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PRIMER NOTE

Development of species-specific markers in an organism with endosymbionts: microsatellites in the scleractinian coral *Seriatopora hystrix*

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Abstract

We report on the development of microsatellites in *Seriatopora hystrix*, a coral with algal endosymbionts. In order to obtain a genomic library free of algal DNA, we conducted a whole genome preamplification from minute amounts of symbiont-free tissue. The resulting fragments were cloned into pUC18, and *Escherichia coli* were transformed with the recombinant plasmids. Twenty-nine microsatellites were isolated from a library screen with a fluorescently labelled $(CA)_{15}$ probe. Five of these yielded reliable polymorphic markers.

Keywords: clonal structure, Cnidaria, corals, genome amplification, microsatellites, *Seriatopora hystrix*

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Reef-building corals (Scleractinia) show an extraordinary diversity of reproductive strategies. Various combinations of sexual and asexual modes of propagation as well as variation in dispersal capabilities within each mode have been documented (Harrison & Wallace 1990). Abundant intraspecific variation allows for direct functional analyses of these key life history traits. A good candidate for such analyses is the widely distributed species *Seriatopora hystrix* (Ayre & Hughes 2000). In order to obtain a set of highly variable single-locus markers for a detailed investigation of its reproductive strategies, we have set out to develop microsatellite loci in *S. hystrix*.

The isolation of molecular markers in corals is hampered by the presence of algal endosymbionts (zooxanthellae). However, if minute amounts of symbiont-free tissue can be obtained, then DNA fragments suitable for the construction of a genomic library can be generated via a whole genome amplification. In a so-called degenerate oligonucleotide primed–polymerase chain reaction (DOP–PCR), we used DOPs (Telenius *et al.* 1992), which include an *Xho*I restriction site that facilitates subsequent cloning of fragments.

Under a binocular microscope (40× magnification), we carefully removed all algal cells from a small piece of fresh *S. hystrix* tissue (100–200 μm²). The absence of symbionts was confirmed at 400× magnification. DNA was extracted from the tissue sample by incubation in 20 μL DNAzol™ and 1 μL Polyacrylcarrier (Molecular Research Center, Inc.) for 10 min at room temperature and precipitated with 50 μL 100% ethanol. After washes in 95% and 70% ethanol, the DNA was air-dried and resuspended in 10 μL H_2O. The genome amplification reaction was set up in a 50-μL volume as follows: 2.0 μL template, 100 pmol of DOPs, 5 μL dNTP (2 mmol/nucleotide), 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 9.0, and 2.5 U *Taq* polymerase (Pharmacia rTaq). The following thermal cycling protocol was used (Hybaid Touchdown™ thermocycler): one cycle at 94 °C for 10 min, eight cycles of 93 °C for 60 s, 30 °C for 90 s and 72 °C for 3 min, then 28 cycles of 93 °C for 60 s, 58 °C for 60 s and 72 °C for 3 min. The polymerase was added after the initial denaturing step. A control reaction without template DNA was run in parallel.

On an agarose gel, the genome amplification generated a thick smear of DNA fragments mainly from 200 to several thousand base pairs (bp), whereas the control reaction showed only a very faint background shadow. *S. hystrix* fragments longer than 300 bp were purified (Qiaquick Gel Extraction Kit, Qiagen), digested to completion with *Xho*I (Gibco), and ligated overnight at 22 °C into *Sal*I-digested, dephosphorylated pUC18 (Appligene). The recombinant

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Table 1 Description of five microsatellite loci developed in *Seriatopora hystrix*

Locus (size of cloned insert, bp)	Primer sequences* (5′–3′)	GenBank accession no.	Repeat motif	T_a† (°C)	No. of genotyped colonies	No. of alleles	Allele size range	H_O	H_E
Sh3.13 (446)	CGTTTATTCCAATCATGAAGC CAGGCAGGCAACTTAACCTC	AF320765	$(CA)_4CGTA\ (CA)_8$	55	69	3	196–204	0.11	0.25
Sh3.32 (539)	CCAAAACCCTGCATTTTGAG* CCCCCTGTAAAAGTGTACCC	AF320766	$(CA)_{20}$	55 (52)	81	9	226–274	0.10	0.56
Sh3.39 (458)	AGTGAAAACACAGCACGTCG* ATTGTTCACACAATGGCGTG	AF320767	$(CA)_{12}$	59 (52)	98	4	217–229	0.42	0.58
Sh4.3 (647)	GGCTAGGATTGCACCAAGTG* CTGCCCCAAACTAAAAGCTG	AF320768	$(CA)_{12}$	60 (54)	31	3	188–200	0.22	0.50
Sh4.28 (140)	TGTGGTCTACAGTATATCTTTTGTG* CAAATTTGAATCTACAGTGGGG	AF320769	$(CA)_{11}$	55 (52)	94	6	102–116	0.47	0.55

*The oligonucleotides marked with *were Cy5-labelled for analysis on an automated sequencer.
†Annealing temperatures in parentheses apply to amplifications with Cy5-labelled primers. Optimal $MgCl_2$ concentrations were 1.5 mmol for loci Sh3.32, Sh3.39 and Sh4.3 and 2.5 mmol for loci Sh3.13 and Sh4.28.

plasmids were used to transform competent *Escherichia coli* (TOP10 One Shot™, Invitrogen), and the culture was grown overnight on selective agar plates.

Colonies were lifted onto nylon membranes, where DNA was immobilized by baking for 2 h at 80 °C. We screened the library with a fluorescently labelled $(CA)_{15}$ probe (ECL 3′-oligolabelling and detection system, Amersham). Hybridization was conducted overnight in a rotary oven at 52 °C. In all subsequent steps of the screen, we followed the manufacturer's instructions, except for the second stringency wash, which we performed in 6× SSC and 0.1% SDS. Colonies that gave a signal on the autoradiograph were streaked out in replicate onto fresh agar plates and subjected to a secondary screen. Plasmid DNA was extracted from overnight cultures of all positives of this second screen (42 clones). Thirty-seven clones were sequenced (ALFexpress™ AutoRead™ Sequencing Kit) and fragments were separated on an automated sequencer (ALFexpress, Pharmacia). We found 29 microsatellites (> 3 repeats). Primers were designed with PRIMER vs. 0.5 (Lincoln *et al*. 1991; Table 1) for nine loci with long repeats [> $(CA)_{10}$] and sufficient flanking sequence.

After some PCR optimization, all nine loci amplified reliably. PCR reactions were carried out in a total volume of 30 µL with 1 µL template (DNA extracted as above), 10 pmol of each primer, 3 µL dNTP (2 mmol/nucleotide), 50 mM KCl, 1.5–2.5 mM $MgCl_2$ (cf. Table 1), 10 mM Tris-HCl pH 9.0 and 0.5 U *Taq* polymerase. The cycling protocol was as follows: one cycle at 95 °C for 3 min followed by 35 cycles at 94 °C for 15 s, 52–60 °C for 30 s (cf. Table 1) and 72 °C for 30 s. In four loci, allelic variation was analysed by using fluorescently labelled primers and separating fragments on an ALFexpress sequencer. For the fifth locus (Sh3.13), electrophoresis was carried out through 10% polyacrylamide gels, and bands were visualized with silver staining. PCR reactions with DNA of *Pocillopora damicornis*, a close relative, did not yield any product. As amplifications of *S. hystrix* from the same aquarium (i.e. a shared pool of zooxanthellae) were successful, these results are in accordance with a species-specific coral origin of our primers. Also, tests with primers Sh3.32 and Sh4.28 failed to amplify DNA from a pure zooxanthellae culture. We genotyped samples of *S. hystrix* colonies from five different sites near Dahab (Gulf of Aquaba, Red Sea, Egypt) located within a 14-km stretch of coastline. In the field, roughly 1 cm long, fresh coral fragments were quickly blow-dried and then stored at ambient temperature until further processing.

Between three and nine alleles were detected per locus. All loci show some heterozygote deficit. At this point, we cannot rule out that null alleles are contributing to this pattern. Likely additional explanations are deviations from the Wright–Fisher model in the form of population subdivision and clonal replication through asexual reproduction. Larger sample sizes are needed to rigorously test these hypotheses.

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