Table 2 Cross-amplification of microsatellite loci from *Suaeda maritima* in three related *Suaeda* species. The absence of a PCR product is symbolized by a slash (/)

	Suaeda spicata (*N* = 5)		*Suaeda salsa* (*N* = 2)		*Suaeda salinaria* (*N* = 2)	
Locus	No. of alleles	Size range	No. of alleles	Size range	No. of alleles	Size range
Sm_a2	8	202–228	4	208–244	4	208–240
Sm_c2	6	250–280	3	244–260	1*	234
Sm_c3	3	299–317	2	297–303	1*	305
Sm_c7	5	139–173	1	139	2	147–149
Sm_c12	/	/	3	201–213	1*	219
Sm_d5	5	196–208	4	210–222	2*	218–226
Sm_d11	5	134–204	4	196–216	1*	158
SmI_a3	5	214–240	2	214–226	1*	218
SmI_d4	5	119–139	3	121–129	2*	123–133
SmI_d7	2	172–174	3	170–174	1	170
SmI_e9	7	136–170	1	148	1*	164
SmI_h7	1	275	1	251	/	/

*Amplification in only one of two individuals.

types of inland habitats were less diverse than individuals from the coast at most of the loci. Populations from natural and anthropogenic inland sites were fixed for a single allele at two and six loci, respectively (Table 1), perhaps as a consequence of founder effects and/or genetic drift.

All 12 primer pairs cross-amplified in the closely related *Suaeda salsa*, and 11 markers each were transferable to *Suaeda salinaria* and *Suaeda spicata* (Table 2). These three species belong to the same section (sect. *Brezia*) as *S. maritima*. No PCR products were produced with template DNA from a single individual of *Suaeda vera* (sect. *Suaeda*). The microsatellite loci described here are being utilized for analysing the genetic diversity and structure of *S. maritima* and closely related species, with a special focus on the colonization dynamics of anthropogenic inland salt sites.

References

Aellen E (1968) *Suaeda*. In: *Illustrierte Flora von Mitteleuropa*, 2nd edn (ed. Hegi G), pp. 734–739. P. Parey, Berlin & Hamburg.

Chapman VJ (1947) *Suaeda maritima* (L.) Dum. *Journal of Ecology*, **35**, 293–302.

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small amount of fresh leaf issue. *Phytochemical Bulletin*, **19**, 11–15.

Fischer D, Bachmann K (1998) Microsatellite enrichment in organisms with large genomes (*Allium cepa*). *BioTechniques*, **24**, 796–802.

Garve E, Garve V (2000) Halophyten an Kalihalden in Deutschland und Frankreich (Elsass). *Tuexenia*, **20**, 375–417.

Markwith SH, Stewart DJ, Dyer JL (2006) TETRASAT: a program for the population analysis of allotetraploid microsatellite data. *Molecular Ecology Notes*, **6**, 586–589.

Prinz K, Weising K, Hensen I (2009) Genetic structure of coastal and inland populations of the annual halophyte *Suaeda maritima* (L.) Dumort in Central Europe inferred from amplified fragment length polymorphism markers. *Plant Biology*, doi: 10.1111/j.1438-8677.2008.00178.x.

Rogstad SH (1992) Saturated NaCl-CTAB solution as a means of field preservation of leaves for DNA analyses. *Taxon*, **41**, 701–708.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, New Jersey.

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Polymorphic microsatellite DNA markers in the mangrove crab *Ucides cordatus* (Brachyura: Ocypodidae)

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Abstract

The isolation and characterization of the first polymorphic microsatellite markers for the mangrove crab *Ucides cordatus* are described. The number of alleles at each locus ranged from three to 25, mean of nine alleles, in 46 crabs captured in two Brazilian mangroves. The markers averaged high levels of observed (0.709 ± 0.183) and expected (0.716 ± 0.170) heterozygosities. Departures from Hardy–Weinberg equilibrium were observed at two loci.

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Linkage disequilibrium tests were not significant and no evidence of null alleles was detected. All these microsatellite loci are expected to be useful in estimating fine-scale population processes of this valuable mangrove species currently subjected to excessive fishing efforts.

Keywords: genetic markers, mangrove, microsatellite loci, population structure

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The mangrove crab, *Ucides cordatus*, is a widespread species occurring along the coastline of tropical and subtropical America (Melo 1996). Despite its extensive distribution, the impact caused by excessive uncontrolled fishing pressure can eliminate well-adapted stocks, which would cause the species to decline (Avise & Hamrick 1996). Microsatellites, short stretches of repeated DNA, are helpful in intraspecific studies as genetic markers for assessing diversity, population structure and gene flow of threatened species (Goldstein & Schlötterer 1999). This work describes the isolation and characterization of the first eight polymorphic and easily scorable microsatellite loci in *U. cordatus*.

Microsatellite isolation was carried out as outlined in Glenn & Schable (2005). Genomic DNA was isolated from the pereiopod muscle using phenol–chloroform–isoamyl alcohol (25:24:1) extraction of the SDS/proteinase-K-digested tissue, followed by ethanol precipitation (Sambrook *et al.* 1989). DNA aliquots of approximately 2 μg were independently digested with *Rsa*I and *Bst*UI and the fragments were ligated to double-stranded SuperSNX linkers with T4 DNA ligase in the presence of *Xmn*I. All restriction enzymes were supplied by New England Biolabs (NEB). The linker-ligated DNA was enriched for repeat motifs, $(GACA)_6$, $(GATA)_7$, $(GGAT)_5$ and $(GTAT)_5$, with biotinylated probes. Double enrichments were also performed for certain samples following Diniz *et al.* (2007). Enriched and double-enriched fragments were recovered and amplified in 50-μL reactions using 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 25 μg/μL BSA, 1.0 mM dNTP mix, 0.8 μM $SuperSNX_F$, 5.0 U of *Taq* polymerase and 10 μL of the enriched DNA. The following programme was used for amplification: 95 °C for 5 min, 20× (95 °C for 45 s, 62 °C for 1 min, 72 °C for 2 min) and 72 °C for 30 min. Ligated enriched DNA was cleaned with Purelink™ PCR purification kit (Invitrogen) and ligated into Qiagen pDrive™ Vectors, which were used to transform NEB 5-alpha competent *Escherichia coli* (DH5α derivative; NEB). Bacteria were inoculated in imMedia™ Amp Blue (Sigma-Aldrich) and grown overnight. After the cloning procedure, 200 positive colonies were identified in a white (positive) and blue (negative) screen. The colonies were transferred to Luria–Bertani (LB) broth with ampicillin (100 μg/mL) and allowed to grow for 15 h with constant shaking (225 rpm). Then, 1.0 μL of LB broth containing positive bacteria was used in PCR amplifications of target sequences, via the universal primers M13F and M13R. Product size was screened in 1% agarose gel and fragments larger than 500 bp were identified in 78% (156) of the clones. The fragments were purified and submitted to sequencing. Microsatellite motifs containing more than five repeats were identified in 34 sequences (24 with tetra- and 10 with tri-repeats) and primer pairs for each loci were designed using Primer 3 (Rozen & Skaletsky 2000). An oligonucleotide tail, corresponding to M13F (5′-GTAAAACGACGGCCAGTG-3′) or T7 promoter (5′-GTAATACGACTCACTATAGGGC-3′), was added to one of the primers of each pair (Diniz *et al.* 2005). Microsatellite amplification was accomplished in a PCR with three primers: a tailed microsatellite primer conjugate, a nontailed microsatellite primer and the fluorescent (HEX)-labelled primer.

The optimization reactions for each primer were performed in 46 crab samples captured in Cananéia (25°02′19″S; 47°56′42″W; 24 individuals) and Fortim (4°27′25″S; 37°47′48″W; 22 individuals), two Brazilian mangroves approximately 3700 km apart. After this procedure, eight polymorphic microsatellite loci were identified. The remaining loci presented high level of stuttering or showed no variation in fragment size after amplification. Amplification reactions for each of the eight polymorphic markers were performed in a 10-μL volume containing 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM of nontailed primer, 0.05 μM of tailed primer, 0.45 μM of fluorescent-labelled primer (HEX-$M13_F$ or HEX-T7 tag), 0.5 U of *Taq* DNA polymerase and 1.0 μL of crab DNA. Reaction conditions were 95 °C for 2 min, 30× (95 °C for 25 s, annealing temperature according to Table 1 for 30 s, 72 °C for 50 s), and 72 °C for 7 min. PCR products were screened in 6% denaturing polyacrylamide gels and visualized using either a FMBIO-II scanner (Hitachi), a LI-COR 4300 DNA Analyzer (LI-COR Biosciences) or silver staining. Product sizes were determined with 10 bp DNA ladder (Invitrogen) and scored manually. The individuals were genotyped to obtain baseline allele frequency information. Observed and expected heterozygosities and tests for departure from Hardy–Weinberg expectations (HWE) were performed using GENEPOP (Raymond & Rousset 1995) as well as the occurrence of linkage disequilibrium between loci. Allelic richness for each locus was calculated using FSTAT 2.9.3 (Goudet 1995) and the presence and frequency of null alleles were determined with the program MICRO-CHECKER 2.2.3 (van Oosterhout *et al.* 2004).

Details of the eight polymorphic microsatellite loci for the mangrove crab *U. cordatus* are presented in Table 1. The fragment sizes ranged from 115 to 317 bp (including the added tail). The average number of observed alleles and allele richness were 9.286 ± 7.319 and 8.978 ± 5.959 respectively.

Table 1 Characteristics of the polymorphic microsatellite markers tested in 46 *Ucides cordatus* samples. The PCR amplifications were performed using a nontailed primer, a tailed primer (containing a M13 or a T7 tail) and a fluorescent-labelled primer (FL)

Locus	Primer sequence (5′→3′)	Core motif of the cloned allele	T_a (°C)	Size range/clone size* (pb)	No. alleles	Allelic richness	H_O	H_E	P_{HWE}	GenBank Accession no.
UcSSR-01	F: CGTCTGCATGCCAATTAGTTC R: $M13_F$-tail—CACGAGGTGAATGTGAGGTG FL: HEX—GTAAAACGACGGCCAGTG	$(CTCG)_7$	54	115–139/123	5	4.466	0.717	0.526	0.059	FJ483820
UcSSR-02	F: T7-tail—TCTGCGTAACTCTGCTTCT R: ACATCATTGAGGAAAGACAG FL: HEX—GTAATACGACTCACTATAGGGC	$(CTGT)_6$	52	131–171/139	10	8.944	0.739	0.765	0.648	FJ483821
UcSSR-03	F: $M13_F$-tail—CCTAGGAGACAGAATAGACAAGC R: ATGGATGGATATGACGAAGC FL: HEX—GTAAAACGACGGCCAGTG	$(ACAG)_7$	57	119–155/123	7	6.642	0.804	0.787	0.435	FJ483822
UcSSR-04	F: $M13_F$-tail—AACAGTCTGTAATCCTACCCATC R: TGCTTTAGACTGGCAAACAG FL: HEX—GTAAAACGACGGCCAGTG	$(TCTA)_6$	54	160–168/160	3	3.000	0.304	0.401	0.161	FJ483823
UcSSR-05	F: $M13_F$-tail—TGTCTGTCCGTTTCTTTGTG R: TTTCTGCTATTCATGGCAAG FL: HEX—GTAAAACGACGGCCAGTG	$(TGTA)_6$	57	161–181/165	6	5.989	0.739	0.786	< 0.010	FJ483824
UcSSR-06	F: $M13_F$-tail—GGCTATGAATCGTGGAACTCA R: CCGCGCCGTAAGTTTAAATA FL: HEX—GTAAAACGACGGCCAGTG	$(ATAG)_6(N)_{12}(GATA)_{12}$	54	174–270/261	25	21.865	0.935	0.932	0.314	FJ483825
UcSSR-07	F: AGACAAGTTACGGAGACACG R: T7-tail—TCAGTCTCTGTTGCTTCCAC FL: HEX—GTAATACGACTCACTATAGGGC	$(CATA)_6\ (N)_2\ (ACAG)_6$	60	177–209/205	9	8.505	0.783	0.811	< 0.010	FJ483826
UcSSR-08	F: T7-tail—GCTGCCATTACATTCACTGC R: GTTCCCTCGTTCACTTCTCG FL: HEX—GTAATACGACTCACTATAGGGC	$(ACT)_6(N)_{22}(CTA)_4$	52	272–317/272	14	12.416	0.652	0.718	0.133	FJ483827

T_a, annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; P_{HWE}, probability of departure from Hardy–Weinberg equilibrium.
*Allele includes the additional size of tails, M13 (18 bp) or T7 (22 bp), added to one of the microsatellite primers.

The mean expected heterozygosity value (0.716 ± 0.170) was similar to the mean observed heterozygosity (0.709 ± 0.183). Departures from HWE were detected for loci UcSSR-05 and UcSSR-07 ($P < 0.01$), possibly an artefact of small sample size or a consequence of Wahlund effect (Lessios 1992). However, when the analysis was carried out separately for samples from each sampling site, none of the markers deviated from HWE after Bonferroni correction. No evidence of null allele was detected. Linkage disequilibrium was not significant for any pair of loci ($P > 0.01$).

The markers described in this study are expected to be useful in estimating fine-scale population processes of this valuable mangrove species, currently subjected to excessive fishing efforts. Ongoing research is involved in applying these markers for population discrimination of *U. cordatus* throughout its range.

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References

Avise JC, Hamrick JL (1996) *Conservation Genetics: Case Histories from Nature*. Chapman & Hall, New York.

Diniz FM, Maclean N, Ogawa M, Paterson I, Bentzen P (2005) Microsatellites in the overexploited spiny lobster, *Panulirus argus*: isolation, characterization of loci and potential for intraspecific variability studies. *Conservation Genetics*, **6**, 637–641.

Diniz FM, Iyengar A, Lima PSC, Maclean N, Bentzen P (2007) Application of a double-enrichment procedure for microsatellite isolation and the use of tailed primers for hight throughput genotyping. *Genetics and Molecular Biology*, **30**, 380–384.

Glenn TC, Schable NA (2005) Isolating microsatellite DNA loci. *Methods in Enzymology*, **395**, 202–222.

Goldstein DB, Schlötterer C (1999) *Microssatellites: Evolution and Applications*. Oxford University Press, New York.

Goudet J (1995) FSTAT: a computer program to calculate F-statistics. *Jounal of Heredity*, **86**, 485–486.

Lessios HA (1992) Testing electrophoretic data for agreement with Hardy-Weinberg expectations. *Marine Biology*, **112**, 517–523.

Melo GAS (1996) *Manual de identificação dos Brachyura (caranguejos e siris) do litoral brasileiro*. Plêiade/FAPESP, São Paulo.

van Oosterhout C, Hutchinson WF, Wills DP, Shipley P (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248–249.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general users and biologists programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (eds Krawetz S, Misener S), pp. 365–386. Humana Press, Totowa, NJ.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

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Isolation and characterization of 12 novel DNA microsatellites in the western rainbowfish, *Melanotaenia australis*

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Abstract

Microsatellites were isolated from the western rainbowfish, *Melanotaenia australis*, for use as molecular markers. Twelve polymorphic loci were found. When characterized using 32 individuals, these had between two and 24 alleles each, and observed heterozygosities ranged from 0.00 to 0.97. Ten loci were in Hardy–Weinberg equilibrium, and all of them showed independent inheritance. These loci will be useful for the study of molecular subdivision in this species.

Keywords: FIASCO protocol, *Melanotaenia splendida australis*, Melanotaeniidae, population genetics

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