Isolation and characterization of microsatellite markers in *Ruditapes philippinarum*

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ABSTRACT. *Ruditapes philippinarum* is considered a commercially valuable species, which is commonly found in tidal flats along West Pacific coasts. In China, it is mainly distributed in the southeast sea. In this study, 16 novel microsatellite loci from the *R. philippinarum* genome were developed, using the protocol of fast isolation by amplified fragment length polymorphism of sequence containing repeats. Thirty-two wild-caught individuals were used to evaluate the degree of polymorphism of these markers. Our results show that there were 10 polymorphic loci and 6 monomorphic loci. The number of alleles per locus and the polymorphism information content ranged from 2 to 6 and from 0.199 to 0.751, respectively. The observed and expected heterozygosities varied from 0.1333 to 0.6207 and 0.1603 to 0.7412, respectively. Of all loci, only one locus was found to deviate significantly from Hardy-Weinberg equilibrium after Bonferroni correction. The loci identified here will provide useful information for future population genetic studies of *R. philippinarum*.

Key words: *Ruditapes philippinarum*; Microsatellites; Genetic markers; FIASCO

INTRODUCTION

Ruditapes philippinarum is considered a commercially valuable species, widely distributed on tidal flats in the West Pacific coasts from Russia to the Philippines (Allam et al., 2000; Mura et al., 2011). In China, it is mainly distributed in the north and south coastal waters (Yan et al., 2006). It belongs to the family Veneridae, and because of its characteristics of rapid growth, short breeding cycle, and strong adaptability to a wide salt and temperature ranges, it is highly suitable for artificial, high-density breeding of shellfish (Park and Choi, 2001). Recently, it has become one of China's four major farmed shellfishes. Being among the most economically important mollusks, its 2009 annual yield was 3 million tons, accounting for 30% of shellfish aquaculture production and close to 20% of the mariculture production (Chong and Wang, 2001). The Chinese market has a huge potential, with the improvement of people's living standards and short supply in both domestic and foreign markets. Due to lack of attention to the natural resource conservation of *R. philippinarum*, its natural production has been declining every year. Therefore, there is currently a serious shortage of seedlings, causing a "bottleneck" that restricts the development of *R. philippinarum* breeding (Kang et al., 2006). There is therefore an urgent need to establish proper molecular genetic marker techniques that can be used to study its genetic structure, test the level of genetic variation, and aid in establishing molecular marker assisted breeding and genetic improvement.

Microsatellite DNA refers to tandem-repeated nucleotides in the DNA sequence, which are also known as simple sequence repeats (SSR) or short tandem repeats (STR) (Cao et al., 2012). Compared with other molecular markers, microsatellite markers are non-coding, highly polymorphic, and have high information content. Microsatellites also exhibit high repeatability, co-dominant inheritance, and are currently widely used in studies of population genetics, population differentiation, linkage analysis, and evolution (Li et al., 2014; Ning et al., 2015). To date, population genetic studies of *R. philippinarum* have mainly been based on RAPD, EST SSR, SRAP, and mitochondrial COI genetic markers, etc. However, there are no studies of the magnetic bead enrichment method that is currently used to develop *R. philippinarum* microsatellite markers. Using this method, this line of research aims at performing clam population genetic analyses and developing and providing technical support to an assisted breeding and germplasm resource protection community. In the present study, a novel marker suite of 16 *R. philippinarum* loci was developed. This may be useful for future population genetic studies and for the protection of the species resources.

MATERIAL AND METHODS

DNA extraction and microsatellite library construction

We report 16 *R. philippinarum* microsatellite markers identified using the fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) protocol (Zane et al., 2002). Firstly, 32 wild, healthy adult *R. philippinarum* individuals were collected from Xiamen, China. Genomic DNA was extracted from adductor muscle tissue, using the Genomic DNA Extraction kit (Tiangen, Beijing, China) following the manufacturer instructions. Enriched DNA was estimated by ultraviolet spectrophotometer analysis and electrophoresis on a 1% agarose gel. The obtained DNA was diluted to 100 ng/μL, followed by digestion with 10U restriction enzyme FastDigest TruI in a 25 μL volume, incubated

for 10 min at 65°C. The digested fragments, ranging from 500 to 1200 bp, were purified and ligated overnight at 22°C to *MseI* adapter 1 (5'-ACGATGAGTCCTGAG-3')/*MseI* adapter 2 (5'-TACTCAGGACTCAT-3') by T4 DNA ligase (Fermentas, Vilnius, Lithuania). The digestion-ligation mixture was subsequently denatured and hybridized to the biotin-labeled oligo-nucleotide probes (CT)₁₅ and (GT)₁₅. Fragments containing microsatellite repeats were captured with Streptavidin-coated Magnetic Sphere Particles (Promega, Madison, WI, USA), and non-annealed DNA was washed away. The recovered products were amplified using *MseI* adapter 1, and the polymerase chain reaction (PCR) products were subsequently purified by removing extra dNTPs and adaptors. Finally, the purified products were ligated to the pMD19-T vector (TaKaRa, Shiga, Japan) and then transformed into *Escherichia coli* (Invitrogen, Carlsbad, CA, USA) for further selection on ampicillin plates.

Gaining SSR fragments and designing specific primers

Positive clones were amplified via colony-PCR with universal M13 primers. DNA fragments >500 bp were sequenced by the Invitrogen Company (Shanghai, China). Specific primers with SSR sequences were designed using Primer Premier v. 5.0 (Singh et al., 1998). The amplification conditions of all primers were selected through optimization in an Eppendorf Mastercycler Gradient System (Eppendorf, Hamburg, Germany).

Polymorphism validation

Primers that amplified successfully were validated in the genomic DNA of the 32 wild-caught *R. philippinarum* individuals. The PCR amplification conditions consisted of a 10-μL volume containing 50 ng genomic DNA, 10X Taq buffer, 2 mM MgCl₂, 0.4 μM each primer, 0.2 mM each dNTP, and 0.25U Taq DNA Polymerase (Fermentas). The PCR was carried out under the following conditions: pre-denature at 94°C for 5 min, followed by 37 cycles of denaturing at 94°C for 40 s, annealing at optimal temperature (Table 1) for 40 s, and 72°C for 1 min, with a final 10 min elongation step at 72°C. The PCR products were electrophoresed on 6% denaturing polyacrylamide gels in a Sequi-Gen Sequencing Cell (Bio-Rad, Hercules, CA, USA) and were then visualized by silver staining.

Statistical analysis and interpretation

The data matrix was analyzed by the statistical software POPGEN 32 (v. 1.32) (Yeh et al., 2000) and CERVUS 3.0 (v. 3.0) (Yeh et al., 2000). The number of alleles per locus and the polymorphic information content (PIC) were calculated using POPGEN 32 (v. 1.32), while the observed and expected heterozygosities were calculated by CERVUS 3.0 (v. 2.2.3). Deviations from Hardy-Weinberg equilibrium and genotypic linkage disequilibrium were tested using POPGEN 32.

RESULTS

Cloning and sequencing results

A total of 180 positive clones were amplified via colony-PCR with universal M13

primers. Of these, 140 microsatellite sequences of >500 bp were successfully obtained. Most of these contained two and four base pair repeats, but a few also contained repeats consisting of three to more than five bases. There were no impure peaks, sets of peaks, or wave peaks appearing in the sequencing results.

Table 1. Statistical information of 10 polymorphic microsatellite loci found in 32 individuals of *Ruditapes philippinarum*.

GenBank accession No.	Locus ID	Primer sequences (5'-3')	Repeat motif	Ta (°C)	N _A	PIC	H _O	H _E	Allele size (bp)
KR704619	F1	F: AAGGAAAGACGGCTTCG R: GTGATTGACCACAGGGAC	(CT) ₁₄	53	4	0.494	0.2105	0.2845	183-210
KR704620	F2	F: ATTCCATTAGCACCTCT R: CACACACACCCACACA	(TGCG) ₅	43	5	0.333	0.2759	0.3091	135-155
KR704621	F3	F: ATATCTGCCTGCTGGTAA R: TCGGTCAAGCCTTCA	(GTCG) ₄	49	4	0.438	0.6207	0.5136	148-174
KR704622	F4	F: GCTTTCAAGCCACATCC R: AGACATTCCAGGTAGTCCAAC	(GAT) ₃	53	6	0.751	0.4400	0.7412	200-231
KR704623	F5	F: CTAATGACTGACTTTGCTCT R: TCTGTAGTCCCAACCC	(GA) ₃₅ (GA) ₅	48	2	0.324	0.3000	0.4130	200-207
KR704624	F6*	F: GAACTTGCAGCCAGAG R: AACACCCCAACGGACTA	(GTT) ₃	50	3	0.199	0.1724	0.1603	216-240
KR704625	F7	F: TTTCATCCACCATCTCC R: CACAAACATAGAAGTCCAGT	(GAT) ₃	52	4	0.566	0.4643	0.5981	275-297
KR704626	F8	F: AGACGGGAACATCACCAT R: AAACCGCACCTGACACC	(GACAAG) ₃	53	5	0.665	0.2800	0.6408	220-243
KR704627	F9	F: TTCCTCCCTCGCTTCAC R: AGATGGATGGCACTGGTT	(CA) ₅	53	3	0.403	0.5357	0.3994	199-218
KR704628	F10	F: ACCGCACCTGACACTCT R: ACGGGAACATCACCATC	(CTTGTC) ₃	50	5	0.206	0.1333	0.2181	230-254

Ta = annealing temperature; N_A = number of polymorphic alleles per locus; PIC = polymorphism information content; H_O = observed heterozygosity; H_E = expected heterozygosity; *high significant deviations (P < 0.001) of locus from Hardy-Weinberg equilibrium after Bonferroni correction (k = 10).

Polymorphic analysis

We designed 45 pairs of specific primers. The optimized PCR products were visualized on 6% denaturing polyacrylamide gels, e.g., the polymorphic locus F2 shown in Figure 1. We were able to successfully amplify 16 novel microsatellite markers, including ten polymorphic microsatellite loci (Table 1) and six monomorphism loci (Table 2).

Figure 1. Electrophoretogram of the amplification of the F2 locus.

The number of alleles ranged from two to six, whereas the PIC ranged from 0.199 to 0.751. The levels of expected and observed heterozygosities varied from 0.1333 to 0.6207 and 0.1603 to 0.7412, respectively. Even after sequential Bonferroni correction (P < 0.0005), most loci were in Hardy-Weinberg equilibrium, except for locus F6. This may be attributed to the limited number of samples tested.

Table 2. Statistical information of 6 monomorphic microsatellite loci identified in 32 *Ruditapes philippinarum* individuals.

GenBank accession No.	Locus ID	Primer sequences (5'-3')	Repeat motif	Ta (°C)	N _A	PIC	H _O	H _E	Allele size (bp)
KR704629	F11	F: TTCCATTAGTACCTCTATAAAA R: GAAACGTCAGTCATCTTC	(AG) ₆ N(GT) ₂₂	46	-	-	-	-	315
KR704630	F12	F: ATCACCAGCTTGAGCAG R: TAAAGCCTCCCGTAAAT	(CA) ₂₉ N(CT) ₂₄ N(TC) ₁₂	47	-	-	-	-	268
KR704631	F13	F: TGTTCCTTTGTTTGTTCGTT R: CCTGTAGGTCACCGTTGC	(GT) ₂₀ (CGTG) ₃ (GT) ₂₅	53	-	-	-	-	192
KR704632	F14	F: GAAACCGCACCTGACACC R: CAACAATCTCAAGGAGGAAAC	(CTTGTC) ₃	55	-	-	-	-	314
KR704633	F15	F: ATCCCTACCCGAAACA R: TCCGATTATTGCTCTATCACTT	(AT) ₅	53	-	-	-	-	274
KR704634	F16	F: TTACCCCTCGTTTCTGC R: CCAAATCTTTATGCTGA	(TCTG) ₄	49	-	-	-	-	330

Ta = annealing temperature; N_A = number of polymorphic alleles per locus; PIC = polymorphism information content; H_O = observed heterozygosity; H_E = expected heterozygosity.

DISCUSSION

Despite the well-known importance of *R. philippinarum*, its genetic information has been studied relatively little. In general, this research field currently mostly uses SSR/SNP markers to study genetic diversity. However, SSR markers are a more appropriate choice when dealing with rare species and requires substantial funding. In this study, we used the FIASCO protocol to identify 10 polymorphic loci and six monomorphic loci. Xing et al. (2014) used 10 published primers to study cultured and wild populations of *R. philippinarum* in China. Therefore, the results of this study can be used as candidate gene loci to study population genetics. We developed a relatively small number of loci, due to the limitation in sample collection from different sites. To investigate genetic diversity of two Korean populations, Kim et al. (2014) developed 10 polymorphic loci from expressed sequence tag-derived microsatellite markers, using next-generation sequencing. Different methods used to develop SSR loci for the same species differ in efficiency. In this study, we had a 77.78% positive rate of cloning, which greatly exceeded the 0.52% achieved in Kim et al. (2014). This phenomenon could be due to the construction of an aimed SSR gene library. Following the library construction, 10 polymorphic loci out of 45 loci (22.22%) were screened that contained two to six different nucleotide repeat motifs. This rate of polymorphism is slightly higher than the 16.1% found in the same species (Kim et al., 2014). In our study, six monomorphic loci were also identified. These could be the result of conserved properties of the loci or low genetic diversity, but in the future, these too may play an important role in genetic studies of *R. philippinarum*.

This study was limited by the number of populations and the methods used for testing for polymorphism. Although the series of procedures used for developing and screening the SSR loci were complicated, the efficiency was satisfactory. In conclusion, these novel microsatellite markers isolated for *R. philippinarum*, will increase and supplement the genetic information background for this species. These loci will be useful for studies of genetic diversity, population structure, the construction of genetic map, and conservation strategy design.

Conflicts of interest

The authors declare no conflict of interest.

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