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A set of microsatellite primers for *Zostera japonica* (Zosteraceae)[1]

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- *Premise of the study:* Polymorphic microsatellite primers were developed in the seagrass *Zostera japonica* to investigate genetic variation and to identify clonal structure.
- *Methods and Results:* Thirteen polymorphic loci and 23 monomorphic loci were developed in *Z. japonica*. Two to 13 alleles per locus were observed at the polymorphic loci across 57 individuals of two *Z. japonica* populations. The observed and expected heterozygosities within populations ranged from 0.0000 to 1.0000 and from 0.0000 to 0.8542, respectively.
- *Conclusions:* Our study showed high-level polymorphism at the polymorphic loci in *Z. japonica*. These primers would be a powerful tool to study genetic variation, clonal structure, and mating systems.

Key words: microsatellite primers; polymorphism; seagrass; *Zostera japonica*.

Zostera japonica Asch. & Graebn. (Zosteraceae), i.e., dwarf eelgrass or Japanese eelgrass, is a widespread monoecious seagrass native to the western Pacific. This species was introduced with oyster aquaculture to native eelgrass beds on the Pacific coast of North America and has become an invasive species (Williams, 2007). Like other seagrasses, this species can reproduce both sexually via seed production and vegetatively by rhizomatic growth. Clonal growth has significant impacts on genetic variation within and among populations, resulting in a presumption of low genetic diversity in seagrass populations as revealed by allozymes (Arnaud-Haond et al., 2005). However, recent studies adopting microsatellites have found high genetic variation in seagrasses comparable to those of nonclonal species (e.g., Arnaud-Haond et al., 2005). Although some primers developed in congeners might be cross-amplified in *Z. japonica* (Reusch, 2000; Coyer et al., 2004), the high possibility of null alleles limits their applications in *Z. japonica* (Coyer et al., 2004), especially in identifying clonal structure and estimating outcrossing rate. In this study, we isolated and characterized microsatellite primers for *Z. japonica* that will be useful in the analysis of genetic variation and mating system of this species.

METHODS AND RESULTS

Total genomic DNA was extracted from silica gel–dried leaves of one *Z. japonica* individual using Plant Genomic DNA Kit (Tiangen Biotech, Beijing, China) to construct a DNA library enriched for microsatellites. The enrichment procedure followed the protocols of Liu et al. (2009) and Xu et al. (2010). About 300 ng of genomic DNA was digested with the enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA) and ligated to an *Mse*I-adapter pair (AdapF: 5′-TACTCAGGACTCAT-3′ and AdapR: 5′-GACGATGAGTCCTGAG-3′). This is followed by concentration of adapter-ligated fragments that were elevated through PCR amplification using *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′). The PCR products were denatured and hybridized to 5′-biotinylated $(AC)_{15}$ or $(AG)_{15}$ probes. Streptavidin-coated magnetic beads (Promega, Madison, Wisconsin, USA) were employed in capturing single-stranded DNA fragments containing microsatellites, and followed by PCR using *Mse*I-N as the primer. The PCR products were ligated into pMD19-T vector (Takara, Dalian, China) and transformed into *E. coli* strain DH5α after they were purified with a multifunctional DNA Extraction Kit (Bioteke Corporation, Beijing, China).

A total of 1436 clones were selected and tested by PCR with $(AC)_{10}$ or $(AG)_{10}$ and primers $M13^{+}/M13^{-}$. Two hundred seventy-two positive clones were isolated and sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA). We obtained 270 sequences, of which 136 contained simple sequence repeats. Some sequences were discarded because they lacked a flanking region or contained few repeats. Finally, 64 sequences containing at least seven simple repeats were used to design primers using Premier 5.0 (Lalitha, 2000). Among the 64 primers, 15 failed to obtain products, 13 produced multiple bands that were difficult to interpret, and 36 produced clear bands with expected sizes on agarose gel. To test the polymorphism, we randomly selected 10 individuals collected from Beigang and 10 collected from Dongjiao, Hainan Province, China (Appendix 1), for PCR using the 36 primers; 13 loci appeared polymorphic in 8% polyacrylamide gel electrophoresis. Subsequently, the forward primers of these 13 primers were labeled with fluorescent dye (5′6-FAM, 5′HEX, or 5′ROX). With the labeled primers, polymorphism was further tested in 28 individuals collected from Beigang and 29 from Dongjiao. Microsatellite loci were amplified under the following conditions: 5 min denaturation at 94°C; 30 cycles of 30 s at 94°C, 30 s at 53–65°C, and 30 s at 72°C; and a final 10 min extension at 72°C. The products were scanned by an ABI 3130 automated sequencer (Applied Biosystems) using an internal lane standard (GS500(–250LIZ)). Allele binning and calling were conducted using GeneMapper 4.0 (Applied Biosystems).

Characteristics of the microsatellite loci of *Z. japonica* are shown in Table 1. Each polymorphic locus had two to 13 alleles, with a mean of 5.1 alleles. At the population level, the observed and expected heterozygosities ranged from 0.0000 to 1.0000 and from 0.0000 to 0.8542, respectively (Table 2), calculated by the software TFPGA v1.3 (Miller, 1997).

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TABLE 1. Characteristics of 36 pairs of microsatellite primers developed for *Zostera japonica.* Forward and reverse primers, fluorescent dye labels of polymorphic loci, repeat motif, allele size range (bp), annealing temperature (T_a), and GenBank accession numbers are shown. Polymorphic loci are shown in bold.

Locus	Primer sequence (5′–3′)	Motif	Size range (bp)	T_a (°C)	GenBank Accession No.
ZJ5	**F: <6-FAM>TGATGGGAGGTTCTTGGT** **R: AAGCTCACAAAGGTAGACAT**	**$(TC)_{11}$**	**144–152**	**65**	**JN052166**
ZJ11	F: GATTAGGTGAGATGGGACA R: TTGGCAGAAGATTGTCG	$(CT)_8$	154	65	JN052167
ZJ48	**F: <HEX>TAGAGGAAGCAAGGGACG** **R: AGATTTGGGCTACTGGGTT**	**$(AG)_7$**	**140–142**	**65**	**JN052168**
ZJ56	F: GCCAATAGAAGAAAATGGAAACC R: AAATCATCCCCAACCCTCAA	$(GA)_7$	299	65	JN052169
ZJ134	F: GGTGGATGTTTAGTAGTTTAGG R: TGCCATTATGCCAACGATA	$(TG)_6AC(TG)_7$	325	65	JN052170
ZJ178	**F: <6-FAM>TCAACAATCCACAGCAAG** **R: TCGGCTTGAAGTATCTAAAC**	**$(AC)_7$**	**215–219**	**65**	**JN052171**
ZJ198	**F: <HEX>GCACTATGACGCACCTATT** **R: GAGCGTTGATGGTATCTATT**	**$(AT)_8…(CA)_6$**	**200–204**	**65**	**JN052172**
ZJ207	**F: <6-FAM>GCTTGATAGATAGAAGTAGATTAG** **R: AGACTGGTATGATTTATTTGG**	**$(TG)_8$**	**211–217**	**61**	**JN052173**
ZJ397	**F: <6-FAM>ATTATCCATACCCACGA** **R: AAGGTTTCTCATATCTTGT**	**$(CA)_7$**	**172–178**	**53**	**JN052174**
ZJ415	F: CAGCACTAACCTGTGATTTC R: AGATGGACTAAAAGTTCTAACC	$(CA)_9$	175	64	JN052175
ZJ419	F: TCCATTAGTCACAGTTACAGG R: AAGTCATTAGTGGCGAAGA	$(AC)_8$	168	65	JN052176
ZJ542	**F: <6-FAM>AGAGCGGAAGATGGAGAA** **R: CACGGTGCAATAGTGTAAC**	**$(GA)_{18}$**	**118–134**	**62**	**JN052177**
ZJ574	F: GATTTAGGCAGATTCAGG R: AAACTTACGGTCAGATGG	$(GA)_9$	278	64	JN052178
ZJ649	F: GAGTGAAAAGCAATCAGGAA R: AGAGTTACCCACCTATTGAGAT	$(AG)_9$	129	64	JN052179
ZJ693	F: GTGTTGAGACCATTGAAGAC R: GCCAGACCCATAATCTAAATG	$(AG)_{19}$	223	56	JN052180
ZJ706	**F: <HEX>AACTCCTGGCTATCCTCTACCT** **R: GAACTATCCAAACTCCGCAAC**	**$(TC)_7$**	**180–186**	**65**	**JN052181**
ZJ799	F: CGCTGAATCCAAATCCTA R: CTGGTATAGAACGAAACCCT	$(GA)_8$	133	65	JN052182
ZJ812	**F: <6-FAM>GCAGCCCTAAAGTAAAT** **R: TTCTAGCCCAAATCAAC**	**$(GA)_7$**	**247–255**	**56**	**JN052183**
ZJ816	F: GTCGTGCTCAAGATC R: ACACTAAAGGCCAGA	$(CT)_{16}$	159	54	JN052184
ZJ827	F: GGTTCACCAATGACAAGTC R: GAGAAGAGGAATACTGTAACG	$(TC)_6TTTC(TC)_9$	179	65	JN052185
ZJ892	F: TTCTGTTCTTCCACCTCT R: CTCACTCGAAATGATGAC	$(GA)_7$	144	61	JN052186
ZJ896	**F: <6-FAM>ATCACGCCCTCCACTAT** **R: ACGATTTGGGGATTTGC**	**$(CT)_{19}$**	**94–122**	**61**	**JN052187**
ZJ906	F: AGAGGAGCGCCGATTGATA R: AAAATGTCCACGACGTGCC	$(GA)_7$	112	65	JN052188
ZJ966	F: AGTCGGAGACGCTTTATTG R: GTCCAGGTGACCATTTCG	$(AG)_7…(GA)_6$	290	64	JN052189
ZJ1069	**F: <ROX>AAGAAGACGCAGGAGACA** **R: CAGTGAAACAGTTAGGGATT**	**$(AG)_9$**	**220–234**	**61**	**JN052190**
ZJ1089	F: CCGCTTCTAGTAAAGGTTCT R: ATAAGGGACATCAGATACCG	$(GA)_9$	223	61	JN052191
ZJ1159	F: GTTCCATACTCGGTCAAT R: AAACTGGAAGAGGCAAA	$(GA)_8$	204	61	JN052192
ZJ1202	F: ATGCTTGCTAGAGGAT R: CTTGATAGGGAGGTTT	$(GA)_9$	240	56	JN052193
ZJ1235	F: AGGGACATCAGATACCGA R: CATGACAATTTCCGCTTC	$(TC)_9$	231	64	JN052194
ZJ1258	F: ACAACTACATCCCCTTTT R: TCCTGTCATTGCTATTCTC	$(GA)_7$	149	56	JN052195
ZJ1285	F: GGGATAGATAGATGTGAAAGC R: AAAGTTGGTCGCAAATAGA	$(GA)_8…(GA)_9$	278	65	JN052196
ZJ1314	F: GCAAAATACAGCAAGAG R: CCACTCAGCTAACCTTA	$(TC)_{11}$	180	63	JN052197
ZJ1345	**F: <6-FAM>AAGTCCTGCTAAGGTGTTGAGA** **R: TCCCACGAAGAGGAAGATAACA**	**$(AG)_{21}$**	**216–234**	**65**	**JN052198**

TABLE 1. Continued.

Locus	Primer sequence (5′–3′)	Motif	Size range (bp)	T_a (°C)	GenBank Accession No.
ZJ1364	F: GAGGAGAAGTGGACGAGAAAT R: GTCGTGCCTGAATCAACTCTA	$(GA)_5…(GA)_8$	290	65	JN052199
ZJ1368	**F: <HEX>ACTTCAGCCACTCGTCTAACC** **R: CAAGTAGAAAGAGGGCAAAGC**	**$(CT)_7$**	**119–123**	**65**	**JN052200**
ZJ1382	F: TCCTGTCATTGCTATTCTC R: AACAACTACATCCCCTTTT	$(TC)_7$	150	61	JN052201

TABLE 2. Characteristics of 13 polymorphic microsatellite loci tested in two *Zostera japonica* populations. Number of samples genotyped (N), number of alleles per locus (N_a), and observed heterozygosity (H_o) and expected heterozygosity (H_e) at each locus are shown.

	Beigang (20°00′44.0″N, 110°33′38.8″E)				Dongjiao (19°31′33.6″N, 110°51′14.5″E)			
Locus	N	N_a	H_o	H_e	N	N_a	H_o	H_e
ZJ5	28	4	0.4643	0.4968	29	5	0.8276	0.6703
ZJ48	28	2	0.1786	0.1656	29	1	0.0000	0.0000
ZJ178	28	1	0.0000	0.0000	29	3	0.3103	0.2789
ZJ198	28	2	0.6071	0.5084	29	3	0.7241	0.5451
ZJ207	28	3	0.3571	0.6130	29	3	0.4483	0.5390
ZJ397	28	3	1.0000	0.5266	29	3	1.0000	0.5420
ZJ542	28	7	0.9643	0.7383	29	9	0.8966	0.8421
Z706	28	2	0.4286	0.3818	29	2	0.6207	0.4356
ZJ812	28	2	0.3929	0.3630	29	2	0.3448	0.3339
ZJ896	28	7	0.8929	0.7974	29	9	0.8621	0.8542
ZJ1069	28	5	0.8214	0.6649	29	4	0.7421	0.6382
ZJ1345	28	10	0.7143	0.7935	29	9	0.7586	0.7629
ZJ1368	28	3	0.0714	0.2539	29	2	0.0345	0.0345
Mean	28	3.9	0.5302	0.4849	29	4.2	0.5809	0.4982

CONCLUSIONS

In the current study, we have successfully developed 13 polymorphic loci in *Z. japonica*. Although 23 loci were found to be monomorphic in the two studied populations, they might be polymorphic in other regions. These polymorphic microsatellites provide a powerful tool in microevolutionary studies of this species. For example, polymorphic microsatellites can be used to identify clonal structure of seagrasses and to estimate seed and pollen dispersal at a fine geographical scale. Furthermore, the developed microsatellites can be applied to *Z. japonica* populations in both invasive and native ranges to locate the origin and invasive route, both of which provide important information in the control and management of alien species.

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APPENDIX 1. Information on voucher specimens deposited at East China Normal University. Information presented: voucher specimen; collection locality.

Zostera japonica:

N. N. Xu et al. BG (HSNU); Beigang, Hainan, China.

N. N. Xu et al. DJ (HSNU); Dongjiao, Hainan, China.