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RESEARCH ARTICLE

GENETIC DIVERSITY PHYLOGENETIC ANALYSIS OF THE GENUS *DAWKINSIA FILAMENTOSA* GROUP (ACTINOPTERYGII: CYPRINIFORMES: CYPRINIDAE) FROM SOUTHERN WESTERN GHATS, INDIA, USING MITOCHONDRIAL GENE SEQUENCES

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ABSTRACT

Genetic diversity and molecular phylogeny among six endemic fishes of the Western Ghats belonging to *Dawkinsia* genus was studied. The partial sequence of Cytochrome Oxidase subunit I (COI) gene sequences were generated for five species namely *Dawkinsia filamentosa*, *D. assimilis*, *D. rohani*, *D. tambraparniei* and *D. exclamatio*. The amplification of COI gene was done using suitable primers, followed by direct sequencing, analysis of nucleotide variation and phylogenetic analysis were carried out. The COI sequence of *Dawkinsia arulius* retrieved from GenBank database for phylogenetic analysis. The mitochondrial COI gene of *Dawkinsia* species exhibited 50 polymorphic and 18 parsimony informative sites between species. The pairwise genetic distance between the studied species were ranged from 0.008 to 0.075. The present study inferred that these species exhibit very close genetic similarity between the *Dawkinsia filamentosa* group.

INTRODUCTION

The Western Ghats situated in India, is one of the hotspots of biological diversity in the world (Myers *et al*, 2000). It is an important watershed area in the Peninsular India, many streams and rivers (East and West flowing) originate from this critical ecosystem. The southern part of Western Ghats has the highest species richness with more endemic freshwater fishes. Among the endemic fishes, *Dawkinsia* (=*Puntius*) *filamentosa* (Valenciennes, 1844) is medium sized barbs (< 115 mm SL) and are easily recognized by the elongated, filament-like extensions of the branched dorsal fin-rays in adult males. They form an important part of the local food and ornamental fishery in most of the area (Pethiyagoda, 1991; Talwar and Jhingran, 1991). Recently a new genus *Dawkinsia* was created and the *P. filamentosus* group was moved on into *Dawkinsia* (Pethiyagoda *et al*, 2012). Currently six valid species are recognized under this genus namely, *Dawkinsia arulius*, *D. exclamatio D. filamentosa*, *D. tambraparniei*, *D. rohani* and *D. assimilis* and the distribution of these species are restricted only in southern part of Western Ghats. *D. arulius*, *D. assimilis* and *D. tambraparniei* have distinct morphotype and one can easily recognize the species. In contrast, the species within the *filamentosus* group: *D. filamentosa*, *D. exclamatio* and *D. rohani* are sharing many morphometric and meristic characters.

Genetic variation within and among populations level studies taking an important role in successful conservation and effective management of a species, including develop strategies for maintaining genetic diversity (Dian-Qiao Sun *et al*, 2011). Mitochondrial DNA (mtDNA) is the genetic material that exists outside the nucleus in eukaryotic cells, evolutionary rate is 5 to 10 times faster than the nuclear genome (Avise, 1994), because of its fast evolution, has been broadly applied in population genetics and conservation biology of animals (Wang *et al*, 2000). Various molecular makers, microsatellite DNA markers have been successfully used in revealing population genetic diversity, because they are co-dominant and highly polymorphic (Selkoe and Toonen, 2006). The phylogenetic relationship study can shows the evolutionary pattern and genetic variability among species, subspecies and populations using mitochondrial DNA sequences. (Alves *et al*, 2001). The present study attempts to address the genetic variation within different populations of *D. filamentosus* and analyse the phylogenetic relationship within the *D. filamentosus* lineage from east and west flowing rivers of southern Western Ghats. Till date, not much has been reported about genetic variation in the genus *Dawkinsia*.

MATERIALS AND METHODS

Sampling

Fish samples were collected from 15 different locations covering east and west flowing stream/ rivers of southern Western Ghats. Twenty six samples of *Dawkinsia filamentosa* were collected from eight different lactations (Manimuthar Thalayanai, Kallidaikurichi, VK puram, Servalar, Thylar, Pabanasam, Bhavani Sagar and Pallipalayam) covering east flowing rivers, Tamiraparani and Cauvery; six samples of *D. tambraparniei* were collected from two different lactations (Kurukuthurai and Melapalayam) in Tamiraparni river; five samples of *D. exclamatio* from one location in Kallada river; six individuals of *D. rohani* from two different lactations (Kalikesam and Pechiparai) a west flowing Chittar river and six individuals of *D. assimilis* from two different lactations

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(Attoor and Themanoor) in Kanyakumari district in Tamil Nadu. Sampling locations, site abbreviations and GPS co-ordinate given in the Table 1 and illustrated in Figure 1. Fish samples were collected by multifilamentous cast nets. After collection, a portion of gill region was taken and fixed in absolute ethanol for molecular study.

DNA extraction PCR amplification and sequencing

The total genomic DNA from the gills was extracted using Phenol-Chloroform method (Sambrook *et al*, 1989). The quality and quantity of the extracted DNA were estimated on 0.8% agarose gels stained with ethidium bromide (EtBr). The partial sequence of COI gene was amplified by polymerase chain reaction (PCR) for each individual, using set of forward and reverse primers - Fish F1 (5' – TCA ACC AAC CAC AAA GAC ATT GGC AC - 3') - Fish R1 (5' – TAG ACT TCT GGG TGG CCA AAG AAT CA - 3') (Ward *et al*, 2005). PCR was carried out in a final volume of 20µl reaction tube containing 10-50 ng of extracted DNA, 10X PCR buffer (Invitrogen), 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 5pmol of each forward and reverse primers, and 0.5 units of AmpliTaq Gold DNA polymerase (Invitrogen). The amplifications of COI gene sequence were carried out by adopting the following procedures: initial denaturation at 95°C for 10 min followed by 35 cycles each at denaturation 94°C for 30 sec, annealing 55°C for 30 sec, extension 72°C for 45 sec, followed by a final extension for 15 min at 72°C. A negative extraction control was used in each PCR run to check the chance of contamination. Amplified PCR products were electrophoresed on 2% agarose gel and visualized under UV light in the presence of ethidium bromide (EtBr). PCR amplicons were treated with Exonuclease-*I* and Shrimp alkaine phosphatase (USB, Cleveland, OH) for 15 min. each at 37°C and 80°C, respectively to remove any residual primer and dNTP's. The purified PCR products were sequenced with an automated sequencer (ABI 3130 Genetic Analyzer) using Big Dye Terminator v. 3.1 sequencing kits (Invitrogen) for both the strands.

Sequences alignment and data analysis

All the sequences were analyzed and cleaned using Sequencer 4.7 (Gene code Corporation). These raw sequences were aligned by the visual method using BioEdit version 7.1.3.0 (Hall, 1999). Mean pairwise differences between species (Kimura's 2-parameter) were generated in MEGA5 (Tamura *et al*, 2011). In this analysis COI sequence data of *D. arulius* (KJ683749**)** was retrieved from the GenBank. Based on the partial sequence of COI sequence phylogenetic trees were constructed using the Neighbor Joining (NJ) (Saitou and Nei, 1987) method at bootstrapping for 1000 replicates using Kimura-2 parameter by MEGA 5 (Tamura *et al*, 2011). The Hasegawa-Kishino-Yano (HKY+G) using a discrete Gamma distribution model has the lowest Bayesian Information Criterion (BIC) score among all the models tested, using MEGA 5. Hence, it is considered the best model for nucleotide substitution.

RESULTS

DNA Sequence variations analysis

The partial fragments of 511bp Cytochrome Oxidase Subunit I (COI) gene were obtained from individuals of *D. filamentosa*, *D. assimilis*, *D. rohani*, *D. exclamatio* and *D. tambraparniei* from various sampling sites of southern Western Ghats. Complete mitochondrial genome of *Pethia ticto* (NC008658.1) was chosen as out group. Clarifying the phylogentic relationship among the main *Dawkinsia* groups, the sequence of *D. arulius* (KJ683749) was taken from NCBI GenBank. The sequence of COI gene of *D. filamentosus* group analysed in present study corresponds to the position 5578 – 6088 of *P. ticto* (Saitoh *et al*, 2006). The mitochondrial COI gene revealed 50 polymorphic and 18 parsimony informative sites in 511bp sequence (Table 2). The number of transitions (ts) and transversion (tv) sites was 47 and 6 respectively. The estimated ts/tv bias (*R*) was 8.74 across the five species. There are no indel (insertion/deletion) observed in any sites. Alignment of 44 samples showed five different haplotypes, one haplotype J1 identified in 24 samples of *D. filamentosa*,, one haplotype J2 was identified in 6 samples of *D. assimilis*, one haplotype J3 was identified in 6 samples of *D. rohani*, one haplotype J4 was found in 5 samples of *D. exclamatio and* another one haplotype J5 were found in *D.tambraparniei* which consist of 6 samples. Average haplotype diversity was high (H = 1.00, SD ± 0.09) in total samples. Nucleotide diversity (per site) was low in all samples (average π = 0.04, SD ±0.006).

Phylogenetic analysis

Neighbor-joining tree (Figure 3) (Carried out using *Pethia ticto* as out group) produced 3 distinct lineages: upper clade I comprising of *D. filamentosa* and *D. assimilis*; the clade II consists of *D. rohani* and *D. exclamatio* and clade III comprising of *D. tambraparniei*. *D. arulius* is placed between the clades of II and III. As the haplotypes from geographically closer localities, they were genetically closer to each other and from their own cluster. In the *D. filamentosa* and *D. assimilis* forms their own cluster had 100% bootstrap value, meanwhile, the *D. rohani* and *D. exclamatio* were closer to each other with 95% bootstrap value. It showed that the members of these lineages shared more similarity in COI sequence. The pairwise genetic distance among the *D. filamentosa* ranges from 0.008 to 0.075 (Table 3) Analysis of genetic distance on the basis of sequence difference shows very little differences. The present finding shows a very close genetic similarity among the *D. filamentosus* group.

Table 1 Stream name, site abbreviations and GPS locations of fish sampling in the southern Western Ghats.

Site Name	Code	Latitude & Longitude	
Manimuthar Thalayanai	MT	$8^0$33'11.7''	$77^0$23' 1.9''
Kallidaikurichi	KK	8^0 41'37.5''	$77^0$27' 43.5"
VK Puram	VK	$8^0$41' 13.8''	$77^0$26' 14.7"
Servalar	SA	$8^0$41' 31.8''	$77^0$19' 28.8"
Thylar	TY	$8^0$41' 24.8"	77^0 19'02.0"
Pabanasam	PB	$8^0$42' 24.0"	$77^0$22' 0.2"
Pechiparai	PP	$8^0$26' 59.4"	$77^0$18' 26.9"
Kallada	KL	$8^0$57' 28.4"	$77^0$3' 52.4"
Attoor	AT	$8^0$19' 44.8"	$77^0$15' 48.7"
Themanoor	TM	$8^0$20' 13.2"	$77^0$14' 57.9"
Melapalayam	MP	$8^0$42' 43.9"	$77^0$41' 52.7"
Kurukuthurai	KUR	$8^0$43' 15.1"	$77^0$42' 18.8"
Kalikesam	KSM	$8^0$24' 35.6"	$77^0$23' 30.3"
Bhavani Sagar	BH	$11^0$28' 21.6"	$77^0$6' 53.9"
Pallipalayam	PLP	$11^0$21' 38.2"	$77^0$44' 38.0"

DISCUSSION

Every species is believed to be undergoing micro and macro evolutionary process resulting in the expression of significant

genetic variations at levels of species specific chromosome morphology/structure, gene controlled protein structure and polygene controlled morphometrics and metrics (Ayala and Keiger, 1980). Mitochondrial DNA has been widely applied in systematics, population genetics, and inference of migration routes and conservation biology of animals, because of its fast

Table 2 Variable positions (511bp) of mitochondrial DNA COI region lies between nucleotide 5578 - 6088 of complete genome of *P.ticto* (Genbank acc. no. NC_008658.1). Nucleotide positions were shown in numeric digit at top. "." Indicating the similar nucleotide position

	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Nucleotide	6	6	6	6	6	6	7	7	7	7	7	7	7	7	7	7	7	7	8	8	8	8	8	8	8
Positions	0	1	2	5	5	7	0	2	3	4	5	5	5	6	7	9	9	9	1	1	1	2	3	3	4
	6	7	7	3	9	4	1	2	4	3	2	5	8	4	9	1	5	7	2	5	8	7	3	9	8
D. filamentosa	C	A	T	T	T	T	A	G	G	A	T	T	C	A	C	C	C	A	C	A	C	C	T	T	G
D. assimilis	.	.	.	C	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.
D. rohani	.	.	C	.	.	.	.	A	A	G	.	C	.	G	T	T	.	.	.	G	T	.	.	.	.
D. exclamato	.	.	.	.	.	C	.	A	A	G	C	C	.	.	T	T	.	.	.	.	T	.	.	.	.
D. tambraparniei	T	.	.	.	C	.	.	.	A	.	C	.	T	.	T	.	T	.	T	.	T	T	C	.	A
D. arulius KJ683749	.	G	.	.	C	.	.	.	A	.	C	C	.	.	T	.	.	G	T	.	T	.	.	.	.

	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	6	6	6	6	6	6	6	6
Nucleotide	8	8	8	8	8	8	8	9	9	9	9	9	9	9	9	9	0	0	0	0	0	0	0	0	0
Positions	6	7	7	7	8	9	9	0	2	3	3	5	5	6	8	9	1	1	2	4	5	6	7	7	8
	0	2	5	8	7	3	9	2	9	2	5	0	3	5	3	8	0	3	2	0	8	4	6	9	5
D. filamentosa	G	T	A	C	A	A	C	C	C	A	C	A	A	A	C	A	G	C	T	C	C	A	C	A	A
D. assimilis	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D. rohani	A	.	.	.	.	.	.	.	.	G	.	G	.	G	T	.	C	.	.	T	.	C	T	.	.
D. exciamatio	A	.	.	.	G	.	.	.	.	.	.	G	.	.	T	.	C	.	.	T	.	C	T	.	.
D. tambraparniei	A	C	C	T	.	G	T	T	T	.	T	.	.	.	.	.	T	T	C	.	T	T	A	T	.
D. arulius KJ683749	A	.	C	.	.	.	.	.	T	.	.	G	G	.	.	G	T	.	.	T	.	T	A	.	G

Table 3 Pairwise genetic distance of COI gene sequence determined between haplotypes from *D*awkinsia

	D. filamentosa	***D. assimilis***	***D. rohani***	***D. exclamatio***	***D. tambraparniei***	***D. arulius* KJ683749**
D. filamentosa	-	0.004	0.009	0.008	0.011	0.009
D. assimilis	0.008	-	0.009	0.008	0.011	0.010
D. rohani	0.038	0.043	-	0.006	0.012	0.010
D. exclamatio	0.032	0.036	0.018	-	0.011	0.009
D. tambraparniei	0.058	0.062	0.075	0.067	-	0.009
***D. arulius* KJ683749**	0.040	0.045	0.045	0.036	0.049	-

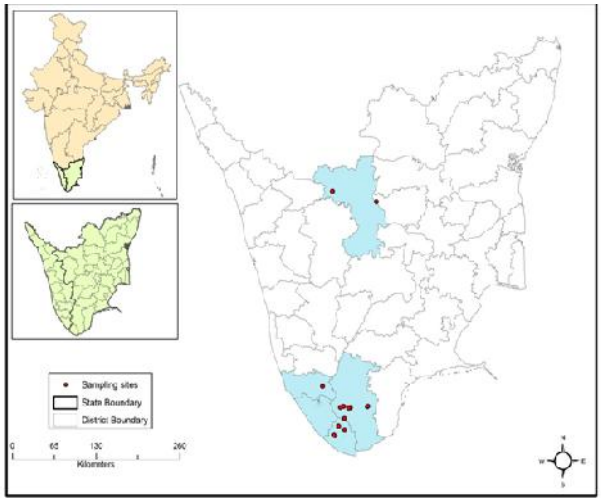

Figure 1 Map indicating locations of different sampling sites in the southern Western Ghats.

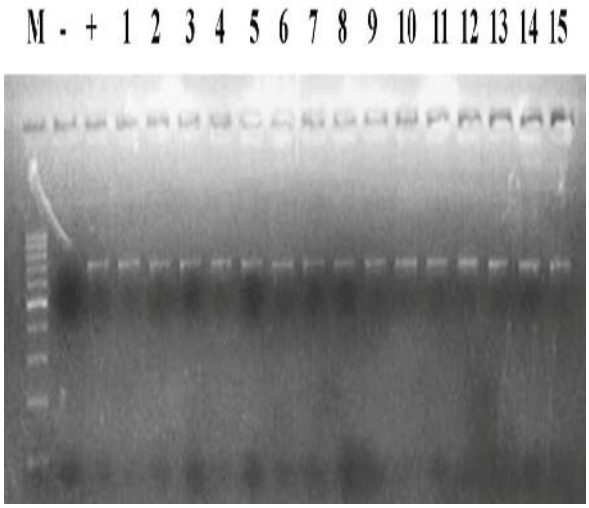

Figure 2 PCR amplification with COI gene, lane M 100bp ladder

Table 4 Molecular Characterization information content of the mtDNA COI region of analyzed *Dawkinsia*

Number Of bases analyzed	Nucleotide Composition				Invariable Sits	Polymorphic Informative Sites	Parsimony Informative Sites	Number of transitions (ts)	Number of transversins (tv)	Estimated tv/ts bias (R)
	%A	%G	%T	%C						
511	28.44	15.85	28.64	27.07	461	50	18	47	6	8.74

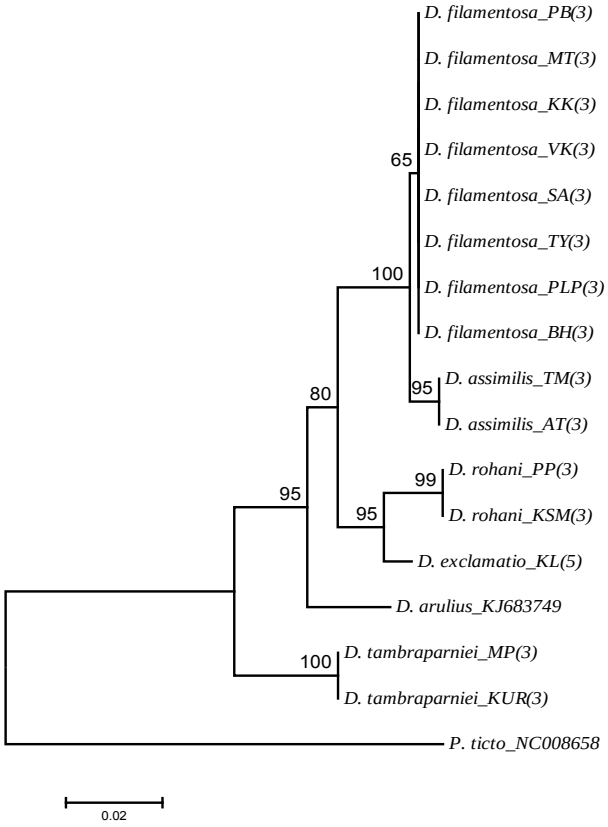

Figure 3 Neighbor Joining Tree of the genus *Dawkinsia* inferred from haplotype variation of the mitochondrial COI. Numbers at nodes shows bootstrap values.

evolution (Avise, 1994). In the result of COI gene sequence indicates that all six species examined have distinct pattern of COI sequences. Further the present study has provided that all these six species are genetically distinct, though they share same morphological characters. The presence of five haplotypes within *filamentosa* group indicates that high level of genetic diversity found within COI sequences of studied species. Further, this study also inferred that species of *Dawkinsia* exhibit very close genetic similarity with *D. filamentosa*.

As cytochrome oxidase subunit I (COI) gene suppose to be evolving faster than 16S rDNA has been used widely in molecular taxonomy to resolve the phylogenetic relationships between species and to study intraspecific population genetic structure in several groups of eukaryotes including fishes. Many workers used the COI sequences along with 16S and 18S rDNA to study the molecular taxonomy of different taxonomic groups (Yamazaki *et al*, 2003; Klinbunga *et al*, 2005; Duran and Rutzler, 2006; Ponniah and Hughes, 2006; Gruenthal *et al*, 2007).

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