Research Article

Stock discrimination of *Barilius vagra* (Hamilton, 1822) (Teleostei: Danionidae) inhabiting the Alaknanda and Chenab River basins of India using mitochondrial COI gene

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Abstract: *Barilius vagra* is an important freshwater fish found in the streams of hilly region of the Indian Himalaya consumed by local inhabitants. Genetic characterization of *B. vagra* was done using COI gene sequences of mitochondrial DNA (mt DNA). The COI gene of 18 individuals collected from two geographically isolated river basins of Indian Himalaya was sequenced. 18 sequences having more than 640 base pairs of *B. vagra* revealed the nucleotide diversity to be 0.00169 and 0.00330, respectively, from the Alaknanda and Chenab River basins. The haplotype gene diversity was found to be 0.806 and 0.833 for *B. vagra* from the two river basins, respectively. The maximum likelihood phylogenetic tree revealed the presence of two separate genetic stocks of *B. vagra* from the Alaknanda and Chenab River basins. The results of the present study suggested that the geographically isolated populations of *B. vagra* have developed significant divergence amidst the basins. This information would be useful for planning effective management strategies for conservation of hill trouts.

Keywords: COI gene, Haplotype, Indian Himalaya, Maximum likelihood tree.


Introduction

*Barilius vagra* (Hamilton, 1822), a member of family Danionidae is called as ‘hill trout’ along with other species of this genus. *Barilius* species, an important candidate for aquaculture is mostly found in shallow, clear and cold water of spring-fed streams of the Himalayan region of India (Gurung et al. 2005). Traditionally morphometric measurements were mostly used by ichthyologists for identification of fishes (Nayman 1965) but presently because of morphological plasticity it is very difficult to identify the fishes only by using morphometric characters (Victor et al. 2009). Sometimes using only morphometric characters for identification of the species of same genus is troublesome as in case of *Schizothorax plagiostomus, S. richardsonii* and *S. progastus*. Mitochondrial DNA analysis of various organisms played a crucial role in solving the taxonomical problems, population characterization, evolution and systematics (Gold et al. 1990; Barat & Sahoo 2007; Sahoo et al. 2007; Pradeep et al. 2011; Barat et al. 2012). The fast rate of mitochondrial DNA evolution coupled with maternal inheritance have made mt DNA an extremely useful genetic system for studying gene flow, hybrid zones, population structure and other population regarded studies. Different mitochondrial genes have been used to determine the variation at the intraspecific and interspecific level in fishes.

Information on stock structure is required for the conservation and management of native populations of *B. vagra*. Muneer et al. (2009) reported that the information of stock structure is vital for planning management and conservation of natural resources, besides it is useful in genetic improvement programs. Till date the studies on the stock discrimination of
Table 1. GPS coordinates of the sampling sites on the Alaknanda and Chenab River basins of the Indian Himalaya.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Alaknanda River (Central Himalaya)</th>
<th>Chenab River (Western Himalaya)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latitude °N</td>
<td>Dugadda 30°15′31″</td>
<td>Khankhra 30°14′32″</td>
</tr>
<tr>
<td>Longitude °E</td>
<td>Khandah 30°11′47″</td>
<td>Dhudhar 32°55′15″</td>
</tr>
<tr>
<td>Altitude (m. a.s.l.)</td>
<td>Jhajjar 78°46′34″</td>
<td>Jhuni 75°01′42″</td>
</tr>
<tr>
<td></td>
<td>Jhuni 78°55′36″</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jhuni 78°55′36″</td>
<td></td>
</tr>
</tbody>
</table>

B. vagra using molecular tools are very limited. Hence, the present study was undertaken with the objective of identification and discrimination of B. vagra stock using COI gene, to infer phylogenetic status and to study genetic divergence among the stocks of the Alaknanda and Chenab Rivers.

Materials and Methods

Sample collection: The 18 samples of B. vagra were collected from two different river basins, Alaknanda and Chenab by hiring local fishermen. Three sampling sites, Dudhar, Jhajjar and Jhuni were selected on the Chenab River basin while other three, Dugadda, Khandah and Khankhra were selected on the Alaknanda River basin. The GPS coordinates of the sampling sites are depicted in Table 1. Specimen identification was done by using the taxonomic keys of Day (1878), Talwar & Jhingran (1991), Mirza (1991) and Kullander et al. (1999). After proper identification the samples were stored in 95% ethanol for molecular analysis.

DNA extraction, PCR amplification and Purification:
The DNA was isolated using Qiagen DNA isolation purification kit following the manual provided by the manufacturer. The mitochondrial gene cytochrome c oxidase subunit I (COI) was amplified with primers FishF1-CGTATTTTTTGGTGGAGCCG and FishR-AATAAGCCCAAACCCGGGAA in a 50ml volume with 100ng template DNA, 10 pmole of each specific primer, 250mM of each dNTPs, 1.0U of Taq DNA polymerase and 1_Taq buffer containing 1.5mM MgCl₂. The PCR conditions were consisted of Initial Denaturation at 94°C for 3min, Denaturation at 94°C for 20s 35 cycles, Annealing at 54°C for 30s, Extension at 72°C for 45s and Final extension at 72°C for 10min. To visualize the PCR products, 1.2% agarose gel was used, the amplicons were purified. After successful amplification of DNA, sequence reactions were performed via outsourcing to Xcelris Lab, Pvt. Ltd. Gujarat, India.

Sequences confirmation, submission and analysis:
The COI sequence confirmation was carried out using BLAST programme of NCBI. Sequin protocol of NCBI was followed properly for the submission of sequences to the NCBI nucleotide data base. Software programme Clustal W (Thompson et al. 1997) was used for trimming and alignment of raw sequences. The number of polymorphic sites, variable sites, parsimony sites and nucleotide composition were determined by DNA sp ver. 3 (Rozas 1999). Estimation of evolutionary distance, sequence divergence and construction of Maximum Likelihood tree was done using MEGA 6.0 (Tamura et al. 2013). Some other sequences of the genus Barilius were taken from NCBI GenBank to increase the resolution among the species.

Results
All the analysed sequences of B. vagra (Table 2) showed more than 98% similarity and query coverage greater than 95% with the previously published COI sequences of B. vagra in the NCBI’s nucleotide data base. The nucleotide composition of B. vagra collected from the Alaknanda River basin was 29.6% (T), 27.5% (C), 24.0% (A), 18.9% (G) while it was 29.6% (T), 27.7% (C), 24.6% (A), 18.2% (G) for the species collected from the Chenab River basin. The nucleotide diversity was found to be 0.00169 and 0.00330 while haplotype gene diversity was 0.806 and 0.833 for B. vagra from the Alaknanda
and Chenab River basins, respectively. The calculated or estimated Transition/Transversion bias (R) was found to be 5.912 (Table 3). After eliminating gaps and missing data total 591 positions in the final data set of *B. vagra* were noticed. The average evolutionary distance among the sequences of *B. vagra* was 0.015. Maximum likelihood type of phylogenetic tree constructed by COI gene sequences revealed that 9 sequences of *B. vagra* collected from the Alaknanda River basin were clustered in a single clade while the other 9 sequences of *B. vagra* formed another clade (Fig. 1). Similar results were obtained through Neighbour joining linkage and minimum evolutionary and phylogenetic method.

**Table 2.** Accession numbers and base pairs of *Barilius vagra* collected from different sampling locations in the Alaknanda and Chenab River basins.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Species name</th>
<th>Voucher ID</th>
<th>Accession number</th>
<th>Base pair length</th>
<th>Location/Basin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Barilius vagra</em></td>
<td>BV1D</td>
<td>MF445037</td>
<td>654</td>
<td>Dudhar stream/ Chenab</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>BV2D</td>
<td>MF445038</td>
<td>645</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>BV3D</td>
<td>MF445039</td>
<td>654</td>
<td></td>
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<tr>
<td>4</td>
<td></td>
<td>BV1J</td>
<td>MF445040</td>
<td>654</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>BV2J</td>
<td>MF445041</td>
<td>647</td>
<td>Jhajjar stream/ Chenab</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>BV3J</td>
<td>MF445042</td>
<td>658</td>
<td></td>
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<tr>
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<td></td>
<td>BV1K</td>
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<tr>
<td>8</td>
<td></td>
<td>BV2K</td>
<td>MF445044</td>
<td>657</td>
<td>Jhuni stream/ Chenab</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>BV3K</td>
<td>MF445045</td>
<td>653</td>
<td></td>
</tr>
<tr>
<td>10</td>
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<tr>
<td>11</td>
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<td>B</td>
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<tr>
<td>12</td>
<td></td>
<td>C</td>
<td>MK188888</td>
<td>667</td>
<td></td>
</tr>
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<td>13</td>
<td></td>
<td>D</td>
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<td></td>
<td>E</td>
<td>MK188890</td>
<td>667</td>
<td>Dugadda stream/ Alaknanda</td>
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<tr>
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<td></td>
<td>F</td>
<td>MK188891</td>
<td>667</td>
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<td>16</td>
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<td>669</td>
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</tr>
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<td>17</td>
<td></td>
<td>H</td>
<td>MK188893</td>
<td>669</td>
<td>Khankhra stream/ Alaknanda</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td>I</td>
<td>MK188894</td>
<td>668</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.** Maximum composite likelihood estimate pattern of nucleotide substitution for *Barilius vagra*.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>T</th>
<th>C</th>
<th>G</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>-</td>
<td>2.06</td>
<td>1.97</td>
<td><strong>23.28</strong></td>
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<tr>
<td>T</td>
<td>1.73</td>
<td>-</td>
<td><strong>15.41</strong></td>
<td>1.29</td>
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<tr>
<td>C</td>
<td>1.73</td>
<td><strong>16.07</strong></td>
<td>-</td>
<td>1.29</td>
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<tr>
<td>G</td>
<td><strong>31.14</strong></td>
<td>2.06</td>
<td>1.97</td>
<td>-</td>
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</tbody>
</table>

**Discussion**

In order to study the population genetics of various fish species molecular markers have been used (Linda & Paul 1995). Mitochondrial DNA is now emerging as the most preferred choice for studying both population structure and genetic variability due to rapid evolutionary rate and complete maternal inheritance (Song et al. 2008). Barat et al. (2013) characterized two coldwater fishes *B. bendelisis* and *Acanthocobitis botia* using cytb gene and karyotyping, while Mishra et al. (2012) did molecular characterization of two hill trouts *B. bendelisis* and *B. barna* using RAPD markers. Sah et al. (2011) postulated that no detail information is available on the genetic diversity of *Barilius* species. The COI gene was also used by Sun et al. (2013) and Peng et al. (2009) to analyse the genetic variation in *Eletheronema rhadinum* and *Pampus argenteus*.

Based on the COI analysis, Ward et al. (2005) reported that the overall GC content was higher 42.2 to 47.1% in fishes. Jones & Avise (1998) found that
the sequences of cytb gene in *B. bendelisis* were A+T rich (51.2%). Sun et al. (2013) estimated the genetic diversity in *E. rhadinum* using COI gene and found average content of A, T, G, and C to be 29, 23, 24.6 and 22%, respectively which was also A+T rich. In the present study, the nucleotide composition of *Barilius vagra* collected from the Alaknanda River basin was 29.6% (T), 27.5% (C), 24.0% (A), 18.9% (G) while it was 29.6% (T), 27.7% (C), 24.6% (A), 18.2% (G) in the fishes collected from Chenab River basin. The nucleotide composition was also A+T rich to be 53.6 and 54.2, respectively from two different river basins of Indian Himalaya.

The transition versus transversion ratio (5.912) in COI sequences of studied *B. vagra* species in the present study was found in harmony with those of many other fishes (Ward et al. 2005; Junbing et al. 2008; Lakra et al. 2011). The average genetic distance among the sequences of *B. vagra* was found to be 0.015. Similar observations were recorded in various studies where they proved the genetic distance to be lesser within the genus than between the genus (Hebert et al. 2003, Borda & Siddall 2004). Lack of genetic differentiation within species on macrogeographic scale in marine fishes was observed by Dudgeon et al. (2000), Bernardi et al. (2001), Palumbi (1994) and Hellberg et al. (2002) while Planes et al. (2001) and Santos et al. (2006) reported deep intraspecific genetic divergence in species showing restricted migratory behaviour.

**Fig.1.** Maximum Likelihood type of phylogenetic tree for *Barilius vagra* collected from two different river basins.
which also limit gene flow among individuals. Certain other studies also proven that intraspecific variation of COI barcodes is small and clearly discriminable from interspecific variation (Hubert et al. 2008; Rock et al. 2008; Swartz et al. 2008; Steinke et al. 2009; Ward et al. 2009; Barbuto et al. 2010; Bucklin et al. 2011; Wong et al. 2011). In the present study, two main clades for B. vagra, one from the Alaknanda River basin and other from the Chenab River basin were shown by Maximum likelihood phylogenetic tree revealing genetic differentiation in B. vagra populations collected from two different river basins of India.

Lakra et al. (2009) reported that the success of conservation programs and effective management policies depend on the level of genetic divergence within and between species and developing strategies to maintain the natural genetic diversity. The mitochondrial DNA (COI gene) represents an important tool for the characterization of distinct populations for conservation, breeding and management programs. Thus, the COI sequences data will have a wide application in planning breeding programs for aquaculture importance and conservation strategies of B. vagra. The results of the present study demonstrated the utility of partial COI mt DNA sequence to determine intraspecific genetic diversity and discriminate genetic stocks in the wild populations of B. vagra which may be useful in case of other fishes.

Acknowledgements
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References


273-290.


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مقاله پژوهشی

تربیک سنی و پارامترهای رشد دو گونه از آب‌های ترنگانو، مالزی (هامورماهیان استخوانی عالی: هامورماهیان)

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چکیده:
سن و رشد دو گونه از هامورماهیان Epinephelus areolatus و E. sexfasciatus از آب‌های ترنگانو در مالزی با استفاده از برش گیری عرضی از اتولیت تخمین گردید. تعداد 795 نمونه به‌طور ماهیانه و به مدت 12 ماه از فوریه 2014 تا ژانویه 2015 از دو اسکله ماهی‌گیر ترنگانو جمع‌آوری گردید. نتایج نشان داد که سن ماهیان مورد مطالعه برای گونه E. areolatus و E. sexfasciatus، 1-9 و 2-9 بودند. متناسب بودن داده‌های طول درسن ماهیان با مدل رشد von Bertalanffy نشان می‌دهد که برای گونه E. areolatus مقدار TL0 = 86.66 [e(0.44(t-1)) - 1] و برای گونه E. sexfasciatus مقدار TL0 = 33.86 [e(0.86(t+2.32)) - 1] است. نسبت بالای ماهی نابالغ در صید و کمبود ماهیان بالغ نشان می‌دهد که جمعیت گونه E. sexfasciatus بالغ در معرض خطر است و اقدامات مدیریتی فوری برای کاهش صید ماهیان نابالغ و نیز برای حفظ ذخایر ماهیان بالغ و سایر هامورماهیانی که ارزش تجاری دارند، مورد نیاز است.

کلمات کلیدی: شیلات مالزی، هامورماهیان، اتولیت، ماهیان صخره‌ای، عملکرد رشد فون برتالانفی.