Molecular phylogeny of Barbin fishes of North - East India based on mitochondrial 16SrRNA gene sequences

N. Sobita* and Ch. Basudha

ICAR-Research Complex for NEH Region, Manipur Centre Lamphelpat, Imphal -795 004, Manipur, India.

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ABSTRACT
The Barbin fishes (Cypriniformes: Barbinae) have achieved great diversity in the world and cosmopolitan in distribution but their phyletic classification is ambiguous. Molecular phylogeny of barbin fishes of North-East India was studied by using mitochondrial 16S rRNA sequences. Partial sequences (540-615 bp) of mit. 16S rRNA genes of four species of barbin fishes – Pethia atra, Puntius chola, P. javanicus, and P. sophore were generated and sequences of 7 species were downloaded from GenBank for present study. The aim of our present study is to resolve the taxonomic relationship and to establish the molecular phylogeny of Barbin fishes of North-East India based on mitochondrial 16S rRNA genes sequences. The Maximum Parsimony Tree shows three clusters with high bootstrap value and present data indicates that all fishes in this group had a common ancestor but now they have separated into distinct evolutionary lineages.

Key words: Barbinae, Mitochondrial 16SrRNA gene, Molecular phylogeny, Phylogeny, Taxonomic relationship.

INTRODUCTION
The fishes of Cyprinidae family are cosmopolitan but their phyletic classification is ambiguous. The present study provides the phylogenetic relationship among the members of Barbinae of North East India and it questions the monophyletic origin of Cyprinidae family. Phylogenetic analysis is conducted to identify the monophyletic analysis of the members of which share a common ancestors. To align a monophyletic group, one or more morphological characters have to be studied due to which the natural classifications of taxa were obtained but the molecular information provided an impartial characters on which the natural classification may be done. The fishes of the subfamily Barbinae have achieved great diversity in the world and it is one of the members of the family Cyprinidae of the order Cypriniformes. A total of 21 species of the Barbin fishes are found in North-East India which comprise of four genera, Hypsibarbus (1 species), Pethia (15 species), Puntius (3 species) and Schizothorax (2 species) (Vishwanath et al., 2014). Among these genera, the genus Puntius Hamilton-Buchanan is represented by a large number of species of Asian tropics. Most of the species have bright colours, can be maintained easily in aquarium, make them one of the most popular fresh water aquarium fishes. Many species are traded internationally as ornamental fishes (Collins et al., 2012). These species also comprise an important category of fish consumed as food either as sun-dried or used in fermented fish preparation. The fermented fishes are used as condiments in all sorts of curry preparations and thus popular and highly priced in North-East India.

*Corresponding author’s e-mail: n_sobita@yahoo.co.in
conservation. These markers generally exhibit 5 to 10 time
greater variability than single copy nuclear genes, and have
been used as a molecular tool for estimating phylogenetic
relations in various groups of species. For the molecular
phylogenetic study, mitochondrial 16S rRNA genes were
used in some fishes (Gilles et al., 1998; Li et al., 2013). These
genes are conserved and non-coding in nature which played
vital role in determination of new phylogenetic relationships
and in checking reliability of earlier established phyletic
classification.

The aim of our present study is to resolve the
taxonomic relationship of some cyprinid fishes of subfamily
Barbinæ under two genera Pethia and Puntiús of Manipur
region and to establish the molecular phylogeny of Barbin
fishes of North-East India based on mitochondrial 16S rRNA
genes sequences.

MATERIALS AND METHODS
Collection of fish samples: Live fish specimens of one
species of Pethia – Pethia atra and three species of Puntiús
- Puntiús chola, P. javanicus and P. sophore were collected
from different water areas of Manipur. A total number of
forty fishes (ten fishes for each species) were collected for
the present study. The morphological identification was done
according to Vishwanath and Laisram (2004). For molecular
identification, muscle tissue samples were collected and
preserved in 70% alcohol until used.

Isolation of genomic DNA: Total DNA were isolated by
using DNeasy Blood & Tissue Kit (Qiagen, Germany).

16S rRNA gene analysis: The mitochondrial 16S rRNA
gene was amplified using universal 16S rRNA primers
described in Palumbi, (1996). Thermal regime consisted of
initial denaturation of 95°C for 5 mins. , followed by 35
cycles of denaturation (95 ºC for 40 sec.), annealing for 30
sec. at 45ºC and extension 72 ºC for 40 sec. and post cycling
extension 72 ºC for 5 min. and held at 4°C.

DNA Sequencing: PCR products were visualized on 1.8%
agarose gels. The products were sequenced bi-directionally
by using an automated ABI 3100 Genetic Analyzer (Merck).

Phylogenetic analysis: DNA sequences were edited
manually and aligned using CLUSTAL W. Alignment was
then manually checked and corrected and deposited in NCBI
Genbank. Phylogenetic and molecular evolutionary analysis
was conducted using MEGA version 5 (Tamura et al., 2011).
The phylogeny was established using the maximum parsimony (MP method). The evolutionary distances were
calculated using the maximum composite likelihood method
which was shown by the units of the number of base
substitutions per site. All position containing gaps and
missing data were eliminated from data set (complete deletion).
The percentage of replicate trees in which the
associated taxa clustered together in the bootstrap test (1000
replicates) was shown next to the branches. The maximum
parsimony tree was obtained using the Sub-tree Pruning
Regrafting algorithm (SPR) with search level 1 in which the
initial trees were obtained with the random addition of
sequence (10 replicates). One outgroup Devario aequipinnatus
was included in alignment as a root of tree.

RESULTS AND DISCUSSION

Out of 21 species of Barbin fishes of North East
India, 4 species were collected from different water areas of
Manipur, India and partial sequences of mitochondrial 16S
rRNA gene sequences (540-615 bp) were generated. The
PCR amplified results are shown in Fig.1. The partial
sequence of mitochondrial 16S rRNA gene of Pethia atra,
Puntiús chola, Puntiús javanicus and Puntiús sophore were
generated and deposited in NCBI GenBank and their
Accession numbers are shown in Table 1. Eight Barbin fishes
namely Pethia chonchonius, P. gelius, P. phutunio, P.
stoliczkanus, Puntiús terio, Schizothorax labiatus and S.
richardsonii were downloaded from NCBI GenBank (Table
1). For rooting of the phylogenetic tree, Devario
aequipinnatus is used as outgroup.

Nucleotide Diversity: The nucleotide sequence length
varied from 406-615 bp. Here 595 characters were included
out of which 456 (76.63%) were conserved sites (monomorphic)
and 121(20.33%) were variable sites (polymorphic). Out of 121 variable sites 26 were parsimony
informative sites. The nucleotide diversity was \( \pi = 0.11315 \).
The nucleotide frequencies are 33.10% (A), 19.90% (T),
21.84% (C), and 25.16% (G). The transition/transversion
rate ratios are \( k_1 = 1.202 \) (purines) and \( k_2 = 4.812 \)
(pyrimidines). The overall transition/transversion bias is \( R
= 1.324 \), where \( R = [\text{A*G*} + \text{T*C*}] / (\text{A+G})(\text{T+C}) \)
and the bias was towards transitional mutations (Table 2) (Tamura
et al., 2011).

Phylogenetic analysis: The phylogenetic tree of Maximum
Parsimony (Fig. 2) was constructed from the combined
dataset of 16S rRNA consisting of 11 sequences aligned with
one outgroup as a root (Devario aequipinnatus). From
dendrogram it is clear that all the genera of Barbinæ were

Table 1: List of fish species and their GenBank Accession Numbers

<table>
<thead>
<tr>
<th>Name of fish species</th>
<th>GenBank Accession Number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pethia atra</td>
<td>KP276243</td>
<td>This study</td>
</tr>
<tr>
<td>Pethia conchonius</td>
<td>PK12683</td>
<td>NCBI GenBank</td>
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<tr>
<td>Pethia gelius</td>
<td>JX16143</td>
<td>NCBI GenBank</td>
</tr>
<tr>
<td>Pethia phutunio</td>
<td>KP712608</td>
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<td>Pethia stoliczkanus</td>
<td>KP71685</td>
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<td>Puntiús chola</td>
<td>KJ751550</td>
<td>This Study</td>
</tr>
<tr>
<td>Puntiús javanicus</td>
<td>KJ611246</td>
<td>This Study</td>
</tr>
<tr>
<td>Puntiús sophore</td>
<td>KJ577617</td>
<td>This Study</td>
</tr>
<tr>
<td>Puntiús terio</td>
<td>KP712675</td>
<td>NCBI GenBank</td>
</tr>
<tr>
<td>Schizothorax labiatus</td>
<td>JX507507</td>
<td>NCBI GenBank</td>
</tr>
</tbody>
</table>
| Schizothorax
richardsonii | JX204412                 | NCBI GenBank |
| Devario aequipinnatus | KJ590087               | NCBI GenBank |
differentiated into 3 clusters with Devario aequipinnatus as root.

In cluster 1 the four species of the genus Puntius were clustered with 88%, 99% and 80% bootstrap values. In cluster 2, five species of the genus Pethia were clustered and among these species, Pethia gelius is closely related with the species of the genus Puntius. In cluster 3, two species of the genus Schizothorax were clustered with bootstrap value of 99%. The maximum parsimony tree is paraphyletic in nature as also reported by Li et al. (2013) and cluster 1 and 2 consist of fishes of interest which shows that these fishes are from same lineage and may share a common ancestors. Genetic diversity among the four species shows that Puntius sophore is closely related with Puntius chola (Table 3) whereas Puntius javanicus is distantly related with Puntius chola. A higher value for nucleotide diversity $\pi = 0.11315$ indicates high genetic diversity among the sequences. The transition/transversion bias $R=1.324$ deviate from the neutral evolution ($R = 0.5$) (Li et al., 2013). The reason behind this deviation may be due to the structure of nucleotide bases and the complementary base pairing as discussed by Topal and Fresco (1976) and this was also established by deviation of bias towards transitional mutation (Table 4). The higher value of transitional mutations reflects that the fishes are under selection procedure as observed by Rosenberg et al. (2003). Phyletic classification based on morphological characters was not clear representative but genetic differentiation was clearly reflected by molecular method. The data gathered is showing that all fishes in this group had a common ancestor but now they have separated into distinct evolutionary lineages.

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REFERENCES


