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# Genetic Identity of an Endangered Fish Species *Tor Mussullah* (Sykes, 1839) (Teleostei: Cyprinidae) Revealed from Mitochondrial DNA Markers

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***Abstract***

*Tor khudree* and *Tor mussullah* (Sykes, 1839) (Pisces: Cypriniformes) are the two endangered Mahseer belonging to the family Cyprinidae, inhabited in fast flowing rivers of Western Ghats which has more taxonomic ambiguities. The taxonomy and phylogenetic relationship of genus *Tor* has more debate due to the different types of morphological variations they exhibit based on the habitat. The taxonomic position of the hump backed mahseer *Tor mussullah* has been extremely confusing. For many years the species had been treated as *Tor mussullah* and later Menon (1992) referred this species to under the genus *Hypselobarbus* and the humpbacked *Tor* from peninsula so far named as *T. mussullah* is not *T. mussullah* and it is considered the same as *T. khudree*. So far no efforts have been made to differentiate these two species from Peninsular India using DNA barcoding. In the present study, the mitochondrial Cytochrome Oxidase Subunit 1 (CO1) gene was used to determine the existence of the two different species. The *Tor* samples of two species were collected from different rivers of Southern Western Ghats and the tissue samples were sequenced and data were analysed. The present study indicates that the distribution of *Tor mussullah* and *Tor khudree* is confirmed in the Southern Western Ghats. The taxonomic ambiguities of the species *mussullah* have been resolved through DNA barcoding. The species *mussullah* is belonging to the genus *Tor* not *Hypselobarbus*.

***Keywords: DNA barcoding, Tor khudree, Tor mussullah, Hypselobarbus, CO1 gene.***

## Introduction

Genus *Tor* Gray, (1834), well known as mahseer, is one of the most diversified groups of fresh water fishes of family Cyprinidae distributed across Asia. It is an important game and food fish and inhabits mountainous streams and rivers as well as fast flowing rivers in the plains, often preferring clear, swift flowing waters with stony, pebbly or rocky bottoms (Lal *et al.*, 2012). They are famous for their size and also recognized for the socio-economic benefits to the poor rural communities through ecotourism based employment opportunities (

Pinder *et al.*, 2018 and Pinder and Raghavan, 2013).The taxonomy and phylogenetic relationship of genus *Tor* is extremely confusing and a subject of debate due to the different types of morphological variations they exhibit based on the habitat (Silas *et al.*, 2005; Mohindra *et al.*, 2007). Many studies (Hora, 1939; 1942; 1943 and Menon, 1992) have been done to review the status of *Tor* in India and concluded that taxonomic status of some species was still ambiguous. The *Tor* species so far reported from Indian region include *Tor khudree* (Sykes), *T. kulkarnii* (Menon), *T. malabaricus* (Jerdon), *T. mosal*(Hamilton-Buchanan), *T. mussullah* (Sykes), *T. neilli* (Day), *T. progenies* (McClelland), *T. putitora* (Hamilton-Buchanan), *T. remadevii* (Kurup and Radhakrishnan) and *T. tor* (Hamilton-Buchanan). There are six species of genus *Tor* have been reported from South India like *Tor khudree* (Hora, 1943; Sen and Jayaram, 1982;Easa and Basha*,1995*; Shaji *et al.*,1995; Arun,1997; Jayaram,1999; Menon,1992; Ajithkumar *et al.*, 2001; Manimekalan, 2000), *Tor mussullah* (Easa and Basha*,1995;*Jayaram,1997; Easa and Shaji, 2003)*, T. tor* (Ajithkumar *et al.*, 2001), *T. malabaricus* (Sen and Jayaram, 1982; Silas, 1951; 2005; Ajithkumar *et al.,* 2001), *T. putitora* (Manojkumar and Kurup, 2004) and *T. remadevii* (Kurup and Radhakrishna, 2010).

*Tor mussullah,* hump backed mahseer, is an endemic and endangered species from Western Ghats (IUCN, 2017). Sykes (1839) described this species from the Ghod River, Sirur, Pune District Maharashtra, India for the first time. He designated the species as B*arbus mussullah* and later Annandale (1919) stated this species as *Tor mussullah*. It has been described from Maharashtra (Sykes,1839; Annandale,1919; Hora,1943; Sutur, 1944; Silas 1953 and Jayaram, 2005) Karnataka (David 1963; Jayaram 2005; Shahnawaz and Venkateshwarlu 2009), Tamil Nadu (Chacko 1952; Manimekalan 1998) and Kerala (Easa and Shaji, 2003; Jayaram 2005). Now this is a very rare species throughout its range (Jayaram 1995; Menon 2004; Shahnawaz and Venkateshwarlu 2009).

There is a great debate on the generic identity of *Tor mussullah* Talwar and Jhingran (1991) and Jayaram (1997; 2005; 2010) were treated this species as *Tor mussullah* by following the discussions given in Hora (1943). Later Menon (1992) referred this species to under the genus *Hypselobarbus* following Rainboth (1989) and the humpbacked *Tor* from Peninsular India so far named as *T. mussullah* is not *T. mussullah* and it is considered the same as *T. khudree*.

The efficacy of mitochondrial molecular markers in identification and Phylogenetic relationship of the fish species with designated barcodes have been proved by many authors (Lakra *et al,* 2009,2011;Smith *et al.,*,2008; Ward *et al.,*2005;Persis *et al.,*2009; Indu *et al.*, 2012). Kushwaha *et al.* (2001) developed species-specific markers for *T. khudree* and *T. mussullah* to resolve the taxonomic ambiguity between these two species. No efforts have been made so far to prove the generic identity of *Tor mussullah* from Peninsular India especially using mitochondrial DNA marker genes. In the present study the partial sequence data of 16S rRNA and COI genes were used to prove the generic identity of *Tor mussullah* and to confirm whether this species come under the genus *Tor* or *Hypselobarbus*.

## Materials And Methods

### Sample collection

The species of *Tor* such as *T. Khudree* (Fig.1)*, T. malabaricus, T. mussullah* (Fig.2)*,* and the *Hypselobarbus* species like *H. periyarensis, H. Micropogan* (Fig.3)*, H. kurali* were collected from Chaliyar, Chalakudi, Kabini and Periyar rivers of Southern western Ghats and identified following Talwar and Jhingran (1991); Jayaram (1999; 2010) and Menon (1999).

**Figure -1**. ***Tor khudree***

**Figure -2.** ***Tor mussullah***

**Figure -3.** ***Hypselobarbus micropogon***

**Isolation of Genomic DNA**

The total DNA was extracted from the tissue (fin clips) samples following Ruzzante *et al.* (1996) with minor modifications. The concentration of isolated DNA was estimated using a UV spectrophotometer. The DNA was diluted to a final concentration of 100ng/ µl.

**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') (Ward *et al*., 2005). 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. The following thermocycler conditions were used: initial preheat at 94ºC for 3 min, followed by a 35 cycles of denaturation at 94ºC for 30 s, annealing 54º C for 30 s, extension $72^0$C for 60s, followed by a 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 (Sanger *et al*., 1977) using ABI Prism Big Dye Terminator v3.1 Cycle Sequencing kit, (Applied Biosystems, USA) and sequenced following manufacturer's instructions.

**Sequence Analysis**

The raw DNA sequences were edited using BioEdit sequence alignment editor (Hall, 1999). Sequences were aligned using CLUSTAL W (Thompson *et al*, 1997). The sequences after their confirmation were submitted in GenBank, using a standalone multiplatform submission programme called "sequin" (www.ncbi.nlm.nih.gov/Sequin/index.html).

**Phylogenetic analysis**

Phylogenetic analysis was conducted using MEGA ver. 4 (Tamura *et al.,* 2007). Sequence data was subsequently analysed using distance (Neighbour-Joining) and Maximum Parsimony methods. Pairwise sequence divergence was calculated according to Kimura two-parameter model (Kimura, 1980). The number and rate of transitions / transversions were also calculated using the program MEGA. Bootstrap analysis was carried out using 1000 pseudoreplications.

## Results

A total of 40 individuals from genus *Tor* and *Hypselobarbus* were used for the partial sequence analysis of CO1 and 16SrRNA genes and the sequences were submitted to Genbank under the accession numbers of JQ585591, JX401291-JX401310, KC445463-445465 and KC559888-KC559891. Simplicity and un-ambiguity were observed among the sequences of the both mitochondrial regions as there were no insertions, deletions and stop codons in the sequences.

### CO1 sequence analysis

Sequencing of the CO1 gene produced an average of 655bp nucleotide base pairs per taxon. No insertions, deletions or stop codons were observed in any sequence. The analysis revealed the nucleotide frequencies as A= 26.77 %, T= 28.41%, G=27.34 % and C= 17.04 % in CO1 region. The CO1 gene fragment showed an A+T bias. More nucleotide changes were observed at the 3rd codon positions than the 1st codon. In the present study the average transitional pairs were more frequent than the average transvertional pairs with an average ratio of 3.97 in CO1 sequence. The overall genetic distance of individuals among species was estimated as 0.043 and within species as 0.002. Interspecies distance ranged from 0.011 to 0.077 and the intraspecies distance ranged from 0.001 to 0.004. The highest interspecies genetic distance (0.077) was observed between the species of *Hypselobarbus periyarensis* and *Tor mussullah*. The Phylogenetic tree (Fig.4) constructed using the Neighbour-Joining method. The phylogenetic relationship among the species was clearly established, and similar species were clustered under same nodes while dissimilar species were clustered under separate nodes. Each clades were supported by high boot strap values.

### 16SrRNA sequence analysis

Sequencing of 16SrRNA produced an average of 550bp nucleotide base pairs per taxon. No insertions, deletions or stop codons were observed in any sequence. All variable changes within species were observed at the third codon position. The nucleotide analysis revealed the frequencies as A= 32.52 %, T= 21.59%, G=23.41 % and C= 22.41 %. As expected, average transitional pairs were more frequent than transversional pairs with an average ratio of 3.18. The overall mean genetic distance of individuals among species was estimated as 0.025 and within species as 0.026. The highest interspecies genetic distance (0.43) was between *Hypselobarbus periyarensis* and *T. mussullah.* The N-J tree constructed with 16SrRNA sequence revealed the identical phylogenetic relationship among the species as with CO1sequence (Fig.5). The species of both genus was formed two distinct clusters in the tree.

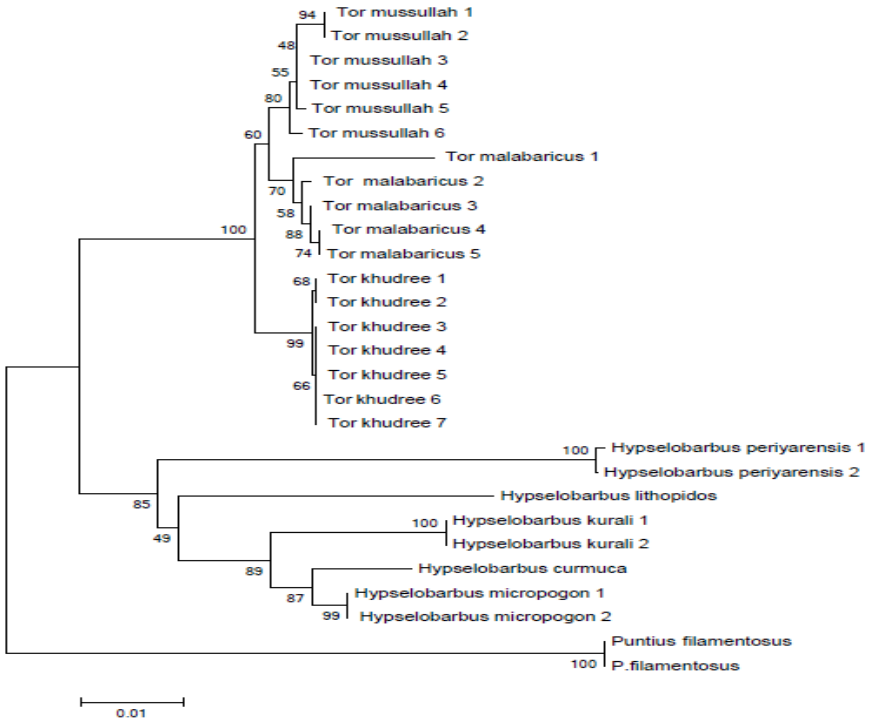


**Figure-4. Neighbour - Joinig tree of *Tor* and *Hypselobarbus* species inferred from the mitochondrial *CO1* gene**

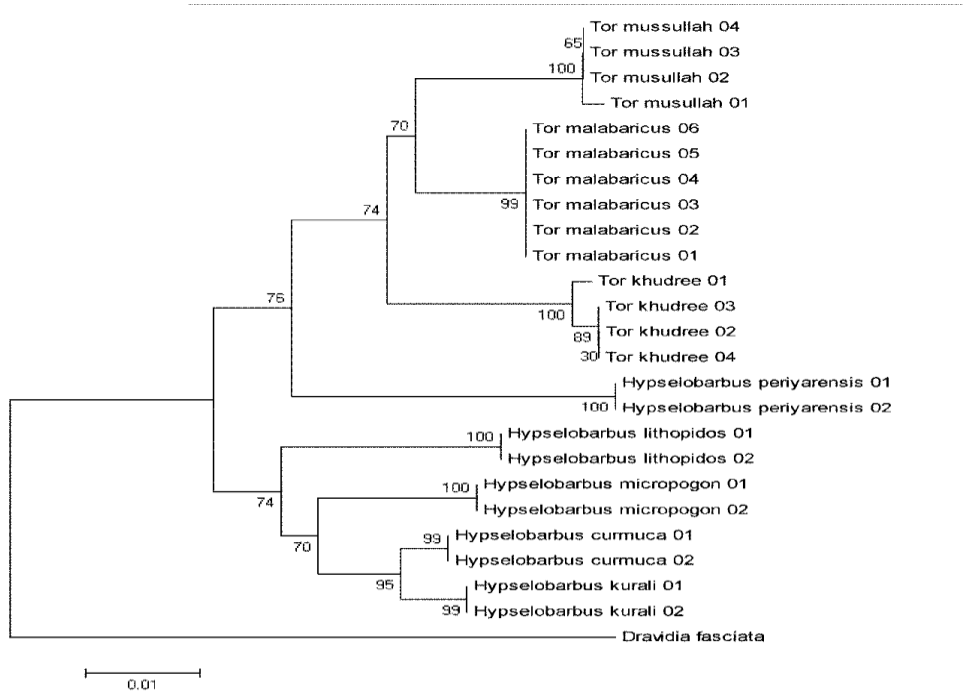


**Figure-5. Neighbour - Joinig tree of *Tor* and *Hypselobarbus* species inferred from the mitochondrial 16SrRNA gene**

## Discussion

The present study clearly discriminated the taxonomic status of all the species examined. The variation was more among the congeneric individuals than among the conspecific individuals. The genetic distances were 0.077 and 0.043 between the species of *Tor* and *Hypselobarbus* using the CO1 and 16SrRNA gene respectively. The intraspecific sequence distance was 0.002 and 0.026 using the CO1 and 16SrRNA gene respectively. DNA based identification system depends on the ability to distinguish intraspecific from inter specific variation (Cywinska *et al.*, 2006). As DNA barcoding requires that intraspecific DNA barcode variation should be substantially less than interspecific variation to allow accurate identification of individuals (Ward *et al.*, 2005; Lakra *et al.,*2011), the present result indicated the effectiveness of COI than 16SrRNA markers in identifying the species and clearly separate the sequences of both genus.

No insertions, deletions or stop codons were observed in any sequence. This supporting the hypothesis that all the amplified sequences derive from a functional mitochondrial CO1 sequences. The absence of stop codons in the amplified sequences suggests that the nuclear DNA sequences originating from mitochondrial DNA sequences (NUMTs- Nuclear Mitochondrial DNA) were not sequenced (Ward *et al.*, 2005; Lakra *et al.*, 2011 and Persis *et al.*, 2009). The occurrence of nuclear DNA sequences originating from mitochondrial DNA sequences has not been reported in Actinopterygii (Bensasson *et al*, 2001). As for other teleosts (Gao *et al*., 2004; Ward *et al*., 2005; Zhang *et al.,* 2009) AT content was higher than the GC content for all the two mitochondrial fragments. More nucleotide changes were observed at the 3$^{rd}$ codon positions than the 1st codon in the present study. This reflected the fact that most synonymous mutations occurred at the 3$^{rd}$ codon, with a few at the 1$^{st}$ codon and none at the 2nd codon (Meyer, 1993; Lakra *et al.,* 2010; Zhang *et al.,* 2009).

The Phylogenetic analysis using MEGA 5.05 with the CO1 and 16SrRNA genes has shown the identical phylogenetic relationship. Mainly two clusters are formed in the tree, one cluster includes the species of *Tor* genus and the other cluster contains the species of genus *Hypselobarbus.* Species in each cluster supported with high boot strap values. *Tor mussullah* is located far away from the clade of *Hypselobarbus* and it is present in the tree with the group of *Tor* species. *T. khudree* is formed a seperate clade away from the *Tor mussullah.* In the present study *Putius filamentosus* was selected as an out group.

The present study is an attempt to resolve the taxonomic ambiguities of the species *mussullah* through DNA barcoding. This indicates that the distribution of *Tor mussullah* and *Tor khudree* is confirmed in the Southern Western Ghats and also supports the views of Jayaram (2005). The differences between the two species are

obvious in this study. Comparative cytogenetic studies of *Tor khudree* and *Tor mussullah* using conventional staining and NOR banding also differentiated the two species of *Tor khudree* and *Tor mussullah* (Kushwaha *et al.*, 2001). It seems that the numbers of ichthyologists who have seen and examined the true *mussullah* are very few and the species is also very rare (Jayaram, 1997). The present study supports the views of Jayaram (1997; 2005 and 2010) who treated this species as *Tor mussullah* by following the discussions given in Hora (1943). Easa and Shaji (2003) also reported the presence of *Tor mussullah* from the River Chaliyar, Southern Western Ghats Kerala. The original suggestion of the genus *Hypselobarbus* by Bleeker (1860) with Syke's *mussullah* as its type species and the transfer of *mussullah* species under genus *Hypselobarbus* by Rainboth (1989) are based on the illustration by Sykes (1839) and not by examining specimens. Suggestions made by Hora (1943) that the species should be called *Tor mussullah* seems more valid as the species called as 'musunda' in the type locality of *Barbus mussullah* and neighboring areas is indeed a *Tor* species (Neelesh Dahanukar pers. obs.) (IUCN, 2017).

## Conclusion

The present study clearly shown that the *Tor mussullah* is a distinct species and which is differed from *Tor khudree.* Hence, it can be confirmed that the species should be called *Tor mussullah* seems more valid than *Hypselobarbus mussullah.* Due to habitat loss, destructive fishing, human interference and heavy utilization of *Tor* for food, sport and traditional medicine, a steady decline has been reported Indian rivers. The *Tor* species are under tremendous stress in Western Ghats and needs urgent attention to conserve this precious national icon for future generation.

## Ethical Statement

The authors are confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Department of Environmental Sciences, Bharathiyar University Coimbatore.

## Author Contributions

**Ambili, T.R**: Conceptualization, Investigation, Experiments, Original draft writing; **Sojomon Mathew**: Resources, Experiments, Supervision, Formal data analysis, Data curation; **Manimekalan A**: Conceptualization, Investigation, Writing - review and editing; **Shiny K. J.**: Formal data analysis, Data curation, Validation; **Elezabeth Basil**: Data curation, data editing, data analysis. All authors have read and agreed to the published version of the manuscript.

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## Conflict Of Interest

The authors declare no conflicts of interest.

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