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PERMANENT GENETIC RESOURCES

Isolation of polymorphic microsatellite loci for the marine invader *Microcosmus squamiger* (Ascidiacea)

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Abstract

The ascidian *Microcosmus squamiger* is native to Australia and has recently spread worldwide. It has become a pest in some littoral communities within its introduced range. An enriched genomic library of *M. squamiger* resulted in a total of eight polymorphic loci that were genotyped in 20 individuals from a population within its introduced range, and 20 individuals more from a native population. The mean number of alleles per locus was 5.33 and mean observed heterozygosity was 0.432. No significant linkage disequilibrium was found among loci pairs. Significant genetic differentiation was observed between populations.

Keywords: ascidian, biological invasion, *Microcosmus squamiger*, microsatellites, population structure

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The solitary ascidian *Microcosmus squamiger* is a sessile marine invertebrate native to Australia (Kott 1985) that spreads through its short-lived planktonic larval stage. However, over the last 50 years, this species has been found in distant places around the globe (Lambert & Lambert 1998; Turon *et al*. 2007) where it was most likely introduced via ship transport (Rius *et al*. 2008). In the localities within its introduced range, it can be found in high densities in open coastal habitats where it affects native biota, as well as in large shipping harbours where *M. squamiger* forms dense clumps that cover the available substratum (Turon *et al*. 2007). High genetic diversity in introduced populations and nonindependent colonization events were found using the COI gene of mtDNA (Rius *et al*. 2008). However, microsatellite loci can refine introduction histories of colonizing species involving complex scenarios (Pascual *et al*. 2007).

One *M. squamiger* specimen collected from a locality within its introduced range (Cubelles, Mediterranean Sea; 41°11′37″N, 1°39′17″E) was used to prepare a microsatellite-enriched genomic library, following the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol proposed by Zane *et al*. (2002). DNA was extracted using QIAamp DNA kit (QIAGEN), digested with *Mse*I and ligated to the adapters *Mse*I-AFLP (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′). The enrichment step was performed following the protocol described in Kijas *et al*. (1994). DNA sequences containing microsatellites were retained with streptavidin-coated magnetic particles (Streptavidin Magnesphere Paramagnetic Particles, Promega) by hybridization with four biotin-labelled probes [$(CA)_{15}$, $(GA)_{15}$, $(CAA)_7$ and $(GATA)_7$]. After eluting the retained DNA, a 15-cycle polymerase chain reaction (PCR) was carried out with *Mse*I-N (5′-GATGAGTCCTGAGTAAN-3′) primers. The amplified DNA was purified and cloned using the P-GEM T Easy Vector II (Promega). Positive clones were detected following the Estoup and Turgeon protocol (http://www.inapg.inra.fr/dsa/microsat/microsat.htm) using the same probes labelled with digoxigenin. Of approximately 1200 colonies plated, only 81 clones showed positive screening signals indicative of low density of microsatellites in ascidians. These positive clones were grown in an LB medium overnight and DNA was extracted using QIAprep Spin Miniprep Kit (QIAGEN). Genomic inserts were amplified using T7 (5′-TAATACGACTCACTATAGGG-3′) and SP6 (5′-ATTTAGGTGACACTATAGAA-3′) primers. Sequencing reactions were run in a 10 μL final volume (3 μL BigDye version 3.1 (Applied

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Table 1 Characteristics of the eight microsatellite loci developed for *Microcosmus squamiger*

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size (bp)	Cubelles				Albany				GenBank Accession no.
					N_A	H_O	H_E	F_{IS}	N_A	H_O	H_E	F_{IS}	
MS6	F: FAM – CCAGCGAAATACAGCAGTCTC R: CAGGTGGGTGATCTTGGACT	$(GT)_8AAGT$	53	149–200	3	0.421	0.428	0.017^{NS}	3	0.353	0.544	0.358^{NS}	EU797420
MS7	F: FAM – CCGAAAAATCGAGACTCAGC R: CATAATCGCAAACACGCACT	$(CAA)_6$	57	252–315	2	0.500	0.467	–0.073^{NS}	4	0.350	0.453	0.231^{NS}	EU797421
MS8	F: HEX – TGACTTCCTGCTCTGTCTTGG R: CTTGCACACGCACACATTC	$(GT)_{15}$	47	218–295	8	0.526	0.795	0.344*	7	0.526	0.556	0.055^{NS}	EU797422
MS9	F: HEX – GGAGGGGCGAAACAGTGTA R: GGATGTAAGAAGAATTAGGAGATGG	$(TC)_2TT(TC)_{13}$	55	253–322	8	0.000	0.815	1*	9	0.150	0.751	0.804*	EU797423
MS10	F: HEX – CTGCCGAAGGGTCTGTATGT R: TTGATTGCTGCTGTTTCGTC	$(CAA)_5$	47	350–440	5	0.421	0.373	–0.134*	4	0.737	0.558	–0.333*	EU797424
MS11	F: NED – CGGACGACACCATAGTAACC R: GCCTTGGCGTGTTTGACTT	$(AAC)_5$	57	201–213	5	0.650	0.796	0.188^{NS}	5	0.350	0.547	0.367^{NS}	EU797425
MS12	F: NED – CAAGTCAAACACGCCAAGG R: GTCAGAAAGGCGCAGAAGC	$(CAA)_7$	57	102–135	5	0.421	0.516	0.189^{NS}	7	0.800	0.760	–0.054^{NS}	EU797426
MS13	F: NED – CTCGATTGGCGCTTCTTATC R: ACAGGAACACGACCAAAACC	$(GTT)_3GCT(GTT)_3$	57	224–239	6	0.400	0.667	0.406*	5	0.300	0.718	0.588*	EU797427
	Total mean				5.250	0.417	0.607	0.318*	5.500	0.446	0.611	0.275*	

T_a, annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity under Hardy–Weinberg equilibrium; F_{IS}, inbreeding coefficient with its significance: NS, non-significant, *$P < 0.05$.

Biosystems), 3 μL DNA, 0.25 μL of either SP6 or T7 primer (10 μM) and 3.75 μL H_2O) and their products analysed with an automated sequencer (ABI PRISM 3100 Genetic Analyser, Applied Biosystems) from the 'Serveis Cientifico-tècnics' of the University of Barcelona. Forty-seven microsatellite loci were identified, of which 53.33% were perfect, 43.33% were imperfect and the rest (3.33%), compound. Of these, dinucleotides were the most abundant accounting for 70% of the total, while trinucleotides accounted for 26.67% and the remaining 3.33% were tetranucleotide repeats. The mean number of repeats for dinucleotides was 19.57 (SD = 12.56), for trinucleotides, it was 6.88 (SD = 3.18), and for the only tetranucleotide, it was 24. Whenever enough flanking region was available, primers were designed with the Primer3 web-based software package (http://frodo.wi.mit.edu/, Whitehead Institute for Biomedical Research). Only eight loci were retained as the remainder were dropped either because they did not have enough flanking regions or because they failed to amplify reliably.

Polymorphism was tested using 20 specimens collected from Cubelles and 20 from a native region (Albany, Western Australia, 35°01′56″S, 117°53′25″E; Table 1). DNA was extracted using the REALPURE extraction kit (Durviz) and amplified by PCR using a 20 μL total reaction volume, with 0.5 μL of each primer (10 μM), 0.5 μL dNTPs (10 mM), 2 μL 10× buffer, 3 μL $MgCl_2$ (25 mM), 1 U *Taq* polymerase (Promega) and approximately 30 ng of DNA. An initial denaturation at 94 °C for 5 min was followed by 30 cycles consisting of a denaturation step at 94 °C for 1 min, an annealing step at the corresponding temperature (Table 1) for 30 s, and an elongation step at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The forward primer of each locus was labelled with a fluorescent dye (FAM and HEX from Isogen, and NED from Applied Biosystems) (Table 1). Allele sizes were estimated based on the standard molecular ladder Rox (70–500 bp, Bioventures) using a 3730 DNA Analyser (Applied Biosystems) from the Serveis Cientifico-tècnics. Allele calls were visually assigned with GeneMapper (version 3.7, Applied Biosystems), while statistical analyses were performed using GENALEX (Peakall & Smouse 2006) and GENETIX (Belkhir *et al*. 2001). Loci possessed two to nine alleles per locus (total mean ± SD = 5 ± 1.996) (Table 1). No pair of loci showed significant linkage disequilibrium after sequential Bonferroni correction. The inbreeding coefficient showed significant positive results (homozygote excess) in MS9, and MS13 in both populations, while MS8 showed significant results only for Cubelles (Table 1). To test the possible presence of null alleles, we used MICROCHECKER version 2.2.3 (2003) (van Oosterhout *et al*. 2004). The results showed patterns congruent with the presence of null alleles in MS9 and MS13 for both localities, as well as in MS8 in Cubelles and in MS11 in Albany. Some caution is therefore necessary when using these loci. However, they are also the ones with higher number of alleles and may therefore be more sensitive to any deviation from panmixia. A wider sampling is necessary to ascertain whether null alleles can explain the patterns found or whether the

populations of this species are truly inbred. The inbreeding coefficient was significantly negative (heterozygote excess) for MS10, which may indicate linkage disequilibrium with a gene under selection. The two populations studied presented significant genetic differentiation ($F_{ST} = 0.105$, $P < 0.001$). The results found here suggest that these polymorphic markers will be useful for population structure and connectivity studies, as well as for understanding the invasion history of *M. squamiger* populations.

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