Development and characterization of 16 polymorphic microsatellite markers for the highly invasive ascidian, *Ciona savignyi*

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Received: 3 September 2014 / Accepted: 8 September 2014 / Published online: 14 September 2014
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Abstract The Pacific transparent ascidian *Ciona savignyi*, which is considered native to Japan and possibly northern Asia, has successfully invaded coasts of temperate regions and even Southern Hemisphere. The significantly negative effects on local biodiversity caused by invasions of this ascidian have resulted in challenges in biological conservation. In order to study its dispersal dynamics, here we developed and characterized 16 polymorphic microsatellite markers. Variation evaluation showed the number of alleles ranged from two to 11, and the observed and expected heterozygosity varied from 0.033 to 0.697 and from 0.033 to 0.867, respectively. These markers will be useful for studying dispersal dynamics and associated population genetic structure during fast spread of *C. savignyi*.

Keywords Biological invasion · *Ciona savignyi* · Invasive species · Microsatellite · Solitary ascidian

Human-mediated introductions and spread of marine non-indigenous species at a global scale have sharply increased in the past decades, making coastal waters as one of the most invaded habitats (Carlton and Ruiz 2005). Among many problem-causing species, the Pacific transparent

ascidian *Ciona savignyi* is one of the most notorious invaders (Lambert and Lambert 1998). This invasive solitary ascidian, which is considered native to Japan and possibly northern Asia, has spread to temperate regions such as Pacific coast of North America, and even Southern Hemisphere such as New Zealand (Lambert and Lambert 1998; Smith et al. 2010). *C. savignyi* is very competitive in invaded habitats, resulting in profound changes to benthic communities by rapidly covering available substratum and excluding native species in a short period of time (Lambert and Lambert 1998).

Although negative effects caused by marine invaders have been well documented, studying their dispersal dynamics poses immense technical challenges, as available information pertaining to dispersal dynamics is often incomplete in aquatic habitats. The use of robust genetic analyses based on polymorphic markers can advance our understanding of both dispersal dynamics and associated population genetic patterns (e.g. Zhan et al. 2010). To investigate the dispersal dynamics, as well as population genetic structure in both native and invaded ranges for the highly invasive *C. savignyi*, here we developed and characterized 16 polymorphic microsatellite markers.

Microsatellite markers were developed based on the *C. savignyi* genome sequence (http://asia.ensembl.org/Ciona_savignyi/Info/Index). The downloaded whole genome sequences were mined for microsatellites using the methods of Zhan et al. (2010). Unlinked microsatellites with sufficient flanking sequences were chosen for primer design using Primer Premier version 5 (Premier Biosoft International). Forward primers were 5'-end fluorescently labeled with one of the following four fluorescent dyes, 6-carboxyfluorescein (6-FAM), hexachlorofluorescein (HEX), carboxy-X-rhodamine (ROX), and carboxy-tetramethylrhodamine (TMR).

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Table 1 Characterization of 16 polymorphic microsatellites for the highly invasive ascidian, *Ciona savignyi*. T_a = optimized annealing temperature; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity

Locus	Repeat motif	Primer sequences	Dye used	Size (bp)	T_a	N_A	H_O	H_E
CS3689	(GAT) ₅	F: CTGAATACAAGAGGCTAA R: GACACAATCTTGCCATCT	ROX	340–364	56	6	0.219	0.259
CS5209	(ACCAC) ₃	F: GTGGATCAGTTTGGTTCCCT R: ATACGACACTCTGTGCTTGTC	6-FAM	256–266	50	2	0.033	0.033
CS6397	(TGA) ₅	F: TGGATAACCTCCGCTTGTTTC R: CATCTGGTTCTGGTGATGTAGTAG	TMR	170–179	56	4	0.609	0.727
CS7688	(TATCC) ₃	F: TGAACCCAGCGGATTAATGG R: GATCCAACGAGTGCTTGAGT	HEX	254–274	62	4	0.328	0.422
CS7928	(AAT) ₅	F: CAGTTCAGACCGGCTAATGA R: CTGGGTAAGGTCATAGGTGAAG	6-FAM	280–283	50	2	0.030	0.088
CS9784	(GAT) ₅	F: CCAAGACTAACCAGGCGATAC R: TGACCCGATCATCCAAATCC	HEX	153–156	50	2	0.257	0.269
CS50352	(ACC) ₅	F: TGGTTTGTGGGTACTTGCTGG R: TCATTGTCGCTGTTTTATCT	ROX	146–165	56	5	0.400	0.485
CS50771	(TATCC) ₃	F: CTATCCCCCTACCCTCTTA R: CGTACCCGAGGGCACCA	6-FAM	151–156	56	2	0.306	0.261
CS52718	(AAC) ₅	F: GAGGCGAATTGTCCCAT R: TCCCTCCAACAATGCGG	TMR	348–357	62	3	0.400	0.540
CS56726	(CACCCC) ₃	F: CGATTGGGTTGACGCTTA R: CTTGGGGGGTTCGGGGTGC	ROX	231–237	62	2	0.111	0.170
CS60921	(TAAAC) ₅	F: CGTCTTATCACGCACAAC R: CCGCAGCAGCATTATCAA	HEX	236–241	56	6	0.214	0.714 ^b
CS4940	(GAG) ₅	F: AAAGAAGTGCTCGCGAAGTC R: AGTCCTCATCGCTCTCATTCT	HEX	116–125	61	3	0.111	0.667 ^b
CS8713	(CAA) ₅	F: TCCCAACAACCTGCCAACT R: GCTCGCATCTGTTGTTCTTG	ROX	148–169	50	5	0.697	0.658
CS61676	(AGG) ₅	F: CCTCCTGCCGCGCTACCTTCT R: TCCTGGCCCCGACTCCATCTT	HEX	142–185	56	11	0.500	0.867
CS62319	(AAGAA) ₃	F: GAGATATCAGTGGGAAGG R: GAATACGATCAGCTCCAG	TMR	251–257	56	3	0.034	0.100
CS63497	(TCA) ₅	F: GGTGGAGGTGGGGTTGTA R: GCCTTGTCGCCGTAGTTG	ROX	276–315	61	7	0.514	0.657

^b illustrates significant deviation from Hardy–Weinberg equilibrium (HWE) after Bonferroni correction

We used a population ($N = 36$) collected from the Sanggou Bay (37°09'N, 122°30'E), Shandong Province, China to characterize variation of these microsatellite markers. The tissue around siphons was used to extract total genomic DNA using the standard phenol/chloroform method. PCRs were performed in a reaction volume of 12.5 μ L containing 1 U *Taq* DNA polymerase (Takara), 0.2 μ M primers, 2 μ M Mg^{2+} , 100 μ M each dNTP, and 50 ng genomic DNA. PCRs were programmed as: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, locus-specific annealing temperature (Table 1) for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. Amplified fragments were separated on an ABI 3130XL automated sequencer with the GeneScanTM–500 LIZTM

internal size standard and scored using GENEMAPPER[®] version 4.0 (Applied Biosystems). Diversity indices including the number of alleles (N_A), observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using Genepop online version (<http://wbiomed.curtin.edu.au/genepop/>). Hardy–Weinberg equilibrium was tested using Genepop for all loci, and sequential Bonferroni correction was applied for multiple tests to avoid Type I errors. The possibility of occurrence of null allele(s) and large allele dropout was tested using Micro-Checker version 2.2.0 (van Oosterhout et al. 2004).

Of the 72 primer pairs examined, 16 produced PCR products that were clear enough to score and were polymorphic. Characterization of these microsatellite markers

in the population showed a moderate level of polymorphism. The number of alleles varied from two to 11, and the observed heterozygosity and expected heterozygosity ranged from 0.033 to 0.697 and from 0.033 to 0.867, respectively (Table 1). After sequential Bonferroni correction for multiple tests, only two loci (CS60921 and CS4940) were found to depart significantly from Hardy–Weinberg equilibrium and showed heterozygote deficiency (Table 1). A further test based on Micro-Checker showed the presence of null alleles at these two loci. The microsatellite markers developed here will be useful for investigating spread dynamics and population genetic structure during biological invasions of *C. savignyi*.

Acknowledgments This work was supported by the National Natural Science Foundation of China (31272665) and 100 Talents Program of the Chinese Academy of Sciences to A.Z., and by Comprehensive Evaluation Methods for Quality of Marine Environments in South China Sea [DOME(PMEA)-01-03].

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