J.Natn.Sci.Foundation Sri Lanka 2010 **38** (3):193-199

RESEARCH ARTICLE

Phylogenetic positions of some species of the genus *Macrobrachium* Bate, 1868 (Crustacea, Decapoda, Palaemonidae) in Sri Lanka

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Revised: 27 April 2010 ; Accepted: 23 July 2010

Abstract: *Macrobrachium* species, are an economically important freshwater prawn group in Sri Lanka. These species are recognized by their local names and many synonyms can be found for one species. Identification of taxonomic positions and species boundaries within the genus is important to obtain reliable information for application in aquaculture and biodiversity conservation programmes.

Approximately 471bp partial sequences from mitochondrial 16S rRNA using seven *Macrobrachium* species were collected during the present study. Six of them were collected from the southern part of Sri Lanka and their phylogenetic positions among the relevant species that have been recorded within the region were determined. The analysis resulted in five clades, of which three showed monophyletic lineages (*M. australe* group, *M. latimanus* group, and *M. latidactylus* group). *M. malcolmsonii* is in a sister clade to *M. rosenbergii* while *M. scabriculum* joined with *M. idae*. The estimated intraspecific nucleotide divergence level varied from 0 − 6.07% while it varied from 5.21 – 10.84% at the interspecific level. The phylogenetic positions of the samples recorded within the region are discussed.

Keywords: Freshwater prawns, *Macrobrachium*, mitochondrial 16S gene region, phylogenetic relationships, Sri Lanka.

INTRODUCTION

Freshwater prawns of the genus *Macrobrachium* Bate, 1868[1] are important macro invertebrates in freshwater and estuarine systems throughout the tropical and warm temperature areas of the world. This genus can be ecologically separated into two groups. Most species are widely distributed and require specific saline concentrations to complete larval development (euryhaline species) and others are land locked species with limited distributions and complete their entire life cycle in freshwater[2,3]. So far more than 200 species have been described around the world and there are more to be described. *Macrobrachium* species have a high commercial value in the fisheries sector [4]. A study[5] listed 86 *Macrobrachium* species that are economically important and among them at least 11 species have gained great commercial value in different countries. The giant freshwater prawn *M. rosenbergii* [6] is farmed commercially, both within its natural range and outside.

Taxonomically, this genus is one of the most challenging decapod crustacean groups. The most distinguishable morphological characters of this genus are the rostrum and the second pereopod, which are highly variable among species[7]. Most of the studies on *Macrobrachium* species are based on morphology[2,3,5,7,8] and in recent years few studies have been published using molecular data. Some previous studies [9-12] have produced important clarifications for Australian *Macrobrachium* species based on 16S mitochondrial gene region. Further, based on the same gene region another study [3] suggested multiple origins of *Macrobrachium* species, region wise. Subsequently, two researchers[14,15] carried out phylogenetic studies using multiple gene regions. For these studies data were collected from a wide geographical range mainly from the south and south-eastern Asian regions. However, Sri Lankan *Macobrachium* samples were not included. Collectively, the above studies produced interesting information and insight regarding *Macrobrachium* taxonomy.

In Sri Lanka, *Macrobrachium* species are an economically important group. Many species are

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recognized by their local names and many synonyms can be found for one species, which makes studies more complicated. Therefore, identification of species boundaries within this genus is important to obtain information for applications in aquaculture and biodiversity conservation.

Two detailed taxonomic studies are recorded from Sri Lankan *Macrobrachium* species. One study[16] described six species from different geographic locations and the other[8] described twelve species collected from all over the country. However, these recorded species are only briefly described and their morphological characters are not properly illustrated using figures. To date no taxonomic studies at molecular level have been conducted with this genus in Sri Lanka. Therefore, the objective of the present study was to collect partial sequences of mitochondrial 16S ribosomal gene region from seven *Macrobrachium* species collected mainly from the southern part of Sri Lanka to determine their phylogenetic positions among the relevant species that have been recorded within the region.

METHODS AND MATERIALS

Seven *Macrobrachium* species were identified based on morphological features[4] and used in this study. Among them 6 species (*M. rosenbergii*, *M. scabriculum*[17], *M. idea*[17], *M. australe*[18], *M. latimanus*[19] and *M. latidactylus*[20]) were collected from the southern part of Sri Lanka mainly from the streams and small rivers of 2 major river basins: Nilwala and Walawe. *M. malcolmsonii* [21] was collected from the Gal Oya river system in the eastern part of Sri Lanka (Figure 1). Three to four specimens from each species were used to extract DNA. Reference samples were stored at the Department of Zoology, University of Ruhuna, Matara for further studies. Sampling localities are given in Table 1.

DNA was extracted using the easy DNA extraction kit (QIAGEN, USA). A fragment of the 16S rRNA mitochondrial gene was amplified by Polymerase Chain Reaction (PCR) using primers 1471- 5'CCTGTTTANCAAAAACAT3' and 1472-

Table 1: Sampling localities and gene bank accession numbers for sequences

Species (abbre:)	Location	Sample code	Genbank accession numbers
M. rosenbergii	Sri Lanka[1]	SriA	FJ595480*
(M. rose)	Sri Lanka[1,2]	SriB	FJ595481*
	India	Ind	DQ004836
	Thailand	Tha	AY203908
	Papua New Guinea	Pap	AY203906
	Australia	Aus	AY203918
M. malcolmsonii	Sri Lanka[3]	Sri	GU987055*
(M.malc)	India	Ind	AY730050
M. scabriculum	Sri Lanka[1]	Sri	GU987059*
(M.scab)	India	Ind	AY730055
M. idea	Sri Lanka[1,2]	Sri	GU987058*
(M.idae)	Taiwan	Twn	DQ194930
	Australia	Aus	AY282777
M. australe	Sri Lanka[1]	Sri	GU987057*
(M.aust)	Philippine	phi	DQ194905
	Taiwan	Twn	DQ194904
	New Guinea	New	DQ681290
M. latimanus	Sri Lanka[1,2]	Sri	GU987056*
(M.lati)	Japan	Jap	DQ194938
	Taiwan	Twm	DQ194936
	Philippine	Phi	DQ194937
M. latidactylus	Sri Lanka[1]	Sri	GU987060*
(M.latid)	Taiwan	Twn	EU493140
	Thailand	Tha	DQ194946
	Philippine	phi	DQ194945
	China	Cha	DQ194943
	Malaysia	Mal	DQ194944
Palaemon			FM986647
(Palmon)			

* Sequences derived from this study

Sampling localities: 1-Nilwala river basin, 2- Walawe river basin, 3- Gal Oya basin (refer to Figure 1)

Table 2: Percentage of sequence divergence (uncorrected 'p' distance) among populations and out group for the 16S gene region. Abbreviations are given in Table 1.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1	M.rose,SriA	-																											
2	M.rose,SriB	0.22	-																										
3	M.rose,Ind	0.00	0.22	-																									
4	M.rose, Tha	0.00	0.22	0.00	-																								
5	M.rose, Pap	5.86	6.07	5.86	5.86	-																							
6	M.rose, Aus	5.21	5.42	5.21	5.21	0.65	-																						
7	M.malc, Sri	6.07	6.29	6.07	6.07	5.42	5.21	-																					
8	M.malc, Ind	6.07	6.29	6.07	6.07	5.42	5.21	0.00	-																				
9	M.scab, Sri	9.58	9.79	9.58	9.58	10.22	9.79	8.06	8.06	-																			
10	M.scab, Ind	9.12	9.33	9.12	9.12	9.99	9.55	7.82	7.82	0.43	-																		
11	M.idae, Sri	7.59	7.82	7.59	7.59	8.46	8.03	8.03	8.03	6.96	6.73	-																	
12	M.idae,Twn	7.59	7.82	7.59	7.59	8.46	8.03	8.03	8.03	6.96	6.73	0.00	-																
13	M.idae, Aus	8.03	8.25	8.03	8.03	9.11	8.68	8.46	8.46	6.75	6.52	0.86	0.86	-															
14	M.aust,sri	10.21	9.99	10.21	10.21	9.99	10.2	10.2	10.2	10.9	10.7	8.90	8.90	9.77	-														
15	M.aust,Phi	9.99	9.78	9.99	9.99	9.78	9.56	10.2	10.2	10.5	10.2	8.69	8.69	9.53	1.52	-													
16	M.aust,Twn	9.99	9.78	9.99	9.99	9.78	9.56	10.2	10.2	10.5	10.2	8.69	8.69	9.53	1.52	0.00	-												
17	M.aus,New	10.21	9.99	10.21	10.21	10.21	9.99	9.78	9.76	10.9	10.7	9.12	9.12	9.53	1.30	2.39	2.39	-											
18	M.lati,Sri	9.33	9.11	9.33	9.33	9.33	9.11	9.54	9.54	8.72	8.46	8.88	8.88	9.32	8.23	8.48	8.48	8.05	-										
19	M.lati,Jap	10.41	10.19	10.41	10.41	8.68	8.46	8.68	8.68	7.63	7.82	8.45	8.45	8.88	8.05	8.26	8.26	7.83	2.82	-									
20	M.lati,Twn	10.63	10.41	10.63	10.63	8.89	8.68	8.89	8.89	7.84	8.03	8.67	8.67	9.10	8.26	8.48	8.48	8.05	3.04	0.22	-								
21	M.lati,Phi	10.41	10.19	10.41	10.41	8.68	8.46	8.68	8.68	7.63	7.82	8.45	8.45	8.88	8.05	8.26	8.26	7.83	2.82	0.00	0.22	-							
22	M.latid,Sri	10.41	10.19	10.41	10.41	10.19	10.2	8.89	8.89	8.47	8.23	9.10	9.10	9.54	8.91	9.13	9.13	8.26	7.59	7.38	7.59	7.38	-						
23	M.latid,Twn	10.84	10.62	10.84	10.84	9.54	9.75	8.89	8.89	8.63	8.44	9.54	9.54	9.97	9.78	9.35	9.35	9.34	7.81	7.59	7.81	7.59	1.95	-					
24	M.latid,Tha	10.41	10.19	10.41	10.41	9.93	9.98	8.89	8.89	8.47	8.23	8.67	8.67	9.11	8.69	8.92	8.92	8.04	7.59	7.38	7.59	7.38	1.29	2.38	-				
25	M.latid,Phi	10.19	9.97	10.19	10.19	9.75	9.98	9.12	9.11	8.91	8.66	9.32	9.32	9.76	9.13	9.35	9.35	8.69	7.59	7.38	7.59	7.38	1.73	0.65	2.17	-			
26	M.latid,Cha	10.62	10.41	10.62	10.62	9.32	9.54	9.12	9.11	8.9	8.66	9.32	9.32	9.76	9.56	9.35	9.35	9.13	7.59	7.38	7.59	7.38	1.73	0.21	2.17	0.43	-		
27	M.latid,Mal	10.41	10.19	10.41	10.41	9.54	9.75	8.89	8.89	8.69	8.44	9.10	9.10	9.54	9.35	9.57	9.57	8.91	7.38	7.16	7.38	7.16	1.52	0.43	1.95	0.22	0.22	-	
28	Palmon	20.32	20.54	20.32	20.32	19.84	19.8	19	19	18.7	18.7	17	16.9	17.2	19	19.2	19.2	18.1	16.6	17.4	17.7	17.4	16.4	16.6	16.8	16.6	16.4	16.38	-

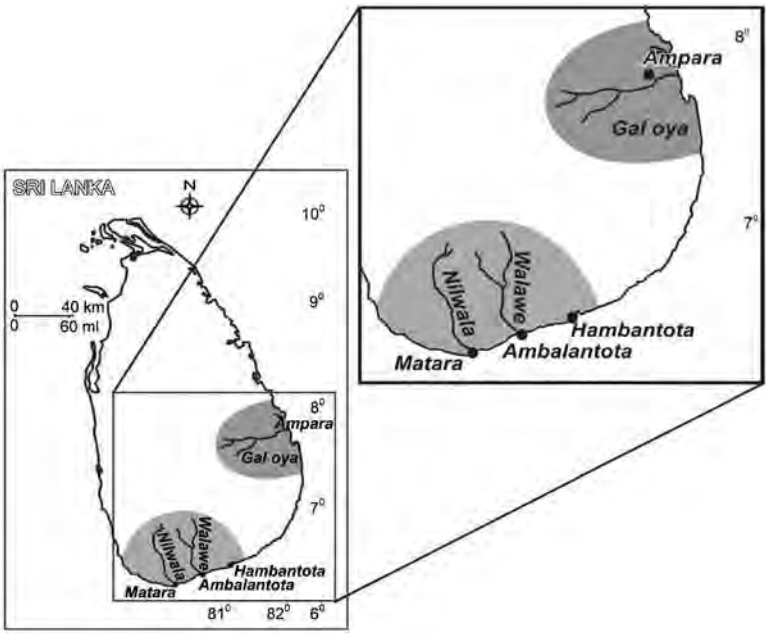

Figure 1: Sampling localities of the current study

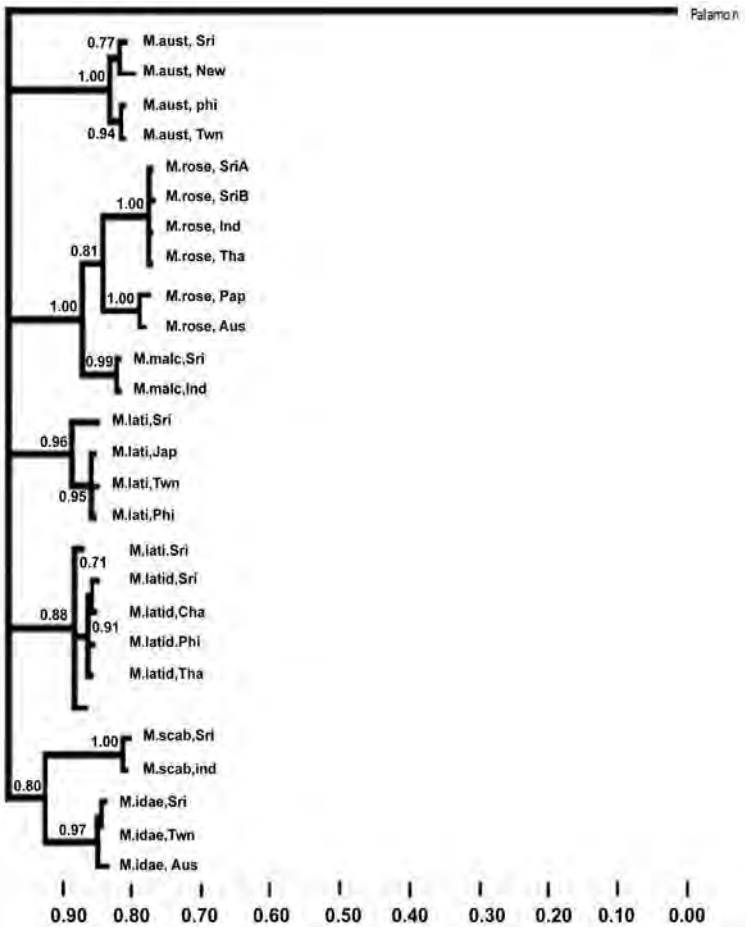

Figure 2: Majority-rule consensus tree derived from Baysian analysis. Numbers indicate posterior probability values (> 0.7) that supported for each relationship. Refer Table 1 for abbreviations.

5'AGATAGAAACCAACCTGG3' described in a previous study[22] which were initially designed for freshwater crayfish. Double stranded PCR products were obtained in a total reaction volume of 25 µL, containing 5 µL of 10X PCR buffer, 0.4 mM of each dNTP, 0.8 µM of each primer, 4 mM $MgCl_2$, 1 unit of Taq polymerase and 2 µL of DNA extract. PCR amplification was carried out using the following temperature regime: an initial denaturation step of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, an annealing temperature of 50°C for 30 s and an extention of 72°C for 30 s. This was followed by an additional extension of 72°C for 3 min. PCR products were purified using a QIAGEN QIAquick PCR purification kit, with a final elution volume of 50 µL per individual. The quality of the PCR products was visualized on 1.5% agarose gels. Sequencing was carried out in both directions using the same primer pairs for PCR. Sequences were obtained using the Big Dye Terminator version 3.1 protocol (Applied Biosystems, USA) and analyzed using an ABI 3130xl Genetic Analyzer (with KB base caller; Applied Biosystems, USA).

Sequence chromatograms were viewed and edited manually using a combination of Edit View and SeqPup programmes[23]. Once edited, multiple alignments were performed using Clustal X programme[24] with multiple alignment parameters of gap penalty equal to 10−15 and gap extension penalty equal to 3−5. Positions of uncertain alignment were excluded to produce a stable data set. Sequences were then imported into PAUP 4.0b10 programme[25] for phylogenetic analysis. Additional sequences for the recorded species within the region and out group species [26] were collected from Genebank (Table 1) and used to justify the phylogenetic positions of Sri Lankan species among them. Sequences derived for this study were deposited in Genebank (Accession number GU987055 - GU987060 and FJ595480 - FJ595481) (Table 1).

Bayesian analyses (BA) were performed with MrBayes version 3[27] using the model selected by MrModeltest programme[28]. Markov chain Monte Carlo (MCMC) chains were run for $1x10^6$ generations, and trees were saved each 100 generations (with the 1st 1000 trees being discarded as 'burn in'). The probability values greater than 95% were considered as significant support for relationships. The neighbour-joining analyses [Minimum Evolution option (ME)] were performed using distance calculated under the same model of evolution as the Bayesian analysis. Maximum Parsimony (MP) analyses were performed with gaps treated as missing data and heuristic search option was used with tree bisection-reconnection (TBR) branch swapping and 100 stepwise random additions. Bootstrapping was performed with 1000 replicates for all analyses. The level of support for each analysis is indicated on clads of the phylogenetic tree.

RESULTS

Sequences of approximately 471bp in length were obtained from 16S rRNA gene. The mean nucleotide composition was A= 36.58% , T= 30.57%, C= 22.57% , G=10.28%. This indicates that the 16S rRNA region of the mtDNA is the adenosine and thymine rich in the palaemonids. On the basis of the ModelTest , the GTR+I+G model of sequence evolution was chosen and the parameters specified by this model [(A-C)=1.1231, (A-G) 20.5595, (A-T)=2.0745, (C-G)=0.0001, (C-T)=9.5918, (G-T)=1.0000 and gamma distribution parameter=0.2301] were used for further analysis. Pairwise distances (uncorrected 'p' distance) for the data set are given in Table 2.

In this study, the estimated intraspecific nucleotide divergence level varied from 0 − 6.07% while it varied from 5.21 – 10.84% for the interspecific level. All three methods of phylogenetic analysis (BA, MP, ME) produced identical tree topologies. Five clades were derived: *M. australe* group, *M. malcolmsonii* + *M. rosenbergii* group, *M. latimanus* group, *M. latidactylus* group and *M. scabriculum* + *M. idae* group. However, the generated dendogram did not resolve the deeper level phylogenetic relationships (Figure 2).

DISCUSSION

This study was based on mitochondrial 16S rRNA gene region of seven *Macrobrachium* species collected from different geographical regions. The analysis resulted in five clades and four of them produced monophyletic lineages. *M. malcolmsonii* made a sister clade to *M. rosenbergii* while *M. scabriculum* joined with *M. idea* (Figure 2).

Congeneric crustacean species commonly exhibit significant differences at the 16S rRNA mtDNA gene ranging from 2 − 17% sequence divergence[29-32]. This divergence range is also supported by other recent studies conducted on *Macrobrachium* species[9-11,14]. Therefore, the divergence levels that became evident in this study between and among species are typical for those observed between crustacean species (Table 2).

M. australe showed the lowest intraspecific nucleotide divergence that varied from 0 − 2.4%. *M. australe* group is defined with high support and

contains two clades. The southeast Asian samples were grouped together while Sri Lankan *M. australe* is grouped with New Guinean sample with lower support (0.77). The nucleotide divergence level between Sri Lankan and New Guinean sample was 1.3% while the Sri Lankan sample varied from the others with 1.5% divergence level.

M. malcolmsonii formed a sister clade which was basal to *M. rosenbergii*. The nucleotide divergence level between the two species varied from 5.2 − 6.4%. Two haplotypes were found within Sri Lankan *M.rosenbergii.* The *M. rosenbergii* samples collected from the South and South-East Asia were grouped together to accept the hypothesis of eastern and western division of this species along the Huxley's line suggested by a previous study [33]. The neucleotide sequence divergence between the two clades varied from 5.2 − 6%. This value was quite similar to the divergence level found between *M. rosenbergii* and *M. malcolmsonii* samples. *M. malcolmsonii* is so far reported from the South Asian region and it is worthwhile to conduct further phylogenetic studies using more gene regions to reveal their relationship.

In *M. latimanus* group, the Sri Lankan sample was positioned basal to the other samples. This separation of Sri Lankan *M. latimanus* from other samples (nucleotide divergence level 3%) was greatly supported by high bootstrap values. This result was also supported by a previous study[14], which reported up to 3.2% intra specific sequence divergence levels for *Macrobrachium* species. Within the *M. latidactylus* group, Sri Lankan and Thailand samples have given basal support to the clade. The Sri Lankan sample differed from the inner group by 1.5 − 2.% nucleotide divergence level while this level was 1.2 − 2.2% for the Thailand sample. *M. idae* made a sister clade to *M. scabriculum* with lower support. Within the *M. idae* clade, two Asian samples were similar to each other and showed 0.9% nucleotide divergence level from the Australian sample. *M. scabriculum* is so far reported from South Asian region and the Sri Lankan sample differs from the Indian sample by 0.4% nucleotide divergence level.

Detailed morphological study of Sri Lankan *Macrobrachium* taxonomy has been conducted previously[8]. In this study, many species have been collected from outside the southern Sri Lanka. From the twelve species described in the above study, the present study has found six species in the southern part of Sri Lanka. However, the previous studies have not highlighted the phylogenetic relationships of the species. The current study is the first molecular based study of the genus *Macrobrachium* in Sri Lanka. More taxon sampling with additional gene regions are required to better understand the phylogenetic relationships among Sri Lankan samples.

Acknowledgement

This study was supported by the UNESCO/Keizo-Obuchi Research Fellowship Programme. The author thanks the following: Professor C.M. Austin, Charles Darwin University, Australia, for providing laboratory facilities, Dr. S. Smith and Dr. M. SchaltZ, Charles Darwin University, Australia for the assistance in data analysis Mr. N. Liyanage, former Field Offcier of the National Aquaculture Development Authority for support in collecting samples and the two anonymous reviewers for their comments that improved the quality of this manuscript.

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