

**MITOCHONDRIAL DNA VARIATION IN THE ENDANGERED FISH
DAWKINSIA TAMBRAPARNIEI (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE)
FROM SOUTHERN WESTERN GHATS, INDIA**

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Background. *Dawkinsia tambraparniei* (Silas, 1954) is confined to an area not exceeding 100 km² within a single watershed—the Tamiraparani River, southern Western Ghats, India. Its populations have recently declined, earning the fish the endangered status. For effective conservation efforts it is important to determine the levels of genetic variation between different populations. Therefore we attempted to quantify the cytochrome *b* (Cyt *b*) and cytochrome *c* oxidase subunit *I* (COI) gene sequence similarity between different populations of *D. tambraparniei*, as well as to study the phylogenetic relation with the closely related species *D. arulius* and *D. filamentosa*.

Materials and methods. Samples were collected from 9 locations and 10 individuals from each were preserved for DNA analysis. The partial sequence of Cyt *b* and COI genes were amplified and sequenced. The sequences were aligned by the visual method using BioEdit version 7.1.3.0 and edited using Sequencer 4.7. The Bayesian consensus tree was constructed using the Monte Carlo Markov Chain (MCMC) method by BEAST v1.7.5.

Results. The partial sequences of Cyt *b* and COI from 90 specimens of *Dawkinsia tambraparniei* representing nine populations were examined. The Cyt *b* gene sequences showed that all populations belong to a single haplotype and no nucleotide variation was observed between populations. In the case of COI gene sequences, two haplotypes were revealed: an additional haplotype with a clear transition of C to T at the nucleotide 58 position was detected within the Thalayanai Stream population. The phylogenetic analysis of COI gene demonstrated that the pair wise genetic distance between *D. tambraparniei* and *D. arulius* was low (0.004 ± SE 0.003) when compared with the distance between *D. tambraparniei* and *D. filamentosa* (0.061 ± SE 0.012).

Conclusion. The mitochondrial DNA analysis revealed very low diversity among investigated populations of *D. tambraparniei* for studied genes, with the only local population from the Thalayanai Stream demonstrating certain divergence. The phylogenetic analysis confirms the concept on the validity of *D. tambraparniei* as a separate species. This phylogenetic study also demonstrated that *D. tambraparniei* is closer to *D. arulius*, followed by *D. filamentosa*.

Keywords: endemic cyprinids, cytochrome *b*, cytochrome *c* oxidase *I*, phylogenetic tree

INTRODUCTION

Dawkinsia tambraparniei (Silas, 1954) is a small fish, commonly known as Tamiraparani barb, and it is very popular in aquarium trade because of its brilliant body and fin colour. It was originally described by Silas (1954) as a subspecies of *Dawkinsia arulius* (Jerdon, 1849) (= “*Puntius arulius*”) occurring in the Cauvery River basin, India. *D. tambraparniei* has been confined to a single watershed, namely the Tamiraparani River in southern Western Ghats, India. This species is distributed in many parts of Tamiraparani River including tributaries, mid-streams, lower stretches of the river, as well as river-associated wetlands. In recent years, populations

of *D. tambraparniei* have declined gradually due to human activities such as sand mining, habitat alterations, and collection of wild stock for the aquarium trade. Currently, the distribution range of this species is restricted to less than 100 km². Because of these threats and restricted distribution, *D. tambraparniei* was assessed as endangered in the IUCN red list (Dahanukar 2011). In order to conserve and effectively manage this endemic species, including the development of strategies to maintain genetic diversity, it is important to determine the levels of genetic variation between different populations. In this context, molecular techniques have provided new and better insights into the fields of taxonomy, population

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structure, and conservation management of species. Different molecular markers have been used for the identification of species, phylogenetic studies, and also to quantify the specific genetic variations between and within populations. Among the markers, the maternally inherited mitochondrial DNA markers like cytochrome *b* (Cyt *b*) and cytochrome *c* oxidase subunit *I* (COI) gene are widely used in molecular studies. 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 (Chan et al. 2009). This molecular information provides a clear understanding of ecological and adaptive characteristics as well as historical and genetic variation among populations, which is necessary for the conservation of species as evolutionary entities. Information on the patterns of genomic diversity of *D. tambraparniei* has not been known, except for a preliminary study on cytogenetic diversity by Arunachalam and Murugan (2007). Thus, the presently reported study aimed to quantify the Cyt *b* and COI gene sequence diversity between different populations of *D. tambraparniei*, as well as to study the phylogenetic relation with the closely related species *D. arulius* and *D. filamentosa* (Valenciennes, 1844).

MATERIALS AND METHODS

Sample collection. Fish were collected between December 2010 and July 2011 from nine sampling sites (Fig. 1), of which six locations were from the main tributaries of the Tamiraparani River, namely Manimuthar, Thalayanai, Karayar, Servalar, Kallar, and Illuppaiyar rivers, and three locations from the downstream reaches of the Tamiraparani River at Kokkarakulam, Chittar, and Marudur. Fish were collected using monofilament gill nets, cast nets, and scoop nets. After collection, a portion of caudal fin (a straight vertical cut approximately half of the fin from each fish) was taken for molecular analysis. At each location samples were taken from 10 individuals and the fin tissues were stored in absolute ethanol. This study has been carried out after obtaining permission from the Wildlife Division of the State Forests Department, Tamil Nadu.

DNA extraction, PCR amplification and gel electrophoresis. Total genomic DNA was extracted from all the samples using the phenol/chloroform method (Sambrook et al. 1989) in a final extraction volume of 200 μ L. The polymerase chain reaction (PCR) was carried out with a final 10 μ L volume reaction mixture containing 10–50 ng of extracted genomic DNA, 10X PCR buffer (Applied Biosystem), 2.0 mM MgCl₂, 0.2 mM of each dNTP, 5pmol of each forward and reverse primers, and 0.5 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). The partial sequences of Cyt *b* (564 bp) and COI (652 bp) were amplified using primers Cyt *b* A 5'-CCA TGA GGA CAA ATA TCA TTY TGR GG-3' and Cyt *b* C 5'-CTA CTG GTT GTC CTC CGA TTC ATG T-3' (Bossuyt and Milinkovitch 2000) and Fish F1 5'-TCA ACC AAC CAC AAA GAC ATTGGCAC-3'

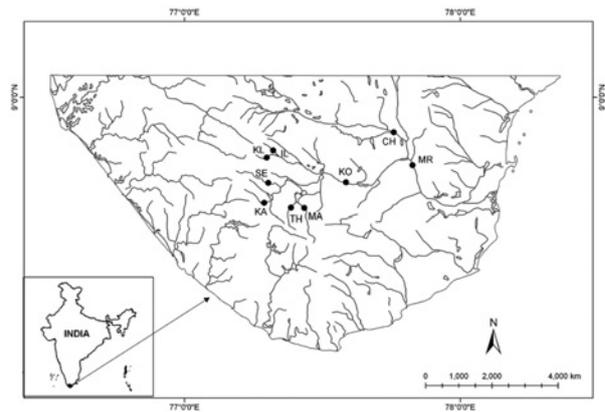


Fig. 1. Sampling locations of *Dawkinsia tambraparniei* in the Tamiraparani River of southern Western Ghats, India; MA = Manimuthar, TH = Thalayanai, KA = Karayar, SE = Servalar, KL = Kallar, IL = Illuppaiyar, KO = Kokkarakulam, CH = Chittar, MR = Marudur

and Fish R1 5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' (Ward et al. 2005), respectively. The following PCR conditions were used for amplification of Cyt *b* and COI partial sequence: initial preheat at 95°C for 10 min followed by 35 cycles at 94°C for 30 s, annealing at 55°C for 30 s, extension 72°C for 45 s, with a final extension at 72°C for 15 min. To check DNA contamination, negative controls omitting template DNA from the reaction mixture were included in each PCR run. The amplified PCR products were visualized under UV light in 2% agarose gel stained with ethidium bromide.

PCR product clean up and DNA sequencing. The amplified PCR products were treated with exonuclease-*I* and shrimp alkaline phosphatase (USB, Cleveland, OH) for 15 min each at 37°C and 80°C, respectively, to remove any residual primer. The PCR products were directly sequenced using the BigDye[®] Terminator kit (v3.1) and analyzed on an Applied Biosystems Genetic Analyzer ABI 3130. All products were sequenced in both directions. The sequences were aligned and edited by using Sequencer 4.7 (Gene Code Corporation). All the raw sequences were aligned by the visual method using BioEdit version 7.1.3.0 (Hall 1999).

Molecular data analyses. Mean pairwise differences between and within groups (Kimura's 2-parameter) were generated in MEGA5 (Tamura et al. 2011). The Bayesian consensus tree was constructed using the Monte Carlo Markov Chain (MCMC) method by BEAST (version 1.7.5; Drummond and Rambaut 2007). Based on the partial sequence of Cyt *b* and COI gene sequence, two different phylogenetic trees were generated using Hasegawa-Kishino-Yano (HKY) model with a constant rate applied across the tree.

The first tree was based on Cyt *b* gene sequence of *Dawkinsia tambraparniei* and the two closely related species of *D. arulius* and *D. filamentosa*, and an additional three cyprinid species were chosen as outgroups: *Pethia*

conchonius (Hamilton, 1822), *Pethia ticto* (Hamilton, 1822), and *Puntius sophore* (Hamilton, 1822). The second tree was based on the COI gene, where the sequence of *D. tambraparniei* was compared with *D. filamentosa* only due to unavailability of the COI gene for *D. arulius* in the open source. The same three cyprinids mentioned above were used as outgroups. The sequence data of the species used in this analysis were retrieved from the GenBank and their accession numbers are given in Table 1.

RESULTS

Mitochondrial DNA sequence. The sequences of the partial cytochrome *b* and cytochrome *c* oxidase gene *I* from 90 specimens of *Dawkinsia tambraparniei* representing nine populations from the Tamiraparani River drainage were examined and the sequences were deposited in the GenBank Database (Table 1). The Cyt *b* partial sequence data revealed that no haplotype and nucleotide variation was observed among studied *D. tambraparniei* populations. In the case of COI gene, sequences showed that 2 haplotypes were present within the populations. Haplotype 1 was shared by all the population examined and another haplotype 2 was found in eight individuals of *D. tambraparniei* population inhabiting in Thalayanai

stream, while the sequences of two remaining individuals were identical with other populations. A clear transition of C to T was detected between haplotypes of *D. tambraparniei* at the nucleotide 58 position that indicates occurrence of a single nucleotide polymorphism (SNP) within population from the Thalayanai Stream.

Phylogenetic analysis. Based on the Cyt *b* partial gene sequence, the phylogenetic tree was constructed using HYK model for different populations of *Dawkinsia tambraparniei* and also closely related species such as *D. arulius* and *D. filamentosa*. The Cyt *b* gene revealed 131 variable sites in 564-bp long sequence, in which 73 sites were found to be parsimony-informative sites. In the case of COI amplicon which had 149 variable sites in 652-bp and 71 parsimony-informative sites. The phylogenetic tree indicates that the Cyt *b* of different populations of *D. tambraparniei* had similar conserved region and they were clustered in a single group. At the same time the populations of *D. tambraparniei* were separated from *D. arulius* and *D. filamentosa* (Fig. 2A). The genetic distance based on Kimura's 2-Parameter Model between *D. tambraparniei* and closely related species, as well as outgroup species, are presented in Table 2. The values indicated that the pair-wise genetic distance between

Table 1

GenBank accession number of mitochondrial gene haplotypes for two regions (Cyt *b*, COI) of selected species of *Dawkinsia*, *Pethia*, and *Puntius*

Species	GenBank Acc. No.	Gene	Sequences source
<i>Dawkinsia tambraparniei</i>	JX049981	Cyt <i>b</i>	Presently reported study
<i>Dawkinsia tambraparniei</i>	JX049983	COI	Presently reported study
<i>Dawkinsia tambraparniei</i>	KC577833	COI	Presently reported study
<i>Dawkinsia filamentosa</i>	JX049982	Cyt <i>b</i>	Presently reported study
<i>Dawkinsia filamentosa</i>	JX049984	COI	Presently reported study
<i>Dawkinsia arulius</i>	EU241450	Cyt <i>b</i>	Fang et al. 2009
<i>Pethia conchonius</i>	JQ795488	Cyt <i>b</i>	Pallavi et al. 2012
<i>Pethia conchonius</i>	JQ667569	COI	Khedkar et al. 2012 (unpubl.)
<i>Pethia ticto</i>	JQ795478	Cyt <i>b</i>	Pallavi et al. 2012
<i>Pethia ticto</i>	NC_008658	COI	Saitoh et al. 2006
<i>Puntius sophore</i>	JQ795440	Cyt <i>b</i>	Pallavi et al. 2012
<i>Puntius sophore</i>	FJ459407	COI	Lakra and Verma 2008 (unpubl.)

Table 2

Matrix of genetic distances between the selected species of *Dawkinsia*, *Pethia*, and *Puntius*, under Kimura's 2-Parameter Model (Cyt *b* gene); The numbers below the diagonal line are pair-wise genetic distances, those above the diagonal line are standard errors

No.	Species	1	2	3	4	5	6
1	<i>Dawkinsia tambraparniei</i>	—	0.003	0.012	0.026	0.028	0.029
2	<i>Dawkinsia arulius</i>	0.004	—	0.012	0.026	0.029	0.029
3	<i>Dawkinsia filamentosa</i>	0.061	0.056	—	0.027	0.029	0.028
4	<i>Pethia ticto</i>	0.175	0.17	0.175	—	0.017	0.027
5	<i>Pethia conchonius</i>	0.0189	0.183	0.181	0.092	—	0.033
6	<i>Puntius sophore</i>	0.195	0.189	0.177	0.189	0.204	—

D. tambraparniei and *D. arulius* was low when compared with the distance between *D. tambraparniei* and *D. filamentosa*. The phylogenetic tree based on COI partial sequence showed that all populations of *D. tambraparniei* and two individuals of the Thalayanai population belong to the same clade in the tree, whereas the remaining eight individuals from the Thalayanai population represented a slightly diverged subclade in the phylogenetic tree. Further, all populations of *D. tambraparniei* were clearly separated from *D. filamentosa* in phylogenetic tree (Fig. 2B). The Kimura's 2-Parameter pair-wise genetic distance between *D. tambraparniei* and *D. filamentosa* was 0.059 (Table 3). Due to the lack of information on the COI gene sequence of *D. arulius* in GenBank, the phylogenetic distance between *D. arulius* and *D. tambraparniei* was not generated.

DISCUSSION

The mitochondrial DNA sequence data revealed that low haplotype diversity was observed in *Dawkinsia tambraparniei* for the studied genes. The only exception was the population from the Thalayanai Stream, which exhibited two different haplotypes from COI gene within the population. This finding suggests that the populations of *D. tambraparniei* probably had a small effective population size historically. In fact this endemic species has very restricted

distribution within the river basin (about 100 km², Dahanukar 2011) and there is chance for a high level of gene flow between the populations. This finding is similar to the results of intraspecific genetic diversity analysis of small or isolated fish populations reported by many studies (Apostolidis et al. 1997, Finne 2001, Tinti et al. 2002, Sah et al. 2011, Marshall unpublished*). The occurrence of two haplotypes in COI gene indicates that the gene pool of Thalayanai Stream population has some variation within the population. However, these findings need further substantiation of sequence data using suitable microsatellite markers.

The result of study of phylogenetic relation among the haplotypes of *Dawkinsia tambraparniei* and also with closely related species provide significant information on the validity of this species. Indeed, *D. tambraparniei* has been confused with the closely related species *D. arulius* over the last five decades; it has long been considered as a junior synonym of *D. arulius* by Talwar and Jhingran (1991) without giving any reason. Moreover, it could have been overlooked by the authors, since *D. tambraparniei* shares several meristic characters (such as number of lateral line scales, lateral transverse scale rows, number of rays in fins) with *D. arulius* (see Jerdon 1849, Silas 1954). Recently, Pethiyagoda and Kottelat (2005) treated

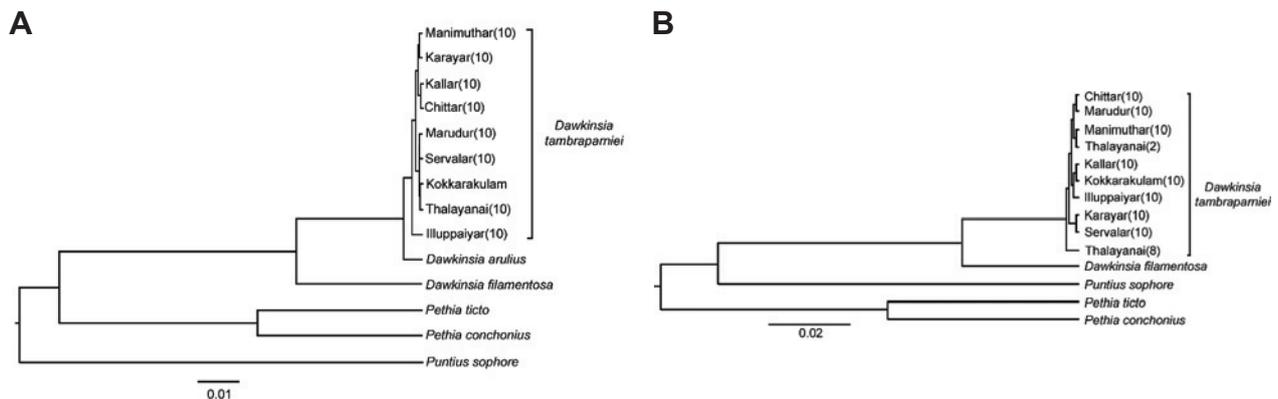


Fig. 2. Bayesian consensus tree (based on HYK model) of the populations of *Dawkinsia tambraparniei* sequence variation of the mt DNA Cyt *b* gene (A) and COI gene (B); Sequence of *Dawkinsia arulius*, *Dawkinsia filamentosa*, *Pethia conchoniuis*, *Pethia ticto*, and *Puntius sophore* were retrieved from GenBank data (Table 1) and they were included as outgroup species with original sequences for easy comparison

Table 3

Matrix of genetic distances between the selected species of *Dawkinsia*, *Pethia*, and *Puntius*, under Kimura's 2-Parameter Model (COI gene); The numbers below the diagonal line are pair-wise genetic distances, those above the diagonal line are standard errors

No.	Species	1	2	3	4	5
1	<i>Dawkinsia tambraparniei</i>	—	0.022	0.152	0.127	0.327
2	<i>Dawkinsia filamentosa</i>	0.059	—	0.171	0.104	0.166
3	<i>Pethia ticto</i>	0.215	0.233	—	0.153	0.044
4	<i>Puntius sophore</i>	0.225	0.203	0.229	—	0.278
5	<i>Pethia conchoniuis</i>	0.25	0.225	0.111	0.252	—

* Marshall C.R.E. 2005. Evolutionary genetics of barramundi (*Lates calcarifer*) in the Australian region. Dissertation, School of Biological Sciences and Biotechnology, Murdoch University, Australia

D. tambraparniei as a valid species. They retained the validity of this species based on its mouth position, length of maxillary barbels, and dorsal fin rays of males (mouth subterminal vs. terminal in *D. arulius*; maxillary greater than ½ eye diameter vs. less than ¼ eye diameter in *D. arulius*; dorsal rays of males prolonged into filament-like extension vs. dorsal rays of males not prolonged into filament-like extensions in *D. arulius*). Further, this species was grouped under the *Dawkinsia filamentosa* (=“*Puntius filamentosus*”) group because of sharing the distinctive colour patterns in juveniles. Their juveniles have three vertical bands on the side of the body: one beneath the dorsal-fin base, one above the anal-fin base, and one at the base of the caudal fin (Pethiyagoda and Kottelat 2005). According to the phylogenetic tree generated for Cyt *b* partial gene sequence data, *D. tambraparniei* is a genetically distinct species and thus our data confirm its validity reported by Pethiyagoda and Kottelat (2005). At the same time, this phylogenetic study also revealed that *D. tambraparniei* is much closer to *D. arulius*, followed by *D. filamentosa*. This result presumes the necessary of further phylogenetic studies within *D. filamentosa* species group.

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