesting that there is a geographical structure between groups, and similar results were also reported from other freshwater fishes (Mohindra et al., 2012; Sahoo et al., 2020). Limited gene flow, widespread geographic distribution, and the occurrence of distinct haplotypes may all be contributing factors to the significant genetic differentiation (Behera et al., 2018a). Comparing populations of freshwater fish to estuary or marine fishes, freshwater fish typically show higher degrees of genetic differentiation among populations (Ward et al., 1994). Similarly, in terms of ecological traits and breeding practices, riverine populations differ considerably from populations that migrate frequently, forming a unique ecological group (Wagh and Ghate, 2003). The pairwise comparison of the genetic differentiation indexes of allele frequency F_{ST} results also supports the fact regarding the presence of greater genetic divergence between populations than within subspecies. There

may be several factors such as geographic segregation, habitat differences, and migration which play significant roles in the genetic divergence of different fish populations. In the present study, genetic differentiation between populations determined by pairwise F_{ST} was moderate to high, which is perhaps attributed to high gene flow (Guo et al., 2014). Despite being from different river basins which are fragmented, the studied fish populations were observed to have moderate to high levels of genetic differentiation, which might indicate a common ancestry in the prehistoric period as observed in other cyprinid fishes (Singh et al., 2010, 2016). However, the low differentiation in the Ganga, Brahmaputra, and Teesta river populations might possibly be due to the ancestral proximity or some gene flow since flood plains of the Ganga River basin possibly serve as the source of dispersal of brood fishes and young ones across the tributaries.

Haplotype phylogeny may differ along the genome regions due to recombination and the process of swapping genome regions between haplotypes during the process of meiosis (Selle et al., 2021). Our aim was to find out haplotype effects by leveraging phylogenetic relationships between haplotypes. The haplotype networks helped us to predict the evolutionary relationship among unique haplotypes and the genealogical history of individuals. In the current study, the haplotype network based on the ATPase 6/8 analysis revealed that H5 is present in the majority of individuals, as evidenced by the median-joining network, which is positioned in the center of the network and has the most connecting nodes. It suggests that H5 is probably the ancestral haplotype from which all the other haplotypes might have originated directly or through subsequent mutations. H5 was shared among all the studied river populations of *L. gonius* (Ganga, Brahmaputra, Teesta, and Mahanadi except Narmada), suggesting a common ancestral origin and subsequent gene flow among these river populations (Das et al., 2018). Some haplotypes were observed to be exclusive or river-specific such as H1–H3 haplotypes observed in the river Narmada, H7 in the river Ganga, H8–H12 in the river Brahmaputra, and H13–H17 in the river Mahanadi. The unique and river-specific haplotypes of the river Narmada population may have arisen due to environmental changes or independent mutation events (Balasubramaniam et al., 2022). Haplotype median-joining network analysis from the D-loop region revealed that haplotypes are exclusively population-specific and not shared by other populations such as H1–H16 in the river Brahmaputra, H17–H38 in the river Ganga, H39–H45 in the river Teesta, H46–H54 in the river Mahanadi, and H55–H58 in the river Narmada. The prevalence of population-specific haplotypes may have evolved due to environmental changes at various sites through mutation or dispersal (Sahoo et al., 2019), as well as the absence of population admixture (Sharma et al., 2019), or independent mutation events (Das et al., 2024). These unique or private haplotypes could be used as a population-specific marker for stock identification. However, certain haplotypes, such as H25, are not closely related to the cluster formed by congeneric species in a haplotype network, implying probable genetic divergence and a distinct evolutionary history for the individual haplotype. The haplotype distribution in the phylogenetic tree based on both

ATPase 6/8 and D-loop analyses showed that haplotypes from four river populations (Ganga, Brahmaputra, Teesta, and Mahanadi) did not cluster into distinct branches, but rather mixed clusters, with the exception of the Narmada population. This indicates that the phylogenetic tree did not show a clear relationship between geographic areas and haplotypes. It could potentially signify considerable genetic divergence in the *L. gonius* population. With respect to phylogeography, our results suggested that *L. gonius* population from the river Narmada has genetic divergence with other studied rivers, and this is in accordance with our previous findings in *L. gonius* using Cyt b analysis (Behera et al., 2018c). Moreover, the haplotype distribution patterns are based on the Indian geographical region, since the rivers Ganga, Brahmaputra, and Teesta that rise from the Himalayas and flow through eastern India are mostly clustered together. The west-flowing peninsular Narmada river population clustered separately from the other river populations. However, in the ATPase 6/8 analysis, H17 was clustered with an outgroup species, and one probable explanation is that H17 underwent a distinct mutational history. Nevertheless, the mitochondrial D-loop region generated more mutations and genetic variation than the ATPase region as reported in earlier studies (Mandal et al., 2012; Lau et al., 2018). Mutation rates in the D-loop and ATPase regions are different in different river populations; therefore, it is logical to analyze genetic diversity using multiple mitochondrial sequences. It is also suggested that the D-loop and ATPase mtDNA regions were effective markers for assessing population genetic diversity (Sivasundar et al., 2001; Divya et al., 2015; Paixão et al., 2018; Sahoo et al., 2020; Balasubramaniam et al., 2022; Fang et al., 2022).

In our previous study, *L. gonius* mitochondrial Cyt b gene analysis exhibited 12 haplotypes with 14 variables from four major Indian river populations (Ganga, Mahanadi, Brahmaputra, Narmada) and one hatchery population (Behera et al., 2018c). In the present study, the analysis of *L. gonius* population with the mitochondrial control region gene from five major Indian rivers revealed 17 haplotypes and 70 polymorphic sites for the ATPase 6/8 region and 58 haplotypes and 67 polymorphic sites for the D-loop region. So, the results clearly demonstrated that the present study on the mitochondrial control region generated more information on mutations and genetic variation. The nucleotide sequences of *L. gonius* were found to be AT-rich as revealed by all mtDNA Cyt b, ATPase 6/8, and D-loop analyses, which is evident in other freshwater fishes and *Labeo* species (Luhariya et al., 2012; Swain et al., 2016; Sahoo et al., 2020). The AMOVA results were also in accordance with the previous findings in Cyt b analysis (Behera et al., 2018c), which also showed significant genetic differentiation among populations. Because of the fragmented populations of fishes especially those that live in freshwater, high genetic differentiation is observed.

In conclusion, the present study demonstrated the utility of the D-loop and ATPase 6/8 regions of mtDNA as potential markers for studying the genetic diversity and population structure of *L. gonius* across major Indian rivers. More specifically, the D-loop region gives a better and more reliable indication for genetic differentiation. Furthermore, specific haplotypes found in the

current study may be useful for differentiating the stocks. The current study rejected the null hypothesis that single panmictic populations of *L. gonius* are present in Indian river water. Moreover, *L. gonius* populations along the east coast especially in Himalayan rivers (Ganga, Brahmaputra, and Teesta river basin) are found to be genetically more diverse. However, the present study revealed that the Narmada population was found to be isolated and genetically divergent from other river populations. Lastly, the study represents a basic understanding of the genetic diversity and phylogeographic features of *L. gonius*, which provides a scientific foundation for the assessment of fisheries resources and the development of management strategies for conservation. The highly divergent Himalayan rivers—Ganga and Brahmaputra—can serve as conservation units. Furthermore, looking at the significance of *L. gonius* for aquaculture, the information produced may additionally be utilized in scientific planning to start a genetic improvement model that includes the stocks from all the sampled areas to form a synthetic population that can then serve as the foundation for long-term selection.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

SR: Conceptualization, Data curation, Investigation, Software, Validation, Writing – original draft. BKB: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. RVL: Data curation, Formal analysis, Investigation, Writing – review & editing. AR: Methodology, Software, Writing – review & editing. VK: Data curation, Writing – review & editing. PP: Methodology, Writing – review & editing. AK: Data curation, Writing – review & editing. PD: Methodology, Writing – review & editing. DK: Formal analysis, Writing – review & editing. DB: Data curation, Investigation, Writing – review & editing. AA: Formal analysis, Writing – review & editing. BKD: Funding acquisition, Validation, Visualization, Writing – review & editing. JJ: Funding acquisition, Visualization, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The editor, TC, declared a past co-authorship with authors, SR, VK, BKB, and BKD.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1345649/full#supplementary-material

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