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Genetic Relationships of Four Korean Oysters Based on RAPD and Nuclear rDNA ITS Sequence Analyses

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ABSTRACT

Random amplified polymorphic DNA (RAPD) marker and sequence analyses of the internal transcribed spacer (ITS) region of ribosomal DNA were used to assess phylogenetic relationships of four Korean oyster species. The average number of species-specific markers identified from five universal rice primers (URPs) by RAPD-PCR was 1.8 for *Crassostrea gigas*, 3.2 for *C. nippona*, 3.6 for *C. ariakensis*, and 4.6 for *Ostrea denselamellosa*. The length of the ITS (ITS1-5.8S-ITS2) region ranged from 1,001 to 1,206 bp (ITS1, 426-518 bp; 5.8S, 157 bp; and ITS2, 418-536 bp), while the GC content ranged from 55.5-61.1% (ITS1, 56.8-61.8%; 5.8S, 56-57.3%; and ITS2, 54.1-62.2%). A phylogenetic analysis of the oysters based on our RAPD, ITS1, and ITS2 sequence data revealed a close relationship between *C. gigas* and *C. nippona* and a distant relationship between the genera *Crassostrea* and *Ostrea*. Our results indicated that RAPD and ITS sequence analysis was a useful tool for the elucidation of phylogenetic relationships and for the selection of species-specific markers in Korean oysters.

Key words: Korean oyster species, random amplified polymorphic DNA (RAPD), internal transcribed spacer (ITS), phylogenetic relationships.

INTRODUCTION

According to fishery statistics (capture production) published by the Korean Ministry of Maritime Affairs and Fisheries (MOMAF, 2006), 31,000 metric tons of oysters worth US$ 20 million are produced annually because of their commercial and biological importance. Four species, *Crassostrea gigas*, *C. nippona*, *C. ariakensis*, and *O. denselamellosa*, have been identified in South Korea based on their habitats and distributions (Lee *et al.*, 2000). The taxonomic niches and systematic relationships among Korean oysters have not been fully resolved, mainly due to morphological problems. Therefore, DNA-based techniques have been used to identify the taxonomic status of individual oyster species (Lee *et al.*, 2000; Boudry *et al.*, 2003; Lam and Morton, 2003; Wang *et al.*, 2004) ; however, a suite of molecular markers suitable for identifying and classifying Korean oyster species and their systematic relationships is needed.

Few genetic studies have been conducted using Korean oysters despite their commercial and biological importance (Park and Kim, 1995; Kim *et al.*, 1997; An *et al.*, 1999). Previous studies aimed at species identification and determining the phylogenetic relationships among the four known Korean oyster species focused on analyses of mitochondrial cytochrome oxidase I (CO I) and 16S rRNA (Park and Kim, 1995; Kim *et al.*, 1997; Lee *et al.*, 2000) ; however, those studies did not provide any clear suggestions for identifying the four species or for clarifying their systematic relationships.

The development of automated PCR-based sequencing has made various types of molecular markers available for use in species identification and

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in the study of the systematic relationships among oysters (Boudry *et al.*, 2003; Lam and Morton, 2003; Liu and Cordes, 2004; Wang *et al.*, 2004; Gaffney *et al.*, 2006; Jung *et al.*, 2006). Random amplification of polymorphic DNA (RAPD) is a particularly useful method for species identification as it allows for the grouping of species at the interspecific and intraspecific levels even when little or no sequence data are available for the organisms in question (Williams *et al.*, 1990; Crossland *et al.*, 1993; Patwary *et al.*, 1994; Klinbunga *et al.*, 2001; Kang *et al.*, 2002; Kim *et al.*, 2003). In addition, the internal transcribed spacers (ITSs) found in nuclear ribosomal DNA (rDNA) have been analyzed for sequence variability, ease of detection and amplification, and usefulness for inferring phylogenies in bivalves (Yu *et al.*, 2000; Kenchington *et al.*, 2002; Insua *et al.*, 2003; He *et al.*, 2005). The aim of the present study was to determine the genetic relationships among four Korean oyster species and to investigate the usefulness of the RAPD technique and ITS sequence analysis as molecular markers for species identification of Korean oysters.

MATERRIALS AND METHODS

1. Oyster samples and DNA extraction

Individuals of four oyster species (*C. gigas*, *C. nippona*, *C. ariakensis*, and *O. denselamellosa*) were collected from four locations (Namhae in Gyungnam Province, Pohang in Gyungbuk Province, Jinju in Gyungnam Province, and Gwangyang in Joennam Province, respectively) in Korea. *Ostreola conchaphila* was used as the outgroup. Genomic DNA was extracted from the adductor muscles of the oysters using the TNES-urea buffer method (Asahida *et al.*, 1996).

2. PCR amplification and sequencing

The primers and PCR conditions used in our RAPD analysis were adapted from those reported by Kang *et al.* (2002). The sequences of the universal rice primers (URPs) used in this study were 5'-ATCCAAGGTCCGAGACAACC-3' (URP 1), 5'-CCCAGCAACTGATCGCACAC-3' (URP 2), 5'-GTGTGCGATCAGTTGCTGGG-3' (URP 3), 5'-ATGTGTGCGATCAGTTGCTG-3' (URP 6) and 5'-AATGTGTGGCAAGCTGGTGG-3' (URP 9). PCR was performed in a 50 μl reaction mixture containing 50 ng of template DNA, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each dNTP, 200 ng of each URP, and 2.5 U of Taq DNA polymerase. Amplification was performed in a PTC-220 thermal cycler (MJ Research) programmed for 5 min at 94°C followed by 35 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C, with a final extension of 10 min at 72℃. The products were separated on 1.5% agarose gels to verify successful amplification. To amplify the nuclear rDNA regions spanning ITS1, ITS2, and 5.8 rDNA, universal primers (ITS5, 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS4, 5'-TCCTCCGCTTATTGATATGC-3') complementary to the conserved 18S and 28S regions were used (White *et al.*, 1990). PCR was performed in a PTC-220 thermal cycler (MJ Research) programmed for 5 min at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C with a final extension of 10 min at 72°C. The reactions were performed in a 10 μl volume containing 50 ng of genomic DNA, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 5 pmol of each primer, and 0.5 U of Taq DNA polymerase. The products were cloned using the pGEM-T Easy system (Promega) according to the manufacturer's protocol. To facilitate the sequencing of the products, the internal primers ITS1-S1 (5'-CCTTAARTACAGACGAGCTCG-3') and ITS2-S2 (5'-CTGCATTTAAGGCGAAGKAGC-3') were used. Sequencing was achieved using an ABI 3100xl Genetic Analyzer (Applied Biosytems). In all cases, three clones for each individual were sequenced in both directions.

3. Data analysis

Phylogenetic analysis of the RAPD data was performed using the method of Nei (1987), which scores PCR products as present (value = 1) or absent (value = 0). The similarity coefficient (F) was calculated based on the fraction of shared fragments between each species pair. For each pair of oyster species (X and Y), we used the equation F=2Nxy/ (Nx + Ny), where Nxy is the number of DNA fragments shared by species X and Y, while Nx and Ny are the numbers of fragments scored from species X and Y, respectively. On the basis of the similarity coefficients, a dendrogram was constructed using the program NTSYSpc (version 1.70, Fixer Software) by the unweighted pair-group method with arithmetic averages (UPGMA; Sneath and Sokal, 1973).

The ITS regions of the four species were sequenced using an ABI 3100xl Genetic Analyzer; the sequence of ITS2 from *O. conchaphila* (EF035118) was obtained from GenBank. The sequences were edited and aligned using SeqMan II (DNASTAR, Inc., Madison, WI). The boundaries of the various regions were deduced by comparing them to data from various invertebrate phyla, including Annelida, Mollusca, and Cnidaria (Ursi *et al.*, 1983; Chen *et al.*, 1996; Odorico and Miller, 1997). Our results indicated that the amplified regions corresponding to the partial 18S, 5.8S, and 28S sequences were sufficiently conserved, thus permitting unambiguous alignments. Genetic relationships among the haplotypes were reconstructed by maximum parsimony (MP) and the neighbor-joining (NJ) method using MEGA 4.0 (Kumar *et al.*, 2004). Genetic distances were generated by Modeltest 3.7 (Posada and Crandall, 1998). The best fit for the ITS1, ITS2, and complete ITS region (partial 18S, 28S, and 5.8S) was determined using the HKY + G model with invariable sites (I) = 0 and gamma shape parameter (Γ) = 0.9268, 0.9010, and 0.4982, respectively. The reliability of the phylogenetic relationships was evaluated using 1,000 bootstrap replications (Felsenstein, 1985).

RESULTS

1. RAPD analysis

DNA amplification using five URPs, which were pre-screened from 12 primers, was performed for four Korean oyster species. High-stringency conditions were employed during the annealing step to ensure specificity between the template DNA and RAPD primer. In total, 102 bands were reproductively generated from the five URPs (Fig. 1). The number of fragments amplified varied between 15 and 36 for each primer, with an average of 20.4 bands per primer. A total of 66 polymorphic bands, varying in length from 250 to 4,500 bp, was produced from the pre-selected primers (URP1, 2, 3, 6, and 9; Fig. 1). Differences were observed between *Crassostrea* and *Ostrea* with each URP. Nine specific RAPD markers were generated for *C. gigas*, 16 for *C. nippona*, 18 for *C. ariakensis*, and 23 for *O. denselamellosa*. The average number of species-specific bands per primer was estimated to be 1.8 for *C. gigas*, 3.2 for *C. nippona*, 3.6 for *C. ariakensis*, and 4.6 for *O. denselamellosa*. The level of genetic similarity, based on Nei's estimation, between *Crassostrea* and *Ostrea*

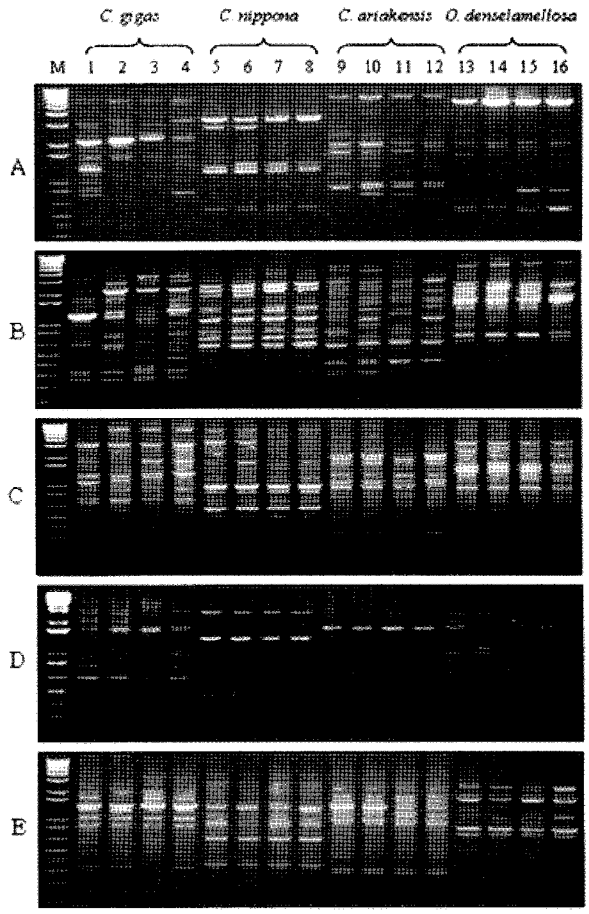

Fig. 1. RAPD profiles of four oyster species with URP1 (A), URP2 (B), URP3 (C), URP6 (D) and URP9 (E) primers. M, 1 kb plus ladder; Lanes 1-4, *C. gigas*; Lanes 5-8, *C. nippona*; Lanes 9-12, *C. ariakensis*; Lanes 13-16, *O. denselamellosa*.

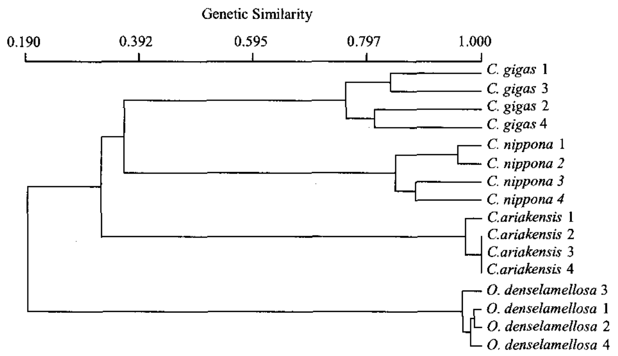

Fig. 2. Dendrogram illustrating genetic relationships among four oyster species generated by UPGMA cluster analysis calculated 102 RAPD bands produced by 5 primers. Number indicates the individuals from each species used.

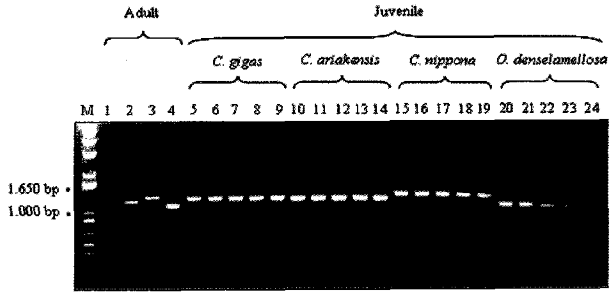

Fig. 3. Species identification of each juvenile oyster by ITS amplification. Lane 1-4, oyster adult shell; Lanes 5-24, oyster juveniles. Lane 1, *C. gigas*; lane 2, *C. ariakensis*; lane 3, *C. nippona*; lane 4, *O. denselamellosa*; lanes 5-9, *C. gigas*; lanes 10-14, *C. ariakensis*; lanes 15-19, *C. nippona*; lanes 20-24, *O. denselamellosa*. M, 1 kb plus DNA ladder (bp).

ranged from 0.097 to 0.192, whereas the similarity among the *Crassostrea* spp. ranged from 0.228 to 0.331. The level of genetic similarity between *C. gigas* and *C. nippona* (0.331) was slightly higher than that between *C. gigas* and *C. ariakensis* (0.301).

A genetic similarity matrix calculated from the RAPD fingerprinting data was used to estimate the phylogenetic relationships among the four species. A dendrogram generated using the genetic distances specified by our URP-PCR data and based on the similarity index is presented in Fig. 2. As expected, two groups, *Crassostrea* and *Ostrea*, were clearly separated. The *O. denselamellosa* individuals evaluated showed 97-99% similarity and were distantly related to *Crassostrea* spp. with 19% similarity. The level of genetic similarity among the *C. gigas* specimens was the lowest for all species analyzed (71-82%). The relationships detected among the four species indicate that *C. gigas* is more closely related to *C. nippona* than to *C. ariakensis*.

2. rDNA ITS sequence analysis

The universal primers ITS4 and ITS5 were used to amplify the partial 18S, ITS1, 5.8S, ITS2, and partial 28S regions of the Korean oyster genome. Using this set of primers, we consistently obtained distinct fragments that ranged in size from 1,114 to 1,319 bp, and which included partial 18S (54 bp) and 28S (59 bp) rDNA sequences. Due to the shortage of morphological characters available for taxonomic identification, four juveniles were analyzed by PCR based on the size differences of the ITS sequences. The species of each juvenile was determined based on a comparison to the PCR products from four adult oysters (Fig. 3). The length of the ITS-5.8S-ITS2 region varied from 1,001 to 1,206 bp, while the GC content ranged from 55.5 to 61.1%. The length of ITS1 ranged from 426 to 518 bp, and the GC content ranged from 56.8 to 61.8%. *Crassostrea nippona* had the longest ITS1 sequence (515-518 bp), and its GC content ranged from 57.8 to 58.4%. *Ostrea denselamellosa* had the shortest ITS1 sequence (426-427 bp), and its GC content ranged from 61.3 to 61.8%. The length of ITS2 varied from 418 to 536 bp, while the GC content ranged from 54.1 to 62.2%. *Crassostrea gigas* had the longest ITS2 sequence (535-536 bp), and its GC content ranged from 54.1 to 54.2%. *Ostrea denselamellosa* had the shortest ITS2 sequence (418 bp), and its GC content ranged from 61.9 to 62.2%. The sequence of the 5.8S rRNA gene was highly conserved, with a length of 157 bp in each species and a GC content of 56-57.3% (Table 1). The ITS1-5.8S-ITS2 sequences from the four species were aligned using the program MegAlign. The level of interspecific sequence divergence was greater in the ITS1 region than in the ITS2 region (from 10.8% between *C. nippona* and *C. ariakensis* to 71.6% between *C. gigas* and *O. denselamellosa* in the ITS1

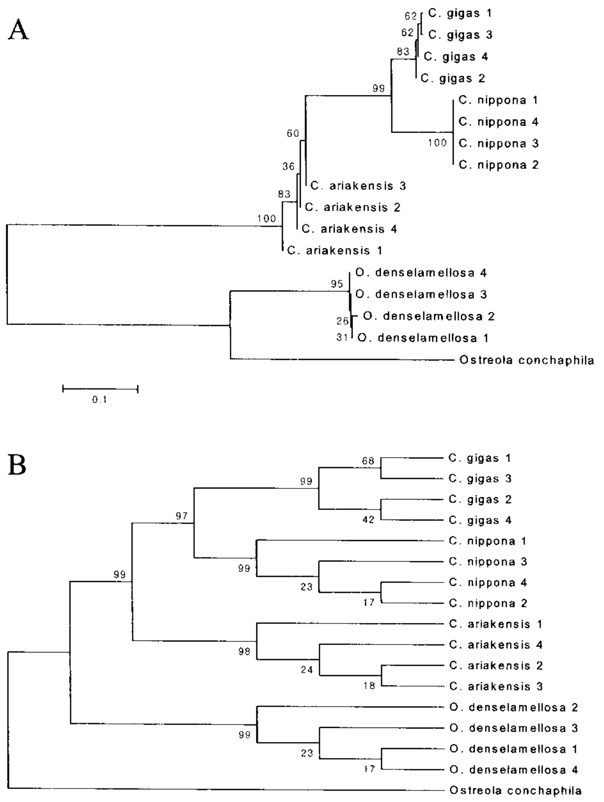

Fig. 4. Phylogenetic relationships of four oyster species based on ITS2 sequences, with *Ostreola conchaphila* as an outgroup species using Neighbor-Joining (NJ) method (A) and maximum parsimony (MP) method (B). Bootstrap values are shown as percentages at each node based on 1000 replicates.

region and from 15.8% between *C. gigas* and *C. ariakensis* to 45.5% between *C. nippona* and *O. denselamellosa* in the ITS2 region). The average sequence divergence (> 44%) between *Crassostrea* and *Ostrea* was remarkable regardless of the presence of insertions/deletions; moreover, clear genetic differentiation was detected between the genera. The average intraspecific sequence divergence ranged from 0.35% (*O. denselamellosa*) to 1.01% (*C. gigas*) in the ITS1 region and from 0.22% (*O. denselamellosa*) to 0.53% (*C. gigas*) in the ITS2 region. Unlike the considerable sequence variation observed in the ITS1 and ITS2 regions, only four variable sites were identified in the 5.8S rRNA gene. Moreover, the 5.8S rRNA genes in *C. gigas* and *C. nippona* were identical.

A phylogenetic tree was constructed for the four Korean oyster species using the ITS2 sequences by the NJ and MP methods using *O. conchaphila* (GenBank accession no. EF035118) as an outgroup species (Fig. 4). MP and NJ analyses of ITS1, ITS2, and ITS1-5.8S-ITS2 produced similar phylogenetic relationships with slight changes in the grouping of the individuals within each species. Because the bootstrap values in ITS2 were higher than in ITS1, ITS2 was used in our phylogenetic analysis. The genetic distances in the ITS2 region, based on pairwise comparisons of the transitions and transversions, ranged from 0.139 (between *C. gigas* and *C. nippona*) to 1.141 (between *C. gigas* and *O. denselamellosa*). *Crassostrea nippona* was placed as a sister taxon to *C. gigas*, and *C. ariakensis* was placed as a subclade relative to them. As expected, the genus *Crassostrea* formed a distinct group from the genus *Ostrea*.

DISCUSSION

Table 1. Sizes in base pairs (bp), percent GC content of ITS1, 5.8S rDNA, and ITS2 and genbank accession numbers of the four oyster species studied

Species	Total		ITS1		5.8S		ITS2		GenBank accession no
	Length (bp)	GC (%)	Length (bp)	GC (%)	Length (bp)	GC (%)	Length (bp)	GC (%)	
C. gigas	1,143-1,148	55.5-55.8	451-455	56.8-57.6	157	56	535-536	54.1-54.2	FJ356675-77, FJ356690
C. nippona	1,202-1,206	56.6-56.7	515-518	57.8-58.4	157	56	530-531	55.1-55.5	FJ356678-81
C. ariakensis	1,102-1,106	57.1-57.3	473-475	58.8-59.5	157	56-56.7	472-475	55.4-56.0	FJ356682-85
O. denselanellosa	1,001-1,002	60.9-61.1	426-427	61.3-61.8	157	56-57.3	418	61.9-62.2	FJ356686-89

RAPD analysis has been used to determine the genetic relationships and develop effective genetic markers for various organisms (Patwary *et al.*, 1994; Tassanakajon *et al.*, 1997; Heipel *et al.*, 1998; Klinbunga *et al.*, 2000), since morphological characters alone are often insufficient. Furthermore, the taxonomic identification of larval marine invertebrates at the species level or higher by morphological examination is notoriously difficult (Morgan and Rogers, 2001). Despite these problems, a simple molecular genetic method involving the use of three novel microsatellite loci was used as a highly sensitive and specific taxonomic indicator to identify larvae of the European oyster, *Ostrea edulis* (Morgan and Rogers, 2001). Prior to our study, the morphological classification of similar oyster species distributed or cultured in Korean waters was unclear, limiting the determination of new species and the development of hybrids for the improvement of specific oyster breeds.

RAPD analyses using random oligonucleotide primers that are ten bases long often produce uncertain results with a limited number of repeats and unexpected band appearance. However, the 12 URPs used in this study were 20 bp long and proved to be useful for species identification with as few as five URPs (URP1, 2, 3, 6, and 9), as each URP had species-specific bands. Similar to the report of Klinbunga *et al.* (2001), which showed that species-specific markers were required to unambiguously identify commercial oyster species in Thailand, our study was carried out to identify genetic markers that may be used for the identification of Korean oysters. The average number of bands per primer and the percentage of polymorphic bands were much greater for *Saccostrea* and *Striostrea* oysters than for *Crassostrea*, indicating a low level of genetic diversity in the latter group (Klinbunga *et al.*, 2001). The average number of bands per primer in our study was 20.4, which is lower than the number reported previously in *Saccostrea* and *Striostrea* oysters (Klinbunga *et al.*, 2001) as a result of the use of URPs that were relatively long (20 bp). Caetano-Anolles *et al.* (1992) reported that the use of 10-mers resulted in the amplification of non-specific bands leading to low reproducibility because of inadequate stringency at the annealing stage. Thus, the species-specific bands obtained in our study using URP1, 2, 3, 6, and 9 may be used as genetic markers for the discrimination of Korean oyster species. An UPGMA tree constructed from our genetic distance data illustrated clear separation between *Crassostrea* and *Ostrea* spp. According to our RAPD data, *C. gigas* is more closely related to *C. nippona* than to *C. ariakensis*. This result is consistent with speculations based on mitochondrial 16S rRNA and COI sequencing (Lee *et al.*, 2000).

The ITS regions were used to evaluate the possibility as markers for species identification and the determination of phylogenetic relationships among Korean oysters. Considering the condition of Korean coastal waters, possible hybridization due to similar spawning seasons, morphological similarities, and co-inhabitants makes the application of DNA sequence-based methods difficult. Especially, the identification of the larval or juvenile stages in bivalves is a huge challenge when there are no available external morphological characteristics. In this study, differences in length of ITS region makes identification of Korean four oyster larva or juvenile possible by simple and accurate PCR method. Other researchers who have used the ITS region have suggested that it is a good molecular marker for accurate and reliable species identification, especially for closely related species and individuals of the same species (Takabayashi *et al.*, 1998; Hsueh *et al.*, 2001; Kuwahara *et al.*, 2001).

The GC contents of the ITS1 (56.8-61.8%) and ITS2 (54.1-62.2%) regions in our study were similar and showed very little variation among the four species. The GC contents of the four Korean oyster species were higher than those of scallops (Insua *et al.*, 2003; ITS1 = 43-49% and ITS2 = 44-49%), but similar to those of the pearl oyster (He *et al.*, 2005; ITS2 = 51.9-55.5%). In bivalve species, the GC content is between 45 and 66% in ITS1 and between 45 and 68% in ITS2 (Freire, 2002); thus, the four Korean oyster species fall within the average range. The presence of microsatellites throughout the ITS region has been reported in a number of bivalve species (King *et al.*, 1999; Cheng *et al.*, 2006); however, no microsatellites were detected in the current study. In our analysis of the sequence of the 5.8S region, the Korean oyster species showed little variation, which is different from the result reported for three clam species (Fernández *et al.*, 2001). A high level of conservation was reported in the 5.8S region, which averages about 157 bp in length, as a common phenomenon in bivalves (Nazar, 1984; Insua *et al.*, 2003; He *et al.*, 2005; Cheng *et al.*, 2006).

Recently, molecular techniques such as DNA sequencing have been successfully used for intraspecific identification, to determine interspecific relationships, and to construct phylogenetic trees for populations and species. ITS regions are particularly useful for comparing closely related groups, such as species within a genus (Fernández *et al.*, 2001). Chu *et al.* (2001) showed that ITS1 is highly divergent among crustaceans and could be an appropriate marker for molecular systematic studies at the species and population levels, although the presence of intragenomic variation must be taken into consideration. The ITS1 and ITS2 regions of rDNA are widely used for phylogenetic analyses at and below the species level (Morgan and Blair, 1998; Yu *et al.*, 2000; Insua *et al.*, 2003; Cheng *et al.*, 2006) owing to the homogeneous characteristics of nuclear ribosomal sequences within species (Hills and Davis, 1998). The relationship observed between *Crassostrea* and *Ostrea* was clear in our ITS analysis. The close relationship between *C. gigas* and *C. nippona* was confirmed with high bootstrap values in both our ITS1-5.8S-ITS2 and ITS2 analyses, although the bootstrap value (47%) was low in ITS1. The previous phylogenetic data for 16S rRNA and COI showed the same sister groupings (Lee *et al.*, 2000). In this study, ITS1 had weak phylogenetic discrimination power in Korean oysters, whereas ITS2 and the ITS1-5.8S-ITS2 region were useful for identifying phylogenetic relationships among Korean oyster species. Therefore, ITS2 may be used for the efficient reconstruction of evolutionary relationships among these organisms. Considering our results, RAPD and ITS sequence analyses are useful tools for the identification of phylogenetic relationships and the selection of specific markers in Korean oyster species.

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