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ORIGINAL ARTICLE

Phylogenetic analysis of *Tor putitora* (Hamilton, 1822) from Rivers Sutlej, Beas and Yamuna using 12S rRNA gene sequences

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Abstract
The term "mahseer" is collectively used for three genera of the family Cyprinidae namely *Tor*, *Neolissochilus* and *Naziritor*. Among mahseers, *Tor putitora*, popularly known as Golden Mahseer, is one of the most important fishes in the Himalayan regions and South Asia. It is commercially important as an edible fish and a popular game fish. The present study is an attempt to understand the phylogenetic relationship of *Tor putitora* with other species of mahseers using the mitochondrial partial 12S rRNA gene. The phylogenetic analyses revealed that *Neolissochilus* is a sister genus of *Tor* and *Tor putitora* is more closely related to *Tor tor* and *Tor mosal mahanadicus* as compared to other species of *Tor*.

Keywords: Mahseer, Taxonomic status, Evolutionary relationships, *Tor putitora*.

INTRODUCTION

Family Cyprinidae is the largest family of freshwater fishes comprising 160 valid genera and about 1790 valid species (Fricke et al. 2023). Among the cyprinids, almost 50 species of the three genera viz., *Tor, Neolissochilus* and *Naziritor* are collectively named "mahseer" (Mani et al. 2009). The species of mahseer are facing a decline in their population (Nautiyal 2014) due to various anthropogenic activities that have led to overexploitation, habitat degradation, and loss of breeding grounds of mahseer species. The term "mahseer" is profoundly used for the genus *Tor* having sixteen recognized species (Pinder et al. 2019) that are endemic in Southeast Asia and a majority of them have been included in the threatened list of IUCN (Lakra et al. 2010). Fishes of the genus *Tor* are considered "true mahseer" due to the presence of the median lobe which is absent in the species of genera *Neolissochilus* and *Naziritor* (Kumar et al. 2013; Jaafar et al. 2021). In order to distinguish different species of genus *Tor*; features such as shape, size and length of the median lobe are often used (Zhou & Cui 1996). However, these classification methods are a source of disagreement among taxonomists (Nguyen et al. 2006) as these characters are highly variable (Roberts 1999) and influenced by environmental factors which makes them less reliable (Ng 2004) for species identification and taxonomical purposes.

Tor putitora, popularly known as Golden Mahseer, is one of the most important fishes in the Himalayan regions and South Asia inhabiting rapid streams with rocky bottoms, riverine pools and lakes. It is a column feeder depending on periphytic algae and diatoms in the juvenile stage and becomes omnivorous when adult (Jha & Rayamajhi 2010). *Tor putitora* is a commercially important edible fish. However, due to its large size, attractive golden colour and the habit of fighting back when hooked, it is also a popular game fish (Yadav et al. 2020; Bhatt & Pandit 2016). Furthermore, *T. putitora* is also one of the important health indicators of the freshwater. Unfortunately, the species is currently under threat due to excessive fishing, building of dams and illegal sand mining activities which have resulted in loss or degradation of habitat and breeding grounds (Sharma et al. 2015). Due to all these reasons, *T. putitora* is regarded as endangered on the IUCN red list (Jha et al. 2018).

In recent years, DNA barcoding has emerged as a reliable and powerful technique for phylogenetic analyses and species identification (Ude et al. 2020; Wang et al. 2020). Complemented with traditional

taxonomy, molecular methods are being employed to resolve the taxonomic ambiguities of cryptic (Jirsová et al. 2019) and polymorphic species (Magalhaes et al. 2015). Despite being a group including commercially important and threatened fish species; very few studies have been done regarding the species characterization of mahseers using molecular methods. Mitochondrial DNA (mtDNA) is widely used as a molecular marker for studying intraspecific as well as interspecies variation in animals (Sati et al. 2013) because of its high copy number, faster mutation rate than nuclear DNA and non-recombinant nature (Brown 1985). Among the mitochondrial DNA markers, the 12S rRNA gene is highly conserved and used for species identification and molecular phylogeny (Panicker et al. 2019). To date, very few studies have been done to classify the mahseers using the 12S rRNA gene (Sivaraman et al. 2009).

The present study is, therefore, an attempt to assess the phylogenetic relationship of *T. putitora* (Hamilton, 1822) with other species of the mahseer group including species of the genera *Tor* and *Neolissochilus* using mitochondrial partial 12S rRNA gene.

MATERIAL AND METHODS

Study area and Sample collection: Samples of *Tor putitora* were collected from Rivers Beas, Sutlej and Yamuna (n=3). For River Beas, sample collection was done from Pong reservoir (32°01'N 076°05'E); for River Sutlej from Gobind Sagar reservoir (31°25′N 76°30′E) and from Paonta Sahib30.438°N 77.624°E) for River Yamuna (Fig.1). All samples were procured from local government fish collection centers meant for collecting fishes for eating purposes and were dead at the time of procurement. Muscle tissue samples were taken near the dorsal fin region; ice-packed and brought to the Department of Zoology, Panjab University, Chandigarh. The samples were then stored in absolute alcohol and kept at -20°C until further analyses.

DNA extraction and purification: DNA extraction was done by using Fast DNA™ Spin Kit for Soil (MP Biomedicals) with minor modifications. The muscle tissue samples were cut into small pieces and mixed

Fig.1. Map showing sampling sites of the present study.

with the Sodium Phosphate Buffer in Lysing Matrix E tube (provided with Kit) and brief vortexing was done followed by Proteinase K treatment at 55°C for 10 minutes. The rest of the protocol was followed as per the manufacturer's instructions. DNA was eluted in nuclease-free water and isolated DNA was then purified using DNA Clean and Concentrator Kit ™ (Zymo Research). The purified DNA was quantified using Nano drop spectrophotometer by measuring the absorption at 260 and 280nm. Good quality DNA having 260/280nm absorption ratio between 1.7 to 1.9 was selected for PCR amplification. The quality of purified DNA was checked on 0.8% agarose gel stained with ethidium bromide.

PCR Amplification: Partial sequences of 12S rRNA gene were amplified by using universal primers (Forward: 5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3' and Reverse: 5'- GAG GGT GAC GGG CGG TGT GT-3') (Sivaraman et al. 2010). The reaction mixture was as follows: 12.5µL (2X) PCR master mix (Thermo Scientific), 5µL of isolated DNA, 2µL (10pM/µL) from each of forward and reverse primers, 0.7µL DMSO and 5.3µL of autoclaved distilled water to make the total reaction volume 27.5µL. The thermal profile used for amplification began with initial denaturation at 94°C for 5 minutes

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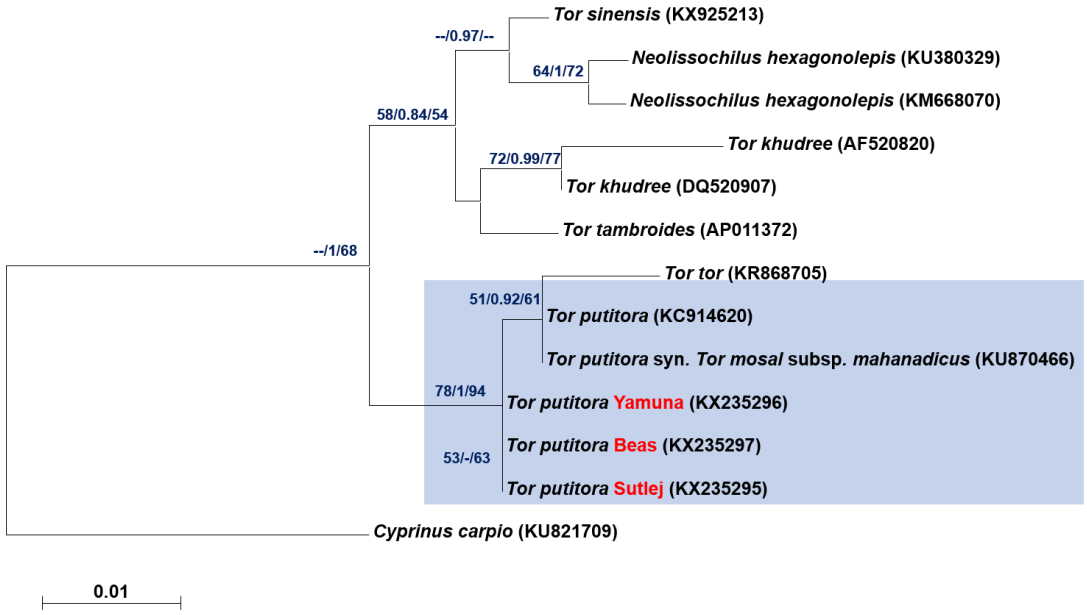

Fig.2. Phylogenetic tree inferred by RAxML and MrBayes and MEGA (Bluebox) using 12S rRNA gene sequences. The isolates from this study (Yamuna, Sutlej, Beas) are highlighted in red font. ML bootstrap support value/Bayesian posterior probability is displayed at the nodes.

followed by 35 cycles of denaturation at 94°C for 45s, annealing at 60.5°C, extension at 72°C for 1 minute and final extension for 10 minutes. The amplified PCR products were then purified using DNA Clean and Concentrator kit (Zymo Research) for the removal of free dNTPs and DNA polymerase. The purified PCR product was resolved under 1.2% agarose gel and visualized under the UV gel documentation system to know the approximate size of the amplicon by using a 100-base pair ladder as marker.

Sequencing and Data Analysis: PCR products were sent for Sanger sequencing to Chromas Biotech, Bangalore. The quality of sequences obtained was analyzed by using BioEdit software. Chromatogram analysis revealed good quality sequences. Forward and reverse sequences obtained in FASTA format were joined manually and curated to get combined sequences of a desired length of ~400 bp. To check the authenticity of sequences obtained, the joined sequences were subjected to BLAST and sequences of the 12S rRNA gene of *T. putitora* showed a number of good matches for other *Tor* species as well as for other related genera. The 12S rRNA gene FASTA sequences from related species showing high sequence similarity were extracted from the GenBank (Top 7 sequences including different species of Tor and 2 of genus Neolissochilus were downloaded from GenBank) followed by multiple alignment using MUSCLE software (Edgar 2004). Phylogenetic tree construction was based on Maximum likelihood and Bayesian analysis. Maximum likelihood tree was generated using RAxML (Kozlov et al. 2019) with default parameters and 100 replicates of bootstrapping. Bayesian analysis was performed using Mr. Bayes v3.2.7 (Ronquist et al. 2012) for 100000 MCMC generations. In addition to this, phylogenetic analysis and pairwise distance calculation were also done using MEGA version 11 (Tamura et al. 2021). For the construction of phylogenetic trees, *Cyprinus carpio* was used as an outgroup. Three sequences obtained from the present study were submitted to the NCBI database and were given the following accession numbers: KX235295 (*T. putitora*, Sutlej); KX235297 (*T. putitora*, Beas) and KX235296 (*T. putitora*, Yamuna).

RESULTS

The phylogenetic tree constructed using partial 12S rRNA gene fragments from River Sutlej, River Beas and River Yamuna showed two main clusters; one including all *T. putitora* and *T. tor* species and another having the rest of *Tor* species (obtained from

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[ 1] #AF520820_Tor_khudree
[ 2] #AP011372_Tor_tambroides
[ 3] #DQ520907_Tor_khudree
[ 4] #KC914620_Tor_putitora
[ 5] #KM668070_N_hexagonolepis
[ 6] #KR868705_Tor_tor
[ 7] #KU380329_N_hexagonolepis
[ 8] #KU821709_Cyprinus_carpio
[ 9] #KU870466_Tor_mosal_mahanadicus
[10] #KX235295_Tor_putitora_Sutlej
[11] #KX235296_Tor_putitora_Yamuna
[12] #KX235297_Tor_putitora_Beas
[13] #KX925213_Tor_sinensis

[         1      2      3      4      5      6      7      8      9     10     11     12     13 ]
[ 1]
[ 2]  0.0216
[ 3]  0.0108 0.0107
[ 4]  0.0328 0.0187 0.0243
[ 5]  0.0189 0.0133 0.0134 0.0214
[ 6]  0.0413 0.0269 0.0326 0.0079 0.0297
[ 7]  0.0244 0.0133 0.0188 0.0215 0.0053 0.0297
[ 8]  0.0624 0.0500 0.0504 0.0527 0.0588 0.0615 0.0588
[ 9]  0.0328 0.0187 0.0243 0.0000 0.0214 0.0079 0.0215 0.0527
[10]  0.0300 0.0214 0.0216 0.0026 0.0242 0.0106 0.0242 0.0499 0.0026
[11]  0.0300 0.0214 0.0216 0.0026 0.0242 0.0106 0.0242 0.0499 0.0026 0.0000
[12]  0.0300 0.0214 0.0216 0.0026 0.0242 0.0106 0.0242 0.0499 0.0026 0.0000 0.0000
[13]  0.0189 0.0133 0.0134 0.0214 0.0106 0.0297 0.0106 0.0472 0.0214 0.0187 0.0187 0.0187
```

Fig.3. Pairwise genetic distance calculation of *T. putitora* and other sequences from NCBI database.

GenBank; *T. khudree, T. sinensis* and *T. tambroides*) along with the related genus *Neolissochilus* (Fig.2). All three samples of *T. putitora* of the present study clustered along with *T. putitora* sequences taken from GenBank with a high level of bootstrap support. Additionally, the sequence of *T. mosal mahanadicus* obtained from GenBank was also clustered along with *T. putitora* sequences. Pairwise genetic distance values revealed that there was no difference among the sequences of *T. putitora* samples from the present study (Fig.3). The highest interspecific distance was found to be 0.0624 between *T. khudree* and *C. carpio*. The pairwise genetic distance values among all the sequences of the current study (from Beas, Yamuna and Sutlej) were found to be zero. The pairwise genetic distance values of *T. mosal mahanadicus* with the current study sequences was 0.0026 and with *T. tor* was 0.0079.

DISCUSSION

Studying morphological characteristics alone make it difficult for the researchers to assign the correct taxonomic status for a particular taxon under study, especially in cases of cryptic species which are morphologically identical but genetically different and also for polymorphic species which are morphologically different type or subpopulations of the same species having same genetic structure. In such cases, if species identification and taxonomic assignment are done solely based on morphological data; misleading interpretations can be made (Ude et al. 2020; Wang et al. 2020).

For the classification of fish species, generally osteological and morphological characters especially the proportion of head length to body depth are used (Talwar & Jhingran 1992) but the plasticity of these characters leads to taxonomic ambiguities regarding the validity of species and subspecies among mahseers (Mohindra et al. 2007). Therefore, while assigning the taxonomic status of any group with ambiguities, morphological data must be complemented with molecular techniques for correct species identification and interpretations of evolutionary relationships. High mutation rate, uniparental inheritance and rare non-coding segments make mitochondrial DNA genes suitable molecular markers for species identification and phylogenetic analysis. The accelerated evolutionary rate of animal mitochondrial DNA results in significant amounts of variation in DNA sequences that can be used to discriminate not only closely related species (Yang et al. 2014) but also cryptic and polymorphic species.

Among the mitochondrial genes, the 12S rRNA gene has a slower evolution rate as compared to

mitochondrial protein-coding genes which makes it a better choice to be used as a phylogenetic marker (Chan et al. 2020). The amplification of conserved regions of the 12S rRNA gene using universal primers not only helps in species identification but can be used for phylogenetic analysis (Panicker et al. 2019) and resolving taxonomic discrepancies.

The results obtained from mitochondrial 12S rRNA gene analysis showed that *Neolissochilus hexagonolopis* clustered separately indicating *Neolissochilus* is a sister genus of *Tor.* This observation is in agreement with the findings of Kumar et al. (2013). The analyses also showed that *T. putitora* and *T. tor* are closely related to each other than other mahseer species which has also been substantiated by other researchers who have described the species primarily on the basis of the morphological features especially Talwar and Jhingran (1991) and Jayaram (2010). These findings are also in accordance with Pawan-Kumar et al. (2016). Furthermore, the results of the study complement the findings of Sati et al. (2013) who studied the phylogenetic relationship of five Indian mahseer species using cytochrome oxidase gene and suggested that *T. putitora* and *T. tor* have closer relationship with each other which has also been validated in the present study.

Tor mosal mahanadicus is endemic to the Mahanadi River, also described as *T. khudree mahanadicus* (Menon 1992), *Tor tor mahanadicus* (Sugunan 1995) and used as a synonym of *T. khudree*. However, molecular analyses such as Random Amplified Polymorphic DNA (RAPD) analysis of *T. mosal mahanadicus* have revealed it to be more similar to *T. putitora* than other *Tor* species (Mohindra et al. 2007). Khare et al. (2014) reported that *T. macrolepis* and *T. mosal mahanadicus* were not distinct from *T. putitora* and considered *T. mosal mahanadicus* as a subpopulation or genetic stock of *T. putitora*. In the present study also, *T. mosal mahanadicus* clustered along with the sequences of *T. putitora* from Beas, Yamuna, Sutlej and with the *T. putitora* sequences obtained from the NCBI database. The pairwise genetic distance values of *T. mosal mahanadicus* with all the *T. putitora* sequences of the present study was 0.0026 and the value for *T. putitora* sequence (KC914620) retrieved from NCBI was zero. Also, the pairwise genetic distance value of *T. mosal mahanadicus* with *T. tor* was 0.0079 which indicates that *T. mosal mahanadicus* is closer to *T. putitora* than *T. tor*. All these findings further strengthen the fact that *T. mosal mahanadicus* could be considered as a subpopulation or genetic stock of *T. putitora*. However, we recommend that more than one molecular marker should be used to resolve the exact position of *T. mosal mahanadicus.*

CONCLUSION

Mahseers are an important commercial group of fishes which are currently under immense pressure due to various anthropogenic activities. Correct species identification and understanding of evolutionary relationships are crucial for the characterization of different populations and frame management strategies for the conservation of species that are under threat (Walton et al. 2017). The present study explores the potential of 12S rRNA gene sequencing to resolve the taxonomic discrepancies in the complex mahseer group. Findings of the present study suggest that *T. putitora* is more closely related to *T. tor* and *Neolissochilus* is the sister genus of *Tor* which is in accordance with the previous findings. Also, the study reconfirms the fact that *T. mosal mahanadicus* is closer to *T. putitora*. So, further studies should be done based on multiple molecular markers with a larger sample size to resolve the taxonomic ambiguity regarding the status of *T. mosal mahanadicus.*

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مقاله کامل

تجزیه و تحلیل تبارشناختی *Tor putitora* (Hamilton, 1822) از رودخانه‌های سوتلج، بیس و یامونا با استفاده از توالی‌های ژن S rRNA۱۲

آنوراجینی کادالیا، فوتسوگ دولما، خوشبو رانا، یوگی کومار راوال، آرچانا چاهار*

گروه جانورشناسی، دانشگاه پنجاب، چندیگر، هند.

چکیده: اصطلاح «مهسیر» در مجموع برای سه جنس از خانواده کپورماهیان به نام‌های *Tor*، *Neolissochilus* و *Naziritor* استفاده می‌شود. در میان ماهی‌ها، ماهی *Tor putitora*، معروف به مهسیر طلایی، یکی از مهم‌ترین ماهی‌های نواحی هیمالیا و جنوب آسیا است. از نظر تجاری به‌عنوان یک ماهی خوراکی و یک ماهی با اهمیت تفریحی (صید) است. مطالعه حاضر تلاشی برای درک رابطه فیلوژنی *Tor putitora* با سایر گونه‌های مهسیر با استفاده از بخشی از ژن S rRNA۱۲ میتوکندری است. آنالیزهای فیلوژنی نشان داد که *Neolissochilus* یک جنس خواهر *Tor* است و *Tor putitora* در مقایسه با سایر گونه‌های *Tor* به *Tor tor* و *Tor mosal mahanadicus* نزدیک‌تر است.

کلمات‌کلیدی: مهسیر، وضعیت تاکسونومیکی، روابط تکاملی، *Tor putitora*.