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Mitogenome of *Neolissochilus pnar*, the largest cavernicolous species of Mahseer

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The study of the mitogenome of *Neolissochilus pnar*, the world's largest cave fish, uncovered its structural features, gene content and evolutionary dynamics within mahseer. Its mitogenome is of 16,440 base pairs, resembling those of the teleost species and exhibits a high degree of conservation in genes arrangement. It comprises 37 mitochondrial genes, including 13 protein-coding genes (PCGs), 22 tRNA genes (tRNAs), 2 rRNA genes (rRNAs) and a control region. Notably, the distribution of genes on the L- and H-strands is consistent with that of the typical teleost. The study reveals the lengths and variations in PCGs in mahseer species, displaying a range from 164 to 11,404 bp. The tRNA and rRNA genes and the control region also demonstrate conservation among the species. A robust phylogenetic analysis, employing Bayesian and ASAP methods, supports the classification of *N. pnar* within the *Neolissochilus* genus and validates the taxonomic status of this species. Selection pressure analyses indicate positive selection in seven genes: COII , COIII, Cytb, ND1, ND2, ND5 and ND6. These findings suggest the dynamic nature of mitochondrial evolution in mahseer species. The purifying selection preserve essential mitochondrial functions, and additionally, the specific sites in ND5 and ND6 genes undergo episodic positive or diversifying selection, likely in response to environmental changes or selective pressures. In conclusion, this research enriches our understanding of *N. pnar* visa-vis other mahseers' mitogenomes, pointing to its possible mitogenome evolution to adaptation to cave environment.

Keywords Largest cave fish, Mitogenome, Genes arrangement, Phylogenetic analysis, Selective pressures

Neolissochilus pnar, identified as the world's largest cave fish, belongs to the genus Cyprinidae and is endemic to one of India's biodiversity hotspots, specifically the caves in Meghalaya state, Northeast India. Predominantly found in the Um Ladaw and the Krem Chympe caves in Meghalaya, this cave fish was initially reported in the limestone caves of the Jaintia Hills of Meghalaya[1], with photographic evidence and collection date back to 2019[2]. The largest individual observed in these caves exceeded 400 mm in standard length, setting a record as the largest known individual of any subterranean fish species globally or cave fish[2]. Notably, *N. pnar* is distinguished from closely related *Neolissochilus hexastichus* by its lack of pigmentation and reduction or absence of eye, which is small in juveniles and completely absent externally in adults[2]. Moreover, it exhibits genetic distinctiveness from its close congeners, with a genetic divergence of 1.1–2.7% in the COI gene compared to putative topotypes of *N. hexastichus* and 2.1–2.6% compared to putative topotypes of *N. hexagonolepis*. Distinguishing characteristics of *N. pnar* from the hypogean *N. subterraneus*, it is known for its lesser pre-pelvic length (47.8–49.4 vs. 50.5–55.3% SL), shorter caudal peduncle (16.1–16.8 vs. 17.8–23.7%SL) and shorter dorsal fin (17.4–20.8 vs. 21.5–26.3%SL)[3].

The prevailing view suggests that troglobitic adaptations arise due to limited food availability in cave habitats. The necessity to locate sparse food reserves is believed to drive the development of enhanced chemosensory capabilities typical of troglobites[4]. Limited food availability is also thought to constrain the body size of fish able to thrive in cave environments[5]. However, given the species' restricted habitat range, it is highly susceptible to extinction risks. As these habitats are particularly vulnerable to various anthropogenic activities[6], there is an urgent need to study the hidden diversity of subterranean species. Safeguarding its unique habitat and implementing conservation measures are crucial to ensure the long-term survival of this world's largest cave fish species.

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In the present study, the mitogenome of *N. pnar* was successfully assembled and its phylogenetic relationships with other *Neolissochilus* and *Tor* species were investigated. Additionally, the presence of selection pressure on the mitochondrial genes was studied to understand the possible evolutionary basis for cave adaptation of the species.

Materials and methods

Specimen collection

Two specimens were collected from Krem Umladaw in East Jaintia Hills district of Meghalaya State, India and were sedated with clove oil[7] and tissue (fin) samples were collected and preserved in 95% ethanol and stored at 4 °C for further applications.

Specimens collection from Krem Umladaw in East Jaintia Hills district of Meghalaya State, India, with permission from Meghalaya Biodiversity Board to access bio-resources (Permit no: SBB.19/ABS/3030 dated 16.01.2017). Specimen collection, care and experimental animal use protocols were approved by the Institutional Ethical Committee of Gauhati University, Guwahati, India (Permit reference number: IAEC/KS/2022/PhD-IAEC/2022-10/15). No living specimens were allowed to expose with any other harmful chemicals. In addition to the ARRIVE guidelines, all the methods were performed in accordance with the relevant guidelines and regulations.

DNA extraction and COI sequencing

Total genomic DNA was extracted, following the modified phenol–chloroform isoamyl alcohol method[8], followed by purification with AMPure PB beads (Beckman Coulter, CA). DNA integrity was evaluated via 0.8% agarose gel electrophoresis and concentration; purity was assessed using a NanoDrop 2000 (NanoDrop Technologies, USA) and Qubit 4.0 fluorometer (Thermo Fisher Scientific, USA). For species validation, the partial sequence of the mitochondrial Cytochrome C oxidase subunit I (COI) gene was amplified using primers: FishF1-5'TCAACCAACCACAAAGACATTGGCAC3', FishR1-5' TAGACTTCTGGGTGGCCAAAG AATCA 3'[9]. The PCR products were sequenced using ABI 3730 capillary sequencer (Thermo Fisher Scientific, USA), following the manufacturer's instructions. The COI sequences were submitted to NCBI (Accession No. OP480824-OP480825) and blasted against the NCBI nucleotide database for validation of species.

Mitogenome sequencing

For long-read sequencing library preparation, genomic DNA from one specimen was used. The 20 kb genomic DNA fragment size was selected using BluePippinTM (Sage Science, Beverly, USA) and the library was prepared using SMRTbell® Express Template Prep Kit 2.0 (Pacific Biosciences, USA). The library was sequenced on the PacBio Sequel system (Pacific Biosciences, USA) and assembly was carried out through Wtdbg2.5[10]. The contigs with full mitochondrial genome fragments were obtained and annotated using MitoFish and MitoAnnotator[11]. And the ORFs of the protein coding genes were confirmed using ExPASy[12]. The complete mitochondrial genome was submitted to NCBI with Accession No. OR766867.

Data processing and phylogenetic analysis

To investigate the phylogenetic position and relationship among the mahseer species, the complete mitogenome sequences of 19 species of mahseer (family Cyprinidae) were collected from NCBI database (Table 1). Analysis of concatenated nucleotide sequences of thirteen protein-coding genes (PCGs), from 17 species was carried out, with *Labeo rohita* and *Cyprinus carpio* as outgroups, utilizing two tree models, MrBayes bayesian inference and ASAP (Kimura (K80) ts/tv) Tools. A rooted phylogenetic tree was constructed using the MrBayes 3.2.6 [http://www.phylogeny.fr/;[13]], using number of 10,000 generations and ample a tree every 10 generations; and FigTree v1.4.4[14] to visualize the resulting evolutionary phylogenetic trees. The genetic distances across all mahseer were calculated with software MEGA 11[15]. To gain insights into the codon bias among the mahseer mitogenomes, the relative synonymous codon usage (RSCU) analysis was done using MEGA 11. ASAP (Assemble Species by Automatic Partitioning) (https://bioinfo.mnhn.fr/abi/public/asap/#)[16] was employed for species delimitation, based on the K80 substitution model. In ASAP analysis, genetic distances are used to identify the transition between intraspecific variation and interspecific divergence, including scoring to identify the best-fitting set of partitions for candidate species.

Selection pressure analysis

A total of thirteen datasets for each gene was aligned and concatenated with the Clustal X algorithm of BioEdit[17]. DATAMONKEY (Adaptive Evolution server)[18] was used to infer the sites/codon undergoing purifying and diversifying selection, various methods followed were: Fixed Effects Likelihood (FEL)[19]; Fast, Unconstrained Bayesian AppRoximation for Inferring Selection (FUBAR)[20] and Mixed Effects Model of Evolution (MEME)[21]. The threshold p-value < 0.1 and the posterior probability value > 0.95 were used for FEL and FUBAR and MEME, methods, respectively. The three-dimensional protein structure of Gene under positive selection were predicted using SWISS-MODEL[22].

Results

Partial COI sequences of *N. pnar* were submitted to NCBI with accession Numbers OP480824-OP480825, and blastn through NCBI revealed similarity with that of *N. pnar* (OQ351360-OQ351362). Through long-read sequencing, a total of 250.687 (148.77X) Gb (Estimated Genome Size for the species: 1.685 Gb) of polymerase read bases were generated with 75,795, mean read length and mean insert length of 18.567 kb and a complete mitochondrial genome of a contig length of 16,440 bp was obtained. Our analysis confirmed that the mitogenome

S. No.	Species	NCBI Accession No.
1	*Neolissochilus pnar* (present study)	OR766867
2	*Neolissochilus stracheyi*	AP011252
3	*Tor putitora*	AP011326
4	*Tor tambroides*	JX444718
5	*Tor sinensis*	KF305826
6	*Neolissochilus hexagonolepis*	KM668070
7	*Tor tor*	KR868704
8	*Tor khudree*	KR868706
9	*Cyprinus carpio*	KU146529
10	*Tor mosal*	KU870466
11	*Tor malabaricus*	MG397041
12	*Labeo rohita*	MW557325
13	*Neolissochilus heterostomus*	MW762597
14	*Tor tambra*	NC036511
15	*Tor douronensis*	NC036512
16	*Neolissochilus benasi*	NC053732
17	*Tor barakae*	NC056296
18	*Neolissochilus hexastichus*	NC056297
19	*Neolissochilus soroides*	OM203154

Table 1. List of mitogenomes of 17 mahseer species and two outgroups of family Cyprinidae analyzed in this study.

of *N. pnar* displays a conserved gene arrangement across 37 mitochondrial genes, and these include 13 PCGs, 22 transfer RNA (tRNA) genes, 2 ribosomal RNA (rRNA) genes (Fig. 1). Eight tRNAs and ND6 gene are encoded on the L-strand (Fig. 1 and Table 2). There was a cumulative length of 11,404 bp for thirteen PCGs of *N. pnar* mitochondrial genes, encoding 3594 amino acids. The PCGs range from 164 to 11,404 bp among the mahseer mitochondrial genomes (Table 3), which include 7 NDH dehydrogenases (ND1-ND6 and ND4L), 3 cytochrome c oxidases (COI-COIII), 2 ATPases (ATP6 and ATP8) and 1 cytochrome b (cytb). The shortest PCG is 164 bp (ATP8) and longest PCG 1823 bp (ND5) (Table 3). Mahseer mitogenomes revealed notable bias of AT content, ranging from 56.5% (*N. pnar, N. heterostomus, and N. soroides*) to 57.2% (*Tor tambroides*) (Tables 4 and 5). AT skew are consistently low across mitogenomes, ranging from 0.1153 (*T. tambroides*) to 0.1272 (*T. douronensis*). The *N. pnar* mitogenome shows an AT skew of 0.1185. Additionally, the thirteen protein-coding genes exhibit AT skews 0.085 (*N. hexastichus*) to 0.1 (*N. soroides*).

In mitogenome of *N. pnar*, the size of 16S rRNA and 12S rRNA genes are of 2708 bp, which reside between trnL2 and trnF, separated by trnV. The set of 22 tRNAs was identified, ranges 66 (trnC) to 76 bp (trnL2), with H-strand encoding 14 tRNAs and the remaining on the L-strand. Total lengths of the 22 tRNA genes among the 39 Mahseer species range varies from 1562 to 1565 bp (Table 2).

The *N. pnar* mitogenome PCGs predominantly initiate with ATG, except for COX1, which commences with GTG (Table 2). Most PCGs terminate with stop codon TAR (TAA/TAG) and an incomplete codon (TA_, T__). The RSCU analysis revealed that out of the 3797 codons of mitogenome PCGs (Fig. 2a), the most predominant codon families are of leucine, followed by sxerine and exhibit the highest usage bias (Fig. 2b). The most commonly used codon across these species is consistently CUA (RSCU:2.02).

The present analysis of mitochondrial genes in Mahseer fish reveals a complex pattern of selection, with a combination of purifying selection and positive/episodic positive or diversifying selection. Evidence of episodic positive or diversifying selection at 16 sites was obtained in MEME ($p < 0.1$). FUBAR inferred 7 sites subject to diversifying positive selection with posterior probability > 0.9. FEL found evidence of pervasive positive or diversifying selection at 1 sites across. Among the 24 codon identified by all three methods, 2, 1, 3, 2,2, 4, 9, 1 codons were located in COII, COII, Cytb, ND2, ND4, ND5 and ND6, respectively. Unconstrained Bayesian AppRoximation for Inferring Selection (FUBAR) detected positive selection in 4 genes, ie., COII (1 codon), Cytb (3 Codon), ND4 (1 codon)and ND5 (2 codon). Furthermore Mixed Effects Model of Evolution (MEME) detected episodic positive/diversifying selection 7 genes ie., COII (1 codon), COIII(1 codon), ND (2 codon)1, ND2 (2 codon) and ND4 (3 codon), ND5 (6 codon), and ND6 (1 codon). The ND5 gene showed sites under strong positive/diversifying selection, among all mitochondrial genes examined, with all the three analysis. The ND5 codon position 563 coding with Amino acid substitution (isoleucine's , Methionine , Alanine and Valine) in mahseer ND5 genes shows consistency FUBAR, FEL and MEME analysis enhances the confidence in the identification of positive selection on these genes (Table 6). Additionally ND4 Gene codon position 51 with Amino acid substitution (Threonine, Alanine and Valine) in mahseer ND5 gene shows consistency and evidence for positive selection with FUBAR, and MEME (Fig. 3a,b). The three-dimensional protein structure of ND4 and ND5 were predicted. The 3D structure structure of ND4 genes with transmembrane segment shows the codon position 51 is outside the membrane, where as the ND5 codon postion 563 is locateted in transmembrane helices (Fig. 4a,b).

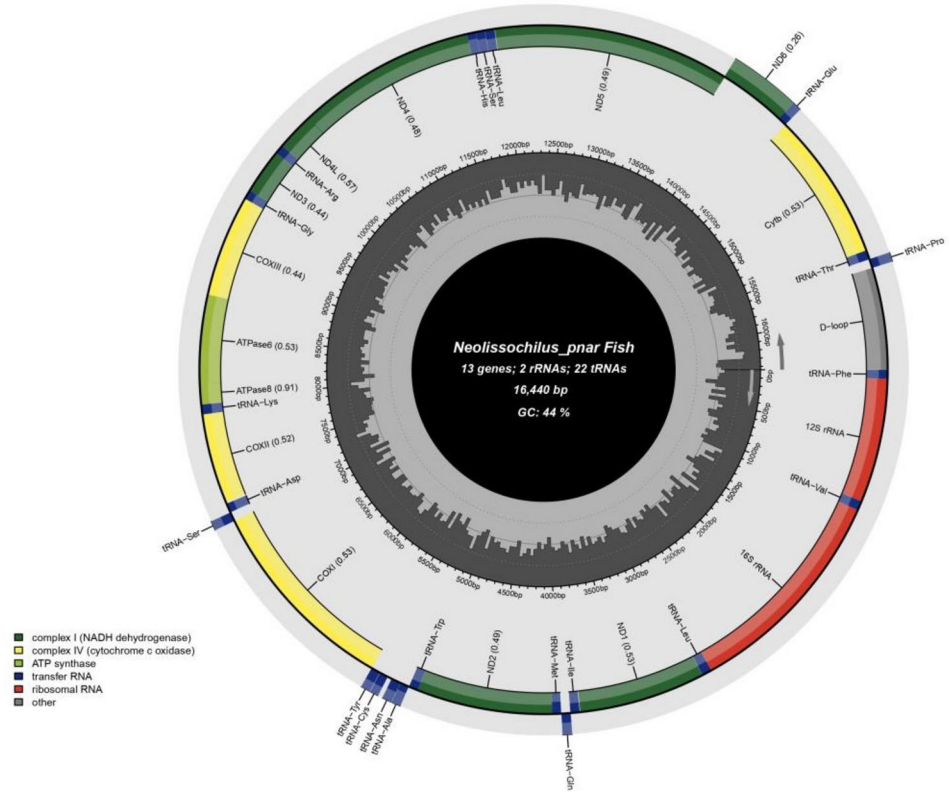


Fig. 1. The organization of the mitochondrial genome of *Neolissochilus pnar*, the largest cavernicolous species of Mahseer.

The pairwise genetic distance analysis (K2P) of *N. pnar* PCGs with all other mahseer species showed a range of 0.011 (*N. hexasticus*) to 0.69 (*Tor douronensis*) (Table 7). To decipher the relationship of *N. pnar* and members of Mahseer family, the phylogenetic tree analysis of 17 Mahseer species based on the mitochondrial genes set showed robust support to the topologies, with all major clades (Figs. 5 and 6). Additionally, the ASAP analysis also could differentiate all the species in the study with significant ASAP scores 2.5 (Fig. 6). It establishes well-supported clades with other documented mahseer species (BP = 1). The target species *N. pnar*, in conjunction with other *Neolissochuilus* species, led to their clustering into a single cluster, supported by robust clades and nodes.

Discussion

The present study successfully assembled the mitogenome of *N. pnar* and studied its phylogenetic relationships with other *Neolissochilus* and *Tor* species. Additionally, the presence of selection pressure on the mitochondrial genes was studied, to understand the cave adaptation of the species. The partial COI sequence established the fish species under study as *N. pnar* and the results of analysis of all thirteen PCGs unequivocally reaffirm the classification of *N. pnar* within the Mahseer family in line with previous findings[3].

The arrangement of protein coding sequences as well as tRNAs and rRNAs were found to be in par with the typical teleost mitochondrial genome structure observed[23]. The pattern of biasness of AT content in Mahseer as well as the *N. pnar* mitogenome showing an AT skewness are also in resemblance with teleost[24,25].

In *N. pnar*, 16S rRNA and 12S rRNA genes showcase a high degree of conservation among mahseer mitogenomes, as compared to other published fish mitogenomes[26–28]. The incomplete stop codon in protein coding genes is frequently observed in Mahseer mitogenomes and other fish species[3], likely due to post-transcriptional modifications during mRNA maturation. Results also suggested biased synonymous codon usage for most amino acids, that are conserved across the Mahseer species, likely due to their close familial relationships and possible environmental selection pressure on mitochondrial genes during their adaptation, for enhanced efficiency of translational machinery[29].

The construction of phylogenetic trees using MrBayes bayesian inference and ASAP (Kimura (K80) ts/tv) models, confirmed the *N. pnar* differentiation from other species of Mahseer family. The phylogenetic tree depicts *N. pnar* and *N. hexasticus* to be the nearest species, supported by strong nodal values (1). This unequivocally reaffirms the classification of *N. pnar*, consistent with Dahanukar et al.[3]. Dahanukar et al.[3] based on partial COI sequence reported that *N. pnar* is genetically and morphologically distinct from its closest relatives, *N. hexastichus* (genetic distance: 1.1–2.7% based on COI gene) and *N. hexagonolepis* (genetic distance: 2.1–2.6%). Based on the 13 PCGs incurrent study, *N. pnar* showed genetic distances of 1.1% with *N. hexastichus*

Gene	Strand	Position		Length (bp)	Start codons	Stop codons	Anticodon
		From	To				
trnF	H	1	69	68			GAA
rrnS	H	70	1026	956			
trnV	H	1027	1098	71			TAC
rrnL	H	1099	2777	1678			
trnL2	H	2778	2853	75			TAA
nd1	H	2855	3829	974	ATG	TAA	
trnI	H	3834	3905	71			GAT
trnQ	L	3974	3904	70			TTG
trnM	H	3976	4044	68			CGT
nd2	H	4045	5089	1044	ATG	T	
trnW	H	5090	5161	71			TCA
trnA	L	5164	5232	68			TGC
trnN	L	5234	5306	72			GTT
trnC	L	5340	5405	65			GCA
trnY	L	5408	5478	70			GTA
cox1	H	5480	7030	1550	GTG	TAA	
trnS2	L	7031	7101	70			TGA
trnD	H	7103	7174	71			GTC
cox2	H	7188	7878	690	ATG	T	
trnK	H	7879	7954	75			TTT
atpase8	H	7956	8120	164	ATG	TAG	
atpase6	H	8114	8796	682	ATG	TA	
cox3	H	8797	9580	783	ATG	T	
trnG	H	9581	9652	71			TCC
nd3	H	9653	10001	348	ATG	T	
trnR	H	10002	10071	69			TCG
nd4l	H	10072	10368	296	ATG	TAA	
nd4	H	10362	11742	1380	ATG	T	
trnH	H	11743	11811	68			GTG
trnS1	H	11812	11880	68			GCT
trnL1	H	11882	11954	72			TAG
nd5	H	11958	13781	1823	ATG	TAA	
nd6	L	13778	14299	521	ATG	TAA	
trnE	L	14300	14368	68			TTC
cob	H	14373	15513	1140	ATG	T	
trnT	H	15514	15585	71			TGT
trnP	L	15585	15654	69			TGG
Control region		15655	16440	785			

Table 2. Gene annotations of the complete mitogenome of *Neolissochilus pnar* (present study).

and 3.5% with *N. hexagonolepis*, Thus, re-confirming the taxonomic position of *N. pnar* based on the 13 PCGs of mitochondrial genome.

The mahseer populations in present analysis reveal a complex pattern of selection pressure exerted on mitogenomes, which included a combination of purifying selection and episodic positive/diversifying selection. In the present study, observation of episodic (series of events) positive or diversifying selection at ND2,ND4 ND5 and ND6 genes may suggest a response to the local adaptation to the cave environment and indicate the presence of adaptive evolution in mahseer species[30,31]. The NADH dehydrogenase complex (complex I) is the first and largest multimeric enzyme among the five complexes that make up the oxidative phosphorylation pathway[32]. This complex is responsible for transferring electrons from NADH to quinone, producing quinol while simultaneously translocating four protons (H^+) across the inner mitochondrial membrane. As the subunits ND2, ND4 and ND5, members of in oxidative phosphorylation (OXPHOS) complex I, function directly as proton pumps for H^+ ions, variations observed in their amino acid sequences may confer adaptive advantages. Numerous studies have found that sites of positive selection are notably concentrated in OXPHOS complex I across various fish species[33–36], which may be linked to protein function[37,38], given that this complex accounts for approximately forty percent of the proton pumping necessary for ATP synthesis. Similar polymorphisms have also been observed in other groups, including hares[39], mammals[37] and birds[38,40]. Additionally, it has been reported that mutations or dysregulation in the mitochondrial-ND genes can lead to dysfunction of complex I

Species	Accession No.	Size of mito genome (bp)	PCGs (bp)	Ribosomal RNA (12S + 16S) (bp)	tRNAs (bp)	Control region (bp)
Neolissochilus pnar	OR766867	16440	11404	2708	1563	784
Neolissochilus stracheyi	AP011252	16584	11397	2708	1564	928
Tor putitora	AP011326	16570	11397	2703	1563	925
Tor tambroides	JX444718	16690	11397	2708	1562	922
Tor sinensis	KF305826	16579	11398	2707	1564	921
Neolissochilus hexagonolepis	KM668070	16563	11397	2704	1563	911
Tor tor	KR868704	16571	11397	2705	1563	923
Tor khudree	KR868706	16573	11397	2703	1563	917
Cyprinus carpio	KU146529	16581	11398	2706	1563	840
Tor mosal	KU870466	16572	11397	2703	1564	926
Tor malabaricus	MG397041	16580	11397	2703	1563	930
Labeo rohita	MW557325	16609	11399	2717	1564	937
Neolissochilus heterostomus	MW762597	16585	11397	2708	1563	813
Tor tambra	NC 036511	16581	11397	2708	1562	926
Tor douronensis	NC 036512	16586	11397	2711	1565	925
Neolissochilus benasi	NC 053732	16583	11397	2708	1563	925
Tor barakae	NC 056296	16780	11397	2705	1564	1126
Neolissochilus hexastichus	NC 056297	16538	11397	2707	1563	883
Neolissochilus soroides	OM203154	16584	11397	2707	1563	815

Table 3. Characteristics of complete mitogenomes of 17 mahseer species and two outgroup species of family Cyprinidae analyzed in this study.

Accession No.	Species	T(U)	C	A	G	Total	A + T	G + C	AT_skewed
OR766867	*Neolissochilus pnar*	24.9	27.6	31.6	15.9	16440	56.5	43.5	0.118584
NC56297.1	*Neolissochilus hexastichus*	25	27.5	31.6	15.9	16538	56.6	43.4	0.116608
KM668070.1	*Neolissochilus hexagonolepis*	25	27.4	31.8	15.8	16563	56.8	43.2	0.119718
MW762597.1	*Neolissochilus heterostomus*	24.8	27.7	31.7	15.8	16585	56.5	43.5	0.122124
AP011252.1	*Neolissochilus stracheyi*	24.8	27.7	31.8	15.7	16584	56.6	43.4	0.123675
NC56296.1	*Tor barakae*	25.1	27.3	31.9	15.7	16780	57	43	0.119298
KU870466.1	*Tor mosal*	25	27.5	31.9	15.6	16572	56.9	43.1	0.121265
AP011326.1	*Tor putitora*	25	27.5	31.9	15.6	16570	56.9	43.1	0.121265
KR868704.1	*Tor tor*	25.1	27.4	31.9	15.6	16571	57	43	0.119298
KR868706.1	*Tor khudree*	24.8	27.7	31.9	15.6	16573	56.7	43.3	0.12522
MG397041.1	*Tor malabaricus*	24.9	27.6	31.9	15.7	16580	56.8	43.3	0.123239
NC53732.1	*Neolissochilus benasi*	25	27.4	31.8	15.7	16583	56.8	43.1	0.119718
NC36511.1	*Tor tambra*	25.1	27.4	31.9	15.6	16581	57	43	0.119298
JX444718.1	*Tor tambroides*	25.3	27.3	31.9	15.6	16690	57.2	42.9	0.115385
KF305826.1	*Tor sinensis*	24.9	27.4	31.9	15.7	16579	56.8	43.1	0.123239
NC36512.1	*Tor douronensis*	24.7	27.7	31.9	15.7	16586	56.6	43.4	0.127208
KU146529.1	*Cyprinus carpio*	24.8	27.5	31.9	15.8	16581	56.7	43.3	0.12522
MW557325.1	*Labeo rohita*	24.3	27.8	32.6	15.2	16609	56.9	43	0.14587
OM203154.1	*Neolissochilus soroides*	24.7	27.8	31.8	15.8	16584	56.5	43.6	0.125664
Avg		24.9	27.5	31.9	15.7	16586.8	56.8	43.2	0.123239

Table 4. Base composition of complete mitogenomes of 17 mahseer species and 2 outgroup of family Cyprinidae analyzed in this study.

and impaired mitochondrial respiration. Such mutations are associated with a variety of mitochondrial disorders and diseases, such as Leber hereditary optic neuropathy (LHON), a rare mitochondrial disorder in human, which causes visual loss due to optic nerve degeneration[41]. In mammals, ND4 together with ND2 and ND5 are considered to be the actual proton pumps in complex 1. Thus, variation in these subunits may affect the efficiency of the proton-pumping process, might be through chemical changes that may alter proton translocation[37].

Cytochrome b, a component of respiratory complex III in the mitochondrial respiratory chain, facilitates electron transfer from ubihydroquinone to cytochrome c, generating an electrochemical gradient. Mutations at specific amino acid positions can affect cytochrome b's structure and function, altering its coupling efficiency.

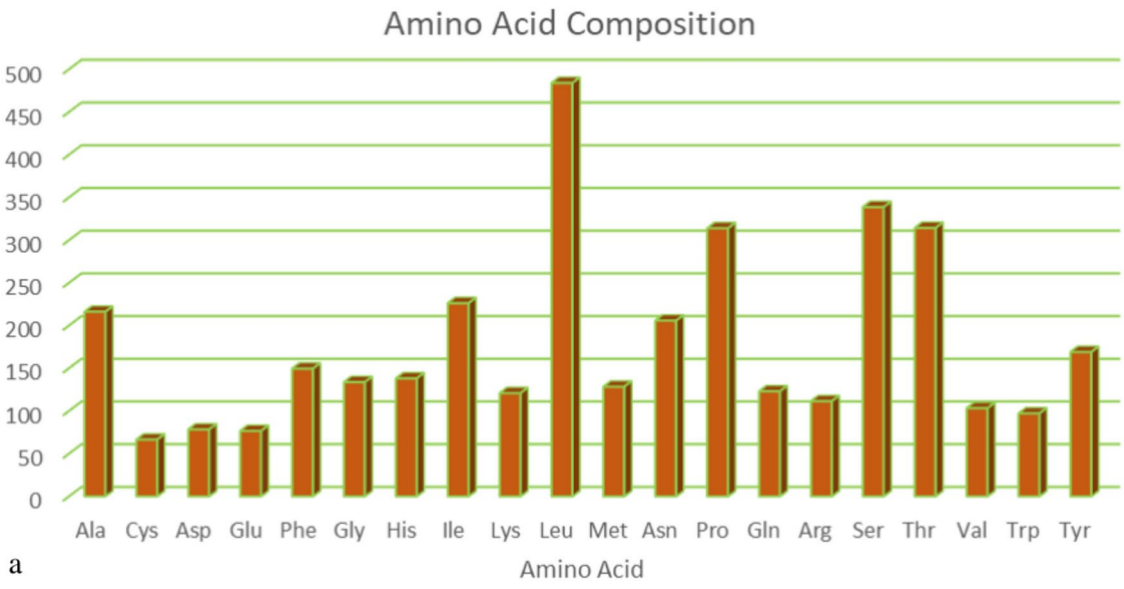
Accession No.	Species	T(U)	C	A	G	Total	A + T	G + C	AT_skewed
OR766867	*Neolissochilus pnar*	25.7	29.1	30.7	14.5	11404	56.4	43.6	0.0887
NC56297.1	*Neolissochilus hexastichus*	25.8	29	30.6	14.6	11397	56.4	43.6	0.0851
KM668070.1	*Neolissochilus hexagonolepis*	25.8	28.9	30.9	14.5	11397	56.7	43.4	0.0899
MW762597.1	*Neolissochilus heterostomus*	25.4	29.3	30.7	14.6	11397	56.1	43.9	0.0945
AP011252.1	*Neolissochilus stracheyi*	25.4	29.3	30.9	14.5	11397	56.3	43.8	0.0977
NC56296.1	*Tor barakae*	25.7	28.9	30.9	14.4	11397	56.6	43.3	0.0919
KU870466.1	*Tor mosal*	25.7	29	31.1	14.3	11397	56.8	43.3	0.0951
AP011326.1	*Tor putitora*	25.6	29.1	31.1	14.2	11397	56.7	43.3	0.0970
KR868704.1	*Tor tor*	25.7	28.9	31	14.3	11397	56.7	43.2	0.0935
KR868706.1	*Tor khudree*	25.4	29.2	31	14.4	11397	56.4	43.6	0.0993
MG397041.1	*Tor malabaricus*	25.6	29.1	30.9	14.4	11397	56.5	43.5	0.0938
NC53732.1	*Neolissochilus benasi*	25.6	29	30.9	14.5	11397	56.5	43.5	0.0938
OM203154.1	*Neolissochilus soroides*	25.2	29.5	30.8	14.6	11397	56	44.1	0.1000
NC36511.1	*Tor tambra*	25.9	28.9	31	14.3	11397	56.9	43.2	0.0896
JX444718.1	*Tor tambroides*	25.8	28.9	31	14.3	11397	56.8	43.2	0.0915
KF305826.1	*Tor sinensis*	25.5	29.1	31	14.3	11398	56.5	43.4	0.0973
NC36512.1	*Tor douronensis*	25.3	29.3	30.9	14.5	11397	56.2	43.8	0.0996
KU146529.1	*Cyprinus carpio*	25.4	29.2	31.1	14.3	11398	56.5	43.5	0.1009
MW557325.1	*Labeo rohita*	24.8	29.6	32	13.7	11399	56.8	43.3	0.1268
Avg		25.5	29.1	31	14.4	11397.6	56.5	43.5	0.0973

Table 5. Base composition of 13 protein coding genes of mitogenomes of 17 mahseer species and 2 outgroups of family Cyprinidae analyzed in this study.

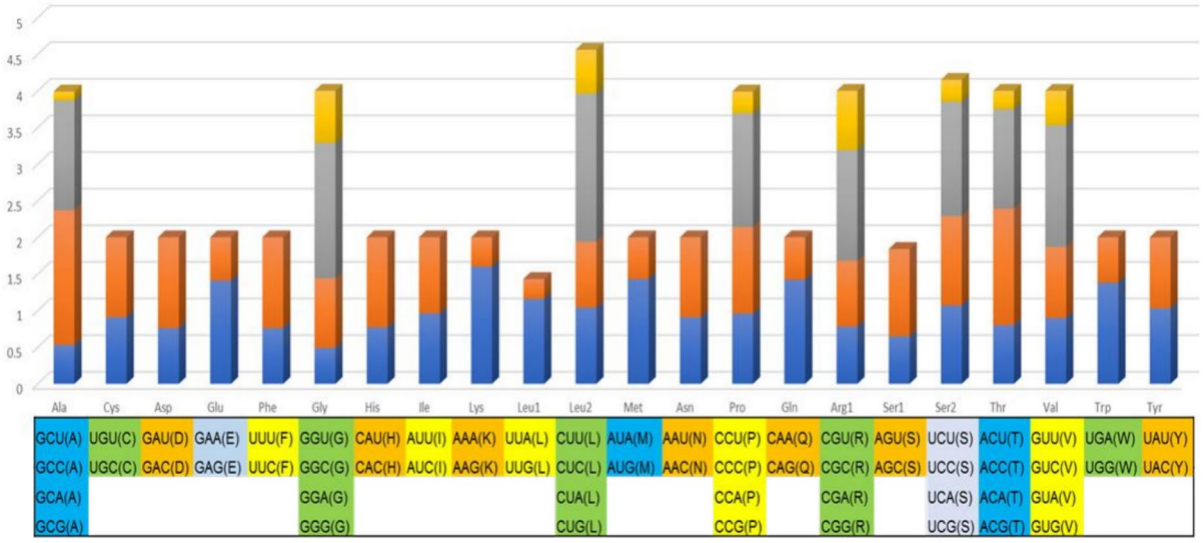
In humans, mutations enhancing water binding at the Qi site are linked to increased longevity[42], while in yeast, mutations at the Qo site are associated with reduced catalytic efficiency and increased oxygen radical production[43]. This suggests that substitutions at Cyt *b* binding sites may have functional significance warranting further study.

Cytochrome c oxidase (complex IV) catalyzes the final step of the electron transfer chain, receiving electrons from four cytochrome c molecules and transferring them to oxygen, producing water and translocating protons across the membrane[44]. Key amino acid residues involved in electron transfer and proton pathways are highly conserved, indicating functional constraints. The observation of diversifying positive selection in COII, COIII and Cytb Supports adaptive evolution in the mitochondrial genes of mahseer species, highlighting natural selection as a driving force for diversification in the oxidative phosphorylation (OXPHOS) system[45].

The findings from our analysis highlight the dynamic nature of mitochondrial evolution in mahseer. While purifying selection acts to preserve essential mitochondrial functions, specific sites in ND2 ND4, ND5 and ND6 genes undergo episodic positive or diversifying selection. Overall, the selection in genes of OXPHOS Complexes (Complex I: NADH dehydrogenase ND2 ND4, ND5 and ND6), Complex III: Cytochrome b (Cyt b) and Complex IV: Cytochrome C Oxidase COII, COIII)) coinciding with wide divergence of geographical and climatic condition of different species of mahseer is likely in response to environmental changes or selective pressures, result adaptive evolution in blind fish populations. These results can add to our understanding of the molecular mechanisms underlying adaptation in extreme environments and emphasize the importance of mitochondrial genetic diversity in facilitating survival and adaptation in Mahseer fish populations. Further research is warranted to elucidate the functional significance of sites under selection and their implications for the biology and ecology of blind fish.



a

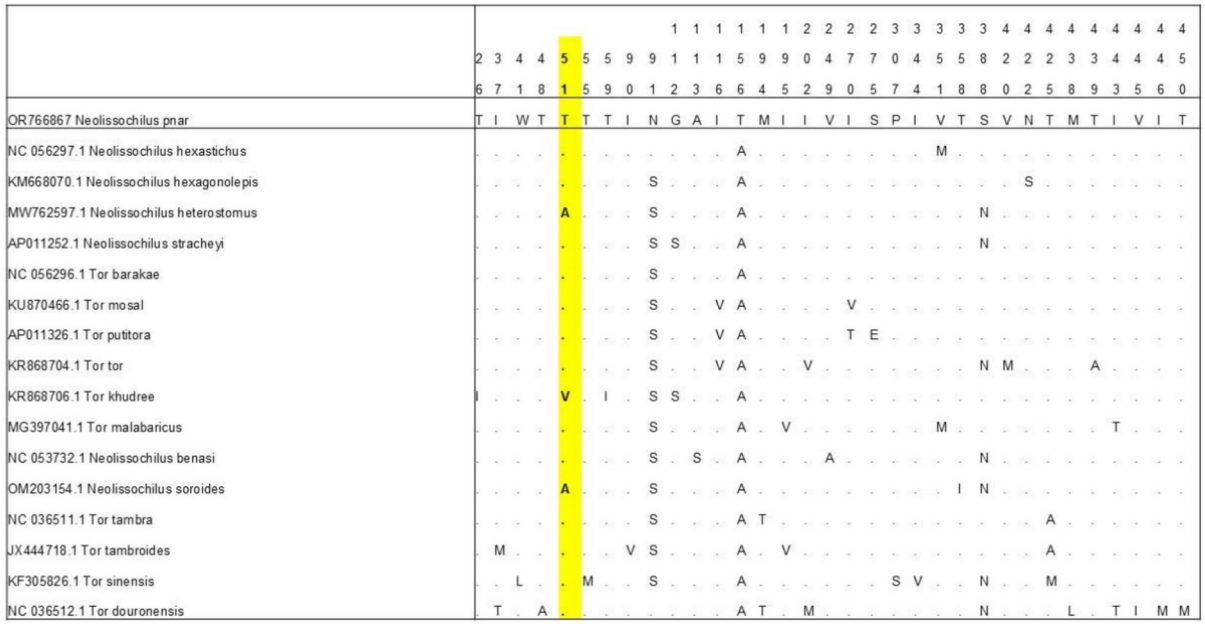


b

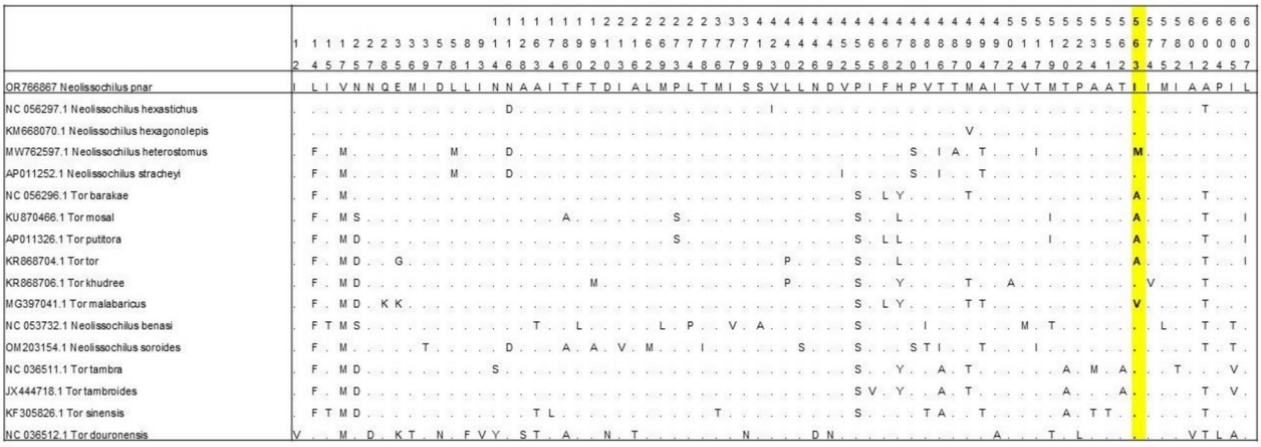
Fig. 2. (**a**) Amino acid composition of protein coding genes of *Neolissochilus pnar* mitogenome. (**b**) Codon usage bias/Alternate codon usage in mitogenome of *Neolissochilus pnar*.

Genes	Sequences in the alignment	Codon sites in the alignment	Median branches/ partition used for testing	Non-invariant sites tested	FUBAR Evidence, positive/diversifying selection at 0.9 posterior probability	Codon position of positive/ diversifying selection	FUBAR Evidence, purifying	FEL evidenc, sites under diversifying positive selection selection at $p \leq 0.1$ (p-value)	Codon position of positive/ diversifying selection	FEL evidence, sites under purifying selection at $p \leq 0.01$(p-value)	MEMEevidence of episodic positive/ diversifying selection at $p \leq 0.1$ (p-value)	Codon position of positive/ diversifying selection
ATPASE6	17	227	29	134	0		92	0		71	0	
ATPASE8	15	54	24	26	0		7	0		9	0	
COI	17	516	31	241	0		221	0		182	0	
COII	17	230	29	102	1	214	51	0		61	1	146
COIII	17	261	29	110	0		80	0		86	1	168
Cytb	17	380	31	216	3	14, 333,356	172	0		158	0	
ND1	17	324	31	228	0		187	0		175	2	55, 275
ND2	17	348	30	187	0		123	0		119	2	156, 246
ND3	16	116	27	59	0		40	0		34	0	
ND4	17	460	31	271	1	51	222	0		191	3	51, 270,275
ND4L	16	98	26	44	0		28	0		28	0	
ND5	17	607	31	344	2	25, 563	270	1	563	226	6	58,468,497,534, 563, 607
ND6	17	173	30	57	0		55	0		57	1	92

Table 6. Analysis of mitochondrial genes showing positive/diversifying selection in mitochondrial genes in Mahseer mitogenome.



a



b

Fig. 3. Codon position and Amino acid at site of Evidence, positive/diversifying selection in (**a**) ND4 and (**b**) ND5 Genes.

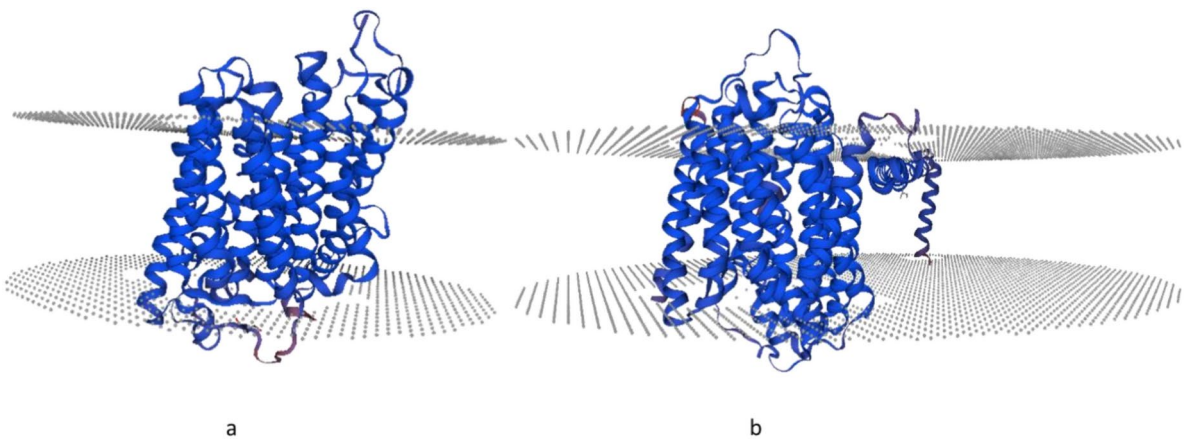


Fig. 4. The predicted three-dimensional protein structure (**a**) ND 4 gene, (**b**) ND 5 gene.

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	*Neolissochilus pnar*	–	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.002	0.0020	0.002	0.003	0.003	0.004	0.004
2	*N. hexastichus*	0.011	–	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.003	0.003	0.002	0.0020	0.002	0.003	0.003	0.004	0.004
3	*N.hexagonolepis*	0.035	0.037	–	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.0020	0.002	0.003	0.003	0.004	0.005
4	*N. heterostomus*	0.052	0.051	0.054	–	0.001	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.0202	0.002	0.003	0.003	0.004	0.004
5	*N. stracheyi*	0.051	0.051	0.053	0.013	–	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.002	0.002	0.003	0.003	0.004	0.004
6	*Tor barakae*	0.058	0.060	0.055	0.058	0.057	–	0.002	0.002	0.002	0.002	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.004	0.004
7	*Tor mosal*	0.060	0.062	0.058	0.058	0.057	0.039	–	0.001	0.001	0.002	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.004	0.004
8	*Tor putitora*	0.061	0.063	0.058	0.057	0.057	0.040	0.008	–	0.001	0.002	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.004	0.004
9	*Tortor*	0.061	0.063	0.060	0.059	0.058	0.041	0.021	0.021	–	0.002	0.002	0.003	0.002	0.002	0.002	0.002	0.002	0.004	0.004
10	*Tor khudree*	0.062	0.064	0.061	0.062	0.061	0.035	0.047	0.046	0.048	–	0.002	0.003	0.002	0.002	0.002	0.002	0.003	0.004	0.005
11	*Tor malabaricus*	0.062	0.063	0.060	0.060	0.060	0.033	0.045	0.044	0.047	0.038	–	0.003	0.003	0.002	0.002	0.002	0.003	0.004	0.004
12	*N. benasi*	0.069	0.070	0.071	0.069	0.067	0.067	0.066	0.066	0.065	0.070	0.068	–	0.003	0.003	0.003	0.003	0.003	0.004	0.004
13	*N. soroides*	0.058	0.057	0.059	0.030	0.030	0.063	0.062	0.062	0.063	0.067	0.066	0.072	–	0.002	0.002	0.003	0.003	0.004	0.004
14	*Tor tambra*	0.058	0.061	0.058	0.058	0.058	0.029	0.042	0.042	0.043	0.036	0.037	0.066	0.063	–	0.001	0.002	0.002	0.004	0.004
15	*Tor tambroides*	0.059	0.061	0.057	0.058	0.058	0.030	0.043	0.043	0.043	0.038	0.038	0.066	0.062	0.017	–	0.002	0.002	0.004	0.004
16	*Tor sinensis*	0.068	0.070	0.069	0.067	0.066	0.052	0.051	0.051	0.055	0.056	0.056	0.074	0.070	0.049	0.051	–	0.002	0.004	0.005
17	*Tor douronensis*	0.070	0.070	0.071	0.069	0.069	0.056	0.055	0.056	0.057	0.063	0.059	0.078	0.072	0.057	0.058	0.064	–	0.004	0.004
18	*Cyprinus carpio*	0.156	0.157	0.156	0.154	0.154	0.154	0.150	0.151	0.150	0.154	0.156	0.154	0.154	0.154	0.153	0.152	0.159	–	0.004
19	*Labeo rohita*	0.154	0.155	0.155	0.155	0.154	0.153	0.150	0.150	0.152	0.155	0.156	0.156	0.155	0.154	0.152	0.152	0.157	0.143	–

Table 7. K2P genetic distance based on 13 protein coding genes of *Neolissochilus pnar*, and other mahseer species and 2 outgroup species of family Cyprinidae. Below diagonal: genetic distance; above diagonal: *p* value.

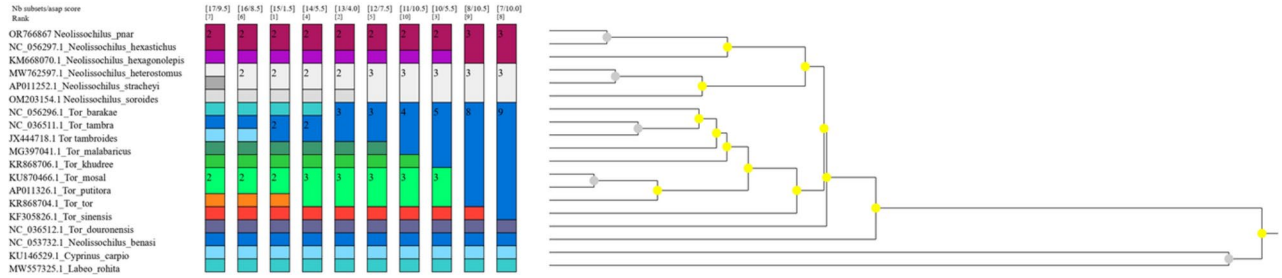


Fig. 5. Phylogenetic trees derived from ASAP approaches based on the 13 protein coding genes of mitochondrial genomes, along with that of *Neolissochilus pnar*.

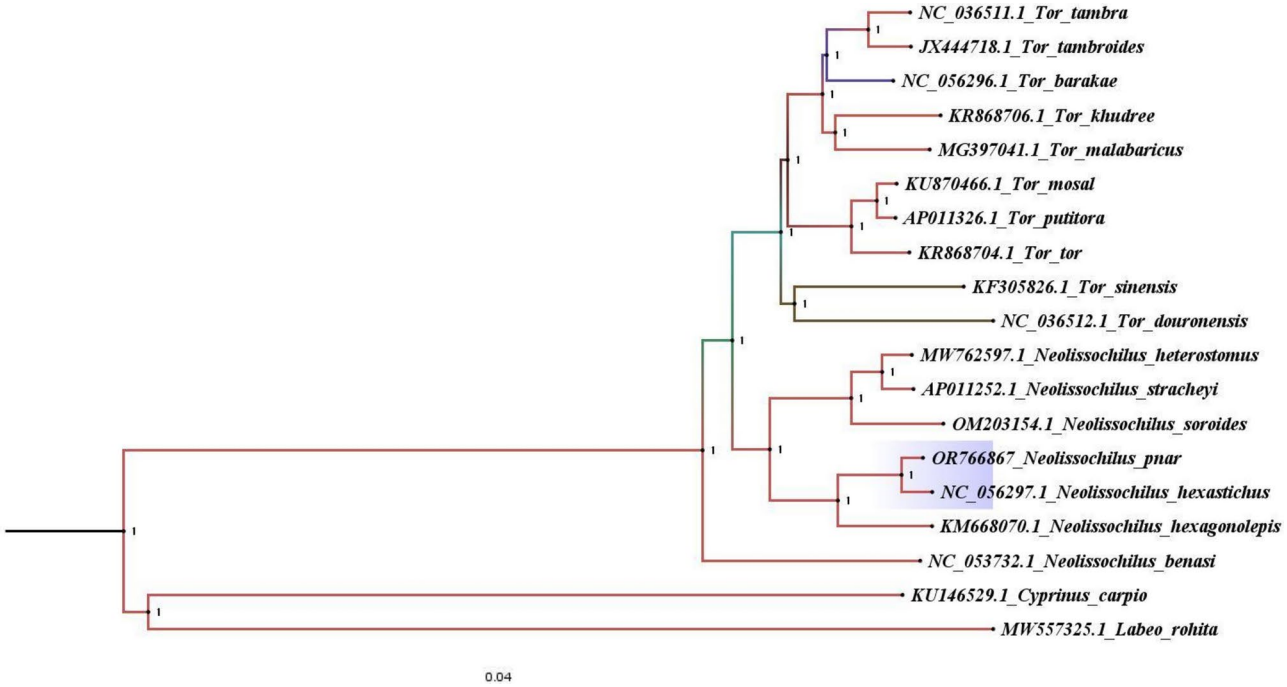


Fig. 6. Phylogenetic trees derived from MrBayes approaches based on the 13 protein coding genes of mitochondrial genomes, along with that of *Neolissochilus pnar*.

Data availability

The Mitogenome sequence is submitted to NCBI with Accession No. OR766867 and partial COI sequences OP480824-OP480825.

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Author contributions

JKJ, VM and LMC designed and conceptualized the project. DKBM, KS, DPW, DS collected the specimens and tissue samples. LMC and VM generated the data; LMC, DS and VM analysed the data, LMC and VM wrote the manuscript. All authors read and approved the final manuscript.

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Declarations

Competing interests
The authors declare no competing interests.

Ethical approval
Specimens collection from Krem Umladaw in East Jaintia Hills district of Meghalaya State, India, with permission from Meghalaya Biodiversity Board to access bio-resources (Permit no: SBB.19/ABS/3030 dated 16.01.2017). Specimen collection, care and experimental animal use protocols were approved by the Institutional Ethical Committee of Gauhati University, Guwahati, India (Permit reference number: IAEC/KS/2022/PhD-IAEC/2022-10/15). No living specimens were allowed to expose with any other harmful chemicals. In addition to the ARRIVE guidelines, all the methods were performed in accordance with the relevant guidelines and regulations.

Additional information
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