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SHORT COMMUNICATION

Microsatellites within genes and ESTs of the Pacific oyster *Crassostrea gigas* and their transferability in five other *Crassostrea* species

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Keywords: indwelling medical devices, infections, surface attachment.

Abbreviations: AFLP: amplified fragment length polymorphism
EST: expressed sequence tags
H_e: expected heterozygosity
H_o: observed heterozygosity
HWE: Hardy-Weinberg equilibrium
PCR: polymerase chain reaction
SSR: simple sequence repeat

We developed 15 novel polymorphic microsatellites for the Pacific oyster *Crassostrea gigas* by screening genes and expressed sequence tags (ESTs) found in GenBank. The number of alleles per locus ranged from two to 24 with an average of 8.7, and the values of observed heterozygosity (H_o) and expected heterozygosity (H_e) ranged from 0.026 to 0.750 and from 0.120 to 0.947, respectively. No significant pairwise linkage disequilibrium was detected among loci and eight loci conformed to Hardy-Weinberg equilibrium. Transferability of the markers was examined on five other *Crassostrea* species and all the markers were amplified successfully in at least one species. These new microsatellites should be useful for population genetics, parentage analysis and genome mapping studies of *C. gigas* and closely related species. The nine markers identified from known genes are expected to be especially valuable for comparative mapping as type I markers.

The Pacific oyster, *Crassostrea gigas*, naturally distributed in Japan, China and Korea, has become a worldwide aquaculture species because of its fast growth rate, high disease resistance and adaptability to different environments. It has had the highest worldwide production of any cultured aquatic species since 1993; in 2006, world production of this species was 4.6 million metric tons (Food and Agriculture Organization, 2008). Still, oysters are in an early stage of domestication. To improve the increasingly valuable Pacific oyster industry, genetic improvement programs have been initiated in several countries (Langdon et al. 2003), and to date two genetic linkage maps that would facilitate marker-assisted selection, quantitative trait locus (QTL) mapping, and functional genomic research have been developed using amplified fragment length polymorphism (AFLP) (Li and Guo, 2004), and microsatellite markers (Hubert and Hedgecock, 2004). However, the AFLP maps are poorly transferable, and the microsatellite map is only moderately. There is a great need for developing codominant markers in the Pacific oyster.

Microsatellites, or simple sequence repeats (SSRs), are

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Table 1. Characterization of 15 novel polymorphic microsatellites within genes and ESTs for the Pacific oyster *Crassostrea gigas*.

Locus	Repeats	Primer sequence (5'-3')	T_a	N_a (size range, bp)	Gene identity	Location in the gene	H_o	H_e	P	Accession no.
CGG001	$(AT)_6$	F: GTGCCTCCTCTATGTGGG	60	3 (320-340)	Alpha amylase A	1st intron	0.050	0.441	0.000*	AF320688
		R: GGATCGCTGTTTCACTCG								
CGG002	$(AG)_5$	F: CAAACAAGGTATCATCCG	54	16 (204-244)	5S ribosome RNA	NTS	0.256	0.888	0.000*	AY765366
		R: AATAATAGCAGCTTCCATC								
CGG003	$(AG)_{16}$	F: GGAGGACAAAGAGGAGGT	58	9 (405-413)	Metallothionein	1st intron	0.525	0.812	0.001	AJ242657
		R: CCAGTCCCGCATGTCTAG								
CGG004	$(GA)_{12}$	F: GAGTATTGAGGAGACGGAC	56	13 (631-639)	Activin-like type 1 receptor	1st intron	0.105	0.903	0.000*	AJ309316
		R: GACAGTTCATCAGGTGCTTC								
CGG005	$(GA)_{26}$	F: AGCACGGCAGAGGATAGG	56	19 (361-415)	Chitinase-like protein 1	3rd intron	0.180	0.939	0.000*	AJ971241
		R: ACTATTCGGCGGGTCTCA								
CGG006	(AG)18	F: AGCCTTGAATAATCGGTC	56	7 (430-448)	Metallothionein 2	1st intron	0.750	0.594	0.374	AJ297818
		R: ATTTACACCCGTCACCAC								
CGG007	$(GA)_{22}$	F: ATTGTTTCTGTCTTCGTC	50	3 (345-349)	LPS-binding protein	3'UTR	0.026	0.285	0.000*	AY165040
		R: TCGTACAAGAATAAAGGAT								
CGG008	$(AG)_{20}$	F: TCTCCTCTACCCCGACAG	56	24 (176-254)	Iron regulatory protein 1-like protein	1st intron	0.650	0.947	0.000*	AJ579915
		R: GTGATGAACAAACCACCAAC								
CGG009	$(AT)_5$	F: CCTTAACTGCACTGGTCCC	60	2 (154-160)	Heat shock protein 68	3'UTR	0.083	0.178	0.021	AB122062
		R: CGTGCAATGATAAATGATGC								
CGE027	$(AG)_5$	F: GCCGCCTTTCAGACTTTC	54	8 (278-298)	Unknown	Unknown	0.675	0.761	0.274	ES789161
		R: GATGGGACAAACAACGACA								
CGE028	$(CT)_8$	F: CATCTTGTTGTAACCCACG	54	3 (246-254)	Unknown	Unknown	0.125	0.120	1.000	DV736587
		R: GCTTCCCTTTAATGATTCTC								
CGE030	$(AC)_5$	F: TGCTGCCTGTGGAATGTT	52	2 (193-195)	Unknown	Unknown	0.375	0.392	1.000	BQ427330
		R: TTGTGTTGAGTCGCAAGAGT								
CGE031	$(CT)_5$	F: GATCATTGAAAGAAAGTC	48	3 (116-124)	Unknown	Unknown	0.359	0.403	0.295	BQ426470
		R: CATTGTTGAATAGGTTGT								
CGE032	$(AG)_7$	F: TCGTTGAAGGTGACAAGT	55	15 (140-172)	Unknown	Unknown	0.667	0.899	0.000*	CX068987
		R: AACCGAACCATTTACATC								
CGE033	$(TA)_5$	F: AGTGCCTTCCTATTGTTG	55	4 (174-182)	Unknown	Unknown	0.263	0.411	0.411	BQ426509
		R: GGCGATGATAATGATGAC								

T_a, optimal annealing temperature (°C); N_a, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; P, exact P value for Hardy-Weinberg equilibrium test; *, statistically significant after Bonferroni correction ($P < 0.01/15$).

extremely useful markers for genetic linkage mapping because of their high polymorphism, abundance, codominance and small length, which facilitates genotyping using polymerase chain reaction (PCR) (Liu et al.1999; Wang et al. 2007). For *C. gigas*, numerous microsatellites have been developed recently (Li et al. 2003). But, the pace of development has been limited by the time-consuming and labor intensive requirement to construct, enrich and sequence genomic libraries (Edwards et al.1996). Recently, identification of microsatellites from expressed sequences has been extensively used as an alternative strategy. In addition to requiring less time and money to develop, expressed sequence tags (EST)-derived microsatellites have a number of intrinsic advantages. They tend to be more widely transferable between species, and even genera (Bouck and Vision, 2007). More importantly, because they represent genes, they serve as type I markers, which are more valuable for comparative gene mapping (Liu et al. 1999). To date, a large number of EST-SSRs have been successfully developed from public sequence databases in several aquatic animals, including fish (Serapion et al. 2004), shrimp (Perez et al. 2005), sea urchin (Kong and Li, 2008) and molluscs such as the Eastern oyster (Wang and Guo, 2007) and Pacific oyster (Yu and Li, 2007; Yu and Li, 2008; Wang et al. 2008; Sauvage et al. 2009). Although detection of microsatellites within ESTs generates potential type I markers (Serapion et al. 2004), usually only a small proportion of EST-SSRs shows high identity to previously annotated genes in aquatic species. For example, 16 of 65 (24.6%) showed high identity to annotated genes and was confirmed as type I markers in Atlantic salmon, and similar results have been reported for Eastern oyster (32.1%) and Pacific oyster (37.5%) (Ng et al. 2005; Wang and Guo, 2007; Yu and Li, 2008). In contrast, microsatellites identified from known genes are all type I markers. The identification of specific SSRs in known genes permits not only the location of the genes in linkage maps, but also the unraveling of the biological significance of SSR distribution, expansion, and contraction on the function of the genes themselves (Li et al. 2004). In the present study we report the identification of microsatellites within all known genes and ESTs from *C. gigas*, as well as their characterization and cross-species amplification in five other commercially important *Crassostrea* species.

MATERIALS AND METHODS

All the Pacific oyster gene sequences and 1168 EST sequences were downloaded from GenBank (NCBI, http://www.ncbi.nlm.nih.gov/sites/entrez). The SSRHUNTER program (Li and Wan, 2005) was used to search for SSRs. The criteria used in SSRHUNTER to identify SSRs were as follows: five repeats for di-, tri- and tetranucleotide repeats. EST sequences containing microsatellites were assembled using SeqMan II sequence assembly software (DNASTAR Inc. Madison, USA) for clustering analysis. Sequences containing SSRs were annotated using BLAST software. Primers flanking microsatellites were designed using the Primer Premier 5.0 program.

To evaluate polymorphism, 40 individuals of *C. gigas* collected from coastal waters in Hiroshima, Japan, were used. Genomic DNA was extracted from adductor muscle by standard proteinase K digestion, phenol-chloroform extraction, and DNA precipitation. PCRs were performed in 10-μL volumes containing 0.25 U *Taq* DNA polymerase (Takara Inc.), 1 x PCR buffer, 0.2 mM dNTP mix, 1 μM of each primer set, 1.5 mM $MgCl_2$ and about 100 ng template DNA. PCR was performed on a GeneAmp 9700 PCR System (Applied Biosystems) as follows: 3 min at 94ºC; 35 cycles of 1 min at 94ºC, annealing (Table 1, annealing temperatures) for 1 min, 72ºC for 1 min per cycle; followed by 5 min at 72ºC. Amplification products were resolved via 6% denaturing polyacrylamide gel, and visualized by silver-staining. A 10-bp DNA ladder (Invitrogen Inc.) was used as a reference marker for allele size determination. The number of alleles (N_a), expected heterozygosity (H_e) and observed heterozygosity (H_o) were calculated using Microsatellite Analyser software (Dieringer and Schlötterer, 2003). Tests for linkage disequilibrium and deviations from Hardy-Weinberg equilibrium (HWE) were performed using the GENEPOP program. Significant levels were calculated per locus using sequential Bonferroni method (Rice, 1989).

RESULTS AND DISCUSSION

A total of 327 gene sequences and 1168 EST sequences were screened for microsatellite repeats, from which 21 gene sequences and 37 EST sequences harbored microsatellite-type repeats. From the sequences containing microsatellites, 41 primer pairs were designed for microsatellite marker optimization. Of the 41 primer pairs, 12 were not easily amplified, 14 were monomorphic (Appendix A) and 15 produced polymorphic profiles of the expected size. All these loci are different from the published EST-SSRs (Yu and Li, 2007; Yu and Li, 2008; Wang et al. 2008; Sauvage et al. 2009). Characterizations of these polymorphic loci are summarized in Table 1. The numbers of alleles ranged from 2 to 24 with an average of 8.7 alleles per locus. The observed and expected heterozygosities ranged from 0.026 to 0.750 and from

Table 2. Cross-species amplification of 15 microsatellites from *C. gigas* in five other Crassostrea species including *C. plicatula*, *C. hongkongensis*, *C. ariakensis*, *C. nippona* and *C. sikamea*.

Locus	*C. plicatula* (n = 30)	*C. hongkongensis* (n = 30)	*C. ariakensis* (n = 30)	*C. nippona* (n = 30)	*C. sikamea* (n = 30)
CGG001	5 (300-316)	—	—	—	6 (310-332)
CGG002	7 (206-238)	6 (214-236)	7 (216-236)	5 (210-226)	5 (216-234)
CGG003	7 (405-421)	1 (330)	1 (330)	1 (310)	5 (407-421)
CGG004	—	6 (631-651)	3 (641-651)	3 (639-643)	4 (628-641)
CGG005	8 (381-399)	6 (379-397)	5 (381-399)	8 (381-397)	3 (387-395)
CGG006	3 (436-446)	—	—	1 (436)	4 (434-446)
CGG007	3 (174-180)	3 (184-188)	1 (186)	1 (186)	1 (216)
CGG008	14 (190-240)	2 (162-172)	1 (172)	2 (330-336)	7 (196-236)
CGG009	4 (150-160)	—	—	—	—
CGE027	5 (282-294)	3 (288-298)	2 (294-298)	3 (288-298)	2 (276-282)
CGE028	1 (248)	4 (246-254)	3 (248-254)	2 (248-254)	2 (248-254)
CGE030	1 (195)	2 (195-197)	2 (195-197)	1 (195)	2 (189-195)
CGE031	3 (116-120)	2 (118-122)	3 (116-122)	2 (116-118)	5 (114-124)
CGE032	6 (154-168)	3 (150-156)	—	2 (156-158)	4 (166-174)
CGE033	3 (178-182)	8 (174-194)	4 (176-184)	—	3 (184-190)

Numbers of alleles and allele size range (in parentheses); n, number of samples.

0.120 to 0.947, respectively. No significant pairwise linkage disequilibrium was found among loci. Seven of the 15 microsatellites significantly deviated from the HWE after correction for multiple tests, and all of them showed heterozygote deficiency. We used MICRO-CHECKER (Van Oosterhout et al. 2004) to estimate the most probable cause for departures from HWE. The MICRO-CHECKER analysis suggested there were no indications for scoring error due to stuttering or for large allele dropout. All the seven loci were prone to null alleles ($P < 0.01$). In fact, null alleles are very common in the Pacific oyster (Li et al. 2003; Hedgecock et al. 2004); due to extremely high levels of DNA sequence variation.

Among the 15 polymorphic markers, nine were identified from known genes, and the other six from ESTs. GenBank (BLASTX) searches indicated that the six SSR-bearing ESTs had no significant matches to genes of known functions at E values less than 10^{-4}. The nine microsatellites derived from known genes are all type I markers. Of them, six were located in introns, two (CGG007 and CGG009) in the 3′ untranslated region (3′UTR), and one (CGG002) in the non-transcribed spacer (NTS) of 5S ribosome RNA gene (Table 1). Goldstein and Schlötterer (1999) suggest that null alleles in EST-derived SSRs are usually lower than in genomic SSRs due to lower mutation at spicing sites. The fact that all the microsatellites from known genes are located at non-coding sequences might be responsible for high frequency of null alleles (7 of 15 or 46.7%) detected in this study.

Cross-species amplification was examined in five other *Crassostrea* species, including *C. plicatula*, *C. hongkongensis*, *C. ariakensis*, *C. nippona* and *C. sikamea*, which were collected from their typical habitats. *C. plicatula* and *C. hongkongensis* were collected from Fujian and Guangxi provinces in China, respectively; *C. ariakensis* and *C. nippona* were sampled from the Sea of Ariake and Okatsu Bay, Japan; *C. sikamea* which originated from Japan were collected from Newport, Oregon, USA. Thirty individuals of each species were used for the examination of the transferability of genic microsatellites. All the primer sets amplified successfully at least one species, with *C. plicatula* and *C. sikamea* sharing 14 primer pairs, *C. hongkongensis* and *C. nippona* 12, and *C. ariakensis* 11, indicating the high rate of transferability across *Crassostrea* species (Table 2).

In summary, these genic SSR markers showed adequate level of polymorphism and high rate of transportability. Thus, they can be used as molecular markers for population genetics, pedigree analysis, and genome mapping studies not only in the Pacific oyster but also in related species. The ones located in genes, as type I markers, are expected to be especially useful for mapping these genes in linkage maps and comparative mapping.

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APPENDIX

Appendix A. Primer sequences, repeat types and annealing temperatures for 26 unamplified and monomorphic microsatellite loci in this study.

Locus	Repeats	Primer sequence (5'-3')	T_a	Amplification	Accession no.
Cgjy11	$(AG)_5$	F: CACGAAGGGACCCAAAGA	56	Unamplified	AY551097
		R: GGGACAATGCCAGTCACTAAA			
Cgjy12	$(TAG)_5$	F: TGGTGCCGTTGGATTTGA	54	Unamplified	AB262086
		R: TCACGCTTCCCCATTCAGA			
CgET1	$(CAG)_5$	F: GTCCCCATCATTACCTCAA	54	Monomorphic	ES789767
		R: CTGTATTCATTTCGTTCCCTA			
CgET2	$(TG)_5$	F: TTTCTCACGGATAACGACC	54	Unamplified	ES789749
		R: GCAAACAATCAGGCGATG			
CgET3	$(GA)_5$	F: GACTCAAGGTCGCCAACG	58	Monomorphic	ES789722
		R: TCTCTGAGCCTCTGTTCCAT			
CgET4	$(CA)_5$	F: CACAAACATCGGTAAAGG	54	Monomorphic	ES789663
		R: GTTGCCAGGATAGTTCTTC			
CgET5	$(GTA)_6$	F: AGACGATAACGACCACGAC	56	Monomorphic	ES789514
		R: GACTATGGCATCCACCCT			
CgET6	$(CCG)_5$	F: AAGTTGGTGGAGGAGCAG	56	Monomorphic	ES789479
		R: AAGGTCCGATATTTGAAGC			
CgET8	$(GT)_5$	F: GGGGAGGGGAAAGAAAAC	52	Unamplified	ES789233
		R: CGACTTGACACGGGAATGA			
CgET9	$(TC)_5$	F: GGGGAGGGGAAAGAAAAC	52	Unamplified	ES789175
		R: CGACTTGACACGGGAATGA			
CgET10	$(ATG)_5$	F: TGTCCTACGGATTCAAGATT	54	Unamplified	ES789168
		R: CCATAGACTGGATAGCACCT			
CgET12	$(GT)_5$	F: AGGACGGTCAGTTCTTGT	56	Monomorphic	DV736792
		R: TTGGTGTAATGTTGTCCC			
CgET16	$(AT)_5$	F: GACTTCACCAAGAAATACG	52	Monomorphic	CX068904
		R: TCACTATCGGTTACAGCAC			
CgET17	$(GT)_5$	F: CTGCCTTCGGTGTTCCTT	58	Unamplified	CB617346
		R: CACATAGACGGGCTGAGAAC			
CgET18	$(AGA)_5$	F: ACATGCAACTACAACCCCC	56	Monomorphic	AJ565768
		R: TGGATGCCCAATCACAAG			
CgET19	$(AC)_5$	F: TCAGTCTGGTGATTTCGTG	52	Unamplified	BQ427090
		R: AGTGGCGTGACTTCCTGT			
CgET20	$(TA)_5$	F: TTCCATCTTCAAGTTCGG	52	Unamplified	BQ426771
		R: GAGCACATATTTTGCGTTA			
CgET23	$(AG)_6$	F: CAGATTTGATTGTAGTTC	54	Monomorphic	CX739659
		R: CAAGATTGAGCAGAGTTA			
CgET24	$(CA)_5$	F: CAGACTACCAACCCACCT	56	Unamplified	BQ427097
		R: CCACAGAATGACGCAAGT			
CgET25	$(TC)_5$	F: TCAACTACAAAATGCTCCTC	46	Unamplified	BQ426985
		R: ATACGGTGGTGACGATTA			
CgET27	$(TG)_5$	F: GTTGAGACATTAGGGAGAT	55	Monomorphic	BQ426493
		R: TGTGTAAACAGAGGGTAGTG			
CgET28	$(AG)_5$	F: GAGTATGAGGCAGCAGAG	54	Monomorphic	BQ426346
		R: GTATCGTTTGCTTTGTGC			
CgET29	$(GAA)_5$	F: AGATGCTGATGGGTTTAT	55	Monomorphic	CK172355
		R: CCCTGTTTCACTCTTTTC			
CgET30	$(AG)_5$	F: ATACCATTCATACAACCGAT	55	Monomorphic	CK172332
		R: TTCAACTACAAAATGCTCCT			
CgET31	$(CT)_6$	F: TTGGACTATCACATCTCA	58	Monomorphic	DV736587
		R: ACAGTTTATTACCTACTTCTA			
CgET32	$(CAA)_6$	F: AGGCAGAGGCAACAAACT	55	Unamplified	DV736339
		R: CGTGATGAACAATAGCGAAA			

T_a, optimal annealing temperature (°C).