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0.17 to 0.89 (Table 1). Statistical tests for Hardy–Weinberg and linkage equilibrium were conducted in GenePop (Raymond & Rousset 1995). A significant heterozygote deficiency was observed for two loci, Crest1T2.83a and Crest1T4.76, suggesting null alleles. All pairwise tests of linkage disequilibrium between loci were nonsignificant after sequential Bonferroni correction. These microsatellite loci are highly polymorphic and suitable for population studies in *Cx. restuans.*

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

DELPHINE LALLIAS,* RUTH STOCKDALE,* PIERRE BOUDRY,† ANDY R. BEAUMONT* and SYLVIE LAPÈGUE‡

**School of Ocean Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey LL59 5AB, UK, †Ifremer, UMR M100 Physiologie et Ecophysiologie des Mollusques Marins, Plouzané, France, ‡Ifremer, Laboratoire Génétique et Pathologie, Ronce-les-bains, 17390 La Tremblade, France*

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

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Correspondence: Dr Delphine Lallias, Fax: (+44) (0) 1248 716367, E-mail: d.lallias@bangor.ac.uk

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

Table 1 Repeat structure, primer sequences, amplification conditions and summary statistics for microsatellite loci developed for *Ostrea edulis*. Four labelled universal-tailed primers (Schuelke 2000) were used: FAM (5′-TGTAAAACGACGGCCAGT-3′), VIC (5′-GCCGCTCTAGAACTAGTG-3′), NED (5′-TAGAAGGCACAGTCGAGG-3′) and PET (5′-GCAGGAAACAGCTATGAC-3′). *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 heterozygosities; *P*, *P* values of fit to Hardy–Weinberg , Bonferroni-adjusted significant *P* values (< .01285) in bold. Thirty-two 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 (°C)	Protocol	n_a	H_O	H_E	*P*
Oed 144 (32)	JF236811	$(GT)_5(AT)_6(GT)_{22}$	FAM	F: GTCGTTGAAAGTGCCTGGAT	126–180	63	A	20	0.875	0.950	0.105
				R: ACAATAAATCTGTAGCAAATTTAGT					1.000	0.936	0.882
Oed 149 (32)	JF236812	$(GT)_3GC(GT)_2T$ $(GT)_9(GC)_4(GT)_3$	PET	F: CCATGAACAGCTAAAAAGTGATG	138–154	65	B	8	0.563	0.790	0.068
				R: TTGGTCTCTACCCAGAGTTATCG					0.875	0.845	0.940
Oed 165 (30)	JF236813	$(AG)_{29}$	VIC	F: CCGTGTTGGTCCAAACTCTT	171–207	65	B	13	0.571	0.852	0.017
				R: GCGCGCATCAATTCTTTTAT					0.438	0.825	**0.000**
Oed 174 (29)	JF236814	$(CT)_{14}$	VIC	F: AAGGAGACGAAATTTTAAAGCA	241–273	60	A	13	0.533	0.876	**0.005**
				R: GCAGGGAATTATTTTGAAAGCA					0.429	0.839	**0.006**
Oed 177a (32)	JF236815	$(AG)_{17}$	FAM	F: TGCAAGATTAAAAGGCAGCA	170–194	60	A	13	1.000	0.879	0.930
				R: TCTGCACCTAATAGACTGTTCTGA					0.875	0.887	0.682
Oed 180 (32)	JF236816	$(AG)_{22}$	NED	F: GCGACTGTTAAAAGCCACAT	186–230	58	A	17	0.875	0.909	0.664
				R: TGATGAATCGATTAAGAAGTAAAAACA					0.938	0.927	0.688
Oed 181 (32)	JF236817	$(AT)_6(AG)_{31}$	NED	F: TGGTCAGCTGAAACTGTTCAA	176–222	63	A	17	0.938	0.936	0.268
				R: CAAGGCCTTTTCAATAATGTACTGT					0.750	0.917	0.032
Oed 199 (31)	JF236818	$(CT)_{28}$	FAM	F: TTCGGGTCAAATAACGCAAG	184–250	60	A	21	1.000	0.947	0.847
				R: TGCCCGACTATGTCTTAGCA					0.875	0.913	0.624
Oed 202a (32)	JF236819	$(AG)_{27}$	VIC	F: AAATTCAAATCACCGGAGGA	233–269	63	B	16	0.812	0.921	0.147
				R: TCCTCCCTGAATATCTGTCCA					0.938	0.837	0.908
Oed 202b (32)	JF236819	$(AG)_{22}$	FAM	F: GCGGGTATTACATTAGCAATCC	232–270	63	A	15	0.875	0.913	0.860
				R: TTGTACATGGAAGTAGGACAGTCA					0.875	0.893	0.551
Oed 212a (31)	JF236820	$(CT)_{19}TT(CT)_6$	NED	F: TCTACAGCCAGGCACATCAG	188–246	60	A	19	0.875	0.919	0.446
				R: CGTCCAGTCCTCCAGAGAAT					0.800	0.821	0.297
Oed 212b (32)	JF236820	$(GA)_{21}$	VIC	F: TTGAAATGCCGATGTCTGTC	206–244	55	A	15	0.875	0.925	0.063
				R: TGCCTCTTTGTAAAGTCTTTGTATATT					0.938	0.887	0.967
Oed 219 (32)	JF236821	$(TC)_{10}T(TC)_{15}$	PET	F: CTCCACATTCCTCAGCAAGAG	187–239	60	B	17	1.000	0.923	0.465
				R: CAAAAGCAAAAGTTTGAAAAACAA					0.688	0.825	0.040
Oed 234 (30)	JF236822	$(CA)_{18}$	NED	F: GTTGAAACTTTAACTTCCGATTATTT	221–279	65	A	19	0.938	0.923	0.965
				R: TCAAACGAGACGTTAAGCAAGA					0.786	0.773	0.484
Oed 240 (31)	JF236823	$(GA)_{30}$	NED	F: GACTTACATAAGCAAACTCTT	137–165	63	A	13	0.933	0.926	0.657
				R: ACTGGGCGGTCACCACCTTGGGCC					0.750	0.810	0.743
Oed 243 (29)	JF236824	$(AG)_{21}$	PET	F: GCCGCGAGCTGTAATCATA	243–273	60	A	14	0.750	0.887	0.075
				R: CGGCTGACCGCTATATTTGT					0.692	0.861	0.046
Oed 258 (30)	JF236825	$(AC)_{11}$	PET	F: AGTCTGCGTTGCAGATTAGTG	222–240	63	A	7	0.688	0.792	0.499
				R: TAGGGTGTGGTTGGGTTTTC					0.500	0.688	0.019
Oed 268 (32)	JF236826	$(AG)_{18}$ ... $(AG)_{15}$	VIC	F: TGACGCAAGGTTACCATTCA	134–260	63	A	25	0.938	0.948	0.770
				R: ATTCACGCATGAGAGTCGTG					0.938	0.958	0.260
Oed 269 (32)	JF236827	$(TC)_5(TG)_{14}$ $G(TG)_5$	FAM	F: GGGATTGAGCGCAGTAAAGA	190–226	60	A	9	0.375	0.617	**0.009**
				R: ATTTTCGGACGGAACGTTTA					0.750	0.748	0.449
Oed 273 (32)	JF236828	$(GA)_{13}$	NED	F: CGCCTAACGTCTAGGTTTGC	205–223	60	A	6	0.688	0.736	0.014
				R: TGCATCTGGAATAAACTTGTCA					0.500	0.613	0.693
Oed 315 (32)	JF236829	$(CT)_{21}$	NED	F: TCTAACCTTCAATTGCTTGCTG	209–255	63	A	16	0.688	0.897	0.082
				R: TGGTTGGCGTAGGTTTGAAT					0.937	0.881	0.925
Oed 319 (32)	JF236830	$(AG)_{21}$	VIC	F: CAAGTAGTTGCGGCCAGATT	209–259	65	B	19	0.875	0.933	0.584
				R: TTCATCGTTGTACACGTAGAATAAA					0.938	0.952	0.721
Oed 321 (32)	JF236831	$(GA)_{23}$	FAM	F: GGACGAGAAATGGTGCTTTC	195–235	60	A	16	0.875	0.929	0.673
				R: CGAAATTCGGAATGTGGATAA					0.875	0.929	0.026
Oed 325 (30)	JF236832	$(CT)_{27}$	VIC	F: GAGACCTTGATTCGAAACTTCTTT	154–188	63	A	16	0.750	0.919	0.052
				R: CACGACATATCTAGCACTTTTCA					1.000	0.923	0.678
Oed 327 (32)	JF236833	$(TC)_{26}$	FAM	F: CCGTTAGCCCCATCAGATAA	165–195	63	A	15	0.687	0.881	**0.006**
				R: TGGGGTGTAAAGTAATCTTCCAG					0.938	0.929	0.441
Oed 328b (32)	JF236834	$(GA)_{11}GC(GA)_7$	NED	F: AGAGATTTAGGGGCCACACC	210–240	63	A	15	0.937	0.917	0.583
				R: CACTTTGGGATGTTGAGTGTTG					0.813	0.921	0.085
Oed 331 (31)	JF236835	$(GA)_{27}$	VIC	F: TTGCATTTTAGCCGCGTTAT	224–268	65	B	15	0.467	0.926	**0.000**
				R: GCCAGGGCTAGTAGGAATGC					0.563	0.859	0.025

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 1980s, 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 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 (Primer 3; www.genome.wi.mit.edu/cgi-bin/primer/primer 3_www.cgi). Using four oysters, polymerase chain reaction (PCR) was first optimized for 76 primer pairs. Further optimization was done on eight oysters. PCR amplifications were conducted in Mastercycle thermal cyclers (Eppendorf) using universal fluorescent-labelled 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 mixtures contained 100 ng genomic DNA, 1× GoTaq Flexi Buffer (Promega), 80 μM of dNTP, 0.1 μM of unlabelled reverse primer and 1 U of GoTaq Flexi DNA Polymerase (Promega) in a 15-μL final volume. For protocol A, PCR mixtures contained 2 mM $MgCl_2$, 0.04 μM of unlabelled forward primer with a tail at the 5′-end and 0.17 μM of labelled tail. Initial denaturation at 96 °C for 5 min was followed by 30 cycles of 96 °C for 30 s, annealing temperature (T_a, see Table 1) for 45 s, 72 °C for 45 s; followed by eight cycles of 96 °C for 30 s, 50 °C (annealing temperature of the universal tailed primer) for 45 s, 72 °C for 45 s; final elongation at 72 °C for 30 min. For protocol B, PCRs contained 1 mM $MgCl_2$, 0.02 μM of unlabelled forward primer with a tail at the 5′-end and 0.1 μM of labelled tail. An initial denaturation at 96 °C for 5 min was followed by 12 cycles of 96 °C for 30 s, T_a (Table 1) for 1 min 30 s, 72 °C for 1 min; followed by 30 cycles of 96 °C for 30 s, 50 °C for 1 min 30 s, 72 °C for 1 min; final elongation at 72 °C 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).

Table 2 Estimations of null allele frequencies (Micro-Checker, Van Oosterhout *et al.* 2004) at microsatellite loci in *Ostrea 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

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 data set. 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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Isolation and characterization of microsatellite loci for alligator gar (*Atractosteus spatula*) and their variability in two other species (*Lepisosteus oculatus* and *L. osseus*) of Lepisosteidae

GREGORY R. MOYER,* BRIAN L. SLOSS,† BRIAN R. KREISER‡ and KEVIN A. FELDHEIM§
**Warm Springs Fish Technology Center, Conservation Genetics Laboratory, US Fish and Wildlife Service, 5308 Spring Street, Warm Springs, GA 31830, USA, †US Geological Survey, Wisconsin Cooperative Fishery Research Unit, College of Natural Resources, 800 Reserve Street, University of Wisconsin-Stevens Point, WI 54481 USA, ‡Department of Biological Sciences, 118 College Drive #5018, University of Southern Mississippi, Hattiesburg, MS 39406, USA, §Pritzker Laboratory for Molecular Systematics and Evolution, The Field Museum, 1400 S. Lake Shore Drive, Chicago, IL 60605, USA*

Abstract

We report on the isolation of 17 polymorphic microsatellite loci from alligator gar (*Atractosteus spatula*), a large-bodied species that has experienced population declines across much of its range. These loci possessed 2–19 alleles and observed heterozygosities of 0–0.974. All loci conformed to Hardy–Weinberg equilibrium expectations, and none exhibited linkage disequilibrium. Nine and eight of these loci were found to be polymorphic in the related species *Lepisosteus oculatus* and *L. osseus*, respectively. These microsatellite loci should prove useful in conservation efforts of *A. spatula* through the study of population structure and hatchery broodstock management.

Keywords: alligator gar, *Atractosteus spatula*, cross-species amplification, Lepisosteidae, microsatellite

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Correspondence: Brian Kreiser, Fax: +1601 2665797; E-mail: brian.kreiser@usm.edu