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Identification of Novel Microsatellite Loci in the Pacific Oyster (*Crassostrea gigas*) by Magnetic Bead Hybridization Selection

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Summary

Four microsatellite loci were isolated from the Pacific oyster, *Crassostrea gigas*, using an enrichment method based on magnetic/biotin capture of microsatellite sequences from a size-selected genomic library. The method can rapidly recovers microsatellite from oyster genomic DNA. Forty-one unique microsatellite loci (20.5% of white colonies screened) containing 5 or more dinucleotide repeats were identified. Primers designed to PCR amplify the microsatellite loci were used to screen 30 individuals from a natural Pacific oyster population in Onagawa Bay, Miyagi Prefecture, Japan. The four microsatellite loci were all polymorphic, with an average of 6.5 alleles per locus (range 3-10). The mean observed and expected heterozygosities were 0.60 (range 0.48-0.80) and 0.73 (range 0.65- 0.88), respectively. Significant deviations from Hardy-Weinberg expectations were observed at one locus, due to homozygote excess. The expected heterozygosity values were considerably higher than those previously found for allozymes (range 0.19-0.21), suggesting that these microsatellite loci should provide useful markers for studies of trait mapping, kinship, and population genetics.

Key words: microsatellite, Pacific oyster, genetic variability

Introduction

Microsatellites are tandemly repeated array of short nucleotides motifs found in all prokaryotic and eukaryotic genomes analyzed to date (1). As they are evenly dispersed throughout the genomes, and usually characterized by high length polymorphism, microsatellite markers have been widely used for genomic mapping, linkage analysis, pedigree analysis, and population genetics of biological resources (2-5). The Pacific oyster, *Crassostrea gigas*, is of great economic value in the world. To assist in selective breeding programs and population genetic

studies, the microsatellite markers were isolated in the oyster by traditional method (6–8). The traditional approach to obtain microsatellites is to create a size-selected genomic library in a plasmid or phage vector and then screen clones by oligo probes containing different repeat motifs. For microsatellite repeats which are less abundant in the genome, it is difficult to isolate using the method. As numerous microsatellite markers are needed for the studies such as linkage analysis and genomic mapping, it is necessary to establish an efficient method to clone oyster microsatellite sequences. Recently, different approaches have been developed with the aim to enrich for microsatellites (1). One method is based on selective hybridization, and its protocols appear to be extremely popular in those employing enrichment procedures (1, 9–13). In the present study, we isolated CA repeat DNA loci in the Pacific oyster using magnetic bead hybridization selection, and assess polymorphism at microsatellite loci in individuals from a natural population.

Materials and Methods

DNA extraction from oyster

DNA was extracted from a live Pacific oyster from Onagawa Bay, Miyagi Prefecture, Japan. The muscle tissue was removed from the oyster, and ground with dry ice in a mortar. About 100 mg of the tissue was digested overnight at 37°C in 0.7 ml of lysis buffer (6 M urea, 10 mM Tris-HCl, 125 mM NaCl, 1% SDS, 10 mM EDTA, pH 7.5) and 35 μl of proteinase K (20 mg/ml). The reaction mixture was extracted with phenol:chloroform (1 : 1), precipitated with isopropanol, and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA was treated with DNase-free RNase (20 μg/ml) at 37°C for 1 h, and purified again using a phenol/chloroform extraction.

Digestion of genomic DNA, size fractionation and ligation of adapters

Extracted DNA was digested with *Hae*III and *Dra*I using 150 U each for 50 μg of oyster DNA. The restricted DNA (5 μg) was electrophoresed on a 2.5% NuSieve GTG agarose gel (FMC Bioproducts, Maine, USA), and fragments of 300–1,000 bp were excised and purified using a Qiagen column (QIAquick Gel Extraction Kit, QIAGEN, Hilden, Germany). The fragments (1 μg) were ligated with 200 pmol of the *Eco*RI-*Not*I-*Bam*HI adapter (TaKaRa, Ootu, Japan) using a DNA Ligation Kit (TaKaRa), collected by ethanol precipitation, and resuspended in 20 μl H_2O.

Magnetic isolation of target sequences and adapter PCR

One 0.6 ml tube of the Streptavidin MagneSphere® Paramagnetic particles (Promega, Madison, USA) was washed according to the manufacturer's instruc-

tions, resuspended in 300 μl of 5×SSC (1×SSC=150 mM NaCl, 15 mM sodium citrate), and mixed with 300 pmol of biotinylated oligoprobe: 5′-$(CA)_{12}$ GCTTGA-biotin. The six-base noncomplementary region at the 3′ end was used to prevent the probe, copurified with target DNA during magnetic isolation, from acting as primers in subsequent adapter PCR (13). The beads and probe were incubated for 15 min at room temperature, then washed three times in 5× SSC and resuspended in 100 μl of hybridization solution (0.5 M NaCl, 4% polyethylene glycol 8,000) at 56°C (13). The fractioned DNA (20 μl) was mixed with 80 μl of the hybridization solution, denatured by incubating at 95°C for 5 min, added quickly to the beads, and incubated at 56°C for 20 min. The beads were then washed 4 times at room temperature in 200 μl 2×SSC followed by 4 times at 30°C in 200 μl 1×SSC. Immobilized DNA fragments were eluted from the beads in 50 μl 0.15 M NaOH at room temperature for 20 min. The beads were then removed, and the supernatant was neutralized by addition of 5.5 μl 10×TE, 3.25 μl 1.25 M acetic acid. DNA was purified by a Qiagen column (QIAquick PCR purification kit, QIAGEN) and eluted in 50 μl TE buffer. PCR amplification was performed in 50 μl volumes containing 5 μl of the released DNA, 1.25 U of AmpliTaq Gold (Perkin Elmer, CA, USA), 5 μl of GeneAmp 10×PCR buffer (Perkin Elmer), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and 0.6 μM of the adaptor sequence primer, 5′-CGGCGGCCGCGGATCC-3′. Reactions were denatured at 95°C for 11 min before 35 cycles of 94°C for 1 min, 63°C for 1 min and 72°C for 1 min, followed by a 5 min, 72°C final extension. PCR products were purified using a QIAquick PCR purification kit (QIAGEN).

Cloning of the PCR-amplified DNA fragments

The purified PCR products were digested with *Not*I, inserted into the *Not*I site of the pBluescript II SK(+) vector (Stratagene, CA, USA), and the recombinant plasmid vector was transformed into XL1-Blue MRF′ supercompetent cells (Stratagene) following the manufacturer's protocol. Recombinant clones were selected by plating on LB media containing ampicillin (100 μg/ml), IPTG (100 μl of 10 mM stock solution) and X-Gal (100 μl of 2% stock solution).

PCR screening of microsatellite-containing clones

A small portion of a white colony was transferred to 10 μl of 10 mM Tris-HCl (pH 8.5) with a toothpick, incubated at 95°C for 10 min, and then used as template (1 μl) in the PCR reaction with two vector primers (T3 and T7) and the nonbiotin-labeled $(CA)_{12}$ primer. Each 10 μl reaction mixture contained 0.25 U of AmpliTaq Gold (Perkin Elmer), 1× the supplied buffer (Perkin Elmer), 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, and 0.2 μM of each primer. Screening amplifications were performed as follows: 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C, followed by 5 min at

72°C. PCR products were electrophoresed on 1.5% agarose gels. Inserts producing two or more bands were considered likely to contain a microsatellite locus. Positive plasmid DNAs were purified using Qiaprep spin columns (QIAGEN), and then double sequenced on a Shimadzu DSQ-2000L DNA sequencer (Shimadzu, Kyoto, Japan) using ThermoSequenase cycle sequencing kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) in combination with the universal forward (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and reverse (5′-GAGCGGATAACAATTTCACACAGG-3′) primers, respectively. PCR primers for each microsatellite locus were designed using the OLIGO software package (National Biosciences Inc.).

Assessment of polymorphism in microsatellite loci

Primer pairs were tested on a random sample of 30 Pacific oysters from Onagawa Bay. Oyster DNA was extracted by the method as described above. PCRs were performed in 10 μl volumes containing 0.25 U of AmpliTaq Gold (Perkin Elmer), 1×PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 1 μM forward FITC labeled primer and reverse primer, and about 100 ng template DNA. The PCR conditions for all loci were 11 min at 95°C followed by 35 cycles of 1 min at 94°C, 1 min at the annealing temperature listed in Table 1, and 1 min at 72°C, with a final extension of 5 min at 72°C. Amplification products were resolved via 6% denaturing polyacrylamide gel on a Shimadzu DSQ-2000L DNA sequencer equipped with DSQ-SA program (Shimadzu), and a pBluescript II SK(+) sequencing reaction was co-electrophoresed as a size marker.

Statistical analysis

Number of alleles per locus, expected and observed heterozygosities, and chi-square tests of deviations from Hardy-Weinberg expectations were calculated using GENEPOP 3.1 software (14).

Results

Isolation of microsatellites in C. gigas

Two hundred white colonies from the CA repeat-enriched library were screened by PCR, and 67 clones (33.5%) produced two or more bands corresponding to a putative CA repeat motifs. Sequencing these clones revealed 41 (20.5% of white colonies screened) that contained CA repeat sequences with more than five repetitions and appeared promising for further development (unique, flanking sequences intact), 15 that did not have sufficient quality sequence on both sides of the repeat to allow design of PCR primers, and 11 that did not contain an obvious microsatellite. We designed PCR primer sets for 20 candidate loci, and 4 loci were successfully optimized.

Table 1. Characterization of four polymorphic microsatellite loci in the Pacific oyster, Crassosterea gigas.

Locus	Repeat motif	Primers (5′-3′)	Annealing temp. (°C)	Size (bp)	No. of alleles	H_O	H_E	GenBank Acc.no.
Cg521	$(CA)_{18}(CGCA)_8$	GGGATCCACAAGCCGTGAC TCAAACCTGCCAACACAGTGA	53	157-233	8	0.57	0.70	AB084072
Cg326	$(CA)_{36}$	TGTTAGGACTAGTCCAAGCAACCA ACTGCCAATATCTTCAACAATCCA	55	162-196	3	0.53	0.65	AB084073
Cg428	$(CA)_{27}TA(CA)_{22}$	CCTGCATTCGCCATACGGTCG ACAGGTGGAGATGGGCGGATACAG	53	290-302	5	0.48*	0.67	AB084074
Cg433	$(CA)_{32}CG(CA)_{11}$	CATACACACCATCCCCAACATAC CATTAGAAACTGCCAAGTCCAAA	51	258-284	10	0.80	0.88	AB084075

H_O, observed heterozygosity; H_E, expected heterozygosity; $^*P<0.01$.

Table 2. Allele frequencies of four microsatellite loci of Pacific oyster, C. gigas, from Miyagi Prefecture.

Allele	*Cg521*	Allele	*Cg326*	Allele	*Cg428*	Allele	*Cg433*
157	0.017	162	0.317	290	0.033	258	0.083
169	0.033	182	0.450	296	0.250	262	0.100
171	0.467	196	0.233	298	0.133	264	0.167
189	0.200			300	0.483	266	0.167
191	0.217			302	0.100	270	0.017
201	0.033					272	0.050
205	0.017					274	0.217
233	0.017					276	0.033
						278	0.117
						284	0.050

Genetic variability of microsatellites in C. gigas

Primer sequences, repeat motif, annealing temperature, number of alleles, amplified product size range, the observed (H_O) and expected (H_E) heterozygosities for the four microsatellite loci are summarized in Table 1. The four microsatellite loci were all highly polymorphic, while the degree of variability was different at each locus. *Cg433* had the highest number of alleles (10), while number of alleles at *Cg521*, *Cg326* and *Cg428* was 8, 3, and 5, respectively. The expected heterozygosity ranged from 0.65 at *Cg326* to 0.88 at *Cg433*. Significant deviations from Hardy-Weinberg expectations for the observed heterozygosities were observed in the *Cg428* ($P<0.01$). Allele frequencies of the four microsatellites for samples from Miyagi Prefecture are shown in Table 2. At loci *Cg521*, *Cg326*, *Cg428*, and *Cg433*, the frequencies of major alleles 171, 182, 300, and 274 were 0.467, 0.450, 0.483, and 0.217, respectively.

Discussion

In this study, PCR primer design for unique microsatellites was possible for 20.5% of the total colonies screened. The enrichment efficiency shown here is similar to other reports by Gardner et al. (16.7%) (13) and Refseth et al. (33%) (12). In the European flat oyster, the percentage of positive clones containing dinucleotide repeat was 0.52% in the pBKS library and 0.66% in the pUC library (15). Compared with the traditional colony hybridization method, the enrichment procedure, being based on the magnetic bead hybridization selection, is very efficient.

Although the variability observed in the microsatellite loci (average 6.5

alleles per locus) is possibly underestimated due to small sample size (30 individuals), it was still much higher than that of allozymes in the Pacific oyster population (16). Ozaki and Fujio (16) surveyed 10 allozyme loci using 398 Pacific oyster individuals in four wild populations from coastal waters of Japan, and found the average expected heterozygosity in total populations was 0.199 (range 0.191-0.211). The high level of length variation of the microsatellites found here is similar to previous reports in the oyster (6, 7).

There were significant deviations from Handy-Weinberg expectations for the observed genotype frequencies in the *Cg428* ($P<0.01$), perhaps due to the limited sample size and/or the presence of null alleles which were reported in the oyster microsatellite loci (8). The high variability of these microsatellites will make them excellent tools for assessment of genetic variability.

In this study we created CA repeat-enriched microsatellite library in the Pacific oyster. The library will be useful resource for developing additional anonymous microsatellite markers for oyster in the future.

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