Assessment of Morphometric and Genetic Variation in Three Freshwater Fish Species of the Genus *Garra* (*Osteichthyes: Cyprinidae*)

Arulraj DHINAKARAN[1], Nabeel Mannalamkunnath ALIKUNHI[1], Selvaraj CHINNATHAMBI[2], Ramaiya SORNAM[2], Murugaiyan KALAISELVAM[1]

Ramadoss RAJASEKARAN[1], Subramanian MANIVANNAN[3]

[1]*Annamalai University, Centre of Advanced Study in Marine Biology, Parangipettai, 608502, Tamil Nadu, India; smanmbbt@gmail.com*
[2]*Manonmaniam Sundaranar University, Department of Biotechnology, Sri Paramaayani Centre for Environmental Sciences, Awarkurichi, 627412, Tamil Nadu, India*
[3]*Sathak College of Arts and Science, Department of Biotechnology, Mohammed, Sholinganallur, Chennai-119, Tamil Nadu, India*

Abstract

The present study evaluated the patterns of morphometric and genetic variation using RAPD-PCR techniques for the first time on three species of *Garra, viz. G. mullya, G. kalakadensis* and *G. gotyla stenorhynchus*, collected from various river basins of South-India. The results of morphological analysis revealed that *G. mullya* and *G. kalakadensis* hold many similar characters compared to the other congener, *G. gotyla stenorhynchus*. However, the *G. gotyla stenorhynchus* fish species exhibited distinct variation in the morphological characters such as snout length, pre-nasal length, inter-nasal width, gap width, lower jaw to isthmus, head depth at pupil, dorsal fin length and disc width from the other two species of *Garra*. However, certain morphometric characters overlapped. Hence the RAPD finger printing was used to assess the levels of genetic variation in *Garra* spp. using RAPD-PCR technique. A total of 72 reliable fragments were detected using 10 Operon primers, ranging from 2600 molecular weight to 3100. The shared RAPD fragments found in both *G. mullya* and *G. kalakadensis* with fixed frequencies were observed with all the investigated primers, implying their genetically closer relationship. However, the similarity index observed for *G. gotyla stenorhynchus* was less with the other two species specifying a genetically distant link. The present investigation thus contribute to the knowledge on morphological and genetic variation in these three *Garra* species.

Keywords: fresh water fishes, genetic variations, morphological changes, PCR , RAPD

Introduction

India is a land of 'biological paradise' which supports a great variety of flora and fauna with its diverse topography, climate and natural resource. The fresh water resources of Indian subcontinent are very rich but largely unexplored. An estimate states that there are 12 major rivers and 48 lesser rivers with a total catchment area of 277.6 million hectares (Ghosh and Ponniah, 2001). Out of the 2500 species of fishes, recognized in the Indian subcontinent, 930 are categorized as freshwater species (Jayaram, 1999) and account for 9.3% of global inland fish production (Ghosh and Ponniah, 2001). However, studies on freshwater fishes in the Indian subcontinent have been limited to scattered works on commercial fisheries and have been largely restricted to some of the major river systems. The fin fish population in many fresh water resources of the country still remains unexplored for their taxonomical delineation.

Taxonomy is the pioneering exploration of life on earth, which lays the foundation for the phylogenetic tree of life (Wilson, 2004). It provides requisite database for ecology and conservation science and makes access to the vast and still largely unexplored biodiversity to humanity (Wilson, 2004). For ichthyo-taxonomical studies, morphometric characters are generally being used in discriminating many fish species (Anyanwu and Ugwumba, 2003; Eyo 2002, 2003; Teugels, 1992). They are measurable features which are helpful for separating closely related genera, species and even populations within them (Cadrin, 2000).

The development of molecular techniques has received considerable attention for investigating the genetic diversity of the fishes. Advances in molecular techniques increased the availability of different DNA-based markers, which has become efficient tools in conservation genetic studies (Haig, 1998; Avise, 2004). Random amplified polymorphic DNA (RAPD) is a simple and straightforward PCR-based technique that enabled dramatic improvements in genetic diversity analysis within the past decade. This technique uses arbitrary primers for amplification of discrete regions of genome (Williams *et al.*, 1990). RAPD markers have been used to evaluate the genetic diversity in numerous organisms and on fish populations belonging to the same family or genus (Ali *et al.*, 2004; Cooper, 2000; Lockley and Bardsley, 2000).

The *Garra* Hamilton-Buchanan genus, belonging to the *Garrinae* subfamily, is widely distributed in fresh water basins of the world.

According to Talwar and Jhingran (1991), this genus is represented by 21 species in the Indian subcontinent. Among this three closely related species, *Garra mullya* (Sykes, 1839), *Garra kalakadensis* (Rema Devi, 1993) and *Garra gotyla stenorhynchus* (Jerdon, 1849) are abundantly present in various fresh water basin of Tamil Nadu, South India. They slightly differ in their morphological features but certain characters overlap each other. The purpose of this study was to evaluate patterns of morphological and genetic variation in the primary range of the species, by examining banding pattern in several mitochondrial and nuclear genes, using RAPD-PCR techniques.

Materials and methods

Fish samples were collected from three geographically isolated river systems of the Western Ghats (Fig. 1). The *Garra kalakadensis* was collected from Kalakkad Mundandhurai Tiger Reserve (site 1); *G. mullya* from upstream of Hanumannadhi, one of the sub-basins of river Tamiraparani (site 2); *G. gotyla stenorhynchus* from Bhavani River near Mettupalayam (site 3). Fifteen individuals of each fish species were collected and preserved in 10% formalin (i.e., 4% formaldehyde solution) for morphometric studies and fresh fish samples were preserved in 90% ethanol for isolation of genomic DNA. The morphometric measurements viz. standard length, preanal length, predorsal length, prepelvic length, prepectoral length, preoccipital
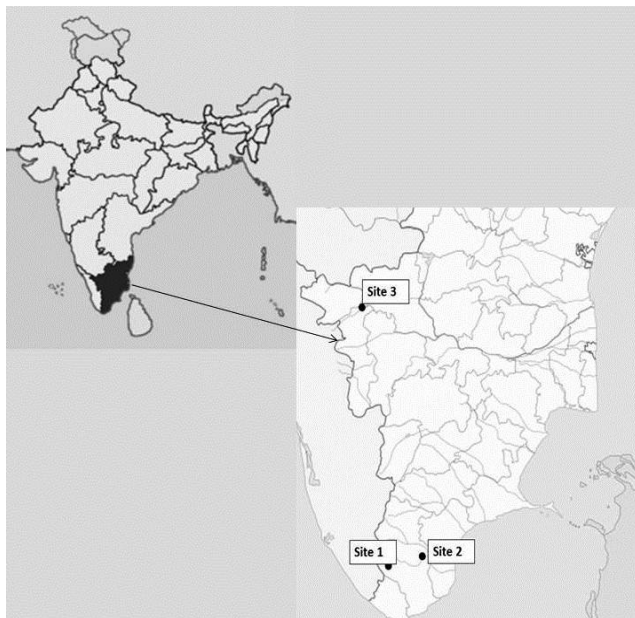

Fig. 1. The map showing various sites of fish collection for the experiment

length, snout to opercle distance, upper jaw length, snout length, prenasal length, orbit width, interorbital width, internasal width, head width, gape width, peduncle length, anal fin height, head depth at nostril, head depth at pupil, head depth at occiput, caudal peduncle depth, caudal fin length, dorsal fin height, pectoral fin length, pelvic fin

length, pelvic axillary scale length, maxillary barbel length, rostral barbel length, distance b/w pect fin and vent, distance b/w pelc fin and vent and disc width distance b/w vent and anal fin were taken. They were examined using correlation matrix in PCA and cluster analysis by the PAST software.

The total genomic DNA was extracted from muscle tissue following standard Phenol-Chloroform protocol (Sambrook *et al.,* 1989). The quantification of DNA was done by UV spectrophotometer analysis. The quantity of DNA was measured by obtaining the absorbance reading at 260 nm and the purity of DNA was checked by calculating the ratio of absorbance readings at 260 nm and 280 nm. After isolation, the DNA samples were taken out and mix with 7 μl Bromophenol blue (sample loading dye) and a 15 μl of mixed DNA product was loaded in 1.5 % Agarose gel (50 ml) containing Ethidium bromide at the concentration of 20 μl per 50 ml of gel. The electrophoresis was carried out for 1 to 2 hours at 50 volts. After electrophoresis gel was placed in the UV transilluminator and bands were visualized and were photographed in gel documentation system.

For the RAPD analysis, the DNA extracts were subjected to PCR amplification with primers purchased from Operon Technologies, U.S.A. Ten different primers were tested on fish samples and four better responding were selected for further studies. The RAPD profile generated by each set of primer was scored for presence or absence of an amplification product using agarose gel. The presence of a band was scored as 1 and the absence of it as 0. Cluster analysis were performed and dendrograms plotted based on pair wise genetic distance estimated using the unweighed pair group method with arithmetic mean (UPGMA) based on Nei (1978).

Results and discussion

Morphometric measurement values obtained for the three species of *Garra* are shown in the Tab. 1. *Garra mullya* and *G. kalakadensis* are similar regarding morphological characters comparing to other congener, *G. gotyla stenorhynchus*. *G. gotyla stenorhynchus* exhibited distinct variation in the morphological character such as snout length, pre-nasal length, inter-nasal width, gap width, lower jaw to isthmus, head depth at pupil, dorsal fin length and disc width. The principale components analysis revealed that *G. mullya* and *G. kalakadensis* are more similar species than *G. gotyla stenorhynchus* (Fig. 2). The cluster analysis also exhibited a similar trend with *G. gotyla stenorhynchus,* distinguishable from the other two *Garra* species, *G. mullya* and *G. kalakadensis.* (Fig. 3). The morphometric results are insignificant to support the established genetic structure of the population that often leads to taxonomic uncertainty (Daniel, 1997; Ponnian and Gopalakrishnana, 2000; Garg *et al.*, 2009b). The investigation was further extended to analyze the genetic variation.

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Tab. 1. The proportional values of morphometric measurements of the three species

No.	Morphometric measurements	*G. mullya*	*G. kalakadensis*	*G. gotyla stenorhynchus*
1	Standard length	527.245	666.54	878.455
2	Snout to urocentrum	499.593	643.08	817.7092
3	Pre-anal length	413.107	532.59	673.5358
4	Pre-dorsal length	267.896	533.235	426.8375
5	Pre-pelvic length	278.871	520.56	464.5125
6	Pre-pectoral length	117.579	158.22	204.8717
7	Pre-occipital length	129.907	152.205	205.195
8	Snout to opercle	99.325	94.5	169.2817
9	Upper jaw length	66	64.515	90.6
10	Snout length	81.392	94.56	129.0333
11	Pre-nasal length	53.368	64.845	75.79667
12	Orbit width	39.348	43.035	53.21
13	Inter orbital width	72.788	82.365	112.4533
14	Inter nasal width	47.608	56.22	78.13667
15	Head width	116.088	144.72	173.38
16	Gape width	75.2	91.44	115.1833
17	Peduncle length	74.134	82.125	134.6658
18	Anal fin height	115.392	137.235	189
19	Head depth at nostril	65.972	76.71	126.2533
20	Head depth at pupil	84.472	95.085	144.0167
21	Head depth at occiput	96.912	112.74	157.71
22	Peduncle depth	69.396	82.515	127.1275
23	Caudal fin length	169.384	194.445	329.7167
24	Dorsal fin height	141.612	162.675	241.06
25	Pectoral fin length	151.052	173.955	223.7933
26	Pelvic fin length	129.472	148.2	196.4667
27	Pelvic axillary scale length	42.984	36	82.21
28	Maxillary barbell length	25.084	15.12	28.74333
29	Rostral barbel length	36.088	36	42.70667
30	Distance b/w pect fin/vent	270.648	325.545	428.71
31	Distance b/w pelc fin/vent	111.704	124.905	156.3933
32	Disc width	61.024	57.195	70.85667
33	Distance b/w vent/anal fin	42.708	47.055	70.88

Tab. 2. Nucleotide sequences of RAPD primers (OPA-01-10) showing amplification status with the three fish species (best performing primers are in bold)

Primer code	Primer sequence (5' to 3')	Molecular Weight (Da)	Amplification	Polymorphism
OPA-01	CAGGCCCTTC	2985	ND	ND
OPA-02	**TGCCGAGCTG**	**3035**	**+**	**++**
OPA-03	**AGTCAGCCAC**	**2987**	**+**	**++**
OPA-04	AATCGGGCTG	2964	ND	ND
OPA-05	AGGGGTCTTG	3048	ND	ND
OPA-06	**GGTCCCTGAC**	**3056**	**+**	**++**
OPA-07	**GAAACGGGTG**	**3108**	**+**	**++**
OPA-08	GTGACGTAGG	3012	ND	ND
OPA-09	GGGTAACGCC	2978	ND	ND
OPA-10	GTGATCGCAG	2992	ND	ND

ND: Not Detected; **+**: Amplification present; **++**: Polymorphic Bands Present; **-**: No Polymorphic Bands Present; ******: Selected primers for this study

Tab. 3. The pair wise comparison of Nei's genetic identity of three species of *Garra*

Species	*G. mullya*	*G. kalakadensis*	*G. gotyla stenorhynchus*
G. mullya	****	0.2369	0.3544
G. kalakadensis	0.2369	****	0.5912
G. gotyla stenorhynchus	0.3544	0.5912	****

The isolation of high quality DNA is essential for many molecular biology applications using polymerase chain reaction (Chakraborty *et al.*, 2008). This systematics realm of methods offers new suites of characters for analyzing the relationship among the fishes (Hillis, *et al.*, 1996; Carvalho and Pitcher, 1995). In the present study, an optimum quantity of DNA was found in all the extracts of the fish
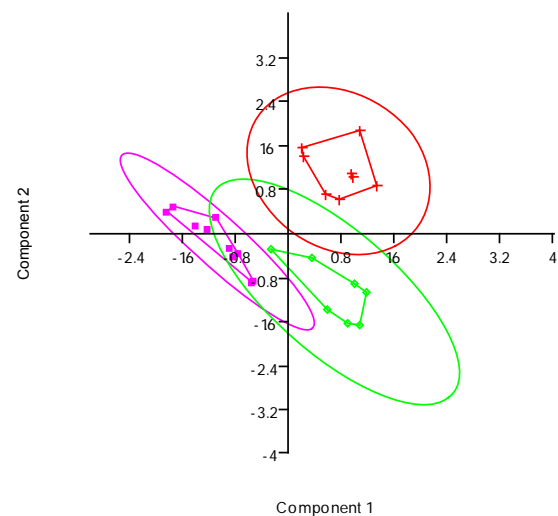

Fig. 2. The PCA scatter diagram showing the variation of the three *Garra* species (red for *Garra mullya,* green for *Garra kalakadensis* and pink for *Garra gotyla stenorhynchus*)
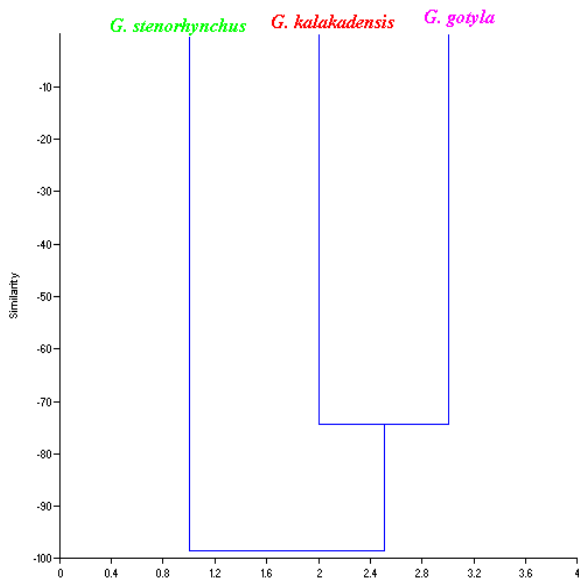
Fig. 3. Cluster analysis of three *Garra* species

OPA-6 primers produced the maximum number of amplified products. Reproducible polymorphic bands from the RAPD analysis were screened qualitatively for presence or absence in each sample. The shared RAPD fragments found in both *G. mullya* and *G. kalakadensis* with fixed frequencies were also observed in all investigated primers, implying their genetically close relationships. The pair wise comparison of genetic distance of three species revealed that *G. mullya* are more similar, considering the *G. kalakadensis* index, with less genetic distance (Tab. 4). However, *G. gotyla stenorhynchus* was showing higher genetic distances with two species, which was higher with *G. kalakadensis*. A similar observation was found with cluster analysis, in which *G. gotyla stenorhynchus* was separated from the other two species of *Garra* (Fig. 5). Hence, the present investigation revealed the taxonomical relation between three *Garra* species, which will contribute much to the least studied fresh water fishes of India.

Genetic approaches offer powerful tools for examining the current status of populations, for understanding the population changes for its conservation (Belfiore and Anderson, 2001). RAPD technique is one of the most frequently used molecular methods for taxonomic and systematic analyses of various organisms (Garg *et al.*, 2009a; 2010). The present study evaluated morphometric characters and patterns of genetic variation in three *Garra* species. Both the morphological and genetic analysis revealed that *G. mullya* and *G. kalakadensis* had many similar char-
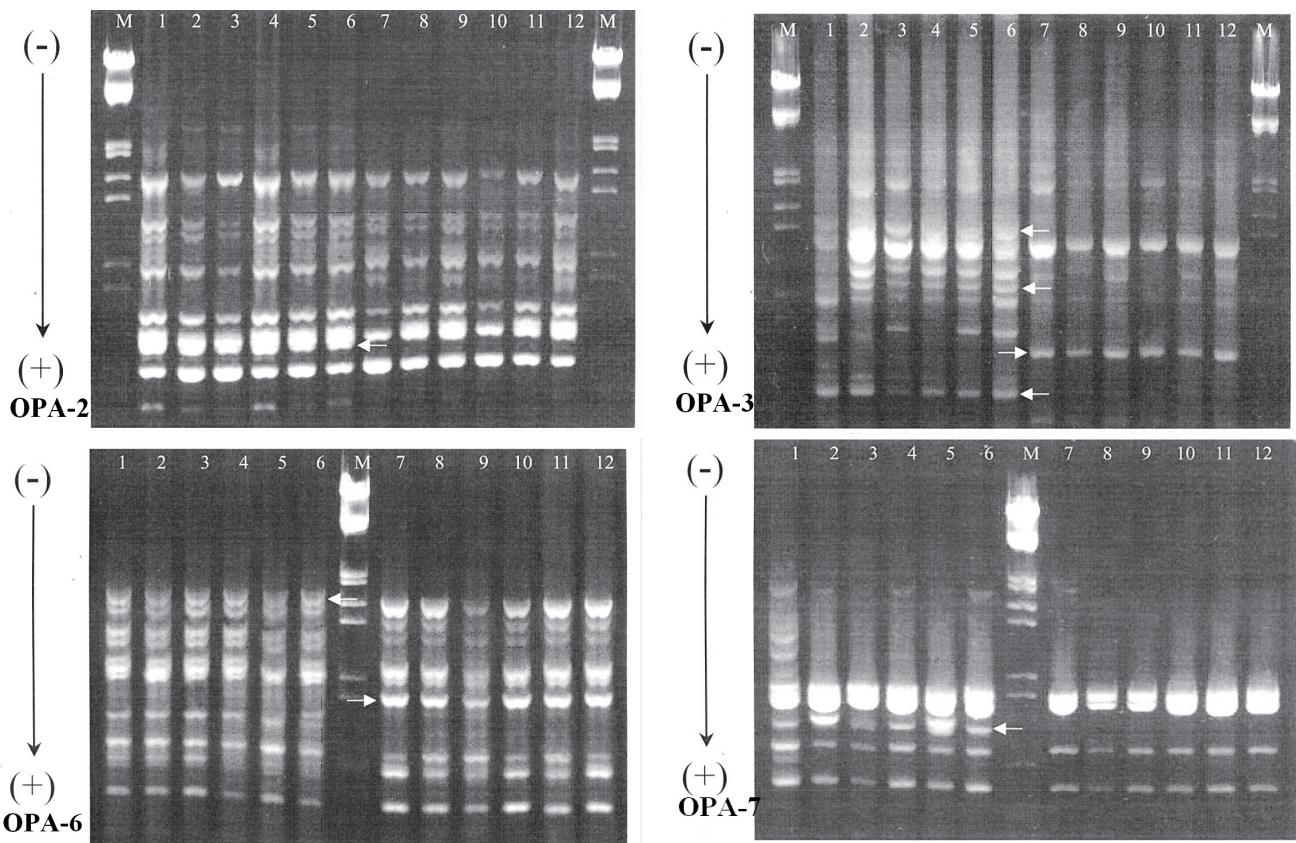
species with various primers. A total of 72 reliable fragments were detected and observed using 10 Operon primers ranging from 2600 molecular weight to 3100. Thus 4 of the better responded primers viz. OPA-02, OPA-03, OPA-06 and OPA-7 were used for further studies (Tab. 2). Each of the random primers produced distinct polymorphic banding patterns at all of the fishes (Fig. 4). The

Fig. 4. RAPD pattern with various primers tested for the three species of *Garra*. Lane no. (1-4) different individuals of *Garra mullya,* Lane no. 5-8 different individuals of *Garra Kalakadensis* and Lane no. 9-12 different individuals of *Garra gotyla stenorhynchus*. Arrows indicate the species specific markers
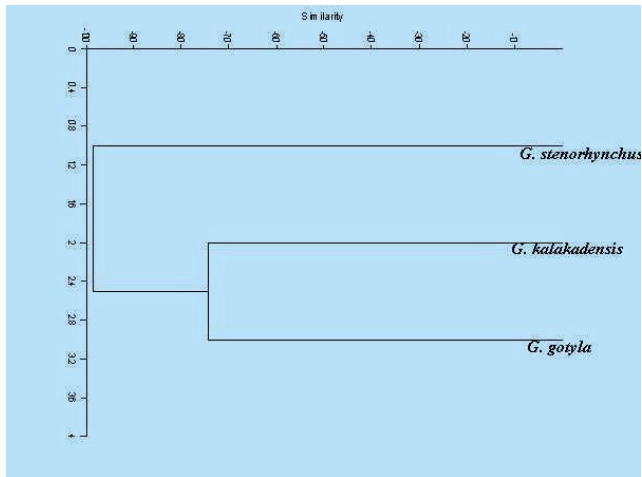
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Fig. 5. The cluster analysis showing the genetic relation of the three species of *Garra*

acters, whereas *G. gotyla stenorhynchus* exhibited distinct variations. The present structure of genetic diversity is the invisible dimension of biological diversity, being the result of the evolutionary history of the species exposed to natural selection pressures in variable environmental conditions. Natural selection at the local level is an evolutionary force opposed to gene flow. The combination of the two forces creates a powerful mechanism for maintaining within-species diversity (Edward *et al.*, 2002).

Conclusions

The present investigation revealed the morphometric and genetic variation of three *Garra* species. The results of morphological approach revealed that *G. mullya* and *G. kalakadensis* are more similar in comparison to the other congener, *G. gotyla stenorhynchus*. The latter exhibits distinct variation both in the morphological character and genetic fragments. The present investigation contribution to the knowledge on morphological and genetic variation to the *Garra* species. However, much specific molecular biomarkers are required for understanding the taxonomical relations of many other species of this group, which are widely distributed in various fresh water basins of India.

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