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

Arulraj DHINAKARAN¹, Nabeel Mannalamkunnath ALIKUNHI¹, Selvaraj CHINNATHAMBI², Ramaiya SORNAM³, Murugaiyan KALAISELVAM¹ Ramadoss RAJASEKARAN¹, Subramanian MANIVANNAN³

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

Abstract

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). For ichthy-taxonomical studies, morphometric characters are generally being used in discriminating many fish species (Anyawu 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 Hanumammadhi, 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, prepectorlal length, preoccipital 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 pelvic 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, internasal width, gape width, head depth at pupil, dorsal fin length and disc width. The principal 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.
The isolation of high quality DNA is essential for many molecular biology applications using polymerase chain reaction (Chakraborty et al., 2008). This systems 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 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          | 75.7966                |
| 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      | AATCGGCGCTG                | 2964                  | ND            | ND           |
| OPA-05      | AGGGGTCTTG                 | 3048                  | ND            | ND           |
| OPA-06      | GGTCCCTGAC                 | 3056                  | +             | ++           |
| OPA-07      | GAAACGGGTTG                | 3108                  | +             | ++           |
| OPA-08      | GTGACGTAGG                 | 3012                  | ND            | ND           |
| OPA-09      | GGTAACGCCT                 | 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 systems 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 species.
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 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-
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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