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Comparative analysis of genetic diversity among three species of the freshwater fish genus *Garra* (Osteichthys: Cyprinidae) using restriction fragment length polymorphism

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Abstract

The genetic variation in three species of the freshwater cyprinid *Garra* was studied using the traditional morphometric, meristic and Restriction fragment length polymorphism as molecular tool analysis. Samples were collected from their respective geographic locations of southern Western Ghats. Based on the 46 morphometric and 18 meristic characters employed during this study 23 characters showed variation among the three species and hence were utilized for the PCA ordination. The principal component analysis was performed using 15 morphometric and 8 meristic characters of which 12 components were extracted and the first three axes showed eigenvalues >1 and they explained the variance about 81.46 % of the total variance. The genome size of the species *Garra mullya* ranged from 3.8-6.15 µg/mg, *Garra kalakadensis* ranged from 3.25- 6.3 µg/mg and *Garra gotyla stenorhynchus* ranged from 3.9-6.15 µg/mg. Based on the electrophorogram, different bands of fragments in each lane and band volume were analyzed, According to *Hind III* enzyme the electrophorogram analysis showed maximum fragment length polymorphism in *Garra mullya* which had four fragments and the total volume of bands was 12.582 nmoles. Based on the *Eco R1* enzyme digestion the electrophorogram analysis revealed that the maximum fragment length polymorphism in *Garra mullya* composed of four fragments and the total volume of bands in the entire lane was 10.5965 nmoles. Based on the *Hind III* and *Eco R1* restriction enzymes, the cluster analysis clearly showed that the *Garra mullya* and *Garra kalakadensis* grouped together while *Garra gotyla stenorhynchus* with distinct genetic distance did not cluster with the other two species. *Garra gotyla stenorhynchus* can also be distinguished morphologically from *Garra mullya* and *Garra kalakadensis*.

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Introduction

Fishes are the most diverse group of living vertebrates, with more than 24,600 extant species currently known. The Order Cypriniformes is the planet's largest monophyletic group of freshwater fishes, with over 400 genera and 3000 species native to Asia, Europe, Africa, and North America (Nelson, 2006). Cypriniformes in peninsular India is represented by 4 families, 8 sub families, 40 genera and 166 species. The family Cyprinidae has 4 subfamilies, 27 genera and 135 species (Arunachalam *et al.,* 2008). Historically the morphology of fishes is the main source of information for taxonomic and evolutionary studies. Systematic ichthyologists still rely on morphology for taxonomic characters as species have characteristics shape, sizes, pigmentation patterns, disposition of fins and other external features which help in identification and classification. Meristic traits are often considered to be the most reliable taxonomic characteristics, because most are easy to determine.

All the organisms are subjected to mutations as a result of normal cellular operations or interactions with the environment, leading to genetic variation. In conjunction with selection and genetic drift, there arises genetic variation within and among individuals, species and higher order taxonomic groups. This variation are useful to geneticists, but it must be heritable and discernable to the researcher, whether as a recognizable phenotypic variation or as a genetic mutation distinguishable through molecular techniques. Several marker types are highly popular in genetics.

The development of molecular techniques has helped to investigate fish systematics. The realm of methods developed for molecular studies offers new suites of characters for analyzing relationships among fish systematics (Hillis, *et al.,* 1996; Ferraris and Palumbi, 1996) and hence have been effectively applied from the level of populations to orders. A restriction fragment length polymorphism or RFLP, is a variation in the DNA sequence of a genome that can be detected by breaking the DNA into pieces with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis.

DNA-based techniques for species identification have recently started to be applied towards a wide variety of fish species, including closely related species belonging to the same family or genus (Davidson 1998; Bossier 1999; Lockley and Bardsley 2000). The information on genetic resource of Indian fish fauna is very limited, especially native cyprinids. The genetic variation in different populations of the freshwater cyprinid species *Puntius filamentosus* was studied using Restriction Fragment Length Polymorphism (RFLP) analysis. The genomic size of the different populations of *P. filamentosus* was found between 3.45 and 3.80ng/mg. The study could prove one population as distinct. (Johnson *et al.,* 2007).

The cyprinid fish genus *Garra* includes bottom dwelling fishes usually found in fast flowing streams where they cling to rocks using the highly modified mouth which acts as a sucker. *Garra* is a common cyprinid and are mostly inhabitants of rapid running waters, adapted with suctorial disc to attach themselves to the substratum in swift currents and horizontally placed paired fins, especially the pectorals. The flattening of the head and anterior part is as a result of efforts to utilize more and more of the anterior part of the body for adhesion. The proboscis and the tubercles help to lessen the velocity and impact of the rushing torrents. Adaptive features in all the species being similar most of the morphological features show overlap and hence the taxonomic characters commonly used are not helpful in clear cut diagnosis of the species and hence taxonomic ambiguities exist.

The genus is widely distributed from southern China, across South East Asia, India and the Middle East to northern and central Africa. Because of the extreme

morphological divergence of the genus *Garra*, the sister group relationships of this genus is of extreme interest to systematic ichthyologists, fish biologists, and evolutionary biologists alike.

In the present study, the aim is to analyze the overall features of morphometric, meristic characters, and Restriction pattern of nuclear DNA using the restriction enzymes *Hind III*, and *Eco RI* regions in order to evaluate patterns of genetic variation between species of *Garra* and to determine the phylogenetic relationship of three species of the endemic genus *Garra* in selected streams and rivers of the peninsular India.

Materials and Methods

Fish samples were collected from three geographically isolated east flowing river systems of the Western Ghats. *Garra mullya* was collected from the upstream of Hanumannadhi, which forms a sub basin of Tamiraparani river and *G. kalakadensis* was collected from Pachiyar in Kalakad Mundandhurai Tiger Reserve and *G. gotyla stenorhynchus* collected from Nellithurai (Bhavani River) near Mettupalayam (Fig. 1.) These native cyprinids were collected using mono filamentous, multi filamentous gill nets of different mesh sizes from 8-28 mm and cast nets. Fishes were identified in the field and a portion of gill and muscle tissues were fixed in 95% ethanol and were kept in the ice cubes for molecular analysis. Few individuals were preserved in 10% formalin for morphological studies. Large specimens were injected with 15% formalin carefully through the vent prior to preservation. Specimens were kept preserved in Manonmaniam Sundaranar University Museum of Natural History (MSUMNH). Measurements were made point to point using digital caliper. The meristic and morphometric measurements are based on the method by Hubbs and Lagler (1956).

The genomic DNA was isolated by phenol-chloroform method (Sambrook *et al.,* 1989). Amount of DNA present in each sample were determined using UV spectrophotometer. Isolated DNA samples were subjected to restriction enzyme digestion at 37° C for 2 h using *EcoR1* and *Hind III* enzyme. After incubation each samples were loaded in to 1% agarose gel and electrophoresed at 50 – 100 V for one and half hours. After electrophoresis, gel was placed in the gel document unit (BIORAD) and bands were visualized and were photographed. The DNA fragment in each lane was viewed and number of band and band volume were documented using TOTAL LAB gel analyzing software. The band volume data were used for construction of similarity cluster using PAST software (Free ware: version. 1.83).

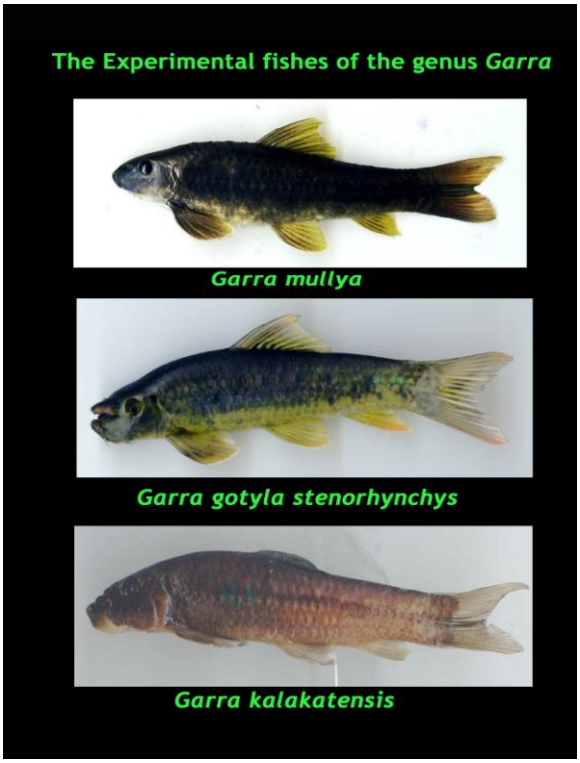

Fig. 1. The Experimental fishes of the genus *garra*.

Results

In the present study the Principal component analysis based on the 46 morphometric and 18 meristic characters were employed. As 23 characters showed variation among the three species and hence these characters were used for PCA ordination of which 15 are morphometric and 8 were meristic characters

(Table 1). Of these 12 components were extracted and the first three axes showed the eigen value >1 and also variation is 81.46 % of the total variance (Table 2). The first component alone explained about 53.7 % variance in characters such as: Snout length, Pre nasal length, Head width, Transverse breast rows, Pre anal scales, Pelvic insertion to anal origin, Lower transverse rows, Circumpeduncular scales, Head depth at occiput and Peduncle depth showed higher factor loadings (> 0.8). The second axis explained 18.38 % variance and higher loadings were noted in two characters Disc width and branched pelvic fin rays. The 3rd and 4th components together explained 9.28 % variance among the characters but none of them was significant in factor loadings.

Characters such as head length and predorsal scales varied between *Garra gotyla stenorhynchus* and *Garra kalakadensis* (Head length 20.56 – 25.48 vs. 26.37 - 28.32; predorsal scales 10 vs.10 - 12) as shown by the PCA (Fig. 2) Disc width and pelvic fin rays of *Garra mullya* were lower in values compared to the other two species and hence it has been plotted between the *G. kalakadensis* and *G. gotyla stenorhynchus* in the ordination.

Table 1. Morphometric and meristic measurements of *Garra mullya, Garra kalakadensis* and *Garra gotyla stenorhynchus*.

Characters	*Garra mullya* (n=10)		*Garra kalakadensis* (n=10)		*Garra gotyla stenorhynchus* (n=10)	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Snout length (SL)	45.54 - 52.39	48.89 ± 1.98	41.38 - 46.98	44.85 ± 1.61	49.26 - 58.43	54.88 ± 2.77
Pre nasal length (PNL)	32.1 - 38.09	35.57 ± 2.13	25.89 - 33.8	29.48 ± 2.73	34.29 - 42.28	38.31 ± 2.22
Head width (HW)	68.79 - 76.58	72.95 ± 2.73	64.54 - 69.12	66.82 ± 1.66	69.78 - 78.75	74.96 ± 2.85
Head depth at nostril (HDN)	34.56 - 45.32	39.45 ± 3.52	33.1 - 40.23	37.68 ± 2.36	45.39 - 53.00	47.36 ± 2.69
head depth at pupil (HDP)	45.08 - 57.41	52.07 ± 3.66	48.22 -56.13	51.81 ± 2.96	54.79 - 62.93	59.11 ± 2.58
Head depth at occiput (HDO)	63.21 - 71.73	66.66 ± 3.45	55.84 - 65.4	61.24 ± 2.97	70.73 - 79.14	74.65 ± 2.64
Peduncle depth (PD)	9.82 - 13.1	12.18 ± 0.88	9.82 - 10.59	10.24 ± 0.27	12.70 -18.07	14.76 ± 1.41
Dorsal origin to anal origin (DA)	31.22 - 40.89	37.89 ± 2.58	31.3 - 35.6	33.5 ± 1.06	35.28 - 47.12	39.5 ± 2.97
Dorsal fin base (DFB)	15.03 - 20.06	17.03 ± 1.54	11.76 -15.71	13.44 ± 1.12	16.73 - 23.10	18.3 ± 1.77
Pectoral insertion to anal origin (PECA)	48.78 - 57.43	54.62 ± 2.36	48.53- 56.22	52.16 ± 2.40	54.25 - 61.90	58.01 ± 2.92
Pelvic insertion to anal origin (PELA)	21.61 - 26.12	24.39 ± 1.18	19.41- 24.38	22.13 ± 1.49	23.75 - 34.33	27.25 ± 2.89
Head length (HL)	20.39 - 25.93	24.52 ± 1.63	26.37- 28.31	27.48 ± 0.68	20.56 - 32.60	24.05 ± 2.93
Body depth (BD)	18.23 - 25.3	22.56 ± 1.96	16.22- 21.16	19.24 ± 1.60	19.21 - 29.70	22.88 ± 2.74
Disc length (DL)	22.93 - 29.91	26.11 ± 2.76	26.52- 34.39	30.52 ± 2.68	28.27- 35.27	33.12 ± 2.19
Disc width (DW)	37.16 - 46.27	41.75 ± 3.06	48.49 - 55.39	51.01 ± 2.07	46.47- 54.31	50.26 ± 2.80
Branched pelvic fin rays (pelvic)	7 - 8	7.3 ± 0.45	8	8± 0.00	8	8 ± 0.00
Branched pectoral fin Rays (pectoral)	11-13	12± 0.43	12 - 14	12.90 ± 0.57	12 - 15	13.92 ± 0.79
Pre dorsal scales (predor)	10-11	10.9 ± 0.29	10 - 12	11.10± 0.88	10	10 ± 0.00
Lower transverse rows (Lowtr)	3-4	3.8 ± 0.45	3	3± 0.00	4	4 ± 0.00
Circumpeduncular scales (circp)	15-16	15.8± 0.39	12 - 14	12.60 ± 0.70	16	16 ± 0.00
Circumferential scales (circf)	22	22 ± 0.00	16 - 20	19.60 ± 1.26	22	22 ± 0.00
Transverse breast rows (breast)	5	5 ± 0.00	5	5± 0.00	6	6 ± 0.00
Pre anal scale (pre anal)	16-21	17.6 ± 2.02	14 - 17	15.70± 1.06	21-23	21.75 ± 0.62

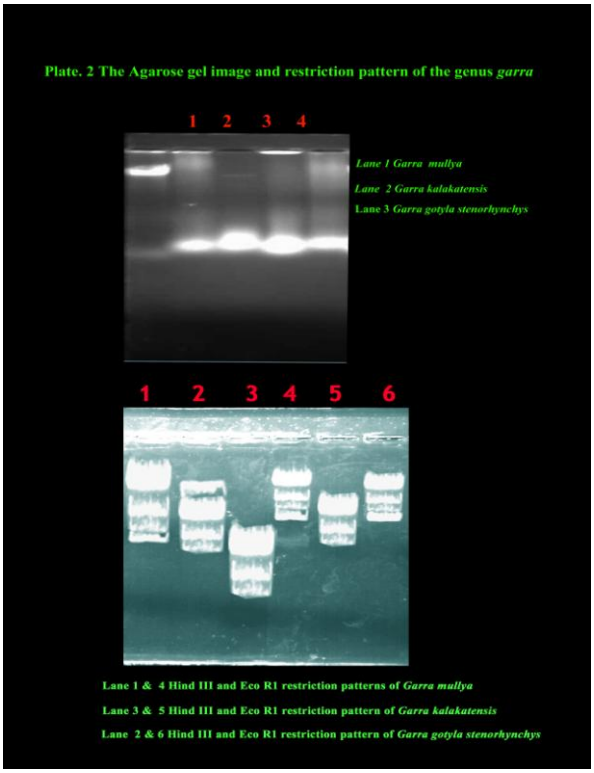

Fig. 2. Agarose gel image and restriction pattern of the genus *garra*.

The DNA content of each species and the corresponding O.D. values are given in Table 3. The genome size of *Garra mullya* ranged from 3.8-6.15 µg/mg, while *Garra kalakadensis* showed the range from 3.25- 6.3 µg/mg and *Garra gotyla stenorhynchus* showed the range from 3.9-6.15 µg/mg. The result of the restriction analysis showed that there was clear separate DNA banding pattern for the three species and the fragment migration were ranged from 5000-2000bp. Based on the electrophorogram, different bands of fragments in each lane and band volume were analyzed (Table 4, 5). According to *Hind III* enzyme the electrophorogram analysis showed maximum fragment length polymorphism in *Garra mullya* which had four fragments 1.056 nmoles, 5.842 nmoles, 3.216 nmoles and 2.468 nmoles respectively and the total volume of bands along the entire lane was 12.582 nmoles. *Garra kalakadensis* had three restriction fragments 4.236 nmoles, 3.924 nmoles and 3.124 nmoles and the total volume of bands in the entire lane was 11.284. *Garra gotyla stenorhynchus* had four restriction fragments with corresponding band volumes of 1.649 nmoles, 0.926 nmoles, 3.418 nmoles and 2.236 nmoles and the total volume of bands in the entire lane was 8.229 nmoles.

Table 2. Component matrix of four axes from PCA of morphological data.

Characters	Axis 1	Axis 2	Axis 3	Axis 4
Snout length (SL)	0.874*	-0.139	0.194	-0.162
Pre nasal length (PNL)	0.864*	0.206	0.088	0.191
Head width (HW)	0.813*	0.198	0.350	0.020
Head depth at nostril (HDN)	0.787	-0.281	-0.100	0.232
head depth at pupil (HDP)	0.709	-0.401	0.110	0.021
Head depth at occiput (HDO)	0.900*	-0.057	0.032	-0.098
Peduncle depth (PD)	0.935*	0.041	-0.190	0.031
Dorsal origin to anal origin (DA)	0.772	0.359	-0.247	0.000
Dorsal fin base (DFB)	0.766	0.335	0.026	0.221
Pectoral insertion to anal origin (PECA)	0.755	-0.097	-0.424	0.070
Pelvic insertion to anal origin (PELA)	0.827*	-0.004	-0.314	-0.095
Head length (HL)	-0.729	-0.057	-0.576	0.122

Body depth (BD)	0.607	0.447	-0.290	-0.395
Disc length (DL)	0.288	-0.676	-0.293	-0.140
Disc width (DW)	0.006	-0.924*	-0.097	0.107
Branched pelvic fin rays (pelvic)	0.018	-0.844*	0.250	-0.236
Branched pectoral fin Rays (pectoral)	0.424	-0.761	-0.081	-0.053
Pre dorsal scales (predor)	-0.673	0.277	-0.012	-0.565
Lower transverse rows (Lowtr)	0.834*	0.204	-0.108	-0.323
Circumpeduncular scales (circp)	0.835*	0.460	0.049	0.079
Circumferential scales (circf)	0.747	0.431	0.078	0.050
Transverse breast rows (breast)	0.827*	-0.502	0.018	-0.019
Pre anal scale (preanal)	0.842*	-0.244	0.250	-0.149
Percent of variance explained	53.79	18.38	5.35	3.93

Table 3. DNA Purity and Genome size of the three species of genus *Garra*.

Species	No.	Absorbance at		Purity of DNA	Total amount µg/mg
		260nm	280nm		
Garra mullya	1	0.084	0.1377	1.64	4.2
	2	0.076	0.1284	1.69	3.8
	3	0.092	0.1490	1.62	4.6
	4	0.102	0.1693	1.66	5.1
	5	0.123	0.2115	1.72	6.15
Garra kalakadensis	1	0.126	0.2179	1.73	6.3
	2	0.108	0.1825	1.69	5.4
	3	0.089	0.1441	1.62	4.45
	4	0.085	0.1385	1.63	4.25
	5	0.065	0.1040	1.60	3.25
Garra gotyla stenorhynchus	1	0.078	0.1263	1.62	3.9
	2	0.089	0.1450	1.63	4.45
	3	0.096	0.1622	1.69	4.8
	4	0.123	0.2152	1.75	6.15
	5	0.110	0.1870	1.70	5.5

Table 4. Number of fragments and band volume of Electrophorogram for the three species of genus *Garra* based on the *Hind III* restriction pattern.

Fish species	Number of Fragments	Band volume (n moles)	Total band volume (n moles)
Garra mullya	1	1.056	12.582
	2	5.842	
	3	3.216	
	4	2.468	
Garra kalakadensis	1	4.236	11.284
	2	3.924	
	3	3.124	
Garra gotyla stenorhynchys	1	1.649	8.229
	2	0.926	
	3	3.418	
	4	2.236	

Table 5. Number of fragments and band volume of Electrophorogram of the genus *Garra* based on the *Eco RI* restriction pattern.

Fish species	Number of Fragments	Band volume (n moles)	Total Band volume (n moles)
Garra mullya	1	2.3645	10.5965
	2	3.1004	
	3	4.1252	
	4	1.0064	
Garra kalakatensis	1	4.5692	11.4773
	2	3.6541	
	3	3.2540	
Garra gotyla stenorhynchys	1	1.2301	7.1364
	2	0.6359	
	3	3.1540	
	4	2.1164	

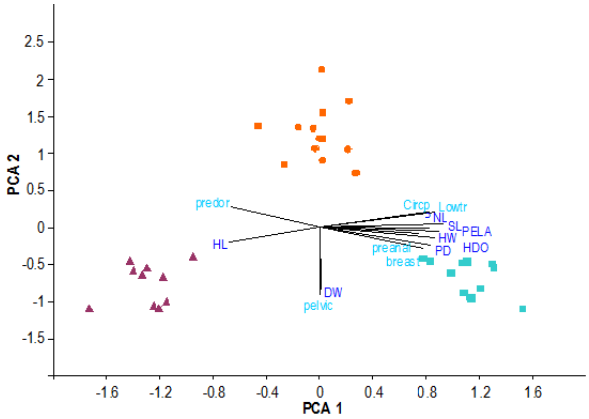

Fig. 3. Principal Component Ordination for three garrine species *Garra mullya* (●), *Garra gotyla stenorhynchus* (■) and *Garra kalakadensis* (▲) based on morphological characters.

Based on the electrophorogram, different bands of fragments in each lane and band volume were analyzed (Table 5). Based on the *Eco R1* enzyme digestion, the electrophorogram analysis revealed that the maximum fragment length polymorphism in *Garra mullya* composed of four fragments with each band volume of 2.3645 nmoles, 3.1004 nmoles, 4.1252 nmoles and 1.0064 nmoles respectively and the total volume of bands in the entire lane was 10.5965 nmoles. *Garra kalakadensis* possessed three restriction fragments 4.5692 nmoles, 3.6541 nmoles and 3.254 nmoles and the total volume of bands in the entire lane was 11.4773 nmoles. *Garra gotyla stenorhynchus* had three restriction fragments each with a band volume of 1.2301 nmoles, 0.6359 nmoles, 3.1540 nmoles, and 2.1164 nmoles and the total volume of bands in the entire lane was 7.1364 nmoles.

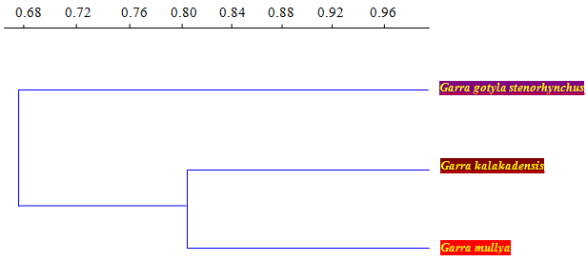

Fig. 4. Genetic distance among the three species of *Garra* based on the RFLP band volume.

Based on the *Hind III* and *Eco R1* restriction enzymes, the cluster analysis clearly showed that *Garra mullya* and *Garra kalakadensis* grouped together while *Garra gotyla stenorhynchus* with distinct genetic distance did not cluster with the other two species (Fig. 3) *Garra gotyla stenorhynchus* can also be distinguished morphologically from *Garra mullya* and *Garra kalakadensis* by the presence of proboscis.

Discussion

The genus *Garra* known for its higher degree of plasticity ascribed to its adaptive features comprises a number of species some with distinct characters and few others with close resemblances owing to the more similar morphological characters. Most of the morphological traits of fishes are similar and often

overlap among the three species under study though *G. gotyla sternorhynchus* is distinct with its protuberant proboscis. Data on morphological features both morphometric characters and meristic counts are not enough to support the established genetic structure of the different species and hence leads to taxonomic uncertainty (Ponniah and Gopalakrishnan, 2000).

The genome size of the three species (*Garra mullya, Garra kalakadensis,* and *Garra gotyla stenorhynchys*) ranged from 3.25 to 6.3 µg/mg and much variation was observed among the three species. The results reported here regarding the genetic variation found among the three species are in agreement with morphological data. *Garra kalakadensis* (Pachaiyar river basin) was earlier thought of as a geographically isolated population of the commonly available species *G. mullya* and hence formerly ascribed to the species *G. mullya* which was later been recognized and described as distinct species in this genus that comprises of at least 12 species and subspecies in peninsular India that share a number of morphological traits.

Restriction fragment length polymorphism analysis has been used for the analysis of genetic structures and diversity or to distinguish among related species (Takahashi and Ohara 2004). During the present study, with respect to the degree of differences among the three species, molecular data based on electrophorogram analysis showed significant variation in fragment length among the three species and have been congruent with morphological results. *Garra mullya* and *Garra kalakadensis* show closer resemblance with each other and *Garra gotyla stenorhynchus* was distinctly separate as evident from the cluster based on genetic linkage.

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