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TECHNICAL NOTE

Isolation and characterization of eight polymorphic microsatellite loci for the Mediterranean gorgonian *Paramuricea clavata*

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Abstract The gorgonian *Paramuricea clavata* is a benthic organism often included in conservation management plans since it creates complexity in the ecosystems and is extremely vulnerable to disturbances. Eight microsatellite markers isolated from an enriched genomic library were characterized in a total of 50 individuals from two north-western Mediterranean populations. All loci were polymorphic and the number of alleles per locus ranged between 2 and 14. Significant genetic differentiation was observed between populations. The polymorphic markers presently isolated will allow for the assessment of the spatial genetic structure between and within populations of this umbrella species of the Mediterranean Sea.

Keywords Microsatellites · Gorgonian · *Paramuricea clavata* · Genetic structure · Mediterranean Sea

Gorgonians are marine invertebrates with a long lifespan and slow population dynamics which makes them especially vulnerable to severe disturbances (Linares et al. 2008). These species are considered to be ecosystem engineers as they create complexity and structure, and normally host a rich associated community. Thus, gorgonians are important to the maintenance of biodiversity of benthic communities and need to be protected (Bavestrello et al. 1997). In the north-western Mediterranean Sea this species has recently been affected by mass mortality events, which are most likely due to anomalous seawater temperatures (Cerrano et al. 2000). Thus, a good understanding of the spatial genetic structure between and within populations of *P. clavata* is required in order to establish adequate management plans for the conservation of this species and its associated community. Calderón et al. (2006) failed to detect genetic variability in this species using mitochondrial COI and nuclear ITS-2 sequences, indicating that more polymorphic markers are needed. The aim of this study was to isolate microsatellite loci for *P. clavata* for future intra- and inter-population studies.

Total genomic DNA was extracted from one individual from Columbretes archipelago, Spain (39°55′N, 0°40′E) using QIAamp minikit-colums (QIagen). The DNA was used to construct a partial genomic microsatellite enriched library following the FIASCO protocol (Zane et al. 2002). DNA was enriched for AC_n, AG_n, CAA_n and $GATA_n$ motifs. DNA enriched fragments were cloned using the P-GEM®-T Easy Vector System II (Promega). Detection of positive clones from the colonies using digoxigenin labelled probes was performed following the Estoup and Turgeon protocol (http://www.inapg.inra.fr/dsa/microsat/microsat.htm). Positive clones were sequenced on an ABI Prism 3700 sequencer from the "Serveis Científico-Tècnics" of the University of Barcelona.

Approximately 1,200 colonies were screened for microsatellites yielding 45 positive clones. A total of 36 microsatellite loci were identified, of which 56% where perfect, 31% imperfect and 13%. Trinucleotide repeats

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Table 1 Microsatellite loci isolated from the gorgonian *Paramuricea clavata*

Locus (dye) (accesion no.)	Primer sequence F and R (5′–3′)	Repeat motif	T (°C)	Size range	CORSICA					CABRERA				
					N	N_A	H_O	H_E	F_{IS}	N	N_A	H_O	H_E	F_{IS}
Par_a (VIC) (FJ560968)	F:TGGCAAACATAATTCCACCA R:CAAATGATGCTATTGAAGCCAGT	$(GTT)_9$	59	158–208	31	8	0.710	0.806	0.122	19	6	0.737	0.768	0.042
Par_b (NED) (FJ560969)	F:TTTCGAAGGGCAGACAGAGT R:TTGGAAGCCTAACGCAACTT	$(CAA)_{21}$	47	147–168	30	3	0.400	0.337	−0.192*	19	2	0.737	0.478	−0.565*
Par_d (NED) (FJ56070)	F:GGGCTGTGGTGTATTCCATT R:AATGGCTTTAGGGCCGTAGT	$(GTT)_6$ GTGTC$(GTT)_3$ AT$(GTT)_2$	51	338–356	30	4	0.600	0.620	0.032	19	4	0.632	0.700	0.100
Par_e (PET) (FJ560971)	F:TGTGGCTTACTGGGTGATGA R:CCACGCTTCTAGGCAAAGTC	$(GT)_8$	55	154–160	26	3	0.192	0.520	0.635*	19	2	0.105	0.193	0.463
Par_f (PET) (FJ560972)	F:ATGCACGTACATGGCTGAAA R:CTTAGCCGATGGGAAATGAA	$(GT)_{15}$	49	250–263	26	3	0.385	0.540	0.292	19	4	0.368	0.474	0.227
Par_g (6 FAM) (FJ560973)	F:ATGTGACTCGAACCCAGGTC R:TAGATCAATTGTCCGCATCG	$(CT)_{11}$	51	245–289	30	4	0.300	0.273	−0.101*	19	2	0.263	0.235	−0.125*
Par_k (VIC) (FJ560974)	F:TTGTTGCTGCTACGGTTGTT R:GGGGGATCTGAATGTCTGTG	$(GTT)_6$	51	197–239	30	3	0.467	0.389	−0.203*	19	3	0.474	0.397	−0.200*
Par_m (NED) (FJ560975)	F:AGAACATGACAACGACAACGA R:CGTACATTTGCGAAGCAAGA	$(CAA)_{29}$	49	172–337	25	12	0.480	0.809	0.412*	19	5	0.263	0.616	0.580*
Total mean						5	0.442	0.537	0.180*		5.6	0.479	0.483	0.075

N number of amplified individuals, N_A number of alleles, T annealing temperature, H_O observed heterozygosity, H_E expected heterozygosity, F_{IS} inbreeding coefficient

* significant departure of Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.05$). Forward primers are 5′ fluorescently labelled with VIC, NED, PET or 6-FAM (Applied Biosystems)

were the most abundant constituting 79% of the total, while the remaining were dinucleotides. Whenever there was enough flanking region, primers were designed with Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Finally, eight loci yielded good amplification and its allelic variation were assayed in a total of 50 individuals from two populations of *P. clavata*: Corsica Island, France (48°49′ N, 2°29′ E) ($N = 31$) and Cabrera Island, Spain (39°09′N, 2°57′E) ($N = 19$). Genomic DNA for each individual was extracted following Pascual et al. (1997). The forward primers of each locus were labelled with fluorescent dyes for screening (Table 1). Amplification reactions were performed in a final volume of 25 µl containing 10–30 ng of DNA, 0.25 µM of each primer, 0.25 mM of dNTP mix (Sigma–Aldrich), 1 U of Taq polymerase, 1× reaction buffer and 4 mM of $MgCl_2$ (all of them from Bioron). Samples were amplified in a thermocycler (MWG Primus 96) with an initial 8 min denaturation step at 95°C, and 35 amplification cycles: 60 s at 95°C, 90 s at the locus specific annealing temperature (Table 1) and 60 s at 72°C, followed by 5 min extra final extension at 72°C. The resulting amplified products, ranging from 147 to 356 bp, were visualised in a 1.6% agarose gel to assess amplification success before being genotyped using an ABI 3700 automated sequencer. Allele sizes were assigned with GeneMapper software (version 3.7) according to an internal size standard (LIZ 500, Applied Biosystems).

Statistical analyses were conducted using GENEPOP v.4 (Rousset 2008). No linkage disequilibrium was observed between pairs of loci after Bonferroni correction. All loci showed high amplification success and proved to be highly polymorphic, between 3 and 14 alleles were detected per locus (total mean ± SD = 5.625 ± 3.926). The inbreeding coefficient (F_{IS}) showed significant homozygote excess for locus Par_e from Corsica and Par_m from both populations. Results obtained with Micro-Checker v.2.2.3 (van Oosterhout et al. 2004) indicated that these populations are possibly at Hardy Weinberg equilibrium with signs of null alleles in these two loci (frequencies ranging between 0.174 and 0.210). Interestingly, significant heterozygote excess was found for loci Par_b, Par_g and Par_k in both populations. The fact that most loci presented high F_{IS} values either negative and positive is very intriguing and promising for understanding the mass mortality events that have recently affected this species. A combination of inbreeding and overdominant selection could explain the results observed in these populations and should be further investigated using these polymorphic loci. Significant genetic differentiation was observed between these two western Mediterranean islands ($F_{ST} = 0.0606$, $P < 0.001$). The results found in this study indicate that the microsatellites isolated for *P. clavata* are polymorphic and can be a useful tool to study connectivity between populations as well as genetic spatial structure within populations and local selection.

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