

Novel polymorphic microsatellite markers developed for a common reef sponge, *Stylissa carteri*

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Abstract Despite the ubiquitous role sponges play in reef ecosystem dynamics, little is known about population-level connectivity in these organisms. The general field of population genetics in sponges remains in its infancy. To date, microsatellite markers have only been developed for few sponge species and no sponge population genetics studies using microsatellites have been conducted in the Red Sea. Here, with the use of next-generation sequencing, we characterize 12 novel polymorphic loci for the common reef sponge, *Stylissa carteri*. The number of alleles per loci ranged between three and eight. Observed heterozygosity frequencies (H_o) ranged from 0.125 to 0.870, whereas expected (H_e) heterozygosity frequencies ranged from 0.119 to 0.812. Only one locus showed consistent deviations from Hardy-Weinberg equilibrium (HWE) in both populations and two loci consistently showed the possible presence of null alleles. No significant linkage disequilibrium was detected for any pairs of loci. These microsatellites

will be of use for numerous ecological studies focused on this common and abundant sponge.

Keywords Microsatellites · Sponges · Population genetics · Connectivity · *Stylissa carteri*

Introduction

In the field of benthic reef ecology, considerable effort has been made to study coral population dynamics (Van Oppen and Gates 2006; Davies et al. 2012), while sponge ecology and population genetics remain in their infancy (Becerro 2008; Uriz and Turon 2012). Sponges are among the most diverse and ecologically important benthic organisms to reefs (Sarà and Vacelet 1973; Uriz and Turon 2012). Sponges are important to reef ecosystems as primary producers and bioeroders (Rützler 1975; Diaz and Rützler 2001). In addition, sponges increase overall habitat availability for reef microfauna (Beaulieu 2001; Rützler 2004; Henkel and Pawlik 2011). They remove organic matter from the reef and increase the cycling rate of carbon and important nutrients (Reiswig 1971; Pile et al. 1997; Diaz and Rützler 2001; Jiménez and Ribes 2007). Furthermore, having arisen in the Precambrian era (Finks 1970; Li et al. 1998), sponges are among the most ancient metazoans, making them target organisms for evolutionary studies on such varied topics as speciation, cell and tissue development, and the origin of the immune system (Schütze et al. 1999; Müller and Müller 2003; Srivastava et al. 2010). Lastly, sponges produce important secondary metabolites for possible pharmaceutical applications (Munro et al. 1999; Mayer et al. 2010). Our understanding of the basics of sponge population ecology and genetics is thus crucial to understanding how sponge populations function, how they evolve, and how resilient they can be to perturbations that affect reef ecosystems. Like many benthic reef organisms,

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sponges have a complex life history with a juvenile larval stage and a sedentary adult stage. While a given sponge species might reproduce both sexually and asexually, sponge populations rely heavily on larval dispersal for genetic exchange (Maldonado and Uriz 1999; Maldonado 2006). Incorporating knowledge of genetic connectivity in sponges into the design of marine protected areas will be crucial for developing conservation schemes that will maintain ecosystem stability and diversity (McCook et al. 2009; Arrieta et al. 2010).

To broaden our knowledge of population genetics in sponges, we developed 12 new polymorphic microsatellite markers for a common reef sponge, *Stylissa carteri* (Table 1). *S. carteri* has a widespread distribution (Hooper and van Soest 2002) and has been found to be abundant in coastal Red Sea reefs typically between 5 and 15 m in depth (M.L.B. and E.C. G., unpublished data). As a member of the family Axinellidae, *Stylissa carteri* has indirect and external larval development (Maldonado 2006), and the morphology of the larva is most likely of the parenchymella form (Ereskovsky 2010). To date, genetic resources for sponges are extremely limited. For instance, microsatellite markers have only been characterized for eight species not including *Stylissa carteri* (Duran et al. 2002; Knowlton et al. 2003; Blanquer et al. 2005; Hoshino and Fujita 2006; Noyer et al. 2009; Dailianis and Tsigenopoulos 2010; Gigliarelli et al. 2010; Guardiola et al. 2011). The purpose of this study was to identify polymorphic loci that are informative for determining the population structure, connectivity patterns, and possible reproductive capabilities of *S. carteri*.

Methods

Sample collection, DNA extraction and sequencing

Samples of *Stylissa carteri* were collected by SCUBA at Fsar Reef (22°23'N; 39°03'E), $n=24$, and Abu Shoosha Reef (23°305'N; 39°049'E), $n=24$, in the central Red Sea at a depth between 5 and 15 m. Samples were collected in November 2010, June 2012, and August 2012 and immediately placed in 70 % ethanol and calcium-magnesium-free artificial seawater (CMF-ASW) upon surfacing. The sample taxonomy was verified as *S. carteri* by Nicole de Voogd at The Netherlands Centre for Biodiversity (NCB) Naturalis.

The total holobiont DNA was extracted from 200–250 mg sponge tissue with an initial cell lysis step where the tissue was immersed in 600 μ l of 1 % 2-mercaptoethanol RLT buffer (Qiagen) and shaken using a FastPrep Instrument (MP Biomedicals). Following cell lysis, the DNA extraction method proceeded via the Allprep DNA/RNA mini Kit (Qiagen). The quality and quantity of

extracted DNA was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific).

Next-generation sequencing was done for one *S. carteri* sample using a Roche 454 GS-FLX (titanium) sequencer at the King Abdullah University of Science and Technology Bioscience Core Lab. Over 2 million reads with an average length of 230 bp were generated. Raw, unassembled reads were used to search for putative microsatellite loci.

Characterisation of microsatellite loci and PCR optimization

Microsatellites were mined from the generated 454 data using the software MSATCOMMANDER v 1.0.8 (Faircloth 2008) with Primer3 plug-in (Rozen and Skaletsky 2000). MSATCOMMANDER default settings were used for screening reads for perfect, dinucleotide motifs. A total of 2,417 putative microsatellite loci were obtained and 96 were selected for polymerase chain reaction (PCR) trials. PCR reactions were set up following protocols associated with the Multiplex PCR kit (QIAGEN). Primers were tested at annealing temperatures ranging from 55 to 63 °C. PCR reaction volume was 10 μ l, consisting of 5 μ l Multiplex Mix (Qiagen), 1 μ l primers (2 μ M), 3 μ l water, and 1 μ l genomic DNA (30–100 ng/ μ l). PCRs were performed using an Applied Biosystems GeneAmp PCR 9700 system using the following parameters: 15 min at 95 °C, followed by 30 cycles of 30 s at 94 °C, 90 s at 55–63 °C, 90 s at 72 °C and a final extension of 72 °C for 10 min. The 96 designed primer pairs were first tested on eight samples to identify polymorphic loci. PCR products were run on a QIAxcel genetic analyzer (Qiagen) using a high-resolution cartridge to check for primer specificity (clean bands) and to identify polymorphic loci. Of the 96 loci tested, 31 showed clear unambiguous and polymorphic PCR bands. Primers for these loci were labeled with ABI fluorescent tags (6-FAM, PET, VIC, and NED) and PCRs were performed as described previously for all 48 samples. Diluted PCR products were mixed with Hi-Di formamide (Applied Biosystems) and GeneScan 500-LIZ size standard (Applied Biosystems) and run on an ABI 3730xl genetic analyser (Applied Biosystems). Microsatellite loci were scored using the software GENEMAPPER 4.0 (Applied Biosystems).

Data analysis

Allelic frequencies, number of alleles (N_a), observed (H_o) and expected heterozygosities (H_e) were estimated for each reef using the software Genalex v 6.5. We tested for the presence of linkage disequilibrium and deviations from Hardy-Weinberg proportions using Genepop (Raymond and Rousset 1995; Rousset 2008). Genepop was also used to calculate F_{is} using the Weir and Cockerham estimate

Table 1 Microsatellite markers isolated from *Stylyssa carteri*. Presented here are the 12 polymorphic microsatellite markers isolated for *Stylyssa carteri* from two populations, Fsar and Abu Shoosha

Locus (accession no.)	Primer sequence F and R (5'-3')	Ta (°C)	Repeat motif	Size range (bp)	FSAR					ABU SHOOSHA						
					N	Na	Ho	He	Fis	p value	N	Na	Ho	He	Fis	p value
sc58 (KC315709)	F: TGTGGCTTCTGTCTATGCC R: CTAGCGTTTGACTCATGCCG	57	(AC) ₁₄	90–112	24	7	0.333	0.576	0.439	0.001	24	8	0.542	0.707	0.254	0.006
sc65 (KC315719)	F: CGTAGAAAGTCTGCCCAAATG R: ACTCGGCCGTAAGTCATAGG	57	(TG) ₁₅	184–232	24	8	0.667	0.619	-0.056	0.782	24	7	0.583	0.604	0.056	0.071
sc72 (KC315710)	F: CATGGCATGCTTCACCTGAG R: TCAAGTGCCGTTGTCCCTCTTC	60	(AC) ₁₃	147–159	24	5	0.208	0.194	-0.050	1.000	22	4	0.565	0.753	0.270	0.001
sc88 (KC315711)	F: AGTCAGGGATGTGGCTAGAG R: AGGAAGTAGCCAAACGGAGAC	60	(AC) ₁₂	190–224	24	6	0.792	0.744	-0.043	0.038	23	8	0.167	0.419	0.616	0.005
sc90 (KC315712)	F: CACACTCATGATCACACGCG R: ACATTCGCTACACTTGTGGG	60	(AC) ₁₂	108–130	24	5	0.167	0.415	0.612	0.000	24	5	0.591	0.741	0.224	0.006
sc56 (KC315715)	F: CCATCTCAGATCCCTCTGG R: TCGGTGACCCCTTCTCTGTC	60	(TG) ₁₅	122–136	22	5	0.273	0.525	0.498	0.017	22	6	0.591	0.741	0.224	0.006
sc75 (KC315716)	F: CAACAGAGTAGCGCAACACC R: AGTTGACCACCTGCACAGAG	60	(AC) ₁₃	93–133	24	9	0.167	0.608	0.735	0.000	23	7	0.174	0.557	0.699	0.000
sc64 (KC315717)	F: CAGGTCATGCTTTACACGGC R: TGTGTGGGAGTCTGTAGGG	60	(AC) ₁₄	128–142	24	6	0.667	0.778	0.163	0.012	23	7	0.870	0.812	-0.049	0.672
sc85 (KC315720)	F: CTGGATGTGACTTACGGCAC R: CCTCAACAGCAAGCTTTGG	60	(AG) ₁₂	142–158	24	5	0.458	0.662	0.327	0.023	24	6	0.375	0.737	0.507	0.000
sc83 (KC315713)	F: GAACCTGCAGGAAGGTGAGTG R: ACGTGAGGGAAGACAGTGAC	60	(AC) ₁₂	173–189	20	6	0.750	0.776	0.059	0.099	24	6	0.833	0.755	-0.082	0.798
sc82 (KC315714)	F: GTAAGTTGGTGAGCGTGATGG R: TACCGCCTCAAATGGCAAC	63	(TG) ₁₃	152–162	24	5	0.583	0.702	0.189	0.079	24	5	0.333	0.685	0.529	0.000
sc96 (KC315718)	F: AGTGTGCCCTTCTCTCAAG R: ACCCTACAGTCACATGCACC	63	(TG) ₁₁	190–226	24	3	0.125	0.119	-0.030	1.000	24	8	0.167	0.301	0.464	0.000

Significant *p* values after Bonferroni corrections ($p < 0.004$) are shown in *italic*

Ta Annealing temperature, N number of individuals, Na number of alleles, Ho observed heterozygosity, He expected heterozygosity, Fis inbreeding coefficient—Genepop W&C

(Weir and Cockerham 1984). MICROCHECKER (Van Oosterhout et al. 2004) was used to verify if deviations from HWE could be explained by the presence of null alleles.

Results and discussion

Of the 31 primer pairs that were used to amplify all 48 samples, only 12 yielded clear fragment peaks that could be reliably scored and amplified in all samples. An average of 73.5 alleles (3–8 per loci/population) was found per population analyzed. None of the samples that were genotyped shared the same multilocus genotype, indicating the absence of clones within our samples. Observed heterozygosity frequencies (H_o) ranged from 0.125 to 0.870, while expected (H_e) heterozygosity frequencies ranged from 0.119 to 0.812. Results from Genepop suggested that a total of seven loci deviated from Hardy-Weinberg equilibrium (HWE) after Bonferroni correction for at least one population (Fsar: sc58, sc90, sc75; Abu Shosha: sc72, sc75, sc85, sc82, sc96). However, only one locus (sc75) deviated from HWE in both populations ($p_{\text{Fsar}} < 0.001$, $p_{\text{Abushosha}} < 0.001$). Results from MICROCHECKER showed the possible presence of null alleles for seven loci (Fsar: sc58, sc90, sc56, sc75; Abu Shoosha: sc90, sc75, sc85, sc82, sc96). Yet, only two of these loci showed consistent evidence for null alleles in both populations (sc90 and sc75). No significant linkage disequilibrium was detected for any pairs of loci ($\alpha = 0.05$).

While it is noted that several markers developed here showed deviations from HWE, the disparity of these deviations among populations in all but one locus (sc75) suggests that other contributing factors different from null alleles might be the cause of this phenomena. Deviations from HWE linked to reduced heterozygosities seem to be rather common in other sponge species (Duran et al. 2004a; Whalan et al. 2005; Duran and Rützler 2006; Noyer 2010; Guardiola et al. 2011; Uriz and Turon 2012), and might be a consequence of limited dispersal leading to inbreeding, the presence of clonality, or both, rather than the presence of null alleles. Overall, it seems that heterozygote deficits might be a natural trend of sponges and, in the case of this study, it does not seem to be an artifact due to primer binding errors. However, further studies are warranted to test these hypotheses in *S. carteri*. The markers described in this study will certainly provide a valuable tool for this purpose.

Conclusion

The 12 microsatellite markers developed here will make useful contributions to the future studies of sponge population genetics. Until now, microsatellites have only been developed for eight species within all the Porifera (Duran

et al. 2002; Knowlton et al. 2003; Blanquer et al. 2005; Hoshino and Fujita 2006; Noyer et al. 2009; Dailianis and Tsigenopoulos 2010; Gigliarelli et al. 2010; Guardiola et al. 2011) and have only been used for population-wide analyses in few species (Duran et al. 2004a; Calderón et al. 2007; Hoshino et al. 2008; Blanquer et al. 2009; Blanquer and Uriz 2010, 2011; Noyer 2010; Dailianis et al. 2011; Guardiola et al. 2011). The highly variable markers presented herein will be useful for determining fine scale variability between sponge populations and tools for determining species identification for cryptic taxa within this group. Due to strong spatial structure reported in some sponges (Duran et al. 2004b; Blanquer and Uriz 2010), microsatellite markers will be especially important for making strong ecological and evolutionary inferences.

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