Mol Biol Rep (2013) 40:7039–7043
DOI 10.1007/s11033-013-2823-9

Polymorphic microsatellite *loci* for two Atlantic oyster species: *Crassostrea rhizophorae* and *C. gasar*

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Received: 29 December 2012 / Accepted: 25 October 2013 / Published online: 1 November 2013

Abstract Using a CA/CAA enriched library screening procedure, we isolated and characterised a total of seventeen polymorphic microsatellite *loci* for two species of *Crassostrea* with recognised economic importance. Eleven microsatellite *loci* were developed for *C. rhizophorae*, a Western Atlantic species for which no microsatellites were previously known. Another six *loci* were developed for *C. gasar*, a species that occurs on both sides of the South Atlantic, adding to the ten *loci* previously described for the species. The levels of polymorphism were estimated using 24 *C. rhizophorae* from Southeast Brazil (São Paulo) and 23 *C. gasar* individuals from North Brazil (Maranhão). The number of alleles per polymorphic *locus* varied from 3 to 27, and the observed and expected heterozygosities ranged between 0.174 and 0.958 and between 0.237 and 0.972 in *C. rhizophorae* and *C. gasar*, respectively. No linkage disequilibrium was found between any *locus* pair, and four of them exhibited deviations from Hardy–Weinberg expectations. Of the 17 *loci* developed, 8 cross-amplified in *C. gigas* and 13 in *C. virginica*. These markers are useful for evolution and population genetics studies of *Crassostrea* species and may provide fundamental data for the future cultivation of native oysters in Western Atlantic.

Keywords SSR · Population structure · Stock · Aquaculture · Mollusc

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Introduction

Crassostrea species are responsible for almost all of the worldwide oyster production [1]. Oyster production in the South Atlantic is dominated by three species: the native *Crassostrea gasar* (Adanson, 1757; sin. *C. brasiliana*, [2]) and *C. rhizophorae* (Guilding, 1828), which are extracted from natural banks, and the cultivated *C. gigas* (Thunberg, 1793), responsible for 91 % of the total Brazilian oyster production [3] and recently becoming invasive in natural banks [4]. Native oyster species have a wide distribution along the Atlantic coast of Latin America and Africa [5], but their cultivation is hampered by the uncertainty about the existing stocks and their limits. To date, the few studies that have analysed the genetic structure of *C. gasar* and *C. rhizophorae* used mitochondrial (cytochrome oxidase I) and allozyme markers [2, 4, 6]. Recently, ten microsatellite *loci* were developed for *C. gasar* [7] and will allow new genetic structuring studies with more polymorphic markers. In contrast, microsatellite *loci* for *C. rhizophorae* are still lacking.

According to Beck et al. [8], over 100 years of intense and unregulated exploitation has eliminated more than 99 % of the oyster reefs of many bays around the world, and thus, the worldwide oyster populations can be considered functionally extinct. In some cases, oyster populations have vanished altogether, such as for *C. rhizophorae*, which has not been found in Ciénaga Grande

de Santa Marta (Colombia) since 1996, most likely due to the combined effects of uncontrolled extraction and alterations in freshwater flow [9]. Aquaculture is accepted as an alternative to this uncontrolled oyster extraction; however, to avoid the risks of outbreeding depression and loss of locally adapted gene traits, it relies on knowledge of the number and limits of genetic stocks. This knowledge can be greatly improved by population genetics studies based on highly polymorphic *loci*. Here, we describe the isolation and characterisation of microsatellite *loci* for *C. gasar* and *C. rhizophorae* and test their cross amplification in the cultivated *C. gigas* and the native *C. virginica* (Gmelin, 1791).

Materials and methods

DNA was extracted from the adductor muscle according to the modified cetyltrimethylammonium bromide (CTAB) protocol, as previously described [10]. Microsatellite DNA was isolated from an enriched partial genomic library following the protocol of Bloor et al. [11]. A pool of high-quality genomic DNA from four individuals (10 μg) was digested with *Sau*IIIA and ligated to phosphorylated double-stranded linkers; the fragments (between 500 and 1,000 bp) were excised from a 2 % agarose gel. The DNA fragments were hybridised with biotinylated $(CA)_{12}$ and $(CAA)_8$ probes and isolated using streptavidin-coated magnetic beads. A PCR primed with the forward linker oligonucleotide was used for enrichment of the DNA containing microsatellites. The enriched fragments were cloned using the pGEM-T vector (Promega) for posterior insertion in One Shot TOP10 competent cells (Invitrogen). White colonies were selected during screening on S-gal (Sigma)/agar/ampicillin plates. The presence of a microsatellite insert was confirmed by two or more PCR products after amplification using the forward linker oligonucleotide and (non-biotinylated) microsatellite oligonucleotides as primers. 48 positive clones from each species were sequenced in both directions using an ABI3500 automated sequencer (Applied Biosystems). The sequences were edited using SEQMAN (Lasergene Inc.). 19 and 47 sequences from *C. gasar* and *C. rhizophorae*, respectively, were selected for the design of primer pairs flanking the microsatellite regions using WEBSAT [12]. Primers were initially screened for quality of PCR amplification on a Veriti® Cycler (Applied Biosystems), using eight individuals of each species. Those primers that did not amplify, or that produced multiple bands on high-resolution agarose gels were discarded. The 22 *loci* selected were tested for polymorphism through genotyping on an ABI3500 automated sequencer using the tailed primer method [13], whereby all the forward primers were synthesised with a M13 tail at their 5 end to which oligonucleotides labelled with different dyes (6-FAM, VIC, NED, or PET) could anneal. The best annealing temperature (Ta) for each set of primers was estimated in a 54 to 64 °C temperature gradient PCR. Subsequently, thermocycling was performed using an initial denaturing at 94 °C for 3 min, 30 cycles of 45 s at 93 °C, 45 s at the specific Ta, and 1 min at 72 °C, followed by 8 cycles identical to the 30 initial cycles but with the Ta fixed at 53 °C (as in [13]). The cycling was finalised with a 15 min extension at 72 °C. The PCR reactions included 20 ng template DNA, 1 U GoTaq® DNA Polymerase (Promega), 200 μM dNTPs, 0.2 μM tailed forward primer, 0.4 μM labelled primer, 0.8 μM reverse primer, 2.5 mM $MgCl_2$, and 15 μg BSA in a 15 μL total volume. The PCR products were pooled with a GSLIZ-600 size standard (Applied Biosystems) and were denatured in formamide prior to capillary electrophoresis. Allele sizing and genotype confirmation were performed using the GENEMARKER 1.97 programme (SoftGenetics—free trial version). After the analyses, 5 monomorphic *loci* were discarded, so 17 polymorphic *loci* (6 for *C. gasar* and 11 for *C. rhizophorae*) were selected. Binning of the alleles was performed using FLEXIBIN software [14]. The number of alleles was determined, and tests of linkage disequilibria were performed using FSTAT [15]. The observed and expected heterozygosities and tests of Hardy–Weinberg equilibrium were calculated using ARLEQUIN 3.5 [16]. The presence of null alleles was investigated with the MICRO-CHECKER programme [17].

Results and discussion

Twenty-four individuals of *C. rhizophorae*, from Southeast Brazil (São Paulo: 25°01S, 47°55W) and 23 individuals of *C. gasar* from North Brazil (Maranhão: 02°41S, 41°58W) were genotyped. Linkage disequilibrium was not found for any pair of *loci* after Bonferroni correction for either species, but four *loci* (GASA5, RHIZ7, RHIZ12, and RHIZ18) exhibited significant heterozygote deficiencies, possibly due to the presence of null alleles (Table 1). A high frequency of null alleles has been previously verified for microsatellite markers in molluscs [18]. The analysed *loci* demonstrated high polymorphism, ranging from 5 to 27 alleles, and the observed and expected heterozygosities ranged between 0.609–0.958 and 0.582–0.972 in *C. rhizophorae* and *C. gasar*, respectively (Table 1). An exception was GASA15, which had only three alleles and observed and expected heterozygosities of 0.174 and 0.237, respectively.

Cross-amplification was tested for all the *loci* in two other species of *Crassostrea*: *C. virginica* and *C. gigas*. Seven *C. virginica* individuals from Panamá (8°58′N, 79°31′W) and eight *C. gigas* individuals from an oyster farm in South Brazil (Santa Catarina: 26°15′S, 48°41′W) were analysed. 11 *loci* amplified well for *C. virginica*,

Table 1 Microsatellite *loci* developed for *C. gasar* and *C. rhizophorae*

Primer (5′–3′)		GenBank Accession n°.	Motif	*Ta* (°C)	Size range (bp)	*Na*	H_o	H_e	*P* value (HW)	Null freq.
GASA5	CTTGTAGAATGAGCTTGGCCTT	KC335527	(GT)17	54	216–240	7	0.435	0.755	0.00030*	0.200
	GATCTGAGTGTCTGTGAG									
GASA6	TTCTTTTCAACGAATTGAATCC	KC335530	(GTT)57	54	156–261	10	0.609	0.782	0.11901	0.093
	GACCTTTTCTGAACCAAACACA									
GASA9	TGTTAGGTTTGTAGGTGTACTG	KC335528	(GTT)10	54	329–350	5	0.652	0.582	0.95081	0
	GACGTGGTCTACTGTCTGAG									
GASA11	GGCTCGACATTTTCTATTCCAG	KC335531	(CT)32	60	235–305	19	0.826	0.902	0.16830	0.030
	AAAGTCTAATCACGGTCAACCC									
GASA15	ACAACATTGGTCTTTTGTGGCT	KC335529	(ACA)4	60	276–300	3	0.174	0.237	0.31082	0.095
	AGCGAGTGTTTGTAAGGAGGAG									
GASA25	GGGGTGGGGTAGACAATTAAC	KC335532	(CAA)4CATCAT(CAA)7	60	157–310	16	0.783	0.829	0.57381	0.010
	CAATTCCTTTTGTTTGTGTTGC									
RHIZ3	TACATGAAGCAACACCCTTGTG	KC335533	(TG)14	62	135–191	12	0.833	0.881	0.23220	0.017
	CGGCACTTCTTATGCCCTATAC									
RHIZ7	ATATTTGTCATCACCGAGCAGG	KC335534	(TG)25	64	310–362	13	0.667	0.890	0.00075*	0.112
	CTGAATGTCCATAGCATACCAGAA									
RHIZ8	TGGGCTTAAATTGATAAGACGG	KC335535	(CA)21	60	223–293	19	0.833	0.933	0.25509	0.017
	CTTGTGCTGTATGGGTTTTCGT									
RHIZ11	GATTACAAAGGGGTAAACAAGACAG	KC335543	(TTG)12(GTT)11	54	163–223	15	0.875	0.930	0.37966	0.019
	TCAAGTGAAAACAAGTTCGCTC									
RHIZ12	TCGGTAGGTCAAAACCTTCAGT	KC335541	(CA)22	58	294–378	25	0.583	0.972	0.00000*	0.019
	TTACACGCTCCCAAATGTTGT									
RHIZ13	ATGGTAGTGAGAGCCACCAATC	KC335539	(TG)6(GT)11	58	228–314	27	0.958	0.971	0.73694	0
	CATCCAGACCTCAATGTTACGA									
RHIZ18	GATCATTTTAACACAACACCCC	KC335542	(AC)14	58	248–310	14	0.583	0.911	0.00000*	0.171
	CATCATCTACTTCCTGCGAGAC									
RHIZ19	CTCCGAACTGTTCCACACAAGT	KC335540	(TG)21A(GA)16	60	135–190	12	0.833	0.881	0.25756	0.017
	GATCATGGAGGGTGTATCATCGTGT									
RHIZ42	TTTGCAGAGAGTGGACGTAAG	KC335536	(CA)5(CA)5(AC)7(CA)5(CA)7 (CA)5(AC)7(CA)9(CA)5	64	183–341	17	0.708	0.890	0.03268	0.093
	AGTAAATGCTGTTCATACTTGGC									
RHIZ49	TGAACTGCATCAACTGAGGAAA	KC335537	CAA(6)*n*(CAC)4TAC(CAA)7	64	188–239	9	0.583	0.827	0.01822	0.141
	GCCAACCTGACCCACTATTTTA									
RHIZ56	CTCCTTACAGACCATGTGTTGC	KC335538	(TTG)4(TCG)5	62	247–349	13	0.583	0.817	0.00908	0.131
	CAAAACCCAAGTTTACCACGTT									

Ta annealing temperature, *Na* number of observed alleles, H_o observed heterozygosity, H_e expected heterozygosity, *Null freq* null allele frequency

*$P < 0.05$, for tests of Hardy–Weinberg (HW) equilibrium after Bonferroni correction

Table 2 Cross-amplification of the developed *loci* in *C. virginica* and *C. gigas*

	C. virginica					*C. gigas*				
	Ta (°C)	*Na*	H_o	H_e	*P* value (HW)	*Ta* (°C)	*Na*	H_o	H_e	*P* value (HW)
GASA5	56	7	0.867	0.802	0.744	54	4	0.885	0.692	0.091
GASA15	60	2	0.143	0.143	1.000	–	–	–	–	–
RHIZ3	54	3	0.439	0.385	1.000	–	–	–	–	–
RHIZ7	60	9	0.714	0.912	0.034	–	–	–	–	–
RHIZ8	60	2	0.143	0.143	1.000	–	–	–	–	–
RHIZ11	54	6	0.714	0.681	0.551	56	3	0.635	0.585	1.000
RHIZ12	54	2	0.600	0.477	1.000	–	–	–	–	–
RHIZ13	54	5	1.000	0.768	0.517	–	–	–	–	–
RHIZ19	58	5	0.800	0.822	0.341	–	–	–	–	–
RHIZ42	62	3	0.500	0.449	1.000	–	–	–	–	–
RHIZ49	56	4	0.571	0.780	0.358	–	–	–	–	–

Ta annealing temperature, *Na* number of observed alleles, H_o observed heterozygosity, H_e expected heterozygosity

while just two *loci* worked for *C. gigas*. No pairwise linkage disequilibrium was found between *loci* of either species, nor did they present any significant deviations (after Bonferroni correction) from Hardy–Weinberg equilibrium. Values of annealing temperature, heterozygosity and number of alleles for cross-amplification are shown in Table 2. All *loci* exhibited high levels of polymorphism, except for RHIZ8 in *C. virginica* (H = 0.14). Surprisingly, none of the *loci* from *C. rhizophorae* could be used in *C. gasar* and vice versa: some heterologous *loci* did amplify between the two species, but they did not pass the stringent conditions set for the selection of *loci* for the two species that were the main objective of this work.

The new markers developed here will be useful for population and aquaculture studies. More specifically, they will help with the delimitation of the genetic stocks along the coast, which will provide critical information for the cultivation of native species, contributing both to their protection and to habitat restoration.

Acknowledgments We would like to thank AV Vasconcellos and G Rodriguez-Rey for assistance during the microsatellite development process. This work is part of the PhD thesis of Nathalia Cavaleiro at the Universidade do Estado do Rio de Janeiro, with scholarships from Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and was supported by grants from the Brazilian Ministério da Pesca e Aquicultura (MPA), Ministério da Ciência, Tecnologia e Inovação (MCTI; CT-AGRO/CT-HIDRO), CNPq, CAPES, and Agência Brasileira da Inovação (FINEP).

References

1. FAO (2012) Fishstat Plus 2.30: Universal software for fishery statistical time series. Fisheries Department, Fishery Information, Data and Statistics Unit, Rome
2. Lazoski C, Gusmão J, Boudry P, Solé-Cava AM (2011) Phylogeny and phylogeography of Atlantic oyster species: evolutionary history, limited genetic connectivity and isolation by distance. Mar Ecol Prog Ser 426:197–212
3. Oliveira-Neto FM (2008) Síntese informativa da produção de moluscos (mexilhões, ostras e vieiras) comercializados em 2007 no Estado de Santa Catarina (Epagri, Artigos, Produção da Maricultura). http://www.epagri.rct-sc.br/. Accessed 15 Dec 2012
4. Melo CMR, Silva FC, Gomes CHAM, Solé-Cava AM, Lazoski C (2010) *Crassostrea gigas* in natural oyster banks in southern Brazil. Biol Invasions 12:441–449
5. Lapègue S, Boutet I, Leitão A, Heurtebise S, Garcia P, Thiriot-Quiévreux C, Boudry P (2002) Trans-Atlantic distribution of mangrove oyster species revealed by 16S mtDNA and karyological analyses. Biol Bull 202:232–242
6. Ignacio BL, Absher TM, Lazoski C, Solé-Cava AM (2000) Genetic evidence of the presence of two species of Crassostrea (Bivalvia: Ostreidae) on the coast of Brazil. Mar Biol 136:987–991
7. Melo MAD, Silva ARB, Varela ES, Sampaio I, Tagliaro CH (2012) Development and characterization of ten microsatellite markers for population studies of the native Brazilian oyster *Crassostrea gasar*. Cons Gen Resour 4:583–586
8. Beck MW, Brumbaugh RD, Airoldi L, Carranza A, Coen LD, Crawford C, Defeo O, Edgar GJ, Hancock B, Kay MC, Lenihan HS, Luckenbach MW, Toropova CL, Zhang G, Guo X (2011) Oyster reefs at risk and recommendations for conservation, restoration, and management. Bioscience 61:107–116
9. Valero AL, Caballero YQ (2003) A practitioner's guide for the culture of marine bivalves in the Colombian Caribbean Sea: pearly oysters, oysters and scallops. Serie de Documentos Generales Invemar, Cargraphics, Bogota
10. Gusmão J, Solé-Cava AM (2002) Um sistema de diagnóstico molecular para a identificação de espécies comerciais de camarões marinhos brasileiros. In: CIVA 2002: Comunicaciones y Foros de Discusión Primer Congresso Iberoamericano Virtual de Acuicultura, Zaragoza, pp 754–764
11. Bloor PA, Barker FS, Watts PC, Noyes HA, Kemp SJ (2001) Microsatellite Libraries by Enrichment. http://www.genomics.liv.ac.uk/animal/Protocol1.html
12. Martins WS, Lucas DCS, Neves KFS, Bertioli DJ (2009) WebSat—a web software for microsatellite marker development. Bioinformation 3:282–283

13. Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234
14. Amos W, Hoffman JI, Frodsham A, Zhang L, Best S, Hill AVS (2006) Automated binning of microsatellite alleles: problems and solutions. Mol Ecol Notes 7:10–14
15. Goudet J (1995) FSTAT (Version 1.2): a computer program to calculate F-statistics. J Hered 86:485–486
16. Excoffier L, Lischer HE (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564–567
17. Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-checker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538
18. McInerney CE, Allcock AL, Johnson MP, Bailie DA, Prodöhl PA (2011) Comparative genomic analysis reveals species-dependent complexities that explain difficulties with microsatellite marker development in molluscs. Heredity 106:78–87