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Full Length Research Paper

Genetic population structure of the Japanese mitten crab *Eriocheir japonica* in Japanese islands

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Fragment of 376 bp at the mitochondrial ND2 gene was sequenced for 133 individuals of Japanese mitten crab, *Eriocheir japonica* from 17 localities of Japan and 30 individuals of Chinese mitten crab, *E. sinensis* from 2 localities of China. In Japanese mitten crab, sequence comparison of this segment revealed 23 polymporphic sites, defining 8 haplotypes. However, 30 individuals of Chinese mitten crab from Dandong and Shanghai shared one haplotype. Haplotype diversity and nucleotide diversity, for the17 populations of Japanese mitten crab ranged from 0 to 0.5833 and from 0 to 0.0025, respectively. AMOVA and pairwise F_{ST} revealed significant genetic differentiation between the Okinawa Island and main islands of Japan, and lack of genetic structure among main islands of the Japan populations. Compared with the genetic distance among populations of two species, the degree of genetic divergence between these two groups (Okinawa and main islands of Japan) is equivalent to the genetic distance between congeneric species. Our results failed to demonstrate significant geographical structure in main islands of Japan, indicating that populations of Japanese mitten crab are capable of extensive gene flow among estuaries along the coastal waters.

Key words: Japanese mitten crab*, Eriocheir japonica*, *Eriocheir sinensis,* mitochondiral ND2, genetic structure.

INTRODUCTION

The Japanese mitten crab, *Eriocheir japonicus* (de Haan, 1835), which belongs to the family Grapsidae, is a subtropical species widespread in the rivers and estuary complexes in the Northeastern Pacific Coast from the Japanese Islands to the Sakhalin Island, including the Maritime Territory (Kalinina et al., 2008). In the Japanese archipelago, it can be found from Hokkaido in the north to Okinawa and Ogasawara Islands in the south (Yamasaki et al., 2006). The Japanese mitten crab is a catadromous species that lives most of its life in fresh water, but migrates downstream to reproduce and spawn in higher salinity waters. There are five zoeal stages and one megalopal stage, and the juveniles migrate upstream and grow to the maximum carapace width of 10 cm (Kobayashi and Matsuura, 1995). This crab is one of the most important crustaceans harvested for food from rivers and coastal areas in Japan (Kobayashi et al., 1997). Because of this economic importance and the need for conservation of natural populations, there have been many reports on the general ecology of this species covering reproduction, ethology and growth (reviewed in Yamasaki et al., 2006).

However, few studies have focused on the population genetic structures of this species (Gao and Watanabe, 1998; Yamasaki et al., 2006). Horizontal starch gel electrophoresis has been used to investigate the genetic stock structure of the Japanese mitten crab (Gao and Watanabe, 1998). Among 22 sample sites from Hokkaido to Okinawa in Japan, two alleles at *GPI** locus served as

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the diagnostic character of the Okinawa populations. Okinawa is geographically isolated from the main islands of Japan. Yamasaki et al. (2006) used RFLP analysis of COI gene to detect the amount of genetic variation in this crab among 19 sample sites. Three main groups among Japanese mitten crab populations in Japan were identified by this study, namely; the main islands, Okinawa and Ogasawara groups. Like allozyme data, Okinawa populations showed population specific dominant haplotypes.

Adults of Japanese mitten crab live in burrows in rivers, and the genetic exchange between neighboring river populations is done primarily through their larval forms in coastal waters, which are planktonic. There are two main demographic strategies for benthic estuarine macroinvertebrates that have a dispersive planktonic larval phase (Bilton et al., 2002): (1) retention of larval stages within the estuary; and (2) export of newly hatched stages from the estuary into shelf waters, with subsequent return migration to the same or other estuaries by a late larval or early post-larval stage. Both strategies have important consequences within and between estuaries or as the homogenization of genetic variation due to extensive gene flow, respectively (Oliverira et al., 2007).

In order to warrant adequate management of natural populations, it is imperative to understand in detail the biological characteristics of the managed species. In particular, it is important to understand the geographical distribution of the genetic variability among its subpopulations. However, the potential power of allozyme and RFLP markers in revealing genetic variation is relatively low compared to more recently developed markers and techniques, such as direct sequencing of mtDNA and SSR (Liu et al., 2004). The amount and pattern of polymorphism in mtDNA sequences is a robust tool for population genetic analysis, and has proved effective to identify reproductive isolation among populations and for bringing an evolutionary perspective to conservation, management and speciation (King et al., 2001). Analyses of mtDNA markers have been used extensively to investigate stock structure in a variety of aquatic animals reviewed in Liu and Cordes (2004). In this study, we analyzed the sequences of mtDNA NADH dehydrogenase 2 (ND2) gene in 17 populations of *E. japonicus* collected from Japan to detect the population genetic structure and estimate the larval dispersal ability of this species over different geographic scales.

MATERIALS AND METHODS

Sampling and sequencing

One hundred and thirty three individuals of *E. japonicus* were collected from 17 rivers or lakes from main islands of Japan and two rivers from Okinawa Island (Henoko River and Genka River) from October 1992 to October 1996 (Table 1). Thirty specimens of *E. sinensis* collected from Dandong and Shanghai served as out groups (Table 1). Muscle samples were preserved or frozen in 70 - 90% ethanol before DNA extraction.

Genomic DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method. Mitochondrial ND2 gene was amplified with primers ND2-F and ND2-R. The primer sequences are ND2-F: 5'-TCTGATCAAAATAGAACACT -3' (forward) and ND2-R: 5'-AAGCTTTGAAGGCTTTTAG -3' (reverse) (Liu et al., 2005).

Each PCR reaction was performed in a volume of 50 µL containing 20-50 ng template DNA, 5 µL of 10 × reaction buffer, 5 µLof $MgCl_2$.(25 mM), 1 µL of dNTPs (10 mM), 10 pM of each primer, and 2.5 units of *Taq* DNA polymerase (Promega) in an Eppendorf Mastercycler 5333. Initial denaturation was for 3 min at 94°C, followed 40 cycles off 45 s at 94°C for denaturation, 45 s at 50°C for annealing, and 45 s at 72°C for extension; and a final extension at 72°C for 10 min. All sets of PCR included a negative control reaction tube in which all reagents were included, except template DNA. PCR product was separated on a 1.5% agarose gel and purified with the Gel Extraction Mini Kit (Watson BioTechnologies Inc., Shanghai). The purified product was used as the template DNA for cycle sequencing reactions performed using BigDye Terminator Cycle Sequencing Kit (ver. 2.0, Applied Biosystems, Foster City, California), and sequencing was conducted on an ABI Prism 3730 (Applied Biosystems) automatic sequencer with both forward and reverse primers. The primers used for sequencing were the same as those for PCR amplification.

Data analyses

Sequences were edited and aligned using Dnastar software (DNASTAR, Inc., Madison, USA). Molecular diversity indices such as number of haplotypes, polymorphic sites, transitions, transversions, and indels were obtained using the program ARLEQUIN (Ver. 2.0) (Schneider, 2000). Haplotype diversity (h), nucleotide diversity (π) and their corresponding variances were calculated after Nei (1987) as implemented in ARLEQUIN.

Genetic relationships among haplotypes were reconstructed using the neighbour-joining method (Saitou and Nei, 1987) implemented in MEGA 2.0. Genetic distances were generated for phylogenetic reconstruction using Kimura's two-parameter substitution model. We used bootstrap analysis with 1000 replicates to evaluate support for phylogenetic relationships.

Population structure was measured with an analysis of molecular variance (AMOVA) incorporating sequence divergence between haplotypes. The significance of the covariance components was tested using 1000 permutations. In addition, pair wise genetic divergences between sample sites were estimated using the fixation index F_{ST}, which included information on mitochondrial haplotype frequency and genetic distances. The significance of the F_{ST} was tested by 1000 permutations for each pairwise comparison.

RESULTS

A PCR fragment of 376 bp from the mitochondrial ND2 gene were sequenced and obtained for 163 individuals of two species from 19 populations. In Japanese mitten crab, sequence comparison of this segment revealed 23 polymporphic sites with 21 transitions and 2 transversions. These polymorphic sites defined 8 haplotypes, giving an overall haplotype diversity of 0.4319±0.0457 and nucleotide diversity of 0.0174±0.0092. The most common haplotype was shared by 76.18% of individuals. In the Chinese mitten crab, 30 individuals from Shanghai and Dandong shared one haplotype (Table 2), and no

Table 1. Sampling data of *Eriocheir japonica* and *E. sinensis* including sample size, data of collection and several diversity indexes.

Species	Sample	Date of collection	Sample size	Number of haplotypes	Haplotype diversity	Nucleotide diversity
Eriocheir japonica	Ishikari River (Is)	1996.7	12	3	0.3182±0.1637	0.0012±0.0003
	Jusan Lake (Ju)	1996.5	4	1	0.000	0.000
	Miomote River (Mi)	1996.5	6	2	0.3333±0.2152	0.0001±0.0001
	Oyabe River (Oy)	1996.2	5	1	0.0000	0.0000
	Takeno River (Te)	1994.11	9	2	0.2222±0.1662	0.0012±0.0013
	Takatsu River (Ta)	1994.11	6	1	0.000	0.000
	Kamo River (Ka)	1996.9	8	2	0.2500±0.1802	0.0013±0.0014
	Shisa River (Sh)	1996.9	9	4	0.5833±0.1833	0.0025±0.0021
	Izaku River (Iz)	1995.10	8	1	0.000	0.000
	Ogawara Lake (Og)	1996.5	6	1	0.000	0.000
	Kesen River (Ke)	1996.10	5	1	0.000	0.000
	Hinuma Lake (Hi)	1995.5	8	1	0.000	0.000
	Banda River (Ba)	1996.6	7	1	0.000	0.000
	Hamana Lake (Ha)	1996.4	7	1	0.000	0.000
	Koza River (Ko)	1996.9	5	1	0.000	0.000
	Henoko River (He)	1996.10	13	2	0.1538±0.1261	0.0004±0.0006
	Genka River (Ge)	1996.9	15	2	0.1333±0.1123	0.0003±0.0002
E. sinensis	Dandong (Da)	1999.7	15	1	0.000	0.000
	Shanghai (Sg)	1996.11	15	1	0.000	0.000

Table 2. Distribution of haplotypes among localities.

Location	H1	H2	H3	H4	H5	H6	H7	H8	H9
Is	10					1	1		
Ju	4								
Mi	5						1		
Oy	5								
Te	8					1			
Ta	6								
Ka	7							1	
Sh	6					1	1		1
Iz	8								
Og	6								
Ke	5								
Hi	8								
Ba	7								
Ha	7								
Ko	5								
He	0		12		1				
Ge	0		14	1					
Da	0	15							
Sg	0	15							
Total	97	30	26	1	1	3	3	1	1

polymorphic site was detected. The haplotype diversity (*h*) and nucleotide diversity were low for all populations of Japanese mitten crab, with values ranging from 0 to 0.5833±0.1833 and 0 to 0.0025±0.0021 (Table 1).

The NJ tree constructed with163 individuals of the two species revealed two highly divergent clades in Japanese mitten crab (Clades A and B) (Figure 1). The Clades A and B included 7 and 2 haplotypes comprising 105 and 28 individuals, respectively. These two clades of Japanese mitten crab have significant geographic structure, showing strong correspondence with the geographic regions. The clade A included 15 populations in the main islands of Japan, whereas the clade B comprised two sites in Okinawa. However, the topology of the neighbor-joining tree of clade A was shallow, and there were no significant genealogical branches or clusters of samples corresponding to sampling locality. The minimum spanning tree also identified two clades, corresponding to those defined in the NJ tree (Figure 2).

Significant separation of the two clades in Japanese mitten crab was also supported by AMOVA, with 98.91% of all variance being partitioned between the two clades (F_{CT}=0.9891, P=0.004). The genetic structures of the populations within clade A and B were also investigated by AMOVA. In clade A, the genetic variation found among populations was negative (F_{ST}=-0.0091, P=0.52), indicating no significant population structure in the main islands of Japan. AMOVA analyses in the clade B (Okinwawa group) indicated that the genetic variation between popu-

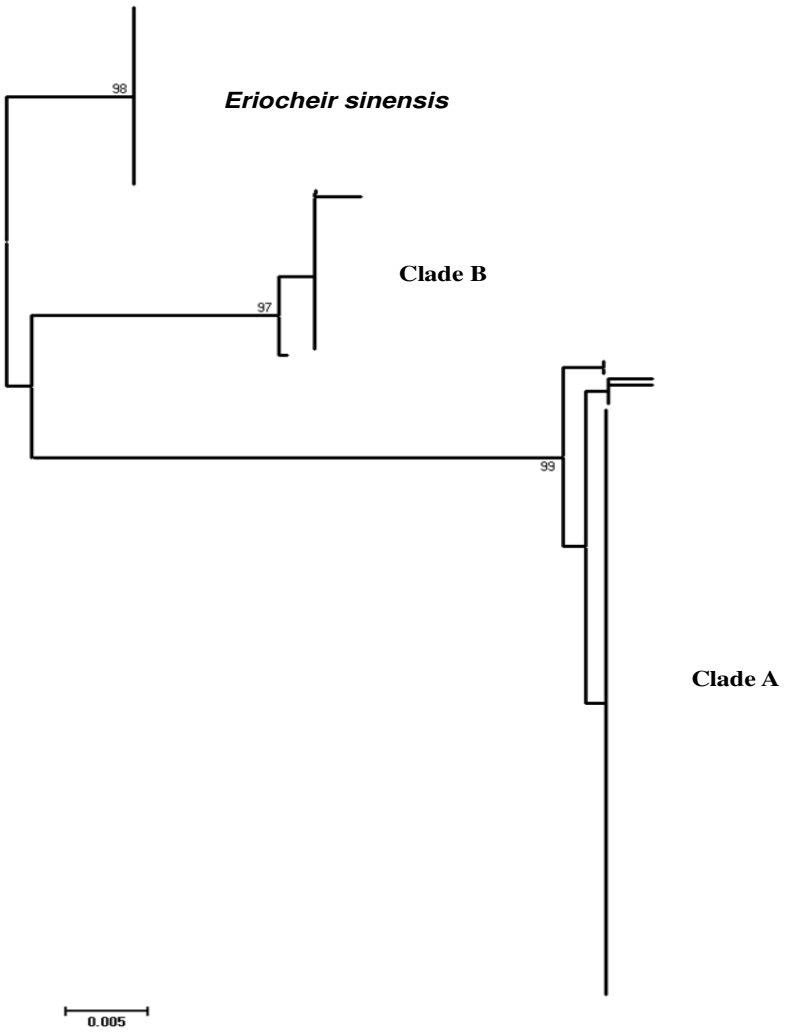

Figure 1. NJ tree for ND2 sequence data of two species based on K-2P substitution model. Bootstrap values >70% are showed at nodes, 1000 replicates.

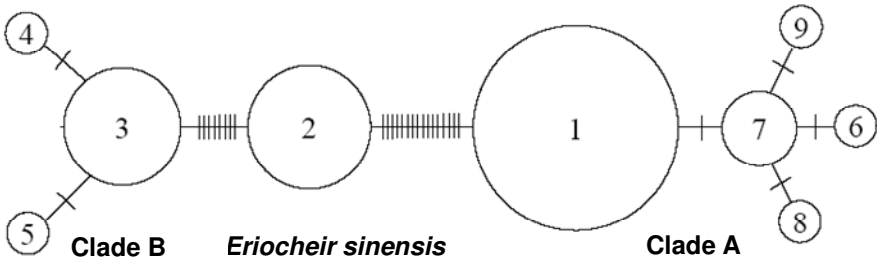

Figure 2. Reduced median-network showing genetic relationship in two speices. The sizes of circles are proportional to haplotype frequency. Perpendicular tick marks on the lines joining haplotypes represent the number of nucleotide substitutions. The number in each circle represents the haplotype name.

lations was 0.10% (P=0.71), indicating little genetic differentiation existed in the two populations of Okinawa. Pairwise F_{ST} values among populations of the two clades of Japanese mitten crab were high and significant, sup-

Table 3. Pairwise F_{ST} values among populations of mitten crab.

	Is	Ju	Mi	Oy	Te	Ta	Ka	Sh	Iz	Og	Ke	Hi	Ba	Ha	Ko	He	Ge	Da
Ju	-0.0782																	
Mi	-0.1220	-0.0811																
Oy	-0.0426	0.0000	-0.345															
Te	-0.0975	-0.1163	-0.1119	-0.0778														
Ta	-0.0177	0.0000	-0.0000	0.0000	-0.0511													
Ka	-0.0567	-0.1089	-0.1181	-0.0687	-0.0618	-0.0403												
Sh	-0.0375	0.0170	-0.0694	0.0568	-0.0228	0.0867	-0.0045											
Iz	0.0166	0.0000	0.0514	0.0000	-0.0141	0.0000	0.0000	0.1317										
Og	-0.0177	0.0000	0.0000	0.0000	-0.0511	0.0000	-0.0403	0.0867	0.0000									
Ke	-0.0426	0.0000	-0.0345	0.0000	-0.0778	0.0000	-0.0687	0.0568	0.0000	0.0000								
Hi	0.0166	0.0000	0.0514	0.0000	-0.0141	0.0000	0.0000	0.1317	0.0000	0.0000	0.0000							
Ba	0.0012	0.0000	0.0278	0.0000	-0.0307	0.0000	-0.0182	0.1109	0.0000	0.0000	0.0000	0.0000						
Ha	0.0012	0.0000	0.0278	0.0000	-0.0307	0.0000	-0.0182	0.1109	0.0000	0.0000	0.0000	0.0000	0.0000					
Ko	-0.0426	0.0000	-0.0345	0.0000	-0.0778	0.0000	-0.0687	0.0568	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000				
He	0.9848*	0.9939*	0.9939*	0.9942*	0.9865*	0.9946*	0.9861*	0.9768*	0.9952*	0.9946*	0.9952*	0.9949*	0.9949*	0.9942*	0.0000			
Ge	0.9859*	0.9945*	0.9945*	0.9948*	0.9875*	0.9951*	0.9873*	0.9787*	0.9955*	0.9951*	0.9955*	0.9953*	0.9953*	0.9948*	0.0096	0.0000		
Da	0.9876*	1.0000*	1.0000*	1.0000*	0.9903*	1.0000*	0.9901*	0.9797*	1.0000*	1.0000*	1.0000*	1.0000*	1.0000*	1.0000*	0.9923*	0.9928*	0.0000	
Sg	0.9876*	1.0000*	1.0000*	1.0000*	0.9903*	1.0000*	0.9901*	0.9797*	1.0000*	1.0000*	1.0000*	1.0000*	1.0000*	1.0000*	0.9923*	0.9928*	0.0000	0.0000

*Highly significant values (P<0.01).

porting the significant separation of the two clades (Table 3). Among 15 populations from main islands of Japan, all pair wise F_{ST} values were lower and not significant, indicating no significant genetic structure in this clade. For Okinawa populations, the genetic differentiation between He and Ge was also no significant (Table 3).

DISCUSSION

Two deeply divergent clades were found in Japanese mitten crab reflecting extremely restricted gene flow between the main islands of Japan and Okinawa Island. AMOVA analysis and pair wise F_{ST} values also indicated the complete isolation between Okinawa Island populations and other populations of Japanese mitten crab. This result is consistent with the previous studies based on allozyme and RFLP analysis of COI gene (Gao and Watanabe, 1998; Yamasaki et al., 2006). Previous allozyme analyses of Japanese mitten crab revealed rare alleles in populations from Okinawa, but could not establish clear genetic differences among populations from different geographic areas. RFLP analysis of COI gene of Japanese mitten crab showed that the specimens from the Okinawa populations did not share any haplotype with other populations. Like our study, strong genetic break between the main lands of Japan and Okinawa Island populations also have been reported in fiddler crab *Uca arcuata* (Aoki et al., 2008). In the fiddler crab, the Okinawa Island population is isolated geographically from others, and showed a marked low genetic variability and significant differentiation from other population samples in haplotype composition. Genetic drift under the conditions of small population size and low gene flow from other populations were responsible for the isolation of Okinawa population in the fiddler crab.

However, in this study no significant differentiation was detected among populations collected from the main islands of Japan. The lack of genetic structure in main islands of Japan indicated high gene flow among populations. The results of the present study are consistent with the preponderance of the larval export strategy through

out the evolutionary history of *E. japonica*, as revealed by low degree of differentiation over a wide coastal area of Japan.

Japanese mitten crab has a long time of planktonic larvae stage (about 1-2 months) before settling on the shore (Yamasaki et al., 2006). Many marine organisms have pelagic larvae that can potentially interconnect distant populations through dispersal on ocean currents and generally show low levels of genetic differentiation over large geographical distances (reviewed in Liu et al., 2007). The main islands of Japan are surrounded by the Kuroshio Current, Oyashio Current and Tsushima Current (Oba et al., 2006). These currents might transport the larva to distant populations. Larval dispersal by ocean current has been reported in many marine organisms (Aoki et al., 2008; Suzuki et al., 1993; Hayashi et al., 1992; Inoue and Sekiguchi, 2002). For example, the larval stages of *U. arcuata* are composed of five zoeal stages and one megalopa stage, and the completion of the five zoeal stages requires 18 days. The Kuroshio Current can carry larvae of this species from the south of Taiwan around the coast of Japan. Moreover, there are many reports of tropical prawns, spiny lobster and oval squid around main islands of Japan, which are attributed to transportation by the Kuroshio Current (Suzuki et al., 1993; Hayashi et al., 1992; Inoue and Sekiguchi, 2002). However, the present Kuroshio Current passes offshore on the western side of Okinawa Island, and the Okinawa Island populations might experience little influence from the Kuroshio Current.

Compared with allozyme and RFLP studies, our result based on ND2 gene showed a low level of haplotype diversity (0-0.5833) and nucleotide diversity (0-0.0025) in Japanese mitten crab. Heterozygosity and genetic distances between populations of Japanese mitten crab estimated by allozyme data ranged from 0.010 to 0.039 and from 0 to 0.0013, respectively. The haplotype diversity and nucleotide diversity revealed by RFLP analysis of COI gene were from 0.1210 to 0.8306 and 0.0098 to 0.0384, respectively. Because of the differences in methodology, a direct comparison with these values may be difficult, but this result may demonstrate lower mutation rate of ND2 gene compared to COI gene.

This present study confirms complete isolation between main islands of Japan and Okinawa Island. Considering the genetic distance between Japanese mitten crab and Chinese mitten crab, the levels of interregional divergence between Okinawa and the Main islands appear closer to the levels of genetic distance observed between species. RFLP analysis of COI gene also sup-ported the sequence divergence between Okinawa and main islands of Japan were at the species level (Yamasaki et al., 2006).

The lack of significant genetic differentiation among estuaries in main islands of Japan has important implications for the management of natural populations of *E. japonica*. The considerable gene flow among populations from different estuaries indicates that local populations are demographically interdependent, with substantial exchange of immature forms among adjacent estuaries. Compared with different genetic structures among and within clades of Japanese mitten crab, Japanese mitten crab may choose the life strategy, which exports newly hatched stages from the estuary into shelf waters, with subsequent return migration to the same or other estuaries by a late larval or early post-larval stage. The sharp shelf waters between Okinawa Island and main islands of Japan may limit the offshore dispersal of larval. The ecological study also revealed that Chinese mitten crab had chosen the same strategy (Huang et al., 1999). Considering the conservation of species, it is important to decide management units within species. If a population has significant ecologic and genetic differences, it should be managed as a separate unit. The Okinawa Island population of Japanese mitten crab showed the typical population genetic structure. It must be treated as an evolutionarily significant unit (ESU). A rigorous evaluation of the status of Okinawa populations will need multilocus nuclear DNA, morphological and mark-recapture studies. Based on these findings, conservation efforts are necessary to guarantee the genetic integrity of these distinct groups.

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