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Molecular Systematics of the Freshwater Prawn Genus *Macrobrachium* Bate, 1868 (Crustacea: Decapoda: Palaemonidae) Inferred from mtDNA Sequences, with Emphasis on East Asian Species

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Min-Yun Liu, Yi-Xiong Cai, and Chyng-Shyan Tzeng (2007) Molecular systematics of the freshwater prawn genus *Macrobrachium* Bate, 1868 (Crustacea: Decapoda: Palaemonidae) inferred from mtDNA sequences, with emphasis on East Asian species. *Zoological Studies* **46**(3): 272-289. Based on the mitochondrial DNA fragment of the large subunit (16S) ribosomal RNA gene, the monophyletic phylogeny of the genus *Macrobrachium*, including both land-locked and euryhaline species, was supported. There was, however, poor support for the internal structure. Results suggest that the evolution of this group was marked by rapid radiation. The 2 hypotheses that the prawns originated from marine ancestors and subsequently migrated towards fresh water in more than 1 wave of migration and that the abbreviated larval development of land-locked species represents adaptive convergence are both supported. It appears that most of the morphological characters commonly used to determine the taxonomy of genus evolved independently during the invasion of inland waters. Based on a fragment of 16S and a fragment of the cytochrome oxidase subunit I (COI) gene, several species groups, including species from different geographic regions and endemic groups were recognized from various continents (or regions). The presence of 5 cryptic species was indicated, and the identities of 4 supposedly undescribed species are confirmed. Localized speciation events in East Asia can be correlated with morphological similarities, notably members of the *M. asperulum* species group. Morphological characters tend to be conserved within species but quite variable between geographically distant populations, making species identification difficult. The present molecular results combined with morphological datasets can be used to help reorganize the taxonomy of various species groups. http://zoolstud.sinica.edu.tw/Journals/46.3/272.pdf

Key words: Molecular systematics, Phylogeny, Land-locked species, *Macrobrachium*, Cryptic species.

Freshwater prawns of the genus *Macrobrachium* Bate, 1868 (Crustacea: Palaemonidae) are a highly diverse group of decapod crustaceans thought to have originated from marine ancestors, some of which subsequently migrated towards fresh water in more than 1 wave; hence its members are known to inhabit the entire range of habitats from purely marine areas to inland hill streams and impounded water bodies (Tiwari 1955, Shokita 1979, Jalihal et al. 1993). To date, approximately 210 species are recognized (Short 2004); and there are numerous yet undescribed cryptic species (Chace and Bruce 1993, Wowor and Choy 2001, Cai and Ng 2002, Cai et al. 2004, Short 2004, D. Wowor, pers. comm., Y. Cai, pers. obs.).

The genus *Macrobrachium* can be ecologically separated into 2 groups: most species are widely distributed and require a certain saline concentration (i.e., 10‰-35‰) to complete their larval development, as euryhaline species; others are land-locked species, with limited distributions and complete their entire life cycle in fresh water (Holthuis 1950, Johnson 1973, Shokita 1979). As *Macrobrachium* migrated towards fresh water, the prawns gradually evolved several adaptive fea-

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tures. One of them is the abbreviated larval development achieved by reducing both the number of larval stages and the duration of the larval period (Shokita 1979, Jalihal et al. 1993). The abbreviated development of larvae in land-locked species was suggested to be a result of selective pressures for becoming established in freshwater environments (Shokita 1979, Magalhães and Walker 1988), and is a convergent phenomenon overriding phylogenetic relationships even above the generic level (Magalhães and Walker 1988). On the contrary, Pereira and Garcia (1995) suggested that since primitive palaemonids, like *Troglocubanus, Palaemonetes*, and *Pseudopalaemon*, possess abbreviated development, it can possibly be considered a primitive trait, and the abbreviated development took place early in the origin of the family Palaemonidae, rather than being a recent process.

Because of the degree of conservation of morphological characteristics, much debate has surrounded the systematic relationships of many species within this group, and its taxonomy and phylogenetic inferences have until recently been exclusively based on comparisons of external morphological characters (Holthuis 1950 1952, Johnson 1973, Pereira 1997). Some species groups were proposed based on morphological similarities, mainly of the rostrum and 2nd pereiopod (Holthuis 1950, Johnson 1973). The phylogenetic significance of these groupings remains to be tested. Apart from their taxonomy, the phylogenetic affinities among *Macrobrachium* species are poorly understood. Pereira (1997) carried out the first phylogenetic study based on morphological characters on the family Palaemonidae. In recent years, Murphy and Austin (2002 2003 2004 2005) published a series of results for the phylogeny of *Macrobrachium* species based on the mitochondrial (mt)DNA fragment of the large subunit (16S) ribosomal (r)RNA gene marker. Their studies produced some interesting results and led to the generic clarification of some local species.

East Asia has a large landmass that resides within both the tropics and subtropics and has a long history of overland connections with tropical areas possessing rich species pools (Hamilton 1983, Guo et al. 1998). This region exhibits high species diversity of plant taxa (Guo et al. 1998, Qian and Ricklef 2000), and its freshwater fish fauna also represents one of the richest ichthyofaunas in the Sino-Indian region (Koltelat 1989, Banarescu 1990). Climatic changes and geographic heterogeneity have played major roles in the diversification and speciation of East Asia's biota, and it is a superior continental model for studying increases in regional diversity through allopatric speciation (Qian and Ricklef 2000).

Previous studies of the genus *Macrobrachium* in East Asia comprised regional species surveys or zoogeographic distributions, including in China (Yu 1936, Dai 1984, Liu et al. 1990, Cai and Dai, 1999), Taiwan (Hwang and Yu 1982 1983, Shy and Yu 1998), and Japan (Hayashi 2000a b c). In total, about 37 species of *Macrobrachium* are found in China (Li et al. 2003), Taiwan (Shy and Yu 1998), and Japan (Hayashi 2000a b c), and there is a relatively high level of endemicity. Some population studies of *M. nipponense* were based on allozyme variations and reproductive traits (see Mashiko and Numachi 2000 for review). Shokita (1979) inferred the speciation and origin of land-locked species based on prawns from the Ryukyu Archipelago, southern Japan, and discussed the biogeography of the genus *Macrobrachium* with special reference to larval dispersal (Shokita 1985). However, little work has been carried out on the phylogenetic relationships among *Macrobrachium* species of East Asia.

For this study utilizing the mtDNA 16S rRNA marker, we attempted to investigate the phylogeny and evolution of land-locked species of the genus *Macrobrachium*, based on species from the Indo-West Pacific region and by using sequences available from GenBank. Then, we focused on species distributed in East Asia, including China, Taiwan, and Japan, including the Ryukyus. We used a combination of the 16S rRNA fragment and a fragment of the cytochrome oxidase subunit I (COI) gene to elucidate the phylogenetic relationships of East Asian species, to test if speciation patterns of endemic species in the region resulted from multiple lineages or from a single event, and to reveal any cryptic species that are difficult to distinguish using more-traditional techniques (Kowlton 2000, Hendrixon and Bond 2005, Ellis et al. 2006).

MATERIALS AND METHODS

Collection of materials

In total, 238 specimens, representing 34 putative and 4 undescribed species of *Macrobrachium*, were collected from East and Southeast Asia for the sequence analysis. Moreover, for 15 species, multiple individuals were sequenced from geographically distant populations to assess the monophyly of the putative species (Sites and

Marshall 2003, Peters et al. 2005). One to 8 specimens were analyzed per locality (Table 1). Specimens used in the present study (Table 1) were caught in the wild and preserved in 75%-95% ethanol. Five species of 3 closely related genera in the same family (the Palaemonidae), namely *Exopalaemon modestus*, *E. orientis*, *Palaemonetes sinensis*, *P. atrinubes*, and *Palaemon siuenus*, together with an atyid shrimp, *Caridina pseudodenticulata*, were included as the outgroup. Additional mtDNA 16S sequences available from GenBank were included in this analysis (Table 2), to encompass a total of 62 *Macrobrachium* species.

DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from the abdominal muscle by proteinase K/sodium dodecylsulfate (SDS) dissolution, phenol-chloroform extraction, and ethanol precipitation according to standard procedures (Sambrook et al. 1989). Fragments of 2 mitochondrial genes, the 16S rRNA and COI genes, were amplified from total genomic (g)DNA by a polymerase chain reaction (PCR) using the conserved primers 1471 (5'-CCT GTTTANCAAAAACAT-3') and 1472 (5'-AGATA GAAACCAACCTGG-3') (Crandall and Fitzpatrick 1996) for 16S rRNA, and COI-a (5'-AGTATAAGCGTCTGGGTAGTC-3') and COI-f (5'-CCTGCAGGAGGA GGAGACCC-3') (Palumbi and Benzie 1991) for the COI gene. These primers of 16S and COI did not work well for some species, so a new primer pair, 1471B (5'-CCTGTTTAN CAAAAACATGTCTG-3') and 1472B (5'-AGATA GAAACCAACCTGGCTCAC-3'), was modified for the 16S rRNA gene, and a new COI-fR (5'-CGTCGTGGTATGCCDTTTARWCCTA-3') primer replaced the COI-a primer.

The amplification (50 μl) of 16S rRNA used 1 mM of each primer, 0.2 mM of each dNTP, 1 unit of *Taq* polymerase (Promega, Madison, WI, USA), template DNA (50-100 ng), and 1X amplification buffer containing 1.5 mM $MgCl_2$ and either the

Table 1. Species of *Macrobrachium* and the outgroup included in this study. Sample localities, the number of samples examined, distribution, life cycle, and GenBank accession nos. are provided

Species	Sample locality	Locality code	Number examined	Distribution	Life cycle	Accession nos.	
						16S rRNA	COI
M. anhuiense	Anhui Prov., China	CN	1	East Asia	Land-locked	DQ194909	AB235240
M. asperulum	Fujian Prov., China	CN	6	East Asia	Land-locked	DQ194908	AB235241
	Ilan Co., Taiwan	N-TW[a]	7			DQ194906	AB235242
	Pingtung Co., Taiwan	S-TW[b]	5			DQ194907	AB235243
M. australe	Hualien Co., Taiwan	TW	6	Indo-West Pacific	Euryhaline	DQ194904	AB235245
	Cebu Is., the Philippines	PH	1			DQ194905	AB235244
M. edentatum	Sichuan Prov., China	CN	3	Indo-West Pacific	Land-locked	DQ194912	AB235247
M. equidens	Ilan Co., Taiwan	TW	4	Indo-West Pacific	Euryhaline	DQ194918	AB235250
	Bohol , the Philippines	PH	3			DQ194916	AB235248
	Seletor Is., Singapore	SG	5			DQ194917	AB235249
M. esculentum	Hualien Co., Taiwan	TW	6	Indo-West Pacific	Euryhaline	DQ194913	AB235252
	Cebu Is., Philippines	PH	1			DQ194914	AB235251
M. formosense	Yamaguchi Pref., Japan	JP	2	East Asia	Euryhaline	DQ194920	AB235253
	Okinawa I., Ryukyus, Japan	RK	3			DQ194922	AB235255
	Hainan Prov., China	CN	2			DQ194921	AB235254
	Hualien Co., Taiwan	TW	5			DQ194919	AB235256
M. fukiense	Fujian Prov., China	CN	2	East Asia	Land-locked	DQ194923	AB235257
M. gracilirostre	Hualien Co., Taiwan	TW	5	Indo-West Pacific	Euryhaline	DQ194924	AB235258
M. grandimanus	Okinawa I., Ryukyu, Japan	RK	2	Indo-West Pacific	Euryhaline	DQ194926	AB235260
	Hawaii Is., USA	HW	3			DQ194925	AB235259
M. hainanense	Guangdong Prov., China	CN	3	East Asia	Euryhaline	DQ194927	AB235261
	Hainan Prov., China	CN	2				
M. cf. *horstii*	Hualien Co., Taiwan	TW	9	Indo-West Pacific	Euryhaline	DQ194928	AB235291
M. idae	Khanom, Thailand	TH	2	Indo-West Pacific	Euryhaline	DQ194930	AB235262
M inflatum	Anhui Prov., China	CN	3	East Asia	Land-locked	DQ194931	AB235263

Table 1. (Cont.)

Species	Sample locality	Locality code	Number examined	Distribution	Life cycle	Accession nos.	
						16S rRNA	COI
M. japonicum	Yamaguchi Pref., Japan	JP	2	East Asia	Euryhaline	DQ194934	AB235264
	Okinawa I., Ryukyu, Japan	RK	4			DQ194935	AB235265
	Hualien Co., Taiwan	TW	8			DQ194933	AB235266
M. jaroense	Hualien Co., Taiwan	TW	6	Indo-West Pacific	Euryhaline	DQ194932	AB235267
M. lar	Iriomote I., Ryukyus, Japan	RK	2	Indo-West Pacific	Euryhaline	DQ194941	AB235269
	Hualien Co., Taiwan	TW	8			DQ194939	AB235270
	Cebu Is., the Philippines	PH	2			DQ194940	AB235268
M. lanatum	Myanmar (Burma)	IN	3	Indo-West Pacific	Euryhaline	DQ194911	AB235246
M. latidactylus	Hainan Prov., China	CN	1	Indo-West Pacific	Euryhaline	DQ194943	AB235271
	Hualien Co., Taiwan	TW	8			DQ194942	AB235275
	Bohol Is., the Philippines	PH	2			DQ194945	AB235273
	Tioman Is., Malaysia	MY	4			DQ194944	AB235272
	Dodaga Halmahera, Thailand	TH	1			DQ194946	AB235274
M. latimanus	Okinawa I., Ryukyu, Japan	RK	1	Indo-West Pacific	Euryhaline	DQ194938	AB235277
	Hualien Co., Taiwan	TW	3			DQ194936	AB235278
	Cebu Is., the Philippines	PH	3			DQ194937	AB235276
M. lepidactyloides	Hualien Co., Taiwan	TW	7	Indo-West Pacific	Euryhaline	DQ194929	AB235279
M. maculatum	Anhui Prov., China	CN	4	Indo-West Pacific	Land-locked	DQ194910	AB235280
M. malayanum	Gunonghedang, Malaysia	MY	2	Indo-West Pacific	Land-locked	DQ194947	AB235281
M. mammillodactylus	Bohol Is., the Philippines	PH	1	Indo-West Pacific	Euryhaline	DQ194915	AB235282
M. meridionalis	Hainan Prov., China	CN	6	Indo-West Pacific	Euryhaline	DQ194948	AB235283
	Tiomam I., Malaysia	MY	1			DQ194949	AB235284
M. naso	Yaungwhe, Myanmar	BM	3	Indo-West Pacific	Euryhaline	DQ194950	AB235285
M. neglectum	Langkawi I., Malaysia	MY	1	Indo-West Pacific	Euryhaline	DQ194953	AB235286
M. nipponense	Guangxi Prov., China	CN	5	Indo-West Pacific	Land-locked	DQ194952	AB235287
	Hualien Co., Taiwan	TW	6			DQ194951	AB235288
M. pinguis	Fujian Prov., China	CN	2	East Asia	Land-locked	DQ194958	AB235289
M. placidulum	Leyte, the Philippines	PH	4	Indo-West Pacific	Euryhaline	DQ194956	AB235290
M. placidum	Leyte, the Philippines	PH	3	Indo-West Pacific	Euryhaline	DQ194957	AB235292
M. platycheles	Nee Soon, Singapore	SG	3	Indo-West Pacific	Land-locked	DQ194955	AB235294
	Johor, Malaysia	MY	2			DQ194954	AB235293
M. rosenbergii	Kaohsiung Co., Taiwan	TW	2	Indo-West Pacific	Euryhaline	DQ194959	AB235295
M. shokitai	Iriomote I., Ryukyu, Japan	RK	5	East Asia	Land-locked	DQ194961	AB235296
M. yui	Yunnan Prov., China	CN	2	South East Asia	Land-locked	DQ194960	AB235297
*M. sp.*1	Hualien Co., Taiwan	TW	1	East Asia	?	DQ194962	AB235298
*M. sp.*2	Guangxi Prov., China	CN	5	East Asia	?	DQ194963	AB235299
*M. sp.*3	Jiangxi Prov., China	CN	1	East Asia	?	DQ194964	AB235300
*M. sp.*4	Siem Reap, Cambodia	KH	1	South East Asia	?	DQ194965	AB235301
*M. sp.*5	Hualien Co., Taiwan	TW	8	East Asia	?	DQ194966	AB235302
*M. sp.*6	Bohol, the Philippines	PH	3	Indo-West Pacific	?	DQ194967	AB235303
*M. sp.*7	Okinawa I., Ryukyus, Japan	RK	1	East Asia	?	DQ194968	AB235304
*M. sp.*8	Okinawa I., Ryukyus, Japan	RK	1	East Asia	?	DQ194969	AB235305
Outgroup							
Exopalaemon modestus	Jiangxi Prov., China	CN	2	East Asia	Land-locked	DQ194971	AB235307
Exopalaemon orientis	Taipei Co., Taiwan	TW	3	East Asia	Euryhaline	DQ194972	AB235306
Palaemonetes sinensis	Jiangxi Prov., China	CN	1	East Asia	Land-locked	DQ194970	-
Caridina pseudodenticulata	Hsinchu Co., Taiwan	TW	3	East Asia	Land-locked	DQ194973	AB235308

[a]Ilan County is in northeastern Taiwan. [b]Pingtung County is in southern Taiwan.

primer pair 1471+1472 or 1471B+1472B. The amplification conditions involved an initial cycle of denaturation at 94°C for 5 min, and then 35 cycles of denaturation at 94°C for 1 min, annealing at 48-55°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. For the COI gene, the amplification used either primer pair COI-f+COI-a or COI-f+COI-fR. A similar profile to that of 16S rRNA was employed except that the annealing temperature ranged from 45 to 50°C. The size and quality of the PCR products were visualized on 1.5% agarose gels.

Prior to sequencing, the PCR products were purified using a gel purification kit according to the manufacturer's instructions (QIAGEN, Valencia, CA, USA). In order to control the sequence accuracy and resolve any ambiguous bases, sequencing was carried out in both directions using the same primer pairs for PCR by cycle sequencing using the ABI PRISM Dye-Terminator Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and electrophoresis on an Applied Biosystems Automated Sequencer (model 377 or 3100).

Data analysis

Forward and reverse sequences for an individual were edited using SeqMan (DNASTAR, LaserGene, Madison, WI, USA). All sequences were aligned using MegAlign (DNASTAR, LaserGene), with checks and adjustments made by eye using BioEdit v5.0.9 (Hall 1999). Exploratory data analysis of the sequences was performed using MEGA vers. 2.1 (Kumar et al. 2001) and DnaSP 4.00 (Rozas et al. 2003). Pairwise sequence comparisons provided an assessment of levels of saturation by plotting the number of transitions and transversions against the uncorrected proportional distances (p-distances) for each pair of unique sequences of *Macrobrachium* species (Morrison et al. 2004). Inter- and intraspecific genetic distances were calculated using the Kimura (1980) 2-parameter model with the pairwise deletion option in the MEGA program.

For the 16S sequences, the *Macrobrachium* species collected in this study, including 4 undescribed species, 7 species in the outgroup (Table 1), and additional sequences available from GenBank (Table 2), were analyzed to determine the molecular systematics of the genus. To elucidate the phylogenetic relationships of East Asian species, we analyzed the COI sequences and the combination of 16S and COI sequences only for species collected in this study (Table 1); species containing only 16S sequences from GenBank (Table 2) were not included in this analysis. Sequences of both the 16S and COI genes were combined (Table 1) as both genes are in effect linked, and this is an appropriate way of dealing with random topological differences that are attributable to sampling error (Hipp et al. 2004). In order to test for the consistency of the phylogenetic signals in the data, phylogenetic relationships were inferred using 4 different analytical approaches. Maximum-parsimony (MP) (Camin and Sokal 1965) analysis was conducted assuming equal weightings for all characters, and results were compared when gaps were treated either as missing data or as a 5th character state. Neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-likelihood (ML) (Felsenstein 1981) analyses were carried out using appropriate DNA substitution models calculated with ModelTest vers. 3.5 (Posada and Crandall 1998). In the MP analysis, the heuristic search option with tree-bisection-reconnection (TBR) branch swapping and 100 stepwise random additions of taxa was used. In the NJ and MP analyses, levels of branch support were assessed using bootstrap resampling (Felsenstein 1985) with 1000 replicates to evaluate the reliability of the inferred topologies. Bootstrap resamplings were run with the "fast" stepwise addition algorithm and 100 replicates for ML, because of the large number of taxa involved and the computational time requirements. The NJ, MP, and ML analyses were carried out with PAUP* vers. 4.0b10 (Swofford 2000). Bayesian analyses (BI) were performed with MRBAYES vers. 3.0 (Ronquist and Huelsenbeck 2003) using the models selected by MrModeltest (Nylander 2004). Markov chain Monte Carlo (MCMC) chains were run for 1 x 10^6 generations, and trees were saved each 100 generations (with the 1st 1000 trees being discarded as "burn-in"). In BI, posterior probabilities are true probabilities of clades, and those with values of 95% or greater were deemed to be significantly supported.

RESULTS

Sequence characteristics and variations

The 16S rRNA sequence amplified by the 1471B+1472B primer pair varied from 524 to 533 bp in length (Table 1). Additional sequences obtained from GenBank (Table 2) were shorter;

final truncated lengths for the multiple alignments were 442 bp, including 20 sites with gaps, 191 variable sites, with 160 parsimoniously informative sites. The numbers of transitions outnumbered transversions in all comparisons by a factor of approximately 1.7. The COI sequences of species collected in this study (Table 1) and amplified by the COI-f+COI-fR primer pair contained 608 bp, including 276 sites which were variable and 258 which were parsimoniously informative. No stop codon was revealed when the COI sequences were translated into amino acids. The numbers of transitions outnumbered transversions by an average of 2.2. Base frequencies in both mtDNA

Table 2. Additional 16S sequences of *Macrobrachium* and the outgroup used in this study obtained from GenBank

Species	Sample locality	Locality code	Distribution	Life cycle	Accession no.
M. acanthochirus	Mexico	MX	South/Central America	Euryhaline	AY377837[c]
M. acanthurus	Mexico	MX	South/Central America	Euryhaline	AY282780[b]
M. aemulum	Australia	AU	Indo-West Pacific	Euryhaline	AY282769[b]
M. auratum	Australia	AU	Indo-West Pacific	Euryhaline	AY282775[b]
M. australiense	Australia	AU	Indo-West Pacific	Land-locked	AY282764[b]
M. brasiliense	Brazil	BR	South/Central America	Land-locked	AY377839[b]
M. bullatum	Australia	AU	Indo-West Pacific	Land-locked	AY282778[b]
M. carcinus	Puerto Rico	PR	South/Central America	Euryhaline	AY282779[b]
M. crenulatum	Puerto Rico	PR	South/Central America	Euryhaline	AY377840[c]
M. equidens	Australia	AU	Indo-West Pacific	Euryhaline	AY282773[b]
M. gangeticum	India	IN	Indo-West Pacific	Euryhaline	AY730054[d]
M. hainanense	Hong Kong	HK	Indo-West Pacific	Euryhaline	AY377841[c]
M. handschini	Australia	AU	Indo-West Pacific	Land-locked	AY282781[b]
M. heterochirus	Puerto Rico	PR	South/Central America	Euryhaline	AY377842[c]
M. idea	Australia	AU	Indo-West Pacific	Euryhaline	AY282777[b]
M. intermedium	Australia	AU	Indo-West Pacific	Marine[e]	AF439515[a]
M. koombooloomba	Australia	AU	Indo-West Pacific	Land-locked	AY282767[b]
M. lamarrei	India	IN	Indo-West Pacific	Land-locked	AY730051[d]
M. lar	Australia	AU	Indo-West Pacific	Euryhaline	AY282766[b]
	Indonesia	ID			AY377843[c]
M. latimanus	Australia	AU	Indo-West Pacific	Euryhaline	AY282765[b]
M. latidactylus	Australia	AU	Indo-West Pacific	Euryhaline	AY282770[b]
M. malcolmsonii	India	IN	Indo-West Pacific	Euryhaline	AY730050[d]
M. malayanum	Singapore	SG	Indo-West Pacific	Land-locked	AY377844[c]
M. mammillodactylus	Indonesia	ID	Indo-West Pacific	Euryhaline	AY377845[c]
	Australia	AU			AY282776[b]
M. neglectum	Indonesia	ID	Indo-West Pacific	Euryhaline	AY377846[c]
M. novaehollandiae	Australia	AU	Indo-West Pacific	Euryhaline	AY282772[b]
M. olfersii	Brazil	BR	South/Central America	Euryhaline	AY377848[c]
	Mexico	MX	South/Central America		AY377849[c]
M. platycheles	Singapore	SG	Indo-West Pacific	Land-locked	AY377850[c]
M. potiuna	Brazil	BR	South/Central America	Land-locked	AY377851[c]
M. rosenbergii	Australia	AU	Indo-West Pacific	Euryhaline	AY282774[b]
M. sankollii	India	IN	Indo-West Pacific	Land-Locked	AY730052[d]
M. scabriculum	India	IN	Indo-West Pacific	Euryhaline	AY730055[d]
M. tolmerum	Australia	AU	Indo-West Pacific	Euryhaline	AY282768[b]
M. trompii	Singapore	SG	Indo-West Pacific	Land-locked	AY377852[c]
M. zariqueyi	Annobon	AN	West Africa	Euryhaline	AY377847[c]
Palaemon serenus	Australia	AU	Indo-West Pacific	Euryhaline	AF439518[a]
Palaemonetes atrinubes	Australia	AU	Indo-West Pacific	Euryhaline	AF439520[a]
Palaemonetes australis	Australia	AU	Indo-West Pacific	Euryhaline	AF439517[a]

[a]Murphy and Austin 2003. [b]Murphy and Austin 2004. [c]Murphy and Austin 2005. [d]available in GenBank. [e]the only known species of the genus that spends its entire life in the sea (Holthuis 1952).

genes showed an AT bias (with G+C contents of 35.8% for 16S and 40.7% for COI). The combined data set of 16S+COI multiple alignments was 1142 bp in length including 20 sites with gaps, 472 variable sites, and 410 parsimoniously informative sites. All mtDNA sequences determined in this study were deposited in the GenBank/DDBJ databases under accession numbers DQ194904-DQ194973 and AB235240-AB235308 (Table 1).

In the gene-saturation analyses, substitutions of transitions and transversions for 16S were approximately linear in distribution with a positive slope for the regression (R^2 = 0.714 and 0.660 for transitions and transversions, respectively, data not shown), indicating that 16S rRNA is not saturated. For the COI data, transitions and transversions were plotted by separate codon position, and a saturation tendency was shown for transitions in the 3rd position, not for the 1st or 2nd positions, which appeared to reach a plateau at p-distances above 20% (Fig. 1). Sequence divergence estimates among the *Macrobrachium* species ranged 0.47%-22.44% for 16S and 0.16%-25.54% for COI; for conspecific individuals from the same locality, they ranged 0.00%-0.11% for 16S and 0.00%-3.70% for COI. Between populations (or individuals) from different localities of conspecifics, they ranged 0.00%-3.20% for 16S and 0.00%-12.63% for COI, (the most-distant populations of *M. grandimanus* being from the Ryukyus and Hawaii in this study). Four undescribed species were confirmed to be genetically distinct from other species, with interspecific divergences of 3.5%-19.9% for 16S and 9.48%-27.67% for COI. These will, hereafter, be referred to as *Macrobrachium* sp.1, *M.* sp.2, *M.* sp.3, and *M.* sp.4. We also found some discordant cases in which intraspecific divergences (4.9%-9.2% for 16S and 13.23%-17.24% for COI) greatly exceeded the usual ranges in this study, and might therefore reflect interspecific differences. Such discordant situations were found in samples of *M. latidactylus*, *M. latimanus*, *M. jaroense*, *M. placidum*, and *M. equidens* taken from multiple localities. According to these results, we believe such specimens may represent "cryptic species" (sensu Gusmão et al. 2000, Knowlton 2000, Hendrixon and Bond 2005, Ellis et al. 2006). These species were thus respectively referred to as *M.* sp.5, *M.* sp.6, *M.* sp.7, and *M.* sp.8 for *M. latidactylus*, *M. latimanus*, *M. jaroense*, and *M. placidum* (Figs. 2, 3). *Macrobrachium equidens* is discussed below. In contrast, the divergence between 2 morphologically distinct species, *M. formosense* and *M. hainanense* (0.01%-0.02% for 16S and 0.16%-0.66% for COI), was as close as that of the intraspecific level. This was also found for *M.* cf. *horstii* and *M. placidulum* (0.37% for 16S and 1.33% for COI) (Table 3).

Phylogenetic analyses

Based on results from Modeltest, the best-fit models in the NJ and ML analyses were as follows: 16S, HKY+I+G (Hasegawa et al. 1985), with a correction for the among-site rate variation estimation (G) of 0.6420 and a proportion of invariable sites (I) of 0.4499; COI: GTR+I+G (general time reversible model; Rodriguez et al. 1990), with a correction for G of 0.4023 and I of 0.4704; and for the combined dataset: TrN+I+G (Tamura-Nei model; Tamura and Nei 1993) with a correction for G of 0.7019 and I of 0.5354. For BI, the best-fit models selected by the Akaike information criterion (AIC) in MrModeltest were as follows: 16S: GTR+I+G, with a correction for G of 0.5897 and I of 0.4106; COI: GTR+I+G, with a correction for G of 0.4133 and I of 0.4770; and for the combined dataset: GTR+I+G with a correction for G of 0.6176 and I of 0.4675.

For 16S, 103 haplotypes for *Macrobrachium* species, including 4 undescribed species, 7 species of the outgroup (Table 1), and sequences available from GenBank (Table 2), were analyzed. A 50% majority consensus tree was obtained from the MP analyses. All 4 phylogenetic analyses generated similar tree topologies when gaps were treated as a 5th character (Fig. 2). The major differences between the 4 analyses lay in the levels of support provided for the various clades. Tree topologies supported the monophyly of the genus

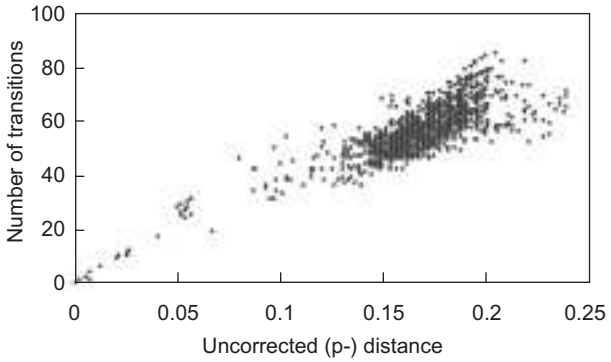

Fig. 1. Saturation test of cytochrome oxidase subunit I (COI), relationships of uncorrected p-distances between pairs of taxa, and the number of transitional changes at the 3rd codon position. A saturation tendency was shown to reach a plateau at p-distances above 20%.

Macrobrachium with high bootstrap values in the NJ, MP, and BI analyses. The outgroup genera of *Palaemonetes*, *Palaemon*, and *Exopalaemon* formed a monophyletic group with high bootstrap support, and were paraphyletic to *Macrobrachium*. *Exopalaemon modestus* and *E. orientis* formed a sister taxon pair with high bootstrap support in all analyses. *Palaemonetes atrinubes* and *P. sinensis* formed a sister taxon pair with weak bootstrap support, *Palaemon serenus* and *M. intermedium* formed a sister taxon pair with high bootstrap support. The species *M. intermedium*, located outside the *Macrobrachium* clade, was more-closely related to *Palaemon serenus* than to any other species (Fig. 2).

The deeper internal nodes were generally unresolved in the 16S analysis (Fig. 2). Some species groups, with good support for many terminal clades, were revealed giving some support or revealing some incongruence with earlier classifications. In addition to the Central/South American and Central/South American/West African species clades as reported by Murphy and Austin (2005), another 4 monophyletic groups were revealed (Fig. 2). The India group (INDIA) contains *Macrobrachium rosenbergii*, 2 Indian euryhaline species, *M. gangeticum* and *M. malcolmsonii*, and 2 Indian land-locked species, *M. lamarrei* and *M. sankollii*. The Indo-West Pacific group (IWP) includes several widely distributed euryhaline species, namely *M.* cf. *horstii*, *M. placidulum*, *M. placidum*, and 1 cryptic species, *M.* sp.8, which is morphologically very close to *M. placidum*. The next 2 distinct groups are endemic to East Asia; East Asia group I (EA I) is the land-locked *M. asperulum* species group, containing *M. asperulum*, *M. anhuiense*, *M. pinguis*, *M. shokitai*, and *M. maculatum*. East Asia group II (EA II) contains the euryhaline species *M. formosense* and *M. hainanense* and the land-locked species, *M. nipponense*, *M. inflatum*, and an undescribed species, *M.* sp.4 (Fig. 2).

Multiple samples from distant geographic populations of putative species were grouped into species-specific monophyletic groups with low to high bootstrap support (i.e., bootstrap values of 51-100), with the exception of 2 species (Fig. 2). The *M. equidens* species group, suggested by Johnson (1973), including *M. equidens*, *M. idae*, *M. mammillodactylus*, and *M. novaehollandiae*, did not form a monophyletic group in our study (Fig. 2). Moreover, specimens of *M. equidens* from 4 different localities, those of Taiwan, the Philippines, and Australia, formed a lineage and were separated, with distinct genetic distances (16.7%-17.4%), from specimens from Singapore, the type locality of *M. equidens*. Such disagreement was more evident in other species. Two populations of *M. hainanense* from China (Guangdong Prov. and Hainan I., Table 1) had an inter-population distance of 0.08%. When we compared our *M. hainanense* (*M. hainanense* (ML)) sequences with M. hainanense (HK) (accession no.: AY377841), collected from Hong Kong (Murphy and Austin 2005), there were inconsistencies with a significant dis-

Table 3. Genetic distance matrix of the large subunit (16S) (lower left) and cytochrome oxidase subunit I (COI) (upper right) among species of 4 species groups. SpG 1-4 are species groups 1-4, as revealed in figure 3

	SpG 1										Sp G 2	
16S \ COI	*M. asperulum*[a]	*M. anhuiense*	*M. asperulum*[b]	*M. pinguis*	*M. asperulum*[c]	*M. shokitai*	*M. maculatum*	*M.* sp. 2	*M.* sp. 3	*M.* edentatum	*M.* sp. 4	*M. hainanense*
SpG 1												
M. asperulum[a]		0.0252	0.0534	0.0537	0.0555	0.0922	0.1522	0.1559	0.1382	0.1248	0.1713	0.1737
M. anhuiense	0.0090		0.0588	0.0553	0.0413	0.0998	0.1498	0.1581	0.1463	0.1248	0.1757	0.1848
M. asperulum[b]	0.0261	0.0284		0.0408	0.0532	0.0998	0.1521	0.1537	0.1526	0.1245	0.1562	0.1651
M. pinguis	0.0236	0.0235	0.0164		0.0393	0.1059	0.1518	0.1622	0.1485	0.1206	0.1711	0.1891
M. asperulum[c]	0.0283	0.0257	0.0256	0.0205		0.0694	0.1359	0.1255	0.1718	0.1311	0.1711	0.1822
M. shokitai	0.0406	0.0381	0.0332	0.0405	0.0405		0.1539	0.1454	0.1741	0.1557	0.1774	0.1842
M. maculatum	0.0555	0.0529	0.0579	0.0630	0.0631	0.0554		0.0948	0.1446	0.1565	0.1718	0.1851
M. sp. 2	0.0657	0.0631	0.0630	0.0605	0.0631	0.0604	0.0358		0.1549	0.1457	0.1588	0.1918
M. sp. 3	0.0734	0.0707	0.0784	0.0783	0.0759	0.0757	0.0710	0.0789		0.1404	0.1902	0.2115
M. edentatum	0.0657	0.0607	0.0656	0.0707	0.0682	0.0504	0.0711	0.0736	0.0761		0.1793	0.1982
SpG 2												
M. sp. 4	0.1086	0.1116	0.0999	0.1053	0.1140	0.0998	0.1001	0.1139	0.1191	0.1062		0.1302

Table 3. (Cont.)

	SpG 1										Sp G 2	
16S \ COI	*M. asperulum*[a]	*M. anhuiense*	*M. asperulum*[b]	*M. pinguis*	*M. asperulum*[c]	*M. shokitai*	*M. maculatum*	*M.* sp. 2	*M.* sp. 3	*M.* edentatum	*M.* sp. 4	*M. hainanense*
M. hainanense	0.0769	0.0741	0.0687	0.0712	0.0794	0.0633	0.0714	0.0713	0.0791	0.0766	0.0791	
M. formosense	0.0769	0.0741	0.0687	0.0712	0.0794	0.0633	0.0714	0.0713	0.0792	0.0766	0.0792	0.0021
M. nipponense	0.0716	0.0688	0.0687	0.0712	0.0741	0.0582	0.0662	0.0713	0.0737	0.0713	0.0791	0.0141
SpG 3												
M inflatum	0.0689	0.0662	0.0661	0.0685	0.0714	0.0608	0.0636	0.0687	0.0712	0.0687	0.0763	0.0117
M. lanatum	0.0975	0.0946	0.0945	0.0998	0.0892	0.0916	0.0949	0.1002	0.0945	0.0893	0.1221	0.0981
M. latidactylus	0.1021	0.1019	0.0886	0.0965	0.1074	0.1046	0.1049	0.1158	0.1209	0.1158	0.1438	0.1104
SpG 4												
M. sp. 5	0.1184	0.1210	0.1184	0.1239	0.1239	0.1264	0.1270	0.1297	0.1406	0.1268	0.1583	0.1356
M. esculentum	0.0895	0.0893	0.0838	0.0839	0.0839	0.0837	0.0815	0.0814	0.0919	0.0814	0.1109	0.0954
M. cf. horstii	0.1082	0.1053	0.1135	0.1078	0.1053	0.1025	0.1161	0.1274	0.1299	0.0888	0.1327	0.1197
M. placidulum	0.1111	0.1081	0.1107	0.1105	0.0998	0.0970	0.1133	0.1188	0.1299	0.0942	0.1299	0.1112
M. placidum	0.1216	0.1243	0.1242	0.1297	0.1158	0.1186	0.1243	0.1302	0.1499	0.1104	0.1495	0.1304
M. sp. 8	0.1051	0.1049	0.1048	0.1049	0.0940	0.0915	0.0942	0.1105	0.1102	0.0993	0.1294	0.1191
M. lepidactyloides	0.1294	0.1293	0.1406	0.1376	0.1433	0.1290	0.1495	0.1464	0.1521	0.1321	0.1726	0.1212

	Sp G 2			Sp G 3				Sp G 4				
16S \ COI	*M. formosense*	*M. nipponense*	*M inflatum*	*M. lanatum*	*M. latidactylus*	*M.* sp. 5	*M. esculentum*	*M.* cf. *horstii*	*M. placidulum*	*M. placidum*	*M.* sp. 8	*M. lepidactyloides*
SpG 1												
M. asperulum[a]	0.1759	0.1651	0.1715	0.1662	0.2165	0.2115	0.1635	0.2171	0.2193	0.2151	0.1916	0.2102
M. anhuiense	0.1870	0.1759	0.1825	0.1579	0.2146	0.2120	0.1638	0.2077	0.2241	0.2174	0.1938	0.2102
M. asperulum[b]	0.1671	0.1612	0.1675	0.1468	0.2094	0.2096	0.1530	0.2081	0.1943	0.1943	0.1656	0.2102
M. pinguis	0.1914	0.1670	0.1823	0.1638	0.2234	0.2043	0.1787	0.2144	0.2075	0.2172	0.1868	0.2288
M. asperulum[c]	0.1842	0.1820	0.1864	0.1701	0.1863	0.2108	0.1633	0.2003	0.2137	0.2163	0.1928	0.2051
M. shokitai	0.1864	0.1798	0.1754	0.1638	0.1934	0.2094	0.1651	0.2277	0.2373	0.2281	0.1996	0.2279
M. maculatum	0.1872	0.1633	0.1676	0.1871	0.2115	0.1932	0.1809	0.2212	0.2224	0.2344	0.2038	0.2027
M. sp. 2	0.1941	0.1764	0.1809	0.2083	0.2197	0.2099	0.1832	0.2115	0.2229	0.2162	0.2057	0.2069
M. sp. 3	0.2138	0.1822	0.1733	0.2066	0.2372	0.2201	0.1848	0.2284	0.2366	0.2663	0.1980	0.2563
M. edentatum	0.2005	0.1757	0.1823	0.1943	0.2049	0.1914	0.1673	0.1834	0.1961	0.2267	0.1914	0.2011
SpG 2												
M. sp. 4	0.1323	0.1137	0.1117	0.1787	0.1964	0.1611	0.1642	0.1754	0.1947	0.1909	0.1506	0.1809
M. hainanense	0.0016	0.0591	0.0591	0.1722	0.1943	0.1742	0.1597	0.1923	0.2029	0.2176	0.1901	0.2155
M. formosense		0.0610	0.0573	0.1711	0.1966	0.1720	0.1618	0.1945	0.2052	0.2201	0.1922	0.2179
M. nipponense	0.0141		0.0271	0.1678	0.2059	0.1832	0.1610	0.1991	0.2011	0.2083	0.1813	0.2274
SpG 3												
M inflatum	0.0117	0.0070		0.1678	0.2012	0.1787	0.1601	0.1945	0.2032	0.2106	0.1904	0.2274
M. lanatum	0.0981	0.0898	0.0898		0.1801	0.1664	0.1463	0.1821	0.2043	0.1883	0.2008	0.1999
M. latidactylus	0.1104	0.1076	0.1132	0.0863		0.1772	0.1861	0.1914	0.2141	0.1864	0.1866	0.1999
SpG 4												
M. sp. 5	0.1356	0.1242	0.1299	0.0941	0.0482		0.1702	0.1755	0.2087	0.2003	0.2165	0.2057
M. esculentum	0.0954	0.0872	0.0927	0.0611	0.0839	0.0891		0.1731	0.1889	0.2045	0.1881	0.1835
M. cf. *horstii*	0.1197	0.1084	0.1056	0.1167	0.1413	0.1442	0.1084		0.0133	0.1293	0.1943	0.1777
M. placidulum	0.1112	0.1056	0.1028	0.1139	0.1384	0.1413	0.0946	0.0037		0.1426	0.1811	0.2045
M. placidum	0.1304	0.1191	0.1161	0.1249	0.1384	0.1355	0.1193	0.0636	0.0584		0.1989	0.1926
M. sp. 8	0.1191	0.1024	0.1051	0.0808	0.0992	0.1074	0.0732	0.0791	0.0790	0.0868		0.2033
M. lepidactyloides	0.1212	0.1211	0.1239	0.1409	0.1571	0.1720	0.1382	0.1186	0.1158	0.1297	0.1132	

[a]Samples collected from northern Taiwan. [b]samples collected from southern Taiwan. [c]samples collected from China.

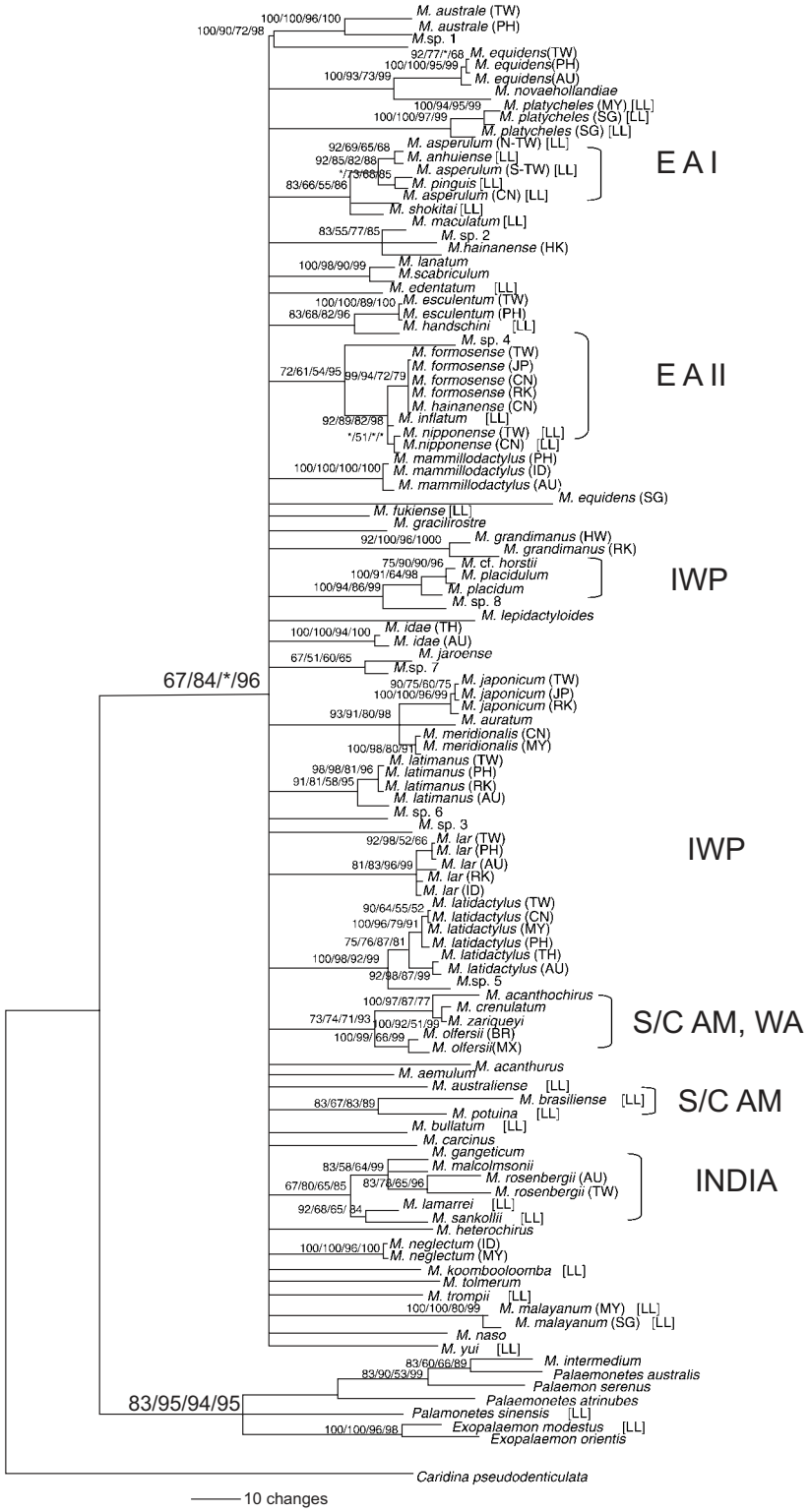

Fig. 2. Phylogenetic relationships based on large subunit (16S) sequences represented by a maximum parsimony (MP) 50% majority tree. Numbers on the branches indicate bootstrap values for the Neighbor-joining (NJ), MP, and maximum-likelihood (ML) analyses, and Bayesian analysis (BI). LL, land-locked species; EA, East Asia; IWP, Indo-West Pacific; S/C AM, South/Central America; WA, West Africa. EA I and EA II, 2 species groups found in East Asia. Abbreviations in brackets after the species names are the locality codes, which are given only when multiple samples of species were collected from different localities to assess the monophyly of the putative species. Locality codes are explained in tables 1 and 2. An asterisk (*) indicates a bootstrap value of < 50.

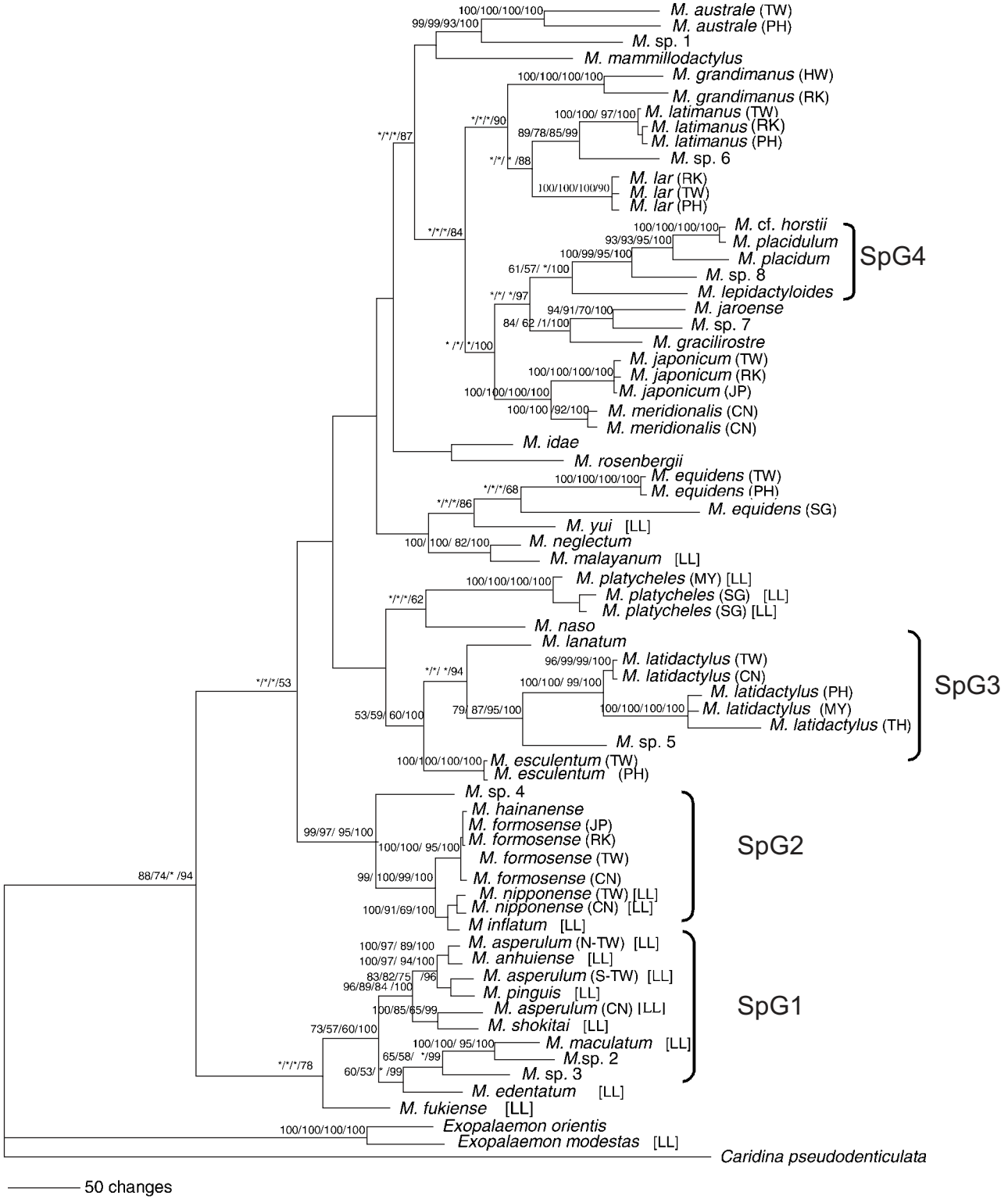

Fig. 3. Phylogenetic relationships based on combined large subunit (16S) and cytochrome oxidase subunit I (COI) sequences represented by a maximum-parsimony (MP) consensus tree. Numbers above the branches indicate bootstrap values for the Neighbor-joining (NJ) and MP analyses, while numbers below the branches indicate maximum-likelihood (ML) bootstrap values and Bayesian analysis (BI). LL, land-locked species; SpG1-4, species group 1-4. Abbreviations in brackets after the species names are the locality codes, which are given only when multiple samples of species were collected from different localities to assess the monophyly of the putative species. Locality codes are explained in table 1. An asterisk (*) indicates a bootstrap value of < 50.

tance of 7.6%. The *M*. *hainanense* (HK) sequence was placed in a different clade with land-locked species and was closely correlated to *M*. *maculatum* with a divergence of 3.8%, wherein our *M*. *hainanense* sequences formed a clade with the euryhaline species group and was closely correlated with *M*. *formosense*.

Species containing only 16S rRNA sequences in previous studies or only available in GenBank (Table 2) were not included in constructing the COI phylogenetic tree (COI tree not shown). Most of the species groups revealed above (Fig. 2), including the 4 undescribed *M*. spp1-4, 4 cryptic species, *M*. spp.5-8, and the incongruence of *M*. *equidens*. The phylogenetic tree of COI sequences revealed similar topologies as constructed by the phylogenetic analyses of the 16S sequences (Fig. 2). The monophyly of the *Macrobrachium* species clade could not be confirmed using COI data, as outgroup species of *Exopalaemon modestus* and *E*. *orientis* nested in one of the polyphyletic clades of *Macrobrachium* species taxa. The non-monophyletic structure of *Macrobrachium* species may be attributed to saturation of the 3rd codon of the COI gene (Fig. 1).

Analysis of the combined dataset

We used a combination of 16S and COI fragments of species collected in this study (Table 1) to elucidate the phylogenetic relationships of East Asian species. Although the deeper internal nodes were generally unresolved and the relationships among the species were not well-resolved by the combined sequences, certain phylogenetic relationships of the species complexes were shown to be well supported (Fig. 3). For example, the morphologically similar land-locked species endemic to East Asia, including the *M*. *asperulum* species group (*M*. *anhuiense*, *M*. *pinguis*, and *M*. *shokitai*) and other land-locked species of *M*. *edentatum* and *M*. *maculatum* and 2 undescribed species of *M*. sp.2 and *M*. sp.3 formed a monophyletic group (species group 1; SpG 1). The morphologically dissimilar land-locked species, *M*. *fukiense*, and Southeast Asian species, *M*. *malayanum*, *M*. *platycheles*, and *M*. *yui*, were not included. *Macrobrachium nipponense*, *M inflatum*, *M*. *formosense*, *M*. *hainanense*, and 1 undescribed species, *M*. sp.4, formed a clade as a species group (species group 2; SpG 2). Another species group (species group 3; SpG 3) with a similar morphology of an unequal 2nd periopod, containing *M*. *esculentum*, *M*. *lanatum*, *M*. *latidactylus*, and cryptic species *M*. sp.5, formed a monophyletic group. Species *M*. *lepidactyloides* formed a clade with the morphologically similar species of *M*. cf. horstii, *M*. *placidulum*, *M*. *placidum*, and cryptic species *M*. sp.8 forming the monophyletic species group 4 (SpG 4).

The incongruence of the non-monophyly of *M*. *equidens* in the 16S (Fig. 2), COI (tree not shown) and 16S+COI (Fig. 3) analyses implies the existence of a 5th cryptic species.

DISCUSSION

Phylogenetic relationships

Although we have included more species distributed in East Asia and the Indo-West Pacific region than Murphy and Austin (2005) did, the phylogeny of *Macrobrachium* species based on mtDNA 16S rRNA still showed poorly resolved "starburst" relationships, which lack internal structure with short internal branch lengths and longer tips among species of *Macrobrachium* (Fig. 2). Such phylogenetic relationships may have been caused by a weakness of the marker when it reaches saturation or by a lack of power of the data to resolve relationships among taxa (Albertson et al. 1999). 16S rRNA, used in this study, is not saturated (data not shown). Alternatively, the unresolved phylogeny detected herein may also be explained by rapid radiation, as suggested by earlier studies of *Sebastes* rockfishes (Johns and Avise 1998), fairy shrimp (Daniels et al. 2004), Caribbean sponge-dwelling snapping shrimp (Morrison et al. 2004), squat lobsters (Machordoma and Macpherson 2004), and freshwater crayfish (Shull et al. 2005). In all of those cases, resolution and/or support for the nodes in question were poor, suggesting a real phenomenon resulting from rapid radiation, rather than simply a paucity of appropriate data. When we focused on prawn species distributed in East Asia, the combined dataset of 16S and COI sequences revealed the same pattern of phylogenetic relationships (Fig. 3), whereas relationships among many of the terminal taxa were moderately to well supported which indicates several important features.

The monophyly of the genus *Macrobrachium* with the outgroup was supported by the phylogenetic analyses (Figs. 2, 3); *M*. *intermedium* was excluded from the genus *Macrobrachium* in earlier studies (Pereira 1997, Murphy and Austin 2002 2003, Short 2004). Multiple origins of

Macrobrachium fauna on various continents (or regions), like Central and South America, East Asia, the Indo-West Pacific, and India (Fig. 2), are suggested, supporting the results of Murphy and Austin (2005). The planktonic larval stage with salinity tolerance may play an important role in its long-distance dispersal and may have contributed to the widespread distribution of species (Shokita 1985, Jalihal et al. 1993, de Bruyn et al. 2004), such as those of *M. latidactylus*, *M. latimanus*, *M. grandimanus*, *M. rosenbergii*, and *M. lar*. Minor genetic differences (0.00%-3.20% for 16S and 0.00%-12.63% for COI) among conspecific, widespread euryhaline populations over broad geographic areas (Tables 1, 2, Figs. 2, 3), suggest that gene flow has been continually ongoing, and that they tend to have low levels of differentiation compared to species with a non-planktonic larval phase (Cameron 1986).

None of the land-locked species, including *Exopalaemon modestus* and *Palaemonetes sinensis*, formed a monophyletic group, or was located in the basal position, suggesting that they did not diverge from a single marine ancestor, but likely originated from marine ancestors and subsequently moved towards fresh water in multiple waves of migration (Figs. 2, 3) (Tiwari 1955, Jalihal et al. 1993). An abbreviated larval development pattern (ovigerous female with large eggs) has been suggested as being a process which resulted from selective pressures caused by attempts to become established in freshwater environments, and is a result of adaptive convergence (Shokita 1979, Magalhães and Walker 1988). This pattern is parallel to that of another freshwater shrimp in the family Atyidae (Magalhães and Walker 1988) and also in Jamaican's *Sesarma* crabs (Schubart et al. 1998).

Jayachandran's (2001) suggestion that the genus *Macrobrachium* could be grouped into 2 categories based on morphological characters of the 2nd pereiopod is not supported by our findings. Our results (Figs. 2, 3) demonstrated that grouping species according to the most-common morphological characters used in the taxonomy of the genus *Macrobrachium* (e.g., the 2nd pereiopod or the rostrum) does not form a monophyletic group, and these characters do not always have phylogenetic value. Morphological characters (such as the unequal 2nd pereiopod, big robust claws, spine, etc.) likely independently developed during the invasion of inland waters.

Taxonomic implications

Based on mtDNA sequences, 11 of 15 species we obtained from geographically distant populations formed monophyletic lineages; 4 undescribed species (*M.* spp.1-4) were identified, and 4 cryptic species (*M.* spp.5-8), respectively grouped with *M. latidactylus*, *M. latimanus*, *M. jaroense*, and *M. placidulum*, were inferred according to the phylogenetic reconstructions and sequence divergence levels. The incongruence of the non-monophyly of *M. equidens* in the 16S, COI, and 16S+COI analyses (Figs. 2, 3, COI tree not shown) implies the existence of a 5th cryptic species.

Some misidentifications or invalid species were also revealed. The species *M. hainanense* (accession no.: AY377841), collected from Hong Kong by Murphy and Austin (2005) was inconsistent with our specimens from 2 Chinese populations (sample localities of Guangdong Prov. and Hainan I., Table 1) with a significant genetic distance (7.6% in 16S) and different position in the phylogenetic tree (Fig. 2). Holthuis (1950) commented that *M. hainanense* was so closely related to *M. formosense* that it should perhaps only be considered a subspecies. According to the divergence and phylogenetic tree (Fig. 2), *M. hainanense* used by Murphy and Austin (2005) is most probably a "misidentified species". Examination of Hong Kong specimens by the second author showed that there is an undescribed species of the *Macrobrachium asperurum* species group in Hong Kong. The subadult specimens of this species could easily be confused with those of *M. hainanense*, which also occurs in Hong Kong.

Macrobrachium anhuiense and *M. pinguis* formed sister pairs to *M. asperulum* distributed in northern and southern Taiwan (Table 1), respectively, with high bootstrap support, and showed intraspecific levels of genetic divergences (0.90%-1.64% for 16S and 2.52%-4.08% for COI) (Table 3) in the 16S and combined dataset analyses (Fig. 2, 3). Liu et al. (1990) suggested that *M. pinguis* is an invalid species described based on undeveloped males, and was synonymous with *M. asperulum*. *Macrobrachium anhuiense* was also excluded from the species list of Chinese palaemonoid fauna (Li et al. 2003). The present DNA data support both taxonomic actions. Neither species, based on morphological and phylogenetic evidence, could be separated from *M. asperulum*, and both should be treated as invalid species.

Macrobrachium shokitai has a restricted distribution on Iriomote I. of the southern Ryukyus, Japan and is closely related morphologically and ontogenetically to *M. asperulum*. When these 2 species were crossed, their hybrid was found to be sterile. Shokita (1979) considered *M. shokitai* to be an offshoot of *M. asperulum*.

When the combined dataset was analyzed, a species group containing *M.* cf. *horstii*, *M. placidulum*, *M. placidum*, and *M. lepidactyloides* formed a monophyletic group, species group 4 (SpG 4) (Fig. 3). Molecular studies did not support the close relationship of *M. lepidactyloides* and *M. placidum* (with genetic distances of 12.97% for 16S and 19.26% for COI) (Table 3) as indicated by morphological similarities. This contradicts a suggestion by Chace and Bruce (1993) that *M. lepidactyloides* may be synonymous with *M. placidum* based on morphology. The species *M.* cf. *horstii* (cf Shy and Yu 1998) was closely allied to *M. placidulum* with an intraspecific level of genetic divergence (0.37% for 16S and 1.33% for COI) (Table 3), and it is also morphologically similar to the latter species; thus, it should be regarded as conspecific with *M. placidulum*.

The 4 cryptic species, (*M.* spp.5-8, and the incongruence of the non-monophyly of *M. equidens* (Figs. 2, 3) imply the existence of a 5th cryptic species, which showed minor morphological differences of "intraspecific" variations. However, they were genetically very distinct from other populations (localities) with values of interspecific divergences (4.9%-9.2% for 16S and 13.2%-17.2% for COI) and were closely allied with high bootstrap values. This also suggests that the use of traditional morphological characters alone is insufficient to accurately diagnose natural species groups of *Macrobrachium* (Holthuis 1950 1952, Johnson 1973).

In our study, the intraspecific 16S sequence divergence estimates between populations (or individuals) from different localities ranged 0.0%-3.2%. The significant divergence of 16S (5.1%-6.2%) between eastern and western *M. rosenbergii* clades along Huxley's line (de Bruyn et al. 2004) is far beyond the ranges of intraspecific divergence determined in the present study when compared with *M. lar*, *M. latidactylus*, *M. mammillodactylus*, *M. latimanus*, and *M. grandimanus* which are also distributed across Huxley's line. Genetic evidence in the present study supports the suggestion of previous studies (Johnson 1973, Lindenfelser 1984, Wowor and Ng 2001, de Bruyn et al. 2004) that *M. rosenbergii* may actually represent 2 distinct taxa: eastern and western forms.

Macrobrachium is a notoriously difficult genus taxonomically, as the morphological plasticity of taxonomically important traits (e.g., the rostrum and/or the 2nd pereiopod) change so much and so gradually during their growth (Holthius 1950) and are influenced by environmental parameters (Dimmock et al. 2004). Morphologically similar species are often quite genetically distinct. However, this might not be reflected in the phylogenetic relationships, as shown in the *M. equidens* species group (Johnson 1973) (Figs. 2, 3), suggesting that conservative systematic traditions or morphological stasis may be involved (Knowlton 2000). Most genetic analyses of species boundaries in marine crustaceans (see Knowlton 2000 for review) and freshwater macroinvertebrates (Baker et al. 2004, Shih et al. 2004 2005 2006) confirm or reveal the existence of cryptic species that are difficult to distinguish using traditional techniques. Some cryptic species are distinguished by surprisingly large genetic differences (Kowlton 2000, Hendrixon and Bond 2005, Ellis et al. 2006). This problem highlights a number of features of species group in these analyses. First, despite the diversity of species of this genus, they are all relatively conservative in general appearance, and taxonomic mistakes are easily made. Second, it shows the considerable value of having multiple samplings within each taxonomic group of interest, so that possible errors can be detected (Sites and Marshall 2003, Peters et al. 2005). It also suggests that using a single example of an individual or population to represent a species in the overall analysis is not justified and should be treated with caution. It is necessary to reevaluate the practical species concept based on such a multiple-sample analysis (Figs. 2, 3). The cryptic species detected here (Figs. 2, 3) suggest that the use of molecular techniques will be a significant help in delimiting species and understanding their relationships (Knowlton 2000, Hendrixon and Bond 2005, Ellis et al. 2006).

Some of the species groups, including species spanning different geographic regions, could be suggested from Central and South America, India, the Indo-West Pacific, and East Asia (Fig. 2). *Macrobrachium rosenbergii*, the widely distributed euryhaline species (De Man 1879, Johnson 1973) with an extended type of larval development and regarded as probably fairly "ancient" in nature by Johnson (1973), was not placed at the basal position of the *Macrobrachium* species clade. However, it formed a species group, as suggested by Johnson (1973), together

with 2 Indian euryhaline species, *M. gangeticum* and *M. malcolmsonii*, and 2 land-locked species endemic to India, *M. lamarrei* and *M. sankollii* (Fig. 2). Species groups containing such a mixture of species with different life cycles suggests that the species group may have evolved from a single ancestral lineage. Such a lineage was also found in species group 2 (SpG 2) containing *M. nipponense*, *M. inflatum*, *M. formosense*, *M. hainanense*, and 1 undescribed species *M.* sp.4 (Figs. 2, 3), and species group 3 (SpG 3), another euryhaline species group with an asymmetrical 2nd pereiopod, containing *M. lanatum*, *M. esculentum*, *M. latidactylus*, and cryptic species *M.* sp.5, (Fig. 3). This euryhaline species group contains a land-locked species endemic to Australia, *M. handschini*, which formed a sister pair with *M. esculentum* (specimens collected from Taiwan and the Philippines) in the 16S analysis (Fig. 2).

An endemic speciation event in East Asia was suggested in the land-locked species group, including the *M. asperulum* species group (species group 1) (Fig. 3). This land-locked species group did not form a monophyletic group with the land-locked species endemic to Southeast Asia (*M. yui*, *M. malayanum*, and *M. platycheles*), which implies a single lineage. Among them, *M. asperulum* is the most widely distributed species, being known from southern Siberia to southeastern China (Holthuis 1950). The other land-locked species have restricted distribution ranges. For freshwater-dependent prawns, factors responsible for dispersal generally involve land continuity and therefore river confluences (e.g., during sea-level lowering in glacial maxima), as well as river capture in headwaters (Banarescu 1990). The best explanation is that it represents fragmentation of a widespread ancestral taxon (vicariance) through allopatric speciation (Qian and Ricklef 2000), rather than a dispersal phenomenon from a more-restricted "center of origin" (Wiley 1988).

In addition to the ancient speciation by radiation, there is evidence for ongoing freshwater invasion and recent speciation in East Asia. The processes of freshwater invasion and penetration of cool temperate areas (at high latitudes) may be represented by *M. nipponense*. *Macrobrachium nipponense* is distributed along the coastline from northern Southeast Asia north to East Asia and Japan (Liu et al. 1990, Cai and Dai, 1999). Its narrow salinity tolerance, and relatively shorter zoeal period may have limited the dispersal distance to a dispersal pattern from estuary to estuary, apparently not a transoceanic dispersal in which larval stages of euryhaline species can disperse across the ocean by ocean currents as can *M. gradimanus* (Shokita 1985) and *M. rosenbergii* (de Bruyn et al. 2004). Some *M. nipponense* populations, on the basis of allozyme variation and reproductive traits, have apparently split into freshwater and estuarine populations in the same river in Japan (see Mashiko and Numachi 2000 for review), while populations in China and Taiwan are now land-locked species (Liu et al. 1990, Cai and Dai, 1999). This probably represents different steps in the process of inland water invasion. The land-locked populations of *M. nipponense* in China and Taiwan represent an advanced state of freshwater invasion, while populations of *M. nipponense* in Japan may represent both the advanced state (the land-locked populations inhabiting fresh water) and a euryhaline state. *Macrobrachium hainanense* and *M. formosense* are closely related species morphologically; Holthuis (1950) commented that *M. hainanense* should perhaps only be considered a subspecies. These 2 species exhibited the lowest interspecific DNA divergence (Table 3) and are closely related (Figs. 2, 3). Based on the fact that *M. formosense* has a more-restricted distribution than *M. hainanense* (Li et al. 2003); we believe that *M. formosense* may represent a newly derived species in recent geological times.

This study was based on only 2 mitochondrial markers. Further studies, particularly using other mitochondrial or nuclear sequence data and including more species, are obviously required to further investigate the evolutionary history of this genus. New data on the biology and ecology of these species and their habitats, and updated knowledge of the paleogeographical history will help clarify the possibility of microhabitat and behavioral specialization giving rise to radiation by *Macrobrachium*.

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