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Genetic diversity of the Asian shore crab, *Hemigrapsus sanguineus*, in Korea and Japan inferred from mitochondrial cytochrome *c* oxidase subunit I gene

Moongeun Yoon[a], Sung-Eic Hong[b], Yoon Kwon Nam[a] and Dong Soo Kim[a]*

[a]*Institute of Marine Living Modified Organisms (iMLMO), Pukyong National University, Busan 608-737, Republic of Korea;* [b]*Faculty of Marine Bioscience and Technology, Gangneung-Wonju National University, Gangneung 210-702, Republic of Korea*

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The genetic diversity and population history of the Asian shore crab, *Hemigrapsus sanguineus*, were investigated with a nucleotide sequence analysis of 536 base pairs (bp) of the mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) in 111 samples collected from four populations in Korea and one in Japan. In total, 28 haplotypes were defined by 27 variable nucleotide sites in the *COI* region examined. The observed haplotypes had a shallow haplotype genealogy and no geographical associations. Most of the populations had high haplotype diversity (0.656–0.788) and low nucleotide diversity (0.00165–0.00244), and significant negative values for Fu's F_S, suggesting rapid and recent population growth from an ancestral population and sudden population expansion. The pairwise fixation indices (F_{ST}) estimated with the exact test and the migration rates indicate that substantial gene flow occurs among these populations as a result of sea currents, except between the Yellow Sea coast of Korea (BUA) and the Pacific Ocean coast of Japan (JPA). These two populations (BUA and JPA) showed significant genetic differentiation and low migration rate.

Keywords: Asian shore crab; *Hemigrapsus sanguineus*; cytochrome *c* oxidase subunit I gene; population structure; sea currents

Introduction

The Asian shore crab, *Hemigrapsus sanguineus*, is widely distributed in the western Pacific ranging in latitude from 20° to 50°N, including Hong Kong and Taiwan, in Korean, Chinese, and Japanese coastal waters, and as far north as Sakhalin Island in Russia (Sakai 1976; Fukui 1988; Dai and Yang 1991; Hwang et al. 1993). The species has now been introduced to the temperate coast of the western North Atlantic and the eastern coast of North America (McDermott 1998; Breton et al. 2002). The crabs have rapidly extended their distributional ranges along the coastlines of the introduced areas. In Japan, the breeding season of *H. sanguineus* is from March to October, with the main peak from May to June (Fukui 1988). This species has a long planktonic larval stage of more than a month, before it develops into the juvenile crab (Fukui 1988). Its pattern of larval dispersal and preferred habitats might have caused geographically distinct regional populations to have become homogeneous.

Hemigrapsus sanguineus is abundant in the upper and middle intertidal zones of open coasts and on rocky beaches in the lower reaches of estuaries (Sakai 1976; Kikuchi et al. 1981; Fukui 1988) and is an important predator in coastal ecosystems like other shore crab species. *Hemigrapsus sanguineus* has spread rapidly since it was first found in September 1988 at Townsends Inlet, Cape May County, New Jersey, USA (McDermott 1998). The species is thought to have been introduced into this area from its native provenance in East Asia by vectors that might have included shipping, contaminated packing material, and ballast water. These have recently spread the crab beyond the eastern coast of North America to the Atlantic coasts of France and the Netherlands (Williams and McDermott 1990; Breton et al. 2002). The methodologies used in several studies included analyses of the genetic variation, population structures and migration rates of the crab to identify the mechanisms driving these successful marine biological invasions (Roman and Palumbi 2004). However, information about *H. sanguineus* throughout the world has been insufficient to survey its population genetics.

Population genetic studies of marine crab species, based on molecular markers, have been used to infer their larval dispersal mechanisms, with significant information gained on their population structures and genetic diversity. The population genetic structures of marine species are influenced by their larval behavior and their dispersal patterns, which are determined by oceanographic features, such as sea currents, hydrological conditions, and physical barriers (Doyle

*Corresponding author. Email: dongskim@pknu.ac.kr

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et al. 1993; Hsieh et al. 2010). Population genetic analyses of several crab species were performed in the 1990s using allozyme data (McMillen-Jackson et al. 1994; Creasey et al. 1997). The sequence analysis of mitochondrial DNA (mtDNA) is also considered a useful tool for phylogenic and systematic studies of closely related crab species (Schubart et al. 1998; Imai et al. 2004), and mtDNA has recently been used for intraspecific population genetic analyses (Roman and Palumbi 2004; Pfeiler et al. 2005; Cassone and Boulding 2006). Although DNA markers are expected to overcome the deficiencies of allozyme analyses of population structure by increasing the accuracy and resolution of its analysis in sea crab species (McMillen-Jackson et al. 1994; Creasey et al. 1997), there have been few reports of the use of mtDNA to measure the genetic variation in shore crabs (Cassone and Boulding 2006). Maternally inherited mtDNA has higher sequence variability than most nuclear genes (Brown et al. 1979). Therefore, the analysis of mtDNA has become the method of choice for evolutionary and ecological studies of crab species.

In this study we estimated the present levels of genetic variation and the population structures of *H. sanguineus* for the first time in Korea and Japan using variations in the mtDNA *COI* sequence, to provide baseline data for ecological and biological studies.

Materials and methods

Hemigrapsus sanguineus samples were collected from five rocky shore sites located on three coastlines of South Korea, and at Onagawa on the Pacific Ocean coast of Japan etween 2009 and 2010 (Table 1 and Figure 1) by lifting boulders at the water's edge at low tide. The collected samples were stored at −20°C or kept in 100% ethanol at room temperature until use. Genomic DNA was extracted using the conventional SDS/proteinase K method, followed by organic extraction and ethanol precipitation (Sambrook and Russell 2001). The polymerase chain reaction (PCR) was used to amplify the *COI* gene with the universal primers LCO1490f (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198r (5′-TAAACTTCAGGGTaGACCAAAAAATCA-3′) (Folmer et al. 1994). PCR amplification was performed with a DNA Engine thermocycler (MJ Research, Tokyo, Japan) in 20 μL reaction volumes containing 1–2 μL of genomic DNA, 2 μM each primer, 0.25 mM each dNTP, 1 unit of Takara *LA Taq*™ DNA polymerase (Takara Shuzo, Shiga, Japan), and 2 μL of 10 × *LA Taq*™ reaction buffer (Takara Shuzo). The PCR conditions consisted of preheating at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s, with a final extension at 72°C for 5 min. After cycle sequencing with the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., Foster City, CA, USA), the purified PCR product was directly sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems Inc.) with the same PCR primer set, allowing the direct nucleotide sequence analysis of about 500 bp of the *COI* gene of *H. sanguineus*.

The sequence data thus obtained were aligned with DnaSP ver. 4.90.1 (Rozas and Rozas 1997) to determine the haplotypes of the *COI* gene. A parsimony network connecting the observed haplotypes was plotted with TCS ver. 1.21 (Clement et al. 2000) to resolve their genealogy. Haplotype diversity (h) and nucleotide diversity (π) within the populations were estimated according to Nei (1987), based on Kimura's two-parameter distance method, using K and DA in the REAP program (MacElroy et al. 1993). Pairwise population F_{ST} values were calculated to estimate the genetic differentiation between populations, according to Slatkin and Hudson (1991) and Tajima and Nei (1984), using the Arlequin program ver. 3.1 (Excoffier et al. 2005). The significance of each F_{ST} value was tested using 10,000 random permutations. Analysis of

Table 1. Sampling sites, dates, geographical coordinates, and numbers of individuals examined (N) in five *Hemigrapsus sanguineus* populations.

Sampling site	Abbreviation	Date of collection	Geographical co-ordinates		N
			Latitude	Longitude	
Korea					
Buan	BUA	June, 2009	35°44′21.83″N	126°35′49.50″E	21
Haenam	HAE	June, 2009	34°20′21.05″N	126°29′28.47″E	24
Geoje	GUJ	April, 2010	34°54′18.50″N	128°44′52.55″E	23
Guryonpo	GUR	April, 2010	36°03′33.00″N	129°31′17.12″E	21
Japan					
Onagawa	JPA	June, 2009	38°26′25.62″N	141°27′27.09″E	22
Total					111

molecular variance (AMOVA) was used to test for population structure with Arlequin ver. 3.1.

Neutral expectation and historic demographic expansions were investigated by examining Fu's F_S and Tajima's D and mismatch distributions with the sudden expansion model (Rogers and Harpending 1992). A goodness-of-fit test was used to test the validity of the sudden expansion model using a parametric bootstrap approach based on the sum of squared deviations (SSD) to compare the observed and the estimated mismatch distributions (Schneider and Excoffier 1999). Both the neutrality test and the mismatch distribution analysis were performed in ARLEQUIN ver.3.1. Because the mutation rate of the *H. sanguineus COI* gene over the estimated time since expansion was unknown, a molecular clock was calculated using the sequence divergence rates of the mitochondrial protein-coding regions of other marine crustaceans, which range between 2.2% and 3.1% per million years (Knowlton and Weight 1998; Schubart et al. 1998).

Results

Sequence analysis of a 536-bp fragment of the *COI* gene detected 27 variable nucleotide sites among 111 individuals from five populations, which defined a total of 28 haplotypes (Supplementary Table 1[1]). The nucleotide sequences of the 28 novel haplotypes have been deposited in the GenBank database under accession numbers HQ702865–HQ702892. All the substitutions at each site were biallelic, suggesting the occurrence of a single-base substitution between sequences and no saturation of the substitutions, except at two sites that were triallelic.

The statistical parsimony network of the observed 28 haplotypes is shown in Figure 2, with the circle sizes reflecting the haplotype abundances. Two focal haplotypes, Hap1 and Hap13, were abundant, whereas the other haplotypes (including singletons) were rare and most likely derived from the focal haplotypes. The distributions of the 28 haplotypes among the five populations of *H. sanguineus* are presented in Supplementary Table 2[1]. The number of haplotypes found in each of these populations was usually 8–10, except for the six at Geoje (GUJ). Although the observed haplotypes did not provide evidence of geographical associations (the focal haplotypes commonly occurred in all the populations, and the genealogically related haplotypes were scattered throughout all the populations), the abundance of Hap13 at Buan (BUA) was lower than its abundance in the other populations.

The haplotype diversities and nucleotide diversities are shown in Table 2. The haplotype diversities at Guryonpo (GUR) and Onagawa (JPA) were the highest of the five populations, suggesting the largest genetic variation in these populations of those examined here. Among the populations examined, low haplotype diversity was observed at BUA and GUJ. The average haplotype diversity was 0.7290 and the average nucleotide diversity was 0.0023, indicating that the *H. sanguineus* populations examined have high haplotype and low nucleotide diversities. The neutrality indices and mismatch analysis for each population are shown in Table 2. With both tests used (Fu's F_S and Tajima's D), the populations at BUA, HAE, and JPA deviated significantly from the neutral evolution model. Hence, only Fu's F_S values for the GUJ ($F_S = -2.43805$, $P = 0.014$) and GUR populations ($F_S = -3.70885$, $P = 0.008$) were significant. The mismatch distributions of all populations were unimodal except for JPA (Supplementary Figure 1[1]). Significant differences for the sums of the square deviations ($P_{SSD} < 0.05$) between the observed and simulated mismatch distributions indicate the population to be at equilibrium, which is not an expansion phase. The goodness-of-fit test did not reject the null hypothesis of sudden population expansion for all population (Table 2). The population expansion for *H. sanguineus* was estimated

Table 2. Measures of mtDNA diversity, neutrality indices, and mismatch analysis results for each population of *Hemigrapsus sanguineus*.

Sampling site	No. of haplotypes	Haplotype diversity (h, $\pm$SD)	Nucleotide diversity (Π)	Fu's F_S (P-value)	Tajima's D (P-value)	τ (95% CI)	$P^{\dagger}_{SSD}$	Time since expansion (year)
Korea								
BUA	10	0.686 ± 0.115	0.00214	−7.56628*	−2.19819*	1.084 (0.070–1.998)	0.65	37199–52417
HAE	10	0.739 ± 0.089	0.00240	−6.25556*	−2.06140*	1.268 (0.377–2.215)	0.70	43514–61315
GUJ	6	0.656 ± 0.079	0.00165	−2.43805*	−1.40842	0.992 (0.383–1.854)	0.20	34042–47969
GUR	8	0.776 ± 0.075	0.00244	−3.70885*	−1.37385	1.357 (0.117–2.305)	0.52	46568–65618
Japan								
JPA	9	0.788 ± 0.065	0.00234	−5.16756*	−1.65330*	1.367 (0.457–2.424)	0.27	46911–66102

$P^{\dagger}_{SSD}$, the probability when the simulated sum of squared deviations (SSD) of the mismatched distribution is larger than the observed SSD in the goodness-of-fit test; τ, time since expansion measured in mutational time units; CI, confidence interval. *$P < 0.05$.

using τ to have occurred 0.034–0.066 million years ago (Ma; Table 2).

The pairwise population F_{ST} estimates (Table 3) were generally low, suggesting little genetic differentiation between population pairs, perhaps attributable to high gene flow. However, the pairwise population F_{ST} estimates were large when BUA and JPA were compared with the other population pairs, and the lowest migration rate was estimated from the pairwise comparison of these two populations (Supplementary Table 3[1]). Significant differentiation was observed between BUA on the west coast (Yellow Sea) of Korea and all other populations, except Haenam (HAE; southwest coast of Korea), suggesting that BUA is genetically distinct from the other populations (GUJ, southeast coast of Korea; GUR, east coast [East Sea] of Korea; JPA, Pacific Ocean coast of Japan). AMOVA revealed that 96.7% of the genetic variation occurred within populations, whereas 3.03% ($P < 0.05$) occurred between populations, suggesting significant levels of genetic structure within and among the populations (Supplementary Table 4[1]).

Table 3. Pairwise F_{ST} estimates among the five populations of *Hemigrapsus sanguineus*. Sampling site abbreviations are those listed in Table 1.

	BUA	HAE	GUJ	GUR	JPA
BUA		−0.0053	0.0445*	0.0451*	0.1323*+
HAE	−0.0053		−0.0006	0.0063	0.0661*
GUJ	0.0445*	−0.0001		−0.0088	0.0049
GUR	0.0450*	0.0061	−0.0088		0.0050
JPA	0.1320**+	0.0657*	0.0050	0.0044	

F_{ST} values were calculated from haplotype frequencies (below the diagonal) and from genetic divergence data among the haplotypes calculated with the method of Tajima and Nei (1984) (above the diagonal).
*Significant differentiation ($P < 0.05$); **highly significant differentiation ($P < 0.001$); + significant differentiation ($P < 0.05$) on the exact test (Raymond and Rousset 1995). Probability of differentiation (with P) value was calculated from 1000 replications.

Discussion

The estimation of population structures using molecular markers is now a commonplace technique in evaluating the dynamics of natural populations. However, the intraspecific genetic structures of marine species of crabs are still poorly understood, mainly because of their complex patterns of dispersal traits and the difficulties encountered in directly observing their life histories. Consequently, only a few commercial crab species have been studied. The sequence of the mtDNA *COI* gene has been used as a tool for population genetic studies of several marine crab species, including the green crab (Roman and Palumbi 2004), swimming crab (Pfeiler et al. 2005), lined shore crab (Cassone and Boulding 2006) and hair crab (Azuma et al. 2008). In this study, we used *COI* mtDNA sequences to examine the relative importance of the proposed sea current effects on gene flow, in shaping the population structure of *H. sanguineus* in Korean coastal waters.

A minimum spanning network of 28 haplotypes from five *H. sanguineus* populations revealed a completely star-like shape. Hap1 and Hap13 were clearly the focal haplotypes, with relatively high abundances, whereas the other haplotypes (including singletons) radiated from the focal haplotypes, predominantly with single substitutions. The mismatch distribution analysis, SSD, significant negative values for Fu's F_S, and the estimated expansion time suggest sudden population expansion of all the populations in the last glacial period (0.07–0.01 Ma) (Slatkin and Hudson 1991). Most of the populations had high haplotype diversity (0.7290) and low nucleotide diversity (0.0023), suggesting rapid population growth from an ancestral population with a small population size attributable to glacial bottlenecking (Nei et al. 1975; Avise 1994). The scenario of relatively recent, rapid population growth for *H. sanguineus* populations after glacial bottlenecking is favored by the star-like genealogy of the haplotypes in the sampling areas. This sort of shallow haplotype genealogy has also been reported in some other crab species and is probably the consequence of population reductions during glacial periods and their subsequent recovery during interglacial periods (Azuma et al. 2008).

Recent research has examined the genetic variations in species to determine the present status of populations. Genetic variation is important for the long-term survival of natural populations, because it confers a capacity to adapt to future changes in environmental conditions, thereby increasing the organism's fitness (Frankel and Soulé 1981). The extensive haplotype diversity and limited nucleotide diversity of the *H. sanguineus* populations demonstrated in the present study are similar to the patterns of genetic diversity seen in other marine organisms, particularly marine crab species. This suggests that the migration and reproductive behavior of *H. sanguineus* are similar to those of other marine crabs, in which haplotypes with recently diverged nucleotides are distributed by the long-range dispersal of larvae among populations or regions (Azuma et al. 2008). Among the populations examined here, reduced genetic variation was detected at BUA (west coast of Korea) and GUJ (southeast coast of Korea). These observations suggest that the populations at BUA and GUJ have experienced recent bottlenecks or founder effects. Generally, low haplotype diversity and low nucleotide diversity can result

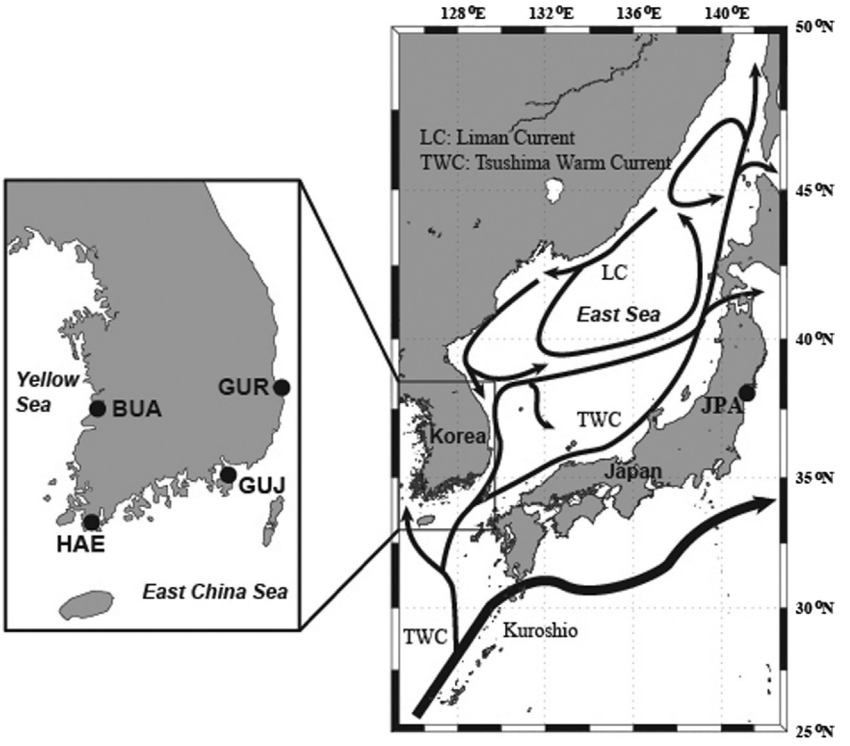

Figure 1. Sampling locations of the five *Hemigrapsus sanguineus* populations analyzed in this study (see Table 1 for site names).

from a transient bottleneck in a large ancestral population, because an extremely short crash can eliminate many haplotypes without necessarily severely affecting nucleotide diversity (Nei et al. 1975; Avise 1998).

The primary aim of many studies of natural marine species is to understand the oceanographic patterns (such as sea currents), climate changes, and vicariant events that affect gene flow and genetic diversity, in relation to the historical patterns of demographic expansion and contraction. The estimated pairwise F_{ST} values calculated with an exact test and the determined migration rates indicate that substantial gene flow has occurred among these populations of *H. sanguineus*, except between the BUA and JPA populations, which showed some genetic differentiation and low migration rates. Generally, marine organisms with passively dispersed planktonic larvae have limited population substructures, and their dispersal may be strongly affected by sea currents (Lessios et al. 2003; Kim et al. 2010). The Tsushima Warm Current (TWC) branches off the Kuroshio Current, with part of the TWC running into the Yellow Sea and the main part entering the East Sea along the Korean Peninsula (Senjyu 1999; Ichikawa and Beardsley 2002) (Figure 1). Therefore, the TWC may transport larvae to the Yellow Sea, near BUA, and to the southern part of the East Sea, near GUR on the Korean coast and JPA on Pacific Ocean coast of Japan. The pairwise F_{ST} values calculated with the exact test indicate a significant amount of genetic differentiation and low migration rates between BUA and JAP compared with those of the other population pairs. This suggests low or restricted dispersal and gene flow between the west coast of Korea and the east coast of Japan. These and our AMOVA results show the possibility of substructures among the *H. sanguineus* populations.

In conclusion, this study has shown a high level of gene flow among populations of *H. sanguineus* and moderately weak genetic differentiation between geographically distant populations on the west coast (Yellow Sea) of Korea and the east coast (Pacific Ocean) of Japan. Our findings should extend our knowledge of the colonization histories and general patterns of migration events in this species. We have also demonstrated that the analysis of the genetic variation in mtDNA *COI* sequences is a useful model for other researchers conducting population-level studies of closely related species. *Hemigrapsus sanguineus* has successfully colonized the eastern coast of the USA, Atlantic France, and the Netherlands over recent decades. The introduction histories of many invader populations remain unclear. In a future study, we will collect more mtDNA sequence information by expanding the samples to include populations from their native and introduced ranges on a global scale to compare their genetic characters. Such a comprehensive analysis should provide useful information about

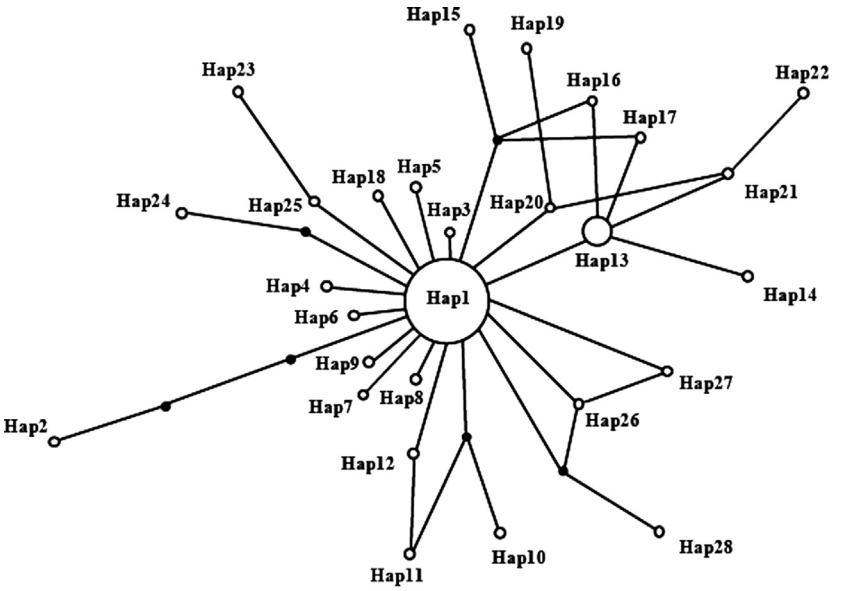

Figure 2. A single minimum spanning tree of the 28 mitochondrial *COI* haplotypes of *Hemigrapsus sanguineus* (Supplementary Table 1[1]). Circle sizes reflect haplotype abundances. Closed circle indicates a missing haplotype.

the introduction history and general patterns of invasion events of *H. sanguineus*.

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Note

1. Supplementary material can be found by clicking on the Supplementary Content tab at http://dx.doi.org/10.1080/19768354.2011.604939.

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