Ocean Sci. J. (2017) 52(1):139–146
http://dx.doi.org/10.1007/s12601-017-0012-9

Available online at http://link.springer.com

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Article

Ocean Science Journal

pISSN 1738-5261
eISSN 2005-7172

New Polymorphic Microsatellite Markers Derived from Hemocyte cDNA Library of Manila Clam *Ruditapes philippinarum* Challenged by the Protozoan Parasite *Perkinsus olseni*

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Received 10 August 2016; Revised 17 November 2016; Accepted 18 December 2016

Abstract – Manila clam *Ruditapes philippinarum* is one of the most important benthic animals in the coastal north Pacific region, where clam populations have been mixed genetically through trade and aquaculture activities. Accordingly, identification of the genetically different clam populations has become one of the most important issues to manage interbreeding of the local and introduced clam populations. To identify genetically different populations of clam populations, we developed 11 expressed sequence tag (EST)-microsatellite loci (i.e., simple sequence repeat, SSR) from 1,128 clam hemocyte cDNA clones challenged by the protozoan parasite *Perkinsus olseni*. Genotype analysis using the markers developed in this study demonstrated that clams from a tidal flat on the west coast contained 6 to 19 alleles per locus, and a population from Jeju Island had 4 to 20 alleles per locus. The expected heterozygosity of the 2 clam populations ranged from 0.472 to 0.919 for clams from the west coast, and 0.494 to 0.919 for clams from Jeju Island, respectively. Among the 11 loci discovered in this study, 7 loci significantly deviated from the Hardy-Weinberg equilibrium after Bonferroni correction. The 5 loci developed in this study also successfully amplified the SSRs of *R. variegatus*, a clam species taxonomically very close to *R. philippinarum*, from Hong Kong and Jeju Island. We believe that the 11 novel polymorphic SSR developed in this study can be utilized successfully in Manila clam genetic diversity analysis, as well as in genetic discrimination of different clam populations.

Keywords – microsatellite loci, simple sequence repeat (SSR), expressed sequence tags (EST), *Ruditapes philippinarum*, *Ruditapes variegatus*, cross-species amplification

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1. Introduction

Indigenous to the west Pacific Ocean, Manila clam *Ruditapes philippinarum* is one of the key species occurring in soft intertidal to shallow subtidal areas with a high density. Manila clams are often cultured on a commercial scale in the coastal Yellow Sea (Park and Choi 2004; FAO 2009; Zhang and Yan 2006; Uddin et al. 2012). Annual landings of Manila clam in Korea have been declining for the past two decades (Park and Choi 2004; Uddin et al. 2012). Consequently, the local clam industries on the west coast are suffering due to the insufficient supply of the seed clams, which is entirely dependent on the catch of wild juveniles. Although the import of juvenile clams helps to compensate for the shortage, the imported clams may interbreed with native populations throughout the year, resulting in genetic hybridization between the native and the introduced populations. As frequency of these types of imports increases, the hybridization processes are accelerating, which may result in the poor genetic diversity of the native clam populations (Chiesa et al. 2011; Kitada et al. 2013).

To understand the genetic structure and gene flow in clam populations, various molecular markers have been developed and applied, including mitochondrial cytochrome oxidase subunit I (Kitada et al. 2013) and mitochondrial 16S rDNA (Hurtado et al. 2011; Chiesa et al. 2014; Nerlovic et al. 2016). In particular, several studies have developed both genomic DNA-based and expressed sequence tags (EST)-based microsatellite DNA markers to investigate the genetic structure and

hybridization of the native and introduced clams in Asia and Europe (Yasuda et al. 2007; An et al. 2009; Chiesa et al. 2011; Kitada et al. 2013; Li et al. 2016). In Korean waters, An et al. (2009) developed genomic DNA-based microsatellite markers, while Kim et al. (2014a, 2014b) developed EST-based microsatellite markers to understand the genetic structure of Manila clam populations.

Microsatellites are short repetitive DNA sequences consisting of 1 to 8 base pairs originating from either genome or expressed genes (i.e., mRNA, Wright and Bentzen 1994). Microsatellites have been used as a molecular marker to locate polymorphism in DNA, including genetic linkage and mapping, and comparative genomics in plants and animals (Liu and Cordes 2004; Ellis and Burke 2007). In shellfish aquaculture, microsatellites have been widely used to evaluate genetic structure (Yu and Guo 2004; Mura et al. 2012), marker-assisted selective breeding (Borrell et al. 2014) and disease-resistance traits (Yu and Guo 2006; Sauvage et al. 2010) of various marine bivalves. Compared to other DNA markers, the microsatellites have many unique features such as high polymorphism, codominance, abundance in the genome, and easy amplification by polymerase chain reaction (PCR) (Liu and Cordes 2004). Recently, microsatellites derived from ESTs have been used extensively, as an alternative approach to the traditional genomic microsatellites (Wang et al. 2009; Yu et al. 2010). ESTs are partial sequences generated from single-pass sequencing of cDNA clones that are associated with functional genes to regulate the genetic characters in a certain tissue or cells. EST-derived microsatellites have a high transferability among species due to the conserved nature of the genes. Moreover, the rapid increase of ESTs registered in the public EST databases makes this a relatively easy and inexpensive way to mine EST derived markers, including EST-simple sequence repeat (SSR) and EST-single nucleotide polymorphism (SNP). Currently, 108 EST markers are available for Manila clam, including 73 EST-SSR (Kim et al. 2014a; Nie et al. 2014; Zhu et al. 2015) and 35 EST-SNP (Kim et al. 2014b; Nie et al. 2015). Although these EST markers of Manila clam have wide allele ranges, most of them are di-nucleotide repeats and only a few are known to be polymeric (i.e., tri-, or tetra-) repeats. Several studies have demonstrated that tri- and tetra-nucleotide microsatellites have a high polymorphism and more reliable allele scoring than di-nucleotide repeats (Somridhivej et al. 2008, Hui et al. 2013). Therefore, it is crucial to utilize more polymorphic microsatellites containing longer repeats in researching genetic diversity.

Perkinsus olseni is a protozoan parasite infecting *R. philippianrum* and *R. decussatus* in European and Asian waters (see review of Villalba et al. 2004 and Choi and Park 2010). Manila clams heavily infected with *P. olseni* often exhibit tissue inflammation, necrosis, hemocyte infiltration, and retarded growth and reproduction (Park et al. 2006; Choi and Park 2010). According to Yang (2011), *P. olseni* is also responsible for mass mortalities of Manila clam during late summer to early fall on the west coast of Korea. In order to understand the gene expression of Manila clam hemocytes engaged in the immune response, Kang et al. (2006) experimentally constructed a cDNA library of Manila clam challenged by *P. olseni*, and they established 1,128 hemocyte cDNA clones.

In this study, we screened the cDNA library of Manila clam as Kang et al. (2006) established, in order to develop genetic markers to be used in the analysis of the genetic structure and gene flow of Manila clams. Here, we report 11 novel polymorphic EST- SSRs derived from the cDNA library, and their application with regard to 2 Manila clam populations in Korean waters.

2. Materials and Methods

Sampling effort

Manila clams were collected from Hwangdo (HD), one of the largest clam culture grounds located on the west coast of Korea. According to Yang (2011), *P. olseni* infection prevalence of the Manila clams at HD are 100%, and the infection intensity ranges from 700,000 to 4,500,000 *P. olseni* cells/g tissues. Manila clams were also collected from Sungsan (SS) on the east coast of Jeju Island, off the south coast of Korea, where the prevalence and infection intensity ranged from 0–100% to 0–380,000 *P. olseni* cells/g tissues, respectively (Yang 2011). For genotyping, total DNA was extracted from fresh or ethanol-fixed adductor muscle of the clams (N = 35 each from HD and SS) using a DNeasy tissue and blood kit (Qiagen, USA). To test cross-species amplification of EST microsatellite marker, *R. variegatus* were also collected from Jeju Island and Hong Kong, and the total DNA was extracted from their adductor muscle (N = 10 from each location). To differentiate *R. philippinarum* from *R. variegatus* at the species level, we also performed polymerized chain reaction (PCR) using the universal primer LCO 1490 and HCO 2198 derived from cytochrome oxidase I gene (Folmer et al. 1994). The PCR confirmed that the 2 populations of *R. variegatus*

collected from Jeju Island and Hong Kong are genetically different from *R. philippinarum*, and those 2 clam populations were confirmed to be *R. varigatus*.

Microsatellite loci screening from Manila clam hemocyte cDNA library

A total of 1,815 EST clones originating from the hemocytes of *R. philippinarum* challenged by *P. olseni* were utilized to search the microsatellite loci (Kang et al. 2006). Before mining the sequence repeats, all EST clones were assembled into 251 contigs and 877 singletons using CAP3 (Huang and Madan 1999). A total of 1,128 unique sequences containing contigs and singletons were screened for the microsatellite loci using WebSat (Martins et al. 2009), TRF (Benson 1999) and SSRIT (Temnykh et al. 2001). From the screened outputs, we selected loci containing di-, tri-, tetra-, penta- and hexa-nucleotides SSR with 5 repeated units and compound SSR with 2 repeated units. As a result, 61 SSR loci were identified and 19 were selected for further primer designing and PCR amplification. For gene annotation, the selected 19 EST sequences were subjected to a similarity search against the database registered in GenBank using BLAST program (Altschul et al. 1997).

Primer design and PCR amplification

Primers were designed for 19 EST-SSRs with sufficiently long sequences using Primer 3 (http://biotools. Umassmed. Edu/bioapps/primer3_www.cgi). PCR amplification of the designed primers was evaluated using 4 Manila clams collected from SS. PCR reaction mixture was prepared in a total volume of 25 μL containing 50 ng of total genomic DNA, 10 × Ex taq buffer, 2.5 mM dNTPs, 50 pmol of each primer, and 0.625 unit of Ex taq polymerase (Takara, Japan). PCR was performed in a Takara PCR thermal cycler TP 600 (Takara, Japan) with a gradient annealing temperature mode. Amplification parameters were programmed with an initial denaturation step at 95°C for 5 min, then repeated 35 cycles of a denaturation at 95°C for 30 sec, 45–55°C of the gradient annealing temperatures for 30 sec and an extension at 72°C for 30 sec. The reaction was terminated with a final extension of 7 min at 72°C. The amplified PCR products were separated on 2% agarose gel and visualized with ethidium bromide staining and UV light. Presence or absence of the PCR product appeared on the agarose gel was confirmed, and finally determined markers are summarized in Table 1.

Microsatellite genotyping and data analysis

A total of 11 microsatellite markers were selected from the primer validation process and used in the analysis of genetic polymorphisms of Manila clams from HD and SS. To test cross-amplification of the developed microsatellite markers to *R. variegatus*, 5 markers (i.e., *Ruph*2, 6, 14, 17, and 19-KR) were selected from the 11 markers, and 20 individuals of *R. variegatus* from Jeju Island and Hong Kong were tested. For PCR amplification, the 5′-end of the forward primer of each primer sets was labelled with fluorescein amidite (FAM) dye, and 10 pmol of the labelled primer was used in each PCR reaction. PCR were performed with the same conditions above (see the annealing temperature of each primer in Table 1). The PCR products were run with a size standard GeneScan-500 LIZ on an ABI 3730XL Genetic Analyzer (Applied Biosystems). Size of the fluorescent-labelled DNA fragments was determined using GeneMapper 4.0 software (Applied Biosystems).

MICRO-CHECKER 2.2.1 software was used to identify the genotyping error and null alleles (Van Oosterhout et al. 2004). Allele number, observed and expected heterozygosity was calculated by POWER MARKER software (Liu and Muse 2005). Exact tests for deviations from the Hardy-Weinberg equilibrium (HWE) were conducted using GENEPOP 4.0 (Raymond and Rousse 1995). Significant levels were calculated per locus using the Bonferroni correction (Rice 1989).

3. Results and Discussion

The screening of the 1,128 unique sequences using three microsatellite prediction programs (WebSat, SSRIT and TRF) identified 61 EST sequences containing di-, tri-, tetra-, and octa-nucleotide SSR with 5 repeated units and two types of compound SSR with 2 repeated units. Of 61 SSRs, 36 (59.0%) were di-nucleotide, 12 (19.6%) were tri-nucleotide, 10 (16.4%) were tetra-nucleotide, 1 was octa-nucleotide, and 2 were combination repeats. Among the di-nucleotide repeats, the motif TA/AT (55.6%) was the most frequent type, accounting for 32.8% of all SSRs. In contrast, the motif CT/TG/CG/GA/TC/TG/AC/GT was a low frequent repeat with one SSR. Nineteen EST sequences selected from the 61 SSRs with sufficient flanking sequences were selected for primer design and PCR amplification. BLAST search identified that 11 of 19 ESTs were unknown genes, while 5 ESTs were known genes (i.e., mitochondrial DNA, oncogene homolog,

Table 1. Characteristics of 11 EST- microsatellite loci in Manila clam, *Ruditapes philippinarum* collected from Hwangdo (HD) and Sungsan (SS). F, forward primer; R, reverse primer; Tm, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HWE}, P value for Hardy-Weinberg equilibrium; *, significant departure after Bonferroni correction ($p < 0.01/11$)

Locus	GenBank	Motif	Primer sequence (5′-3′)	Tm (°C)	Size range (bp) (HD/SS)	Number of alleles (HD/SS)	H_o (HD/SS)	H_E (HD/SS)	P_{HWE} (HD/SS)
Ruph1-KR	KC545801	(CA)6	F:TAGCAACGGATTTTCCTAATGC R:TTTCACGTAAGTAAGCAAACGC	54	303-313/297-313	6/8	0.275/0.181	0.447/0.494	0.0044/0.0000*
Ruph2-KR	KC545802	(AGA)6	F:TTCAATCGGGAAGGTTCTG R:TATCGGGTTATCGTACCCAAA	54	316-347/310-340	11/9	0.375/0.281	0.743/0.830	0.0000*/0.0000*
Ruph6-KR	KC545803	(CA)6	F:ACGGATCTTCCCGAATCGCAGTTA R:GGCAATTGTGCATGTTTCCTAAACC	54	187-249/191-252	19/12	0.441/0.333	0.844/0.820	0.0000*/0.0000*
Ruph7-KR	KC545804	(TTTC) TA(TTTC) G(TA)2	F:GCTTAGGCTCGGAGATTTGCTAAG R:ACGACCAGAATCATAGACGC	52	335-353/338-348	6/4	0.382/0.457	0.472/0.645	0.0163/0.0009
Ruph9-KR	KC545805	(CA)6	F:CAAACAAAACACACTAGCGCA R:TTAAAACTGGGGTAAATCTCGC	54	94-116/98-118	8/10	0.571/0.685	0.764/0.742	0.0083/0.3734
Ruph11-KR	KC545806	(TA)6	F:CCATTTCGTTGCTTTGTCACT R:TTAAATATGTCTCGCGGTTGTG	54	100-121/100-127	6/7	0.176/0.171	0.676/0.671	0.0000*/0.0000*
Ruph12-KR	KC545807	(CA)7	F:AACACACACACAAAACACCTCTG R:TGCACTAAACGACCATACACG	54	203-227/205-223	7/9	0.176/0.142	0.646/0.666	0.0000*/0.0000*
Ruph14-KR	KC545808	(ACT)6	F:GCACGAGGATCATTCACAGG R:CAAATGTCTGTTTCATCACTCACAG	54	300-334/271-324	16/20	0.562/0.636	0.887/0.906	0.0000*/0.0005*
Ruph15-KR	KC545809	(TCA)7	F:AATTATCCTATCAAGGCACCCA R:GGAACAACCTTTTGTATGGAGAC	54	318-348/326-344	9/9	0.657/0.562	0.738/0.721	0.5168/0.0394
Ruph17-KR	KC545810	(TCA)6	F:GCACGAGGCTTCATCATCATCATC R:ACCCAAACTTACAATAGCCATCATTC	52	278-348/262-314	16/16	0.303/0.310	0.893/0.894	0.0000*/0.0000*
Ruph19-KR	KC545811	(TCAA)8	F:GCATTCGTTACGACAGCAT R:GGTTGAAATTCGTTGTTTGGTTGG	54	159-211/163-231	18/19	0.666/0.685	0.919/0.919	0.0000*/0.0000*

homologous mRNA sequences of zebrafish and salmon) and 3 ESTs were hypothetical proteins.

As PCR amplification was tested using genomic DNA of 4 clams, 11 out of the 19 designed primers produced a specific single band of the expected size. In contrast, 5 primers generated non-specific PCR products, and 3 primer pairs failed to amplify any product, indicating that those primers cannot be used as markers. Such amplification failure of the SSR primers designed in this study could be associated with the presence of an intron within the primer sequence. It is also likely that the primer binding site may have sequence polymorphism, resulting in large fragment amplification, or preventing the primer annealing (Wang et al. 2009). Table 1 shows the primer sequences and annealing temperature of the optimized 11 SSR loci.

Table 1 summarizes genotyping results of Manila clams using the 11 SSR loci. The number of allele per locus ranged from 6 to 19 in HD clams, and 4 to 20 in SS clams, with an average of 11.1 in both populations (Table 1). The highest allelic diversity was observed in *Ruph*6-KR locus of the HD population, and *Ruph*14-KR locus of SS clams. The mean observed heterozygosity and expected heterozygosity of HD clams were 0.417 (range of 0.176–0.666), and 0.730 (range of 0.472–0.919), while the mean for clams in SS was 0.404 (range 0.142–0.685), and 0.755 (range 0.494–0.919).

Genotyping analysis of Manila clams from HD and SS using the 11 SSR loci revealed that 7 of the 11 loci significantly deviated from HWE after a Bonferroni correction, due to an apparent heterozygote deficiency. The MICRO-CHECKER analysis indicated that null alleles were present in most of the loci, except 2 loci (*Ruph*7-KR and *Ruph*15-KR) in HD clams and 1 locus, *Ruph9*-KR in SS population. The stuttering pattern was also identified from 2 loci, *Ruph*1-KR and *Ruph*12-KR, *Ruph2*-KR and *Ruph*12-KR, in HD and SS clams, respectively. In contrast, there was no evidence of a large-allele drop in any of the loci.

Several studies have reported heterozygote deficiency in marine bivalves, demonstrating homozygote excess in the HWE test, as the EST-microsatellites exhibit high frequency of null alleles (Carlsson and Reece 2007; Yu et al. 2010; Li et al. 2011). Carlsson and Reece (2007) reported a high level of heterozygote deficiency in the wild Eastern oyster, *Crassostrea virginica* from James River in USA, as evidenced by the high frequency of null alleles in the EST-microsatellite loci. Similarly, Kim et al. (2014a) also reported significant deviation of the 6 loci out of 10 EST-SSR loci of 2 Manila clam populations from the west and south coasts of Korea, from the HWE, suggesting that there is a heterozygosity deficiency phenomenon among the Manila clam populations. In this study, the MICRO-CHECKER analysis demonstrated homozygote excess in the developed SSR loci, suggesting that null alleles are common in Manila clams. Null alleles can be generated by poor annealing primer in one or both primer sequences, differential amplification of size-variant alleles, or PCR failure due to inconsistent DNA template (Dakin and Avise 2004; Lee and Guo 2006; Zhan et al. 2007).

Genetic diversity of *R. philippinarum* analyzed in this study using the 11 SSR loci (i.e., 11.1 as an average with a range of 6 to 19 in HD clams, and 4 to 20 in SS clams) is comparatively lower than the genomic SSRs of Manila clams previously reported. Yasuda et al. (2007) analyzed Manila clam population genetic diversity using 9 SSRs markers, reporting 6 to 22 alleles, with an average of 12.2. Using 13 SSRs markers, An et al. (2009) also identified the number of alleles in Manila clam originating from the west coast of Korea, reporting an average of 14.1 (range of 9–25). However, the mean number of alleles obtained in this study is somewhat higher than the allele numbers previously reported from studies that used EST-SSRs markers, including Kim et al. (2014a), with a mean of 7 (range of 2 to 17), Nie et al. (2014), with an average of 7 (range of 2–17), and Zhu et al. (2015), with a mean of 6 (range of 2–13).

Table 2. Cross-species amplification of 5 EST-microsatellite markers in *Ruditapes variegatus*

Locus	Hong Kong (*R. variegatus*)			Jeju Island (*R. variegatus*)		
	Amplification (success/total)	Number of alleles	Size range (bp)	Amplification (success/total)	Number of alleles	Size range (bp)
Ruph2-KR	1/10	2	179, 200	9/10	9	164–207
Ruph6-KR	10/10	3	203–206	6/10	3	197–205
Ruph14-KR	3/10	3	282–315	1/10	1	293
Ruph17-KR	4/10	5	283–303	1/10	1	329
Ruph19-KR	1/10	2	318, 339	10/10	7	312–336

Compared to genomic SSRs-based markers, the EST derived SSRs marker would have higher transferability to phylogenetically close species, since the ESTs originate from expressed genes (Wang et al. 2009; Yu et al. 2010). To test the transferability, 5 microsatellite loci developed in this study were selected and applied to the congeneric species, *R. variegatus* from Jeju Island and Hong Kong. As Table 1 shows, the 5 selected markers (*Ruph*2-KR, *Ruph*6-KR, *Ruph*14-KR, *Ruph*17-KR, and *Ruph*19-KR) have a comparatively higher number of alleles, compared to the other 7 markers, suggesting that the genetic diversity is well conserved in these markers. As summarized in Table 2, the 5 microsatellite markers successfully amplified *R. variegatus* SSRs in PCR reactions, although the success rate ranges from 1/10 to 10/10, depending on origin of the clam populations. The PCR products also demonstrated that cross-amplification of the *Ruph*14-KR and *Ruph*17-KR loci to *R. variegatus* from Jeju Island yields a monomeric band (i.e., 1 allele), while the same markers applied to *R. variegatus* from Hong Kong resulted in polymeric bands of 3 to 5 alleles. Accordingly, it is believed that the 5 EST microsatellite markers derived from *R. philippinarum* are also useful in the study of stock conservation and genotyping of the congeneric species, *R. variegatus*.

In summary, we developed 11 EST-microsatellite loci from clam hemocyte cDNA clones challenged by *P. olseni*, a protozoan parasite in Manila clam to understand the genetic diversity of Manila clam populations distributed in Korean waters. Among the 11 microsatellite markers, the 5 EST microsatellite markers successfully differentiated *R. philippinarum* from *R. variegatus*, a genetically close species to *R. philippinarum*, suggesting that these markers can be usefully applied in clam stock conservation and genotyping of different species in the genus *Ruditapes*.

Acknowledgements

The authors would like to thank the staff of Shellfish Research and Aquaculture Laboratory at Jeju National University, for their clam sampling and DNA extraction. This research was supported by a grant from National Institute of Fisheries Science of Korea (R2017052), and a grant (11-1541000-001219-01) from the Ministry of Oceans and Fisheries, Korea. This study was also supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2016R1A6A1A03012647).

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