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A N I N T E R N A T I O N A L J O U R N A L

Novel Microsatellite Markers for Brazilian Mangrove Oysters (*Crassostrea gasar*) and their Cross-Amplification in *Crassostrea rhizophorae*

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ABSTRACT

A microsatellite CT/GT enriched genomic library was developed for **Crassostrea gasar** *and twelve new polymorphic loci were isolated and characterized. The markers were successfully amplified from 25 individuals of* **Crassostrea gasar** *and 11 cross-amplified individuals of* **Crassostrea rhizophorae**. *There was no evidence of linkage between loci in either species.*

Key words: SSR; genetic markers; cupped oyster; population structure.

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The cupped oyster genus *Crassostrea* Sacco, 1897, comprises many species distributed in coastal ecosystems worldwide and represents one of the most important resources in terms of global oyster production (FAO 2012). Molecular studies (Varela et al. 2007; Melo et al. 2010a; Melo et al. 2010b; Lazoski et al. 2011) from the Atlantic coast of South America indicated the existence of four species of *Crassostrea* distributed in natural beds, including two native species *Crassostrea gasar* (Adanson, 1757; sin. *Crassostrea brasiliana* Lamarck 1819*)* and *Crassostrea rhizophorae* (Guilding 1828), and two non-native Indo-Pacific oysters, *Crassostrea gigas* (Thunberg, 1793) and *Crassostrea* sp (Gardunho et al. 2012).

Although the native oyster species have a wide distribution along the South Atlantic coast, defined in terms of molecular data (Lapègue et al. 2002), their cultivation is usually restricted to small family groups of fishermen or farmers. Culture of native species is carried out either by extracting young oysters from natural beds for cultivation, or by collecting oyster seed from the water column using artificial substrates, which are then on-grown using suspended lanterns and/or wooden tables in subtidal zones (Galvão et al. 2009; Henriques et al. 2010).

Knowledge of the genetic structure of oyster populations before translocation of individuals among estuaries is important in order to avoid loss of genetic diversity or possible unwanted effects of hybridization among genetically divergent oyster populations (Hurwood et al. 2005). The use of highly polymorphic loci such as microsatellites provide tools for the evaluation of population genetic events, such as structuring and patterns of connectivity among stocks (Rose et al. 2006; Hong et al. 2008; Varney et al. 2009; Xiao et al. 2010), leading to appropriate methods of monitoring the genetic diversity of oyster stocks. The present study describes the isolation and characterization of microsatellite loci for *C. gasar* and *C. rhizophorae*.

We extracted the DNA from a single individual of *Crassostrea gasar* sampled from the municipality of Augusto Corrêa (northern Brazil), according to a phenol-chloroform DNA extraction protocol (Sambrook et al. 1989). This individual was previously identified, in terms of mitochondrial COI sequences (Melo et al. 2010a), as the native species *C. gasar*. DNA digestion was performed with *Rsa*I enzyme and linked to double-stranded previously known sequences. Biotin-linked probes (GT_8 and CT_8) were applied during the enrichment procedure for posterior selection with streptavidin magnetic-coated beads (Promega) and cloning into pGEM-T easy vector (Promega) for posterior insertion into *Escherichia coli* XL 1-Blue competent cells. Transformed clones were loaded onto an automatic sequencer (Applied Biosystems - ABI model 377) according to the manufacturer's protocols and sequences were analyzed in BioEdit 7 (Hall 1999) and FastPCR 5.4 (Kalendar et al. 2009), to search for adaptor sequences and restriction sites, respectively. The Simple Sequence Repeat Identification Tool (SSRIT) software (Temnykh et al. 2001) was used to identify suitable microsatellite sequences and primer design was carried out with Primer3Plus (Untergasser et al. 2007).

A total of 96 clones were initially sequenced and 30 contained repetitive fragments. From these, 24 sequences were suitable for primer design, 12 of which were deemed to be most appropriate, based on peak profile and variability. All 12 selected microsatellite loci (Table 1) were developed according to the M13 tailed primer method (Schuelke 2000). Polymerase chain reactions (PCR) were conducted in a final volume of 13 μL containing 5 ng of DNA, 1 x PCR buffer, 1.5 mM of magnesium chloride, 1.2 mM of dNTP, 8 pM of M13 and reverse primers, 2 pM of forward primer and 1 U *Taq* DNA polymerase. A temperature gradient from 55°C to 65°C was used to identify the correct annealing temperature (*T°m*) for all primers. The PCR profile consisted of an initial denaturing at 94°C for 5 min; 30 cycles of 30 sec at 94°C, 45 sec at "*T°m"*, 45 sec at 72°C; 8 cycles of 30 sec at 94°C, 45 sec at 53°C, 45 sec at 72°C, followed by a final extension at 72°C for 10 min. Positive PCR products were mixed with the GeneScan-500 ROX size standard (Applied Biosystems) and formamide to run on an ABI3500 sequencer. Fragment analysis and genotyping were performed using Genemapper version 4.0 (Applied Biosystems).

Table 1. Locus name, repeat motif, primer sequences and size ranges (bp) of twelve microsatellite loci isolated from Crassostrea gasar and cross-species amplification with Crassostrea rhizophorae.

Locus name	Repeat motif	Primer sequences (5'-3')	Size range (bp) *C. gasar*	Size range (bp) *C. rhizophorae*
CGA07	$(CT)_{23}$	GATACTCTCTCGCTCTCACAGAGTT TCGGGTGGGTAATTTAGAGGTA	164 - 180	166 - 176
CGG08	$(AC)_{10}$	CCGCACTAGTGATTCTCTTGCT CGTGCGGGTATTACTGTCTTGA	106 - 338	126 - 388
CGH05	$(AC)_{17}(AT)_{25}$ $(AC)_5$	AATTCACAAGTAGAATTCCCAAAGA AGTTGAAAAAGGAAAACAGGATGTA	170 - 296	166 - 214
CGB06	$(TC)_7TG(TC)_{14}$	GTAGAGGACATTCCCGAGAAGA CTCCACAGTCGCATACAAGGTT	206 - 286	212 - 268
CGH03	$(CA)_8$	GATCCAGCAGGTGTTAGGAGAT ACACTAAGCATCGGCTTGTTGG	160 - 226	162 - 206
CGA12	$(CA)_9$	CTTGATGCCGACCACTGTAATA TTACCTTGTTTGTGAGGAGAGGTC	180 - 188	180 - 184
CGB09	$(CT)_{16}TT(CT)_7(GT)_{17}GG(CA)_8$	CAGCGAGTCTTAGCAATCTCTC GGCTATGGGAGTCTCGATATAGG	358 - 410	338 - 406
CGE11	$(GT)_4CG(GT)_2GC(GT)_9$	GTAAGTGTAAAACGTCTTTGCTGTC TAGTGCAACCCGTGTTTATATGACT	180 - 202	184 - 202
CGD05	$(TG)_{11}(AG)_{21}$	TAGTCGATCGATCTCTCGTTCC TAGCGGTCTTTGACCAGTATCG	408 - 450	400 - 448
CGG06	$(TC)_3TT(TC)_{22}(C)_{10}(CA)_7$	CCGTTAAGGTGTCCATATACTGC GTAAGAAGGTGGTCGGGGAGTAA	214 - 264	218 - 256
CGH02	$(GT)_{14}$	AGCGTCTTTACGAGTTTGTTGC AGATGAAGACGTCACCGTTACAG	222 - 250	230 - 252
CGF03	$(GT)_8$	AAAGGTGAGATGATCGTAGGAG CGCGTGGACTAACTAAGACAACAG	400 - 452	400 - 436

All 12 loci were polymorphic in the native species of *C. gasar* and *C. rhizophorae* with the number of alleles ranging from 5 to 22 and 3 to 13, respectively (Table 1). Twenty-five individuals of *C. gasar* from northern Brazil (Pará state: 00°43' 29.5"S 47°20'49.3"W) and eleven individuals of *C. rhizophorae* from southeastern Brazil (São Paulo state: 24°57 58.6"S 47°55'18.4"W) were genotyped. Micro-Checker 2.2.1 (van Oosterhout et al. 2004) analysis showed no evidence of genotype errors such as stuttering or allele drop out in both species, and detected potential heterozygosity deficiency in two null allele loci (CGH03 and CGB06) in *C. gasar* and one (CGH03) in *C. rhizophorae*. Two loci (CGH03 and CGG08) in *C. gasar* and only one (CGH03) in *C. rhizophorae* exhibited significant deviation from Hardy-Weinberg equilibrium (Table 2) and there was no evidence of significant linkage disequilibrium between loci of both species using Genepop (Raymond and Rousset 1995). Observed and expected heterozygosities were calculated for all *loci* using the program ARLEQUIN 3.5 (Excoffier and Lischer 2010) ranged from 0.522 to 1.000 (Ho) and from 0.622 to 0.941 (He) for *C. gasar* and from 0.444 to 0.909 (Ho) and 0.380 to 0.913 (He) for *C. rhizophorae* (Table 2). There was a striking difference in number of alleles between *C. gasar* and *C. rhizophorae* for the CGH05 *locus*, which presented nineteen alleles and a 170 to 296 base pair (bp) range for *C. gasar* against six alleles and a 166 to 214 bp range for *C. rhizophorae*. No evidence of a null allele was detected for this locus in both species.

Table 2. Characterization of twelve microsatellite loci isolated from *Crassostrea gasar* and cross-species amplification with *Crassostrea rhizophorae*, including locus name, number of alleles (n), annealing temperature (T), observed (Ho) and expected (He) heterozygosities, and probabilities of deviation from Hardy-Weinberg equilibrium (P_{HW}).

Crassostrea gasar (25 specimens)						*Crassostrea rhizophorae* (11 specimens)					
Locus name	Number of alleles (n)	T (°C)	Ho	He	P_{HW}	Locus name	Number of alleles (n)	T (°C)	Ho	He	P_{HW}
CGA07	8	52.5	0.750	0.839	0.157	CGA07	6	52.5	0.818	0.793	0.190
CGG08	17	55	0.875	0.902	0.000*	CGG08	11	55	0.900	0.855	0.427
CGH05	19	60	0.870	0.922	0.129	CGH05	6	61	0.833	0.792	0.410
CGB06	22	64.5	0.833	0.941	0.027	CGB06	13	64.5	0.818	0.868	0.160
CGH03	16	62	0.720	0.882	0.000*	CGH03	10	61.5	0.444	0.877	0.000*
CGA12	5	51	0.667	0.718	0.629	CGA12	3	51	0.636	0.562	1.000
CGB09	16	60	0.917	0.923	0.224	CGB09	13	60.5	0.818	0.897	0.227
CGE11	10	60	0.680	0.790	0.167	CGE11	8	60	0.636	0.793	0.044
CGD05	15	60	1.000	0.900	0.463	CGD05	13	60	0.909	0.909	0.521
CGG06	21	60	0.840	0.927	0.024	CGG06	13	60	0.818	0.913	0.165
CGH02	13	60	0.875	0.884	0.418	CGH02	10	60	0.909	0.855	0.649
CGF03	6	64	0.522	0.622	0.078	CGF03	4	64	0.455	0.380	1.000

* Significant P_{HW} value after Bonferroni correction $P < 0.005$.

Recently, the availability of microsatellite markers for Brazilian native oysters (genus *Crassostrea*) has been restricted to sixteen *loci* for *C. gasar* and eleven *loci* for *C. rhizophorae* (Melo et al. 2012; Cavaleiro et al. 2013). Seventeen of these loci are inappropriate for cross amplification between both native species (Cavaleiro et al. 2013). All twelve markers described in this study amplified well in *C. gasar* and *C. rhizophorae* samples, taking into account the stringent conditions set during *loci* selection, and increasing the number of these highly polymorphic markers to twenty-eight for *C. gasar* and twenty-three for *C. rhizophorae*. All twelve new markers developed provide a reliable molecular tool for the management and conservation of these tropical commercial species of oysters.

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