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Variable microsatellites in the Pacific Oyster Crassostrea gigas and other cupped oyster species

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Abstract: Source/description : Genomic DNA was extracted from a whole Pacific oyster (C. gigas) after grinding in liquid nitrogen. Purified DNA was then digested by a mix of 3 restriction enzymes (AluI, RsaI, HaeIII). DNA fragments ranging from 250 to 500 bp were size-selected by agarose gel electrophoresis and ligated into the dephosphorylated blunt-ended SmaI-linearised pBKS2 plasmid (Stratagene Cloning System, CA). White colonies (11,500) were screened by hybridisation with double-stranded DNA probes containing dinucleotide repeats. Poly(dAdC), poly(dAdG), poly(dAdT) (Pharmacia Biotech, Sweden) were mixed at equimolar ratio and radiolabelled by [-32P] alpha dATP and dCTP using a random priming labelling kit (Life Technologies, Germany). Filter hybridisation and subsequent washing were carried out at 65°C in 5X SSC. Fifty two positive clones were identified with dinucleotide microsatellites. Plasmid DNA from minipreps performed on these positive clones were cut in the multicloning site of pBKS2 in order to determine the insert length. Plasmid DNA was prepared and sequenced using forward and reverse primers present on each side of the pBKS2 multicloning site. Only 6 positive clones contained a microsatellite motif out of the 51 sequenced. Specific primers were designed for 5 of them.
PCR reaction : Radioactive PCR amplification was carried out in a total volume of 10 µl using 2 µl of extract containing oyster genomic DNA. Reagents included dNTPs (0.074 mM), 1 pmole of each primer, and 0.35 U of Taq polymerase (PROMEGA, WI). Note that the MgCl2 concentration was optimised for each marker (Table 1). Radioactive labelling of the amplification fragment was carried out using 0.25 pmol of a primer phosphorylated with [-33P]-ATP. After a denaturing step of 1 min at 94°C, samples were processed through 30 cycles consisting of 1 min at 94°C, 1 min at the optimal annealing temperature (Table 1) and 1 min 15 sec at 72°C. The last elongation step was lengthened to 5 min.
Polymorphism : Four loci were found to be polymorphic with the primer pairs designed and exhibited a high variability (Table 1). One of the loci (L10) was scored on 324 individuals C. gigas from different geographical origins and displayed 49 alleles. The allelic PCR products differed from each other in multiples of 2 base pairs.
Cross-species amplification : We also examined the conservation of the four polymorphic loci in 10 species of the genus Crassostrea and two of the genus Saccostrea (Table 2). PCR amplifications were performed using the same reaction conditions as above. These microsatellite loci were also amplified in closely related Asian taxa: C. angulata, C. sikamea and C. ariakensis, 3 cupped oysters of interest in aquaculture. These fragments were not sequenced but it is likely that homologous loci were amplified in these species, as they showed similar size and similar allelic variation as those obtained for C. gigas. In addition, the primers for the L48 locus also allowed the amplification of specific products from C. gasar, C. rhizophorae, S. cucullata and S. commercialis but the yields of amplification DNA were too low to allow the identification of allelic variation.

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PCR reaction : Radioactive PCR amplification was carried out in a total volume of 10 µl using 2 µl of extract containing oyster genomic DNA. Reagents included dNTPs (0.074 mM), 1 pmole of each primer, and 0.35 U of *Taq* polymerase (PROMEGA, WI). Note that the $MgCl_2$ concentration was optimised for each marker (Table 1). Radioactive labelling of the amplification fragment was carried out using 0.25 pmol of a primer phosphorylated with [γ-^{33}P]-ATP. After a denaturing step of 1 min at 94°C, samples were processed through 30 cycles consisting of 1 min at 94°C, 1 min at the optimal annealing temperature (Table 1) and 1 min 15 sec at 72°C. The last elongation step was lengthened to 5 min.

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Table 1. Characterisation of four polymorphic microsatellite loci in the Pacific cupped oyster *C. gigas*. Ta is the optimal annealing temperature, He is the expected heterozygosity and Ho the observed heterozygosity. Sample size was 17 individuals for all loci, except for the *L10* locus where it was 468 individuals.

Locus	Repeat array	Primer sequences (5'→3')	GenBank accession number	Annealing temperature (°C)	$[MgCl_2]$ (mM)	Allele number	Ho	He	Mean size range of PCR product (bp)
L8	$(AG)_{22}$	AGAGGTTCAATGACGCTGGTG GATAAACAGTTTTCTGGTGTTAC	AF170849	57	1	14	0.54	0.85	181
L10	$(AG)_{26}$	GGTCAATTCAAAGTCAATTTCCC CATGTTTTCCCTTGACTGATCC	AF170850	55	1	49	0.90	0.96	136
L16	$(AG)_{24}$	CGGACGAATAAGATATTTGGTC TGGATCTGCGCATCATCTCG	AF170851	57	1	15	0.64	0.83	164
L48	$(GA)_{n>30}$	TCAAACCATCTGCTCGTCTACG TCCGAAAATCCAGGAATACCGG	AF170852	60	1.5	13	0.73	0.87	161

Table 2. Cross-species amplification for 4 microsatellite loci

		Locus			
Species	N	L8	L10	L16	L48
Crassostrea angulata	10	+	+	+	+
Crassostrea ariakensis	5	-	1 + of 5 tested	+	+
Crassostrea sikamea	5	-	+	+	+
Crassostrea virginica	5	-	-	1 + of 5 tested	+
Crassostrea iredalei	1	-	-	-	-
Crassostrea belcheria	1	-	-	-	-
Crassostrea margaritacea	3	-	-	-	-
Crassostrea echinata	5	-	-	-	-
Crassostrea gasar	5	-	-	-	?
Crassostrea rhizophorae	4	-	-	-	?
Sassostrea cucullata	5	-	-	-	?
Sassostrea commercialis	4	-	-	-	?

+ amplification, - no amplification, ? slight amplification without reading the genotypes