Genetic Distances of Three White Clam (*Meretrix lusoria*) Populations Investigated by PCR Analysis

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ABSTRACT: The twenty-one individuals of *Meretrix lusoria* were secured from Gunsan, Shinan and Yeonggwang on the coast of the Yellow Sea and the southern sea in the Korean Peninsula, respectively. Amplification of a single COI fragment (720 bp) was imagined, and no apparent size differences were observed in amplified fragments between *Meretrix lusoria* and *M. petechialis* individuals. The size of the DNA fragments also varied excitedly, from 200 to 1,600 bp. The oligonucleotides primer BION-08 produced the least loci (a total of 17), with an average of 2.43 in the Gunsan population, in comparison to the other primers used. Remarkably, the primer BION-13 detected 42 shared loci by the three populations, major and/or minor fragments of sizes 200 bp and 400 bp, respectively, which were identical in all samples. The dendrogram gained by the seven oligonucleotides primers highlight three genetic clusters: cluster 1 (GUNSAN 01 ~ GUNSAN 07), cluster 2 (SHINAN 08 ~ SHINAN 14) and cluster 3 (YEONGGWANG 15 ~ YEONGGWANG 21). The longest genetic distance among the twenty-one *Meretrix lusoria* individuals that displayed significant molecular differences was between individuals GUNSAN no. 01 and SHINAN no. 14 (genetic distance = 0.574). Comparatively, individuals of SHINAN population were fairly closely related to that of YEONGGWANG population. In this study, PCR analysis has discovered significant genetic distances between two white clam population pairs (P<0.05).

Key words: Genetic cluster, Genetic distance, Hierarchical dendrogram, *Meretrix lusoria*, Veneridae, White clam

INTRODUCTION

Asian white clams (*Meretrix lusoria*) is commercially important bivalves, belonging to 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). *Meretrix* is widely distributed in the sandy tidal flat, the intertidal zone and 20-meter depth of seawater areas. Generally, *Meretrix petechialis* can be easily distinguished from *M. lusoria* by morphology, with the posterior dorsal margin of *M. lusoria* being straight, while that of *M. petechialis* is quite swollen, and the apex position of *M. lusoria* is skewed to the anterior side relative to that of *M. petechialis* (Yamakawa & Imai, 2012). But, juveniles of *M. lusoria* and *M. petechialis* have very similar morphologies and shell colors, making species identification difficult at the juvenile stage. Currently, DNA-based techniques for
identifying interspecific variation have been established and applied to some bivalve species, including closely related species belonging to the same Genus. Studies on molecular phylogeny of Veneridae were reported genetic relationship of Veneridae five species (Jung et al., 2004), were announced using mitochondrial 16S rRNA gene or cytochrome oxidase sequencing (Chen et al., 2009). But, no studies have tested for the identification of *M. lusoria* and *M. petechialis* in some Korean localities.

In the present study, to elucidate characteristics of individuals and populations by identifying the genetic distances and geographical variations among three white clam (*Meretrix lusoria*) populations collected from Gunsan, Shinan and Yeonggwang, we performed a clustering analysis by using PCR method and Systat pc-package program.

**MATERIALS AND METHODS**

1. Sample collection and purification of genomic DNA

The twenty-one individuals of *Meretrix lusoria* were secured from Gunsan, Shinan and Yeonggwang on the coast of the Yellow Sea and the southern sea in the Korean Peninsula, respectively (Fig. 1). Muscle tissues was collected in sterile tubes and stored at −40°C until needed. DNA extraction should be carried out according to the separation and extraction methods (Yoon and Kim, 2004). The precipitates obtained were then centrifuged and resuspended in lysis buffer II (10 mM Tris-HCl, pH 8.0; 10 mM EDTA; 100 mM NaCl; 0.5% SDS), and 15 μL of proteinase K solution (10 mg/mL) was added. After incubation, we added 300 μL of 3 M NaCl, and gently pipetted for a few minutes. 600 μL of chloroform was then added to the mixture and inverted (no phenol). 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.

2. Mitochondrial DNA extraction

Muscle was collected in sterile tubes, washed with PBS (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA) was added to the samples. Homogenize the muscle tissue and centrifuge at 740 g for 5 min. Collect the supernatant and discard the pellet and centrifuge again at 740 g for 5 min. Collect the supernatant, discard the pellet and centrifuge at 9,000 g for 10 min. Discard the supernatant and gently resuspend the crude mitochondria. Centrifuge mitochondrial suspension at 10,000 g for 10 min. Remove the supernatant and gently resuspend the crude mitochon-
drial pellet. Centrifuge mitochondrial suspension again
10,000 g for 10 min to remove any microsomal conta-
mination. Get rid of the supernatant and resuspend the
crude mitochondrial pellet. The crude mitochondrial pellet
should be carried out according to the separation and
extraction methods (Wieckowski et al. 2009). Mitochondrial
DNA of crude mitochondrial pellet was extracted using a
DNA blood and tissue kit (Qiagen GmbH, Duesseldorf,
Germany).

3. Mitochondrial DNA assay

Mitochondrial DNA analysis was performed on the
muscle extract of 30 individuals using specific primer. 720
bp fragment of the mitochondrial cytochrome c oxidase
subunit I (COI) was amplified by PCR using Thermal
cyclers (MJ Research, Inc., Watertown, USA; Perkin Elmer
Cetus, Norwalk, USA). PCR reaction was performed in 25
µL samples, which contained 2 µL of template DNA, 20
µL of premix (Bioneer Corp., Daejeon, Korea), and 1 µL
each of 25 pM primers (Forward : 5'-GGT CAA CAA
ATC ATA AAG ATA TTG G-3' and Reverse : 5'-TAA
ACT TCA GGG TGA CCA AAA AAT CA-3'; Folmer et
al., 1994). This premix was followed a pre-denaturation at
94°C for 2 min. The thermal cycler programmed for 30
cycles of denaturation at 94°C for 15 sec, annealing
at 50°C for 15 sec, extension at 72°C for 30 sec, post-extension at
72°C for 7 min.

RFLP surveys of the COI region indicated that Hinc II
(Takara, Inc., Shiga, Japan) digestion produced heterogeneous
distributions of restriction types among samples. RFLP
analysis was performed in 20 µL volume containing 2 µL
10XM buffer (Takara, Inc., Shiga, Japan), 1 µL of Hinc II
and 6 µL of PCR product at 37°C for 2 h. A 20 µL portion
of the reactant was generated electrophoresis on 1% agarose
(VentechBio, Eumsung, Korea) gel containing TBE (90
mM Tris, pH 8.5; 90 mM borate; 2.5 mM EDTA). Gels
were stained with ethidium bromide, illuminated by ultra-

4. Oligonucleotides primers, molecular markers,
amplification conditions and data analysis

Seven oligonucleotides primers BION-01 (5'-CAGGC
CCTTC-3'), BION-08 (5'-TCCGCTCTGG-3'), BION-13
(5'-GTTCGCTCC-3'), BION-17 (5'-TGCTCTGCCC-3'),
BION-47 (5'-CAGCACCCAC-3'), BION-49 (5'-CGGTGG
CGAA-3') BION-69 (5'-GCATCCACCA-3') were shown
to generate average loci per lane and specific loci which
could be clearly scored. Thus, the authors used the primers
to study the genetic variations and DNA polymorphisms of
the Meretrix lusoria. PCR was performed using two Pro-
grammable DNA Thermal Cyclers (MJ Research, Inc.,
Watertown, USA; Perkin Elmer Cetus, Norwalk, USA).
PCR conditions was preheating at 94°C for 5 min followed
by 45 cycles of denaturation at 94°C for 1 min, annealing
at 36°C for 1 min, and extension at 72°C for 1 min, and
then a post-cycle extension at 72°C for 5 min, using
the fastest available transition between each temperature.
Optimal DNA concentrations for amplification were deter-
mined by testing several dilutions, one of which was taken
as the standard for every subsequent amplification. Ampli-
fication products were generated via electrophoresis on 1.4%
agarose (VentechBio, Eumsung, Korea) gel containing
TBE (90 mM Tris, pH 8.5; 90 mM borate; 2.5 mM EDTA).
The 100 bp DNA Ladder (Bioneer Corp., Daejeon, Korea)
was used as a DNA molecular weight marker. Bands were
detected by ethidium bromide staining. The stained agarose
gels were illuminated by ultraviolet rays, and photographed
using a photoman direct copy system (PECA Products,
Beloit, WI, USA).

The degree of variability was calculated by use of the
Dice coefficient \( F \), which is given by the formula:
\[
F = 2 \frac{n_{ab}}{n_a + n_b},
\]
where \( n_{ab} \) is the number of bands shared
between the samples a and b, \( n_a \) is the total number of
bands for sample a and \( n_b \) is the total number of bands for
RESULTS AND DISCUSSION

1. Mitochondrial DNA assay

Amplification of a single COI fragment (720 bp) was imagined, and no apparent size differences were observed in amplified fragments between Meretrix lusoria and M. petechialis individuals. Hinc II digestion of PCR products discovered two unique restriction patterns designated type A (418 bp and 306 bp fragments) and type B (479 and 247 bp), that were completely diagnostic to distinguish M. lusoria from M. petechialis individuals (Yamakawa & Imai, 2012). But no one of the three populations sampled in Korea contained M. petechialis restriction type A phenotype (Table 1 and Fig. 2). In general, the adult shell shape of M. lusoria is a curved triangle, while that of M. petechialis is elliptical. The caudal dorsal edge of M. lusoria is straight, whereas that of M. petechialis is puffy, and the top position of M. lusoria is slanted to the front side relative to that of M. petechialis (Min et al., 2004). Identifying Meretrix lusoria based only on morphological shell shape can be difficult while judgments based on a combination of morphology and genetic information is generally more trustworthy.

2. Data analysis

The amplified products were separated by agarose gel electrophoresis with oligonucleotides primers, and stained with ethidium bromide. Similarity matrix including band-sharing values (BS) and genetic differences was calculated using Nei and Li’s index of the similarity of venerid clam individuals from Gunsan, Shinan and Yeonggwang of the Korean Peninsula, respectively, as illustrated in Table 2. The seven oligonucleotides primers BION-01, BION-08, BION-13, BION-17, BION-47, BION-49 and BION-69 generated the total number of loci, average number of loci per lane and specific loci in Gunsan, Shinan and Yeonggwang

Table 1. Collected Meretrix lusoria samples

| Location | No. | Hinc II restriction type |
|----------|-----|-------------------------|
| GS       | 10  | 0 10                    |
| SA       | 10  | 0 10                    |
| YG       | 10  | 0 10                    |

Frequencies of Hinc II restriction types of the mitochondrial DNA cytochrome c oxidase subunit I.

GS: Gunsan, SA: Shinan, YG: Yeonggwang
population, as summarized in Table 3. Here, the complexity of the banding patterns varied dramatically between the primers from the three populations. The size of the DNA fragments also varied excitedly, from 200 to 1,600 bp, as shown in Fig. 3. The primer BION-01 generated the most loci (a total of 58), with an average of 8.29 in the Shinan population, as revealed in Table 3. The oligonucleotides primer BION-08 produced the least loci (a total of 17), with an average of 2.43 in the Gunsan population, in comparison to the other primers used. In this study, 7 primers generated 34.7 specific loci in the Gunsan population, 42.6 in the Shinan population and 40.1 in the Yeonggwang population. The specific loci generated by oligonucleotides primers demonstrated inter-individual-specific characteristics, thus revealing DNA polymorphisms. Many researchers considered the sizes of DNA fragments

Table 2. Similarity matrix including bandsharing values (BS) and genetic differences calculated using Nei and Li’s index of the similarity of *Meretrix lusoria* from Gunsan, Shinan and Yeonggwang of the Korean Peninsula, respectively

|       | BS from Gunsan | BS from Shinan | BS from Yeonggwang |
|-------|----------------|----------------|-------------------|
| 1     | - 0.710        | 0.545          | 0.543             |
| 2     | - 0.649        | 0.559          | 0.695             |
| 3     | - 0.574        | 0.592          | 0.566             |
| 4     | - 0.591        | 0.722          | 0.697             |
| 5     | - 0.697        | 0.702          | 0.553             |
| 6     | - 0.666        | 0.472          | 0.452             |
| 7     | - 0.480        | 0.513          | 0.450             |
| 8     | - 0.913        | 0.867          | 0.852             |
| 9     | - 0.867        | 0.803          | 0.752             |
| 10    | - 0.820        | 0.731          | 0.723             |
| 11    | - 0.853        | 0.865          | 0.818             |
| 12    | - 0.887        | 0.840          | 0.721             |
| 13    | - 0.918        | 0.782          | 0.759             |
| 14    | - 0.813        | 0.730          | 0.718             |
| 15    | - 0.780        | 0.722          | 0.720             |
| 16    | - 0.820        | 0.725          | 0.696             |
| 17    | - 0.828        | 0.783          | 0.701             |
| 18    | - 0.815        | 0.652          | 0.751             |
| 19    | - 0.662        | 0.815          |                   |
| 20    | - 0.663        |                |                   |
| 21    |                |                |                   |
Fig. 3. PCR-based electrophoretic profiles of individuals *Meretrix lusoria*. DNA isolated from Gunsan population (lane 1 ~ 7), Shinan population (lane 8 ~ 14) and Yeonggwang population of *Meretrix lusoria* (lane 15 ~ 21) were amplified by oligonucleotides decamer primers BION-1 (A), BION-8 (B), BION-13 (C), BION-17 (D), BION-47 (E), BION-49 (F) and BION-69 (G). The PCR products were divided by 1.4% agarose gel electrophoresis and detected by ethidium bromide staining. Each lane displays DNA samples extracted from 21 individuals. 100 bp ladder was used as a DNA molecular size maker (M).
in the PCR profiles of five species of Eastern Pacific abalone (genus *Haliotis*) (Muchmore et al., 1998), the brittle star (*Amphiura filiformis*) (McCormack et al., 2000), oyster (Kim et al., 2004), Korean catfish (Yoon & Kim, 2004), Venus clam (Park & Yoon, 2008) and cockle (Kang & Yoon, 2013).

The seven oligonucleotides primers BION-01, BION-08, BION-13, BION-17, BION-47, BION-49 and BION-69 were used to produce unique shared loci to each population and shared loci by the three populations, as summarized in Table 4. The oligonucleotides primer BION-13 generated 42 unique loci to each population, approximately 200 bp, 400 bp, 800 bp, 900 bp, 1,000 bp and 1,200 bp respectively, in the Shinan population. Remarkably, the primer BION-13 detected 42 shared loci by the three populations, major and/or minor fragments of sizes 200 bp and 400 bp, respectively, which were identical in all samples. With reference to average bandsharing value (BS) results, individuals from Shinan population (0.811) displayed higher bandsharing values than did individuals from Gunsan population (0.626), as summarized in Table 5. In the present study, the dendrogram gained by the seven oligonucleotides primers highlight three genetic clusters: cluster 1 (GUNSAN 01 ~ GUNSAN 07), cluster 2 (SHINAN 08 ~ SHINAN 14) and cluster 3 (YEONGGWANG 15 ~ YEONGGWANG 21), as shown in Fig. 4. Among the seven white clam individuals the shortest genetic distance that displayed significant molecular differences was between individuals 13 and 14 from the Shinan population (genetic distance = 0.036), while the longest genetic distance among the twenty-one *Meretrix lusoria* individuals that displayed significant molecular differences was between individuals GUNSAN no. 01 and SHINAN no. 14 (genetic distance = 0.574). Comparatively, individuals of SHINAN population were

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Table 3. The number of total loci, average loci per lane, specific loci by PCR analysis using 7 decamer primers in *Meretrix lusoria* in Gunsan, Shinan and Yeonggwang of Korea

| Item   | No. of average loci per lane | No. of specific loci |
|--------|------------------------------|----------------------|
|        | GS                           | SA                   | YG       |
| BION - 01 | 4.14 (29)                  | 8.29 (58)            | 6.71 (47) | 15       | 16       | 26       |
| BION - 08 | 2.43 (17)                  | 5.14 (36)            | 6.57 (46) | 10       | 8        | 25       |
| BION - 13 | 7.71 (54)                  | 7.71 (54)            | 6.57 (46) | 29       | 12       | 18       |
| BION - 17 | 3.71 (26)                  | 5.57 (39)            | 5.57 (39) | 19       | 18       | 18       |
| BION - 47 | 5.14 (36)                  | 4.14 (29)            | 5.14 (36) | 36       | 15       | 15       |
| BION - 49 | 5.43 (38)                  | 5.29 (37)            | 4.71 (33) | 17       | 23       | 19       |
| BION - 69 | 6.14 (43)                  | 6.43 (45)            | 4.86 (34) | 29       | 17       | 13       |
| Total No. | 243                        | 298                  | 281      | 155      | 109      | 134      |
| Average No. per primer | 34.7                      | 42.6                 | 40.1     | 22.1     | 15.6     | 19.1     |

GS: Gunsan, SA: Shinan, YG: Yeonggwang
fairly closely related to that of YEONGGWANG population. As above mentioned, a dendrogram disclosed close relationships between individual identities within three geographical bivalve populations (McCormack et al., 2000; Kang & Yoon, 2013). In bivalves, cluster analysis of the pairwise population matrix, created from genetic data, exhibited that geographically close populations have a tendency to cluster together in the blacklip abalone (Huang et al., 2000).

Three *Meretrix lusoria* populations can be evidently distinguished, by PCR-founded approach. The potential of oligonucleotides amplified polymorphic and/or specific DNAs to identify diagnostic markers, species and population identification in shellfish (Callejas & Ochando, 1998; McCormack et al., 2000; Park et al., 2008; Kang & Yoon, 2013) has also been well recognized. PCR fragments discovered in this study may be worthwhile as a DNA marker the three geographical populations to discriminate.

In general, the population classification of venerid clam is constructed on morphological variations in shell body weight, shell color, shell height, shell length, shell type and feet length. It is presumed that differences in such characters reflect diverse origins or genetic identity (Chenyambuga et al., 2004). If systematic research of Korean Veneridae is in additive progress, these data could

**Table 4. The number of unique loci to each population and number of shared loci by the three populations produced by PCR analysis using 7 oligonucleotides primers in Gunsan, Shinan and Yeonggwang population of *Meretrix lusoria*, respectively**

| Primer \ Population | No. of unique loci to each population | No. of shared loci by the three populations |
|---------------------|--------------------------------------|------------------------------------------|
|                     | GS        | SA        | YG        | Three populations (7 individuals per population) |
| BION - 01          | 14        | 42        | 21        | 21                                           |
| BION - 08          | 7         | 28        | 21        | 0                                            |
| BION - 13          | 35        | 42        | 28        | 42                                           |
| BION - 17          | 7         | 21        | 21        | 21                                           |
| BION - 47          | 0         | 14        | 21        | 0                                            |
| BION - 49          | 21        | 14        | 14        | 21                                           |
| BION - 69          | 14        | 28        | 21        | 0                                            |
| Total no.          | 98        | 189       | 147       | 105                                          |
| Average no. per primer | 14        | 27        | 21        | 15                                           |

GS: Gunsan, SA: Shinan, YG: Yeonggwang

**Table 5. Manifold comparisons of average bandsharing values among Korean *Meretrix lusoria* populations from three areas were produced along with the bandsharing values and similarity matrix**

| Populations | GS       | SA       | YG       |
|-------------|----------|----------|----------|
| GS          | 0.626±0.064\(^b\) | 0.491±0.056\(^c\) | 0.482±0.039\(^a\) |
| SA          | -        | 0.811±0.064\(^c\) | 0.653±0.072\(^b\) |
| YG          | -        | -        | 0.729±0.064\(^bc\) |

\(^a-c\): Values with different superscript are significantly different, \(P<0.05\)

Each value is a result of three different experiments.

GS: Gunsan, SA: Shinan, YG: Yeonggwang
Fig. 4. Hierarchical dendrogram of genetic distances, obtained from three populations of Meretrix lusoria. The relatedness among different individuals in the Meretrix lusoria populations from Gunsan, Shinan and Yeonggwang of the Korean Peninsula were created along with the bandsharing values and similarity matrix.

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