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Molecular systematics of the Asian mitten crabs, genus *Eriocheir* (Crustacea: Brachyura)

Boping Tang,[a,1] Kaiya Zhou,[a,*] Daxiang Song,[a] Guang Yang,[a] and Aiyun Dai[b]

[a] *Institute of Genetic Resources, College of Life Sciences, Nanjing Normal University, Nanjing 210097, China*
[b] *Institute of Zoology, Academia Sinica, Beijing 100080, China*

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Abstract

To help resolve phylogenetic relationships among the mitten crabs, complete sequences of the nuclear DNA internal transcribed spacer (ITS) and portions of the mitochondrial genome corresponding to the cytochrome oxidase I (COI), were sequenced for all Asian mitten crabs of the genus *Eriocheir* and seven species of the Grapsoidea. The resulting phylogeny supports the establishment of a separate genus *Neoeriocheir*, but does not provide justification for the recognition of *Platyeriocheir*. A female mitten crab specimen from the Zhujiang River, China, was considered to be *Eriocheir recta* (Stimpson, 1858), a species previously synonymized with *Eriocheir japonica* (de Haan, 1835). In the ITS analysis, a sequence from *Eriocheir formosa* (from Taiwan) falls within a well-supported *E. recta* group, which indicates that *E. formosa* may have to be synonymized with *E. recta*. Three previously recognized members of the genus, *E. japonica*, *Eriocheir sinensis*, and *Eriocheir hepuensis* constitute a monophyletic sister group to *E. recta* in all phylogenetic trees. We provide evidence for the conspecific status of these taxa. Phylogenetic trees based on COI and combined COI and ITS sequences indicate that *E. japonica* consists of three subgroups. Since the name *E. japonica* (de Haan, 1835) takes precedence over *E. sinensis* (H. Milne Edwards, 1853) and *E. hepuensis* Dai, 1991, we suggest that these three subgroups correspond to three subspecies of *E. japonica*: *E. j. japonica*, *E. j. sinensis*, and *E. j. hepuensis*.

Keywords: Mitten crabs; Systematics; rDNA ITS; mtDNA COI

1. Introduction

Mitten crabs (family Grapsidae) are limnic and intertidal crabs distributed in Asia and are one of the most important groups of crabs in the world. The mitten crabs of the varunid genus *Eriocheir* de Haan, 1835, as presently recognized, consist of five taxa, viz., *Eriocheir japonica* (de Haan, 1835), *Eriocheir sinensis* (H. Milne Edwards, 1853), *Eriocheir hepuensis* Dai, 1991, *Eriocheir leptognatha* Rathbun, 1913, and *Eriocheir formosa* Chan et al., 1995. Mitten crabs are restricted to East Asian waters, except *E. sinensis* which naturally occurs in eastern and northern China, but has been introduced into Europe and North America (Cohen and Carlton, 1997; Ingle and Andrews, 1976). Taxonomic boundaries between genera and species, and species and subspecies of the mitten crabs have received some attention from systematists. However, the results have led to some confusion over the taxonomy of *Eriocheir*. The subspecies described as *E. japonica hepuensis* by Dai (1991) was elevated to *E. hepuensis* by Guo et al. (1997). However, it was not recognized as a good taxon by other authors (Chan et al., 1995; Du, 1998; Li et al., 1993; Li and Zou, 1999). *Eriocheir recta* was originally described from Macao located at the Zhujiang River estuary by Stimpson (1858) and recorded subsequently from Guangdong and Taiwan, China, and Japan (Dai et al., 1986; Sakai, 1976). Unfortunately, the type specimen of *E. recta* was lost during the fire in Chicago in 1871, and the original description of *E. recta* by Stimpson was very brief. Chan et al. (1995) synonymized the species with *E. japonica* and described a new species *E. formosa*

* Corresponding author.
E-mail addresses: kyzhounj@jlonline.com (K. Zhou), boptang@sina.com (B. Tang).
[1] Present address: Department of Biology, Yancheng Teacher's College, Yancheng 224002, China.

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based on specimens from Taiwan. Later, Ng et al. (1999) established a new monotypic genus, *Platyeriocheir*, to accommodate *E. formosa* but the validity of *E. formosa* as a species was questioned by Du (1998). *E. leptognatha*, the smallest member of the genus, had been assigned to a new monotypic genus, *Neoeriocheir*, by Sakai (1983), but subsequent authors (e.g., Chan et al., 1995; Kim and Hwang, 1995) did not recognize the genus, while Guo et al. (1997) considered it to be valid. Li et al. (1993) suggested that *E. sinensis* is a junior synonym of *E. japonica* based on morphometric and biochemical data, but this opinion has not been accepted by most authors. Despite the ample availability of taxonomic, morphological, and ecological information on *Eriocheir* species, the phylogenetic relationships among the mitten crabs has not yet been resolved.

In the present study, phylogenetic relationships within Asian mitten crabs of the genus *Eriocheir sensu lato* are inferred, based on DNA sequences of mitochondrial cytochrome oxidase I (COI) and nuclear DNA of internal transcribed spacer (ITS). We address the questions whether present taxonomic relationships within the genus *Eriocheir* are supported by molecular systematics and whether the genera *Neoeriocheir* and *Platyeriocheir* are justified.

2. Materials and methods

2.1. Sample collection

Fifteen individuals, representing five Asian species and subspecies of *Eriocheir* were studied. In addition, three species representing three other genera of the family Varunidae (sensu Schubart et al., 2000, 2002), and four species representing three other families of the Grapsoidea (sensu Schubart et al., 2000, 2002), were also studied. Data for specimens studied are given in Table 1 and a map of collecting localities is given in Fig. 1. Tissue samples, derived from muscle, were preserved in ethanol or stored at −20 °C. Voucher specimens are preserved in the collection of Nanjing Normal University (NJNU), and Institute of Zoology (IZCAS), Chinese Academy of Sciences. Taxonomic assignment of examined species follows Dai (1991), Dai et al. (1986), Dai and Yang (1991).

Table 1
Mitten crab species used in this study with collection locality, catalogue number and GenBank accession number

Species	Collection locality	Catalogue No.	GenBank Accession Nos.	
			ITS	COI
Grapsidae				
Grapsus albolineatus Lamarck, 1818①	Xisha Archipelago	IZCAS741203		AF317338
Varunidae				
Varuna litterata (Fabricius, 1798)①	Fangcheng, Guangxi	IZCAS800606		AF317343
Hemigrapsus sanguineus (de Haan, 1835)	Lianyungang, Jiangsu	NJNU990731		AF317340
Gaetice depressus (de Haan, 1835)	Dongshan, Fujian	NJNU990515		AF317339
Eriocheir japonica (de Haan, 1835)	Okinawa	NJNU98110601	AF316381	AF317329
*Eriocheir japonica*②	Tokushima	IZCAS871003		AF317331
	Osaka	NJNU991123	AF316382	AF317330
*Eriocheir japonica*③	Taizhung, Taiwan	NJNU981101	AF316383	
	Hong Kong	NJNU990813	AF316379–AF316380	
E. hepuensis Dai, 1991	Hepu, Guangxi	NJNU969023	AF316376–AF316378	AF317327
E. hepuensis, Holotype①	Hepu, Guangxi	IZCAS899024A		AF317328
E. sinensis (H. Milne-Edwards, 1853)	Panjin, Liaoning	NJNU951041	AF316391	AF317335
	Gaochun, Jiangsu,	NJNU951067	AF316388–AF316389	AF317333
	Yueqing, Zhejiang,	NJNU9510016	AF316393–AF316394	AF317337
	Minjiang, Fujian	NJNU990521	AF316392	AF317336
	Zhangzhou, Fujian	NJNU990515	AF316390	AF317334
E. leptognatha Rathbun, 1913	Panjin, Liaoning	NJNU961033	AF316384–AF316385, AF316347	AF317337
E. formosa Chan et al., 1995③	Hualien, Taiwan	NJNU981103	AF316375	AF317326
E. recta (Stimpson, 1858)	Zhujiang, Guangdong	NJNU980525	AF316386–AF316387	AF317332
Sesarmidae				
Sesarma haematocheir (de Haan, 1835)①	Haimen, Jiangsu	IZCAS620716		AF317342
Sesarma dehaani H. Milne-Edwards, 1853	Minjiang, Fujian	NJNU990521	AF316395	
Plagusiidae				
Plagusia immaculata Lamarck, 1818①	Xisha Archipelago	IZCAS760402		AF317341

Note. The specimens were identified by ① A.Y. Dai, Institute of Zoology, Chinese Academy of Sciences, ② T. Sakai, Carcinological Society of Japan, ③ T.Y. Chan, Institute of Marine Biology, National Taiwan Ocean University, respectively. Other specimens were identified by B.P. Tang, K.Y. Zhou, and D.X. Song.

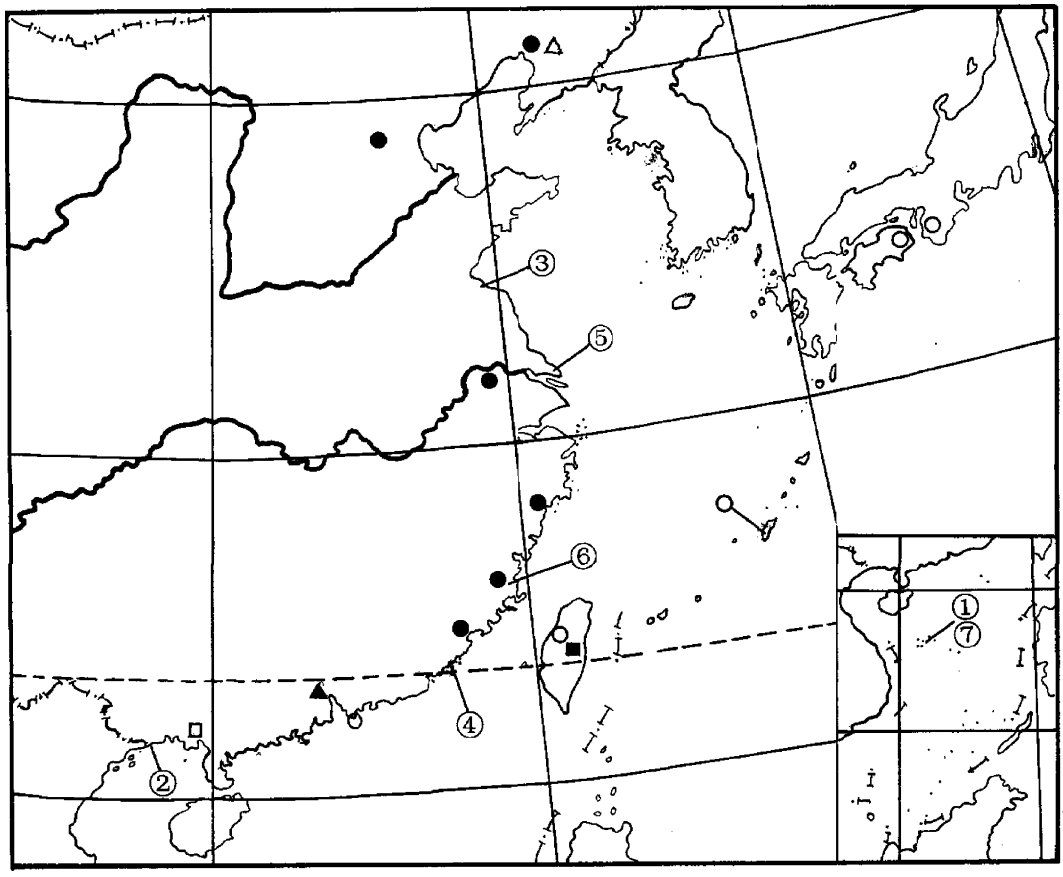

Fig. 1. Map showing collection localities of Grapsoidea crabs studied. □ *Eriocheir hepuensis*, ■ *E. formosa*, ○ *E. japonica*, ● *E. sinensis*, △ *E. leptognatha*, ▲ *E. recta* ① *Grapsus albolineatus*, ② *Varuna litterata*, ③ *Hemigrapsus sanguineus*, ④ *Gaetice depressus*, ⑤ *Sesarma haematocheir*, ⑥ *S. dehaani*, ⑦ *Plagusia immaculate*.

2.2. Laboratory analysis

Total genomic DNA was isolated from muscle tissue of walking legs, following standard proteinase digestion and phenol–chloroform extraction procedures as described in Sambrook et al. (1989).

Primers PT1 and PT3 were designed to amplify the ITS region, based on the 18S and 28S rDNA sequences of crustacean species (Crease, 1993; Friedrich, 1995; Spears et al., 1992). Two other primers, PT2R (paired with PT1) and PT2F (paired with PT3), were designed as internal primers to sequence ITS-1 and ITS-2, respectively (Table 2, Fig. 2). Amplification of ITS was carried out in 30 μl reaction volumes containing: 1.0 U of *Taq* DNA polymerase (Promega), 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 4.0 μl 10 × buffer, 0.25 μM each of primers, and 5–10 ng of template DNA. An initial 2 min predenaturation at 95 °C was followed by 30 cycles of 95 °C denaturation for 45–60 s, 55–61 °C annealing for

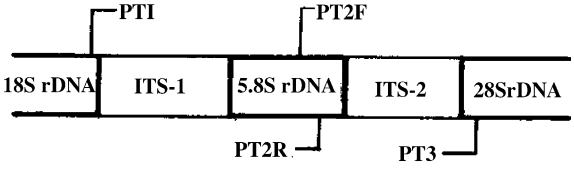

Fig. 2. Diagram showing the nuclear rDNA ITS region of *Eriocheir* crabs and locations of PCR primers used in the present study.

Table 2
Primers used for amplifying and sequencing the nuclear rDNA ITS region, ITS-1, ITS-2, and mtDNA COI gene fragments

Name	Sequence 5′–3′	Reference
PTI	GGAAGTAAAAGTCGTAACAAGG	Present study
PT2R	ATCGACCCACGAGCCGAGTGAC	Present study
PT2F	GTGGGTCGATGAAGACCGCAGG	Present study
PT3	TTCAGTCGCCCTTACTAAGGGAATCC	Present study
PMT1	GGTCAACAAATCATAAGATATTGG	Folmer et al. (1994)
PMT2	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)

30–55 s, 72 °C extension for 1.1–1.4 min. Amplification cycles were followed by a final 7 min extension at 72 °C. Mitochondrial COI segments were amplified under the same reaction conditions (but with a template of 10–45 ng) with some modifications in cycling conditions: 95 °C for 30–45 s, 51–55 °C for 35–45 s, 72 °C for 50–60 s. Some conditions were changed as needed to improve the quality of PCR products. All PCR were carried out on a PTC-200 thermal cycler (MJ Research). PCR products were purified with Wizard PCR Prep DNA Kit (Promega) and then cloned into TA cloning vector (pMD 18-T Vector), prior to automated sequencing using the ABI BigDye terminator mix with an ABI Prism 310 Genetic Analyzer. Sequences of total ITS were obtained by the combination of ITS-1 and ITS-2 sequences. Sequences of COI were also determined for six additional species of crabs representing four families of the Grapsoidea, using the same method as above. All sequences have been deposited in GenBank (Table 1).

2.3. Data analysis

Sequences were aligned using CLUSTAL X (Thompson et al., 1997) and corrected by eye (EMBL Accession Nos.: ALIGN_000452, ALIGN_000453). Nuclear ITS-1, ITS-2, total ITS, mitochondrial CO1, and combined ITS and CO1 data sets were analyzed using two different phylogenetic methods to verify whether alternative topologies support each other. We used Kimura two-parameter distances $(s+v)$ and neighbor-joining (NJ) to analyze distance matrices of sequence divergences with the program MEGA (Kumar et al., 1993). Statistical significance of groups within inferred trees was evaluated by the bootstrap method with 1000 replications. A maximum-likelihood (ML) tree was constructed using the program DAMBE (4. 0. 41). Three models (F84, HKY85, and TN93) were compared. Fixing the most-parsimonious tree as the tree for tests, the HKY85 model was found to be superior to TN93 ($\chi^2 = 103$, $P < 0.001$) and F84 ($\chi^2 = 120$, $P < 0.001$) in ITS data. When the most-parsimonious tree was employed as the tree for tests in analyses of COI data, F84 was found to be superior to TN93 ($\chi^2 = 365$, $P < 0.001$), and was much superior to HKY85 ($\chi^2 = 543$, $P < 0.001$). So maximum-likelihood searches of ITS and COI were then run under the HKY85 and F84 model, respectively (Xia, 2000). *Sesarma* species of Sesarmidae and *Grapsus* species of Grapsidae (Grapsoidea), which diverged earlier to mitten crabs (Grapsoidea), were chosen for the outgroup taxa to increase resolution and support for basal ingroup nodes (sensu Schubart et al., 2000, 2002).

3. Results

3.1. ITS region data set

Twenty-one nuclear rDNA ITS sequences for 13 individuals of 5 species and subspecies of the genus *Eriocheir* were obtained. The aligned 1120 bp of ITS region includes 356 bp ITS-1, 162 bp 5.8S, and 686 bp ITS-2 (Table 3). One hundred and sixty-one sites are variable, and 141 are parsimony informative sites. The G + C composition of the ITS region is significantly higher than that of A + T, and vice versa for COI (Table 3).

NJ and ML trees of the ITS region (Figs. 3A and B) with *Sesarma dehaani* as an outgroup generated very similar tree topologies. The *Eriocheir* crabs consist of three groups, one comprising all populations of *E. japonica*, *E. sinensis*, and *E. hepuensis*, the second *E. formosa* and *E. recta*, and the third *E. leptognatha*. All the bootstrap values for the branches separating these groups are high (> 95). The phylogenetic relationships within *Eriocheir* crabs based on either ITS-1 or ITS-2 sequences are identical to that from the total ITS region, but the latter have higher bootstrap values. Thus, only the phylogenetic trees of the total ITS region are presented here.

Although a subgroup in the first group is supported by 94% bootstrap support, the relationships among the *Eriocheir* populations are not resolved. This may be explained by the reason that intraindividual variations in some individuals are greater than variations among populations of the same species. For example, the intraindividual variations in ITS-2 of Hepu population and Hong Kong population are 0.1–1.0% and 0.0–1.4%,

Table 3
Comparison of the nucleotide composition of different DNA sections among species of *Eriocheir*

	ITS-1 ($N = 21$)	5.8S ($N = 21$)	ITS-2 ($N = 21$)	ITS ($N = 21$)	COI ($N = 13$)
Total aligned sites	356	162	686	1120	529
%A (of total sites)	24.5	24.8	20.7	22.4	27.5
%T (of total sites)	16.4	20.3	21.8	20.1	36.3
%C (of total sites)	32.1	27.2	29.2	29.7	19.2
%G (of total sites)	27.0	27.7	28.3	27.8	17.0
Variable sites	30	2	130	161	111
Parsimony informative sites	27	1	115	141	82

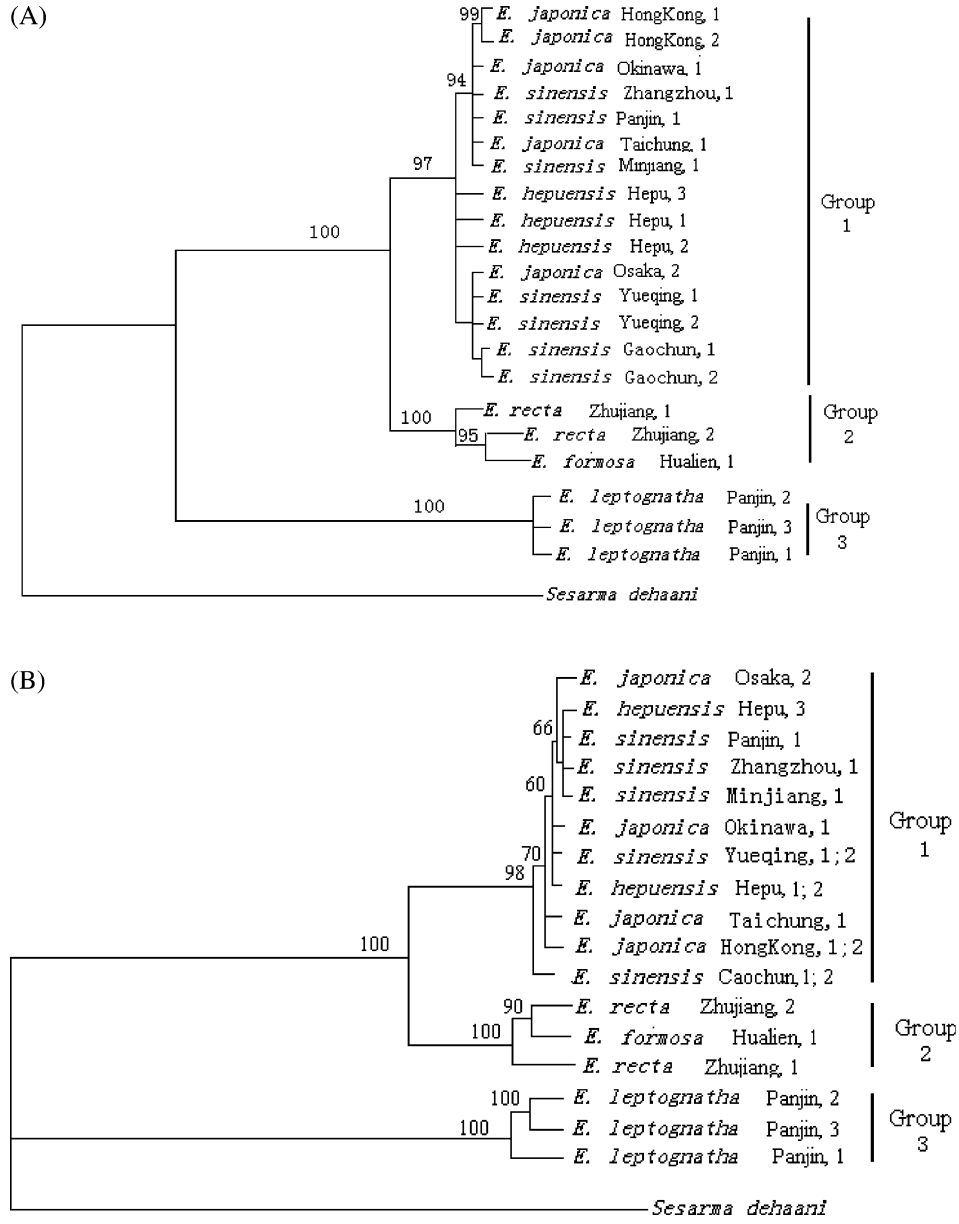

Fig. 3. The neighbor-joining tree (A) and (B) maximum-likelihood tree (ln likelihood = −2797.8367) resulting from analysis of the ITS region sequences of *Eriocheir* species. The number after each species name means different clones of the same individual. *Sesarma dehaani* was used as the outgroup. Bootstrap values for 1000 replicates are shown above branches.

respectively, whereas the divergences between populations, such as Gaochun and Yueqing populations, Panjin and Minjiang populations, Yueqing and Panjin populations, are 0.7%, 0.1–0.5%, and 0.3%, respectively. These results show that the sequence variations in ITS regions of *Eriocheir* crabs could not be used as genetic markers for phylogenetic studies at population level.

3.2. Cytochrome oxidase subunit I data set

The aligned COI fragments of the *Eriocheir* crabs on one hand and with 6 other grapsids on the other hand consist of 529 bp. The former includes 111 variable sites, and 82 parsimony informative sites, whereas the latter includes 185 variable sites and 153 parsimony informative sites (Table 3).

The topology of NJ and ML trees of *Eriocheir* species (Figs. 4A and B) inferred from mitochondrial COI sequences is similar to those of Fig. 3. However, the Okinawa population of *E. japonica* clustered with Jiulongjiang River (Zhangzhou) and Minjiang River populations of *E. sinensis*, and the holotype of *E. hepuensis*, the Osaka population of *E. japonica* clustered with Tokushima population of *E. japonica*, and the Panjin population of *E. sinensis* clustered with Gaochun and Yueqing populations of *E. sinensis*, in

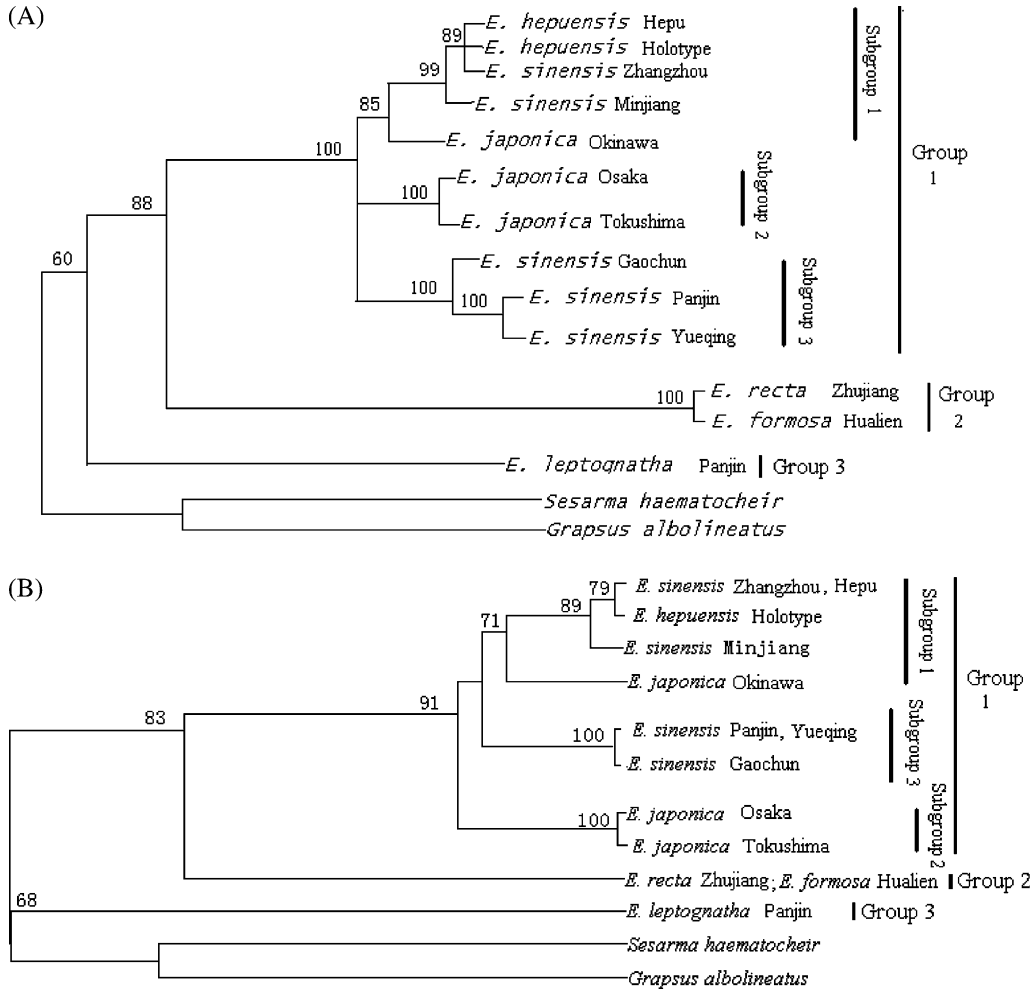

Fig. 4. The neighbor-joining tree (A) and (B) maximum-likelihood tree (ln likelihood = −2194.6321) resulting from analysis of the COI fragment sequences of *Eriocheir* species. *Sesarma haematocheir* and *Grapsus albolineatus* were used as outgroups. Bootstrap values for 1000 replicates are shown above branches.

both COI trees, whereas the relationships among these populations were not resolved in the ITS trees. The topology inferred from combined data set of COI and ITS region of *Eriocheir* species is identical to that from COI.

4. Discussion

4.1. Validity of the genus Neoeriocheir

The genus *Neoeriocheir* was established by Sakai (1983). Specimens of its type species, *E. leptognatha* Rathbun, 1913, are the smallest among specimens of *Eriocheir sensu lato*. This species inhabits the eastern and northern coast of China, the western coast of Korea, and Japan (Dai et al., 1986; Sakai, 1976; Wei, 1991). Chan et al. (1995) and Kim and Hwang (1995) did not recognize the genus, while Ng et al. (1999) considered it valid. Our results show that phylogenetic trees of the *Eriocheir* group derived from ITS-1, ITS-2, ITS, and COI data, as well as combined data of ITS and COI, have a nearly identical conformation, especially in the main groups. In these trees, *N. leptognatha* is always the most divergent group (Figs. 3 and 4). The sequence divergences of nuclear DNA ITS region or Mitochondrial CO1 gene fragment between *N. leptognatha* and other species of *Eriocheir sensu lato* (average 11.5 and 15.6%, respectively) are about 3–5 times higher than those among different species of *Eriocheir* (average 2.5 and 5.5%, respectively), and comparable to those among the 4 studied genera of the same family (average 18.4%, in COI). Additionally, *N. leptognatha* always constitutes the basal branch of the molecular phylogenetic trees of the *Eriocheir sensu lato* group. These results support the establishment of a separate genus *Neoeriocheir*, which was previously synonymized with *Eriocheir*. In the ensuing paragraphs, the specimens assigned to *E. leptognatha* in Table 1 are therefore referred to *Neoeriocheir leptognatha.*

4.2. Eriocheir formosa and Platyeriocheir

On examining the collection of Zhujiang River we came across an adult female specimen assigned to the genus *Eriocheir*, which we declare a neoptype of *Eriocheir recta* Stimpson, based on its fitting with the description of Stimpson and on the fact it was found in the type locality.

Chan et al. (1995) indicated that specimens from Taiwan assigned to *E. recta* by various authors differed from *E. japonica* in characters of frontal margin, lateral margin, setae on chelae, and surface of carapace, and were considered to be a new species. They gave the name *E. formosa* to these specimens. During the present study, the Zhujiang specimen identified as *E. recta* and a Taiwanese specimen labelled as *E. formosa* were studied. The results show that sequences of mt CO1 gene fragment of the Zhujiang specimen are identical to those of *E. formosa*; only 5 and 8 base variations, respectively, were found between *E. formosa* and the two clones of *E. recta* in the ITS region. The nesting of *E. formosa* within the *E. recta* group is strongly supported in the ITS analysis (95% BP), which suggests that the taxon named *E. formosa* may be conspecific with *E. recta*, in which case *E. formosa* would be a junior synonym of *E. recta*.

Ng et al. (1999) noted that *E. formosa* (Chan et al., 1995) differed from its congeners in several characters and transferred it to a new monotypic genus, *Platyeriocheir*. However, as we have shown that *E. formosa* may be conspecific with *E. recta*, the present study does not provide evidence to support *Platyeriocheir* as a valid genus. Thus, the genus *Platyeriocheir* should be regarded as a junior synonym of *Eriocheir* based on these molecular phylogenetic analyses.

4.3. Eriocheir japonica, E. sinensis, and E. hepuensis are conspecific

Eriocheir japonica, the type species of the genus *Eriocheir*, has the widest distribution, ranging from Japan, Korea to Guangdong, Guangxi, Taiwan, and south-eastern China. *E. sinensis* is a commercially important crustacean species cultured extensively in coastal areas of China. Three populations of *E. sinensis* named "Changjiang crab," "Liaohe crab," and "Oujiang crab" with different commercial significance are morphologically difficult to distinguish from one another (Zhou and Gao, 1999). However, no clear separation between *E. japonica* and *E. sinensis* was found by principal component analysis (PCA) of morphometric data, and allozyme electrophoresis (Li et al., 1993). In addition, hybrids were easily produced between cultured *E. japonica* and *E. sinensis* (Peng, 1986; Zhao et al., 1988). On the basis of morphology and hybridization behavior, Dai (1988) suggested that *E. sinensis* was conspecific with *E. japonica*. *E. hepuensis* has only been reported from its type locality so far. PCA of morphometric data of the male first pleopod indicated that *E. hepuensis* overlaps with both *E. japonica* and *E. sinensis* (see Dai, 1991). Our analyses show that these three species constitute a monophyletic sister group to *E. recta* in all phylogenetic trees. The present molecular study gives additional evidence for the conspecific status of these taxa. The name *E. japonica* (de Haan, 1835) takes precedence over *E. sinensis* (H. Milne Edwards, 1853) and *E. hepuensis* (Dai, 1991). In the following, the specimens assigned to *E. sinensis* and *E. hepuensis* in Table 1 will be referred to as *E. japonica.*

The populations of *E. japonica* (including the specimens assigned to *E. sinensis* and *E. hepuensis* in Table 1) consistently cluster together in phylogenetic trees based on sequences of the ITS region, but their relationships are not resolved. This may indicate that the intraindividual variation in ITS region of the mitten crabs could obscure phylogenetic relationships at the population level. This is similar to the result of variation in ITS1 and ITS2 of crayfish (Harris and Crandall, 2000).

However, phylogenetic trees based on COI and combined COI and ITS sequences indicate that *E. japonica* consists of three subgroups. Hepu, Jiulongjiang River (Zhangzhou), Minjiang River, and Okinawa populations grouped in the first subgroup, whereas Osaka and Tokushima populations in the second subgroup, and Oujiang River (Yueqing), Changjiang River (Gaochun), and Liaohe River (Panjin) populations in the third subgroup. We suggest that these three subgroups should be regarded as three subspecies of *E. japonica*, namely, *E. j. japonica*, *E. j. sinensis*, and *E. j. hepuensis*. The type localities of *E. japonica*, *E. sinensis*, and *E. hepuensis* were Japan, China and Hepu, southern China, respectively. The nominal subspecies inhabits the coastal area of Honshu, Japan, whereas *E. j. sinensis* inhabits eastern and northern China, and *E. j. hepuensis* inhabits southern China and Okinawa, Japan.

Here we want to indicate that the sample of *E. japonica* from Okinawa may represent a separate population, because in all trees it does not closely cluster with other populations. It is referred provisionally to *E. j. hepuensis* in the present study.

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