Genetic variation between molly fishes *Poecilia latipinna* and *Poecilia sphenops* using RAPD assay

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Abstract

Investigation of genetic variations for *Poecilia latipinna* and *Poecilia sphenops* from different locations of Chennai, Tamil Nadu was performed using Random Amplified Polymeric DNA (RAPD) assay. High degree of polymorphism was observed, suggesting the degree of genetic variability between *P. latipinna* and *P. sphenops*. The low levels of genetic variation within-species are due to their limited migration and pair fidelity mode of reproduction. Random primer RAPD3 seems to be a good candidate for developing markers. The dendrogram obtained from RAPD clearly depicts that *Poecilia* sp. are closely related to each other where *P. latipinna* shows 57% of variation from dendrogram scale.

Keywords: Genetic variations, *Poecilia* sp., RAPD, polymorphism, random primer, dendrogram, marker.

Introduction

In recent years, a wide range of new molecular techniques have been explored and reported for fishes and shellfishes (Lehmann et al., 2000; Jayasankar, 2004). Several DNA techniques for evaluating genetic variability in fish species are available and are widely used (Harris et al., 1991; Mjolnerod et al., 1997; Coughlan et al., 1998; Wasko and Galetti, 2002; Barman et al., 2003; Matoso et al., 2004; Jayasankar, 2004). One such technique is the Random amplified polymorphic DNA (RAPD) which was first introduced by Williams et al. (1990). It is a technique based on the PCR amplification of discrete regions of genome with short oligonucleotide primers of arbitrary sequence (Welsh and McClelland, 1990; Williams et al., 1990). It utilizes single, arbitrary, decamer DNA oligonucleotide primers to amplify regions of genome based on the polymerase chain reaction (Hadrys et al., 1992; Williams et al., 1993). The characters assessed through RAPD are useful for genetic studies because they provide various types of taxonomic population, inheritance pattern of various organisms including fishes (Brown and Epifanio, 2003; Degani, 2004; David and Pandian, 2006).

Genetic variability on an endangered Neotropical fish species were determined by RAPD analysis which reveals variation between the species (Wasko and Galetti, 2002). Genetic monitoring of the Amazonian fish *Brycon cephalus* were studied using RAPD markers which determine the usefulness of genetic management and biodiversity conservation of this species (Wasko et al., 2004). Genetic diversity of three cultured populations of goldfish were studied which revealed poor genetic diversity of goldfish (Xi-dong et al., 2007).

RAPD analysis of two different populations of cultured Korean catfish *Silurus asotus* were determined which revealed genetic similarity in cultured catfish populations (Yoon and Kim, 2001). Population structure and phylogenetic relationships among the Spanish Barbus species were analyzed RAPD technique (Callegas and Ochando, 2002). The patterns of morphometric and genetic variation of three species of Garra were studied using RAPD analysis which revealed that *G. mulya* and *G. kalakadensis* hold similar characters compared to the other congener, *G. gotylastenorhynchus* (Dhinakaran et al., 2011). Genetic variations among Nile Tilapine fishes were assessed by RAPD revealed that the different primers have different performances for evaluation of genetic polymorphism (El-Alfy et al., 2009). DNA fingerprinting of eight cyprinid fish species of Iraqi Inland waters using RAPD-PCR revealed that the eight cyprinid taxa are distinctive species (Faddagh et al., 2012). Inheritance of RAPD markers in the Guppy fish, *Piecilia reticulata* was assessed using RAPD analysis which determined higher genetic variability between the two varieties and low intra variety genetic variability (Foo et al., 1995). Ten freshwater fishes *Mylopharyngodon piceus*, *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix*, *Aristichthys nobilis*, *Cyprinus carpio*, *Carassius auratus*, *Mygobrama ambycephala*, *Silurus asotus*, *Myxocyprinus asiaticus* and *Peteobargrus fulvidraco* were identified using RAPD analysis (Huai et al., 1998). Morphological and genetic variability of Malaysian *Channa* sp. were assessed using RAPD technique (Husin and Norainy, 2007). The genetic structure of the fish *Astyanax altiparanae* populations were analyzed using RAPD technique which revealed structures regarding fish handling and conservation programs (Leuzzi et al., 2004).
Gene mapping and genetic variation of channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) were determined using RAPD analysis which shows high level of polymorphism (Liu *et al.*, 1999). Genetic diversity of three ornamental reef fishes from the Brazilian coast were determined by RAPD technique revealed genetic variation in the three species were related to intra-population diversity (Affonso and Galetti, 2007). Genetic variation of three Norwegian *Gyrodactylus salaris* populations were determined using RAPD analysis revealed polymorphism between different populations of *G. salaris* (Cunningham and Mo, 1997).

From the above literature, the reports on the genetic variability are very scanty in molly fishes. Hence, the present study is aimed to analyze genetic variability in *Poecilia latipinna* and *Poecilia sphenops* species using RAPD analysis.

**Materials and methods**

*Collection and maintenance of fishes: Poecilia latipinna* and *P. sphenops* were collected at two different geographical locations from Kolathur (13°7'1.25"N, 80°12'41.18"E) and Perungalathur (12°54'18.36"N, 80°5'41.28"E), Chennai. They were brought to the laboratory in a plastic bag containing fresh water with continuous aeration and maintained in glass tank (20 x 10 cm) with 15 cm column of fresh water. The water was aerated continuously and changed every day. These fishes were fed with fish feed and were acclimatized for 2-3 d in prevailing room temperature. Only healthy fishes weighing 1.82 g were used for experimental analysis.

*Quantitative and qualitative determination of DNA:* Approximately 0.3 g of molly fish muscle tissues were dissected and homogenized with 2 mL of lysis buffer and 0.01% of proteinase K using mortar and pestle. The DNA was isolated according procedure of Wu *et al.* (1995). The DNA pellet was washed with 70% ethanol and suspended in 20 µL of 1xTE buffer and stored at -20°C until required. DNA quality was assessed by using Double beam Spectrophotometer R2203 according to Sambrook *et al.*, (1989). DNA concentration of DNA = A\text{260}\times 50 \text{ µg} \times \text{ dilution factor}

*Purity of the DNA:* A\text{260}: A\text{280} ratio = A\text{260}/A\text{280}

DNA fragment was electrophoretically analyzed through 0.8% agarose gel containing ethidium bromide stain at 50 V and visualized using a LARK UV transilluminator (Sambrook *et al.*, 1989).

*Ribonuclease (RNase) treatment:* DNA samples (20 µL) were made up to 400 µL with 1xTE buffer (pH 7) and 20 µg/mL concentration of RNase solution were added to the reaction tubes and the samples were incubated at 37°C for 1 h. The supernatant were extracted once with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged (12000 rpm, 10 min). The samples were precipitated with double the volume of absolute alcohol (12000 rpm, 15 min). The DNA pellets were air-dried and dissolved in 20 µL of 1xTE buffer and stored at -20°C until required. The concentration of extracted DNA was determined spectrophotometrically at 260 and 280 nm and electrophoretically analyzed through 0.8% agarose gel containing ethidium bromide stain and visualized using a UV transilluminator (Sambrook *et al.*, 1989).

*Screening of RAPD primers:* Under optimized PCR conditions, random primers RAPD1, RAPD3, RAPD5, RAPD6, RAPD7, RAPD8, RAPD9 and RAPD10 were used for screening. Primers were chosen for further studies based on the criterion that the DNA patterns were consistent in all trials.

*PCR amplification:* RAPD-PCR was performed in an Eppendorf Master Cycler personnel thermo cycler at 94°C for 3 min, 94°C for 45 sec, 37°C for 1 min, 72°C for 1 min, 94°C for 45 sec (repeated 39 times), 72°C for 7 min and hold at 4°C. Optimal program parameters were selected on the basis of consistent amplification at all DNA concentrations in addition to increased intensity and clarity of the banding pattern. The resulting products were electrophoretically analyzed through 1.5% agarose gels stained with ethidium bromide and visualized using a UV transilluminator.

*RAPD dendrogram:* Dendrogram for RAPD was constructed using SPSS (version 16, 2007) software tool.

**Results**

*Quantitative determination of DNA:* Spectrophotometric analysis of the DNA samples showed the ratio of DNA samples of *P. latipinna* and *P. sphenops* of Kolathur and Perungalathur as 1.7032, 1.7088, 1.7078 and 1.6910 respectively. RNase treatment was done to remove the RNA present along with genomic DNA band. Before RNase treatment there is presence of RNA along with genomic DNA band (Fig. 1) and after RNase treatment there is absence of RNA band and the presence of only the genomic DNA band (Fig. 2) were noted.

*Screening of RAPD primers:* Under optimized PCR conditions, random primers RAPD1, RAPD3, RAPD6, RAPD7, RAPD8, RAPD9 and RAPD10 were used for screening the DNA of *P. sphenops* (Kolathur, Chennai) and *P. latipinna* (Kolathur, Chennai). Among the primers tested RAPD1, RAPD3, RAPD7 were found to be suitable for further investigation (Fig. 3).
Primers were chosen for further studies based on the criterion that the DNA patterns were consistent in each trials.

**RAPD analysis:** A total of 65 scorable bands ranging from 100 to 2500 bp were observed from the RAPD analysis of *P. latipinna* and *P. sphenops* using three oligonucleotide primers RAPD1, RAPD3, and RAPD7 (Fig. 4). The number of amplified bands in all investigated samples was 13 bands for primers RAPD1, 13 bands for RAPD3 and 9 bands for RAPD7. Thirteen polymorphic bands were seen in RAPD1, monomorphic bands are absent; 11 polymorphic band and 2 monomorphic bands were seen in RAPD3; 1 monomorphic band and 8 polymorphic bands were seen in RAPD7. The percentage of polymorphic band of RAPD1, RAPD3 and RAPD7 is given in Table 1.
The genetic distances between the molly fishes within the same species was much lower between different species (Table 3).

**Table 3.** Similarity index, genetic distance and phylogenetic relationships:

<table>
<thead>
<tr>
<th>Species/Location</th>
<th>Similarity index</th>
<th>Genetic distance</th>
<th>Phylogenetic relationship</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perungalathur</td>
<td>0.571</td>
<td>0.63</td>
<td>57%</td>
</tr>
<tr>
<td>Kolathur</td>
<td>0.71</td>
<td>0.55</td>
<td>47%</td>
</tr>
<tr>
<td>Chennai</td>
<td>0.47</td>
<td>0.30</td>
<td>39%</td>
</tr>
</tbody>
</table>

**Discussion**

The present study documents the genetic variability within and between the species of Molly fishes *P. latipinna* and *P. sphenops* from Kolathur and Perungalathur Chennai using RAPD technique. In capture fishery, excessive exploitation combined with poor fishery management results in the depletion of the fishery stocks, such depletions can result in the loss of total gene pool (Nelson and Soule, 1987; Smith et al., 1991). In this investigation, most of the genetic characteristics of fishes were similar and often over-lapped with population. These data are not enough to support the established genetic structure of the population that often leads to taxonomic uncertainty (Daniel, 1997; Ponniah and Gopalakrishnan, 2000).

Allozymes and morphometric analyses were used to discriminate Hilsa populations which were collected from nine different sites within Bangladesh (Salini et al., 2004). RAPD was used in the preliminary inferences concerning genetic variability between two species of crayfish (*Cambarus maculatus* and *Astacus astacus*) and two species of fish (*Carassius auratus* and *Poeckilia latipinna*). This technique is based on the detection of polymorphisms, bands observed in some individuals but absent in others, which are amplified by random primers during the PCR. The primers RAPD1, RAPD3 and RAPD7 which produced different RAPD fragment patterns. The number of amplified bands detected varied, depending on the primers and species. To ensure that the amplified DNA bands originated from genomic DNA, not from primer artifacts, negative control was carried out for each primer species combination. No amplification was detected in the control reactions. All amplification products were found to be reproducible when reactions were repeated using the same reaction conditions. A high degree of polymorphism was observed, suggesting a high degree of genetic variability between *P. latipinna* and *P. sphenops*.

**Table 1.** Sequence of oligonucleotide primers, sizes and numbers of scorable RAPD bands and percentage of polymorphic bands resulting from RAPD analysis using primers RAPD1, RAPD3, and RAPD7.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Size range (no. of Scorable bands)</th>
<th>Percentage of polymorphic band (no. of polymorphic bands)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD1</td>
<td>TCTCAGCATG</td>
<td>200-2500 (13)</td>
<td>100 (13)</td>
</tr>
<tr>
<td>RAPD3</td>
<td>TAGGTCTCTG</td>
<td>100-2500 (13)</td>
<td>84.61 (11)</td>
</tr>
<tr>
<td>RAPD7</td>
<td>CGGATATCG</td>
<td>200-1200 (9)</td>
<td>88.88 (8)</td>
</tr>
</tbody>
</table>

**Table 2.** Molecular diagnostic key of *P. latipinna* and *P. sphenops* based on RAPD analysis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>RAPD marker size</th>
<th>Species/Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD1</td>
<td>300 bp</td>
<td><em>P. sphenops</em> (Perungalathur)</td>
</tr>
<tr>
<td></td>
<td>550 bp</td>
<td><em>P. latipinna</em> (Perungalathur)</td>
</tr>
<tr>
<td></td>
<td>1200 bp</td>
<td><em>P. latipinna</em> (Perungalathur)</td>
</tr>
<tr>
<td>RAPD3</td>
<td>550 bp</td>
<td><em>P. latipinna</em> (Kolathur and Perungalathur)</td>
</tr>
<tr>
<td></td>
<td>1200 bp</td>
<td><em>P. latipinna</em> (Kolathur and Perungalathur)</td>
</tr>
<tr>
<td></td>
<td>2500 bp</td>
<td><em>P. latipinna</em> (Kolathur and Perungalathur)</td>
</tr>
<tr>
<td>RAPD7</td>
<td>550 bp</td>
<td><em>P. latipinna</em> (Kolathur and Perungalathur)</td>
</tr>
<tr>
<td></td>
<td>1200 bp</td>
<td><em>P. latipinna</em> (Perungalathur)</td>
</tr>
</tbody>
</table>

Species specific markers: Several RAPD fragments showed fixed frequencies in each of particular species. Different bands were seen in *P. latipinna* and *P. sphenops* of Perungalathur and Kolathur using primer RAPD1, RAPD3 and RAPD7 which is represented in Table 2.

Similarity index, genetic distance and phylogenetic relationships: Similarity index within the same species of molly fish was between 0.571 and 0.666 (Table 3). The genetic distances between the molly fishes within the same species was much lower between different species (Table 3).

**RAPD dendrogram:** The phylogenetic tree constructed from genetic distances showed that the dendrogram is divided into two major clusters for *P. sphenops* and *P. latipinna* molly fish species. *Poecilia sphenops* of Kolathur and Perungalathur showed 71% relationship whereas *P. latipinna* of Kolathur and Perungalathur showed 57% relationship. *Poecilia sphenops* from Kolathur and Perungalathur forms single cluster and *P. latipinna* from Kolathur and Perungalathur forms another cluster. Genetic difference between *P. latipinna*, *P. sphenops* from Kolathur and Perungalathur is 39% based on the distance scale (Fig. 5).

**Fig. 5.** RAPD dendrogram.
The presence of variability among populations as well as individuals within a population is essential for their ability to survive and successfully respond to environmental changes (Ryman et al., 1995). Intra-population genetic variation in tilapia was studied using different RAPD primers (Bardakci and Skibinski, 1994). This technique is more sensitive than the mt-DNA analysis, which failed to reveal variations within the tilapia populations (Capili, 1990; Seyoum and Kornfield, 1992). Genetic variation was studied between four different populations of Hilsa Sha from Ganga, Yamuna, Hoogly and Narmada rivers of India using RAPD technique (Brahmane et al., 2006). Thus, RAPD has been used in population studies in fisheries and can be used efficiently for variation analysis of populations with differential degrees of geographic isolation.

The present study is the first report on the use of RAPD markers for studying genetic variation in molly fishes. The low levels of within-species genetic variation exhibited in *P. latipinna* and *P. sphenops* are due to their limited migration and pair fidelity mode of reproduction. Similar observations were reported by Barman et al. (2003) in carp species. The main objective of the study was to evaluate the level of variation and to identify species diagnostic markers of *P. latipinna* and *P. sphenops*. RAPD as a rapid method for developing genetic variability developed unique molecular markers for *P. latipinna* and *P. sphenops*. Species-specific RAPD markers were observed using three random primers. Random primer RAPD3 seems to be a good candidate for developing markers. The genetic distance is more between genus than between species and the hypothesis is also proved in this study by this marker. This was also proved in earlier studies in Indian major carps (Barman et al., 2003). Dendrogram was constructed from similarity matrix values using UPGMA algorithm. A statistical software package SPSS version 16 was used to developed dendrogram. The dendrogram obtained from the RAPD data clearly depicts the relationships among *P. latipinna* and *P. sphenops*. The dendrogram divides into two major clusters containing *P. latipinna* and *P. sphenops* together. *Poecilia sphenops* sp. was closely related to each other. *Poecilia latipinna* species shows 57% of variation from dendrogram scale. This again reflects the RAPD results.

SPSS has been used to interpret RAPD results for various organisms like genetic diversity of cultivated olives (Sesli et al., 2010). The SPSS as a ‘system of integrated packages’ can deepen the understanding of the studied topic and give better insight in to solving complex problems.

**Conclusion**

RAPD analysis is a rapid and convenient technique for estimating genetic variation between *P. latipinna* and *P. sphenops* and to generate useful genetic markers in molly fishes. RAPD fragments observed in the two individuals, showed a reasonable degree of genetic variation within and between the species. The population specific bands could not be discerned from the fragment patterns generated. This observation clearly indicated that, both the population’s genetic similarity index and genetic distance within the species.

**Acknowledgements**

Authors are thankful to UGC-SAP, New Delhi, India for financial assistance.

**References**


Table 3. Similarity index and genetic distance within the species of *P. latipinna* and *P. sphenops* analyzed by RAPD1, RAPD3 and RAPD7.

<table>
<thead>
<tr>
<th>Species</th>
<th>Average no. of bands per primer</th>
<th>Total no. of monomorphic/Polymorphic bands</th>
<th>Percentage of polymorphic bands</th>
<th>Similarity index within the species</th>
<th>Genetic distance within the species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. sphenops</em></td>
<td>6.66</td>
<td>10/10</td>
<td>50</td>
<td>0.666</td>
<td>0.334</td>
</tr>
<tr>
<td><em>P. latipinna</em></td>
<td>8.33</td>
<td>10/15</td>
<td>60</td>
<td>0.571</td>
<td>0.429</td>
</tr>
</tbody>
</table>

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