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In silico analysis of mitochondrial DNA genes: implication for conservation of *Tor putitora* (Hamilton, 1822)

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Tor putitora **is an endangered cyprinid fish constrained to cold water and is also considered an indicator of a healthy aquatic ecosystem. The present study aimed to examine the haplotypic diversity, genetic variation and population structure of *T. putitora* isolates using *COI* and *Cyt b* gene sequences submitted in GenBank. Bioinformatic analysis was carried out using 106 *COI* and 183 *Cyt b* gene sequences as well as 2 reference genome sequences. Analysis of *COI* and *Cyt b* gene reveals 18 and 85 haplotypes respectively. Mutation was observed at 44 different sites in *COI* and 173 in *Cyt b* gene sequences. Haplotype 4 and haplotype 37 were considered ancestral in *COI* and *Cyt b* respectively. Analysis of *COI* gene reveals moderate haplotype diversity (0.630) and low Nucleotide diversity (0.00662) whereas *Cyt b* has higher haplotype diversity (0.804) and low Nucleotide diversity (0.00582). Moreover, the neutrality test such as Tajima's D, and Fu's Fs showed negative values in both gene sequences, suggesting population expansion attributed to habitat destruction. So, comprehending the genetic variability within and among the *T. putitora* population is crucial for conserving and managing this species. Integration of genetic diversity into conservation planning can enhance the effectiveness of breeding programs and habitat restoration efforts.**

Keywords *Tor putitora*, *COI*, *Cyt b*, Haplotype, Genetic variation, In silico analysis, Conservation

Tor putitora (Hamilton,1822), widely known as Golden Mahaseer or Himalayan mahaseer, is an important freshwater fish of the Indian subcontinent occupying mostly the rivers of the Himalayan foothills region[1,2]. *Tor putitora* (*T. putitora*) belongs to the family Cyprinidae under the order Cypriniformes[3]. It is reported from Pakistan, Bangladesh, Afghanistan, China, Nepal, Bhutan, Myanmar and India. *T. putitora* is generally distributed all over the major river basins of India viz. Indus, Ganga, Brahmaputra, Krishna, Mahanadi etc.[4,5]. This species is also promoted as a flagship species due to its ecological, and economic significance[6].

T. putitora is a fish of significant economic importance due to its high culinary value, its role in a profitable sport fishery and also its contribution to local employment opportunity[5]. *T. putitora* is an omnivore fish that naturally inhabits running water of the rapid stream with rocky substratum and is also found in lakes and reservoirs[7,8]. *T. putitora* is the state fish in many states of India viz. Arunachal Pradesh, Himachal Pradesh, Uttrakhand, UT of J&K[2]. It is a famous game and food fish[3] which can reach a maximum length of 275 cm and weigh up to 54 kg[9,10].

Due to habitat destruction, loss of breeding grounds, anthropogenic activities such as dam building, pollution, and overfishing, the population of *T. putitora* has sharply declined, leading to its classification as an " endangered" species on IUCN Red List[11–13]. Moreover, the numerous dams constructed in the Himalayas region negatively affect the breeding migration of fish species[5,14,15]. The decrease in the number of individuals in populations living in an area can lead to the extinction of unique genotypes, and once this genetic structure is lost it is almost impossible to restore it[16].

Before implementing a successful management or conservation strategy, it is important to understand the population stock of species. In the present era, molecular markers make it easier to understand stock

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characterisation, as they have been applied in various studies throughout the world to understand population genetics[17]. Mitochondrial DNA (mtDNA) gene sequences show their ability as a marker in various studies on DNA barcoding, phylogenetics, population and conservation genetics[17–19]. mtDNA, due to its small genome size without intron, maternal mode of inheritance, high rate of evolution, and limited recombination, has been providing an opportunity to analyse the population genetic structure[20–22]. Partial sequence of Cytochrome oxidase subunit I gene (*COI*), *D-loop* and Cytochrome b gene (*Cyt b*) have been employed in several research to identify species, for molecular phylogeny and population genetics[18,19,23–25]. Hence these genes are also appropriate for haplotype and genetic diversity. The small number of mtDNA *D-loop* sequences for *T. putitora* population in the NCBI database (GeneBank repository) has led to their exclusion from the current study.

The current study aimed to examine the haplotype diversity, genetic variations and population structure of the mitochondrial (mt) *COI* & *Cyt b* gene sequence of *T. putitora* isolates which had been submitted to GenBank from different regions across countries that might be helpful further in the management and conservation of the species.

Materials and methods

Data collection and alignment

In this study, the sequences of *COI* and *Cyt b* gene fragments of *T. putitora* submitted to the National Center for Biotechnology Information (NCBI) database were used for the in-silico analysis. The data were extracted from the database using specific terms such as "*Tor putitora COI*" "*Tor putitora* COX1" and "*Tor putitora* Cytb". Sequences submitted up to 1 August 2024 were utilised during the present study. Database search yielded 187 sequences for *COI* and 239 sequences for the *Cyt b* gene. Sequences were downloaded in Fasta format, analysed in Bioedit v7.2.5[26] and MEGA 11[27] using ClustalW alignment module. Analysis of the sequences was conducted using multiple sequence alignment tool, taking into account that most sequences were partial so, differ in size. Reference sequences (Accession no. NC021755) and (Accession no. AP011326) were used to align the sequences. After aligning, sequences were trimmed from both ends until the length of the sequences was equalised. After equalising and removing short sequences, 498 bp mt *COI* (n = 106), and 1140 bp *Cyt b* (n = 183) with 2 reference sequences were used for bioinformatic analysis. Phylogenetic tree were constructed for the gene region in MEGA 11 software using the neighbour-joining method (NJ) and Juke cantor nucleotide measure with 1000 bootstrap replicates. Phylogenetic tree was constructed using *Neolissochilus pnar* (Accession no. OQ351362.1 and OQ349707.1) as an outgroup species. The constructed phylogenetic tree were designed using iTol[28].

Data analysis

Equally aligned sequences were exported to nexus format[29]. Haplotypes analysis was carried out using DnaSP6 software[30] and Haplotype network was generated using PopART (Population Analysis with Reticulate Trees) software[31]Haplotype, Haplotype number, Polymorphic sites, Haplotype diversity, Nucleotide diversity, Gene Flow, and Neutrality indices were determined using DnaSP6 software. Minimum Spanning Networks were used to visualise relationships between haplotypes in PopART.

Result

During the present study, we analyse 289 gene sequences of *T. putitora* from the NCBI database. 106 mt*COI* gene and 183 *Cyt b* gene sequences with 2 reference sequences were used in the present study. Sequences belonged to the Indian major riverine systems such as Indus, Ganga, Brahmaputra, Mahanadi, and Krishna, Kosi River of Nepal, Ravi River of Pakistan, Kabul River of Afghanistan and the Tanguar Haor and Someshwari River of Bangladesh (Table 1).

Polymorphism analysis

After analysing 106 sequences of the *COI* gene (498 sites) and 2 reference sequences using DnaSP software, mutations were observed in 44 different points. In *COI* gene sequences, 41 polymorphic sites (segregating sites) with 31 parsimony informative sites were identified and the longest conserved area was detected between 368–410 bp. Moreover, 108 T. *putitora COI* gene isolate sequences were classified into 18 haplotypes (h). *COI* gene region also showed 0.630 Haplotype diversity (Hd), 0.00662 Nucleotide diversity (Pi) and 3.295 average number of nucleotide differences (k) (Table 2). The highest number of haplotypes was found in the Brahmaputra (India) riverine population with 0.83 haplotype diversity (Table 3). Recombination analysis shows 4 minimum number of recombination events (Rm: 4). Recombination has been detected between the following sites: (93,108) (108,129) (195,234) (234,240). Neither of the analysed datasets contained any protein coding region.

Similarly, analyses of 185 *Cyt b* gene sequences (1140 sites) show 160 Polymorphic and 61 parsimony informative sites. Within *Cyt b*, mutations were identified at 173 different sites and the most conserved region was observed between 113–417 bp. Sequence analysis of *Cyt b* gene showed 85 haplotypes (h) with 0.804 haplotype diversity (Hd), 0.00582 nucleotide diversity (Pi) and 6.444 average number of nucleotide differences (k) (Table 2). The highest number of haplotypes was found in the Ganga riverine population (h = 46), followed by the Brahmaputra (India) river population (h = 18). Haplotype diversity was found to be higher in Brahmaputra (1.0000) and Kosi river (1.0000) followed by Ganga (0.96961) and then Krishna (0.74167) river population (Table 3).

Recombinant Analysis revealed 15 minimum number of recombination events (Rm). Recombination has been found between the following sites (15,16) (16,18) (45,92) (145,445) (445,633) (633,730) (730,732) (733,781) (781,819) (864,892) (892,914) (914,950) (971,974) (974,997) (1038,1098).

origin	No. of Isolates	Accession numbers
COI		
India (Brahmaputra)	36	MK599519, MK599518, MK599517, MK599516, MH156967, MN563577, MK714213, MK651786, MN830279, MG769048, MG769047, MG769046, MG736354, MG736353, KT200167, KT200166, KT200165, KT200164, KF410996, KF410995, KF410994, JX127224, JX127225, JX127226, JX127227, JX127228, JX127229, JX127230, JX127240, JX127241, JX127242, GQ469826, GQ469825, OQ874340, OM060681, OM060682
India (Indus)	30	OR148212, OR148213, OR148214, OR148215, OR148217, OR148218, OR148216, OR148219, OR148220, OR148221, OR148222, OR148224, OR148225, OR148226, OR148227, OR148228, OR148230, OR148231, MK804125, MK804124, GQ469802, GQ469803, GQ469804, GQ469805, GQ469806, GQ469807, GQ469808, GQ469809, PP217461, PP217460
India (Ganga)	32	FJ459537, FJ459536, FJ459535, FJ459534, FJ459533, MH283008, KR809787, KR809786, KR809785, KR809784, KR809783, GQ469810, GQ469811, GQ469812, GQ469813, GQ469814, GQ469815, GQ469817, GQ469818, GQ469819, GQ469820, GQ469821, GQ469822, GQ469823, GQ469824, MW871548, MW898441, MW898442, MW898443, MZ519710, MW866825, NC021755
India (Krishna)	2	KP965406, KP965405
India (Mahanadi)	2	OQ118339, OP038557
Bangladesh (KT-Tanguar Haor; OR-Someshwari)	4	KT762379, KT762361, OR794014, OR794015
Nepal (Kosi)	1	AP011326
Pakistan (Ravi)	1	OP575636
Cyt b		
India (Brahmaputra)	18	JX204705, JX204704, JX204703, JX204702, JX204701, JX204700, JX204699, JX204698, JX204697 JX204696, JX204695, JX204694, JX204693, JX204692 JX204691, JX204690, JX204689, JX204688
India (Indus)	83	JX204628, JX204622, JX204617, JX204708, JX204722, JX204721, JX204720, JX204717, JX204706, JX204719 JX204718, JX204715, JX204713, JX204712, JX204711, JX204710, JX204629, JX204627, JX204626, JX204625, JX204624, JX204623, JX204621, JX204620, JX204619, JX204618, JX204616 , JX204615, JX204614, JX204613, JX204612, JX204611, JX204610, JX204609, JX204608, JX204607, JX204606, JX204605, JX204604, JX204603, JX204602, JX204601, JX204600, JX204599, JX204598, JX204597, JX204596, JX204595, JX204594, JX204593, JX204592, JX204591, JX204590, JX204589, JX204588, JX204587, JX204586, JX204716,JX204714, JX204709, JX204707, JX204667, JX204666, JX204665, JX204664, JX204663, JX204662 , JX204661, JX204660, JX204659, JX204658, JX204657, JX204656, JX204655, JX204654, JX204653, JX204652 , JX204651, JX204650, JX204649, JX204648, JX204647 , JX204646
India (Ganga)	59	NC_021755, JX204671, JX204670, JX204669, JX204668 JX204684, JX204675, JX204673, JX204672, MK783224, JX204687, JX204686, MJX204685, JX204683, JX204682 JX204681. JX204680, JX204678, JX204677, MK783225, JX204679, JX204676, JX204674, MK783261, MK783260, MK783259, MK783258, MK783257, MK783256, K783255, MK783254, MK783253, MK783252, MK783251, MK783250, MK783249, MK783248, MK783247, MK783246, MK783245, MK783244, MK783243, MK783242, MK783241, MK783240, MK783239, MK783238, MK783237, MK78326, MK783235, MK783234, MK783233, MK783232, MK783231, MK783230, MK783229, MK783228, MK783227, MK783226
India (Krishna)	16	JX204645, JX204636, JX204644, JX204643, JX204642, JX204641, JX204640, JX204639, JX204638, JX204637, JX204635, JX204634, JX204630, JX204633, JX204632, JX204631
India (Mahanadi)	4	OQ674804, OQ674803, OQ674802, OQ674801
Nepal (Kosi)	2	AP011326, KP712187
Afghanistan (Kabul)	3	KU524918, KU524917, KU524916

Table 1. Accession number of mt COI and Cyt b gene fragments of *T. putitora* isolates with their origin used in this study.

mtDNA	n	H	S	Pi	hD ± Sd	Pi ± SD	k	Tajima's D	*p* value	Fu's Fs	*FLD*	*p* value	FLF	*p* value
COI	108	18	41	44	0.630 ± 0.051	0.006 ± 0.001	3.295	-1.893	* P < 0.05	-3.277	-0.897	P > 0.10	-1.562	P > 0.10
Cyt b	185	85	160	173	0.804 ± 0.031	0.005 ± 0.00063	6.444	-2.48721	***P < 0.001	-83.20	-7.761	**P < 0.02	-6.277	**P < 0.02

Table 2. Analysis of diversity and neutrality indices. *n* Number of isolates, *H* haplotype number, *S* polymorphic sites, *Pi* nucleotide diversity, *hD* haplotype diversity, *SD* standard deviation, *k* average number of nucleotide differences.

Haplotype network analysis and phylogenetic analysis

During the present study, analysis of the *COI* gene revealed 18 haplotypes (Table 4) whereas *Cyt b* gene sequences revealed 85 haplotypes (Table 5). In the *COI* gene, haplotype 4 was the main haplotype with 64 sequences (Fig. 1). Haplotype 4 comprises 59.25% (64/108) of the entire haplotype network followed by haplotype 9, accounting for 11% (12/108). In the *COI* gene sequences, unique single haplotypes were 66.66% (12/18) of the total haplotypes. Unique single haplotypes were from Ganga (India), Indus (India) and Brahmaputra (India) riverine populations. Unique haplotypes, Hap 01, 07, 14, 15 were from Ganga, Hap 05, 06, 10 were from Indus and Hap 11,12,13,17,18 were from Brahmaputra.

COI gene haplotype network comprised 18 haplotypes, which were connected by 1 to 20 mutation steps corresponding to 8 populations. The maximum mutation in nucleotide was found between Hap 08 and Hap 03.

COI (Number of populations included: 6)							
Country	Population (Riverine system)	No. of Sequence	Number of segregating sites (S)	Number of haplotypes (h	Haplotype diversity (Hd)	Average number of differences, K	Nucleotide diversity, Pi
India	Brahmaputra	36	36	11	0.83175	5.04127	0.01012
	Indus	30	3	4	0.19310	0.20000	0.00040
	Ganga	32	5	5	0.23790	0.31250	0.00063
	Krishna	2	0	1	0.00000	0.00000	0.00000
	Mahanadi	2	0	1	0.00000	0.00000	0.00000
Bangladesh	Tanguar haor river	4	13	3	0.83333	8.16667	0.01640
Cyt B (Number of populations included: 7)							
India	Ganga	59	54	46	0.96961	4.75687	0.00429
	Indus	83	50	14	0.34852	2.67881	0.00242
	Mahanadi	4	0	1	0.00000	0.00000	0.00000
	Krishna	16	16	7	0.74167	2.37500	0.00214
	Brahmaputra	18	57	18	1.00000	7.71242	0.00696
Afghanistan	Kabul river	3	1	2	0.66667	0.66667	0.00060
Nepal	Kosi	2	1	2	1.00000	1.00000	0.00090

Table 3. Haplotype of *Tor putitora* isolate in different countries from different river population based on Coi and cyt B gene.

Haplotype (Hap) 4 was found to be the most common and ancestral haplotype due to its maximum abundance in the studied population (Fig. 1). Haplotype 4 shares populations from major Indian riverine systems (Brahmaputra, Indus, Ganga, Krishna, and Mahanadi), as well as from Pakistan (Ravi River) and Bangladesh (Tanguar Haor basin river). Hap1, Hap 5, Hap 6, Hap 8, Hap 12, Hap 14 and Hap 15 were considered as budding haplotypes and found periphery in the network. Hap 02, Hap 03 and Hap 04 share sequences of more than one population. The sequence of the Kosi River (Nepal) shares haplotype (Hap 02) with the population of the Brahmaputra River (India) and Bangladesh River. Hap 3 shares the population of the Brahmaputra River (India) and the Tanguar Haor River of Bangladesh.

Similarly, within the *Cyt b* gene sequence analysis haplotype 37 was the main haplotype (Fig. 2), comprising 43.78% (81/185) of the haplotype network, followed by haplotype 38 which accounts for 7.56% (14/185). Unique single haplotypes contribute 92.94% (79/85) of the total haplotypes. The Ganga riverine population contains 43 (Hap 1- Hap 36, Hap 67, Hap 68, Hap 70, Hap 71, Hap 72, Hap 73, and Hap 85) unique haplotypes, followed by the Brahmaputra riverine population having 17 unique haplotypes (Hap 49- Hap 53, Hap 55- Hap 66). Indus population have 12 unique haplotypes (Hap 39, Hap 41- Hap 48, Hap 80, Hap 81, Hap 82) whereas the Kosi river of Nepal (Hap 84) and Kabul river of Afghanistan (Hap 83) contain a single unique haplotype.

Cyt b gene haplotype network comprised 85 haplotypes connected by 1 to 18 mutation steps corresponding to seven populations. The maximum mutation in nucleotide (18) was found between Hap 61 and Hap 51 and Hap 61 and Hap 54. Hap 37 was the main haplotype having population from Indus (India), Ganga (India), Krishna (India) and Kabul River (Afghanistan). Hap 54, Hap 38 and Hap 37 share sequences of more than one haplotype. Hap 54 share population of Brahmaputra and Kosi River. Similarly, Hap 38 share the population of Ganga and Mahanadi River systems. The phylogenetic tree also reveals the evolutionary relationships between several isolates of *T. putitora* . Phylogenetic analysis of *COI* gene and *Cyt b* are shown in Fig. 3 and Fig. 4. *COI* gene sequence analysis revealed one main clade in phylogenetic tree whereas *Cyt b* analysis revealed two main clades. The phylogenetic tree results were rational with the haplotype network.

Diversity, gene flow and neutrality analysis

The diversity and Neutrality indices of *COI* and *Cyt b* gene are given in Table 2. Tajima's D and Fu's Fs neutrality statistics are generally used in population genetics to examine evolutionary processes, such as to ascertain the presence of selection pressure of DNA sequences in the population. The measurements of Tajima's D and Fu's Fs indicate significant negative values in both *COI* and *Cyt b*, suggesting the presence of an excess number of rare nucleotide site variants. The negative value of Tajima,s D was also statically significant for both *COI* and *Cyt b* gene sequences.

Discussion

The assessment of genetic diversity represents an essential component in diversity assessment and is progressively used for the conservation and management purposes of species[32]. Genetic diversity and population structure of endangered fish species play an important role in their conservation and management[19]. mtDNA genes were the most widely used DNA marker for Population genetics[11]. For this reason, the genetic diversity and population structure of *T. putitora* were investigated through Insilico analysis using two mtDNA genes (*COI* and *Cyt b*). A total of 106 mt *COI* (498 bp) and 183 *Cyt b* (1140 bp) gene sequences of *T. putitora* isolate already submitted in Genebank (NCBI) were utilised in the present study. Overall, the analysis of 289 samples identified 18 haplotypes using mt *COI* gene and 85 Haplotypes using *Cyt b* genes in different populations of *T. putitora*.

Haplotype name	No. Of isolate	Accession numbers
Hap 01	1	NC021755[INDIA (GANGA)]
Hap 02	8	MK599519[INDIA (BRAHMAPUTRA)], MK599518[INDIA (BRAHMAPUTRA)], AP011326[NEPAL (KOSI)], MK599517 [INDIA (BRAHMAPUTRA)], MK599516[INDIA (BRAHMAPUTRA)], KT200166[INDIA (BRAHMAPUTRA)], KT200165[INDIA (BRAHMAPUTRA)], OR794015[BANGLADESH(SOMESHWARI)]
Hap 03	3	MH156967[INDIA (BRAHMAPUTRA)], KT762379[BANGLADESH (TANGUAR HAOR)], KT762361[BANGLADESH (TANGUAR HAOR)]
Hap 04	64	OR148231[INDIA (INDUS)], OR148230[INDIA (INDUS)], OR148228[INDIA (INDUS)], OR148227[INDIA (INDUS)], OR148226 [INDIA (INDUS)], OR148225[INDIA (INDUS)], OR148224[INDIA (INDUS)], OR148221[INDIA (INDUS)], OR148222[INDIA (INDUS)], OR148220[INDIA (INDUS)], OR148217[INDIA (INDUS)], OR148218 [INDIA (INDUS)], OR148215[INDIA (INDUS)], OR148214[INDIA (INDUS)], OR148213[INDIA (INDUS)] OR148212[INDIA (INDUS)], OP575636[PAKISTAN (RAVI)], KR809787[INDIA (GANGA)], KR809786[INDIA (GANGA)], KR809785[INDIA (GANGA)], KR809784[INDIA (GANGA)], KR809783[INDIA (GANGA)], MK804124[INDIA (INDUS)], FJ459537[INDIA (GANGA)], FJ459536[INDIA (GANGA)], FJ459534[INDIA (GANGA)], KP965406[INDIA (KRISHNA)], KP965405[INDIA (KRISHNA)], GQ469825[INDIA (GANGA)], GQ469824[INDIA (GANGA)], GQ469823[INDIA (GANGA)], GQ469822[INDIA (GANGA)], GQ469821[INDIA (GANGA)], GQ469820[INDIA (GANGA)], GQ469819[INDIA (GANGA)], GQ469817[INDIA (GANGA)], GQ469815[INDIA (GANGA)], GQ469814[INDIA (GANGA)] GQ469813[INDIA (GANGA)], GQ469812[INDIA (GANGA)], GQ469811[INDIA (GANGA)]., GQ469810[INDIA (GANGA)], GQ469809[INDIA (INDUS)], GQ469808[INDIA (INDUS)], GQ469807[INDIA (INDUS)], GQ469806[INDIA (INDUS)] GQ469805[INDIA (INDUS)], GQ469804[INDIA (INDUS)], GQ469803[INDIA (INDUS)], GQ469802[INDIA (INDUS)], PP217461[INDIA (INDUS)], PP217460[INDIA (INDUS)], MW871548[INDIA (GANGA)], MW898443[INDIA (GANGA)] MW898442[INDIA (GANGA)], MW898441[INDIA (GANGA)], OQ118339[INDIA (MAHANADI)], OR794014[BANGLADESH (SOMESHWARI)], OQ874340[INDIA (BRAHMAPUTRA)], OP038557[INDIA (MAHANADI)], MZ519710[INDIA (GANGA)], MW866825[INDIA (GANGA)], GQ469818[INDIA (GANGA)], GQ469826[INDIA (BRAHMAPUTRA)],
Hap 05	1	OR148219 [INDIA (INDUS)]
Hap 06	1	OR148216[INDIA (INDUS)]
Hap 07	1	MH283008[INDIA (GANGA)]
Hap08	2	MN563577[INDIA (BRAHMAPUTRA)], MN830279[INDIA (BRAHMAPUTRA)],
Hap09	12	MK714213[INDIA (BRAHMAPUTRA)], MK651786[INDIA (BRAHMAPUTRA)], MG769047[INDIA (BRAHMAPUTRA)], MG769046[INDIA (BRAHMAPUTRA)], KT200164[INDIA (BRAHMAPUTRA)], KF410995[INDIA (BRAHMAPUTRA)], JX127242[INDIA (BRAHMAPUTRA)], JX127241[INDIA (BRAHMAPUTRA)], JX127240[INDIA (BRAHMAPUTRA)], JX127230 [INDIA (BRAHMAPUTRA)], JX127229[INDIA (BRAHMAPUTRA)], JX127228[INDIA (BRAHMAPUTRA)],
Hap10	1	MK804125[INDIA (INDUS)]
Hap11	1	MG769048[INDIA (BRAHMAPUTRA)],
Hap12	1	MG736354. [INDIA (BRAHMAPUTRA)],
Hap13	1	MG736353[INDIA (BRAHMAPUTRA)],
Hap14	1	FJ459535[INDIA (GANGA)],
Hap15	1	FJ459533[INDIA (GANGA)],
Hap16	7	KT200167[INDIA (BRAHMAPUTRA)], KF410996[INDIA (BRAHMAPUTRA)], KF410994[INDIA (BRAHMAPUTRA)], JX127227[INDIA (BRAHMAPUTRA)], JX127226[INDIA (BRAHMAPUTRA)], JX127225[INDIA (BRAHMAPUTRA)], JX127224[INDIA (BRAHMAPUTRA)],
Hap17	1	OM060682[INDIA (BRAHMAPUTRA)],
Hap18	1	OM060681[INDIA (BRAHMAPUTRA)],

Table 4. Haplotypes of COI sequences of *Tor putitora* and accession numbers of isolates forming group.

Haplotype difference is mainly corresponded to the gene length analysed. Therefore, gene fragments with longer length have more haplotypes[24].

The result of the present study reveals high haplotypic diversity in *T. putitora* using the *Cyt b* gene whereas *COI* gene has moderate haplotype diversity. Similarly, Sati et al., (2015) also, identify high haplotypic diversity in *T. putitora* using *Cyt b* gene[11]. Genetic variability (h and Pi) of the *COI* gene is lower because of few polymorphic sites as compared to the *Cyt b* gene. Earlier based on *Cyt b* gene, Yadav et al., (2020) identified 38 haplotypes from Himalayan rivers such as Bhagirathi, Alaknanda, Ganga and Yamuna[19] and Sati et al., (2015) identified 47 haplotypes from rivers like Jia Bhoreli, Satluj, Ravi, Beas, Chenab, Kosi and Indrayani[11]. Our data supplement this information by adding information from other rivers from geographically different locations. There was a high haplotype (*COI* = 0.83175, *Cyt b* = 1.00000 0.01012) and nucleotide diversity (*COI* = 0.01012, *Cyt b* = 0.00696) in the Brahmaputra River population. The high genetic diversity (*COI* and *Cyt b*) in the Brahmaputra River population may be due to more segregating sites than other riverine populations. Substantial population sizes may facilitate the maintenance of elevated haplotype diversity within the population[33]. Sati et al., (2015) also found high genetic variability due to the large number of polymorphic sites[11]. Similarly, the population from Bangladesh's rivers also has high genetic diversity (*COI*), with haplotype diversity = 0.83333 and nucleotide diversity = 0.01640. The high haplotype diversities suggest a rapid population expansion, which is accompanied by the accumulation of new mutations following a period of low effective population size[34]. The migratory behaviour may be the primary cause of genetic variation within a population, rather than among the population[35,36]. The present study reveals less genetic diversity in the Indus riverine population. Inbreeding in the population may be responsible for low genetic diversity[11,37].

Our analysis of the mt *COI* gene identified a total of 18 haplotypes. The predominant haplotype (Hap 4) comprises 59.25% of the total network and there were 12 unique haplotypes. Similarly, *Cyt b* gene analysis reveals 85 haplotypes with the dominance of Hap 37 which contributes 43.78% of the total haplotype network.

Haplotype Name	No of isolates	Accession no
Hap01	1	MK783261[INDIA (GANGA)]
Hap02	1	MK783260[INDIA (GANGA)]
Hap03	1	MK783259[INDIA (GANGA)]
Hap04	1	MK783258[INDIA (GANGA)]
Hap05	1	MK783257[INDIA (GANGA)]
Hapo6	1	MK783256[INDIA (GANGA)]
Hap07	1	MK783255[INDIA (GANGA)]
Hap08	1	MK783254[INDIA (GANGA)]
Hap09	1	MK783253[INDIA (GANGA)]
Hap10	1	MK783252[INDIA (GANGA)]
Hap11	1	MK783251[INDIA (GANGA)]
Hap12	1	MK783250[INDIA (GANGA)]
Hap13	1	MK783249[INDIA (GANGA)]
Hap14	1	MK783248[INDIA (GANGA)]
Hap15	1	MK783247[INDIA (GANGA)]
Hap16	1	MK783246[INDIA (GANGA)]
Hap17	1	MK783245[INDIA (GANGA)]
Hap18	1	MK783244[INDIA (GANGA)]
Hap19	1	MK783243[INDIA (GANGA)]
Hap20	1	MK783242[INDIA (GANGA)]
Hap21	1	MK783241[INDIA (GANGA)]
Hap22	1	MK783240[INDIA (GANGA)]
Hap23	1	MK783239[INDIA (GANGA)]
Hap24	1	MK783238[INDIA (GANGA)]
Hap25	1	MK783237[INDIA (GANGA)]
Hap26	1	MK783236[INDIA (GANGA)]
Hap27	1	MK783235[INDIA (GANGA)]
Hap28	1	MK783234[INDIA (GANGA)]
Hap29	1	MK783233[INDIA (GANGA)]
Hap30	1	MK783232[INDIA (GANGA)]
Hap31	1	MK783231[INDIA (GANGA)]
Hap32	1	MK783230[INDIA (GANGA)]
Hap33	1	MK783229[INDIA (GANGA)]
Hap34	1	MK783228[INDIA (GANGA)]
Hap35	1	MK783227[INDIA (GANGA)]
Hap36	1	MK783226[INDIA (GANGA)]
Hap37	81	[MK783225[INDIA (GANGA)], JX204716[INDIA (INDUS)], JX204714[INDIA (INDUS)], JX204709[INDIA (INDUS)], JX204707[INDIA (INDUS)], JX204679 [INDIA (GANGA)], JX204676 [INDIA (GANGA)] JX204674[INDIA (GANGA)], JX204667[INDIA (INDUS)], JX204666 [INDIA (INDUS)], JX204665[INDIA (INDUS)], JX204664[INDIA (INDUS)], JX204663[INDIA (INDUS)], JX204662[INDIA (INDUS)], JX204661[INDIA (INDUS)], JX204660[INDIA (INDUS)], JX204659 [INDIA (INDUS)], JX204658[INDIA (INDUS)], JX204657[INDIA (INDUS)], JX204656[INDIA (INDUS)], JX204655[INDIA (INDUS)], JX204654 [INDIA (INDUS)], JX204653[INDIA (INDUS)], JX204652 [INDIA (INDUS)], JX204651[INDIA (INDUS)], JX204650[INDIA (INDUS)], JX204649[INDIA (INDUS)], JX204648 [INDIA (INDUS)], JX204647[INDIA (INDUS)], JX204646 [INDIA (INDUS)], JX204644[INDIA (KRISHNA)], JX204643[INDIA (KRISHNA)], JX204642[INDIA (KRISHNA)], JX204641[INDIA (KRISHNA)], JX204640[INDIA (KRISHNA)], JX204639[INDIA (KRISHNA)], JX204638[INDIA (KRISHNA)], JX204637[INDIA (KRISHNA)], JX204629[INDIA (INDUS)], JX204627[INDIA (INDUS)], JX204626[INDIA (INDUS)], JX204625 [INDIA (INDUS)], JX204624 [INDIA (INDUS)], JX204623 [INDIA (INDUS)], JX204621[INDIA (INDUS)], JX204620 [INDIA (INDUS)], JX204619[INDIA (INDUS)], JX204618[INDIA (INDUS)], JX204616 [INDIA (INDUS)], JX204615[INDIA (INDUS)], JX204614 [INDIA (INDUS)], JX204613[INDIA (INDUS)], JX204612[INDIA (INDUS)], JX204611[INDIA (INDUS)], JX204610[INDIA (INDUS)], JX204609. [INDIA (INDUS)], JX204608[INDIA (INDUS)], JX204607[INDIA (INDUS)], JX204606[INDIA (INDUS)], JX204605[INDIA (INDUS)], JX204604[INDIA (INDUS)], JX204603 [INDIA (INDUS)], JX204602[INDIA (INDUS)], JX204601[INDIA (INDUS)], JX204600[INDIA (INDUS)], JX204599[INDIA (INDUS)], JX204598[INDIA (INDUS)], JX204597[INDIA (INDUS)]. JX204596[INDIA (INDUS)], JX204595[INDIA (INDUS)], JX204594 [INDIA (INDUS)], JX204593 [INDIA (INDUS)] JX204592[INDIA (INDUS)], JX204591. [INDIA (INDUS)], JX204590[INDIA (INDUS)], JX204589 [INDIA (INDUS)] JX204588[INDIA (INDUS)], JX204587[INDIA (INDUS)]. JX204586[INDIA (INDUS)], KU524917[AFGANISTAN (KABUL)], KU524916[AFGANISTAN(KABUL)]
Hap38	14	MK783224[INDIA (GANGA)], JX204687[INDIA (GANGA)], JX204686[INDIA (GANGA)], JX204685[INDIA (GANGA)], JX204683[INDIA (GANGA)], JX204682[INDIA (GANGA)], JX204681[INDIA (GANGA)], JX204680[INDIA (GANGA)], JX204678[INDIA (GANGA)], JX204677 [INDIA(GANGA)], OQ674804[INDIA (MAHANADI)], OQ674803[INDIA (MAHANADI)], OQ674802[INDIA (MAHANADI)], OQ674801. [INDIA (MAHANADI)]
Hap39	1	JX204722 [INDIA (INDUS)]
Hap40	4	[JX204721[INDIA (INDUS)], JX204720[INDIA (INDUS)], JX204717[INDIA (INDUS)], JX204706[INDIA (INDUS)]
Hap41	1	JX204719[INDIA (INDUS)]
Hap42	1	JX204718[INDIA (INDUS)]
Continued		

Haplotype Name	No of isolates	Accession no
Hap43	1	JX204715[INDIA (INDUS)]
Hap44	1	JX204713[INDIA (INDUS)]
Hap45	1	JX204712INDIA (INDUS)]
Hap46	1	JX204711[INDIA (INDUS)]
Hap47	1	JX204710[INDIA (INDUS)]
Hap48	1	JX204708[INDIA (INDUS)]
Hap49	1	JX204705[INDIA (BRAHMAPUTRA)]
Hap50	1	JX204704[INDIA (BRAHMAPUTRA)]
Hap51	1	JX204703[INDIA (BRAHMAPUTRA)]
Hap52	1	JX204702[INDIA (BRAHMAPUTRA)]
Hap53	1	JX204701[INDIA (BRAHMAPUTRA)]
Hap54	2	JX204700[INDIA (BRAHMAPUTRA)], AP011326 [NEPAL (KOSI)]
Hap55	1	JX204699[INDIA (BRAHMAPUTRA)]
Hap56	1	JX204698[INDIA (BRAHMAPUTRA)]
Hap57	1	JX204697[INDIA (BRAHMAPUTRA)]
Hap58	1	JX204696[INDIA (BRAHMAPUTRA)]
Hap59	1	JX204695[INDIA (BRAHMAPUTRA)]
Hap60	1	JX204694[INDIA (BRAHMAPUTRA)]
Hap61	1	JX204693[INDIA (BRAHMAPUTRA)]
Hap62	1	JX204692[INDIA (BRAHMAPUTRA)]
Hap63	1	JX204691[INDIA (BRAHMAPUTRA)]
Hap64	1	JX204690[INDIA (BRAHMAPUTRA)]
Hap65	1	JX204689[INDIA (BRAHMAPUTRA)]
Hap66	1	JX204688[INDIA (BRAHMAPUTRA)]
Hap67	1	JX204684[INDIA (GANGA)]
Hap68	1	JX204675[INDIA (GANGA)]
Hap69	2	JX204673[INDIA (GANGA)], JX204672[INDIA (GANGA)]
Hap70	1	JX204671[INDIA (GANGA)]
Hap71	1	JX204670[INDIA (GANGA)]
Hap72	1	JX204669[INDIA (GANGA)]
Hap73	1	JX204668[INDIA (GANGA)]
Hap74	1	JX204645[INDIA (KRISHNA)]
Hap75	1	JX204636[INDIA (KRISHNA)]
Hap76	3	JX204635[INDIA (KRISHNA)], JX204634[INDIA (KRISHNA)] JX204630[INDIA (KRISHNA)]
Hap77	1	JX204633[INDIA (KRISHNA)]
Hap78	1	JX204632[INDIA (KRISHNA)]
Hap79	1	JX204631[INDIA (KRISHNA)]
Hap80	1	JX204628[INDIA (INDUS)]
Hap81	1	JX204622[INDIA (INDUS)]
Hap82	1	JX204617[INDIA (INDUS)]
Hap83	1	KU524918[AFGANISTAN (KABUL)]
Hap84	1	KP71218 [NEPAL (KOSI)]
Hap85	1	NC021755[INDIA (GANGA)]

Table 5. Haplotypes of Cyt b sequences of *Tor putitora* and accession numbers of isolates forming group.

These predominant haplotypes are likely the ancestors from which other haplotypes may have come directly or through mutation[11]. Unique haplotypes originated from mutational events denoted by hatch marks in the branches of the haplotype network[38]. Recently emerged haplotypes show restricted geographical distribution, whereas ancestral haplotypes show their presence in various geographical regions[39,40]. The Kosi River (Nepal) population share the haplotype with the Brahmaputra River population in both *COI* and *Cyt b* gene Haplotype network analysis. In *Cyt b* gene haplotype analysis, Hap 84 which represents Kosi riverine population show their ancestral linkage with Hap 54. Hap 54 shares the population of the Kosi and Brahmaputra Rivers. This sharing can be attributed to ecological and genetic factors. *T. putitora* is widely distributed in the Himalayan River system including Indus, Ganga, Brahmaputra etc. and their tributaries which extend into Nepal [5,41]. Historical connectivity among these river networks facilitates gene flow and genetic exchange among populations. Additionally, habitat fragmentation due to human activity, such as river modification and dam construction also

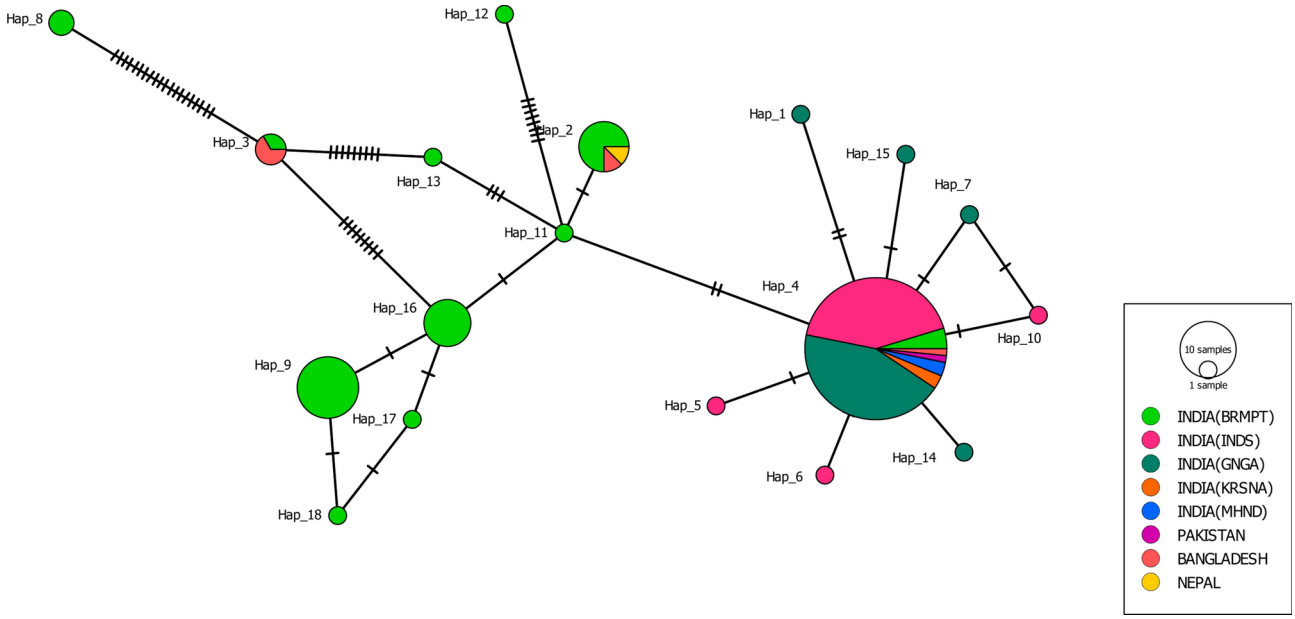

Fig. 1. Haplotype network based on *COI* gene sequences of *T. putitora* isolates. The size of the circle represents haplotype frequency and hatch marks indicate mutation number that discriminate haplotypes. Here, INDIA_BRMPT: Brahmaputra; INDIA_INDS: Indus; INDIA_GNG: Ganga; INDIA_KRSNA: Krishna; INDIA_MHND: Mahanadi River. PAKISTAN represents the Ravi River; Nepal represents the Kosi River and BANGLADESH represents the Tanguhar and Someshwari Rivers.(Created with PopART Qt version 4.8.4).

affects the genetic diversity. Such fragmentation can lead to localised populations that may still retain connection through migration, therefore maintaining haplotype similarity[42].

Neutral theory served has served as a foundation framework for understanding genetic diversity. This theory suggests that the accumulation of genetic differences over time in populations is primarily due to random fixation of these neutral mutations, rather than adaptive change driven by natural selection. However, this theory faces challenges as studies found that many mutations do not evolve neutrally, and selection appears to play a significant role in shaping genome[43,44]. Donati et al. (2021) found that the spatial component of genetic diversity in tropical reef fishes did not align with the expectation of neutral theory. Their finding indicated a negative relationship between genetic diversity and regional abundance, contradicting the theory's prediction that higher abundance should correlate with increasing genetic diversity[45]. Human mtDNA genome is predominantly functional rather than neutral, thereby directly refuting the neutral theory[46]. Similarly, STR repeats were previously considered neutral; however recent finding indicates their ability to bind transcription factors[47]. Also, the maximum genetic diversity theory (MGD) stands in contrast to the neutral theory. This theory emphasizes that higher genetic diversity enhances a population's ability to adapt to environmental changes, thereby increasing its chance of survival and evolutionary success[48,49].

Neutral theory faces challenges which led to the development of the neutrality test. These neutrality tests are statistical tools designed to evaluates whether observed genetic variation fits neutral theory's expectations or if selective forces are also at play. Neutrality tests are essential tools for assessing genetic variation and population dynamics[50]. In this study, we have performed neutrality test to decipher the nature of evolutionary pressure acting on concerned mitochondrial gene, which in turn helped to infer the selective advantages of specific genotype over others, as indicated by their prevalence within population. Tajima's D test compares the number of segregating sites (polymorphic sites) to the average pairwise nucleotide differences among sequences[33,51]. Using the Tajima's D test, populations are evaluated for their deviation from the neutral modal. A positive Tajima's D value signifies heterozygosity which is indicative of selective advantage. On the other hand, a negative Tajima's D value is indicative of selective advantage of a particular allele over another allele and signifies the rapid increase in population[24]. Fu's Fs test is particularly sensitive to recent population expansions[52]. A positive Tajima's D and Fu's Fs indicate an excess of intermediate frequency allele, which may suggest a decline in population[33]. During the present study, the value of Tajima's D and Fu's Fs test for both *COI* and *Cyt b* were negative, suggesting an expansion of the population which could be attributed to habitat destruction[53]. Earlier, Yadav et al. (2020) reported the negative value of Tajima's D and Fu's Fs for *T. putitora* from Himalayan rivers[19]. As shown by the finding obtain in this study maximum genetic variation were observed in the *Cyt b* gene as compared to the *COI* gene, also reject the neutral theory and suggested *Cyt b* gene has considerable role in the evolution and adaptability of newly evolved genotype.

Conclusion

T. putitora is an endangered freshwater fish species restricted to the cold-water habitat having their distribution in the rivers of India, Pakistan, Nepal, Bangladesh, Afghanistan, Bhutan and Myanmar. Habitat destruction,

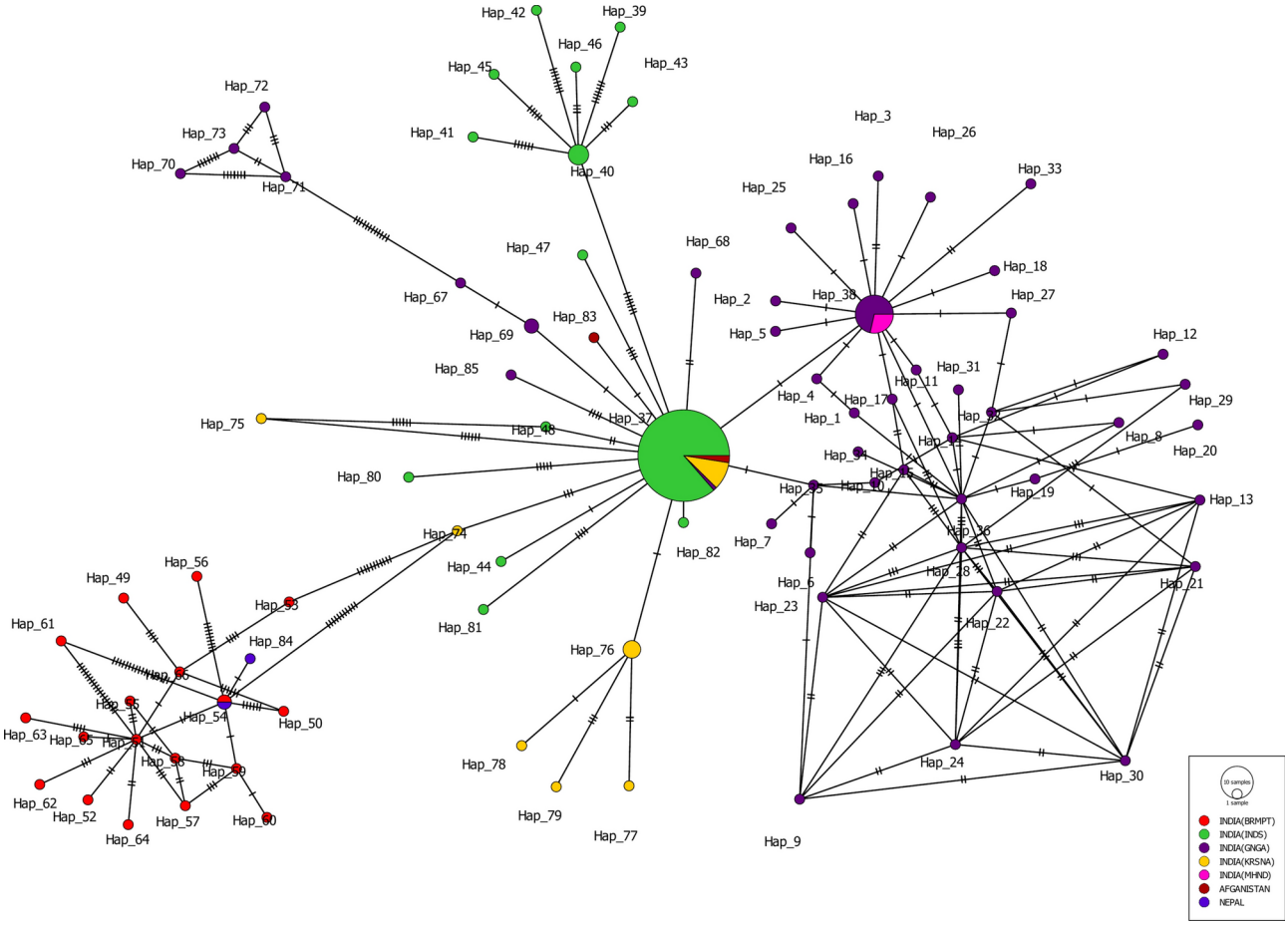

Fig. 2. Haplotype network based on *Cyt b* gene sequences of *T. putitora* isolates. The size of the circle represents haplotype frequency and hatch marks indicate mutation number that discriminate haplotypes. Here, INDIA_INDS: Indus; INDIA_GNG: Ganga; INDIA_KRSNA: Krishna; INDIA_MHND: Mahanadi River. Afghanistan represents the Kabul River and Nepal represents the Kosi River. (Created with PopART Qt version 4.8.4).

introducing exotic species and other anthropogenic activities result in the declining *T. putitora* population in their natural habitat. Mitochondrial DNA genes such as *COI*, and *Cyt b* are the gene loci that are applicable in fish species identification, phylogenetics, population and conservation genetics. All previous research on the haplotype diversity and population study of *T. putitora* has been conducted within the Indian riverine system. This investigation will serve as the basis for subsequent extensive investigation into the population structure and geographical distribution of *T. putitora* on a global scale. The genetic diversity pattern analyses in this study give us information that is helpful in developing conservation and management strategies for *T. putitora* population. To ensure the continuity of *T. putitora* populations, preservation of natural habitat, establishing protected areas in critical habitat, community-based conservation initiatives, and regular monitoring of population dynamics are essential. By implementing these strategies, the long-term continuity of *T. putitora* population can be better supported, preventing further decline and promoting recovery.

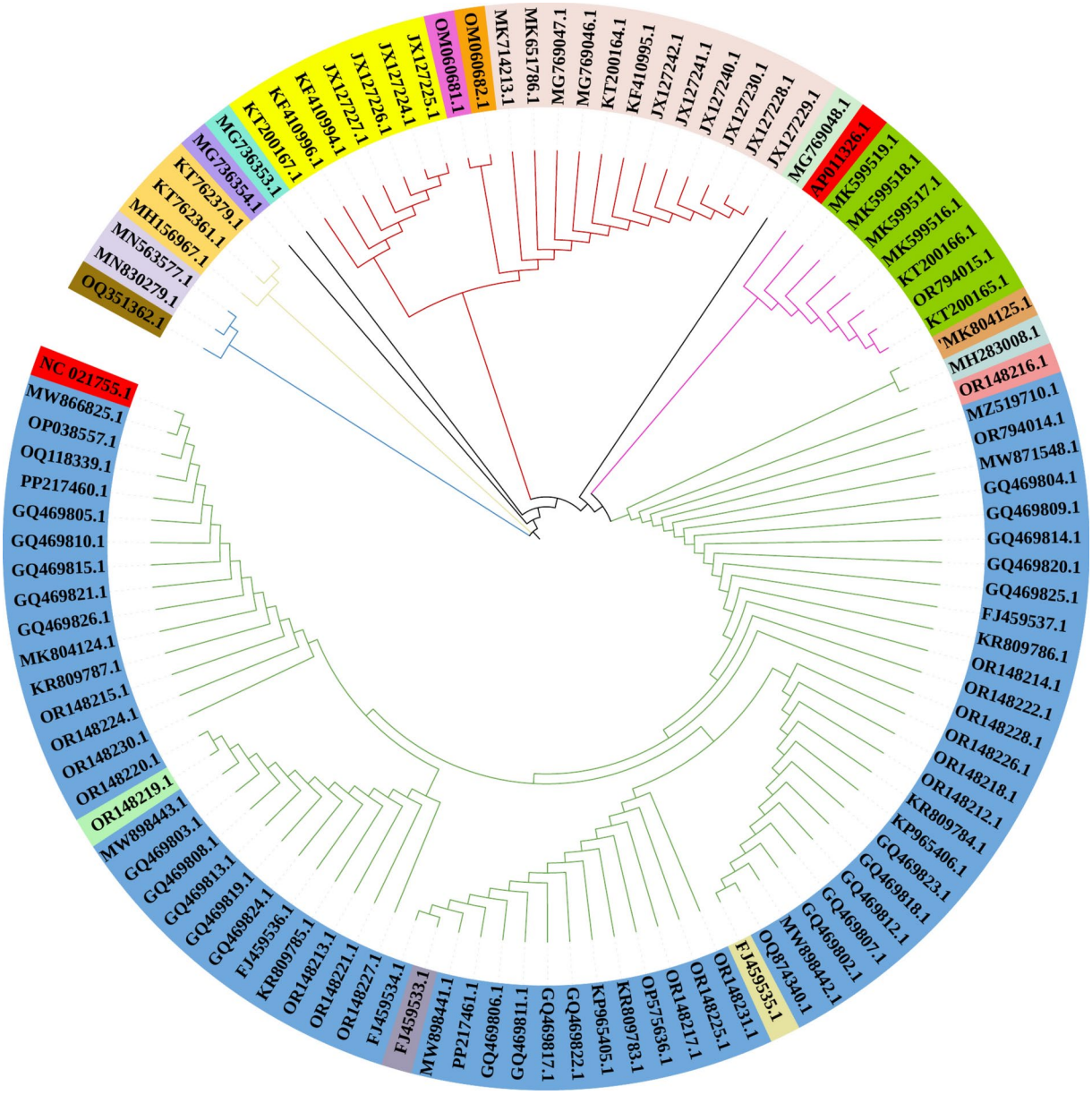

Fig. 3. Phylogenetic relationship of *T. putitora* isolates of *COI* gene sequences. MEGA 11 was used to construct a Neighbour-joining tree based on Juke cantor nucleotide measure with 1000 bootstrap replications. For the construction of phylogentic tree, *Neolissochilus pnar* (Accession no. OQ351362.1*)* was used as an outgroup. (Created with iTol :https://itol.embl.de/itol.cgi).

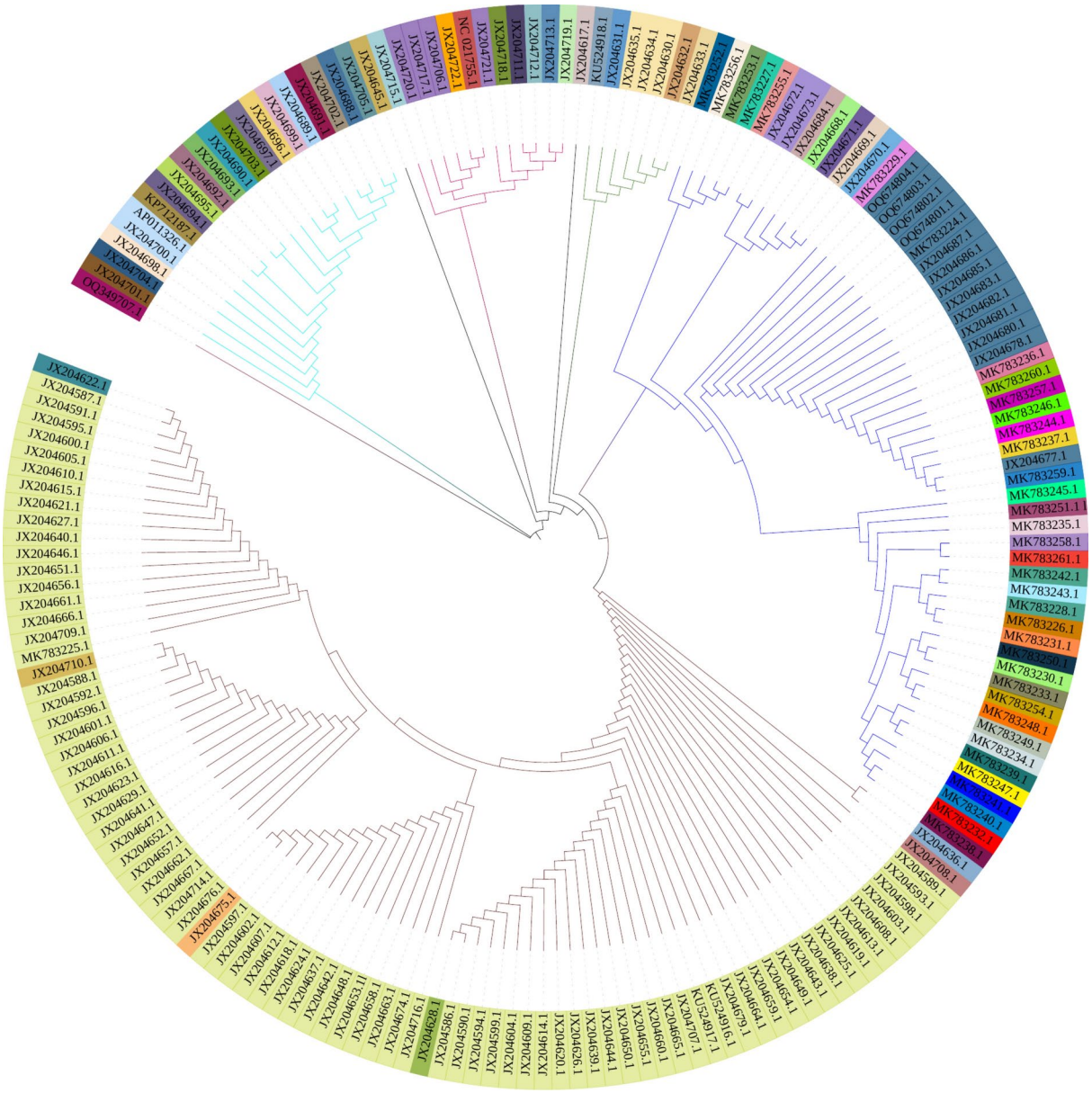

Fig. 4. Phylogenetic relationship of *T. putitora* isolates of *Cyt b* gene sequences. MEGA 11 was used to construct a Neighbour-joining tree based on Juke cantor nucleotide measure with 1000 bootstrap replications. For the construction of phylogenetic trees, *Neolissochilus pnar* (Accession no. OQ349707.1*)* was used as an outgroup. (Created with iTol :https://itol.embl.de/itol.cgi).

Data availability

The datasets used and analysed during the current study available from the corresponding author on reasonable request.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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