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GENETIC DIVERSITY OF GENUS *TOR* IN RIVER CHALIYAR, SOUTHERN WESTERN GHATS, KERALA: THROUGH DNA BARCODING

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ABSTRACT

Genus *Tor* is an ecologically and economically important fresh water fishes under the family Cyprinidae. Taxonomy and phylogenetic relationship of this species are extremely confusing due to the morphological variation and habitat adaptation. *Tor khudree* and *Tor mussullah* was reported from River Chaliyar by earlier workers and presence of *Tor malabaricus* was confirmed through DNA barcoding. *Tor* samples were collected from the different localities of Chaliyar River, one of the west flowing rivers in Western Ghats. DNA barcoding using the mitochondrial COI (Cytochrome Oxidase Subunit 1) gene was carried out. Morphometric analysis was performed using twenty two morphometric and fifteen meristic characters. The existence of *Tor khudree*, *Tor malabaricus* and *T. mussullah* is confirmed through DNA barcoding and it enhance the fish biodiversity of river Chaliyar. Due to habitat change and indiscriminate fishing these species are under tremendous stress and it needs an urgent attention to conserve these threatened species.

Keywords: Genus *Tor*. Taxonomy. DNA barcoding. COI gene. River Chaliyar.

INTRODUCTION

The Western Ghats of Peninsular India is one of the world's richest "biodiversity hotspots" [1]. Kerala part of Western Ghats is endowed with 41 west flowing and 3 east flowing rivers supports richest freshwater fish diversity with a high degree of endemism and proved most fertile fields for ichthyologic discoveries [2,3,4,5,6].

Genus *Tor* [7] is a big scaled carp under the family Cyprinidae; inhabit the mountain streams. *Tor* is well known as an excellent sport and food fish but they are also our national heritage [8]. It is an attraction to anglers as well as naturalists from all over the world since the nineteenth century [9]. *Tor* is consideredas the 'King of Indian aquatic systems' in the bibliography of "Mahseers of the Indian sub-continent" [10]. *Tor* species so far reported from Indian region include *T. tor* (Hamilton), *T. putitora* (Hamilton), *T. mosal* (Hamilton), *T. malabaricus* (Jerdon), *T. neilli* (Day), *T. progenies* (McClelland), *Tor khudree* (Sykes), *T. kulkarnii* (Menon), *T. mussullah* (Sykes), *T. barake* (Arunkumar and Basudha) and *T. remadevii* (Kurup and Radhakrishnan). Among this eleven species six species like *Tor khudree* [11,12,13,14,15,16,17], *T. malabaricus* [2,18,19,20], *T. mussullah* [21,22], *T. putitora* [23], *T. remadevii* [24] and *T. tor* [2,20] have been reported from South India. *Tor* is locally known by different names in different places such as Kadanna, Kuyil, Katti etc., in Kerala.

The biology, distribution, diversity and taxonomic status of genus *Tor* from the Himalayan region is relatively well studied when compared to the same from the peninsular India. Only few reports have explored the presence and taxonomic status of genus *Tor* from the rivers of Southern Western Ghats. Although there have been limited studies on the fish fauna of Chaliyar [25] and the Nilgiri Biosphere Reserve (NBR) had been carried out [12,17,26,27], there is no much study have been done specifically for genetic diversity of the genus *Tor*.

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Species identification in *Tor* group has also been a matter of debate because most of studies are based on either morphological characters or molecular markers only. In such cases, where morphological ambiguities exist, use of DNA markers vis-a-vis morphological characters can be an effective method of species resolution and fixation of species specific molecular signatures forever. DNA barcoding is an initiative that offers for taxon recognition, molecular signature and classification of animal organism based on small sequence fragment (655 base pairs) near the 5'end of mitochondrial gene cytochrome c oxidase I (COI) with universal primers [29]. This region can be used for identification of any organism at the species level [28] and has been successfully tested in a large variety of organisms of both invertebrate and vertebrate, ranging from yeasts to humans [29,30,31,32,33,34,35,36]. Using COI gene for barcode is suitable marker for discriminating between closely related species of fishes [37, 38, 39, 40, 41]. The challenge in use of small DNA barcode (only 655 bp) based phylogenetic study is selection of a nearly perfect nucleotide substitution model for the dataset, so that weakest evolutionary signal is correctly detected. Out of various selection criteria such as likelihood-ratio tests (LRT), hierarchical implementation of the likelihood ratio test (hLRT) [42,43,44], Maximum Likelihood value (lnL) [45], Akaike information criterion (AIC) [46], Akaike Information Criterion, corrected (AICc) [47], Bayesian information criterion (BIC) 48] and performance-based decision theory (DT) [49,50], BIC seems most correctly defining the nucleotide substitution.

The present study was carried out to study the genetic diversity of *Tor* species from the River Chaliyar of Southern Western Ghats using DNA barcoding methodology vis-à-vis morphological character-based criteria to fix the molecular signature for three *Tor* species.

Study Area

This study was carried out in the river Chaliyar which is one of the west flowing rivers from Western Ghats, Kerala, India **(Fig.1).** River Chaliyar flows between latitude 11º 19' N and longitude 75º 51' E. All its tributaries take a very steep course with a series of rapids and falls as they debauch into the foothills and the plains below. The elevation of the basin varies from 100 m to 2200 m in the short distance of 10 km. This river has many tributaries such as Karimpuzha, Punnappuzha, Karuvanpuzha, Tiruvanchipuzha, Cherupuzha, Manjakallanpuzha, Arikkayampuzha and the Panapuzha etc. with a catchment area of 1535 $km^{2.}$ The Chaliyarpuzha arises in the south-west of the Wayanad plateau, while the sources of the Karimpuzha and Punnapuzha are in the Kundah hills [17, 25, 27]. *Tor* fish samples were collected from Cherupuzha, Maanjeeri (Karimpuzha), Punnapuzha and Manjakallanpuzha for morphological and molecular study (Table-1).

MATERIALS AND METHODS

Sample collections

At each sampling site *Tor* species were collected using gill nets of different mesh size ranging from 8 mm to 22 mm, cast net and dip nets depending upon the depth and water velocity. The fishes were identified using the keys described by Talwar and Jhingran, Menon *et al.* and Jayaram [16,51,52]. A small portion of tissue from the right side (fin clips of approximately 5 x 5 mm size) pectoral and pelvic fins was excised in a small tube and preserved in 99% Ethanol and labeled. Further the specimens were labeled and preserved in 10% formalin as voucher specimen for future reference.

Morphological studies

Around 15 specimens from each *Tor* species were collected and twenty two morphometric and fifteen meristic characters were taken from the head and body for the analysis following Rainboth [59]. Principal component analysis (PCA) was performed to know the morphometric characters differ from each species and cluster analysis was performed to know the similarities between the species and dissimilarities between the species using XLSTAT.

Isolation of Genomic DNA

DNA was isolated from approximately 50 mg of pectoral or pelvic fins tissue following standard phenol/chloroform method [54] with partial modifications. Precipitated DNA was resuspended in TE buffer (10mM tris –HCl, 0.1 mM EDTA, pH 8) with a final concentration of 100 ng⁄ µl using Nanodrop 2000 (Thermo Scientific, USA), for all samples.

Amplification and Sequencing

The partial sequence of COI gene was amplified using the primers Fish F1 (5' – TCA ACC AAC CAC AAA GAC ATT GGC AC - 3') and Fish R1 (5' – TAG ACT TCT GGG TGG CCA AAG AAT CA - 3') [39]. The amplifications were performed in 40 µl reactions containing in 4µl of 10X assay buffer, 0.8µl of MgCl2 (25mM), 0.2 µl of each dNTP, 0.4µl of each primer (10mM), 3U of *Taq* polymerase (0.4 µl) and 1.6 µl (50ng/ µl) of genomic DNA. To check DNA contamination, a negative control was set up omitting template DNA from the reaction mixture. Thermocycler conditions were used as initial preheat at 94ºC for 3 min, of denaturation 35 cycles at 94ºC for 30 s, annealing 54º C for 30 s, extension 72^0C for 60s and final extension for 10 min at 72ºC. The PCR products were visualized on 1.2% agarose gels and the most intense product were selected for sequencing. Nucleotide sequencing was performed by the dideoxy chain-termination method [55] using ABI Prism Big Dye Terminator v3.1 Cycle

Sequencing kit, and sequenced following Applied Biosystems, USA.

Diversity Analysis

The raw DNA sequences were edited using BioEdit sequence alignment editor [56], aligned using CLUSTALW [57], refereed against electropherogram and submitted to GenBank (Table-2). To analyze the evolutionary isolation of three species and the level of divergence within species, K2P distance was calculated by averaging pairwise comparisons of sequence difference across all individuals by the Kimura 2-Parameter method [39] under Gama distribution estimated in MEGA 5.1 (Molecular Evolutionary Genetics Analysis) software [58] (Table-3).

Phylogenetic analysis

The phylogenetic and evolutionary history of the genus *Tor* was inferred in a narrower set of sequences by using the Maximum Likelihood (ML), Maximum Parsimony (MP) [53] and Neighbor Joining (NJ) [60] statistical methods in MEGA5.1 [58]. In a total of 16 sequences: 8 sequences were generated in this study from three *Tor* species as well as 9 sequences of *Tor* species from NCBI (from our earlier study as well as other researchers). The substitution rate was modeled by K2+G formula and codon positions included were $1^{st}+2^{nd}+3^{rd}$ with 576 positions in the final dataset. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0500). To assess the reliability of a phylogenetic tree we used 1000 bootstrapre-sampling strategy [61]. In ML the Heuristic Method of Tree Inference, we opted for Nearest-Neighbor Interchange (NNI) and initial NJ tree was made automatically with very strong branch swap filter. In ML initial tree(s) for the heuristic search were obtained automatically by applying NJ algorithm and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (Fig. 5). The evolutionary history was also inferred using the NJ method and the optimal tree with the sum of branch length is shown in Fig. 6. The Maximum Parsimony method generated 12 most parsimonious trees and the consensus tree inferred from is shown in Fig. 7.

RESULTS AND DISCUSSION

There are three species of genus *Tor like Tor khudree* (Sykes), *T. malabaricus* (Jerdon) and *T. mussullah* (Sykes) (Fig. 2) were collected and identified using morphological character.

Morphometric and meristic analysis

There is no overlapping cluster between the three species of *Tor* (*T. khudree*, *T. malabaricus* and *T. mussullah*) in the scatter plot made by the principal component analysis of the morphometric characters (Fig. 4). This analysis indicated that the existence of three morphologically differentiated groups of *Tor* in Chaliyar river. *Tor khudree* could be easily differentiated from the other two species of *Tor* by higher values of ratios PDS, HD, LLS, PreAL, PrePelL, PoDL, PecFUBR, DFUBR, while *T. malabaricus* could be differentiated from other species in the characters CFS, CFR and HL. Dissimilarities between the three species *T. khudree*, *T. malabaricus* and *T. mussullah* are shown in the dendrogram (Fig. 3). *T. mussullah* is differed from other two species in all other characters especially having head length lesser than the body depth. Based on this analysis the distance between *T. khudree* and *T. malabaricus* is 25.35% and between *T. khudree* and *T. mussullah* is 44.47%. *T. malabaricus* and *T. mussullah* is separate each other in a distance of 43.32 %. This shows that *T. mussullah* has an almost equal distance from the other two species. The variables which had higher factor loadings are HL (4.05, 5.33), BD (6.39), DFL (4.79), PrePelL (5.86), PreAL (5.55), PreDL (5.56), HW (8.88), SnL (4.84), UJL (6.02), ED (4.55), AFUBR (9.44), PelFBR (9.37), LLTU (9.37), LLTL (8.25), CFS (9.37), CPS (9.37) and LLS (6.24)**.** The morphometric and meristic analysis shows that several features separate the three closely related species of *Tor*; *T. khudree*, *T. malabaricus* and *T. mussullah* and this analysis also supports the existence of three species of *Tor* in the River Chaliyar.

Molecular studies using COI gene

The efficacy of COI gene in identification and Phylogenetic relationship of the fish species with designated barcodes have been proved by many authors [41,61,63,64]. The universal primers amplified the target region in all species generating the COI barcodes for identification [65]. Sequencing of the mitochondrial COI gene represented an average of 650 nucleotide base pairs per taxon. There were no insertions, deletions and stop codons were observed in the sequences. Analysis of COI revealed that out of 654 positions, 620 were conserved, 34 variable (13 were singleton and 21 were parsimoniously informative, at least two of nucleotides occurring with a minimum frequency of two). The average transition/ transversion ratio (R) over three codon positions is 8.53. The individual wise base composition of Thymine/Uracil (T/U); Cytosine (C); Adenine (A) and Guanine (G) of all the three codon positions combined as well as only third codon position were calculated (Table-3). The average nucleotide frequencies was A= 26.7 %, T=28.3 %, G=17.7 % and C=27.3 % and the average genetic distance within the species was 0.32 %.

In phylogenetic and evolutionary history of the genus *Tor*, MP (Fig. 7) & NJ (Fig. 6) methods grouped the Western Ghats species with other Indian species and two Asian species (China and Malaysia) were on separate

node. But ML (Fig. 5) method which is considered the best among three methods, places the Western Ghats species with two Asian species and other Indian species were on separate node. *Tor malabaricus* and *Tor mussullah* together separated from *Tor khudree* by other Indian *Tor* species (NJ, MP) and other Asian *Tor* species (ML). As both of these observations were weekly supported by bootstrap values, so no further elaborated discussion was done. All other Indian *Tor* remains together in three methods. Initially rooted trees were generated, but for better resolution of individuals and species on phylogenetic tree, root was removed.

Manimekalan [17] and Shaji and Easa [22] reported the presence of *Tor khudree* and *Tor mussullah* from the River Chaliyar. Easa & Basha [12] also recorded *Tor khudree* during the exploration of fish diversity in the Karimpuzha tributary. Baby *et al*. [27] could not identify the *Tor khudree* from River Chaliyar and they mentioned that the *Tor khudree* recorded by Easa and Basha [12] could be *Tor malabaricus* and not *T. khudree*. According to Arunachalam [66] all *Tor khudree* recorded from Kerala, Karnataka and Tamil Nadu are *T. malabaricus* except for three populations in Chalakkudy, Cauvery and Krishna basins. In the present study the molecular analysis using the mitochondrial COI gene is confirmed the presence of three species of *Tor*- *Tor khudree*, *Tor malabaricus* and *Tor mussullah* in the River Chaliyar [22,27].

Ecological observations

Chaliyar River forming a wide array of riverine microhabitats from cascades to riffles and pools [27] and most of the parts are rocky with thick forest cover. The *Tor* species prefer undisturbed ecosystem and clean water [67]. All the three species of *Tor* were present in the Manjeeri part of Karimpuzha might be due to the thick forest cover and undisturbed ecosystem. Due to high run off during the wet months, the water in this river is very low in summer season [25]. *Tor* species are abundantly seen in Chaliyar during the rainy season because of the fast flow of water.

Food and feeding is also an important factor which determines the existence of a particular taxon. According to MacDonald [68] *Tor* is an intermittent feeder. Green filamentous algae, other water plants, slimy matter encrusted on rocks, insect larvae etc., have been recorded from the stomach contents of the *Tor*. The feeding habit for *Tor* with more vegetative preference was reported by many authors [69, 70, 71]. In the case of *T. khudree*, the food items of all age groups include the filamentous algae, benthic diatoms, small crabs, fishes and insects [72]. The availability of micro benthic biota on the river substratum is the main food source for the flourishment of genus *Tor* in Chaliyar River (direct observation).

Fig. 1. Map of Chaliyar river basin showing the sample collection site.

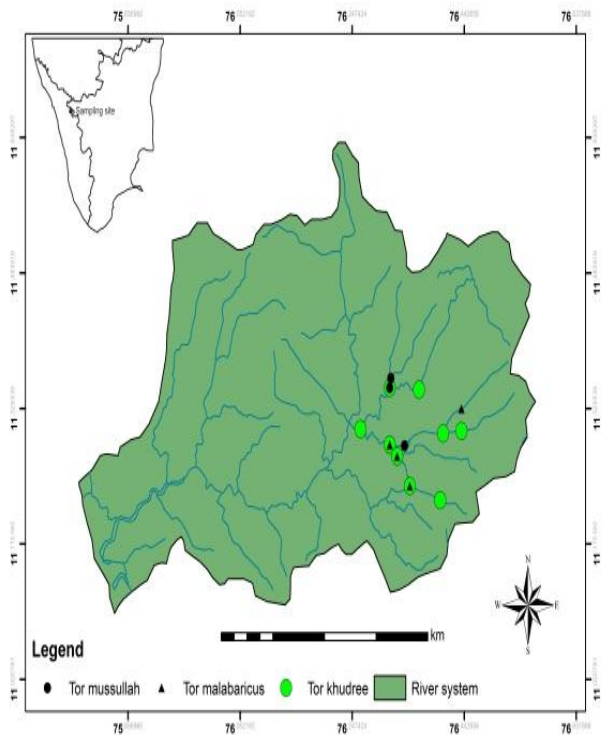

Fig. 2. A. *Tor khudree*, B. *Tor malabaricus*, C. *Tor mussullah*

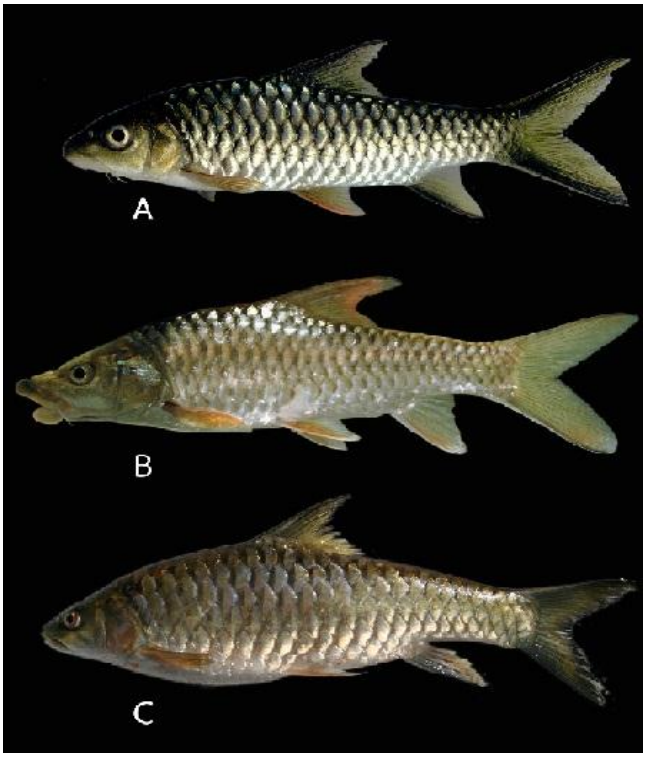

Fig. 3. Dendrogram shows the dissimilarities between *T.khudree, T. malabaricus* and *T. mussullah*

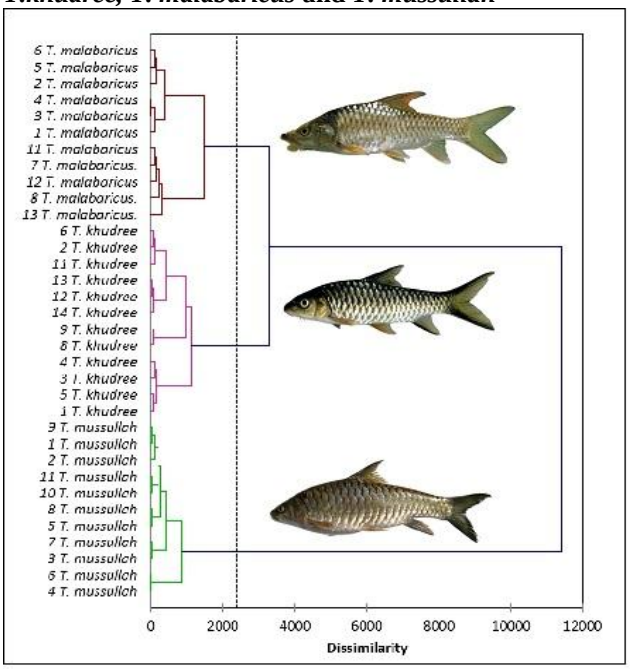

Fig. 4. Scatter plot showing the component score obtained for the morphometric and meristic characters of three *Tor khudree, Tor malabaricus* and *Tor mussullah*

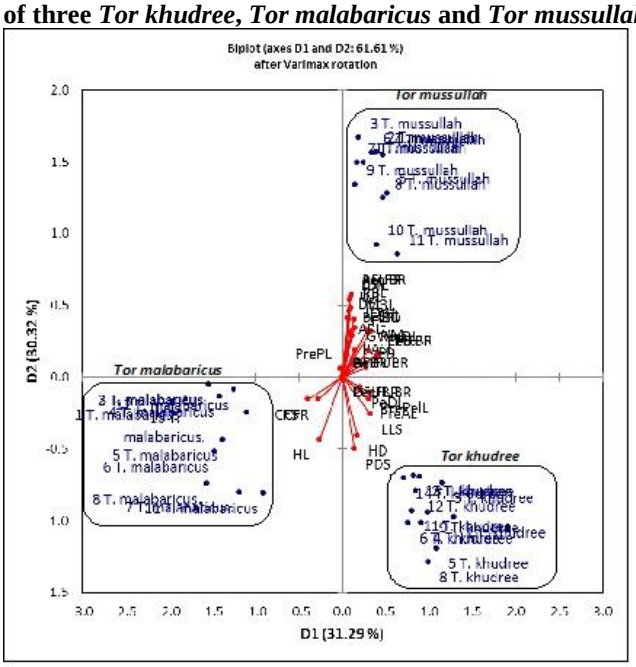

Fig. 5. Molecular Phylogenetic anaylsis by Maximum Likelihood method.

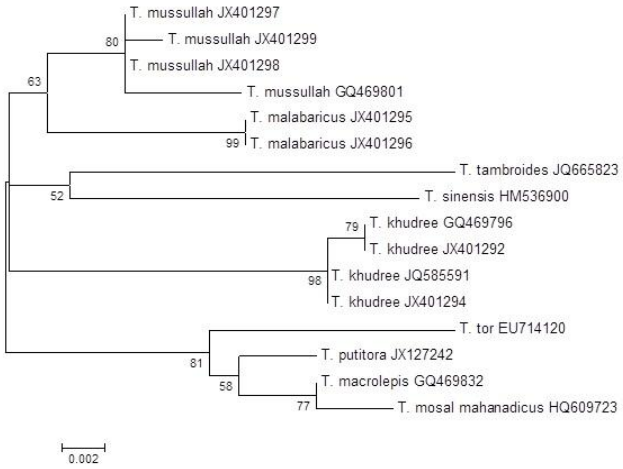

Fig. 6. Evolutionary relationships of taxa by Neighbour Joining method

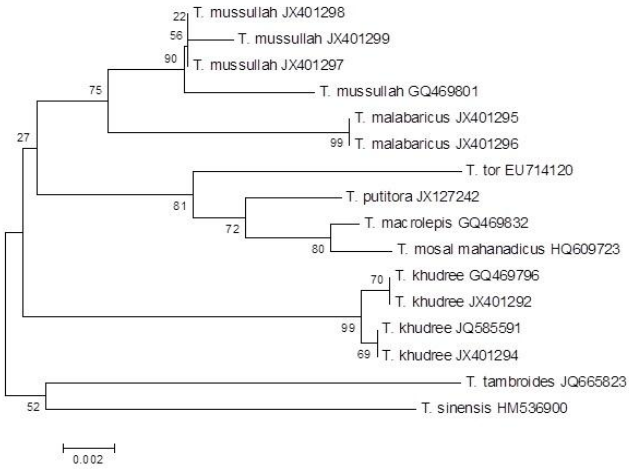

Fig. 7 Maximum Parsimony analysis of taxa by Maximum Parsimony method.

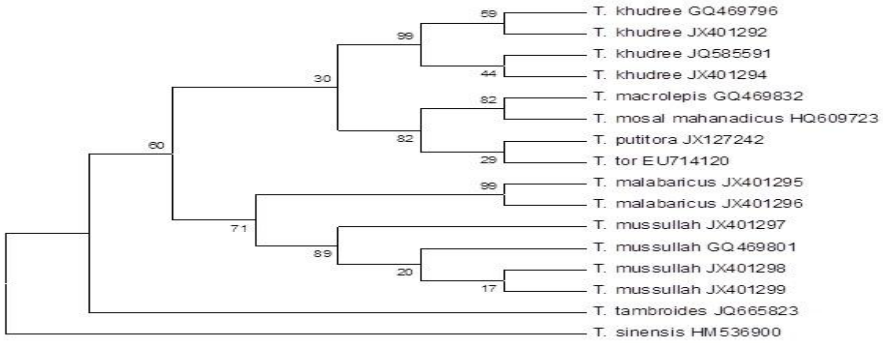

Table 1. Species and location details

Sr. No.	Species	Collection site	Coordinate	IUCNconservation status
1	*T. khudree*	Cherupuzha	11.30N/ 76.35E	EN*
2	*T. malabaricus*	Cherupuzha	11.30 N/ 76.35E	EN
3	*T. khudree*	Maanjeeri (Karimpuzha)	11.31N/ 76.39 E	EN
4	*T. mussullah*	Maanjeeri (Karimpuzha)	11.31 N/ 76.39E	EN
5	*T. khudree*	Punnapuzha	11.35N/ 76.29E	EN
6	*T. khudree*	Manjakallanpuzha	11.31N/ 76.47E	EN
7	*T.malabaricus*	Manjakallanpuzha	11.31N/ 76.47E	EN

EN*- Endangered

Table 2. The mitochondrial COI sequences of Genus *Tor* with the accession number

Sl. No.	Species	Genbank Accession number	Authors
1	*Tor khudree*	JX401292	Present study
2	*Tor khudree*	JX401294	Present study
3	*Tor khudree*	GQ469796	NCBI
4	*Tor khudree*	JQ585591	NCBI
5	*Tor malabaricus*	JX401295	Present study
6	*Tor malabaricus*	JX401296	Present study
7	*Tor mussullah*	JX401297	Present study
8	*Tor mussullah*	JX401298	Present study
9	*Tor mussullah*	JX401299	Present study
10	*Tor mussullah*	GQ469801	NCBI
11	*Tor macrolepis*	GQ469832	NCBI
12	*T. mosalmahanadicus*	HQ609723	NCBI
13	*Tor putitora*	JX127242	NCBI
14	*Tor tor*	EU714120	NCBI
15	*T. sinensis*	HM536900	NCBI
16	*T. tambroides*	JQ665823	NCBI

Table 3. Evolutionary divergence between *Tor khudree, Tor malabaricus* and *Tor mussullah*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	0.027															
3	0.023	0.023														
4	0.030	0.029	0.032													
5	0.027	0.023	0.003	0.032												
6	0.002	0.025	0.025	0.029	0.025											
7	0.032	0.030	0.031	0.030	0.034	0.034										
8	0.023	0.023	0.007	0.029	0.011	0.025	0.027									
9	0.000	0.027	0.023	0.030	0.027	0.002	0.032	0.023								
10	0.002	0.025	0.025	0.029	0.025	0.000	0.034	0.025	0.002							
11	0.027	0.018	0.025	0.027	0.025	0.025	0.031	0.025	0.027	0.025						
12	0.027	0.018	0.025	0.027	0.025	0.025	0.031	0.025	0.027	0.025	0.000					
13	0.021	0.005	0.020	0.023	0.020	0.020	0.025	0.020	0.021	0.020	0.012	0.012				
14	0.021	0.005	0.020	0.023	0.020	0.020	0.025	0.020	0.021	0.020	0.012	0.012	0.000			
15	0.023	0.007	0.021	0.025	0.021	0.021	0.027	0.021	0.023	0.021	0.014	0.014	0.002	0.002		
16	0.032	0.025	0.016	0.038	0.020	0.034	0.032	0.016	0.032	0.034	0.031	0.031	0.021	0.021	0.023	

1. *T. khudree* GQ469796; 2. *T. mussullah* GQ469801; 3. *T. macrolepis* GQ469832; 4. *T. sinensis* HM536900; 5. *T. mosalmahanadicus* HQ609723; 6. *T. khudree* JQ585591; 7. *T. tambroides* JQ665823; 8. *T. putitora* JX127242; 9. *T. khudree* JX401292; 10. *T. khudree* JX401294; 11. *T. malabaricus* JX401295; 12. *T. malabaricus* JX401296; 13. *T. mussullah* JX401297; 14. *T. mussullah* JX401298; 15. *T. mussullah* JX401299; 16. *T. tor* EU714120.

CONCLUSION

The presence of the three species *Tor khudree, Tor malabaricus*and *Tor mussullah*- in the River Chaliyar isconfirmed by DNA Barcoding and morphometric analysis. The nature of ecosystem and the vegetative forest cover makes this river a suitable substratum for the flourishment of the *Tor* species. Many threats are reported against the existence of the fish fauna of this river. Hence, an urgent attention needs to create awareness among local communities and tribes on the importance of the stream habitat and its fish fauna, for conserving these important resources for future generations.

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