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TECHNICAL NOTE

Isolation and characterization of microsatellite loci from the endangered Mediterranean sponge *Spongia agaricina* (Demospongiae: Dictyoceratida)

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Abstract The abundance of the bath sponge *Spongia agaricina* has decreased drastically in recent years and it is now considered an endangered species under Annex 3 of Bern and Barcelona conventions. We describe eight microsatellite markers and present data on their allelic variation and utility as high resolution genetic markers. We analyzed 36 individuals from two populations and found that the number of alleles per locus ranged between 1 and 7. Observed heterozygosity ranged from 0 to 0.72. We found deviations from Hardy–Weinberg expectations for some loci. We exclusively detected null alleles for those loci that deviated from Hardy–Weinberg expectations. Also, distributions of allele frequencies differed significantly between the two populations, making them suitable for population genetic analyses.

Keywords Microsatellites · Population genetics · Porifera · Bath sponge · *Spongia agaricina*

Sponges are one of the most abundant and diverse sessile groups in marine communities worldwide (Sarà and Vacelet 1973). Sponges also are a critical component of the global hidden diversity hosting complex mixtures of microbial communities and natural products with a potential for biotechnological applications (Taylor et al. 2007). Despite their relevance in natural communities and the strong local and global stressors acting on their populations, sponges are underrepresented in ecological studies (Becerro 2008) including molecular ecology. In the Mediterranean, several sponges including the chemically rich and cytotoxic *Spongia agaricina* (Aiello et al. 1988) have been traditionally collected for domestic and ornamental use. As a consequence of overexploitation, habitat degradation, and some recent epidemic events, populations of this species have decreased drastically (Pronzato 1999). *S. agaricina* has been included among marine endangered species in the Annex 3 of Bern and Barcelona Conventions, for specific management requirements (Templado et al. 2004). A good understanding of the spatial genetic structure among and within populations and their connectivity is crucial to establish adequate management programs. Here, we report the isolation of eight microsatellite markers from the endangered Mediterranean sponge *S. agaricina* and preliminary data on their allelic variation in two distant populations.

Total genomic DNA was extracted using QIAamp DNA Minikit columns (QIAGEN) from a subsample of one specimen collected by scuba diving in Ceuta (Spain) 35°53′N 5°18′O. The DNA was used to construct a partial genomic microsatellite enriched library using the FIASCO protocol (Zane et al. 2002) and following the enrichment protocol from Kijas et al. (1994) using four 5′biotinylated probes $(AC)_{15}$, $(AG)_{15}$, $(CAA)_{10}$, and $(GATA)_7$. DNA enriched fragments where cloned using P-GEM®-T Easy Vector SytemII (Promega). Positive clones were screened using digoxigenin labeled probes following Estoup and Turgeon protocol (http://www.agroparistech.fr/svs/genere/microsat/microsat.htm).

We scored as positive 108 clones (out of 720 plated clones), from which 98 were purified and sequenced on an

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Table 1 Microsatellite markers isolated from *Spongia agaricina*

Locus (dye) (Accession no.)	Primer sequence F and R (5′–3′)	T_a (°C)	Repeat motif	Size range (bp)	CEUTA					PHARILLON					
					N	N_A	H_o	H_E	F_{is}	N	N_A	H_o	H_E	F_{is}	F_{st}
SAa (NED) (FJ771993)	F: TCGCTGGTGAACCAATACAA R: ACTGGTCGGTGTCAATAGCC	55	$(GT)_{27}$	205–248	17	4	0.2	0.72	0.7612*	17	1	0	0	–	0.454
SAc (PET) (FJ771994)	F: TACCAGAAAGTGCAGCCTCA R: ATCCCTAGTGTGCGTGTATG	51	$(CA)_7$	158–174	16	5	0.5	0.58	0.146	18	3	0.4	0.34	−0.144	0.071
SAd (PET) (FJ772000)	F: TTGAGGCATTGATTGGATTG R: CTGTCTGTCCGTGTGCTTGT	45–48	$(AC)_{16}$	136–175	0	0	0	0	–	15	2	0.3	0.33	0.200	–
SAf (PET) (FJ771995)	F: AAGGCCCACAATCGTGTAAG R: ATGTAGGGCACTTGCTGACC	55–58	$(CAA)_8$	305–316	18	5	0.7	0.79	0.098	18	2	0.6	0.44	−0.417	0.268
SAg (VIC) (FJ771996)	F: CATAACGATGCTGTCCATGC R: GCCGGCTGTTTAGTTACCTG	40–42	$(CA)_{11}$	205–222	18	4	0.6	0.59	−0.045	16	5	0.3	0.78	0.609*	0.259
SAj (PET) (FJ771997)	F: GAATTGAGACTGGCCAGAGG R: CATCTCCATGCATTCTCGTG	55–58	$(CAA)_6$	204–223	17	4	0.5	0.58	0.089	18	5	0.4	0.62	0.290	0.338
SAk (VIC) (FJ771998)	F: TGAATTTGAGGTTGTGGTCAA R: GTTAGTGTGTGTTCAATTAG	54	$(CA)_{14}$	255–273	11	5	0	0.77	1.000*	17	7	0.4	0.5	0.180	0.302
SAn (NED) (FJ771999)	F: TCCACTCACACCTACACCACA R: CTGAAATGGCAGCATCACTC	52–54	$(CAA)_9$	172–202	16	7	0.4	0.73	0.497*	18	5	0.2	0.63	0.653*	0.206
Total mean						4.25	0.36	0.595	0.356		3.75	0.33	0.53	0.270	0.278

T_a, annealing temperature; N, number of individuals amplified; N_A, number of alleles; H_o, observed heterozygosity; H_E, expected heterozygosity; F_{is}, inbreeding coefficient (*) significant departure of Hardy–Weinberg equilibrium; F_{st}, measure of population structure for the two populations studied

ABI Prism 3700 automated sequencer (Applied Biosystems) at the Scientific and Technical Services of the University of Barcelona. Twenty-two of them contained a complete microsatellite sequence, of which seven were imperfect, thirteen were perfect, and four were compound. Dinucleotide repeats were the most abundant (17), then trinucleotides (4), and just one was a tetranucleotide. Seventeen of these sequences with enough flanking region were used for primer design using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and preliminary tested for amplification and allele size variation. Some loci failed either in amplification (no amplification, wrong size or variable amplification patterns) or presented a pattern of multiples peaks preventing allele size determination. The present work focused on the eight loci that yielded a good amplification and allele size determination.

We assessed the allelic variation in 36 individuals from two populations of *S. agaricina*: Ceuta (Spain) 35°53′N 5°18′O and Pharillon (Marseille, France) 43°18′N 5°22′E. Genomic DNA for each individual was extracted following Pascual et al. (1997). Amplification reactions in a final volume of 20 µl consisted in 1 µl of DNA template, 2 µl of 10× Taq polymerase buffer, 4 mM of $MgCl_2$, 0.25 mM of dNTP, 0.25 µM of each primer, 350 ng/µl of Bovine Serum Albumin and 1U Taq polymerase (Bioron). The forward primer for each locus was labeled with fluorescent dyes (NED, VIC and PET from Applied Biosystems, Table 1). Polymerase Chain Reaction (PCR) was performed in a MWG primus thermocycler or a Mastercycler® Eppendorf. PCR parameters were 5 min denaturation at 95°C, followed by 30 amplification cycles of 1 min at 95°C, 30 s at locus specific annealing temperature (Table 1), and 30 s at 72°C, followed by an extension cycle of 4 min at 72°C. Resulting PCR products were then visualized on a 1.5% agarose gel stained in an aqueous ethidium bromide solution and were then genotyped using an ABI Prism 3,700 automated sequencer (Applied Biosystems) Allele size characterization was performed with an internal standard LIZ with GeneMapper® software (version 3.7, both from Applied Biosystems). Statistical analyses were conducted with GENEPOP 4.0 (Rousset 2008).

The number of alleles per locus ranged from 1 (in Pharillon where one allele is fixed at SAa) to 7. All individuals from Ceuta failed to amplify at the locus SAd, even after several amplification attempts. No significant linkage disequilibrium was detected between the eight loci in either population after Bonferroni corrections. Most loci were at Hardy–Weinberg equilibrium except for SAn in both populations, SAa and SAk in Ceuta, and SAg in Pharillon which showed significant heterozygote deficit ($P < 0.001$). Similar patterns of deviation from HWE due to a high heterozygote deficiency have been reported for various marine organisms (Duran et al. 2002; Perez-Portela et al. 2006). We exclusively found high frequencies of null allele for those loci with heterozygote deficit (frequencies ranging from 0.194 to 0.427). Also, populations of *S. agaricina* from Ceuta and Pharillon differed genetically ($F_{st} = 0.278$, $P < 0.001$). The high and significant F_{st} values, the presence of null and private alleles for some loci suggest that populations of *S. agaricina* are highly structured, which may hinder the capacity of this species to recover after local extinctions.

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References

Aiello A, Ciminiello P, Fattorusso E, Magno S (1988) 3-beta, 5-alpha-dihydroxy-6-beta-methoxycholest-7-enes from the marine sponge spongia-agaricina. J Nat Prod 51:999–1002. doi:10.1021/np50059a036

Becerro MA (2008) Quantitative trends in sponge ecology research. Mar Ecol (Berl) 9:167–177. doi:10.1111/j.1439-0485.2008.00234.x

Duran S, Pascual M, Estoup A, Turon X (2002) Polymorphic microsatellite loci in the sponge *Crambe crambe* (Porifera: Poecilosclerida) and their variation in two distant populations. Mol Ecol Notes 2:478–480. doi:10.1046/j.1471-8286.2002.00285.x

Kijas JMH, Fowler JCS, Garbett CA, Thomas MR (1994) Enrichment of microsatellites from the *Citrus* genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. Biotech 16:657–661

Pascual M, Balanyà J, Latorre A, Serra L (1997) Analysis of the variability of *Drosophila azteca* and *Drosophila athabasca* populations revealed by random amplified polymorphic DNA. J Zool Syst Evol Res 35:159–164

Perez-Portela R, Duran S, Estoup A, Turon X (2006) Polymorphic microsatellite loci isolated from the Atlanto-Mediterranean colonian ascidian *Pycnoclavella* sp. (Ascidiacea, Tunicata). Mol Ecol Notes 6:518–520. doi:10.1111/j.1471-8286.2006.01304.x

Pronzato R (1999) Sponge-fishing, disease and farming in the Mediterranean sea. Aquat Conserv: Mar Freshw Ecosyst 9:485–493. doi:10.1002/(SICI)1099-0755(199909/10)9:5<485::AID-AQC362>3.0.CO;2-N

Rousset F (2008) GENEPOP 2007 a complete re-implementation of the GENEPOP software for Windows and Linux. Mol Ecol Resour 8:103–106. doi:10.1111/j.1471-8286.2007.01931.x

Sarà M, Vacelet J (1973) Ecologie des Demosponges. In: Grasse PP (ed) Traite de zoologie (anatomie, systematique, biologie). Masson, Paris, pp 462–576

Taylor MW, Radax R, Steger D, Wagner M (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. Microb and Mol Biol rev 71:295–347

Templado J, Calvo M, Garvía A, Luque AA, Maldonado M, Moro L (2004) In Guía de invertebrados y peces marinos protegidos por la legislación nacional e internacional. Ministerio de Medio Ambiente, Serie técnica, Madrid, p 214

Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. Mol Ecol 11:1–16. doi:10.1046/j.0962-1083.2001.01418.x