Development of Microsatellite Loci for the Endangered Seagrass Zostera japonica (Zosteraceae)

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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE LOCI FOR THE ENDANGERED SEAGRASS *ZOSTERA JAPONICA* (ZOSTERACEAE)[1]

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- *Premise of the study:* New microsatellite markers were developed for the Asian endangered seagrass *Zostera japonica* (Zosteraceae) to assess genetic diversity and population structure of this species. In China, *Z. japonica* populations have drastically decreased since the 1970s.
- *Methods and Results:* A total of 12 polymorphic tetranucleotide microsatellite loci were isolated and characterized in *Z. japonica.* The number of alleles per locus ranged from one to 11. The expected and observed heterozygosity ranged from 0 to 0.772 and from 0 to 1.000, respectively.
- *Conclusions:* The new microsatellites will be useful in evaluating clonality and population structure of *Z. japonica* and aiding in conservation and management of the endangered seagrass in Asia.

Key words: clonality; population genetics; seagrass; *Zostera japonica*; Zosteraceae.

Seagrass beds are recognized as critical to threatened coastal habitats around the world (Duffy, 2006). The seagrass *Zostera japonica* Asch. & Graebn. (Zosteraceae), an annual/perennial marine flowering angiosperm, is mainly distributed in the intertidal and shallow subtidal zones from temperate to subtropical regions along the North Pacific coast, especially in East Asia (Short et al., 2007). Meanwhile, *Z. japonica* is an introduced species on the west coast of North America (Harrison and Bigey, 1982) and has been reported from British Columbia (Canada) and Washington, Oregon, and California (USA) (Short et al., 2007). *Zostera japonica* can rapidly thrive and form extensive meadows through vegetative reproduction in the intertidal zone (Zhang et al., 2015). However, anthropogenic activities have led to a strong decline of natural populations in Asia.

To conserve and restore *Z. japonica*, much attention should be paid to genetic diversity and population genetic structure, yet few studies have been reported. Microsatellite markers prevail in genetic studies of seagrasses. In the genus *Zostera* L., microsatellites have been developed for quite a few species, such as *Z. marina* Gaertn. (Peng et al., 2012), *Z. muelleri* Irmisch ex Asch. (Sherman et al., 2012), and *Z. nigricaulis* (J. Kuo) S. W. L. Jacobs & Les (Smith et al., 2013), but specialized primers for *Z. japonica* were limited until now. Jiang et al. (2011) have developed a set of dinucleotide microsatellite loci for this species, and the analysis of short tandem repeat (STR) loci by PCR methods has proven to be informative. However, the PCR products of dinucleotide loci often produce multiple visible stutter bands that sometimes complicate the interpretation of alleles. The amplification of tetranucleotides is easier to interpret because only a single stutter band is typically observed (Walsh et al., 1996). Here we report isolation and characterization of the first set of polymorphic tetranucleotide microsatellite loci for *Z. japonica*, which will be used to investigate genetic diversity and population structure of this species.

METHODS AND RESULTS

Genomic DNA was isolated from fresh leaf tissue of a single individual of *Z. japonica* collected from Qingdao, China (36°05′N, 120°34′E) (Appendix 1). Genomic DNA extraction was undertaken using the E.Z.N.A. HP Plant DNA Mini Kit (OMEGA Bio-tek, Norcross, Georgia, USA) according to manufacturer's protocols. A DNA extract of 50 µL with a concentration of 118 µg/µL was obtained. Microsatellites were isolated following the enrichment protocols of Glenn and Schable (2005). Total genomic DNA was digested with *Rsa*I (New England Biolabs, Ipswich, Massachusetts, USA). The digested fragments were ligated to double-stranded SuperSNX-24 linkers and then hybridized with a 5′-biotinylated oligonucleotide probe $(AGAT)_8$ (Life Technologies, Shanghai, China). The DNA fragments containing microsatellite sequences were captured on streptavidin-coated Dynabeads (Invitrogen, Carlsbad, California, USA), and the captured DNA was recovered by PCR using the SuperSNX-24 forward primer (Life Technologies). The PCR products were purified using TaKaRa MiniBEST DNA Fragment Purification Kit ver.3.0 (TaKaRa Biotechnology Co., Dalian, Liaoning, China), ligated into pEASY-T1 cloning vector (TransGen, Beijing, China), and transformed into Trans1-T1 competent cells (TransGen). A total of 201 positive clones were sent to Life Technologies for sequencing. Fragments containing microsatellite repeats were screened using MISA software (Thiel et al., 2003; http://pgrc.ipk-gatersleben.de/misa).

Twenty-five primer pairs were designed by Primer Premier ver.5.00 (PREMIER Biosoft International, Palo Alto, California, USA). The primers were optimized and polymorphisms were tested by genotyping eight individuals collected from Qingdao, China (36°05′N, 120°34′E). The 5′ end of each forward primer was fluorescently labeled (FAM, HEX, or TAMRA; Life Technologies). All loci were amplified separately on a Mastercycler (Eppendorf, Hamburg,

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TABLE 1. Characterization of 15 microsatellite loci for *Zostera japonica.*

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	*A*	GenBank accession no.
Zj001	F: GCAAAAGTGTTGGGTGAAA R: GAAATGGTGATGGGATGGA	$(TATC)_8$	228–236	54	3	KP756928
Zj008	F: ACTTCGGCACCAATCGCAT R: CGCCCCTCCCTCTCCTCTA	$(TATC)_8$	148–160	56	4	KP756929
Zj018	F: CTATGTCGTTGCTCGCACTCT R: CCAAATCAATCCATGTCCCTC	$(TAGA)_{14}$	230–250	56	4	KP985542
Zj023	F: TTTTGGCAAGTGTGGGTT R: CTGTCATGGGTTGAGAGC	$(CTAT)_6$	329	50	1	KP985543
Zj025	F: GCCGACCCTCTCCAGCCT R: CCTTCCATCCACAGCAAT	$(TATG)_6$	360–396	54	5	KP756930
Zj026	F: CGCTCATCAGCATCATCC R: CAAACCCTAAAGCCCAAC	$(TAGA)_6$	104–116	58	4	KP756931
Zj028	F: CTTCTTCCCCTCCCGCCAGT R: TCCAAAAACAACGCAAATCT	$(TCTA)_{15}$	314–350	59	5	KP756932
Zj030	F: GAAGTATCAACGAACCCCA R: CATAAAGAACCGCAGCAGT	$(TAGA)_6$	272–324	54	11	KP756933
Zj033	F: ACAGGACTAACAGGAGAAGC R: GTGAGACAGAGATGAATGGC	$(ATAG)_8$	226	54	1	KP985545
Zj041	F: GGGAAACAAAACAGCACC R: AATGAAAAGAACCCACGC	$(TAGA)_6$	144	58	1	KP985548
Zj042	F: CAAATCCGTCCACAAAAC R: TAGAGTCCCATGCCCACC	$(TAGA)_6$	157–169	50	4	KP756934
Zj011	F: ATCACCAGTTCCTACCTCC R: ATTATTCACACGCTTTCCA	$(TATC)_7$	336–348	56	4	KP985541
Zj029	F: CTCACCTACAACATCCAACA R: GGGAAGAGAATAAGACCGAA	$(ATCT)_6$	149–157	58	3	KP985544
Zj036	F: TTCCCTAACAGCCTAACCCAA R: TCACCCTCTTTTTAACCCATC	$(ATAG)_7$	290–294	56	2	KP985546
Zj037	F: CCCTGCTCTTGTTTTTCT R: TTGCTTGTATTTTTTTCC	$(TATC)_6$	382–460	52	6	KP985547

Note: *A* = number of alleles observed; T_a = annealing temperature of each primer pair.

Germany) in a 10-μL reaction containing 10–50 ng of genomic DNA, 5 μL of PCR mix (Dongsheng Bio Co., Guangzhou, Guangdong, China), 0.25 μM reverse and labeled forward primer, and 3.5 μL of ultrapure water. PCR cycling conditions were 94°C for 3 min; 35 cycles of 30 s at 94°C, 30 s at an annealing temperature gradient of 50–60°C, and 1 min at 72°C; with a final extension at 72°C held for 10 min. PCR products were visualized on a 1.5% agarose gel to judge if the loci were successfully amplified. Pooled products were sent to Life Technologies for genotyping. A final set of 12 microsatellite loci were found to be polymorphic in *Z. japonica* (Table 1).

To assess the genetic diversity of the selected loci, we used 32 individuals of two *Z. japonica* populations from China, i.e., Qingdao (36°05′N, 120°34′E) and Fangchenggang (21°36′N, 108°13′E) (Appendix 1). Samples were collected at least 2 m apart in the field. Allele scoring was performed using GeneMarker 2.2.0 (SoftGenetics, State College, Pennsylvania, USA). The number of alleles per locus ranged from one to 11, and the expected and observed heterozygosity ranged from 0 to 0.772 and from 0 to 1.000, respectively (Table 2). Significant deviation from Hardy–Weinberg equilibrium was observed at three loci ($P < 0.0042$) in the Fangchenggang population after Bonferroni correction using the software program GENEPOP 4.0 (Rousset, 2008). Linkage disequilibrium among loci was also detected using GENEPOP 4.0, with Fisher's method (Raymond and Rousset, 1995; Rousset, 2008), and was detected between locus pairs Zj025 and Zj042, Zj011 and

TABLE 2. Summary genetic statistics for two populations of *Zostera japonica* screened with 12 newly developed polymorphic microsatellites.[a]

	HQ ($N = 16$)						FC ($N = 16$)					
Locus	*n*	*A*	H_e	H_o	PIC	P[b]	*n*	*A*	H_e	H_o	PIC	P[b]
Zj001	16	3	0.621	0.625	0.516	0.2476	15	2	0.517	1.000	0.375	0.0002
Zj008	16	4	0.708	0.750	0.626	0.0287	16	3	0.232	0.250	0.210	1.0000
Zj011	11	3	0.558	0.455	0.432	0.5430	16	1	0.000	0.000	0.000	—
Zj018	11	4	0.710	0.546	0.623	0.1129	16	1	0.000	0.000	0.000	—
Zj025	16	2	0.315	0.375	0.258	1.0000	16	4	0.563	0.250	0.493	0.0019
Zj026	16	2	0.353	0.438	0.283	0.5433	16	3	0.659	0.125	0.567	0.0000
Zj028	16	6	0.772	0.500	0.710	0.0389	16	6	0.585	0.625	0.532	0.9340
Zj029	11	3	0.550	0.455	0.466	0.6040	16	1	0.000	0.000	0.000	—
Zj030	16	2	0.121	0.000	0.110	0.0323	16	3	0.232	0.250	0.210	1.0000
Zj036	16	2	0.515	0.400	0.374	0.6034	16	3	0.179	0.188	0.166	1.0000
Zj037	11	2	0.505	0.400	0.365	0.5736	16	1	0.000	0.000	0.000	—
Zj042	16	2	0.387	0.500	0.305	0.5126	16	5	0.746	0.688	0.675	0.4041

Note: *A* = number of alleles observed; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals genotyped; *N* = number of individuals in the population sampled; PIC = polymorphic information content.

[a] Locality and voucher information for the sampled populations are available in Appendix 1.

[b] *P* values for deviation from Hardy–Weinberg equilibrium.

Zj018/Zj037, and Zj001 and Zj028/Zj029/Zj036 ($P < 0.0042$) after Bonferroni correction (Rousset, 2008), but was most likely due to low polymorphism levels at those loci.

CONCLUSIONS

The new polymorphic microsatellite loci developed in this study have proved to be useful to evaluate genetic diversity of *Z. japonica*. The two studied populations showed different frequencies of alleles at these loci and both displayed fixed alleles. Therefore, it is expected that more alleles will be detected if sampling is conducted more broadly across the species' range. These available microsatellite loci will facilitate future studies of population genetic and clonal structure, connectivity, and gene flow in *Z. japonica*, which will contribute to the conservation and management of this species.

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Appendix 1. Voucher and location information for *Zostera japonica* populations used in this study. One voucher was collected for each population used; all vouchers were deposited in the Marine Biological Museum, Chinese Academy of Sciences, Qingdao, China.

Population code	Collection date	Locality (China)	Geographic coordinates	Herbarium ID
HQ	15 June 2015	Qingdao, Shandong	36°05′N, 120°34′E	MBM283038
FC	5 June 2012	Fangchenggang, Guangxi	21°36′N, 108°13′E	MBMD02001