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Identification of Korean Suminoe Oyster (*Crassostrea ariakensis*) by RFLP Analysis

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The Suminoe oyster, *Crassostrea ariakensis*, occurs in estuaries where rivers meet seawater. In Korea, it is one of the most popular fisheries resources in the Nam River and Sumjin River. However, the genetic identification of this species has been questioned, because specimens are often mis-identified as other species. To identify the species, we conducted polymerase chain reaction (PCR) amplification of the internal transcribed spacer-1 (ITS-1) region, followed by digestion with the restriction enzyme *Hae*III. Restriction profiles for oysters collected from Korea, Japan, and China (north and south) were determined by comparing the PCR-restriction fragment length polymorphism (RFLP) patterns of the ITS-1 regions. Our study verified that the oysters collected from Korea were *C. ariakensis* based on the PCR-RFLP patterns. These results emphasize the value of molecular markers for identifying morphologically uncertain species.

Key words: Suminoe oyster, *Crassostrea ariakensis*, Species identification, RFLP

Introduction

Crassostrea ariakensis is a large flat oyster inhabiting hard intertidal grounds and muddy creeks of warm estuaries (Rao, 1987; Barkati and Khan, 1987). Natural populations of *C. ariakensis* have been reported in southern Japan (Cagbn, 1950; Torigoe, 1981; Quayle and Newkirk, 1989), along the west coast of Korea (Kuroda and Tadashige, 1952; Harry, 1981) and throughout the coastal area of China (Tchang and Lou, 1956; Cai and Li, 1990; Zhuang, 1992). However, the genetic identification of this species in Korea has been questioned because specimens are often mis-identified as *C. rivularis*. Recently, interest in *C. ariakensis* has grown because it is being developed as a native resource in Korea and serves as a substitute species for ecological reconstruction of the Chesapeake Bay in the USA. The increasing interest in this species necessitates accurate identification of *C. ariakensis* in Korea for the provision of fundamental information for resource management.

Genetic studies conducted to characterize species have mainly involved morphometric analyses. Morphology can be a reliable and useful taxonomic tool; however, species delineations based solely only morphology are undermined when taxa display variable or convergent morphologies (Avise, 1994). Reconstruction of phylogenetic relationships in mollusks, especially bivalves, depends heavily on morphological features for species identifications, despite the fact that shell shape can be influenced by environmental conditions (Seed, 1968; Boulding and Hay, 1993).

Various molecular techniques have been used to monitor genetic variation (Avise, 1994). As part of the multi-copy nuclear ribosomal RNA gene complex in eukaryotic organisms (Palumbi, 1996), the first internal transcribed spacer (ITS-1) region is a non-coding region located between the large (18S) and small (5.8S) subunits (Hillis and Dixon, 1991). Variation in ITS-1 presumably accumulates because it is less constrained by selection, making these regions useful for both intra- and interspecific genetic studies between closely related taxa.

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis is a standard technique for investigations of genetic characterization (Avise et al., 1987a, b). Genetic variability among individuals, caused by the gain or loss of sites in the mitochondrial or nuclear DNA, can

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be identified by the presence or absence of fragments after the DNA has been cut with one or more endonucleases and the resulting fragments have been separated by electrophoresis on an agarose gel (Avise, 1994). The RFLP technique is also a cost- and time-efficient approach for species identification.

In this study, we conducted PCR-RFLP analysis of nuclear DNA; ITS-1 markers were used to determine the species of oyster specimens collected from Korea by comparison to wild *C. ariakensis* sampled from Japan and China. In addition, the RFLP markers identified could be used as potential genetic tools to discriminate genetic units of *C. ariakensis*, which would assist genetic-based stock enhancement and breeding program designs.

Materials and Methods

Sample collection

Wild oysters with *C. rivularis* morphology, i.e., roughly round, flat, and thick shell valves and shell heights <10 cm, were collected in coastal areas of western Korea (Kahwa River, KR; Sumjin River, SR; and Kanghwa Island, K) from March to May 2004. Oyster tissues from China and Japan were obtained from colleagues at the Virginia Institute of Marine Science (VIMS) who collected *C. ariakensis* individuals along the coastal areas of northern China (Yellow River, YR), southern China (Shouchang River, S), and the Itoki River (I) in Japan from May to June 1999 (Fig. 1). Oyster tissues of *C. gigas* were also sampled by VIMS.

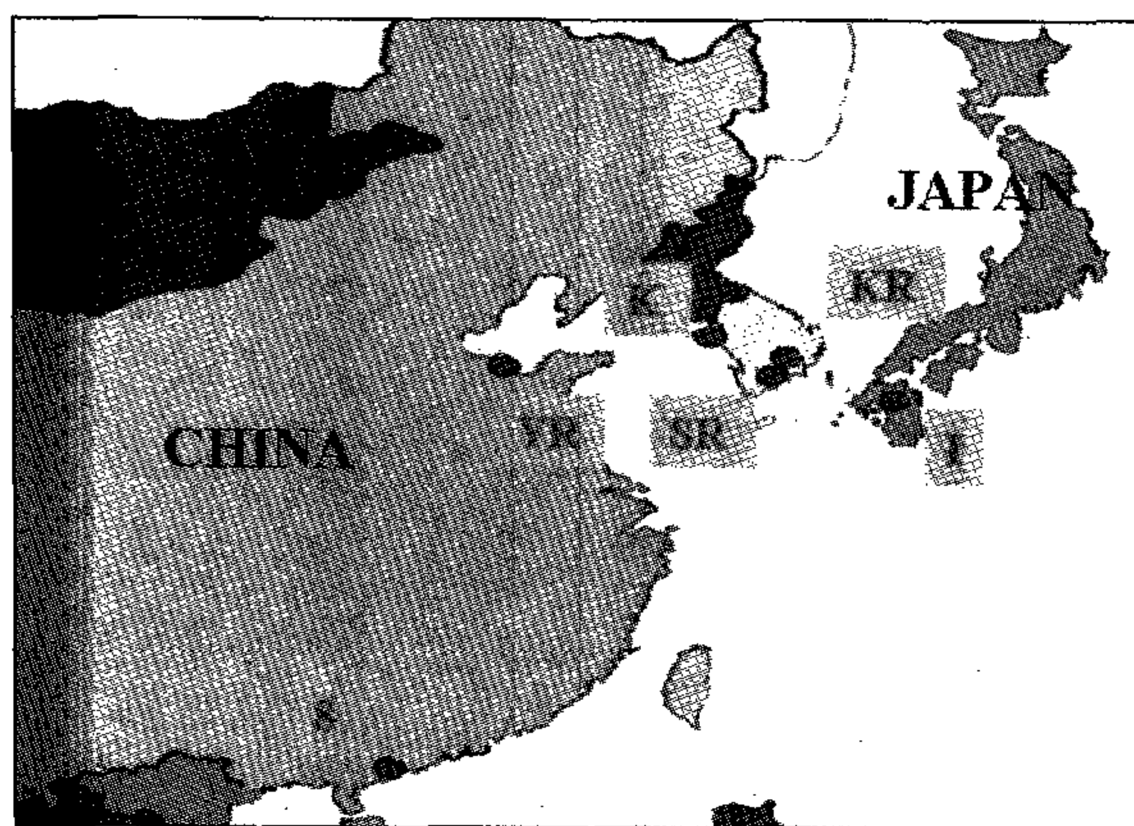

Fig. 1. Map of sampling locations. *K* indicates Kanghwa Island, Korea; *SR*, Sumjin River, Korea; *KR*, Kahwa River, Korea; *I*, Itoki River, Japan; *YR*, Yellow River, China; *S*, Shouchang River, China.

PCR-RFLP screening

Total genomic DNA was extracted from oyster mantle musculature using modified methods of Asahida et al. (1996). DNA was diluted with distilled water to final concentrations of 50 ng/μL. ITS-1 phenotypes of specimens were assayed for species identification. Primers were ITS-A: 5'-GGTTTCTG-TAGGTGAACCTGC-3' and ITS-B: 5'-CTGCGTTC-TTCATCGACCC -3' (Hedgecock et al., 1999). PCRs were performed in 25 μL total reaction volumes. Each reaction contained 2.5 μL of 10×*Taq* buffer (Invi-trogen Life Technologies, Carlsbad, CA, USA), 0.2 mM of each dNTP, 0.4 μM of each primer, 2 units of *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), and 2.5 μL of genomic DNA. Amplification was carried out in a RTC 200 machine (MJ Research Inc.). PCR amplification conditions were as follows: initial denaturation for 3 min at 95°C followed by 30 cycles of 95°C denaturation for 1 min, annealing at 52°C for 1 min, extension at 72°C for 1 min, and a final extension step of 5 min at 72°C. Amplification products were digested with the restriction endonuclease *Hae*III at 37°C for 2 hr. Restriction enzyme digestion was carried out in a 15 μL mixture containing 5 μL of amplified products, 8.2 μL distilled water, 1.5 μL 10x reaction buffer, and 0.3 μL (10 U/μL) *Hae*III (Invitrogen Life Technologies, Carlsbad, CA, USA). The digested fragments were separated on 1% agarose gel. The lengths of the digested fragments were estimated by comparison with a 1-kb plus DNA ladder (Invi-trogen Life Technologies, Carlsbad, CA, USA).

Results and Discussion

ITS-1 regions were amplified for 20 individuals from each of the three Korean populations (KR, SR, and K; Fig. 1). The fragment sizes of the ITS-1 region were approximately 535 bp in the samples from Korea, Japan, and northern China and 520 bp in the samples from southern China (data not shown). The 60 specimens from the three Korean populations showed no within-population variation, with the exception of one individual from the KR population (Fig. 2). One sample showed a different pattern that was consistent with *C. gigas*, the most abundant oyster species in Korea. The RFLP patterns were compared to five samples collected from Korea, Japan, and northern and southern China to confirm the species identification. Species diagnoses by PCR-RFLP patterns were concordant with 15 samples from Korea, Japan, and northern China (Fig. 3). *C. ariakensis* collected from southern China had a significantly different pattern (Fig. 3). This result concurs with a previous report in which significant genetic differentiation was observed between the geo-

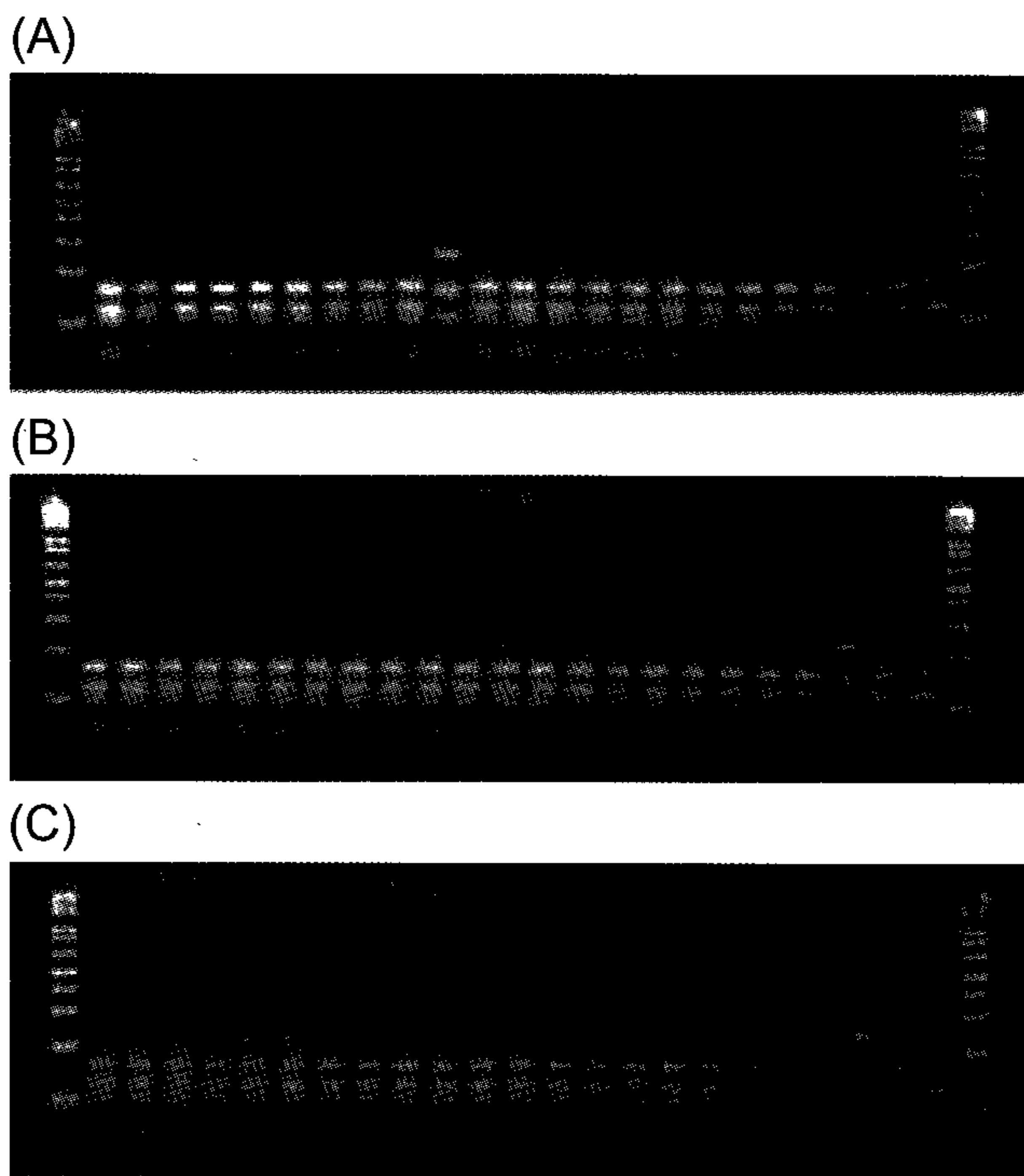

Fig. 2. PCR-RFLP analysis patterns of the partial ITS-1 region RCR-amplified fragments from *C. ariakensis* of three populations in Korea. (A), KR population; (B), K population; (C), SR population. The last three lanes on each gel are *C. gigas*, *C. ariakensis* from northern China and *C. ariakensis* from southern China. M indicates the 1kb plus DNA ladder.

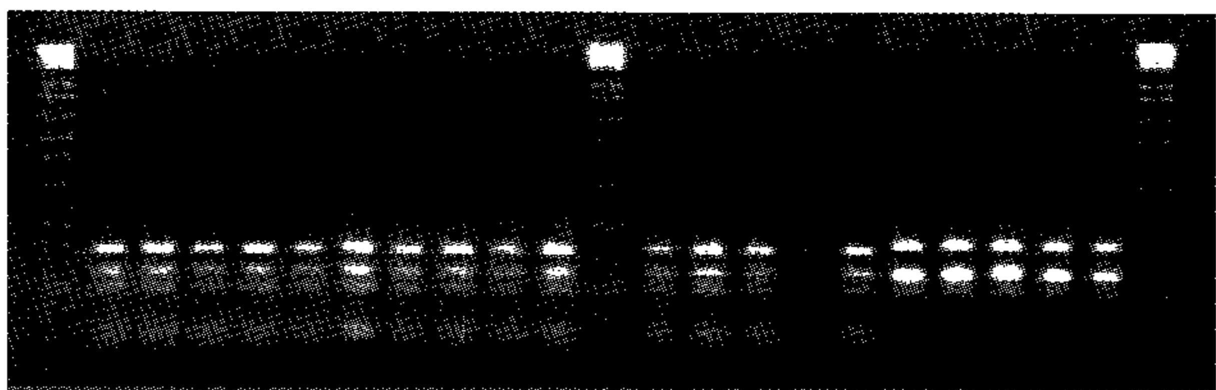

Fig. 3. PCR-RFLP analysis patterns of the partial ITS-1 region RCR-amplified fragments from *C. ariakensis* in Korea, Japan and China. Lane 1-5, Korean population; lane 6-10, IR population; lane 11-15, YR population; lane 16-20, S population. M indicates the 1kb plus DNA ladder.

graphically separated northern (I and YR) and southern (Zhuhai and Yangjiang) specimens that were morphologically identified as *C. ariakensis* (Zhang et al., 2005). Preliminary phylogenetic analyses based on DNA sequence data of mitochondrial cytochrome oxidase I and nuclear ITS-1 regions suggested that the *C. ariakensis* samples from Japan and northern China might show genetic differentiation from southern China individuals, or that they might even represent different subspecies or sister species. Carriker and Gaffney (1996) noted that some *C. ariakensis* distribution reports needed to be viewed with caution since the putative range of distribution could be due to misclassification or incorrect use of the species name. The history of *C. ariakensis* nomenclature is confusing (Zhou and Allen, 2003), but Torigoe (1981) suggested that the species referred to as *C. rivularis* was the same as *C. ariakensis*. However, the identi-fication of *C. ariakensis* in Korea was questioned because of the confusion over scientific names. Therefore, we required genetic analyses to discri-minate these morphologically plastic species and to validate the accurate species name. Most of the *C. rivularis* individuals collected from the three different geographic locations on the western coast of Korea were of the same species as wild *C. ariakensis* oysters collected from Japan and northern China (Figs. 2 and 3).

PCR-RFLP analysis of the ITS-1 region proved very efficient for the identification of *C. ariakensis* individuals in Korea. Although the DNA fragment included only a portion of the ITS-1 region, all of the species examined were clearly distinguishable. Consequently, RFLP analysis through *Hae*III digestion of the ITS-1 region could serve as a useful molecular marker for species identification of *C. ariakensis* in Korea.

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