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Characterization of 27 microsatellite loci in the European flat oyster *Ostrea edulis*

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Abstract:

The flat oyster Ostrea edulis is native to Europe and populations have been severely depleted by the parasite Bonamia ostreae since the 1980s. Additional genetic markers are required to improve population genetics study and linkage map development for selection for B. ostrea-resistance in this species. Here, we characterized 27 novel microsatellite loci for O. edulis. Number of alleles per locus ranged from 6 to 25 and observed heterozygosity between 0.375 and 1. Null alleles were suggested at a few loci but most loci were in Hardy–Weinberg agreement enabling their reliable use in further population and mapping genetics approaches.

Keywords: microsatellites • Ostrea edulis • oysters • universal tailed-primer labelling

The flat oyster *Ostrea edulis* is native to Europe and is distributed from Norway to Morocco, and in the Mediterranean and Black Seas. The nuclear genetic diversity and geographical structure of wild populations were investigated using allozymes (e.g. Saavedra *et al.* 1995), microsatellites (Launey *et al.* 2002) and 12S-rDNA mitochondrial gene (Diaz-Almela *et al.* 2004). Because of the aquacultural importance of the species, selective breeding (Naciri-Graven *et al.* 1998) and genetic mapping of Quantitative Trait Loci (QTL) of resistance to bonamiosis, a parasitic disease that decimated the flat oysters populations in Europe since the 1980's, have been initiated (Lallias *et al.* 2007). A total of 22 microsatellites have previously been published for this species (Naciri *et al.* 1995; Morgan *et al.* 2000; Morgan & Rogers 2001; Sobolewska *et al.* 2001; Launey *et al.* 2002). More microsatellites are required to improve the accuracy of the genetic map and to enhance stock structure studies. Here we report 27 new microsatellites in *O. edulis*.

Genomic DNA was extracted from gill tissue by the chloroform/ isoamylalcohol method and purified with the DNA Clean Up System (Promega). An enriched library was made by *ecogenics* GmbH (Zurich, Switzerland) from size selected genomic DNA ligated into SAULA/SAULB-linker (Armour *et al.* 1994) and enriched by magnetic bead selection with biotin-labelled $(GT)_{13}$ and $(CT)_{13}$ oligonucleotide repeats (Gautschi *et al.* 2000). The enriched fragments were ligated into pUC19 cloning vector from Fermentas. Of 758 recombinant colonies screened with fluorescent probes, 179 gave a positive signal after hybridization (58 GT, 121 CT). Plasmids from 133 positive clones were sequenced. We designed primers for 94 microsatellite sequences (PRIMER3; www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Using 4 oysters, PCR was firstly optimized for 76 primer pairs. Further optimization was done on 8 oysters. PCR amplifications were conducted in Mastercycle thermal cyclers (Eppendorf) using universal fluorescent-labeled tailed primers (Schuelke 2000, Table 1). Optimized microsatellites were genotyped in 32 *O. edulis*, 16 from Loch Ryan (Scotland) and 16 from Grevelingen (the Netherlands). Table 1 shows the 27 new polymorphic microsatellites developed using two different protocols. For both protocols, PCR reactions contained 100 ng genomic DNA, 1X GoTaq® Flexi Buffer (Promega), 80 µM of dNTP, 0.1 µM of unlabeled reverse primer and 1 U of GoTaq® Flexi DNA Polymerase (Promega) in a 15 µl final volume. For Protocol A, PCR reactions contained 2mM $MgCl_2$, 0.04 µM of unlabeled forward primer with a tail at the 5' end and 0.17 µM of labeled tail. Initial denaturation at 96 ºC for 5 min was followed by 30 cycles of 96 ºC for 30 s, T_a (Table 1) for 45 s, 72 ºC for 45 s; followed by 8 cycles of 96 ºC for 30 s, 50 ºC (annealing temperature of the universal tailed primer) for 45 s, 72 ºC for 45 s; final elongation at 72 ºC for 30 min. For Protocol B, PCR reactions contained 1mM $MgCl_2$, 0.02 µM of unlabeled forward primer with a tail at the 5' end and 0.1 µM of labeled tail. An initial denaturation at 96 ºC for 5 min was followed by 12 cycles of 96 ºC for 30 s, T_a (Table 1) for 1 min 30 s, 72 ºC for 1 min; followed by 30 cycles of 96 ºC for 30 s, 50 ºC for 1 min 30 s, 72 ºC for 1 min; final elongation at 72 ºC for 30 min. Products were visualized on an ABI 3130*xl* Genetic Analyser using 36 cm capillary arrays, with POP7 polymer and GeneScan 500 LIZ size standard (Applied Biosystems).

The number of alleles ranged from 6 to 25, and observed heterozygosity from 0.375 to 1. Exact tests of Hardy Weinberg equilibrium (GENEPOP, Rousset & Raymond 1995) revealed significant heterozygote deficiencies at four microsatellites in the Loch Ryan population and two microsatellites in the Grevelingen population after Bonferroni correction (Narum 2006) (Table 1). MICRO-CHECKER (Van Oosterhout *et al.* 2004) analysis suggested null alleles at seven loci in the Loch Ryan population and three loci in the Grevelingen population (Table 2). Therefore the occurrence of null alleles is the most likely explanation for the heterozygote deficiencies observed in the dataset. Significant linkage disequilibrium was detected for three pairs of loci: *Oed* 177a / *Oed* 315 ($P<0.01$); *Oed* 199 / *Oed* 331 ($P<0.05$) and *Oed* 144 / *Oed* 268 ($P<0.05$) (GENEPOP software).

These new microsatellites will strengthen the genetic linkage map (Lallias et al., 2007) that can facilitate the search for QTL of resistance to bonamiosis, leading to marker assisted selection. They will also have value for population genetics studies, parentage analysis and assessment of genetic variability of wild or farmed populations.

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Tables

Table 1. Repeat structure, primer sequences, amplification conditions and summary statistics for microsatellite loci developed for *Ostrea edulis*. Four labeled universal tailed primers (Schuelke 2000) were used: FAM (5'-TGT AAA ACG ACG GCC AGT), VIC (5'-GCC GCT CTA GAA CTA GTG), NED (5'-TAG AAG GCA CAG TCG AGG) and PET (5'-GCA GGA AAC AGC TAT GAC). n: number of successfully genotyped samples; T_a: annealing temperature; Protocol: see text; n_a: number of alleles; H_o and H_e: observed and expected heterozygosity; P: P-values of fit to Hardy-Weinberg , Bonferroni adjusted significant P-values (<0.01285) in bold. 32 oysters were scored: 16 from Loch Ryan (upper row), 16 from Grevelingen (lower row).

Locus (n)	GenBank Accession no.	Repeat array	Label	Primer sequence (5'-3')	Size range (bp)	T_a (ºC)	Protocol	n_a	H_o	H_e	P
Oed 144 (32)	JF236811	$(GT)_5(AT)_6(GT)_{22}$	FAM	F: GTCGTTGAAAGTGCCTGGAT R: ACAATAAATCTGTAGCAAATTTAGT	126-180	63	A	20	0.875 1.000	0.950 0.936	0.105 0.882
Oed 149 (32)	JF236812	$(GT)_3GC(GT)_2T$ $(GT)_9(GC)_4(GT)_3$	PET	F: CCATGAACAGCTAAAAAGTGATG R: TTGGTCTCTACCCAGAGTTATCG	138-154	65	B	8	0.563 0.875	0.790 0.845	0.068 0.940
Oed 165 (30)	JF236813	$(AG)_{29}$	VIC	F: CCGTGTTGGTCCAAACTCTT R: GCGCGCATCAATTCTTTTAT	171-207	65	B	13	0.571 0.438	0.852 0.825	0.017 **0.000**
Oed 174 (29)	JF236814	$(CT)_{14}$	VIC	F: AAGGAGACGAAATTTTAAAGCA R: GCAGGGAATTATTTTGAAAGCA	241-273	60	A	13	0.533 0.429	0.876 0.839	**0.005** **0.006**
Oed 177a (32)	JF236815	$(AG)_{17}$	FAM	F: TGCAAGATTAAAAGGCAGCA R: TCTGCACCTAATAGACTGTTCTGA	170-194	60	A	13	1.000 0.875	0.879 0.887	0.930 0.682
Oed 180 (32)	JF236816	$(AG)_{22}$	NED	F: GCGACTGTTAAAAGCCACAT R: TGATGAATCGATTAAGAAGTAAAAACA	186-230	58	A	17	0.875 0.938	0.909 0.927	0.664 0.688
Oed 181 (32)	JF236817	$(AT)_6(AG)_{31}$	NED	F: TGGTCAGCTGAAACTGTTCAA R: CAAGGCCTTTTCAATAATGTACTGT	176-222	63	A	17	0.938 0.750	0.936 0.917	0.268 0.032
Oed 199 (31)	JF236818	$(CT)_{28}$	FAM	F: TTCGGGTCAAATAACGCAAG R: TGCCCGACTATGTCTTAGCA	184-250	60	A	21	1.000 0.875	0.947 0.913	0.847 0.624
Oed 202a (32)	JF236819	$(AG)_{27}$	VIC	F: AAATTCAAATCACCGGAGGA R: TCCTCCCTGAATATCTGTCCA	233-269	63	B	16	0.812 0.938	0.921 0.837	0.147 0.908
Oed 202b (32)	JF236819	$(AG)_{22}$	FAM	F: GCGGGTATTACATTAGCAATCC R: TTGTACATGGAAGTAGGACAGTCA	232-270	63	A	15	0.875 0.875	0.913 0.893	0.860 0.551
Oed 212a (31)	JF236820	$(CT)_{19}TT(CT)_6$	NED	F: TCTACAGCCAGGCACATCAG R: CGTCCAGTCCTCCAGAGAAT	188-246	60	A	19	0.875 0.800	0.919 0.821	0.446 0.297
Oed 212b (32)	JF236820	$(GA)_{21}$	VIC	F: TTGAAATGCCGATGTCTGTC R: TGCCTCTTTGTAAAGTCTTTGTATATT	206-244	55	A	15	0.875 0.938	0.925 0.887	0.063 0.967
Oed 219 (32)	JF236821	$(TC)_{10}T(TC)_{15}$	PET	F: CTCCACATTCCTCAGCAAGAG R: CAAAAGCAAAAGTTTGAAAAACAA	187-239	60	B	17	1.000 0.688	0.923 0.825	0.465 0.040
Oed 234 (30)	JF236822	$(CA)_{18}$	NED	F: GTTGAAACTTTAACTTCCGATTATTT R: TCAAACGAGACGTTAAGCAAGA	221-279	65	A	19	0.938 0.786	0.923 0.773	0.965 0.484
Oed 240 (31)	JF236823	$(GA)_{30}$	NED	F: GACTTACATAAGCAAACTCTT R: ACTGGGCGGTCACCACCTTGGGCC	137-165	63	A	13	0.933 0.750	0.926 0.810	0.657 0.743
Oed 243 (29)	JF236824	$(AG)_{21}$	PET	F: GCCGCGAGCTGTAATCATA R: CGGCTGACCGCTATATTTGT	243-273	60	A	14	0.750 0.692	0.887 0.861	0.075 0.046
Oed 258 (30)	JF236825	$(AC)_{11}$	PET	F: AGTCTGCGTTGCAGATTAGTG R: TAGGGTGTGGTTGGGTTTTC	222-240	63	A	7	0.688 0.500	0.792 0.688	0.499 0.019

Oed 268 (32)	JF236826	$(AG)_{18}…(AG)_{15}$	VIC	F: TGACGCAAGGTTACCATTCA R: ATTCACGCATGAGAGTCGTG	134-260	63	A	25	0.938 0.938	0.948 0.958	0.770 0.260
Oed 269 (32)	JF236827	$(TC)_5(TG)_{14}G(TG)_5$	FAM	F: GGGATTGAGCGCAGTAAAGA R: ATTTTCGGACGGAACGTTTA	190-226	60	A	9	0.375 0.750	0.617 0.748	**0.009** 0.449
Oed 273 (32)	JF236828	$(GA)_{13}$	NED	F: CGCCTAACGTCTAGGTTTGC R: TGCATCTGGAATAAACTTGTCA	205-223	60	A	6	0.688 0.500	0.736 0.613	0.014 0.693
Oed 315 (32)	JF236829	$(CT)_{21}$	NED	F: TCTAACCTTCAATTGCTTGCTG R: TGGTTGGCGTAGGTTTGAAT	209-255	63	A	16	0.688 0.937	0.897 0.881	0.082 0.925
Oed 319 (32)	JF236830	$(AG)_{21}$	VIC	F: CAAGTAGTTGCGGCCAGATT R: TTCATCGTTGTACACGTAGAATAAA	209-259	65	B	19	0.875 0.938	0.933 0.952	0.584 0.721
Oed 321 (32)	JF236831	$(GA)_{23}$	FAM	F: GGACGAGAAATGGTGCTTTC R: CGAAATTCGGAATGTGGATAA	195-235	60	A	16	0.875 0.875	0.929 0.929	0.673 0.026
Oed 325 (30)	JF236832	$(CT)_{27}$	VIC	F: GAGACCTTGATTCGAAACTTCTTT R: CACGACATATCTAGCACTTTTCA	154-188	63	A	16	0.750 1.000	0.919 0.923	0.052 0.678
Oed 327 (32)	JF236833	$(TC)_{26}$	FAM	F: CCGTTAGCCCCATCAGATAA R: TGGGGTGTAAAGTAATCTTCCAG	165-195	63	A	15	0.687 0.938	0.881 0.929	**0.006** 0.441
Oed 328b (32)	JF236834	$(GA)_{11}GC(GA)_7$	NED	F: AGAGATTTAGGGGCCACACC R: CACTTTGGGATGTTGAGTGTTG	210-240	63	A	15	0.937 0.813	0.917 0.921	0.583 0.085
Oed 331 (31)	JF236835	$(GA)_{27}$	VIC	F: TTGCATTTTAGCCGCGTTAT R: GCCAGGGCTAGTAGGAATGC	224-268	65	B	15	0.467 0.563	0.926 0.859	**0.000** 0.025

Table 2. Estimations of null allele frequencies (MICRO-CHECKER, Van Oosterhout *et al.* 2004) at microsatellite loci in *O. edulis* from Loch Ryan and Grevelingen.

Locus	Loch Ryan	Grevelingen
Oed 149	0.133	-
Oed 165	0.147	0.224
Oed 174	0.192	0.221
Oed 269	0.195	-
Oed 315	0.104	-
Oed 327	0.101	-
Oed 331	0.234	0.161