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Molecular phylogeny of freshwater prawn (Genus: *Macrobrachium*) species based on mtDNA 16S rRNA gene sequencing

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ABSTRACT

Molecular phylogeny of genus *Macrobrachium* was studied using 16S rRNA gene of mitochondrial DNA. The gene was amplified and sequenced to document the sequence variation between nine *Macrobrachium* species. Based on the phylogenetic investigation, *M. rosenbergii, M malcolmsonii, M. gangeticum, M lamarrei* and *M. sankolii* were grouped under one cluster and *M. equidens, M. rude, M. idella* and *M. scabriculum* formed a separate clade. Similarity indices between the sequences indicated maximum similarity between *M. gangeticum* and *M. malcolmsonii* and it was minimum between *M. sankolii* and *M. rude*. The hybridizable species including *M. malcolmsonii, M gangeticum* and *M. rosenbergii*; are very close to each other.

Key words: *Macrobrachium*, Molecular phylogeny, 16S rRNA gene

Freshwater prawn of the genus *Macrobrachium* (Family: Palaemonidae) has emerged as an alternative aquaculture species due to high growth rate, disease resistance as well as its rising market demand in several countries. About 200 species of freshwater prawns of the genus *Macrobrachium* were described by Holthuis (1980) in different continents. The greatest diversity of *Macrobrachium* species occurs in the Indo-Pacific region, particularly in the Indian subcontinent (Murphy and Austin 2005). There have been many problems associated with the classification of the Paleomonidae, which occur at species, genus and family levels (Holthuis 1952, Liendenfelser 1984, Boulton and Knott 1984, Fincham 1987, Pereira 1997 and Short 2000). The systematic studies in the past of *Macrobrachium* species have mainly been based on the analysis of external morphological traits. These traits however have been strongly influenced by the environment in some *Macrobrachium* species and are not indicative of the underlying genetic divergence (Dimmock *et al.* 2002). In addition, allozyme variations in many decapod crustacean species tend to be highly conservative and may not be representative of true molecular divergence. Short (2000) presented the most comprehensive phylogenetic study using morphological, biological, ecological characters to assess the evolutionary relationship of 30 Australian and Non-Australian species of *Macrobrachium* and nine species of related genera of Paleomonidae.

Molecular genetic approaches to resolve systematic ambiguities in *Macrobrachium* have only been applied recently (Murphy *et al.* 2002). The 16S rRNA mitochondrial DNA gene was found extremely useful for studying taxonomic questions and phylogenetic relationship within a number of decapod crustacean groups (Bucklin *et al.* 1995, Kitaura *et al.* 1998, Crandall *et al.* 1999). The 16S rRNA gene has both fast and slow evolving regions and therefore can provide useful information across a broad spectrum from the population to the family level (Murphy *et al.* 2003).

The present study was aimed at establishing phylogenetic relationship between different species of *Macrobrachium* by comparing interspecific genetic divergence.

MATERIALS AND METHODS

Nine species of genus *Macrobrachium* (*M. rosenbergii, M malcolmsonii, M. gangeticum, M. lamarrei, M. sankolii, M. equidens, M. rude, M. idella* and *M. scabriculum*) comprising natural populations were collected from different geographical locations of India. The tissue samples were preserved in 95% alcohol. 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.

A fragment of 16S rRNA mitochondrial gene was

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amplified by PCR using 16 SAR and 16SBR universal primers (Palumbi and Benzie 1991). The PCR was carried out in a reaction volume of 25µl, containing 1×PCR buffer, 0.1 mM of each DNTP, 2.5 pmole of each primer, 1 mM $MgCl_2$ following the temperature cycle involving an initial denaturation step of 95° C for 5 min, followed by 30 cycles of 95° C for 30 sec, an annealing temperature of 55°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 PCR products were directly sequenced using 50.0 ng (2.0 µl) of PCR product, 4 pM (1.0µl) of primer, 4µl of BigDye Terminator ready reaction mix and 3.0 µl of double distilled water to adjust the volume to 10.0µl. Cycle sequencing was carried out in a Gene Amp 9600 thermal cycler employing the conditions: 30 cycles at 96°C for 10 sec 50°C for 5 sec and 60° for 4 min. Extended products were purified by alcohol precipitation followed by washing with 70% alcohol. Purified samples were dissolved in 10µl of 50% Hi-Di formamide and analysed in ABI 3700 automated DNA Analyzer.

A total of 479 bp mitochondrial DNA 16S rRNA gene sequences of nine *Macrobrachium* were downloaded from NCBI and aligned using CLUSTAL X package (Thompson *et al.* 1997). The BioEdit software was used for formatting the sequences to make them compatible with the desired software. Neighbour joining (NJ) was constructed using the PHYLIP Package Ver 3.6 (Felsenstein 1993). Evolutionary distances between sequences were estimated using MEGA 3.0 software and bootstrap analysis was carried out using 1000 pseudoreplications (Kumar *et al.* 2004).

RESULTS AND DISCUSSION

Sequence divergence

A total of 479 bp of the 16S rRNA mitochondrial gene were amplified and sequenced for all the nine species. The mena total nucleotide composition was found to be A=35.50%, T=28.00%, G=11.77% and C=24.73%. Among the nine *Macrobrachium* species, 102 polymorphic sites were observed (Table 1). Out of these sites, transitions were found at 63 sites, transversions at 21 sites and both transitions and transversions at 18 sites. The sequences have been submitted to NCBI data bank (Accession No: AY730049-AY730057).

Genetic distance and number of nucleotide difference

Genetic distance and number of nucleotide difference between the species based on partial 16S sRNA gene (479bp) is given in Table 2. Among the species studied, maximum genetic distance was found between *M. sankolii* and *M. rude* (0.1387). It was minimum between *M. gangeticum* and *M. malcolmsonii* (0.0376). The maximum number of nucleotide (58) difference was found between *M. sankolii* and *M. rude*. It was minimum (17) between *M. gangeticum* and *M. malcolmsonii*.

Table 1. Polymorphin sites

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	5 1 3 4 4 4 5 5 5 5 6 6 6 6 7 7 7 7 8 8 8 8 8 9 9 9 0 0 0 0 1 1 2 3 3 3 3 3 4 4 4 4 4 4 4 4 5 5 5 5 5 6
Nucleotide position	1 3 9 3 4 5 1 4 5 8 6 7 8 9 3 5 8 9 0 1 5 6 8 1 2 6 5 7 6 7 6 9 3 5 7 8 9 0 1 2 3 4 6 7 9 0 1 2 3 7 0
M. gangeticum	G C T C C T T A A A A T A A T A T A C A A A C A G A A A T C G T T A A C C G A A C A A C T A C T C T
M. malcomsonii	. . . . . T . . . . . C . . . . . A . . . . G . . . T . . .
M. lamarrei	. . C . T . C . . . . . A . . . . . C . C T A . . . A . A . . . . .
M. sankolii	A . . . T . C . . . . . C . . . . . C . . . A . . . . T A . . . . . T .
M. rosenbergii	. . . . . C . G . . . TGC . . G . . . TT . T C . . . . . A G . . . . . G
M. scabriculum	. . . T . . A . . . C . . . . TATTT . . A . . . C . A . . . . . A . . . . TCGTC . C
M idella	. . . T . . A . . . . . TA . TT . . AT . . T . A . . . . . CGG . . . . .
M. equidens	A T C T T C A T C C C . A . . . TG . TTTGTTA . C . A . . . . . T T . . AGG . C . . T .
M. rude	. . . TT . A . G . . C A . . CCAGTTT . TT . . . GA . . CAGG . T T C G . G . T C . . . T .

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

Table 2. Genetic distance (upper triangle) and nucleotide difference (lower triangle) between different *Macrobrachium* species based on 16S rRNA gene DNA sequences (479 bp)

	M. gangeticum	*M. malcolmsonii*	*M. lamarrei*	*M. sankolii*	*M. rosenbergii*	*M. scabriculum*	*M. idella*	*M. equidens*	*M. rude*
M. gangeticum		0.0376	0.0516	0.0564	0.0584	0.0822	0.0892	0.1088	0.1220
M. malcomsonii	17		0.0493	0.0662	0.0635	0.024	0.0917	0.1167	0.1329
M. lamarrei	23	22		0.0447	0.0782	0.0927	0.0843	0.1223	0.1277
M. sankolii	25	29	20		0.0756	0.1054	0.1045	0.1064	0.1387
M. rosebergii	26	28	34	33		0.0870	0.0914	0.1296	0.1295
M. scabriculum	36	36	40	45	38		0.0797	0.1071	0.1198
M. idella	39	40	37	45	40	35		0.1026	0.0980
M. equidens	47	50	52	46	55	46	44		0.0948
M. rude	52	56	54	58	55	51	42	41	

Comparision of Indian Macrobrachium rosenbergii population with other species

The similarity between Indian population of *M. rosenbergii* with that of Papua New Guinea population was 93.50% (Table 3). And 94.70% similarity was found between *M. rosenbergii* and *M. gangeticum*. The intraspecific similarity was less as compared to interspecific. *M. rosenbergii* of the above two populations showed more similarity with *M. malcomsonii, M. gangeticum, M. lamarrei* and *M. sankolii in* comparison to other *Macrobrachium* species.

The phylogenetic analysis of 16S RNA gene sequences confirms the geographical, taxonomic and evolutionary complexity within the freshwater prawn genus *Macrobrachium* (Short 2000). The polymorphic sites observed among the different *Macrobrachium* species suggest that *M. gangeticum* is closer to *M. malcolmsonii* whereas *M. lamarrei* is closer to *M. sankolii*. This observation has been supported by the morphological similiarties as both *M. sankolii* and *M. lamarrei* are small sized prawns compared to other species. The Neighbour-Joining [NJ] trees from 16S

Table 3. Similarity matrix showing comparison of *M. rosenbergii* of two populations with other species

	*M.*equidens	*M.rude*	*M.idella*	*M.scabr*	M.lamer	M.sanko	M.gange	M.malco	M.rosen	M.rosep	P.monod
M. equidens	1.0000										
M. rude	0.9144	1.0000									
M. idella	0.9019	0.924	1.0000								
M. scabriculum	0.9099	0.9036	0.9142	1.0000							
M lamarrei	0.931	0.952	0.9182	0.9036	1.000						
M. sankolii	0.994	0.6	0.9015	0.8952	0.9644	1.0000					
M. gangeticum	0.9015	0.9015	0.9224	0.9161	0.9623	0.9560	1.0000				
M. malcomsonii	0.994	0.9015	0.9245	0.912	0.9623	0.9434	0.9665	1.0000			
M. rosenbergii											
Papua New Guinea	0.8826	0.8910	0.9140	0.8994	0.9455	0.9287	0.9602	0.9476	0.9350	1.0000	
P. monodon	0.7578	0.7891	0.7583	0.7484	0.7673	0.7715	0.7704	0.7652	0.7657	0.7715	1.0000

Phylogenetic analysis

The Neighbour-Joining (NJ) tree was constructed with the data from 16S rRNA gene sequences of all the nine Indian species (Fig.1). This tree revealed 2 different groups, one group consisting of *M. rosenbergii, M. malcolmsonii, M. gangeticum, M. lamarrei* and *M. sankolii* and the other group comprised *M. equidens, M rude, M. idella* and *M. scabriculum. M lamarrei* was found close to *M. sankolii,* whereas *M. gangeticum* was close to *M. malcomsonii. M. rosenbergii* was observed to be close to all these 4 species. *M. rude* was seen to be closer to *M. equidens* followed by *M. idella. M. scabriculum was* close to these three species rather than the other group of five species.

rRNA gene also supported this inference. The *Macrobrachium* species included in the present study did not form a monophyletic clade which shows conformity with the findings of Murphy and Austin (2002). This has important implications for both conservation of wild genetic resources and also for potential future directions for the culture industry (Mather and Bruyn 2003). From the phylogenetic tree, it was observed that *M. equidens and M. rude* are genetically closer to each other. *M. idella* was found close to *M. rude* and *M. equidens. M. scabriculum* was found as sister taxon to these three species forming a different clade. The topology of the NJ tree confirms genetic distance and nucleotide difference between the species.

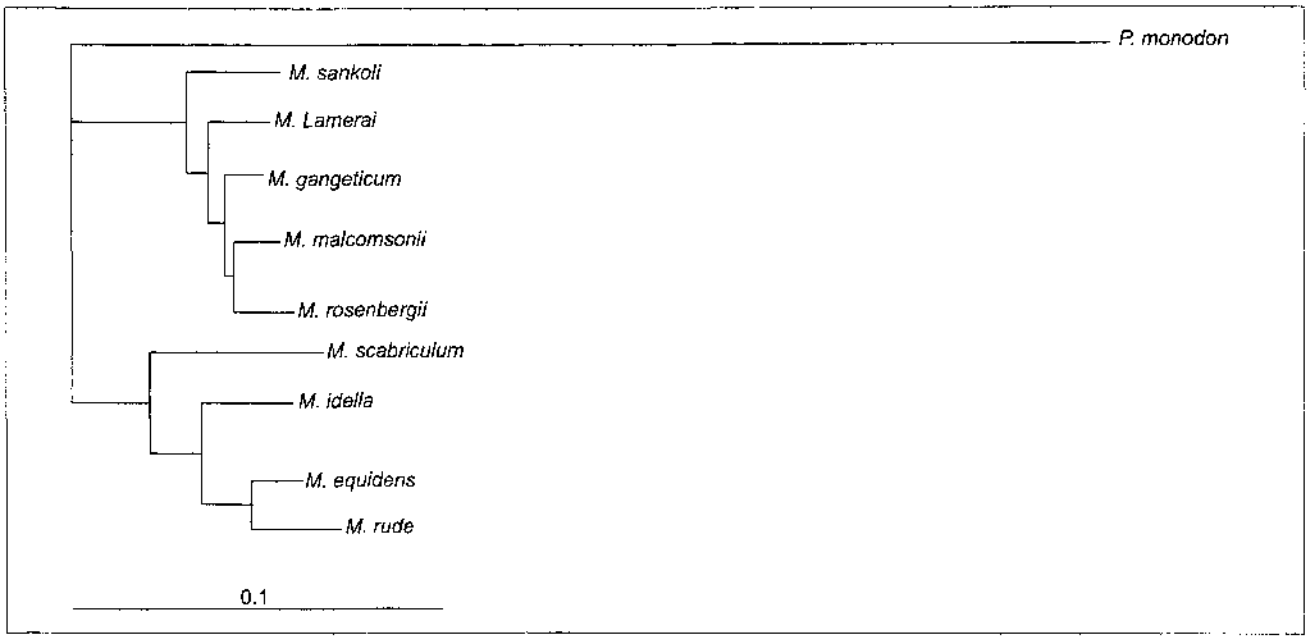

Fig. 1. NJ tree of nine *Macrobrachium* sp based on partial 16S rRNA gene sequence.

The prawn *M. lamarrei, M. sankolii and M. gangeticum* are available only in India whereas, *M. malcolmsoni* is reported from Bangladesh, Pakistan and Myanmar also. These 4 species were close to each other corroborating their biogeographical history. *M. rosenbergii* was closer to the species available only in Indian subcontinent compared to other *Macrobrachium* species. The mean divergence levels amongst *Macrobrachium* species have been reported to be at the higher level of that normally found between species and overlap with commonly found genera in other crustaceans (Fetzner and Crandall 2001).

At the higher taxonomic level (family level), it would appear that the likelihood of hybridization is proportional to genetic similarity. At the species level however, the amount of divergence might not be constraint on the capacity of taxa to hybridize (Smith 1992). It is opined that reproductive compatibility among species may or may not be blocked by subsequent evolution and the potential for gene exchange might thus not be indicative of a close phylogenetic relationship. Our study revealed that *M. malcolmsonii* and *M. gangeticum* are closer to *M. rosenbergii* compared to other species. This observation also gets the support from the fact that all these three species hybridize easily with each other.

In conclusion, *M. malcolmsonii, M gangeticum, M lamarrei* and *M. sankolii,* which are available only in India, are closer to each other. Though *M. rosenbergii* is reported from many countries, it shows more similarity to Indian *Macrobrachium* species. The Indian *M. rosenbergii* populations (Eastern and Western) showed more genetic distance than that of between two Macrobrachium species (*M. malcolmsonii and M. gangeticum*). The understanding of the molecular relationships among the different species of *Macrobrachium* inferred from our study will provide a foundation for future genetic improvement programme in *Macrobrachium* species used in aquaculture.

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REFERENCES

Boulton A J and Knott B. 1984. Morphological and Electrophoretic studies of the Palaemonidae (Crustacea) of the Perth region, Western Australia. *Australian Journal of Marine and Freshwater Research* **35**: 769–83.

Bucklin A, Frost B W and Kocher T D. 1995. Molecular systematics of six calanus and three metridia species. *Marine Biology* **121**: 655–64.

Crandall K A, Fetzner Jr, J W, Lawler S H, Kinnersley, M and Austin C M. 1999. Phylogenetic relationships among the Australian and New Zealand genera of freshwater crayfishes (Decapoda: Parastacidae). *Australian Journal of Zoology* **47**: 199–214.

Dimmock A, Williamson AI and Mather P. 2002. Environmental effects on morphology of mature male *Macrobrachium australiense* (Decapoda: Palaemonidae), In: Seafarming Today and Tomorrow: Extended Abstracts and Short Communications. Special Publication No. 32, European Aquaculture Society, August 2002, Belgium. 208-209 pp.

Felsenstein J. 1993. Phylogeny Inference Package (PHYLIP). Version 3.5. University of Washington, Seattle

Fetzner J W Jr and Crandall K A. 2001. Genetic variation. Holdich D M. *Biology of Freshwater Crayfish*. 291–326pp. (Ed.) Oxford, Blackwell Science.

Fincham A A 1987. A new species of *Macrobrachium* (Decapoda, Caridea, Palaemonidae) from the Northern Territory, Australia and a key to Australian species of the genus. *Zoologica Scripta* **16**: 351–54.

Holthuis L B. 1952. A general revision of the Palaemonidae (Crustacea, Decapoda, Natantia) of the Americas. II. The subfamily Palaemoninae. *Occasional Papers of the Alan Hancock foundation* **12**: 1–396.

Holthuis L B. 1980. Shrimps and Prawns of the World: An annotated catalogue of species of interest to Fisheries. *FAO Fisheries Synopsis* **53** 285–96.

Kitaura J, Wada K and Nishida M. 1988. Molecular phylogeny and evolution of unique mudusing territorial behaviour in ocypod crabs (Crustacea: Brachyura: Ocypodidae). *Molecular Biology and Evolution* **15**: 626–37.

Kumar S, Tamura K, Jakobsen I B and Nei M. 2004. MEGA 3.: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics **5**: 150–63.

Lindenfelser M E. 1984. Morphometric and Allozyme congruence: evolution in the prawn *Macrobrachium rosenbergi* (Decapoda: Palaemonidae). *Systematic Zoology* **33**: 195–204.

Mather P B and Bruyn M. 2004. Genetic divesrity in wild stocks of the giant freshwater Prawn (*Macrobrachium rosenbergii*): Implications for aquaculture and conservation. *NAGA*, N P and Austin C M. 2002. A preliminary study of 16S rRNA sequence variation in Australian *Macrobrachium* shrimps (Palaemonidae: Decapoda) reveals inconsistencies in their current classification. *Invertebrate Systematics* **16**: 697–701.

Murphy N P and Austin C M. 2003. Molecular taxonomy and phylogenetics of some species of Australian palaemonid shrimps. *Journal of Crustacean Biology* **23**: 169–77.

Murphy N P and Austin C M. 2005. Phylogenetic relationships of the globally distributed freshwater prawn genus *Macrobrachium* (Crustacea: Decapoda: Palaemonidae): biogeography, taxonomy and the convergent evolution of abbreviated larval development. *Zoologica Scripta* **34**(2): 17–97.

Palumbi S R and Benzie J. 1991. Large mitochondrial DNA differences between morphologically similar Penaeid shrimp. *Molecular Marine Biology Biotechnology.* **1**: 27–34.

Pereira G. 1997. A cladistic analysis of the freshwater shrimps of the family Palaemonidae (Crustacea, Decapoda, Caridea). *Acta Biologica Venezuelica* **7**: 1–69.

Sambrook J, Fristisch E F and Maniatis T. 199. *Molecular Cloning-A Laboratory Manual* 2nd edn. 6.4-612. Cold Spring Harbor Laboratory Press.

Sambrook J and Russell D W. 2001. *Molecular Cloning: A Laboratory Manual.* 3rd edn 65–66 pp. Cold Spring Harbor Laboratory Press, Cold Spring Harbor New York.

Short J. 2000. 'Systematics and biogeography of Australian *Macrobrachium* (Crustacea: Decapoda: Palaemonidae) with descriptions of other new fresh water decapoda'. Ph.D. Thesis, The University of Queensland, Brisbane.

Smith G R. 1992. Introgression in fishes: Significance for paleontology, cladistics and evolutionary rates. *Systematic Biology* **41**(1): 41–57.

Thompson J D, Gibson T J, Plewniak F, Jeanmougin F and Higgens D G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acid Research* **24**: 476–82.