

Mitochondrial DNA-inferred population structure and demographic history of the mitten crab (*Eriocheir sensu stricto*) found along the coast of mainland China

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Abstract

The mitten crab, *Eriocheir sensu stricto*, is economically important in East Asia, although it is an invasive species in Europe and North America. Little is known about its population structure and historical demography in its native range, especially along the Pacific coast of China. We collected mitochondrial cytochrome oxidase subunit II and cytochrome *b* sequences from 154 individuals distributed in the rivers along the Chinese coast and 15 individuals from Japan. Phylogenetic analysis resulted in three major monophyletic groups: northern China, southern China and Japan. Negligible migration was detected among those groups by coalescent analysis. Hence, we support the recognition of three species: *Eriocheir hepuensis* in southern China, *Eriocheir sinensis* in northern China and *Eriocheir japonica* in Japan. The populations in the middle (the Oujiang and Minjiang Rivers) possess a mixture of haplotypes similar to either the northern or the southern haplotypes. We believe that secondary intergradation as the most likely cause of the clinal variation based on examining the genetic variation in the latitudinal space. The estimated divergence time between *E. sinensis* and *E. hepuensis* is 2.24 million years ago (Ma), while the divergence time between *E. japonica* and *E. sinensis* is 1.83 Ma. Both are in the late Pliocene, suggesting that land bridges associated with low sea level during that time might have severed as vicariant barriers for speciation. The divergence of the northern population and the ‘northern haplotypes’ in the middle population was estimated at 0.12 Ma, while the time separating the southern population and the ‘southern haplotypes’ in the middle populations was estimated as 0.16 Ma, implicating possible secondary contact in the late Pleistocene.

Keywords: *Eriocheir*, gene flow, genetic distance, mitten crab, Pacific coast of China, population

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Introduction

The mitten crab, *Eriocheir sensu stricto*, is commonly referred to as three species (Guo *et al.* 1997; Ng *et al.* 1999; Xu 2005) or subspecies (Tang *et al.* 2003), which includes *Eriocheir sinensis*, *Eriocheir japonica* and *Eriocheir hepuensis*. It has a catadromous lifecycle. Breeding occurs in estuaries in early spring; then juveniles migrate upriver into inland lakes where they become sexually mature. Despite its invasive status in Europe (Panning 1939) and North America

(Rudnick *et al.* 2000), the mitten crab is a popular food in East Asia. In particular, *E. sinensis* is a prized delicacy in China. In 2006, 0.45 million tons of mitten crab valued at nearly RMB40 billion (about US\$5.7 billion) were produced by the Chinese aquaculture industry (Fu & Liu 2006). The commercial importance and significant ecological impact of mitten crabs stimulated interests in population studies in its native range and in the new habitat it has invaded (Li *et al.* 1993; Gao & Watanabe 1998; Li & Li 1999; Li & Zhou 1999; Zhao & Li 1999; Li *et al.* 2001; Zhao *et al.* 2002; Yamasaki *et al.* 2006; Herborg *et al.* 2007). However, the population structure and demographic history of mitten crabs are still not well understood, especially along the Pacific coast of China, which is the centre of its native distribution.

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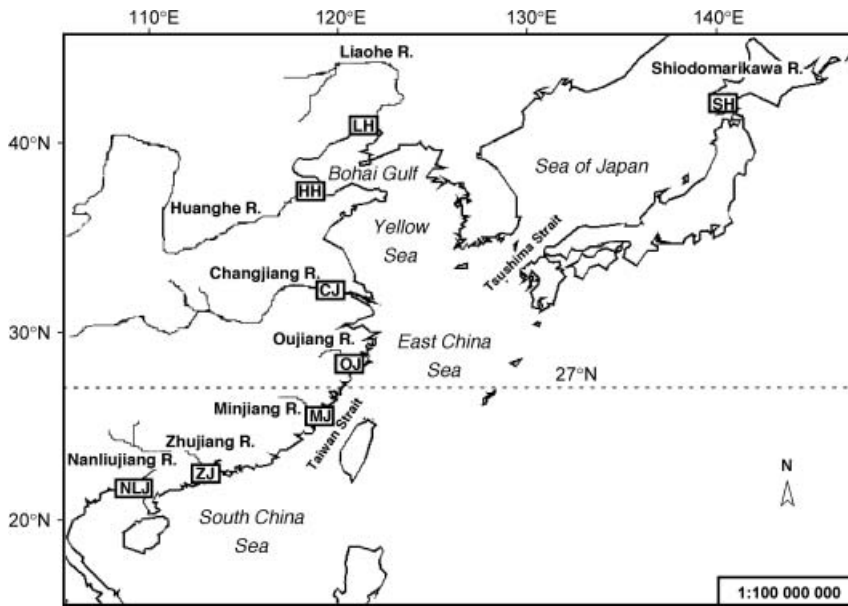


Fig. 1 Sampling sites of the mitten crab, *Eriocheir sensu stricto*. The localities are labeled with the abbreviations of river names. Detailed information about the sites can be found in Table 1. The 27°N parallel was traditionally used to define the border separating *Eriocheir sinensis* and '*Eriocheir japonica*' in China.

It is assumed that *E. japonica* has the widest distribution among the three species, ranging from Japan, the Russian and the Korean coast of the Sea of Japan, to southern China, including Hong Kong and the western coast of Taiwan (Sakai 1935, 1976). The largest and most economically important species in this genus is *E. sinensis*, which is distributed along the coast of the East China Sea, Yellow Sea and Bohai Gulf (Zhao *et al.* 1988). *E. hepuensis* was recently described by Dai (1991) from Hepu, Guangxi Province, on the coast of the South China Sea (Fig. 1).

Along the coast of mainland China, mitten crabs can be found in almost every major river system. Populations from the Changjiang (Yangtze) River, the Liaohe River and the Oujiang River in middle and northern China are most commonly used in aquaculture (Fig. 1). Differences in growth rate, ovarian development and breeding season have been observed between individuals from different populations (Gu & He 1997; Jin & Li 2000; Li *et al.* 2001). Nevertheless, all of the populations in middle and northern China are still treated as *E. sinensis*, because previously observed differences in morphological and molecular characteristics are only slight (Li & Li 1999; Li & Zhou 1999; Zhao & Li 1999; Zhao *et al.* 2002). The 27°N parallel (Zhao *et al.* 1988) is often used to define the border between '*E. japonica*' and *E. sinensis* in China (Fig. 1), with *E. sinensis* living in the north and *E. japonica* in the south. The distribution of *E. hepuensis* centres at the Nanliujiang River in southern China but was documented as overlapping with *E. japonica*, and in some cases even occurred within the distribution area of *E. sinensis* (Dai 1991; Sun *et al.* 2005a).

Three different opinions exist about the status of mitten crabs distributed on the Pacific coast of China (e.g. Sun *et al.* 2002). One view is that the three species of mitten

crabs are indistinguishable from each other based on morphometric characters and allozyme loci; thus, they should be considered as one species (Li *et al.* 1993). A second group of investigators indicate that the mitten crab in China is composed of two distinctive species based on morphometric, allozyme, random amplified polymorphic DNA (RAPD) and mitochondrial sequence analyses. The northern species is *E. sinensis* and the southern species is *E. japonica*, while *E. hepuensis* is genetically indistinguishable from *E. japonica* collected from the Zhujiang (Pearl) River (e.g. Li & Li 1999; Li & Zhou 1999; Zhao & Li 1999; Zhao *et al.* 2002). A third view is that *E. hepuensis* differs from both *E. japonica* and *E. sinensis* morphologically (Dai 1991; Guo *et al.* 1997); therefore, three species (Guo *et al.* 1997) or subspecies (Tang *et al.* 2003) should be recognised.

The possible reasons for the discrepancies among the hypotheses delineating the mitten crab along the coast of China could be traced back to three roots: (i) Probably influenced by Sakai (1935, 1976), most studies assumed that the samples collected from the sites in the Zhujiang River drainage (e.g. Zhujiang, Aotou, Jiulongjiang) were *E. japonica* (e.g. Li *et al.* 1993; Li & Li 1999; Li & Zhou 1999; Zhao & Li 1999; Xu 2005), although some evidence suggested that these individuals formed a monophyletic group with *E. hepuensis* exclusive of other *E. japonica* collected from Japan (Xu 2005). (ii) Mixed populations have been identified in the Oujiang and Minjiang Rivers between the commonly recognised range of the northern and southern species (Sun *et al.* 2005a; Xu 2005). Failing to dissect the signal in those populations and simply treat them as homogenous groups could lead to wrong conclusions, especially when frequency data alone is used. (iii) Most of the previous studies relied heavily on using phylogenetic methods to

Collection site	River	Site abbreviation	<i>n</i>	No. of haplotypes
Panjin, Liaoning	Liao He	LH	20	12
Dongping, Shandong	Huang He	HH	19	15
Zhenjiang, Jiangsu	Changjiang	CJ	35	31
Wenzhou, Zhejiang	Oujiang	OJ	19	13
Fuzhou, Fujian	Minjiang	MJ	21	19
Shunde, Guangdong	Zhujiang	ZJ	20	17
Hepu, Guangxi	Nanlijiang	NLJ	20	15
Hakodate, Hokkaido	Shiodomarikawa	SH	15	13

Table 1 Sampling localities, abbreviation (see Fig. 1), number of individuals sampled (*n*), and number of haplotypes in each population

interpret the pattern of genetic variation among species and often include only a few individuals per locality (Zhao *et al.* 2002; Chu *et al.* 2003; Tang *et al.* 2003; Xu 2005). Those approaches are not appropriate for examining the interrelationships and dynamics among populations from different river drainages because the divergence between species is small and lineage sorting might be incomplete; hence, a bifurcating tree would not be apposite to describe the potentially network-like relationships.

In this study, we aimed to estimate the population structure and interrelationships of mitten crabs along the Pacific coast of China, and to evaluate the historical and current forces shaping it. We combined the merit of both phylogenetic methods and coalescent population approaches. Phylogenetic methods would be more useful for diverged taxa, often shown as reciprocal monophyly, so we use the results of phylogenetic analyses to understand the relationships between monophyletic groups. For groups with intermingled relationships, we performed coalescence-based analyses, which are more appropriate for evaluating the status of unsorted groups. In doing so, we estimated such parameters as the population divergence time and gene flow. We also discussed the possibility of postglacial dispersal, selection, and secondary contact as the sculpting forces for the clinal change in mitten crab populations which has been documented using physiological traits (Gu & He 1997; Jin & Li 2000) and molecular allele frequencies (Li & Zhou 1999).

Materials and methods

Sample collection, PCR amplification and sequencing

We sampled 154 individuals from seven river drainages along the coast of China, ordered from north to south as Liao He River (LH), Huang He (Yellow) River (HH), Changjiang River (CJ), Oujiang River (OJ), Minjiang River (MJ), Zhujiang River (ZJ), and Nanlijiang River (NLJ), along with 15 individuals of *Eriocheir japonica* from Shiodomarikawa River (SH) in Japan (Table 1, Fig. 1). Leg muscles were excised and preserved in 95% ethanol. A

standard phenol–chloroform method was used to extract the DNA. We collected sequences for two mitochondrial genes, cytochrome oxidase subunit II (COII) and cytochrome *b* (cyt *b*), for all 169 individuals (EU315942–EU316184). Sequences of *Geothelphusa dehaani* (NC_007379) were retrieved from GenBank to be used as the outgroup. COII and cyt *b* were amplified and sequenced using primers following Sun *et al.* (2005b). All amplified products were purified using a 3S Spin PCR Product Purification Kit (Biocolor Inc.). The purified COII products were cloned into the plasmid pUG-T18 and sequenced using M13 primers, while the cyt *b* products were directly sequenced using the PCR primers on an Applied Biosystems ABI 3730 DNA sequencer.

Sequence alignment, data partitioning and phylogenetic analysis

Nucleotide sequences were translated into amino acids and aligned using CLUSTAL W (Thompson *et al.* 1994) implemented in MEGA 3.1 (Kumar *et al.* 2004), then translated back into nucleotides for subsequent analyses. The identical haplotypes in the aligned matrix were identified and collapsed using COLLAPSE version 1.2 (Posada 2006). Instead of testing the compatibility of the two mitochondrial genes, we performed partitioned analysis (combined analysis; Nylander *et al.* 2004). Because each data partition was fitted with its own model, conflicting signal would be low between two linked mitochondrial genes. The concatenated sequence was partitioned by gene and codon position, with the 1st and 2nd codon positions grouped into one block and the 3rd codon position as the other block. We compared three data partitioning schemes: no partition, partition by codon and partition by both gene and codon. The final scheme was chosen based on their Bayesian information criterion (BIC) values. The partitioned phylogenetic analysis was carried out using a Bayesian approach (Nylander *et al.* 2004). Each data partition was fitted with an independent GTR + Γ model, with the topology held as the same and branch length set as *prset ratepr = variable* for each partition (Ronquist & Huelsenbeck 2003). Three million Markov

chain Monte Carlo (MCMC) generations were run for the Bayesian analysis with a sampling frequency of 1 in 100 generations. Two independent runs were carried out to allow additional confirmation of the convergence of MCMC runs. Consensus tree was constructed from the saved trees with the trees before reaching the stationarity discarded. We adopted two methods to root the phylogeny. First, we use an outgroup taxa (*G. dehaani*) to root the tree. Because the genetic distances among the three putative species are fairly small (Chu *et al.* 2003; Tang *et al.* 2003; Xu 2005), any outgroup taxa could be too distant and very likely lead to wrong rooting (Huelsenbeck *et al.* 2002). The other way to root the phylogeny is to enforce the molecular clock when constructing the phylogeny. If the molecular clock assumption is not seriously violated by the data, this method has a high probability of finding the correct root (Huelsenbeck *et al.* 2002). We tested the molecular clock hypothesis using a likelihood-ratio test in PAUP (Swofford 2003), with $s - 2$ degrees of freedom, where s is the number of taxa (Felsenstein 1981). Besides the bifurcated tree, haplotype network is often used to describe intraspecific relationships. We constructed the statistical parsimony haplotype network using TCS (Clement *et al.* 2000) and compared the result with the resulting phylogenetic tree.

Intra- and interpopulation (drainage) variation, neutrality test, divergence time, gene flow and spatial analysis of molecular variance

Gene and nucleotide diversities were calculated for each population (drainage) using ARLEQUIN (Excoffier *et al.* 2005). Tajima's D (Tajima 1989) and Fu's F (Fu & Li 1993) were examined to evaluate the demographic history in each population. Mismatch distribution tests implemented in ARLEQUIN were performed against the null model of sudden population expansion. Pairwise F_{ST} and averaged pairwise population distances among populations were estimated using ARLEQUIN. Between reciprocally monophyletic groups, the net averaged population genetic distance, $D_A = d_{iXY} - (d_{iX} + d_{iY})/2$ (Nei & Li 1979) is proportional to the population divergence time since it accounts for the polymorphism within populations (Edwards & Beerli 2000). For populations with incomplete lineage sorting, coalescent methods are more appropriate for inferring divergence time (see below). To estimate the absolute population divergence time, the evolutionary rate of the gene markers should be known. Because the rate for COII and *cyt b* are not available in the mitten crab, we used an estimated divergence time between *E. japonica* in Japan from *Eriocheir* in southern China of 2.31 million years ago (Ma) (Xu 2005) as the calibration, which was based on a rate of 1.66% per million years for COI gene of grapsid crabs (Schubart *et al.* 1998). We calculated the divergence time of other nodes using D_A and the calibration.

To evaluate divergence time between groups without reciprocal monophyly, we used the isolation with migration program, IMA (Hey & Nielsen 2007). Divergence time and gene flow between the northern (LH, HH and CJ) and the middle population (OJ and MJ) and between the southern (ZJ and NLJ) and the middle population were estimated separately. In the middle population, some haplotypes were found to be north-like and some haplotypes were south-like. If we treated the haplotypes in the middle population as a single distinct group and assessed their divergence time from the northern and the southern populations, the estimates would be confounded by combined effects of the old divergence between the northern and the southern populations and the recent dispersal from the northern and the southern populations into the middle region. Thus, we performed the IMA analysis using the northern population with the 'northern' haplotypes in the middle population, and the southern population with the 'southern' haplotypes in the middle population, respectively. In this way, the estimates should yield insights into the dispersal events from the northern and the southern populations that led to the observed genetic signal. Fortunately, a lack of recombination and exclusive maternal inheritance of mitochondrial gene made this unique case of coalescent analysis feasible.

For the IMA analysis, we performed three independent runs. The first run was used to explore the signal in the data and to find out the appropriate priors and running time for the analyses. The last two runs were carried to check for consistency in the results. To calculate population divergence time and gene flow, we first estimated a mitochondrial substitution rate of 1.28×10^{-5} per year by using 2.31 Ma as the calibration time as used above (Xu 2005). Then we used this substitution rate and a generation time of 2 years for *Eriocheir* to convert the estimates to demographic parameters.

To assess the gene flow between the three groups identified in the phylogenetic analysis, northern China, southern China and Japan, we also carried LAMARC analysis (Kuhner & Smith 2007). Because these three groups are old lineages suggested by their reciprocal monophyletic relationship, we think LAMARC is more appropriate to be used here than IMA, which is often used to model recently diverged populations. Negligible gene flow among these groups would suggest that they are separately evolving entities, whereas significant gene flow would argue against them as distinctive species. To increase the statistical power of LAMARC analysis, we randomly pruned the number of individuals in each group to 20 if it was larger than 20 as suggested by Kuhner & Smith (2007). We ran the simulations under the coalescent model with migration to estimate the population parameters M ($M = m/\mu$, where m is the immigration rate per generation and μ is the mutation rate). We performed the Bayesian strategy in LAMARC (Kuhner & Smith 2007). The final estimates were based on a run of one short chain

Table 2 Gene diversity, nucleotide diversity, mismatch analysis and neutrality test results for each population

	LH	HH	CJ	OJ	MJ	ZJ	NLJ	SH
Gene diversity	0.8789	0.9766	0.9899	0.9474	0.9905	0.9789	0.9421	0.981
Nucleotide diversity	0.0026	0.0035	0.0028	0.0221	0.0245	0.0025	0.0033	0.0037
Tajima's <i>D</i>	-0.18 ^{NS}	-0.28 ^{NS}	-1.80*	1.90 ^{NS}	1.85 ^{NS}	-2.01**	-1.57*	-1.58*
Fu's <i>F</i>	-4.02*	-7.16**	-25.82**	2.64 ^{NS}	-1.70 ^{NS}	-13.42**	-6.97**	-6.21**
<i>P</i> (Simulated SSD ≥ Observed SSD)†	0.24	0.88	0.82	0.40	0.03	0.81	0.808	0.02

†*P* (Simulated SSD ≥ Observed SSD), the probability when simulated sum of squared deviation (SSD) of mismatched distribution larger than observed SSD. **P* < 0.05; ***P* < 0.01; NS, not significant.

sampling 500 trees and one long chain sampling 15 000 trees; the sampling interval for each was 20. Multiple simultaneous searches with heating were carried with 4 chains to improve the mixing. Three independent runs (with different seeds) were performed to check the consistency in the results.

Defining groups of populations is often based on a priori assumptions, such as presumed isolation between river drainages. Because mitten crabs are catadromous, migration is possible between populations from adjacent rivers. Therefore, methods to identify genetic barriers based on the data, such as spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.* 2002) should provide a more objective solution to group populations than simply defining the group using drainage. This approach is based on a simulated annealing procedure that aims at maximising the proportion of total genetic variance due to differences between groups of populations (Dupanloup *et al.* 2002). We ran SAMOVA with the *K* value (number of populations) from 2 to 7 and selected the best *K* that resulted in the greatest variation among groups (*F*_{CT}).

Results

Sequence alignment and phylogenetic relationships among populations

Sequence alignment resulted in 693 bp from the COII and 762 bp from *cyt b*, and no indels were created in the alignment. The concatenated sequences resulted in a total of 1455 bp, of which 193 bp were variable and 120 bp were parsimony informative. From the 169 individuals, individuals with identical haplotypes were detected but no haplotypes are shared between the north (LH, HH and CJ) and south China populations (ZJ and NLJ). There were 123 unique haplotypes after the redundant ones were collapsed.

Partitioned phylogenetic analyses were carried out on the 123 haplotypes with the outgroup (*Geothelphusa dehaani*) using MRBAYES. The data were partitioned as (1st + 2nd) codon positions and 3rd codon position. This partitioning

scheme was preferred over no partitioning or partitioning by both codon and gene, as suggested by BIC values (results not shown). The consensus tree from the posterior tree distribution has three major groups (Fig. 2). The individuals from the northern populations (LH, HH and CJ) were grouped together as *Eriocheir sinensis*, while the southern ZJ (*Eriocheir japonica*) and NLJ (*Eriocheir hepuensis*) populations were grouped together. The middle populations, OJ and MJ, were split into two distinctive clades, either with the northern or southern populations. *E. japonica* from Japan formed a monophyletic group. Contrary to the traditional view, *E. japonica* from Japan was found to be more closely related to the northern populations of Chinese mitten crab than to the southern populations, suggesting that '*E. japonica*' in south China should not be considered as true *E. japonica*. Because the outgroup species *G. dehaani* is too divergent from the mitten crabs, there could be a high risk of obtaining the incorrect root. Thus, we also used the molecular clock method to root the tree without outgroup. The likelihood-ratio test could not reject a molecular clock in this group (*P* = 0.97), and the molecular clock method also place the root between the northern and southern populations of mitten crab in China (figure not shown). Three groups were also identified in the statistical parsimony network, with 47 steps of mutations between the Japan and the northern China populations and 57 steps between the northern and the southern China populations. Within each subnetwork, there was no clear structure (figure not shown).

Genetic variation within and among populations and divergence time and gene flow

The gene diversities ranged from 0.8789 in LH to 0.9905 in MJ (Table 2). The nucleotide diversities are similar in the Japan (SH), the north China (LH, HH, CJ) and the south China (ZJ and NLJ) populations, but the middle populations (OJ and MJ) have 10-fold higher nucleotide diversity than the other populations (Table 2). Negative value obtained for Tajima's *D* and Fu's *F* suggest population expansion or selection for all populations except for OJ and MJ.

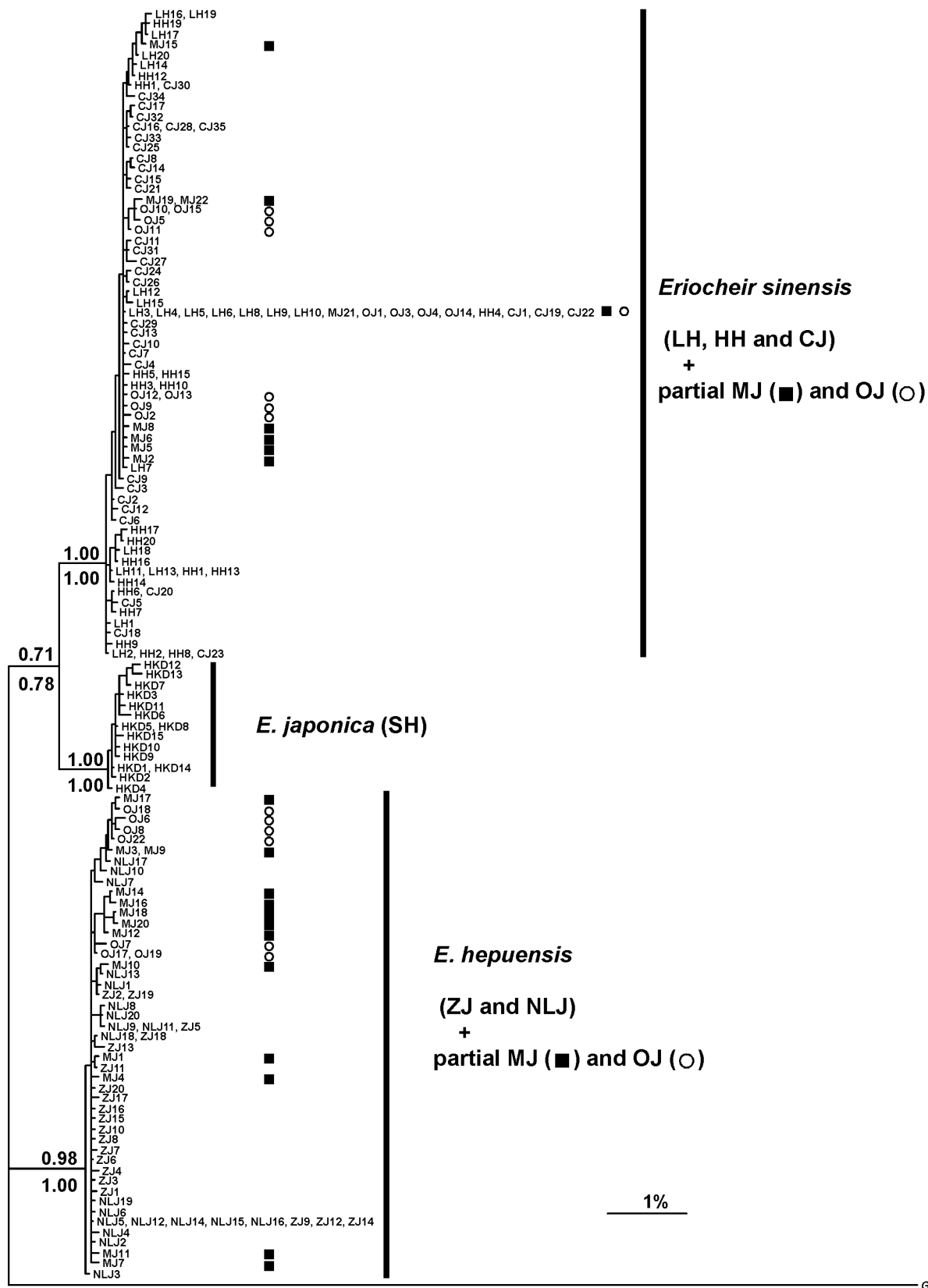


Fig. 2 Phylogram of mitten crabs, *Eriocheir sensu stricto* based on partitioned Bayesian analysis of COII and cyt *b* sequences. Numbers above the branches are posterior probabilities of Bayesian run without assuming the molecular clock was enforced.

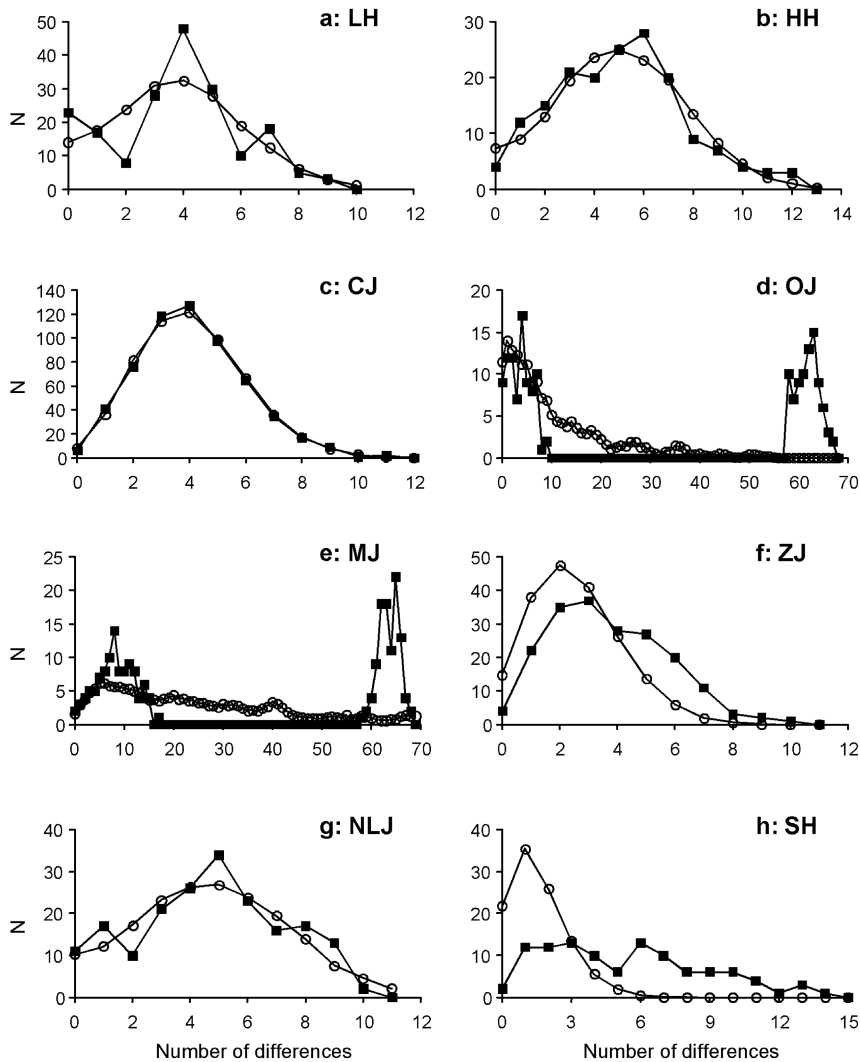


Fig. 3 Mismatch distributions of mitten crab haplotypes in each river drainages. Circles are simulated values and squares are observed values. Specific statistics are listed in Table 2.

Mismatch distribution tests could not reject the null hypothesis of sudden population expansion for all river drainages except for MJ and SH ($P < 0.05$). There are two distinct peaks in the observed distance distributions of OJ and MJ, whereas the distribution of SH is relatively flat and the distribution of the rest of the populations has one prominent peak (Fig. 3).

Both pairwise F_{ST} and D_A suggest that all populations are significantly different from each other, with the exceptions between HH and LH, OJ and MJ, and ZJ and NLJ (Table 3). The largest genetic distance is between SH and ZJ (4.086), while the smallest distance is between ZJ and NLJ (0.000).

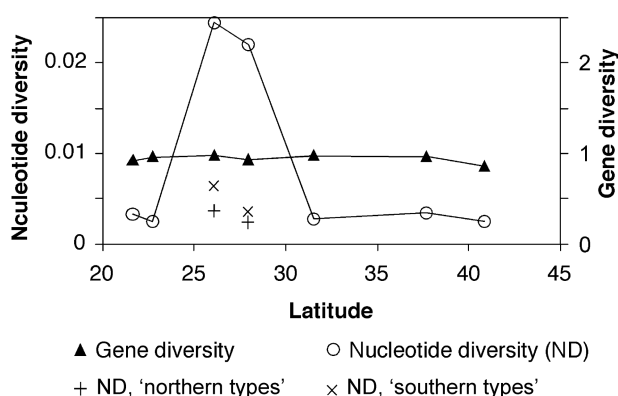
The averaged net pairwise distance (D_A) is 4.053% between the southern China and the Japan populations, 3.933% between the southern China and the northern China populations, and 3.214% between the northern China and the Japan populations. If we use 2.31 Ma as the calibration point between the southern China and the Japan

populations (Xu 2005), the divergence time between the southern China and the northern China populations should be 2.24 Ma, while the divergence time between the northern China and the Japan populations is 1.83 Ma. We also estimated the divergence time between the northern population, the southern population and the middle population in China using the isolation-with-migration model (Hey & Nielsen 2007). The estimated divergence time is 0.16 Ma (95%CI = 0.09–0.28) between southern population and the 'southern' haplotypes in the middle population, while the divergence time between northern population and the 'northern' haplotypes in the middle population is 0.12 Ma (95%CI = 0.07–0.17). The estimated migration rate is 7.6×10^{-7} per year from the southern population to the middle population and 7.4×10^{-8} vice versa. The estimated migration rate is 1.3×10^{-9} from the northern population to the middle population and 1×10^{-8} vice versa.

Table 3 Genetic differences between populations. Above diagonal: pairwise F_{ST} between populations; below diagonal: net average p-distance [$P_{XY} - (P_{IX} + P_{IY})/2$] between populations

	SH	LH	HH	CJ	OJ	MJ	ZJ	NLJ
SH		0.9140**	0.9007**	0.9130**	0.6418**	0.6319**	0.9312**	0.9213**
LH	3.239**		0.0551 ^{NS}	0.0845**	0.3010**	0.5084**	0.9395**	0.9313**
HH	3.232**	0.017 ^{NS}		0.1310**	0.2972**	0.4968**	0.9295**	0.9213**
CJ	3.212**	0.025**	0.045**		0.3573**	0.5761**	0.9360**	0.9300**
OJ	2.533**	0.518**	0.541**	0.504**		0.0684 ^{NS}	0.5702**	0.5594**
MJ	2.753**	1.431**	1.440**	1.452**	0.172 ^{NS}		0.3051**	0.2957**
ZJ	4.086**	3.963**	3.932**	3.955**	1.598**	0.608**		0.0006 ^{NS}
NLJ	4.019**	3.946**	3.923**	3.936**	1.576**	0.596**	0.000 ^{NS}	

* $P < 0.05$; ** $P < 0.01$; ^{NS}, not significant.

**Fig. 4** Plots of nucleotide diversity and gene diversity along the latitudinal space.

The migration parameter M ($M = m/\mu$) estimated using LAMARC is extremely low among the northern, the southern and the Japan groups, ranging from 9.5 to 23, which is from 0.04 to 0.08 immigrants per generation ($2Nm$). Comparing to the migration parameters M estimated within each group (400 to 900, analyses not shown), the gene flows between the three groups are negligible, suggesting that these three groups are highly isolated.

Spatial patterns and latitudinal genetic variation

Combining the eight rivers into five groups maximised the variation among groups (F_{CT}) suggested by the SAMOVA (Table 4). The five groups proposed by SAMOVA are: Japan (SH), northern China (LH + HH + CJ), OJ, MJ, and southern China (ZJ + NLJ). Analysis of molecular variance using the optimal five grouping scheme indicates most of the variance exists among groups (75.12%), while very little variation is contributed by among populations with groups (0.01%; Table 4).

We examined the genetic variation of mitten crabs in the seven rivers in China in the context of latitudinal space. The gene diversity approximates a flat line along different latitudes; the nucleotide diversity, however, is 10 times higher in MJ and OJ than all other populations (Table 2; Fig. 4). When the nucleotide diversity of the middle populations were calculated separately for the 'northern' and the 'southern' haplotypes, lower nucleotide diversity were seen in the middle populations in the same range as the northern or southern populations (Fig. 4). The NLJ and CJ have the highest nucleotide diversity in the southern and northern populations, respectively.

Discussion

Species delineation and phylogeny

Phylogenetic analysis identified three monophyletic groups: the Japan population, the northern China populations with some individuals from middle China, and the southern China populations with some individuals from the middle China populations (Fig. 2). The statistical parsimony network showed substantial genetic distances among the three groups, with 47 steps of mutations between the Japan and the northern China populations and 57 steps between the northern China and the southern China populations (figure not shown). Five groups, the Japan, the southern and northern China in addition to the two middle populations, were identified as the best scheme in the SAMOVA analyses. In addition, the LAMARC analyses suggested that there is negligible gene flow among the Japan, the southern China, and the northern China groups. Based on these results, we suggest that three distinct species should be recognised. The northern China populations are *Eriocheir sinensis*, including LH, HH and CJ in this study. The southern China populations are *Eriocheir hepensis*, including NLJ and ZJ populations, and contain no *Eriocheir japonica*. The populations in middle China are of mixed haplotypes

	Group composition	$F_{SC}^{\dagger}$	$F_{ST}^{\ddagger}$	$F_{CT}^{\S}$
Two groups	1. NLJ + ZJ 2. MJ + OJ + CJ + HH + LH + SH	0.568**	0.806**	0.551*
Three groups	1. NLJ + ZJ + MJ 2. OJ + CJ + HH + LH 3. SH	0.286**	0.785**	0.699**
Four groups	1. NLJ + ZJ 2. MJ + OJ 3. CJ + HH + LH 4. SH	0.068**	0.757**	0.740**
Five groups	1. NLJ + ZJ 2. MJ 3. OJ 4. CJ + HH + LH 5. SH	0.0002**	0.751**	0.751**
Six groups	1. NLJ + ZJ 2. MJ + OJ 3. CJ 4. HH 5. LH 6. SH	0.126**	0.727**	0.687 ^{NS}
Seven groups	1. NLJ 2. ZJ 3. MJ 4. OJ 5. CJ 6. HH + LH 7. SH	-0.009**	0.720**	0.723 ^{NS}

$\dagger F_{SC}$, proportion of total genetic variance due to differences between populations within each

group. $\ddagger F_{ST}$, proportion of total genetic variance due to differences between populations.

$\S F_{CT}$, proportion of total genetic variance due to the differences between groups.

* $P < 0.05$; ** $P < 0.01$; NS, not significant.

Table 4 Fixation indices corresponding to groups of populations inferred by SAMOVA using mitochondrial COII and cyt *b* sequences. The best number of groups (five) has the largest F_{CT} value

from the north and the south populations; hence, they should not be considered a distinct species. One caveat of this study is that only mitochondrial loci were used. More nuclear loci (e.g. microsatellites or amplified fragment length polymorphisms) should be used in future studies to scrutinise the species composition more definitively in the middle populations. Finally, populations found in the main islands of Japan are *E. japonica*, while the population in ZJ should not be considered as *E. japonica* since the genetic distance between them is actually the largest among all other pairs in this study. Li *et al.* (1993) concluded that *E. sinensis*, *E. japonica* and *E. hepuensis* should be classified as one species base on morphometric and allozyme data, while Chan *et al.* (1995) suggested that *E. hepuensis* might be a southern population of *E. sinensis* or a hybrid population of *E. sinensis* and *E. japonica*. Our results clearly illustrated that *E. hepuensis* is distinct from both *E. sinensis* and *E. japonica*. Low resolution in the genetic markers used might lead to the former conclusion of no genetic difference in these three species suggested by Li *et al.* (1993). Tang *et al.* (2003) and Sun *et al.* (2005a) proposed the recognition

of three subspecies – *E. japonica sinensis*, *E. japonica japonica* and *E. japonica hepuensis* – since they formed a monophyletic clade based on cytochrome oxidase I sequences and 16S rDNA RFLP, respectively. Their results are in fact consistent with ours. Instead of recognising three species, they proposed three subspecies under *E. japonica*. Their reluctance to recognise the taxa at species level may be due to the small number of samples they used and the low genetic distances they found among the three taxa. Our results, however, were based on a more thorough sampling effort (at least 15 individuals for each site) and clearly illustrated distinctiveness of the three species. In addition, other evidence supporting three diverged groups lies in our population coalescent analyses (i.e. essentially no gene flow was found between the three taxa); therefore, we argue that these species designation for the three taxa would be the most appropriate.

Guo *et al.* (1997) also suggested three distinct species based on thorough examination of morphological characters of adults, but their treatment of samples collected from southern China was different from our study. In their

study, along with many others (e.g. Dai 1991; Tang *et al.* 2003; Xu 2005), samples collected from Zhujiang River drainage (particularly from Aotou and Hong Kong) were termed as *E. japonica*, while our results indicate that the individuals from ZJ are closely related to *E. hepuensis* from NLJ. Although we collected samples from Shunde (Table 1) instead of Aotou or Hong Kong to represent the ZJ population, it has been shown that samples from those places are not different from each other in mitochondrial sequences (Tang *et al.* 2003; Xu 2005). The species identity of ZJ populations will lead to different conclusions for the phylogenetic relationships among the three species. If the ZJ populations belong to *E. japonica*, *E. hepuensis* would be more closely related to *E. japonica* than to *E. sinensis*, and actually not genetically distinct from one another, which supports the hypothesis that *E. hepuensis* is a subspecies of *E. japonica* (Dai 1991). In addition, this classification imply paraphyly of *E. japonica* in our results (Fig. 2) and others (Xu 2005), because our results suggest that *E. japonica* from Japan is more closely related to the northern China populations than to the southern China populations (Fig. 2). To rule out possible rooting errors from using a highly divergent outgroup, we also rooted the tree using the molecular clock method (Huelsenbeck *et al.* 2002). This method supported the same root. Based on these results, we propose that all populations in southern China are *E. hepuensis* and they form the basal group to *E. sinensis* and *E. japonica*. More thorough sampling in southern China should be done in the future to test if *E. japonica* exists in that region.

Clinal variation

Species with wide latitudinal distributions often exhibit clinal patterns in genetic variation, usually attributed to adaptive selection under the climate gradients (e.g. Eanes 1999; Baines *et al.* 2004; Grant *et al.* 2006). Alternative neutral processes such as restricted gene flow (Vasemägi 2006), secondary intergradation (e.g. González-Vilaseñor & Powers 1990; Turgeon & Bernatchez 2001), postglacial dispersal and founder effects (Hewitt 1996) can also result in similar patterns of genetic variation among populations.

Clinal variation has been frequently documented in many vertebrates (González-Vilaseñor & Powers 1990; Turgeon & Bernatchez 2001; Grant *et al.* 2006) and invertebrates species (Eanes 1999), including the blue crab, *Callinectes sapidus* (McMillen-Jackson & Bert 2004). Clinal variation in the mitten crabs along the coast of China has already been observed in many respects. Ovarian development of mitten crabs was found to be correlated with latitudes, with the higher latitude population developing earlier than their southern counterparts (Gu & He 1997). In a study of four populations from different latitudes that were stocked into similar lakes at the middle reaches of

Changjiang River, a strong correlation ($r = -0.96$) was detected between the time of peak catch and the latitude of the population origin (Jin & Li 2000). This reflected the clinal variation of the breeding season of different populations, because the peak catch with trammel nets coincided with the onset of breeding migration.

There are many potential causes that might produce the clinal structure. First, the clines could be a result of postglacial dispersal from the southern population to the north followed by restricted gene flow (Xu 2005). The signature of a postglacial expansion from refuge populations in the Northern Hemisphere is typically characterised by high diversity in the southern populations and low variation in the north (Hewitt 1996). In a comparative case, McMillen-Jackson & Bert (2004) detected a significant decline in haplotype diversity with increasing latitude for the blue crab along Atlantic Ocean coastal waters of North America. They proposed population range expansion northward as a result of Pleistocene-era climatic fluctuations. Similarly, Xu (2005) postulated northward range expansion of mitten crabs through the Taiwan Strait to explain the genetic variation among populations. However, we did not detect a higher diversity in the southern populations relative to those in the north (Fig. 4). This contradicts the postglacial dispersal hypothesis and indicates that mitten crabs might have survived the Pleistocene fluctuations in their current area of distribution in northern China. Figure 4 also shows a spike in nucleotide diversity in the middle populations (OJ and MJ), but no large difference was observed for gene diversity in those same populations. The high nucleotide diversity is an artefact of their heterogeneous haplotypes composition (i.e. they include haplotypes from both northern and southern populations). On the contrary, gene diversity, which is usually very high for mitochondrial haplotypic data, is 0.88–0.99 in this study; thus, it is insensitive to additional aforementioned heterogeneity in the haplotypes of the middle populations. When the nucleotide diversities were calculated for the 'northern-type' and the 'southern-type' haplotypes separately in the middle populations, the values of nucleotide diversity in the middle population are in the same range as that of the northern and the southern population.

The clinal variation could also be a consequence of adaptive selection, from which a high correlation between the latitudinal change and genetic change should be observed. Although the effect of this type of selection is often difficult to be distinguished from isolation by distance alone (Vasemägi 2006), no correlation between the geographical distance and genetic distance could reject both scenarios. Instead of seeing a gradual genetic change along the latitudes, we found an abrupt change in the middle China, which speaks for itself that a secondary intergradation could be a more reasonable explanation for the clinal pattern seen in the mitten crab. We also caution that selection could

play important role in producing difference among the populations within the north and the south group and maintaining the large genetic difference between the north and the south group. More independent nuclear loci and some neutral markers should be used to investigate the role of selection in shaping the genetic structure of mitten crab. Other potential factors, such as translocation by aquaculture activities, should also be tested in future studies.

Historical biogeography

The effects of Pliocene and Pleistocene climate oscillations on population structure in European and North American flora and fauna have been extensively documented (reviewed by Hewitt 2000) while only a few studies have focused on the East Asia region, especially the Pacific coast of China (e.g. Wang *et al.* 1999; Lin *et al.* 2002; Tzeng *et al.* 2004; Liu *et al.* 2006; Long *et al.* 2006; Shih *et al.* 2007). An enclosed sea in the current East China Sea area (Kimura 1996) and a semiclosed Japan Sea (Tada 1994; Kitamura *et al.* 2001) in the middle Pliocene (3.5 Ma) were proposed as the vicariant forces that yielded the deep divergence in two closely related fish species (Liu *et al.* 2006). The estimated divergence time between *E. sinensis* and *E. hepuensis* is 2.24 Ma and the divergence time between *E. sinensis* and *E. japonica* is 1.83 Ma, both within Pliocene (5.2 to 1.64 Ma). Thus, the sea level changes in the late Pliocene might be the vicariant events that led to the divergence of the three species.

During the last glacial maximum (LGM), the sea level was 120–140 m lower than the current level (Lambeck *et al.* 2002). The Tsushima and Taiwan Straits, the entire Bohai Gulf and the Yellow Sea were exposed (Fig. 1), and the East China Sea was reduced into an elongated trough (Boggs *et al.* 1979; Wang & Sun 1994). The exposed land might have served as bridges for dispersal of inland organisms to islands (e.g. Wang *et al.* 1999; Long *et al.* 2006; Shih *et al.* 2007). Likewise, those land bridges could have acted as barriers for marine and estuarial species, blocking the gene flow between the Japan Sea, the East China Sea and the South China Sea (e.g. Tzeng *et al.* 2004). The dispersal and secondary contact of the northern and southern populations in the middle China region should be recent; that is, after *E. sinensis* and *E. hepuensis* were established in the northern and southern China. A coalescent technique using the isolation-with-migration model obtained a divergence time of 0.16 (0.09–0.28) Ma between the southern population and the southern haplotypes in the middle population, and 0.12 (0.07–0.17) Ma between the northern population and the northern haplotypes in the middle population. This time frame indicates that the onset of secondary contact event probably occurred in the late Pleistocene (1.64 to 0.01 Ma), but earlier than the LGM (about 20 000 years ago).

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The author research interests include population genetics and evolution of aquatic animals, especially fish and crab. Meanwhile, selection and breeding of fish and crab are also the research interests of the authors.
