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Claudio A. Valero-Jiménez, Rocío Pérez-Portela & Susanna López-Legentil

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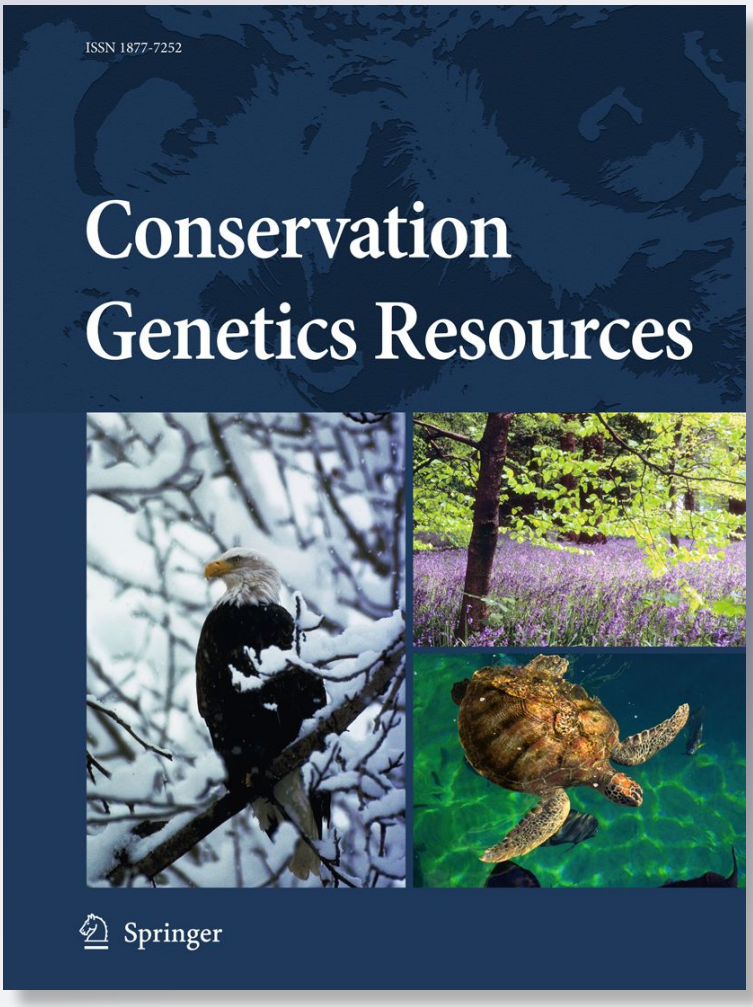

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TECHNICAL NOTE

Characterization of novel microsatellite markers from the worldwide invasive ascidian *Styela plicata*

Claudio A. Valero-Jiménez · Rocío Pérez-Portela · Susanna López-Legentil

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Abstract Ten polymorphic microsatellites loci were isolated and characterized from the invasive ascidian *Styela plicata* by 454 pyrosequencing. From over a hundred potential microsatellite regions, 18 were selected for screening and 10 were retained based on amplification success and marker polymorphism in a sample of 39 individuals from Spain and the United States of America. The number of alleles per locus ranged from 3 to 10, with an average of 6.4 (±2.5 SD). None of the loci showed significant linkage disequilibrium or evidence of large allele drop out or scoring errors. Departure from Hardy–Weinberg equilibrium was observed for two loci in the Spanish population and five loci in the American population, where drastic annual shifts in population genetic structure have been observed. These markers will facilitate further studies on the genetic structure of this species at small spatial and temporal scales.

Keywords Sea-squirt · Tunicata · Pyrosequencing · Excess homozygosity · Genetic structure

Ascidians are sessile marine invertebrates often found in artificial substrates such as harbors and aquaculture facilities (Lambert 2002, 2007). However, some species have spread to the surrounding natural habitat, becoming invasive and causing ecological and economic concerns (Lambert 2007). Until recently, the worldwide distribution of the solitary ascidian *Styela plicata* (Lesueur, 1823) was considered restricted to harbors and man-made structures (Barros et al. 2009; Pineda et al. 2011) but recent observations have reported this species in natural habitats from Sakushima Island, Japan (Nishikawa personal comm.; Pineda et al. 2011), the Ebro Delta in Spain (Pérez-Portela pers. obs.), and Urca Beach, Rio de Janeiro, Brazil (Paiva pers. comm.). The success of *S. plicata* has been attributed to high tolerance of heavy metals (Galletly et al. 2007), the ability to cope with significant temperature and salinity fluctuations (Thiyagarajan and Qian 2003), and fast growth rates to maturity (Yamaguchi 1975).

Here we used 454 pyrosequencing to isolate novel microsatellite loci in *Styela plicata*. Genomic DNA was extracted using the QIAamp® DNA Mini kit (QIAGEN) to a final DNA concentration of 5 ng/µl and distributed in two physically separated lanes of a plate. Pyrosequencing was performed on a Roche Life Science GS-FLX 454 System at the Scientific-Technical Services of the University of Barcelona. A total of 159,832 reads passed quality filtering with a mode length of 330 base pairs (average 277.88 ± 118.62 SD) and were imported into Geneious v 5.4.0 (Drummond et al. 2011). Sequences were screened for perfect microsatellites (di-, tri-, and tetra- nucleotides) with at least five repeats using Phobos v 3.3.12 (Mayer 2009) and over a hundred potential microsatellites were detected. 18 primer sets were designed using the software Primer3 (Rozen and Skaletsky 2000) and, from these, one was monomorphic, seven were difficult to score and ten were polymorphic.

Amplification success and marker polymorphism was tested in 20 specimens from Wilmington NC (USA; 34°8′24″N/77°51′44″W) and 19 specimens from Vilanova i la Geltrú (Spain; 41°12′51″N/1°44′07″E). Samples were collected in 2008 and 2009 and mantel tissue was preserved in absolute ethanol and stored at −20°C. Samples from

C. A. Valero-Jiménez · R. Pérez-Portela · S. López-Legentil (✉)
Department of Animal Biology (Invertebrates), University of Barcelona, 643 Diagonal Avenue, 08028 Barcelona, Spain
e-mail: slopez@ub.edu

Table 1 Characteristics of 10 microsatellite markers isolated for the ascidian *Styela plicata*

GenBank accession number	Locus	Primer sequences including fluorescent dye (5′–3′)	Repeat motif	Ta (°C)	Size range (bp)	Vilanova i la Geltrú				Wilmington NC			
						N	N_A	H_O/H_E	H–W	N	N_A	H_O/H_E	H–W
JQ231089	SPM1	F: [HEX]GTACCGGATGATACTTTGGA R: GTTCGAATCCTACCCACAG	$(AT)_{12}$	50	191–201	19	3	0.211/0.285	0.1954	16	6	0.750/0.710	0.0682
JQ231090	SPM2	F: [PET]GTAACATATGGCTGGTCGAT R: TCAATGCAAGAGAAGAACCT	$(TGT)_7$ $(TGC)_7$	52	188–218	19	5	0.474/0.602	0.2137	19	5	0.526/0.629	0.3292
JQ231091	SPM3	F: [6-FAM]AGATAAACCCAGCACTTACG R: AAACGTGACGAAGACTATGTT	$(TTA)_7$	50	144–153	18	2	0.444/0.356	0.5287	20	3	0.400/0.535	0.2490
JQ231092	SPM4	F: [NED]GCACTGTTTGAACCAACTTT R: TGTTATGCATTGTTGTCCAT	$(CA)_{10}$	51	143–395	17	3	0.118/0.116	1.0000	20	7	0.200/0.774	0.0000*
JQ231093	SPM9	F: [6-FAM]GAAGTGTGTTTCCAACGATT R: ATCTTTCACAAATCCACGAA	$(TTGA)_5$	56	139–177	18	6	0.556/0.552	0.0088	19	5	0.368/0.686	0.0015*
JQ231094	SPM10	F: [6-FAM]ATTCTGTTGCGTCTTGCTAT R: AGCACTCGACTGAATACTGG	$(TTCA)_5$	50	310–324	19	4	0.526/0.730	0.2909	20	3	0.500/0.488	1.0000
JQ231095	SPM13	F: [HEX]AACCAAGTTTATGCACAGGA R: CCGTATGCTCGTATAGCTTC	$(GA)_{10}$	48	307–386	13	3	0.421/0.428	0.1317	19	8	0.474/0.551	0.3346
JQ231096	SPM14	F: [6-FAM]AAACCATCGTGAGCTAAGAA R: GCAGTTTCAAGAAATGGTAGA	$(GTAT)_5$	48	352–364	18	3	0.111/0.427	0.0009*	14	3	0.071/0.474	0.0003*
JQ231097	SPM16	F: [6-FAM]TTTTCAATGGAGTGGTATTT R: TAACACCTGTGTGCAGTACG	$(AT)_{11}$	48	108–128	15	2	0.000/0.239	0.0039*	13	4	0.000/0.578	0.0000*
JQ231098	SPM18	F: [6-FAM]AATACCGACGAATCTCTTGA R: CAAAAGACGAGAGTGACAGC	$(AT)_{11}$	54	182–259	17	5	0.647/0.510	0.8671	18	5	0.111/0.432	0.0000*

N total of individuals, N_A number of alleles, *Ta* annealing temperature, H_O observed heterozygosity, H_E expected heterozygosity

* Significant departure of Hardy–Weinberg equilibrium (H–W) after sequential Bonferroni correction ($P < 0.005$)

Vilanova i la Geltrú and Wilmington NC were extracted and amplified using the REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich), 5 pmol of each primer, and water to a final reaction volume of 10 µl. Forward primers for each locus were labeled with a fluorescent dye (Table 1). Samples were amplified on a PCR System 9700 (Applied Biosystems) with an initial 1 min denaturation step at 95°C; followed by 35 cycles of 95°C for 30 s, 48–56°C for 15 s (depending on each locus; Table 1) and 72°C for 15 s, followed by a 3 min final extension at 72°C. Amplification products were analyzed on an Applied Biosystems 3730xl Genetic Analyzer at the Scientific-Technical Services of the University of Barcelona. The length and allele scoring of PCR products was estimated relative to the internal size standard GeneScan 600LIZ using the software PEAKSCANNER (Applied Biosystems).

Linkage disequilibrium, observed and expected heterozygosity, and deviation from Hardy–Weinberg (H–W) equilibrium were calculated using the program ARLEQUIN v3.5.1.2 (Excoffier and Lischer 2010; Table 1). No evidence of linkage disequilibrium was detected across all pairwise comparisons. Allelic diversity ranged from 3 to 10 alleles per locus, with an average of 6.4 (±2.5 SD). Mean observed and expected heterozygosities were 0.35 (±0.20 SD) and 0.42 (±18 SD), respectively, for the Vilanova i la Geltrú population, and 0.34 (±0.24 SD) and 0.59 (±0.11 SD) for the Wilmington NC population. After sequential Bonferroni correction, two loci significantly deviated from H–W equilibrium for the Vilanova i la Geltrú population, and five for the Wilmington NC population (Table 1). MICRO-CHECKER v2.2.3 (Van Oosterhout et al. 2004) detected potential null alleles for these loci but found no evidence of large allele drop out or scoring errors.

Excess homozygosity and low genetic diversity in introduced populations are generally interpreted as a measure of inbreeding or the result of a founder effect (Holland 2000). Significant inbreeding coefficients have been found in several populations of *S. plicata*, including the population in Wilmington NC studied here (Pineda et al. 2011). In addition, large mortality events decimated this population in June 2008 and 2009, followed by rapid recruitment and population reestablishment (López-Legentil pers. obs.). Thus, inbreeding or founder effects resulting from this re-colonization process may explain the heterozygosis deficiency observed here for some microsatellite loci. The development of ten microsatellite markers for *S. plicata* will now allow investigating the population dynamics of this species at smaller spatial and temporal scales.

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