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TECHNICAL NOTE

Development and characterization of EST-derived microsatellite makers for Manila clam (*Ruditapes philippinarum*)

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Abstract Twenty-five polymorphic microsatellite makers were developed and characterized from expressed sequence tag sequence of the Manila clam, *Ruditapes philippinarum*. The number of alleles at each locus ranged from 3 to 20 with an average of seven alleles per locus. The observed and expected heterozygosity varied from 0.081 to 0.730 and from 0.127 to 0.926, respectively. Thirteen loci were found deviate significantly from Hardy–Weinberg equilibrium. These microsatellite loci will be useful for further studies on the population structure and genetic variation of this species.

Keywords *Ruditapes philippinarum* · Manila clam · Microsatellite · Polymorphism

The Manila clam, *Ruditapes philippinarum*, which is widely distributed on tidal flats in the West Pacific coasts from Russia to the Philippines, is one of the important commercial resources for the coastal fisheries. However, the wild stocks of *R. philippinarum* have been declining dramatically for last decades due to over-exploitation and the deterioration of environmental conditions in China. In recent years, recovery efforts such as artificial breeding program and stock enhancement are in progress (Zhang and Yan 2009). But, the genetic effects of hatchery individuals on wild populations of *R. philippinarum* have not yet been fully evaluated. Therefore, reasonable stock management and genetic improvement are required for sustainable development of *R. philippinarum* aquaculture industry.

Microsatellites or simple sequence repeats (SSRs) have become one of most commonly used DNA markers in population genetics and evolutionary biology research, and they have been widely applied in studies of biological breeding, and genetic linkage maps. Although some microsatellite markers have been developed in *R. philippinarum* (Yasuda et al. 2007; An et al. 2009), more polymorphic microsatellites are still required in *R. philippinarum* to enable parentage, population genetics and genome mapping studies. In this study, we report 25 novel polymorphic microsatellite markers developed from expressed sequence tags (ESTs) of the *R. philippinarum* that will be useful for genetic research of this species.

A total of 5,844 *R. philippinarum* ESTs obtained from GenBank (Sep 20, 2012) were scanned and assembled using SeqMan II sequence assembly software (DNASTAR Inc., Madison, WI) and 4,549 potential unigenes that contain contigs and singletons were generated. SSRHUNTER program (http://www.biosoft.net/dna/SSRHunter.htm) was used to find regions containing microsatellites. Parameters were set for the detection of di-, tri-, tetra-, penta-, and hexanucleotide motifs with a minimum of five repeats. Primers flanking microsatellites were designed using the PRIMER 5.0 program (http://www.premierbiosoft.com/).

Polymorphism evaluation was tested by 38 wild individuals of *R. philippinarum* collected from Dalian, Liaoning province, China. Genomic DNA of each specimen was extracted from adductor muscle tissue by standard proteinase K digestion, phenol–chloroform extraction, and

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Table 1 Characterization of 25 EST-SSR markers in *R. philippinarum*

Locus	Accession Number	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	N_a	Size range (bp)	H_o	H_E	P value
RpN03	AM875256	$(TGTA)_5$	F: TCTCCTGCCTTAACCACA R: GGCTCCCACATTCTCATT	57	6	330–356	0.500	0.482	0.0996
RpN04	AM873802	$(ACT)_5$	F: AATACTAACGCTGTGGAT R: CTAATGACTTAATGAGCAAA	58	15	292–402	0.167	0.853	0.0000*
RpN07	AM875824	$(AT)_5$	F: TACCAACGCTCCTACAACTG R: CCATTCACTTTCCAGCAATA	58	3	177–213	0.132	0.127	1.0000
RpN10	AM872973	$(AT)_5$	F: GGCTCGTGTCTTATTGTCT R: TAAACATACTTCTGAAATGCA	58	20	380–530	0.200	0.926	0.0000*
RpN12	AM877175	$(ATG)_5$	F: CCAACGTATAGTACCCGTAAC R: CCATGTAGACAAGTTTGAACCC	63	3	360–364	0.263	0.406	0.0193
RpN13	AM873183	$(TA)_5$	F: ATTTTGCCAGTAATGTACAGTA R: ACACATGGTCGATAATACGC	53	16	360–490	0.486	0.782	0.0000*
RpN14	AM872742	$(TG)_5$	F: AATTTTACATCACGCATTTACAG R: TCACACAAACGACATTTCAATAC	58	3	380–384	0.189	0.496	0.0000*
RpN15	AM875337	$(TA)_5$	F: AGTGTTTCATAGGATTGGTTTA R: TACACAGTAGTCAGATACACAGCA	61	4	525–610	0.639	0.591	0.0745
RpN21	AM876481	$(AT)_6$	F: AAGAAACACGCCAACCTC R: TTGCACGCATAAACCTTA	57	4	130–170	0.263	0.383	0.1017
RpN25	AM877179	$(AT)_6$	F: GTTATCGACTTGATACGTGGTC R: TGAAAGGTTAAGACATCAACAG	61	3	257–261	0.297	0.472	0.0046
RpN27	AM877536	$(TA)_6$	F: TGAGAATGACGACCTGAC R: CTAGAAATACAAAGCAAACA	60	8	300–410	0.316	0.575	0.0000*
RpN30	AM875354	$(TTA)_6$	F: CTCAAGAAATAGTGGGATTT R: TTACATGGTTTCGGTTCA	47	5	225–290	0.189	0.640	0.0000*
RpN35	AM876061	$(TA)_6$	F: CAAAAGAAAAGAAGCAAAGA R: ATTGTGCAATAACTGTCTCAT	58	6	226–290	0.083	0.574	0.0000*
RpN36	AM873978	$(TGT)_6$	F: TGGATATGGTGCCTGTTG R: GATGTGAGGGCTCGGTTT	61	6	330–358	0.162	0.596	0.0000*
RpN37	AM875292	$(TA)_6$	F: TGAACAGCCATGTCCAAT R: ACCACTAGGCGGAGCATT	61	10	240–290	0.684	0.772	0.0216
RpN38	AM874695	$(ATG)_6$	F: TCAGGAACGGTGACTATCA R: AGCACATCGCCTTCTTTA	58	6	166–192	0.371	0.391	0.0264
RpN41	AM873398	$(TTC)_6$	F: CATCACAAGCAACAGAAC R: CCTATAAATAATACCACCAT	49	14	280–360	0.730	0.726	0.0127
RpN44	AM875208	$(TA)_6$	F: AGACTTGGAATGGTGGGT R: GTGATCATGCTTGTTTGGA	61	6	280–310	0.237	0.645	0.0000*
RpN46	AM875126	$(AT)_6$	F: AAGTGCCATTTTCAAGTT R: CAAGCTATTATAGTGTTTTCG	53	3	180–186	0.108	0.320	0.0001*
RpN47	AM875854	$(ACA)_{13}$	F: ATGTAGAATAAAACAGGCAAAAC R: TCATTCAATGTAACGCTGTC	55	10	180–252	0.514	0.606	0.0857
RpN48	AM872844	$(GAAT)_7$	F: CGATTGGCATTCGTCAGG R: AGCCCTCAAATGTCCGTTA	53	9	310–400	0.278	0.671	0.0000*
RpN50	AM873347	$(AC)_9$	F: CTTGGACGGATTTACTTT R: CGTTCAATTCTTTTGCTT	50	13	351–500	0.605	0.889	0.0000*
RpN51	AM873318	$(GT)_6$	F: AGACGTTATGCTGTTAGC R: TTGTTCTTGTTGCGATAT	53	12	230–520	0.474	0.635	0.0044
RpN53	AM874854	$(AT)_6$	F: AGAGGCTTAATAATACGGTTTA R: CATACAAAACATCTGAGGGA	50	5	370–386	0.139	0.595	0.0000*

Table 1 continued

Locus	Accession Number	Repeat motif	Primer sequence (5′–3′)	T_a (°C)	N_a	Size range (bp)	H_o	H_E	*P* value
RpN56	AM872995	$(GT)_5$	F: TTATGACGCCTGGGTTAC R: GCCAATCAGATGGGAATT	48	4	210–230	0.081	0.130	0.1245

T_a annealing temperature of each primer pair, N_a observed number of alleles, H_O observed heterozygosity, H_E expected heterozygosity

* Indicates significant departure from Hardy–Weinberg equilibrium after sequential Bonferroni correction ($P < 0.05/25$)

DNA precipitation. Polymerase chain reaction (PCR) was performed in 10-µl volumes containing 0.5 U easy *Taq* DNA polymerase (TransGen, Beijing), 1 × PCR buffer, 0.2 mM dNTP, 0.4 µM of each primer set, 1.5 mM $MgCl_2$, and about 25 ng template DNA. The reactions were performed using the following parameters: 3 min at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at the annealing temperature listed in Table 1 and 45 s at 72 °C, then a final extension of 5 min at 72 °C. Amplification products were resolved on a 8 % polyacrylamide gel and visualized by silver staining.

A total of 228 microsatellite-containing EST sequences were identified from 5,844 ESTs in the *R. philippinarum* EST database. Of the 228 sequences, 57 were selected for microsatellite marker optimization because of repetition times and flaking sequence priority. Of the 57 potential microsatellite markers, 18 were not easily amplified, 14 were monomorphic, and 25 were found to be polymorphic among 38 individuals of *R. philippinarum*. Of the 57 primer pairs developed, 25 microsatellite loci (43.9 %) showed polymorphism in the population of *R. philippinarum* (Table 1).

The number of alleles, and observed (H_O) and expected (H_E) heterozygosities were estimated by MICROSATELLITE ANALYSER software (Dieringer and Schlötterer 2003). Tests for linkage disequilibrium (LD) and deviations from Hardy–Weinberg equilibrium (HWE) were performed by GENEPOP 4.0 (Rousset 2008). Sequential Bonferroni corrections (Rice 1989) were applied for all multiple tests ($P < 0.05$).

The number of alleles per locus ranged from 3 to 20 with an average of 7.76, and the observed and expected heterozygosities ranged from 0.081 to 0.730 and from 0.127 to 0.926, with an average of 0.324 and 0.571, respectively (Table 1). Tests for linkage disequilibrium showed a nonrandom association ($P < 0.01$) between four pairs of loci (RpN13/RpN14, RpN10/RpN13, RpN37/RpN38, RpN36/RpN56). Thirteen loci (RpN04, RpN10, RpN13, RpN14, RpN27, RpN30, RpN35, RpN36, RpN44, RpN46, RpN48, RpN50 and RpN53) deviated significantly from HWE after correction for multiple tests, which may be due to the presence of null alleles and sampling effect. The results obtained in this study indicated that these SSRs developed from EST in the Manila clam will be a useful tool for the genetic research such as population variation, parentage analysis, stock enhancement evaluation, and the establishment of effective conservation strategy of *R. philippinarum*.

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