

(JMC48 and JMC82, data not shown) while some showed low variation in species A and B (Table 2). Most loci had lower observed heterozygosity than expected under HW equilibrium (Table 2) and significant inbreeding coefficients (F_{STAT} ; Goudet 2002; randomization tests). In fact, pooled loci F_{IS} values were significant for all three species (randomization tests, all $P < 0.001$): 0.31 for *Sycoscapter* A, 0.24 for *Sycoscapter* B and 0.29 for *Sycoscapter* C. Fig-pollinating wasps are noted for their extreme inbreeding, and it is common for all matings in a patch to be between the offspring of a single foundress mother (e.g. Molbo *et al.* 2004). Our unpublished data for *Sycoscapter* nonpollinating wasps reveal similar extreme levels of inbreeding, which cause strong deviation from HW expectations and LD.

These are the first microsatellite loci developed for any of the many thousands of nonpollinating fig wasp species (Cook & Rasplus 2003) and provide valuable tools for evolutionary and population genetics studies of *Sycoscapter* fig wasps.

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Isolation and characterization of microsatellite markers in the Serra Spanish mackerel, *Scomberomorus brasiliensis*

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Abstract

Thirteen nuclear-encoded microsatellites from a genomic DNA library of Serra Spanish mackerel, *Scomberomorus brasiliensis*, were isolated and characterized. The microsatellites include 10 perfect repeats (eight tetranucleotide and two dinucleotide) and three imperfect repeats (two tetranucleotide and one dinucleotide). An additional five microsatellites, isolated originally from two congeneric species (*S. cavalla* and *S. niphonius*), were characterized in *S. brasiliensis*. Serra Spanish mackerel support artisanal fisheries along the Caribbean and Atlantic coasts of Central and South America, from Belize to Brazil.

Keywords: microsatellites, Serra Spanish mackerel

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The Serra Spanish mackerel, *Scomberomorus brasiliensis*, is distributed along the Caribbean and Atlantic coasts of Central and South America (Collete *et al.* 1978) and is an important component of artisanal fisheries in the region (Lucena *et al.* 2004; Shing 2005). Here, we report development of polymerase chain reaction (PCR) primers for 13 nuclear-encoded microsatellites from an *S. brasiliensis* genomic DNA library. Additionally, primers for five microsatellites, developed originally from *Scomberomorus cavalla* (Gold *et al.* 2002) and *Scomberomorus niphonius* (Yokoyama *et al.* 2006), were characterized in *S. brasiliensis*. Nuclear-encoded microsatellites are well suited for population-genetic analysis and identification of stocks that cross political boundaries (Ward 2000).

An enriched microsatellite library was generated using a protocol modified from Bloor *et al.* (2001). Whole genomic DNA was extracted from muscle tissue of a single individual, using a DNEasy Blood and Tissue Kit (QIAGEN); two separate digestions were performed with *Hae*III and *Alu*I (New England BioLabs). Genomic DNA fragments from both digestions were combined together and adaptors attached with T4 DNA Ligase (Promega). Adaptors were prepared beforehand by annealing two complementary oligonucleotide fragments, Oligo A (5'-CTCTTGCTACGCGTGGACTA-3') and Oligo B (5'-PO4-TAGTCCACGCGTAAGCAAGAGCACA-3'). Adaptor/DNA ligation was visualized on a 2% agarose gel (with ethidium bromide staining) and fragments ranging in size from 500 to 1500 bp were excised and cleaned with a QIAquick Gel Extraction Kit (QIAGEN). Size-selected DNA fragments were amplified with polymerase chain reaction (PCR) that employed 25 pM Oligo A as the primer and the following protocol: initial denaturation at 95 °C for 5 min; 31 cycles with denaturation at 95 °C for 50 s, annealing at 54 °C for 1 min, and extension at 72 °C for 2 min; and a final extension at 72 °C for 10 min. A portion of the PCR product was visualized on a 2% agarose gel (with ethidium bromide staining) to verify success of adaptor ligation to genomic DNA fragments.

Amplified, size-selected DNA was hybridized in separate reactions with two 5'-biotin-modified oligonucleotides: (GACA)₉ at 62 °C and (GATA)₉ at 51 °C. Each hybridization mix was then incubated with streptavidin-coated magnetic M-280 Dynabeads (Invitrogen) at room temperature for 30 min and rinsed with the following series of washes to remove non-target DNA fragments: twice with 200 µL of 1× W/B Buffer (1 M NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA) at room temperature; twice with 200 µL of 6× SSC (0.9 M NaCl, 0.09 M sodium citrate) at room temperature with 10-min incubations; twice with 200 µL of 3× SSC (0.45 M NaCl, 0.045 M sodium citrate) at hybridization temperature with 15-min incubations; twice with 200 µL of 6× SSC at room temperature with 10-min incubations; twice with 100 µL of PCR-grade water at room temperature. Microsatellite-containing DNA fragments were eluted at 98 °C with 100 µL

of PCR-grade water. The quantity of enriched DNA was increased with PCR, using 25 pM Oligo A as the primer and the same protocol as above; PCR products were cleaned with QIAquick PCR Purification Kits (QIAGEN).

Cleaned PCR products were ligated into pGEM-T vector (Promega), using T4 DNA Ligase (Promega), and transformed into *Escherichia coli* (JM109 High Efficiency Competent Cells, Promega). Transformed cells were plated on X-Gal/IPTG Luria-Bertani (LB) agar with 100 µg/mL of ampicillin and grown overnight at 37 °C. Positive (white) colonies were picked with sterile toothpicks, placed in 96-well PCR plates with LB broth (containing 100 µg/mL of ampicillin), and incubated at 37 °C overnight to increase density of the cultures. Clones were PCR-screened for microsatellites, using 20 pM Oligo A, 10 pM of the microsatellite oligonucleotide, and the following PCR protocol: an initial denaturation at 95 °C for 3 min; 38 cycles with denaturation at 95 °C for 30 s; annealing [(GACA)₉ at 54 °C and (GATA)₉ at 51 °C] for 45 s; extension at 72 °C for 1 min; and final extension at 72 °C for 10 min. PCR products were visualized on 2% agarose gels (with ethidium bromide staining); negative colonies were represented by a single band (only the entire genomic DNA insert), whereas positive colonies were represented by one or more additional bands, indicating presence of the desired microsatellite motif.

Colonies identified as positive by PCR screening were used to inoculate 1 mL cultures of LB broth selective media (ampicillin) and incubated overnight at 37 °C. Plasmid DNA was isolated (alkaline lysis) with a BioRobot 8000 (QIAGEN). Miniprep DNA was quantified, normalized, and both strands sequenced, using M13 forward and reverse sequencing primers and ABI BigDye Terminator version 3.1. Products were amplified and electrophoresed on an ABI 3100 DNA Analyser (Applied Biosystems). Phred (CodonCode) was used for DNA sequence base-calling and vector-trimming. A total of 192 sequences were checked for similarities using Sequencher (Gene Codes Corporation); more than 70% of clones shared partial complementarity with one other clone. These 'chimeric' clones presumably were generated during one or both of the PCR protocols employed prior to cloning of genomic DNA (Weising *et al.* 2005) and are possibly correlated to the number of cycles used in the PCR protocols. A total of 36 PCR primer pairs flanking tetranucleotide and dinucleotide microsatellite arrays were designed using Primer 3 (<http://frodo.wi.mit.edu/>).

Unlabelled PCR primers for all 36 primer pairs developed here and for 13 primer pairs developed previously for other species of *Scomberomorus* (Gold *et al.* 2002; Yokoyama *et al.* 2006) were purchased from Integrated DNA Technologies (IDT) and tested for amplification in *S. brasiliensis* by screening nine individuals obtained from the southern coast of Trinidad. PCR amplifications were performed in 10 µL reaction volumes containing 1 µL (~100 ng) DNA,

Table 1 Summary data for microsatellites (Msat) characterized for a total of 41 individual Serra Spanish mackerel, *Scomberomorus brasiliensis*

Msat	Primer sequence (5'–3')	GenBank Accession no.	Repeat sequence	Clone size	T_a	N_A	Size range	H_E/H_O	P_{HW}
<i>Sbr6</i>	GTGGTGTGTGTGTGCTGATG (F) AAAGTGGCAGGACACACAAAC	EU426557	(TAGA) ₁₅	244	55	16	217–277	0.931/0.927	0.258
<i>Sbr9</i>	GTCCTTAAACGGAAGTGGGTT (F) GGAGCAGCGTACTACTTTGT	EU426558	(GATA) ₁₀	310	50	10	299–335	0.856/0.878	0.265
<i>Sbr11</i>	CGACCTGTTGCCATCTCATAG (F) CTGACATCACTTTGTTGCCTTG	EU426559	(CAGA) ₁₃	153	53	35	124–296	0.978/0.951	0.078
<i>Sbr14</i>	AAGCCCTGTGCTGCATATTTA (H) AACCTTGTGTGTCAGTGGGAGTG	EU426560	(TAGA) ₂₄	280	52	22	200–308	0.950/0.927	0.306
<i>Sbr16</i>	ACCAACCTGTAACCTGGCCTCT (F) AGCAGGTGTGTGATGAACCTC	EU426561	(CAGA) ₁₄	287	49	12	250–290	0.874/0.854	0.324
<i>Sbr18</i>	TCTGTGTCAGTATGTGAATG (F) TTTGTCCCTCTCCCTAATGCT	EU426562	(GACA) ₆ (GATA) ₁₂	285	50	20	286–390	0.942/0.951	0.789
<i>Sbr19</i>	AGCATTAGGGAGAGGGACAAA (F) AATGGCTGTCTGCATGGTTAC	EU426563	(CA) ₁₀ 2 bp(CA) ₁₄	161	55	17	129–169	0.892/0.927	0.240
<i>Sbr20</i>	GAGGCAAACTGTCTATTGTCAT (H) TCCGATGGAAACATTTAAAGAC	EU426564	(GATA) ₁₁	191	55	23	128–252	0.945/0.561	<u>0.000</u>
<i>Sbr24</i>	ATTTTGATGTTTCGCCATGAA (H) GCCCACCAACAACTGCTATTA	EU426565	(CAGA) ₈	172	55	7	168–192	0.501/0.512	0.748
<i>Sbr26</i>	TACCTGAAAGGCATCCACAC (H) ATTCAGCACTGATGTGTGTC	EU426566	(CA) ₁₅	269	55	14	257–283	0.889/0.927	0.498
<i>Sbr28</i>	TGGCTGATTTTGTGTGATGTTG (H) TGCAGATTCTACAGGCGAGAT	EU426567	(GATA) ₆	321	52	5	323–343	0.342/0.390	1.000
<i>Sbr35</i>	TGCTCCCTCTGCTTCTGTAAC (H) ATGGTACACACATGCACCTCA	EU426568	(CA) ₁₄	121	55	15	109–147	0.889/0.683	<u>0.001</u>
<i>Sbr36</i>	CCTGCTAGGCAGTGCAATATC (H) TCACCCCTAAGAGAGGGAGAGG	EU426569	(GATA) ₅ 4 bp(GATA) ₂	151	55	7	139–163	0.725/0.854	0.435
<i>Sca49</i>	AGATGTGACAACAGTGGG (H) ATGGCAGCAGTAATAAAG		(TG) ₁₇ *		49	3	150–166	0.297/0.244	0.209
<i>Sca61</i>	GGTACTGTCCGGAGAATGAGAT (H) TGAATTTTATATGGAGGGTCTG		(CA) ₆ 4 bp(CA) ₈ *		49	3	212–216	0.118/0.122	1.000
<i>Sni13</i>	AAATGCTCTCAGCTGCCTGGT (F) TCCTGATGAGAATGCATGGAC	AB208740**	(CA) ₁₂ (CA) ₆ **		52	4	108–114	0.245/0.244	0.528
<i>Sni26</i>	AACAACCTCTTAGGAGCTGACG (F) GATCGCTCTCAGATACATGCC	AB208743**	(CA) ₁₅ **		49	13	112–138	0.874/0.854	0.918
<i>Sni29</i>	GCTGGAAACAACAGGGAGAC (F) TGACCTGAAGCATAAATGAACA	AB208744**	(CA) ₁₃ **		52	3	134–138	0.281/0.317	1.000

Primer sequences are forward (top) and reverse (bottom); Repeat sequence indicates repeat motif; Clone size is the allele size of the sequenced clone (in base pairs); T_a is annealing temperature in °C; N_A is the number of alleles detected; Size range refers to alleles thus far uncovered; H_E and H_O are expected and observed heterozygosities, respectively; P_{HW} represents the probability of deviation from Hardy–Weinberg expectations with significant deviations underlined. The fluorescently labelled primer is in bold: 6-FAM^(F) or HEX^(H). Novel microsatellite loci isolated and characterized in the present study are identified with *Sbr*; *Sca** and *Sni*** loci were previously isolated in Gold *et al.* (2002) and Yokoyama *et al.* (2006), respectively, and characterized for *S. brasiliensis* in the present study.

reaction buffer [50 mM KCl, 10 mM Tris-HCl (pH 9.0 at 25 °C), 0.1% Triton X-100], 0.5 U *Taq* DNA polymerase (GenScript Corp.), 0.5 µM of each primer, 200 µM of each dNTP, and 1 mM MgCl₂. PCR conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 38 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were visualized on a 2%

agarose gel (with ethidium bromide staining). Primer pairs that failed to amplify were subsequently tested at annealing temperatures of 52 °C and 49 °C and then dropped from further evaluation if they still failed to amplify. A total of 18 microsatellite repeats (Table 1), consisting of 13 perfect repeats (eight tetranucleotide and five dinucleotide) and five imperfect repeats (two tetranucleotide and three dinucleotide), were chosen for further screening. Length of cloned

alleles ranged in size from 121 to 321 base pairs; optimal annealing temperatures ranged from 49 to 55 °C (Table 1).

DNA was extracted from a total of 41 individuals sampled from the southern coast of Trinidad, presumably representing a single population. One primer from each pair of the 18 microsatellite primers was labelled with one of two fluorescent labels (6-FAM or HEX), also obtained from IDT. Amplified PCR products were run on an ABI-377 automated sequencer. Alleles were sized using the Genescan-500 ROX Size Standard (Applied Biosystems); allele sizing and calling were performed using Genescan 3.1.2 and Genotyper version 2.5 software. Genetic variability of the microsatellite markers was measured by the number of alleles, gene diversity (expected heterozygosity), and observed heterozygosity. Wright's F_{IS} , estimated as Weir and Cockerham's f in GDA (Genetic Data Analysis; Lewis & Zaykin 2001), was used to measure departure of genotype proportions from Hardy-Weinberg (HW) expectations at each microsatellite. Fisher's exact test, as performed in GDA, was used to test significance of departures from HW equilibrium (genotype) expectations at each microsatellite and for departure from genotypic equilibrium at pairs of microsatellites. The effect of HW departures (within-locus disequilibrium) on significance of between-locus linkage disequilibrium tests was removed by preserving genotypes in GDA (Lewis & Zaykin 2001). Evidence for occurrences of null alleles and/or large-allele drop-out was explored using MICRO-CHECKER (Van Oosterhout *et al.* 2004).

Genotypes were successfully obtained from all 41 individuals at all 18 loci; summary data are presented in Table 1. The number of alleles detected per microsatellite ranged from three (*Sni29*, *Sca49*, and *Sca61*) to 35 (*Sbr11*). Expected heterozygosity ranged from 0.118 (*Sca61*) to 0.978 (*Sbr11*), while observed heterozygosity ranged from 0.122 (*Sca61*) to 0.951 (*Sbr11* and *Sbr18*). Genotypes at two microsatellites (*Sbr20* and *Sbr35*) deviated significantly from HW expectations following sequential Bonferroni correction (Rice 1989); analysis with MICRO-CHECKER indicated the possible occurrence of null alleles at both microsatellites. All pairwise comparisons of microsatellites did not deviate significantly from genotypic equilibrium following Bonferroni corrections (Rice 1989). The 18 microsatellites developed in this study will prove useful for future population-genetic research of *S. brasiliensis*.

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