Phylogenetic relationship of Sailfin (Poecilia latipinna) and Shortfin (Poecilia sphenops) mollies (Order: Cyprinodontiformes) as inferred from the mitochondrial cytochrome gene sequence

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Poeciliid fishes are used as model organisms for experimental studies on natural and sexual selection. The present study is aimed at phylogenetic analysis of Poeciliid fishes, focussing on P. latipinna (Sailfin) and P. sphenops (Shortfin). This paper reports result of phylogenetic analyses based upon nucleotide sequence data from the mitochondrial COI gene of Poeciliids. Briefly, PCR amplification of DNA was carried out using the COI gene primers and sequences were analysed using BioEdit, ExPASy, GeneDoc and Mega 5.0. Partial COI amplicon of 595 bp could be recovered from P. sphenops (JF752336) and 645 bp amplicon from P. latipinna (JF752337) respectively. Phylogenetic relationship of 13 species of Poeciliid fishes, were constructed employing both neighbor joining and maximum likelihood methods. The results of phylogenetic analysis confirmed the basal placement of mollies in the fish phylogeny. The phylogenetic relationships among the Poeciliid fishes in the ML tree were virtually identical to those in the NJ tree. The present study shows that P. sphenops and P. latipinna are more closely related to each other than to other Poeciliid fishes. Molecular phylogenetic arrangements as inferred from the present study also suggest the presence of single origin of Sailfin species from a Shortfin ancestor.

Key words: Phylogenetic analysis, cytochrome oxidase I, Poecilia, cytochrome oxidase I, ornamental fish.

INTRODUCTION

The Poeciliidae is one of the most extensively studied fish families, and its representatives, which include the well-known guppy (Poecilia reticulata), mollies (Mollienesia spp.), swordtails (Xiphophorus spp.), and mosquito fishes (Gambusia spp.) are often used as model systems in evolutionary biology theory, especially for experimental studies on natural and sexual selection, and comparative studies of life-history evolution.

Members of this family viz., swordtails, guppies and mollies are also commonly found in the pet trade. The group of Poeciliid fishes commonly known as the mollies (genus Poecilia, group Mollienesia; Rauchenberger, 1999) are an ideal system for the study of speciation because of their enormous interspecific diversity in male secondary sex characters, male behaviour and mating system dynamics. One of the reasons why Poeciliids are so appealing to evolutionary biologists is their short generation time, ease of culture and staggering diversity in reproductive adaptations found in this family, which include internal fertilization, oviparity, clonality, viviparity, lecithotrophy, matrotrophy and superfetation (Meffe and
Snelson, 1989). They are one of four groups of cyprinodontiform fishes that evolved internal fertilization, and one of three groups that evolved live-bearing (Parenti, 1981; Meyer and Lydeard, 1993).

Family Poeciliidae (Rosen and Bailey, 1963) is widespread and includes diverse group of small-sized fishes comprising 220 species in 28 genera (Lucinda, 2003; Lucinda and Reis, 2005). Rosen and Bailey (1963) have placed several well-established genera, including Mollies, Allopoecilia, Limia, Pamphorichthys, Lebistes and Micropterus into one genus Poecilia, retaining four subgenera. The genus, Poecilia, is a complex and widely distributed group found in a wide range of habitats. Mollies have fascinated biologists for decades because of their diversity in habitat, body size, male secondary sex characters and mating behaviour. Species of Poecilia have been studied extensively for the effects of natural and sexual selection. The genus Poecilia, commonly referred to as mollies, contains 20 named species, which can be divided into two readily distinguishable species complexes the Sailfins of the P. latipinna complex and the Shortfins of the P. sphenops complex (Hubbs, 1933; Miller, 1983).

The primary goal of the present study is to analyse the molecular phylogeny of the Poeciliid fishes focusing on the group Mollies especially P. latipinna and P. sphenops. This paper reports results of phylogenetic analyses based upon nucleotide sequence data from the mitochondrial cytochrome oxidase gene of these fishes. The specific aims in recovering the molecular phylogeny were: to test the classification of Family Poeciliidae especially group Mollies; to test the taxonomic divisions of Shortfin species that is, P. sphenops and Sailfin species that is, P. latipinna; and to examine the relationships of Sailfin species of mollies to the Shortfin species.

MATERIALS AND METHODS

Experimental animals and tissue collection

Mitochondrial DNA sequences were obtained from two species of Mollies viz., Poecilia sphenops (Shortfin species) and Poecilia latipinna (Sailfin species) collected from an ornamental fish aquarium in Kochi, India. The muscle samples were dissected out and transferred to TEN buffers and subjected to DNA extraction.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was isolated from a piece of muscle tissue taken from fresh specimens. Tissue was digested by proteinase K/SDS solution at 37°C. DNA was purified by standard phenol: chloroform extraction and ethanol precipitation techniques. DNA was quantified and qualified by spectrophotometry and agarose gel electrophoresis. PCR amplification was carried out to obtain sequences of the partial mitochondrial COI gene, in a total of 25 μl volume containing 1x standard Taq buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3), 3.5 mM MgCl₂, 200 μM dNTPs, 0.4 μM each primer, 1U Taq DNA polymerase (Fermentas, Inc.) and 1μl DNA template (100 ng). The primer pairs used were COI- F (5'-tcaaccaaccacagacagatgc-3') and COI- R (5'-tagactctgggtgcccagaataca-3'). The thermal profile used was 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 55°C for 30 s and 68°C for 30 s and a final extension at 68°C for 10 min. 10 μl of the amplified PCR product was analyzed by electrophoresis in 1.5 % agarose gel in TBE buffer, stained with ethidium bromide and visualized under UV light. Gel documentation was performed using GelDocXR BioRad unit. Purified PCR products were bidirectionally sequenced using COI primers at Scigenom, Kochi, India.

Sequence analysis

The sequence homology and the deduced amino acid sequence comparisons were carried out using BLAST algorithm at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast). Gene translation and prediction of deduced protein were performed with ExPASy (http://www.au.expasy.org/). The multiple sequence alignments were performed for these sequences using ClustalW and GeneDoc software programs. COI nucleotide sequences were retrieved from the NCBI GenBank and phylogenetic tree was constructed by two independent methods viz., the Maximum Likelihood method and the Neighbor-Joining method based on the nucleotide sequences. Phylogenetic tree was drawn based on the COI sequences using MEGA version 5.0. Confidence in estimated relationships of maximum likelihood and neighbor joining tree topologies were evaluated by a bootstrap analysis with 100 and 1,000 replicates respectively with MEGA version 5.0. The nucleotide and deduced amino acid sequences were submitted to GenBank.

RESULTS

Partial COI amplicon of 595 bp could be obtained from the experimental sample, P. sphenops (JF752336) and 645 bp amplicon from P. latipinna (JF752337) (Figure 1A and B). Analysis of BLAST results obtained for the nucleotide sequences of P. sphenops (JF752336) confirmed the organism to be P. sphenops. The COI sequence also showed identity to COI sequence of other Poeciliid fishes viz., P. velifera (100%), P. latipinna (98%), P. petenensis and P. mexicana (96%), Pamphorichthys hollandi (89%), Limia vittata and Heterandria jonesii (88%), Xiphophorus variatus and Belonesox belizanus (86%) and Gambusia panuco and Girardinus microdactylus (84%) respectively (Table 1).
Figure 1A. Nucleotide sequence and deduced amino acid of (A) *Poecilia sphenops* (JF752336).

Figure 1B. *Poecilia latipinna* (JF752337).
Table 1. Result of BLASTn analysis of COI nucleotide sequence of *Poecilia sphenops* (JF752336).

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Table 2. Result of BLASTn analysis of COI nucleotide sequence of *Poecilia latipinna* (JF752337).

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Figure 2A. Multiple alignment of (A) nucleotide sequence.

BLAST results obtained for the nucleotide sequences of *P. latipinna* (JF752337) confirmed the identity of the experimental organism to be *P. latipinna*. The COI sequence of *P. latipinna* also showed identity to COI sequence of other Poeciliid fishes viz., *P. sphenops* and *P. velifera* (98%), *P. petenensis* (96%), *P. mexicana* and *P. orri* (95%), *L. vittata* and *P. hollandi* (89%), *C. decemmaculatus* (87%), *H. jonesii*, *X. variatus* and *P. reticulata* (86 %) and *B. belizanus* and *G. falcatus* (85%) (Table 2).

Multiple alignments performed for the nucleotide sequence of the COI gene of *P. sphenops* (JF752336) and *P. latipinna* (JF752337) showed variations in the nucleotide sequences at 11 positions viz., 12th, 129th, 156th, 336th, 366th, 378th, 402nd, 438th, 471st, 537th and 582nd positions (Figure 2A). Translation of the nucleotide
sequences were performed using ExPASy tools. BLASTp analysis of the deduced amino acid sequences of COI gene was carried out, which confirmed the presence of COI super family domain, possessing heme binding sites in the translated sequences (Frame-1) of *P. sphenops* (JF752336) and *P. latipinna* (JF752337). Multiple alignments performed for the amino acid sequence of the COI gene of *P. sphenops* (JF752336) and *P. latipinna* (JF752337) also showed great similarity. Though 11 variations could be noticed in the nucleotide sequences of *P. sphenops* and *P. latipinna*; only one variation could be noticed at the amino acid level that is, at 43rd position methionine in *P. sphenops* being replaced by isoleucine in *P. latipinna* (Figure 2B). Multiple alignments performed for the nucleotide sequence and amino acid sequence of *P. sphenops* (JF752336) and *P. latipinna* (JF752337) with other Poeciliid fishes showed that the sequences shared high degree of similarity among Poeciliid fishes (Figure 3).

The phylogenetic relationships of *P. sphenops* and *P. latipinna* were also established, based on nucleotide sequence comparison. The molecular phylogenetic trees were compared with morphology based classification. As shown in Figure 4A, the phylogenetic relationships among the *Poecilia* species in the maximum likelihood tree were virtually identical to those in the neighbor joining tree (Figure 4B). The neighbor joining tree could be broadly divided into two clusters. Cluster 1 included fishes viz., *P. latipinna*, *P. sphenops*, *P. petenensis*, *P. mexicana*, *P. hollandi*, *L. vittata*, *P. reticulate*, *B. belizanus*, *H. jonesii*, *Xiphophorus* sp. and *C. decemmaculatus*. *P. sphenops* (JF752336) and *P. latipinna* (JF752337) were found to be more closely related to each other. Cluster 1 could again be divided into two subclusters. Subcluster 1 included *Poecilia*, *Limia* and *Pamphorichthys* sp.; Subcluster 2 included *Cnesterodon*, *Belonesox*, *Heterandria* and *Xiphophorus* sp. Cluster 2 was found to include only two genera of Poeciliid fishes viz., *Girardinus* sp. and *Gambusia* sp. (Figure 4A).

However, in case of maximum likelihood, the tree could be broadly divided into three clusters. Cluster 1 included fishes viz., *Poecilia*, *Limia* and *Pamphorichthys* sp. and Cluster 2 included *Cnesterodon*, *Girardinus* and *Gambusia* whereas, Cluster 3 included *Belonesox*, *Heterandria* and *Xiphophorus* sp. (Figure 4B).

**DISCUSSION**

Several species of Poeciliid fishes, in particular mollies, are used as model systems for studying the effects of sexual and natural selection on the evolution of natural populations because of their extensive morphological and behavioural variation within and between species; short generation time and ease of culturing this genus in the lab (Endler, 1983; Houde, 1997; Schluter et al., 1998; Hamilton, 2001). Genus *Poecilia* includes at least 20 named species that are commonly referred as mollies and have been divided into two species complexes: *P. latipinna* (Sailfin) and *P. sphenops* (Shortfin) (Hubbs, 1933; Miller, 1983). There are only a few morphological characters that distinguish Sailfins readily from Shortfin species. Sailfin species are characterized by a sexual dimorphism in which males have a greatly enlarged dorsal fin that is erected and presented to the female in a courtship display. Whereas, Shortfin species show...
neither sexual dimorphism in fin morphology nor perform courtship display behaviours. These variations imply an important role for sexual selection in promoting premating reproductive isolation. The *P. latipinna* complex, or Sailfin mollies, includes three species, *P. latipinna*, *P. petenensis*, and *P. velifera*; and males of all three species are sexually dimorphic. Possession of the Sailfin distinguishes these species from the remaining Shortfin molly species in the *P. sphenops* complex. The *P. sphenops* complex includes *P. sphenops* and *P. Mexicana*.

In the present study molecular phylogeny for 13 species of Poeciliid fishes has been done, based on nucleotide sequence data from the mitochondrial genome of the COI gene.

The neighbor-joining and maximum likelihood trees has been presented (Figure 4A) from 1000 and 100 bootstrap iterations respectively. Neighbor-joining and maximum
likelihood analyses recovered similar topologies (Figure 4A and B).

In the present study, approximately 595 and 645 bp COI fragments were obtained from *P. sphenops* (JF752336) and *P. latipinna* (JF752337) respectively (Figure 1A and B). Deduced amino acid sequences showed the presence of conserved COI domain with heme binding sites, during BLASTp analysis of the deduced amino acid sequences. The COI nucleotide sequence obtained for *P. sphenops* and *P. latipinna* along with 11 other COI sequences obtained from GenBank were included for analysis in the present study.

Multiple alignments (Figure 3) were performed with COI nucleotide sequences from the GenBank database of other Poeciliid fishes that was obtained during the BLAST analysis and the molecular phylogenetic trees were
constructed using MEGA 5.0 software (Figure 4A and B). Multiple alignments performed for the nucleotide sequence of the COI gene of *P. sphenops* (JF752336) and *P. latipinna* (JF752337) showed variations at 11 positions. Whereas, multiple alignments, performed for the amino acid sequences of the COI genes showed variation at only one position, which shows that the COI genes of *P. latipinna* and *P. sphenops* are highly conserved (Figure 2B). Multiple alignments were performed for the nucleotide sequence and amino acid sequence of *P. sphenops* and *P. latipinna* to other Poeciliid fishes also showed that the sequences shared high degree of similarity among Poeciliid fishes. However, higher degree of similarity could be noticed within the genus *Poecilia*.

Phylogenetic analysis was performed for the COI sequences of 13 Poeciliid fishes, obtained during BLAST analysis. For a robust and reliable phylogenetic analysis,
Figure 3. Multiple alignment of nucleotide sequence of *Poecilia sphenops* (JF752336) and *Poecilia latipinna* (JF752337) with other similar sequences obtained using GenDoc programme Version 2.7.0. Black and grey indicates conserved sequences.

Two methods were used, viz., neighbor joining and maximum likelihood approaches. Neighbor joining and maximum likelihood trees was constructed using bootstrap test by 1000 and 100 replicates respectively (Figure 4A and B).

The results of phylogenetic analysis based on the COI
sequences confirmed the basal placement of mollies in
the fish phylogeny. Analysis of the results from neighbor
joining and maximum likelihood analyses for 13 species
of Poeciliids support the categorisation by Rosen and
Bailey (1963). The present study shows that P. sphenops
and P. latipinna are more closely related to each other
than to other Poeciliid fishes. The phylogenetic
relationships among the Poeciliid fishes in the maximum
likelihood tree were virtually identical to those in the
neighbor joining tree.

The neighbor joining tree could be broadly divided into
two clusters. Cluster 1 included fishes viz., P. latipinna, P.
sphenops, P. petenensis, P. mexicana, Pamphorichthys
hollandi, L. vittata, P. reticulate, Belonesox belizanus,
Heterandria jonesii, Xiphophorus sp. and C.
decemmaculatus. P. sphenops (JF752336) and P.
latipinna (JF752337) were found to be more closely
related to each other. Cluster 1 could again be divided
into two subclusters. Subcluster 1 included Poecilia,
Limia and Pamphorichthys species. Subcluster 2
included Cnesterodon, Belonesox, Heterandria and
Xiphophorus species. Cluster 2 included Girardinus
calculus and Gambusia panuntridrial. However, in case
of maximum likelihood, the tree could be broadly divided
into three clusters. Cluster 1 included fishes viz., Poecilia,
Limia and Pamphorichthys species. Cluster 2 included
fishes viz., Cnesterodon, Girardinus and Gambusia,
whereas, Cluster 3 included Belonesox, Heterandria and
Xiphophorus species.

Several groups within the genus Poecilia viz., four

Figure 4A. A bootstrapped neighbour-joining tree obtained using MEGA version 5.0 illustrating
relationships between the nucleotide sequences of Poecilia sphenops (JF752336) and Poecilia
latipinna (JF752337) with other Poeciliid fishes. Values at the node indicate the percentage of
times that the particular node occurred in 1000 trees generated by bootstrapping the COI
nucleotide sequences.
species of *Poecilia*, *Limia* and *Pamphorichthys* were found to be supported at the 99 to 100% level in both neighbour joining and maximum likelihood analyses. Within this *Mollienesia* group, the sailfin mollies, *P. latipinna*, *P. petenensis* and *P. mexicana*, form a strongly supported group (94 to 100%). Also, according to both neighbor joining tree and maximum likelihood analysis, *P. sphenops*, *P. latipinna*, *P. petenensis* and *P. mexicana* were found to be closely related to each other than to *P. reticulata*. Interestingly, *P. sphenops* and *P. latipinna* were found to be more closely related to *Limia* sp. and *Pamphorichthys* sp. than to *P. reticulata* belonging to the same genus. These results are in agreement with the observation by Breden et al. (1999) and with the definition of subgenera of *Poecilia* by Rosen and Bailey (1963), which state that they are morphologically quite different from each other, and also that there are monophyletic assemblages within *Poecilia* (e.g., *Limia*, *Pamphorichthys*). Analysis of the phylogenetic tree also showed that the inferred position of *Cnesterodon*,...
**Girardinus, Gambusia, Belonesox, Heterandria and Xiphophorus** sp. also changes depending on the approach taken. Only the maximum likelihood criterion places this species in a group when compared to that of neighbour joining analysis and this relationship is upheld by very low bootstrap support (10 to 40%).

Molecular phylogenetic arrangements as inferred from the present study suggest the presence of single origin of Sailfin species from a Shortfin ancestor. Several lines of evidence implicate an important role for sexual selection in the divergence between Shortfin and Sailfin species. Hence, the enlarged dorsal fin and associated courtship behaviour found in males of all three Sailfin species evolved from a common ancestor (1999).

**Conclusion**

The present study reports results of phylogenetic analyses based on nucleotide sequence data from the mitochondrial cytochrome oxidase gene of two Poeciliid fishes viz., *P. latipinna* (Sailfin species) and *P. sphenops* (Shortfin species). The phylogenetic relationships among the *Poeciliid* fishes in the maximum likelihood tree were virtually identical to those in the neighbor joining tree. The results of phylogenetic analysis based on the COI sequences confirmed the basal placement of mollies in the fish phylogeny. The molecular data was found to support a close relationship between Sailfin species and Shortfin species. Molecular phylogenetic arrangements as inferred from the present study suggest the presence of single origin of Sailfin species from a Shortfin ancestor. The study also shows that the faster evolving genes viz., COI, is useful in differentiating closely-related species and may thus be more used as a universal marker for fish identification.

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