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Note

Molecular phylogeny of *Macrobrachium* species of India: habitat preference, biogeography and taxonomy

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ABSTRACT

The freshwater prawn genus *Macrobrachium* (Bate) is one of the most diverse, abundant and widespread crustacean genera. Different species of this genus have different habitat preference, as some species require access to the sea during their larval development whereas some species are restricted to inland waters with abbreviated larval development. The greatest diversity of the genus occurs in the Indo-Pacific region, in particular the Indian subcontinent. To examine the phylogenetic relationships of *Macrobrachium* species of this region with reference to their habitat preference, biogeography and taxonomy, the cytochrome b gene of mitochondrial DNA was amplified and sequenced. Based on the phylogenetic investigation, it was found that the species having same habitat preference were close to each other. However based on the genetic distance and nucleotide difference, it was further observed that species restricted to Indian subcontinent shared close relationship irrespective to their habitat preference leading to the conclusion that preference of habitat for reproductive strategy has evolved independently within a particular geographical area.

Keywords: Cytochrome b gene, Habitat, *Macrobrachium*, Molecular phylogeny,

Freshwater aquaculture has expanded rapidly in the Asia-Pacific region over the last thirty years, both in terms of the size of the industry and the diversity of species that are cultured. While marine penaeid prawns remain the major crustacean group used in culture in the region, freshwater prawns of the genus *Macrobrachium* have seen a dramatic increase in production. About 200 species of freshwater prawns of genus *Macrobrachium* were listed by Holthuis (1980) in different continents. There have been many acknowledged problems associated with the classification of the freshwater prawns. These occur at the specific, generic and family level (Pereira, 1997; Short, 2000; Murphy and Austin, 2003). The problems have been attributed to difficulties in the interpretation of the significance of morphological characteristics used to classify palaemonid prawn and have hindered the development of stable classification systems. Like their wide diversity in distribution, *Macrobrachium* prawns also inhabit a wide variety of environments from mountain streams and lowland rivers to estuaries and coastal lagoons. This is due to their three types of reproductive strategies. The first type has extended larval development that depends on marine access; the second includes species with distributions including inland and coastal waters and their larval development is more or less extended; and the third type includes species with abbreviated larval development that are independent of marine influence and are restricted to inland waters (Bueno and Rodrigues, 1995; Alekhnovich and Kulesh, 2001).

With the development of molecular biology techniques, several sequence based markers located in mitochondria are used as important tools for studying genetic relationship between different species as well

as for comparison of life history and physiological traits between them. Mitochondrial DNA provides a potential tool for studying species evolution, in outlook of characteristics such as maternal inheritance, high mutation rate (Brown *et al*., 1979), high copy number and lack of recombination (Meyer *et al*., 1993). Most of the mitochondrial protein coding genes have been used to examine phylogenetic relationships. The mitochondrial cytochrome b (cyt b) gene is widely used in systematic studies to resolve divergences at many taxonomic levels and this gene is chosen as a phylogenetic probe due to its ease in aligning protein coding sequences that has evolved over a long period.

Among 60 species of *Macrobrachium* available in India (Jayachandran, 2003, Jayachandran and Indira, 2010) nine species (Table 1) were selected in the present study for their fisheries importance in the country. Further these species have different distribution pattern as some of these species are restricted to Indian subcontinent while some are widely distributed. They also differ in habitat preference according to their reproductive strategy. In the present study, focus is mainly on phylogenetic relationships of these nine *Macrobrachium* species with reference to their habitat preference, biogeography and taxonomy based on cytochrome b gene sequence. A total of 132 specimens (Table 1) of nine *Macrobrachium* species were collected from wild populations of different geographical locations in India and identified with the help of key following Jayachandran (2003). The tissue (pleopods or whole individual) was preserved in absolute ethanol. The total genomic DNA was isolated from tissue or pleopod following Sambrook *et al*. (1989). The concentration of isolated DNA was estimated using UV spectrophotometer. The DNA was diluted to get a final concentration of 100 ng μl^{-1}. A fragment of cytochrome b gene (cyt-b) mtDNA was amplified by PCR using mcb398 and mcb869 universal primers (Verma *et al*., 2003). The PCR was carried out in a reaction volume of 25µl, containing lxPCR buffer, 0.1 mM of each dNTP, 2.5 pmole of each primer, 1 mM $MgCl_2$ following the temperature cycle of an initial denaturation step of 95 °C for 5 min, followed by 30 cycles of 95 °C for 30 sec, an annealing temperature of 58 °C for 30 sec and an extension temperature of 72 °C for 30 sec and finally an additional extension of 72 °C for 10 min. The PCR products were checked in agarose gel and quantified. The products were purified using shrimp alkaline phosphatase enzyme (Sambrook and Russel, 2001). The purified PCR products were directly sequenced using 50 ng (2 µl) of PCR product, 4 pM (1 µl) of primer, 4 µl of BigDye™ Terminator ready reaction mix and 3 µl of double distilled water to adjust the volume to 10 µl. Cycle sequencing was carried out in a Gene Amp 9600 thermal cycler (Perkin-Elmer) employing

Table 1. Species collected for the study

Species	Location	Number of individuals used
Macrobrachium rosenbergii (De Man, 1879)	Champakera, Kochi, Kerala	8
	Danu, Maharashtra	9
	Kakinada, Andhra Pradesh	9
	Bhubaneswar, Odisha	6
M. malcolmsonii (Tiwari, 1952)	Bhubaneswar, Odisha	13
	Rajahmundry, Andhra Pradesh	10
M. gangeticum (Bate,1868)	Lalgola, West Bengal	13
M. lamarrei (H. Milne Edw.1837)	Balasore, Odisha	10
M. sankolii (Jalihal et Shenoy, 1988)	Thane, Maharashtra	10
M. idella (Hilgendorf, 1898)	Champakera, Kochi, Kerala	11
M. equidens (Dana, 1852)	Tevera, Kochi, Kerala	12
M. rude (Heller, 1862)	Berhampur, Odisha	11
M. scabriculum (Heller, 1862)	Thane, Maharashtra	10
Total		132

the conditions: 30 cycles at 96 °C for 10 sec, 58 °C for 5 sec and 60 °C for 4 min. Extended products were purified by alcohol precipitation followed by washing with 70% alcohol. Purified samples were dissolved in 1 µl of 50% HiDi formamide and analysed in ABI 3700 automated DNA Analyser. Sequencing was done from both sides of PCR product using both forward and reverse primers. The BioEdit software was used for formatting cytochrome b gene sequences to make them compatible with the desired software. Edited sequences were aligned using CLUSTAL X package (Thompson *et al*., 1997). Phylogenetic tree was constructed by Neighbour Joining (NJ) method using the PHYLIP Package Ver 3.6 (Felsenstein, 1993). Polymorphic sites, nucleotide difference among the sequences and genetic distances between the species were estimated using MEGA 3.0 software and bootstrap analysis was carried out using 1000 pseudoreplications (Kumar *et al*., 2004).

The present work reports mtDNA diversity in 9 *Macrobrachium* species providing information about their phylogenetic relationship with reference to their habitat preference, biogeography and taxonomy. The sequence variation in the mitochondrial cyt b gene at certain common sites was observed for each of the species. Sequence divergence out of 440 bp of cyt b gene amplified, 395 bp was used in sequence analysis. The mean nucleotide composition was found to be A = 30.76%, T = 28.27%, G = 26.70% and C = 15.84%. A total of 135 polymorphic sites were seen among all 9 species. Out of them, transitions were 80, transversions were 10 and both transition and transversion were 45 (Table 2). Nucleotide difference between species in cytochrome b gene varied from 35 to 90 and genetic distance varied from 0.0958 to 0.2777. From the polymorphic sites, genetic distance and number of nucleotide difference between the species for cytochrome b genes, it was observed that *M. gangeticum* is close to *M. malcomsonii* where as *M. lamarrei* is closer to *M. sankolii*. *M. rosenbergii* was found closer to these four species. *M. equidens* showed more genetic relationship to *M. rude* while *M. scabriculum* was close to *M. idella*. The similarity between *M. gangeticum* and *M. malcolmsonii* and that between *M. lamarrei* and *M. sankolii* is supported by the morphological similarities as both *M. sankolii* and *M. lamarrei* are small sized prawns and rest two are larger in size.

The phylogenetic conclusion is supported by Bueno and Rodrigues (1995); Alekhnovich and Kulesh (2001); as well as Jayachandran and Indira (2010). According to them the above species can be categorised based on their habitat preferences. Category I: Prawns living and completing their larval life cycle in saline water (*M. equidens*, *M. rude*), Category II: Prawns living in estuaries and/or lower stretches of the rivers with or without salinity, but completing their larval life cycle in saline water (*M. idella, M. scabriculum, M. gangeticum*, *M. malcomsonii* and *M. rosenbergii*) and Category III : Prawns living in interior water logged areas (ponds and lakes) with limited distribution (*M. lamarrei* and *M. sankolii*). The Neighbor-Joining [NJ] tree was constructed with the data from cytochrome b gene sequences of all the nine species of the genus *Macrobrachium* (Fig. 1). *M. lamarrei* and *M. sankolii* were found to be close to each other while *M. gangeticum* was closer to *M. malcomsonii*. *M. rosenbergii* was found close to these two species. *M. idella* and *M. scabriculum* shared a common clade and *M. rude* and *M. equidens* were found to be close to each other, showing less similarity with other group which consisted of *M. rosenbergii, M. malcolmsonii, M. gangeticum, M. lamarrei* and *M. sankolii*. *M. lamarrei* is close to *M. sankolii* (category I) whereas *M. equidens* and *M. rude* are genetically close to each other (category III). *M. rosenbergii*, *M. malcomsonii, M. gangeticum, M. idella* and *M. scabriculum* (category II) are seen to be closer to each other compared to other species.

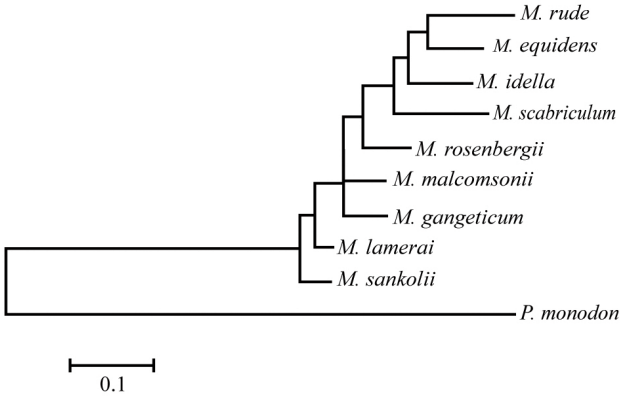

Fig. 1. NJ tree of 9 *Macrobrachium* spp. based on partial Cyt b gene sequence

Genetic distance and number of nucleotide difference between the species based on partial cytochrome b gene (395bp) is given in (Table 3). Among the species of the present study, maximum genetic distance was found between *M. sankolii* and *M. rude* (0.2777) and minimum genetic distance was found between *M. sankolii* and *M. lamarrei* (0.0958) followed by *M. gangeticum* and *M. malcomsonii* (0.1035). Maximum number of nucleotide difference was found between *M. sankolii* and *M. rude* (90) and minimum number of nucleotide difference was found between *M. sankolii* and *M. lamarrei* (35). Based on the genetic distance and nucleotide difference, it is

Table 2. Polymorphic sites of cytochrome b gene between different *Macrobrachium* species

Nucleotide position	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 2 2 2 3 3 3 3 3 4 4 5 5 5 6 6 7 7 7 8 8 8 9 9 9 9 9 9 0 0 0 0 1 1 1 1 1 2 2 2 2 2 3 3 3 4 5 5 5 6 6 6 6 6 7 7 7 7 3 6 9 5 8 1 4 7 0 3 6 8 9 5 8 1 4 7 3 6 2 5 7 1 4 8 0 1 3 4 7 9 0 2 3 9 2 5 7 8 9 0 1 3 7 3 6 9 5 1 4 7 0 3 6 9 1 2 5 8
M. lamarrei	A T T T A G G C G G T A G A T G G G G A C T G G G A G A G T G G G T A A G T G T G T G C A T G A A G A A A A T G T T T T
M .sankolii	. . . . . A . T . . C . A . . . A . T G A . . A . . . . A . . T . . A . . . A T . . A G . . . . G C
M. malcomsonii	. . . C . A A T . . . G T . . . . . T G A G . A A . . . . . . T A . . G C A . . . . G . . . . . G . A . . . .
M. gangeticum	. . . . . . A T . . C G . G . . A . T . A . . A . . . G . . A . . . G T A . . . . C A G . . . . A . . . .
M. rosenbergii	G C . . . . . . A . . G . G . . . . T G G G . A A G . G . . . . G G . C . . . . G G . G G G . G A . . . .
M. equidens	G . . C . . T . T . . G . G A A . A T G T . A T . T A . . A A . . C G G T . A . A . A . G C A . . A G T G . . T C . . .
M .rude	G . C C . . C A T C . G . G . A A A A G T . . . . C . . . G A . C C G C T C . . A . A . G C A G . A G G G . G A C C C .
M. scabriculum	G . . . . . C T . A . G C G A . A . A G T . . . A T . C A . T . . . . . T . A A A C T . . . . G . A G T G G . C C . . .
M. idella	. . C . G . . T T A C G T . . A A A T G T C A T . T . . . G T A . . G G . . . . G . A . G G G . A C C . .

	1 1 1 1 1 1 1 1 1 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 3 3 3 3 3 3 3 3 3 3 3 3 3 3 8 8 8 8 9 9 9 9 9 0 0 1 1 1 2 2 2 2 2 3 3 3 3 4 4 4 5 5 5 5 6 6 6 6 7 7 7 7 8 8 8 9 9 9 0 0 0 1 1 1 1 2 2 2 3 3 4 4 4 4 0 4 6 7 0 3 6 8 9 2 8 1 4 7 0 3 5 6 9 1 2 5 8 4 6 7 0 1 6 9 1 2 5 8 1 3 4 8 1 4 8 1 4 7 3 4 7 0 3 6 9 2 5 8 1 4 0 3 6 8
M. lamarrei	A T T A A T T A G G A T T T A T A G T A G A G A T G A G A A T G T G T G G G G G G A T T A T T G A T A G T T T A T T T A
M .sankolii	G . . . . A A . . . . A . . . . A . . . . . . G . C . A A . A . . . . . . C A . . G . . G . G . C . .
M. malcomsonii	G G . . . . . G T A . . . . A . . A G . T . A . . . . . . C A C A . . . . . C . . . . G
M. gangeticum	G C . G G . . G . A . . C . . C . A . . A G . . A . . . . C . . C . . . . G
M. rosenbergii	G . . . G . . G T . . C . C . . . A . . A . . G . . . . A . . . . G C . . . . T . . C . . . C . G
M. equidens	G . C T . G . . . A . . . . . A G A . G A G A . A C G . G . G . G A G . A . . T . . C A . G . . C . . A . . . G . G . G
M .rude	G C C . G . C G . A . . . . G G G A . G A . . . A T T T . . . A . A . A . . . T A G C G G C A A T . G A . . . G C G . G
M. scabriculum	G A . G G G . G . . . . G . . G A G T T A T T . G G . A . A C . A . . T A G C A . G . A T C G A . . . . C G . G
M. idella	G C . C . A A G A . T C C C G . . A C G A G . T A . T . . . . A . . . A . . A T A G . A G A G A T . C . G G C . C A C G

	3 3 3 3 3 3 3 3 3 3 3 3 3 3 3 4 5 6 6 6 7 7 7 7 8 8 8 9 9 9 9 5 1 4 7 0 3 6 9 2 5 8 1 4 7
M. lamarrei	G T G T A T A T T A A A T A G
M .sankolii	. . . . G . . . .
M. malcomsonii	A C . . . C . . . G . . . . .
M. gangeticum	A A T . G . C . C G . . . . .
M. rosenbergii	. . A . . . . . . G G G . . .
M. equidens	. . A G G . T . . G T . . G T
M .rude	. G A G . G C C A G T . C T T
M. scabriculum	. . A . . A T C G . G . . . .
M. idella	. . A A . A T . . G G . . . C

Table 3. Genetic distance (upper triangle) and nucleotide difference (lower triangle) between different *Macrobrachium* species based on cytochrome b gene sequences (472 bp)

	M. lamarrei	*M. sankolii*	*M. malcomsonii*	*M. gangeticum*	*M. rosenbergii*	*M. equidens*	*M. rude*	*M. idella*	*M. scabriculum*
M. lamarrei		0.0958	0.1132	0.1132	0.1202	0.2368	0.2713	0.2115	0.2147
M. sankolii	35		0.1356	0.1356	0.1660	0.2477	0.2777	0.2242	0.2102
M. malcomsonii	41	48		0.1035	0.1392	0.2233	0.2563	0.2111	0.2279
M. gangeticum	41	48	38		0.1418	0.2180	0.2286	0.2170	0.2102
M. rosenbergii	43	57	49	50		0.2000	0.2349	0.2203	0.2279
M. equidens	79	82	76	74	77		0.1709	0.1878	0.2546
M. rude	88	90	85	77	79	60		0.1968	0.2111
M. idella	72	76	72	74	75	65	68		0.2154
M. scabriculum	73	73	73	72	69	83	72	73	

observed that *M. malcomsonii* and *M. gangeticum* are less close to *M. idella* and *M. scabriculum* compared to the group of *M. lamarrei* and *M. sankoli* even though they share same habitat. This may be due to their geographical distribution. *M. lamarrei, M. sankolii* and *M. gangeticum* are available only in India while *M. malcolmsoni* is reported from India, Bangladesh, Pakistan and Myanmar *i.e.* Indian subcontinent, where as *M. scabriculum* is commonly found in the Indo-west pacific: East Africa, Madagascar, India and Sri Lanka and *M. idella* is found in Indo-west pacific, East Africa upto Madagascar through Indian coast to Java, Sumatra and Malaya Archipelago (Jayachandran, 2003). Our result of similarity on the basis of geographical distribution rather than habitat preference is in agreement with the findings reported by Murphy and Austin (2005). By analysing abbreviated larval development (ALD) in various freshwater prawns and its correlation with phylogenetic relationship, they concluded that independent evolution of this trait has occurred more than once in different geographically isolated places. The observation in the present study that *M. malcomsonii* and *M. gangeticum* are more close to *M. lamarrei* and *M. sankoli* compared to *M. idella* and *M. scabriculum* irrespective of the habitat preference, leads to the conclusion that evolution of habitat preference as a reproductive strategy has evolved independently within different regions. The phylogenetic relationship among different species of the genus *Macrobrachium* indicates that even though they show similarity for having same habitat, geographical distribution is the better predictor for relatedness among them. Within a particular geographical area this habitat preference might have evolved between different species.

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