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Two Genetic Lineages of Sea Slaters, *Ligia* (Crustacea: Isopoda) in South Korea: a Population Genetic Approach

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In this study, the species composition and population genetic properties of the sea slater, *Ligia*, in South Korea were investigated using mitochondrial and nuclear gene sequences. Two groups of sea slaters, genetically isolated from each other, a Western Group (WG) and an Eastern Group (EG) were identified. These groups exhibited considerable genetic divergence from *Ligia exotica,* previously recorded as a species inhabiting this country. These results indicate that there may be two species of *Ligia* in South Korea, but there is a small probability that both groups are *L. exotica.* A comparison of their genetic properties indicates that WG has a higher effective population size than EG, and that EG may have experienced a recent expansion, implying that it has a shorter history in South Korea than WG. These findings suggest that the South Korean sea slater populations may have been established as a result of several colonization events that can be traced on a continental scale by phylogeographic studies of sea slaters.

Keywords: 16S rDNA; 18S rDNA; *Ligia*; Molecular Identification; Sea Slaters; South Korea.

Introduction

Although morphology-based taxonomic studies have numerous advantages, they are limited in some situations, such as when there is phenotypic plasticity and cryptic taxa exist (Hebert et al., 2003). Molecular approaches to taxonomy such as DNA barcodes, short stretches of DNA sequence used to identify species, are flourishing as a way to overcome these difficulties (Blaxter, 2003; Schander and Willassen, 2005; Yoo et al., 2006). Generally, as a result of genetic isolation, members of a species exhibit genetic cohesion that distinguishes them from other species; that is to say, the gene pool of each species has its own unique composition due to microevolutionary processes taking place after speciation. The molecular identification of species is clearcut and efficient, irrespective of phenotypic variation, except in cases of incomplete lineage sorting among recently separated species (Nielsen and Matz, 2006). This method has another strong point in that it reveals cryptic species that are hard to discriminate by traditional approaches (Hebert et al., 2004). If the molecular data reveal adequate intra-specific variation, enough additional information can be obtained by population genetic analyses to infer the evolutionary pathways of the species concerned, such as their population structure, demographic history and phylogeography.

The sea slater, *Ligia*, has an ecologically important role in nutrient recycling in marine ecosystems (Zimmer, 2002). Moreover, being a successful group obligately occupying seashore habitats, they are also evolutionarily significant for studying the adaptation of isopods from aquatic habitats to terrestrial environments (Farr, 1978). Thirty seven species of *Ligia* have been identified worldwide (Schmalfuss, 2003), and one species, *Ligia exotica* Roux, 1828 has been recorded in South Korea (Kwon, 1993). However, there is still uncertainty about the identity of the species and its composition that cannot be resolved by traditional approaches due to the great morphological variation and large number of individuals. Here, we have investigated the genetic composition of South Korean sea slaters and their population genetic properties using mitochondrial and nuclear sequences.

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Abbreviations: EG, Eastern Group; Theta(HOM), theta estimate based on expected homozygosity; Theta(Pi), theta estimate based on the mean number of pairwise differences; Theta(S), theta estimate based on the number of segregation sites; WG, Western Group.

Table 1. Geographical and sample information of sampling locations in this study.

Sampling locations	Coordination	WESTERN GROUP			EASTERN GROUP		
		Number of individual	Number of haplotype	Nucleotide diversity (average per site)	Number of individual	Number of haplotype	Nucleotide diversity (average per site)
GANGHWA2	37°38′24″N 126°23′17″E	7	2	0.003935 ± 0.002907	1	1	–
GANGHWA1	37°35′41″N 126°26′46″E	30	4	0.002782 ± 0.001972			
ERWANG	37°26′37″N 126°22′38″E	28	4	0.000885 ± 0.000918	1	1	–
ANSAN	37°17′04″N 126°34′09″E	57	6	0.001471 ± 0.001244			
TAEAN	36°48′44″N 126°18′37″E	12	6	0.006230 ± 0.003932			
BORYEONG	36°18′28″N 126°30′53″E	6	2	0.004821 ± 0.003521	15	1	–
SEOCHEON	36°09′32″N 126°29′34″E	17	4	0.002304 ± 0.001767			
SEONYUDO IS.	35°49′16″N 126°25′15″E				2	1	–
BUAN	35°42′17″N 126°35′19″E	1	1	–	13	2	0.000793 ± 0.000895
HAMPYEONG	35°05′40″N 126°27′42″E	21	6	0.002239 ± 0.001710			
BOSEONG	34°40′47″N 127°06′34″E	17	1	–	2	2	0.002062 ± 0.002916
HADONG	34°57′09″N 127°46′40″E	1	1	–	13	5	0.003965 ± 0.002706
MASAN	35°06′12″N 128°29′54″E	7	4	0.010015 ± 0.006363	1	1	–
GIJANG	35°13′05″N 129°14′03″E				11	5	0.002024 ± 0.001670
YEONGDEOK	36°22′39″N 129°24′26″E				16	1	–
ULJIN	36°59′15″N 129°25′04″E				15	2	0.000275 ± 0.000485
SAMCHEOK	37°20′43″N 129°15′46″E	2	2	0.008264 ± 0.009240	9	3	0.002864 ± 0.002194
JEJUDO IS.	33°13′04″N 126°30′49″E				26	2	0.000159 ± 0.000352
ULLEUNGDO IS.	37°29′07″N 130°54′23″E				19	5	0.004485 ± 0.002904
TOTAL		206	22	0.004430 ± 0.002734	144	17	0.001874 ± 0.001448

Materials and Methods

Sampling, DNA extraction, PCR and sequencing Samples were collected by hand from nineteen locations in South Korea (Fig. 1 and Table 1) and preserved in absolute ethanol. DNA was extracted from walking legs following the method of Sambrook and Russel (2001). Partial segments of the mitochondrial 16S ribosomal RNA gene and 18S ribosomal RNA gene were amplified using primers 16SAR-SF and 16SBR-SF (Palumbi et al., 1991), and primers I- and D (Park et al., 2006) respectively. Polymerase chain reaction (PCR) mixtures consisted of 1× PCR buffer, 0.2 mM dNTP mix, 2.5 mM $MgCl_2$, 0.2 μM of each primer, 2.5–250 pg of total DNA and 0.04 unit/ul of *Taq* polymerase (Promega, Madison). PCR was performed using a GeneAmp® PCR system 9700 (Applied Biosystems, Foster City) with the following cycle conditions: 94°C for 3 min, followed by 30 cycles at 94°C for 30 s, 47°C for 50 s and 72°C for 50 s, and a final elongation at 72°C for 7 min. The amplified products were purified from the PCR mixtures using a QIA quick PCR purification kit (Qiagen, Spain).

The PCR products of the 16S ribosomal RNA gene were sequenced with both amplification primers using a Big-Dye terminator sequencing kit (Applied Biosystems). Unincorporated dideoxynucleotides, primers and enzymes were removed by ethanol precipitation, and the products were analyzed on an ABI PRISM® Genetic Analyzer 3730 (Applied Biosystems). Bidirectional sequences were aligned and visually checked using SeqEd 1.03 (Applied Biosystems). Amplified fragments of the 18S

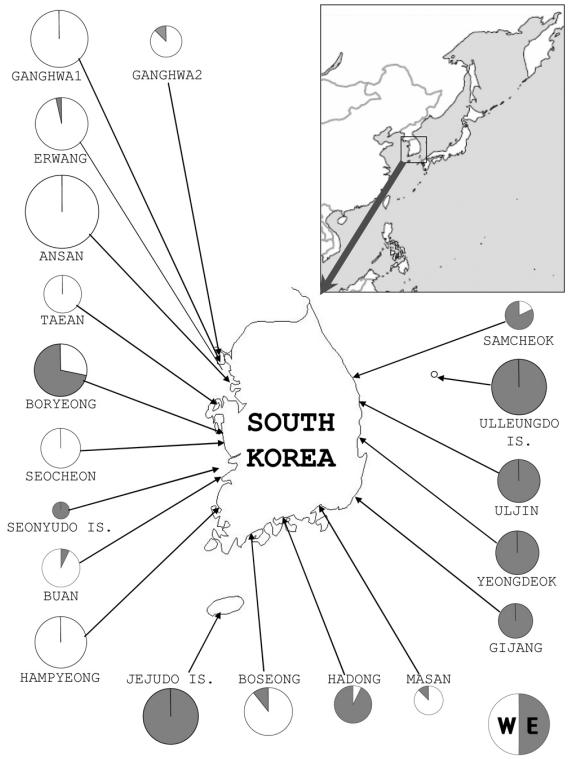

Fig. 1. Sampling locations of *Ligia* in South Korea. The proportions of individuals belonging to the Western (W) and Eastern (E) Groups are represented in the pi-graph.

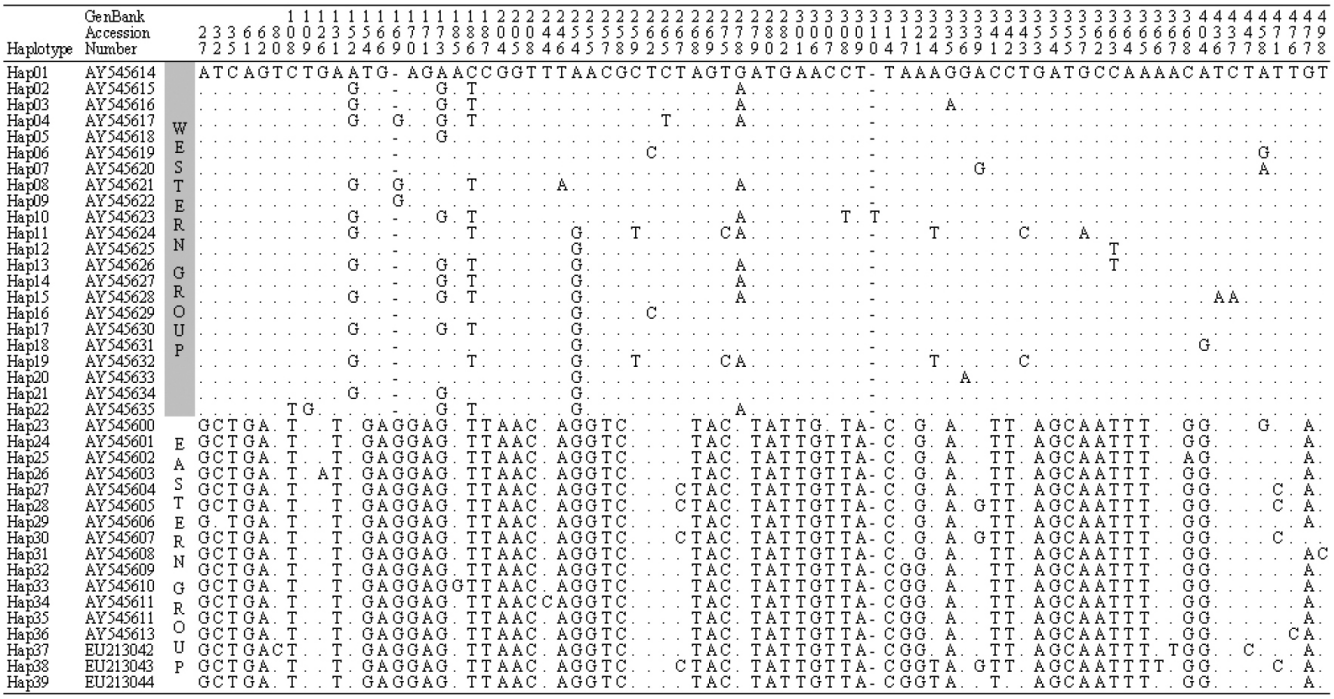

Fig. 2. Alignment of the haplotype sequences (16S rDNA) of *Ligia* in South Korea, with GenBank accession numbers.

ribosomal RNA gene were electrophoresed on 2% agarose gels to compare fragment lengths.

Data analyses After multiple alignment of sequences with CLUSTAL X 1.83 (Thompson et al., 1997), the data were exported to NEXUS and PHYLIP formats for further analyses. We used TCS 1.18 (Clements et al., 2000) to define haplotypes from all the 16S rDNA sequences and to calculate haplotype networks. MEGA 4.1 (Tamura et al., 2007) was used to infer neighbor joining trees among the Korean haplotypes and the sequences of *Ligia exotica* retrieved from GenBank (accession number: AF260861 and AY051339) according to the Kimura-2-parameter model. Using the same program, the p-distance between sequences was computed. The nucleotide diversity of each sampling location was calculated using ARLEQUIN 3.11 (Excoffier et al., 2005). Mantel's test (Mantel, 1967), also using ARLEQUIN 3.11, was performed to look for significant relationships between population genetic distance (linearized F_{ST}) (Slatkin, 1995) and geographical distance. The latter was calculated along the coastline between the sampling locations, but island populations and sites consisting of only one individual were excluded to make the analysis robust. Analysis of molecular variance (AMOVA) (Weir and Cockerham, 1984) was used to measure the extent to which genetic variance could be assigned to the hierarchical structure of the population organization and to estimate F_{ST} values. The statistical significance of the AMOVA was evaluated using over 10,000 permutations. Recent demographic changes were inferred from the mismatch distribution (Harpending, 1994; Rogers, 1995; Rogers and Harpending, 1992) and Tajima's neutrality tests (Tajima, 1989) performed with ARLEQUIN 3.11 (Excoffier et al., 2005). Population demographic parameters (tau, theta initial and theta final) were estimated under the sudden expansion model (Schneider and Excoffier, 1999). Differences between expected and observed mismatch patterns were tested by calculating the sum of the squared deviations (Rogers and Harpending, 1992). The *p*-value of the sum of the squared deviations was calculated by 100 bootstrap replications. The significance of Tajima's *D* values was evaluated by comparison with randomly generated values based on the observed theta (S) with 10,000 repeats.

Results

Two genetic lineages of *Ligia* in South Korea We obtained the partial 16S ribosomal RNA gene sequences of 350 individual organisms representing a total of 39 haplotypes (AY545600−AY545635 and EU213042−EU213044). Sequence alignment identified 529 nucleotide sites of which 76 sites, including two gaps, were variable (Fig. 2). Hap 1, occurring in 131 individuals (ind.), was the most frequent haplotype, followed by Hap 24 (111 ind.) and Hap 2 (45 ind.) (Fig. 3). Hap 24, the most widely distributed haplotype, was found at 14 locations in the sampling sites. Two distinct groups of haplotypes were identified in the haplotype network drawn by TCS 1.18 (Fig. 3). In the neighbor joining trees, the two groups are separated from each other with deep divergence and both of them are also clearly distinguished from sequences of *Ligia exotica*, the sea slater species described in South Korea (Kwon, 1993)

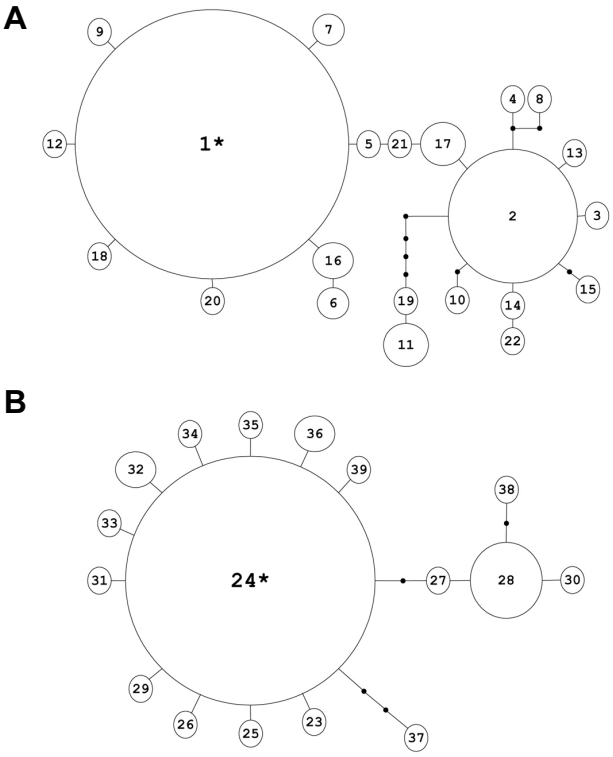

Fig. 3. The 95% plausible set of haplotype networks of the Western Group (WG) (A) and Eastern Group (EG) (B).

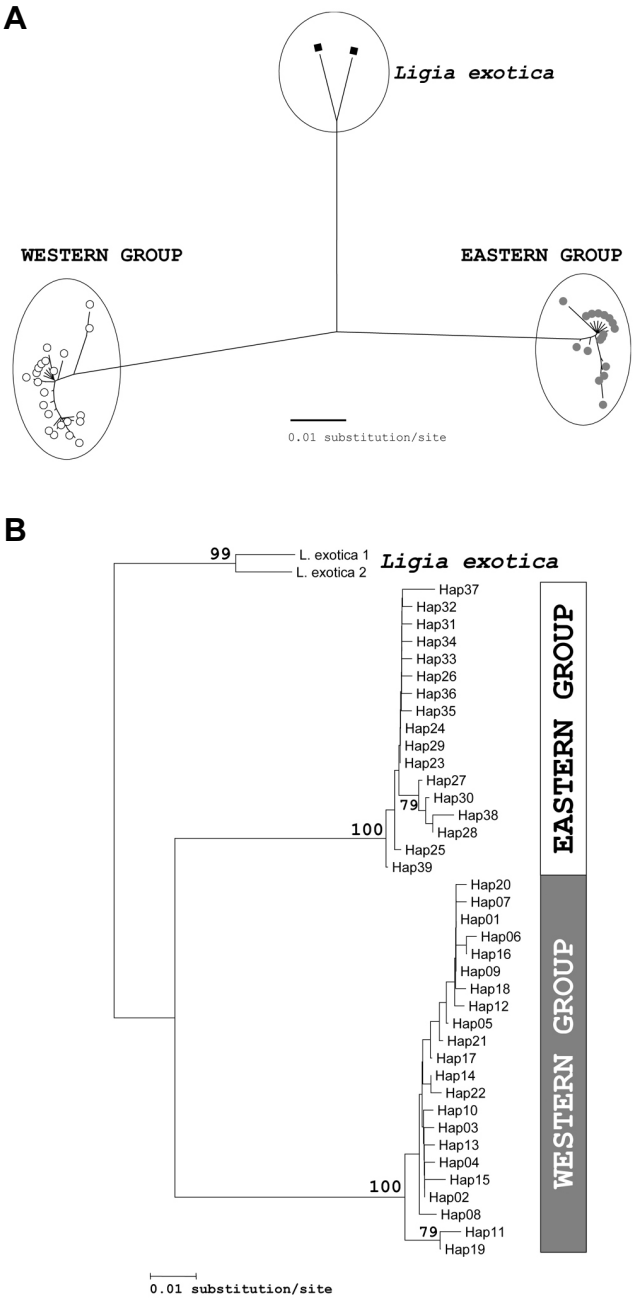

Fig. 4. Unrooted (A) and rooted (B) neighbor joining trees for Korean haplotypes, and the sequences of *L. exotica* (AF260861 and AY051339). Bootstrap values are calculated from 500 replicates; values above 70% are shown on the nodes.

(Fig. 4). Each lineage was monophyletic, supported by a high bootstrap value (100%). The two groups were named Western Group (WG) and Eastern Group (EG), taking into consideration their geographic distribution in South Korea, and were composed of 22 and 17 haplotypes, respectively. The considerable divergence between these groups was confirmed by the pairwise genetic distance values. Genetic differences were less than 2.1 and 1.1% within WG and EG, respectively; however, 8.7–10.4%, 9.2–10.1% and 9.6–11% differences were observed between WG and EG, WG and *L. exotica*, and EG and *L. exotica,* respectively. The patterns of nuclear DNA variation conformed to those of the mitochondrial sequences, based on the finding that a 49 bp difference was observed between WG and EG in the segment of 18 rRNA gene amplified by primers I- and D (Fig. 5). The analysis of nuclear and mitochondrial DNA showed that there cannot be any gene flow between the two groups, i.e. the two groups are genetically isolated. Hence the 18S ribosomal region generating a size difference can be used as an effective molecular marker for distinguishing the two groups of sea slaters in South Korea. These results strongly suggest that the groups of sea slaters observed in South Korea are independent species separated by genetic isolation.

Genetic properties of the two groups of sea slaters As a whole, individuals belonging to WG were distributed over the southwest coast of South Korea and those belonging to EG were found on the southeast coast (Fig. 1). However, the distinction based on the geographic distribution of these two groups is somewhat arbitrary because there were many exceptions that did not correspond to these designations and both groups coexisted at nine locations without any pattern to their distribution (Fig. 1 and Table 1). Moreover Mantel's test revealed no significant correla-

Table 2. Results of AMOVA tests and F_{ST} values of Eastern and Western Groups of *Ligia.*

	Degree of freedom	Sum of squares	Variance components	Percentage of variation (%)
WESTERN GROUP				
Among populations	12	110.921	0.59236	51.01
Within populations	193	109.783	0.56882	48.99
Total	205	220.704	1.16119	
F_{ST} = 0.51014 (P = 0.00000)				
EASTERN GROUP				
Among populations	13	18.695	0.10983	23.58
Within populations	130	48.285	0.35604	76.42
Total	143	64.979	0.46587	
F_{ST} = 0.23576 (P = 0.00000)				

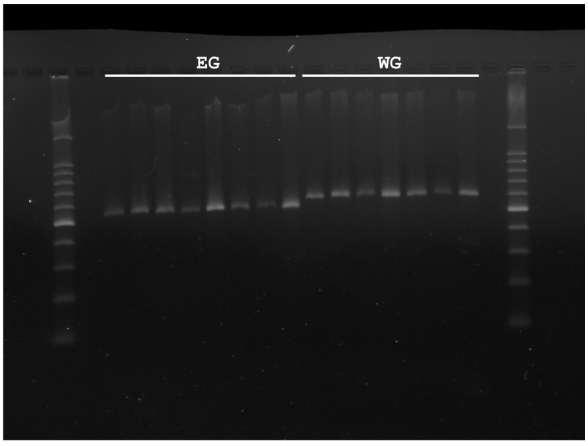

Fig. 5. Size of the fragments of the 18S ribosomal DNA gene amplified in the WG (Western Group) and EG (Eastern Group) of *Ligia* and run on a 2% agarose gel. Size marker: 100 bp ladder marker.

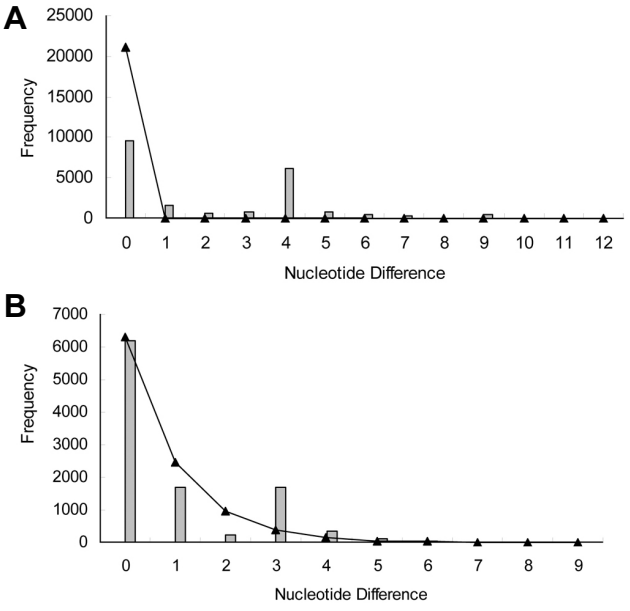

Fig. 6. Mismatch distributions (bar) of the two Korean lineages, with the expected distributions (triangles) under the sudden expansion model of population fitted to the observed distributions.

tion between the genetic differences (linearized F_{ST}s) and geographic distances in the two groups (P_{WG} = 0.198 and P_{EG} = 0.627). AMOVA tests (Table 2) indicated that the genetic variation of EG resided more within populations than between populations, whereas WG varied more between populations. The F_{ST} values of EG inferred from the AMOVA tests were less than those of WG (Table 2). The smaller values of F_{ST} observed in the former may derive from greater gene flow between subpopulations of this group, or may be due to a large proportion of private alleles. The mismatch distribution of WG was significantly different from the distribution simulated under a sudden expansion model, as assessed by the sum of the squared deviation between the two values; however, this was not true for EG (Table 3 and Fig. 6). This conclusion is also supported by Tajima's neutrality tests where an insignificant negative D value was observed in WG whereas the negative value in EG was significant (Table 3), indicating that the EG populations have undergone more recent expansion. These results suggest that the EG populations have a shorter history in South Korea than those of WG.

Discussion

The sea slaters found in South Korea are composed of two genetic lineages with discrete genetic gaps in their mitochondrial and nuclear DNA sequences. Although the genetic divergence between the two groups in the 16S rDNA sequence is less than the value (17.4%) between two separate species of *Ligia* (Wetzer, 2001), each group deserves to be considered a distinct species owing to an additional gap in the nuclear DNA sequences, which implies absence

Table 3. Results of historical demographic analyses of Western and Eastern Groups of *Ligia* in South Korea.

Statistic	WESTERN GROUP	EASTERN GROUP
THETA ESTIMATES		
Theta (HOM)	0.911508 ± 0.130143	0.491552 ± 0.104513
Theta (S)	3.896547 ± 1.145875	3.607795 ± 1.129838
Theta (Pi)	2.153209 ± 1.328591	0.908800 ± 0.702354
MISMATCH DISTRIBUTION ANALYSIS		
Mismatch observed mean	2.153	0.909
Mismatch observed variance	5.727	1.839
Tau (τ)	0.000	2.750
Theta initial (θ_0)	0.000	0.330
Theta final (θ_1)	99999.000	0.635
Sum of squared deviation	0.396	0.027
p-value of sum of squared deviation	0.000	0.270
TAJIMA'S TEST OF SELECTIVE NEUTRALITY		
Tajima's D	−1.24629	−2.07438
p-value	0.08440	0.00110

of genetic exchange across a genetic barrier. Both of the Korean groups also show considerable genetic differentiation from *L. exotica* (8.7−11%). The deep genetic divergence between WG, EG and *L. exotica* suggests that there exist at least two genetically isolated groups, implying separate species, and indicates that the two groups may not be *L. exotica*, the previously reported South Korean sea slater species (Kwon, 1993).

The phylogeographic patterns of the Korean sea slaters, presenting deep genetic divergence of sympatric lineages, are usually observed in animal groups that have experienced allopatric evolution followed by secondary contact (Avise, 2000). In a comparison of genetic properties, all of the theta estimates calculated from EG were lower than from WG (Table 3); however, little difference was found in theta (S), probably as a result of the proportion of singleton haplotypes, which has a great influence on theta (S), and was higher in EG. The higher theta estimates for WG point to a larger effective population size, on the assumption that differences of mutation rates between the two lineages are insignificant. The populations of WG seem not to have undergone recent expansion, in view of the result of the mismatch distribution analysis in which the values expected under a sudden expansion model were significantly different from the observed values. Unlike WG, EG fitted the mismatch distribution of the model, and may have experienced recent expansion. In Tajima's neutrality analysis, EG presented a significantly negative *D* value, indicating recent population expansion, unlike WG which gave an insignificant value. The results of the historical demographic analyses strongly indicate that the populations of EG were established in the Korean Peninsula much more recently than those of WG.

The two groups of sea slaters in South Korea may have resulted from several colonization events with time gaps between them, consistent with geological processes such as land bridge formation between nearby lands and islands during the Pleistocene. Owing to the huge and shallow continental shelves, the shape of the coastlines in the East Asian region has been greatly affected by changes of sea level as glacial and interglacial periods alternated (Chough, 1983; Kaizuka, 1980; Kimura, 2000). Geological changes during this period, therefore, inevitably had a great influence on the biogeography of plants and animals in the region (Kaizuka, 1980). Sea slaters must be one of the most sensitive of the animal groups to geological changes of coastline, for most species of *Ligia* are confined to beach habitats, and they have no larval stage in their life cycle, which makes them incapable of long distance colonization via ocean currents (Tsai and Dai, 2001). Thus the phylogeographic study of *Ligia* in the East Asia region illuminates the speciation and evolution of sea slaters, including the two Korean genetic groups, in the context of geological changes of the coastline.

Recently, several cases of cryptic species have been reported in the East Asia region. The cryptic species *Macrophthalmus banzai* was recently differentiated from *M. japonicus*, a dominant species in mud flats in Korea and Japan, by an analysis of its behavioral traits (Wada and Sakai, 1989), an identification which was later confirmed by molecular evidence (Kitaura et al., 2006). Until very recently, a penicillate shore crab, common in rocky shore intertidal habitats, was recognized as *Hemigrapsus penicillatus*, but a new species, *H. takanoi,* was indicated

by close examination of its morphological characters with the help of allozyme analysis (Asakura and Watanabe, 2005), and this discovery was verified by ecological criteria (Mingkid et al., 2006). Although cryptic species have often been identified, their speciation processes and geographical distribution have barely begun to be studied on a continental scale in the East Asia region. Combined with recent geological history, comparative phylogeographic studies of several species should enlighten the evolution of marine organisms in this region.

The results of our study do not agree with those of a previous study (Kwon, 1993) in which *L. exotica* was recorded as the Korean sea slater species. *L. exotica* has a circum-tropical distribution (Kwon, 1993) and is famous as an introduced species in many countries (Jass and Klausmeier, 2000). However, there is only a remote possibility that either WG or EG is *L. exotica*, as can be inferred from our finding that the two genetic lineages in South Korea showed considerable molecular divergence from *L. exotica*. Our sampling efforts in fact suggest that *L. exotica* may not exist in South Korea, so that taxonomic revision of the species of sea slaters may be needed.

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