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Genetic distances of three venerid species identified by PCR analysis

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ABSTRACT

The seven selected primers BION-13, BION-29, BION-61, BION-64, BION-68, BION-72 and BION-80 generated the total number of loci, average number of loci per lane and specific loci in *Meretrix lusoria* (ML), *Saxidomus purpuratus* (SP) and *Cyclina sinensis* (CS) species. Here, the complexity of the banding patterns varied dramatically between the primers from the three venerid clam species. The higher fragment sizes (> 1,000 bp) are much more observed in the SP species. The primer BION-68 generated 21 unique loci to each species, which were ascertaining each species, approximately 150 bp, 300 bp and 450 bp, in the ML species. Remarkably, the primer BION-80 detected 7 shared loci by the three clam species, major and/or minor fragments of sizes 500 bp, which were matching in all samples. As regards average bandsharing value (BS) results, individuals from CS clam species (0.754) exhibited higher bandsharing values than did individuals from SP clam species (0.607) ($P < 0.05$). In this study, the dendrogram obtained by the seven oligonucleotides primers indicates three genetic clusters: cluster 1 (LUSORIA01-LUSORIA07), cluster 2 (PURPURATUS08-PURPURATUS14), cluster 3 (SINENSIS15-SINENSIS21). Among the twenty one venerid clams, the shortest genetic distance that displayed significant molecular differences was between individuals 18 and 20 from the CS species (genetic distance = 0.071), while the longest genetic distance among the twenty-one individuals that displayed significant molecular differences was between individuals LUSORIA no. 02 and PURPURATUS no. 09 (genetic distance = 0.778). Relatively, individuals of SP venerid species were appropriately closely related to that of CS species, as shown in the hierarchical dendrogram of genetic distances. Eventually, PCR fragments exposed in the present study may be worthwhile as a DNA marker the three venerid clam species to discriminate.

Key words: *Cyclina sinensis*, Genetic cluster, Genetic distance, *Meretrix lusoria*, *Saxidomus purpuratus*, Venerid.

INTRODUCTION

Meretrix lusoria (ML) is commercially important bivalves, belonging to order Veneroida, family Veneridae, widely distributed on the coast of the Yellow Sea, the southern sea and Jeju island in the Korean Peninsula and the several sea areas in China under the natural ecosystem (Min *et al.*, 2004). They widely distributed in the sandy tidal flat, the intertidal zone and 20-meter depth of seawater areas. This shellfish eat sessile diatoms and microscopic algae.

Saxidomus purpuratus (SP) is, environmentally warmwater bivalve species, belonging to order Veneroida, family Veneridae, widely distributed on the coast of the Yellow Sea, southern sea and Jeju Island in the Korean Peninsula and the Bo Hai of China under the natural ecosystem. Under the natural ecosystem, the clams inhabit in the estuary flats consisting of a lot of mud, sand and slime in the coastal tidal wetland where the freshwater is flowed momentarily. Like other clams basically, the rate at which the clam grows depends very much on water quality. This shellfish is black and grey in shell color with ridges and growth rings on shell surface. Their

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larvae eat sessile diatoms, microscopic algae and some debris.

Cyclina sinensis (CS) is one species of an economically important bivalve species, belonging to order Veneroida, and family Veneridae. They mainly dwell in the estuary flats consisting of a lot of sand, mud and slime in the coastal tidal wetland. This clam is widely distributed in the field of reeds and seawater habitats of the Yellow Sea and the southern sea such as the coasts of Incheon, Taean, Seocheon, Gunsan, Suncheon, and Namhae in the Korean Peninsula. The clams are silvery white and coarse in the shell surface under natural conditions. The ribs of the shell surface are compact and yellowish brown or light gray.

As a rule, there are marked differences of the size, color and shape in three shellfish species according to the environmental factors such as geography, water depth, water temperature, nutrition, and winterization period. There is a need to understand the genetic traits and composition of their shellfish so as to evaluate exactly the patent genetic effect. Our authors perform clustering analyses to elucidate the genetic distances among three malacological species of *Meretrix lusoria*, *Saxidomus purpuratus* and *Cyclina sinensis* in the Yellow Sea of the Korean peninsula. However, these species of Korean shellfish, which are well-known important physiologically (Ju and Lee, 2011), reproductively (Shin *et al.*, 2007), economically (Kim *et al.*, 2001; Kim *et al.*, 2007; Jin *et al.*, 2011) as well as biochemically (Mahmoud *et al.*, 2010; Kim *et al.*, 2011) are not genetically studied or researched like other crustaceans. Therefore, in the present study, genomic DNAs isolated from three venerid clam species were analyzed by seven oligonucleotides primers so as to make out the genetic distances through investigating their genetic similarity and diversity.

MATERIALS AND METHODS

1. Sample collection and purification of genomic DNA

Adductor muscle tissues were collected separately from three ML, SP and CS species, respectively. These shellfish muscles were collected in sterile tubes, placed on ice immediately, and stored at - 40℃ until needed. PCR analysis was performed on DNA samples extracted from a total of 21 individuals using seven oligonucleotides primers. DNA extraction should be carried out according to the separation and extraction methods (Yoon and Kim, 2004; Park *et al.*, 2005). After several washings, lysis buffer Ⅰ (155 mM NH_4Cl; 10 mM $KHCO_3$; 1 mM EDTA) was added to the samples, and the mixture tubes were gently inverted. Ice-cold 70% ethanol was added, and then the samples were centrifuged at 19,621 g for 5 minutes to extract the DNA from the lysates. The DNA pellets were incubation-dried for 2 hrs, held at - 40℃ until analysis, and then dissolved in the TE buffer (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentrations of the extracted genomic DNA samples were estimated based on the absorbance at 260 nm by a spectrophotometer (Beckman Coulter, Buckinghamshire, UK).

2. Amplification conditions and data analyses

Amplification products were separated by electrophoresis in 1.4% agarose gels with TBE, using 100 bp DNA ladder (Bioneer Corp., Daejeon, Korea) as DNA molecular weight marker and detected by staining with ethidium bromide. The electrophoresed agarose gels were illuminated by ultraviolet rays, and photographed using a photoman direct copy system (PECA Products, Beloit, WI, USA). We used the oligonucleotides primers to elucidate the genetic distances and geological variations of shellfish individuals. Seven primers, BION-13 (5'-CAGCACCCAT-3'), BION-29 (5'-GACATCTCGC-3'), BION-61 (5'-GACCGCTTGT-3'), BION-64 (5'-CCACTCACCG-3'), BION-68 (5'-CCTTGACGCA-3'), BION-72 (5'-CTTAGGGCAC-3'), and BION-80 (5'-GGGAGGCAAA-3') were shown to create the unique shared loci to each species and shared loci by the three clam species which could be manifestly counted. Thus, we used the oligonucleotides primers to determine the genetic differences and variations of the three shellfish species. PCR was performed using programmable DNA Thermal Cycler (MJ Research Inc., Waltham, MA, USA). Similarity matrix including bandsharing values between different individuals in the three venerid clam species was

generated allowing formula of Jeffreys and Morton (1987) and Yoke-Kqueen and Radu (2006). The degree of variability was calculated by use of the Dice coefficient (*F*), which is given by the formula: $F = 2\ n_{ab} / (n_a + n_b)$, where nab is the number of bands shared between the samples a and b, na is the total number of bands for sample a and nb is the total number of bands for sample b. A hierarchical clustering tree was assembled using similarity matrices to yield a dendrogram, which was assisted by the Systat version 10 (SPSS Inc., Chicago, IL, USA). The Systat software was also used to analyze genetic differences, Euclidean genetic distances within and between venerid clam species, means, standard errors, and *t*-test scores.

RESULTS AND DISCUSSION

The number of total loci, average loci per lane, specific loci was calculated by analytical method using 7 oligonucleotides primers from venerid clam individuals from ML, SP and CS species, respectively, as showed in Table 1. The seven selected primers BION-13, BION-29, BION-61, BION-64, BION-68, BION-72 and BION-80 generated the total number of loci, average number of loci per lane and specific loci in ML, SP and CS species. Here, the complexity of the banding patterns varied dramatically between the primers from the three venerid clam species. The higher fragment sizes (> 1,000 bp) are much more observed in the SP species, as shown in Fig. 1. The number of unique shared loci to each clam species and number of shared loci by the three clam species generated by PCR analysis using 7 oligonucleotides primers in the three venerid clam species, respectively, as summarized in Table 2. The primer BION-68 generated 21 unique loci to each species, which were ascertaining each species, approximately 150 bp, 300 bp and 450 bp, in the ML species. Remarkably, the primer BION-80 detected 7 shared loci by the three clam species, major and/or minor fragments of sizes 500 bp, which were matching in all samples. As regards average bandsharing value (BS) results, individuals from CS species (0.754) exhibited higher bandsharing values than did individuals from SP species (0.607) ($P < 0.05$), as demonstrated in Table 3.

In the present study, the dendrogram obtained by the seven oligonucleotides primers indicates three genetic clusters: cluster 1 (LUSORIA01-LUSORIA07),

Table. 1. The number of total loci, average loci per lane, specific loci by PCR analysis using 7 oligonucleotides primers from three venerid clam species in the Yellow Sea

Item	No. of average loci per lane			No. of specific loci		
Primer	ML	SP	CS	ML	SP	CS
BION-13	6.86 (48)	7.71 (54)	6.29 (44)	41	47	23
BION-29	6.14 (43)	7.71 (54)	7.86 (55)	29	54	34
BION-61	2.71 (19)	5.43 (38)	4.86 (34)	12	38	27
BION-64	6.29 (44)	5.86 (41)	6.29 (44)	37	41	30
BION-68	6.57 (46)	7.14 (50)	8.71 (61)	25	43	47
BION-72	5.14 (36)	9.57 (67)	10.42 (73)	29	53	52
BION-80	3.71 (26)	4.71 (33)	7.14 (50)	19	26	36
Total no.	262	337	361	192	302	249
Average no. per primer	37.4	48.1	51.6	27.4	43.1	35.6

The total number of loci generated by 7 primers in *Meretrix lusoria* (ML), *Saxidomus purpuratus* (SP) and *Cyclina sinensis* (CS).

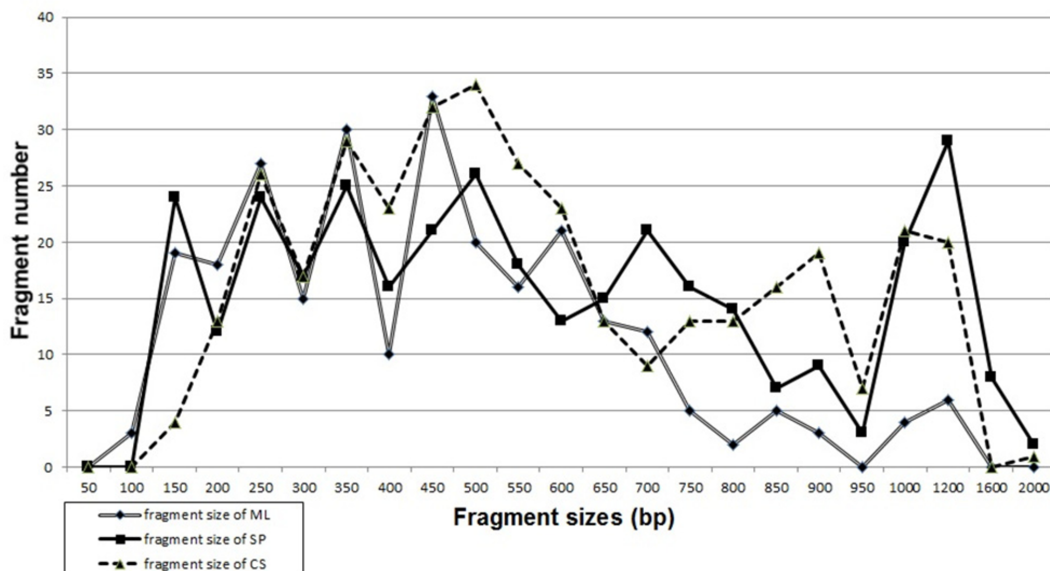

Fig. 1. Distribution of fragment sizes of ML, SP and CS venerid clam species. Solid lines: SP. Dotted lines: CS species. Thin lines: ML species. The fragment numbers in each size interval have been computed from the pooled fragments obtained with all the primers. The higher fragment sizes (> 1,000 bp) are much more observed in the SP species.

Table. 2. The number of unique shared loci to each clam species and number of shared loci by the three clam species generated by PCR analysis using 7 oligonucleotides primers in the three venerid clam species, respectively

Item	No. of unique loci to each clam species			No. of shared loci by the three clam species
Primer \ Species	ML	SP	CS	Three species (7 individuals per species)
BION-13	7	7	21	0
BION-29	14	0	21	0
BION-61	7	0	7	0
BION-64	7	0	14	0
BION-68	21	7	14	0
BION-72	7	14	21	0
BION-80	7	7	14	7
Total no.	70	35	112	7
Average no. per primer	10	5	16	1

ML: *Meretrix lusoria*, SP: *Saxidomus purpuratus*, CS: *Cyclina sinensis*

cluster 2 (PURPURATUS08-PURPURATUS 14), cluster 3 (SINENSIS15-SINENSIS21), as demonstrated in Fig. 2. Among the twenty one venerid clams, the shortest genetic distance that displayed significant molecular differences was between individuals 18 and 20 from the CS species (genetic distance = 0.071), while the longest genetic distance among the twenty-one individuals that displayed significant molecular differences was between individuals LUSORIA no. 02 and PURPURATUS no. 09 (genetic distance = 0.778). Relatively, individuals of SP species were appropriately closely related to that of CS species, as shown in the hierarchical dendrogram of genetic distances. Above-mentioned, a dendrogram

Table 3. Multiple comparisons of average bandsharing values among three shellfish species were created according to the bandsharing values and similarity matrix (P < 0.05)

Species	ML	SP	CS
ML	0.678 ± 0.201[b]	0.402 ± 0.057[d]	0.360 ± 0.056[d]
SP	-	0.607 ± 0.244[c]	0.384 ± 0.058[d]
CS	-	-	0.754 ± 0.159[a]

ML: *Meretrix lusoria*, SP: *Saxidomus purpuratus*, CS: *Cyclina sinensis*

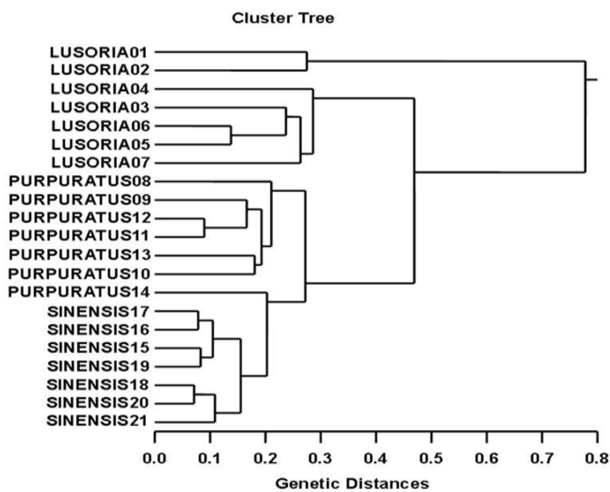

Fig. 2. Hierarchical dendrogram of genetic distances obtained from three venerid clam species. The relatedness among different individuals of *Meretrix lusoria* (LUSORIA01-LUSORIA07), *Saxidomus purpuratus* (PURPURATUS08-PURPURATUS14) and *Cyclina sinensis* (SINENSIS15-SINENSIS21) generated according to the bandsharing values and similarity matrix.

revealed close relationships between individual features within three geographical bivalve populations (McCormack *et al*., 2000). In invertebrates, cluster analysis of the pairwise population matrix, generated from genetic data, showed that geographically close populations be inclined to cluster together in the blacklip abalone (Huang *et al*., 2000).

Three venerid species can be evidently discriminated, by PCR-based approach. The potential of oligonucleotides amplified polymorphic DNAs to discover diagnostic markers for breed, species and population identification in shellfish (Huang *et al*., 2000; McCormack *et al*., 2000; Kim *et al*., 2004; Park *et al*., 2005) has also been well established. PCR fragments revealed in this study may be valuable as a DNA marker the three venerid species to segregate. Overall, the grouping of venerid species is founded on morphological variations in shell type, shell color, shell length and adductor muscle length. It is implicated that differences in such traits reflect distinctive origins or genetic identity (Chenyambuga *et al*., 2004). High levels of a significant genetic distance among three venerid clam species exhibited this PCR approach is one of the most suitable tools for individuals and/or populations biological DNA studies (Tassanakajon *et al*., 1998; Yoon and Kim, 2003). Therefore, this process can also be applied to other family of Veneridae and make technically-convenient the analysis of many samples in a short time.

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