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New polymorphic microsatellite markers for the Korean manila clam (*Ruditapes philippinarum*) and their application to wild populations

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ABSTRACT. Manila clam (*Ruditapes philippinarum*) is a valuable and intensively exploited shellfish species in Korea. Despite its importance, information on its genetic background is scarce. For the genetic characterization of *R. philippinarum*, expressed sequence tag-derived microsatellite markers were developed using next-generation sequencing. A total of 5879 tandem repeats containing di- to hexanucleotide repeat motifs were obtained from 236,746 reads (mean = 413 bp). Of the 62 loci screened, 24 (38.7%) were successfully amplified, and 10 were polymorphic in 144 individuals from 2 manila clam populations (Incheon and Geoje, Korea). The number of alleles ranged from 2 to 17 in the Incheon population and from 3 to 13 in the Geoje population (overall A_R = 7.21). The mean observed and expected heterozygosities were estimated to be 0.402 and 0.555, respectively. Hence, there is less genetic variability in the Geoje population than

in the Incheon population, although no significant reductions of genetic diversity were found between the populations (P > 0.05). However, significant genetic differentiation was detected between the populations (F_{ST} = 0.064, P < 0.001). Significant deviations from Hardy-Weinberg equilibrium and high inbreeding coefficients (mean F_{IS} = 0.22-0.26) were detected in both populations. The 10 novel polymorphic microsatellite loci used in this study will be useful for future genetic mapping studies and for characterizing population structures, monitoring genetic diversity for successful aquaculture management, and developing conservation strategies for manila clam populations in Korea.

Key words: *Ruditapes philippinarum*; Expressed sequence tags; Manila clam; Microsatellites marker; Genetic characterization

INTRODUCTION

The manila clam, *Ruditapes philippinarum*, belonging to the family Veneridae, is one of the most popular commercially important shellfish resources in Korea. The species is widely distributed worldwide in coastal regions, including Korea, Japan, and China. However, in recent years, populations have declined because of habitat destruction, overfishing, and abnormally high mortality rates. To protect and improve populations of this species in Korea, it is necessary to determine their genetic diversity and the relationships among populations. Knowledge of the genetic structure within and among populations can provide useful information for various other applications, including studies on genetic mapping, pedigree, stock structure, conservation, and status improvement (Reiss et al., 2009).

Despite strong commercial interest in the manila clam, few studies have investigated the genetic structure of this species in Korea. Recently, An et al. (2012b) reported the genetic relationship of 5 local manila clam populations using 7 microsatellite loci, which suggested 3 genetically distinct populations along the western and southern coasts of Korea. Many previous studies were conducted using allozyme markers (Oniwa et al., 1988; Vargas et al., 2008), mitochondrial DNA (Sekine et al., 2006), amplified fragment length polymorphisms (Liu et al., 2007) and random amplification of polymorphic DNA (Yoon and Kim, 2004).

Among the various molecular markers that are available to analyze genetic population structure, microsatellites are very useful because of their high level of polymorphism and mutation rates, co-dominance, and large number and even distribution throughout the genome; they are also simple to analyze (Sunnucks, 2000). To date, only 16 genome-derived microsatellite markers are available for *R. philippinarum* (Yasuda et al., 2007; An et al., 2012b). The statistical power depends not only on the number of scored loci but also on other factors, such as the degree of polymorphism of each locus and the sample size because the use of a limited number of loci might fail to offer sufficient information. The number of microsatellite markers for the Korean manila clam is too small to conduct further population genetic analyses such as mapping, pedigree analysis, and assignment tests. Therefore, new highly informative microsatellite markers need to be developed.

The traditional process of isolating microsatellite markers is time consuming, labor-

intensive, and expensive due to repetitive screening and subsequent sequencing of a large number of clones that may contain microsatellite regions (Hamilton et al., 1999). In contrast, microsatellite markers derived from expressed sequence tags (ESTs) could offer additional advantages, such as low cost, high transferability among phylogenetically related species, the probability of being associated with functional regions of the genome, and an effective and stable approach for the development of polymorphic DNA markers (Serapion et al., 2004). Presently, next-generation sequencing (NGS) is a valuable tool for efficient development of EST-simple sequence repeat (SSR) markers and could overcome such a lack of sequence information in bivalves (Wang et al., 2011; Dong et al., 2012).

In this study, 10 novel polymorphic EST-SSR markers were developed from *R. philippinarum* using 454 GS-FLX pyrosequencing, and the genetic variability of, and relationships between, 2 populations in Korea were examined at these microsatellite loci. This information will be useful for future genetic studies to understand the status of population genetic structure and facilitate the conservation and management of *R. philippinarum* in Korea.

MATERIAL AND METHODS

Sample collection and NGS

For microsatellite isolation, Korean manila clams from Namhae (N = 6) were used. Tissue samples were excised from gill, digestive tract, intestine, spleen, and muscle. Total RNA was isolated using the TRIzol® procedure (Invitrogen, USA) and purified with TRIzol® reagent (Invitrogen). The mRNA was prepared from total RNA using a PolyA Tract mRNA Isolation System II (Promega, USA). The cDNA library was produced using 5 mg mRNA and a cDNA synthesis kit (Stratagene, USA), and purified using a QIAquick polymerase chain reaction (PCR) purification kit (Qiagen, USA). Approximately 5 mg cDNA was used to generate a cDNA library using 454 pyrosequencing via GS-FLX-titanium chemistry (Genome Sequencer FLX Titanium platform; Roche, Germany) at Macrogen Inc. (Korea).

For genotyping, 144 wild *R. philippinarum* samples were collected from 2 coastal areas in 2011: western coastal waters (Incheon; IC, N = 66) and southern coastal waters (Geoje; GJ, N = 78). Species were identified based on cytochrome oxidase subunit I nucleotide sequences (Zhang and Hewitt, 2003).

Muscle tissue samples were preserved in 99.99% ethanol at the sampling site and then transported to the laboratory for DNA extraction. Total DNA from each sample was extracted using an automated DNA extraction system, the MagExtractor MFX-6100 (Toyobo, Japan) with a MagExtractor-Genomic DNA purification kit (Toyobo). Extracted genomic DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20°C until further use.

De novo assembly, microsatellite discovery, and primer screening

Raw reads for *R. philippinarum* from GS-FLX 454 were assembled using the CLC Genomics Workbench software v4.0.2 (CLC Bio, Denmark). Selected candidate marker sequences were longer than 334 bp with a minimum of 4 repeats of di- and trinucleotide repeat motifs. For these reads, the CLC Genomics Workbench software v4.0.2 was also used

to design primers with the following criteria to identify loci with a high likelihood of reliable amplification: 1) GC content 10-70%, 2) product size 80-300 bp, 3) primer length 18-24 bp, and 4) melting temperature 48°-62°C.

PCR and genotyping

All of the newly designed PCR primer pairs were tested to optimize annealing temperatures and confirm consistency of the PCR amplification, which was performed on 8 individuals collected from the GJ population in the southern coast of Korea.

PCR amplification was performed in a 10-µL reaction mixture containing 0.1 µL high-fidelity Hs Taq DNA polymerase (2.5 U/µL, TNT Research, Korea), 1 µL 10X reaction buffer, 0.2 µL 10 mM deoxynucleotide triphosphate (dNTP) mixture, 10 pmol of each primer, and 50 ng template DNA using a PTC 200 DNA Engine (MJ Research, USA). The forward primer of each pair was 5'-end-labeled with 6-FAM, NED, and HEX dyes (Applied Biosystems, USA). PCR conditions were as follows: 11 min at 95°C; 35 cycles of 1 min at 94°C, 1 min at the optimal annealing temperature, and 1 min at 72°C; and a final extension of 5 min at 72°C. PCR products were analyzed based on the presence of a visible band after running 3 µL PCR product on a 1.5% agarose gel. The 1-kb Plus DNA ladder molecular weight marker (Invitrogen, USA) was used as a standard to assess PCR product size. Microsatellite polymorphisms were tested using an ABI PRISM 3130 XL automated DNA sequencer (Applied Biosystems), and alleles were designated by PCR product size relative to a molecular size marker [GENESCAN 400 HD (ROX); Applied Biosystems]. Fluorescent DNA fragments were analyzed using the GENESCAN ver. 3.7 and the GENOTYPER ver. 3.7 software packages (Applied Biosystems).

Statistical analysis

Alleles were scored using GeneMapper version 4.0 (Applied Biosystems) with a size standard as an internal control for allele calling, and each allele was coded by its size of nucleotide base pairs (bp). MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004) was used to test the presence of genotyping errors due to null alleles, stuttering, or allele dropout (1000 randomizations) at a 95% confidence interval. For genetic diversity parameters, the number of alleles per locus (N_A) and allelic richness per locus (A_R) were determined using FSTAT version 2.9.3 (Goudet, 1995). The A_R was corrected for the smallest sample size (N = 66) using FSTAT and could be directly compared between populations, regardless of the sample size (El Mousadik and Petit, 1996). Genetic differentiation between populations was calculated with the Weir and Cockerham (1984) estimator of the fixation index (F_{ST}) using FSTAT. The significance levels of F_{ST} values were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989). The overall inbreeding coefficients (F_{IS}) (Weir and Cockerham, 1984) were estimated for each population and locus using the GENEPOP version 4.0 computer package (Rousset, 2008). The significance levels were adjusted for multiple tests using the sequential Bonferroni correction (Rice, 1989). The polymorphic information content (PIC; an indicator of the utility of the marker for linkage or population genetic studies), based on the allele frequencies, was determined using CERVUS version 3.03 (Kalinowski et al., 2007). The observed heterozygosity (H_O) and expected heterozygosity (H_E) were determined

for each population and locus using the ARLEQUIN version 3.1 software (Excoffier et al., 2005). Deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) for each locus were also determined using ARLEQUIN.

RESULTS

Pyrosequencing

Raw sequence data from 454 pyrosequencing were about 323 Mbp containing 780,000 reads or sequences with an average length of 413 bp (maximum = 1941 bp; minimum = 30 bp). A total of 579,952 reads (~74.4%) were assembled into 46,405 contigs with an average length of 616 bp (maximum = 6611 bp; minimum = 30 bp), leaving 190,341 singletons. This process eliminated repetitive sequences and created longer reads. The mean length of these 236,746 sequences (46,405 contigs + 190,341 singletons) was 487 bp, which was longer than that of the raw sequences. The 454 pyrosequencing results are summarized in Table 1. Of the 236,746 sequences, 1237 (0.52%) could be used to design microsatellite marker primers. This is lower than previous studies using NGS, which reported 30.09% in the bream *Megalobrama pellegrini* (Wang et al., 2012), 2.26% in *Haliotis diversicolor supertexta* (An et al., 2012a), and 1.45% in *Mytilus coruscus* (An and Lee, 2012).

Table 1. Summary of the generated *Ruditapes philippinarum* expressed sequence tags (ESTs) and assembly results.

Feature	Value
Total number of ESTs (from 454 reads)	780,000
Average EST length	413 bp
Number of putative transcripts	236,746
Number of singletons	190,341
Number of contigs (next-generation sequencing assembly)	46,405
Average contig length	616 bp
Average number of sequences per contig	7.4

Microsatellite loci isolation

Sixty-two microsatellite loci were selected from the contigs and singletons that were longer than 400 bp for subsequent polymorphism screening in *R. philippinarum*. Among them, 24 (38.7%) loci were successfully amplified for initial evaluation of the microsatellite primers. This result is comparable to those for *H. diversicolor supertexta* (28.3%, An et al., 2012a) and *Miichthys miiuy* (24.4%, Xu et al., 2011), but lower than that obtained for *Mytilus coruscus* (90.2%, An and Lee, 2012) and *Mercenaria mercenaria* (50%, Wang et al., 2010). Subsequently, further screening revealed that 10 (16.1%) loci (2 di- and 8 trinucleotide repeat motifs) were polymorphic in the 8 individuals of *R. philippinarum*. The remaining 14 primer sets gave inconsistent results, despite adjusting the dNTP concentrations and using an annealing temperature gradient. Primer sequences, repeat motifs, annealing temperatures, fluorescent labels, and GenBank accession numbers for the 10 novel microsatellite loci are summarized in Table 2.

Table 2. Primer sequences and information about microsatellite loci from *Ruditapes philippinarum*.

Locus	Primer (5'-3')	Repeat unit	Tm (°C)	Size ranges (bp)	GenBank accession No.
KRph 16989-1	F: TTATTGGATCAACATTTTTATTA ned R: TGAAGTTGAGAGCATTTTCTTTC	$(CA)_9$	52	115-143	KC753223
KRph 9118-1	F: ATAATTAACAACAACCAGTGTGA 6-fam R: AGACCCAATAAAACAAAAGACGG	$(CA)_9$	52	175-225	KC753224
KRph 28486	F: GAACACCGCTCCTGCTAACTTTC hex R: CGTAGCAGAGGGGCCTGACTGTG	$(CAA)_3GAA(CAA)_2$	52	239-260	KC753225
KRph 39753-1	F: GTAGTTTGTTAATTTCTGAGTTT ned R: AGTTTTCTTTTTCTGCTGTTTTT	$(CAA)_4$	52	82-142	KC753226
KRph 7182	F: CTACGATTCCGAAAGTAATGATA 6-fam R: TCGGCTTCTATTTCTGCTGAACT	$(CAA)_5$	50	169-190	KC753227
KRph 14377	F: TTGAAGAATATTACCGACAACTG hex R: GACTGAAATCCATTTGGCTGTGT	$(CAA)_4$	50	203-221	KC753228
KRph 28654	F: CAATAAAAAGCAATAACAACATC 6-fam R: TTTTAATGCAAGGACTGAACACC	$(CAA)_5$	50	126-186	KC753229
KRph 7347	F: AAATACTAACGGACAATCAACAA hex R: TGTACTGAATCCCGGATACGAAG	$(CAA)_4$	50	224-251	KC753230
KRph 41013	F: CCCCTCAGCTTTTACAGTTGTTG hex R: TACACCTGATGCTGCCGCTTGAC	$(CAA)_8$	50	243-276	KC753231
KRph 11536-1	F: TGCCCATAACATTTTTCTGTGAG ned R: GTTGTTTTGTTGTCGGGGGAAGC	$(CAA)_5$	50	116-140	KC753232

Tm = melting temperature.

Comparative genetic variability between 2 manila clam populations

MICRO-CHECKER analysis revealed that 7 of the 10 loci could have been affected by 1 or more null alleles in both populations; our data indicated that loci KRph 169891-1, KRph 9118-1, KRph 39753-1, KRph 7182, and KRph 14377 in the IC population and loci KRph 16989-1, KRph 9118-1, KRph 39753-1, KRph 28486, and KRph 28654 in the GJ population might be affected. Thus, loci KRph 16989-1, KRph 9118-1, and KRph 39753-1 appeared to be influenced in both populations, which indicated that population genetic analyses that assume HWE may be problematic. Accordingly, a global multilocus F_{ST} value was estimated with and without the 3 loci. However, loci KRph 7182, KRph 14377, KRph 28486, and KRph 28654 were affected by null alleles in only 1 sample; thus, these were included in further analyses.

No genotyping errors from allele dropouts or stuttering affected allele scoring. Samples that failed to amplify after the rerun were not included, making it unlikely that poor DNA quality affected the results.

Statistical results for these 10 polymorphic loci are presented in Table 3. All 10 microsatellite loci were determined to be polymorphic in both populations. A total of 146 alleles were detected across 144 individuals. The average N_A per locus was 7.30 and ranged from 2 at locus KRph 28486 to 17 at locus KRph 9118-1 (Table 3). Not all loci were equally variable. Specifically, KRph 9118-1 and KRph 39753-1 displayed a high level of allelic diversity and heterozygosity. A moderate level of allelic diversity was detected in each population (IC, mean N_A = 7.20; GJ, mean N_A = 7.40), which was dramatically lower than that for many other populations of marine bivalves, including pen shell (mean N_A = 20, Liu et al., 2009), small abalone (mean N_A = 14.73, Zhan et al., 2009), and manila clam reported from An et al. (2012b) (mean N_A = 14.17). Overall, the A_R varied from 2 to 17 (Table 3). A higher mean A_R was observed in the IC population (A_R = 17) than the GJ population (A_R = 12.80). However, the difference was not significant (Wilcoxon signed-rank test, P > 0.05).

The H_O per locus ranged from 0.115 at locus KRph 16989-1 to 0.758 at locus KRph 41013, and the H_E per locus ranged from 0.183 at locus KRph 28654 to 0.871 at locus KRph 9118-1 (Table 3). The average H_O and H_E were 0.430 and 0.600 in the IC samples and 0.373 and 0.509 in the GJ samples, respectively. There were no significant differences in these parameters between the populations (Wilcoxon signed-rank test, $P > 0.05$).

The PIC ranged from 0.18 to 0.83 (mean = 0.51), and it was moderately high (0.62-0.85) for 5 loci (KRph 16989-1, KRph 9118-1, KRph 39753-1, KRph 14377, and KRph 41013).

The F_{IS} values varied among markers from -0.072 (KRph 7347) to 0.618 (KRph 39753-1) in the IC population (mean = 0.257) and from -0.134 (KRph 7182) to 0.731 (KRph 9118-1) in the GJ population (mean = 0.222) (Table 3).

In HWE tests, 12 (60%) of 20 locus-population combinations were in accordance with the HWE proportions, and 8 (40%) exhibited significant deviation from HWE using the sequential Bonferroni method for multiple observations (Rice, 1989) (Table 3). Five deviations from equilibrium were observed in the IC population, while 3 cases were found in the GJ population. This deviation was detected at 6 loci (KRph 16989-1, KRph 9118-1, KRph 39753-1, KRph 7182, KRph 14377, and KRph 11536-1), which were affected by null alleles except KRph 11536-1.

Examination of LD using a likelihood-ratio test revealed that all 10 microsatellite loci were in linkage equilibrium ($P > 0.05$). All loci showed significantly different distributions between the 2 populations ($P < 0.05$). Genetic differentiation between the populations was also detected in F_{ST} values. The global multi-locus F_{ST} value was significantly different between the populations using all 10 microsatellite markers ($F_{ST} = 0.064$, $P < 0.001$). In addition, when the KRph 16989-1, KRph 9118-1, and KRph 39753-1 loci were excluded, the global multi-locus F_{ST} was 0.072 ($P < 0.001$).

Table 3. Statistical summary for 10 polymorphic microsatellite loci in 2 *Ruditapes philippinarum* populations.

Population	Microsatellite loci										Mean
	KRph 16989-1[a]	KRph 9118-1[a]	KRph 28486[a]	KRph 39753-1[a]	KRph 7182[a]	KRph 14377[a]	KRph 28654[a]	KRph 7347	KRph 41013	KRph 11536-1	
Incheon (66)											
N_A	10	17	2	10	5	7	5	4	7	5	7.20
A_R	10.00	17.00	2.00	10.00	5.00	7.00	5.00	4.00	7.00	5.00	7.200
H_E	0.697	0.871	0.345	0.749	0.408	0.692	0.509	0.537	0.764	0.429	0.600
H_O	0.561	0.364	0.318	0.288	0.212	0.424	0.424	0.576	0.758	0.379	0.430
P	0.010	0.000	0.486	0.000	0.000	0.000	0.426	0.091	0.890	0.001	
F_{IS}	0.196 (0.015)	0.584 (0.000*)	0.080 (0.494)	0.618 (0.000*)	0.482 (0.000*)	0.389 (0.000*)	0.168 (0.417)	-0.072 (0.099)	0.009 (0.896)	0.118 (0.001*)	0.257
PIC	0.64	0.85	0.28	0.71	0.38	0.65	0.43	0.49	0.72	0.40	0.56
Geoje (78)											
N_A	8	13	3	11	7	6	8	5	7	6	7.40
A_R	7.624	12.797	2.846	10.951	6.800	5.977	7.620	4.842	6.840	5.823	7.212
H_E	0.367	0.854	0.509	0.785	0.339	0.701	0.183	0.377	0.659	0.318	0.509
H_O	0.115	0.231	0.346	0.359	0.385	0.718	0.128	0.372	0.718	0.359	0.373
P	0.000	0.000	0.003	0.000	1.000	0.732	0.005	0.757	0.050	1.000	
F_{IS}	0.687 (0.000*)	0.731 (0.000*)	0.321 (0.007)	0.544 (0.000*)	-0.134 (1.000)	-0.024 (0.727)	0.299 (0.009)	0.015 (0.782)	-0.090 (0.038)	-0.130 (1.000)	0.222
PIC	0.35	0.83	0.38	0.76	0.32	0.65	0.18	0.34	0.62	0.30	0.47

N_A = number of alleles per locus; A_R = allelic richness; H_E = expected heterozygosity; H_O = observed heterozygosity; F_{IS} = inbreeding coefficient; PIC = polymorphism information content are given for each population and locus. *Not in conformity with Hardy-Weinberg equilibrium ($P < 0.005$, Bonferroni-corrected value). [a]Microsatellite loci revealed the presence of null alleles with MICRO-CHECKER 2.2.3.

DISCUSSION

Despite its importance as a highly valuable fishery resource in Korea, *R. philippinarum* has been the subject of only a few studies (Yasuda et al., 2007; An et al., 2012b). Microsatellite markers have many advantages over other molecular markers for research on population structure and genetic diversity (Guichoux et al., 2011). However, a relatively large number of microsatellite markers may be necessary for further genetic studies on aspects such as genome mapping, parentage, kinships, and stock structure. Recently, the development of NGS technology has provided a much more efficient method for the development of genetic microsatellite markers by reducing associated costs, labor, and time, even in organisms for which adequate databases are not currently available (Kircher and Kelso, 2010). In this study, we identified new microsatellite loci from large-scale ESTs in this clam using 454 pyrosequencing, and we genetically characterized the populations.

Only 0.52% of all contigs that were obtained contained a microsatellite, which was fewer loci than that reported in other studies (Wang et al., 2012; An et al., 2012a; An and Lee, 2012). However, this is not surprising considering that the previous studies used genomic-derived microsatellites, which are generally less conserved than the EST-derived microsatellites used in this study. In all, 10 (16.1%) polymorphic microsatellite loci were selected from among 62 loci, which contained 2 dinucleotide and 8 trinucleotide repeat motifs. This is similar to that reported for *H. diversicolor supertexta* (20.2%; An et al., 2012a), but it is dramatically lower than those reported for *Meretrix meretrix* (42.5%; Lu et al., 2011) and *Tegillarca granosa* (44.9%; Dong et al., 2012). The remaining primers did not generate the desired amplification products or showed faint bands, which may be due to nonspecific PCR amplification, although microsatellite loci were selected by stringent criteria for clear genotyping results. Dinucleotide repeats are still the predominant markers. However, trinucleotide microsatellites have advantages over dinucleotides in which they are highly polymorphic, more stable, and show clearer bands (Lindqvist et al., 1996). Lu et al. (2011) compared EST microsatellites and genomic microsatellites in *Meretrix meretrix* and found that the EST microsatellites were composed mainly of tri- and tetranucleotide repeats (82.4%), while the genomic microsatellites were composed mainly of dinucleotide repeats (75%).

We detected less genetic diversity than that reported in previous studies on manila clam (An et al., 2012b; Mura et al., 2012). However, our results are similar to those of studies on other species, including Miiuy croaker (*Miichthys miiuy*; N_A = 5.13, Xu et al., 2011), Mediterranean blue mussel (*Mytilus galloprovincialis*; N_A = 3.9, Li et al., 2011), blood clam (*T. granosa*; N_A = 3.9, Dong et al., 2012), and *Meretrix meretrix* clams (N_A = 9.2, Lu et al., 2011). EST-derived microsatellite markers had a lower N_A than genomic-derived microsatellite markers. This indicates that EST-derived microsatellites are less polymorphic than those derived from genomic regions. In addition, EST-derived microsatellite variability was associated with a lower number of repeats than anonymous microsatellites (Thiel et al., 2003).

Significant deviation from HWE was detected in 6 of the 10 loci in both populations (HWE, $P < 0.005$). Generally, the causes of heterozygote deficiencies include factors such as inbreeding, sub-structuring of the population, and the presence of null alleles (Callen et al., 1993). Indeed, our MICRO-CHECKER analysis detected the presence of null alleles at 7 loci (Table 3). Further research is required to establish whether these null alleles are due to population subdivision or inbreeding. The existence of null alleles is considered the most likely cause of heterozygote deficiencies (Callen et al., 1993). Null alleles, a locus-dependent

effect, have been reported in many other mollusks, including Pacific oyster (Hedgecock et al., 2004), Japanese abalone (Sekino et al., 2006), small abalone (Zhan et al., 2009), and bay scallop (Zhan et al., 2007).

The development of reliable microsatellite markers with moderately high PIC (> 0.5) is an essential step for the genetic analysis of *R. philippinarum*. In this study, 10 polymorphic loci were confirmed to have a moderately high degree of polymorphism (mean PIC = 0.52) (Table 3).

This study was limited by the number of screened populations; the genetic diversity estimates, such as the significant deviation from HWE at many loci and high values of inbreeding coefficients, may be explained more precisely by data from additional populations. Therefore, these results should be interpreted with caution.

The significant difference in the genetic differentiation between the populations based on F_{ST} estimates (F_{ST} = 0.064, P < 0.001) may suggest that these 2 populations are genetically distinct.

It should be noted that we also identified annotated genes with unknown functions (data not shown). In type I markers, including EST-derived microsatellites markers, many unknown gene-associated microsatellites can be useful for comparative mapping (Serapion et al., 2004). Therefore, there is a need for further research to investigate the effects of these genes detected.

In conclusion, we developed 10 polymorphic EST-derived microsatellite markers from the economically important *R. philippinarum* clam using 454 sequencing technology. Using these markers, we demonstrated moderate genetic diversity and significant genetic differentiation within and between 2 populations of *R. philippinarum*. Our results should be useful for future genetic mapping studies, characterization of fine-scale population structures, and the conservation and the development of stock enhancement programs for *R. philippinarum* in Korea.

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