**DEVELOPMENT OF MICROSATellite LOCi FOR THE ENDANGERED SEAGRASS ZOSTERA JAPONICA (ZOSTERACEAE)**

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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* Irmsch 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 *RsaI* (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-TI cloning vector (TransGen, Beijing, China), and transformed into Trans1-Ti 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, Germany).

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of individuals in the population sampled; PIC = polymorphic information content.

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

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | T° (°C) | A | GenBank accession no. |
|-------|--------------------------|--------------|-----------------------|--------|---|----------------------|
| Zj001 | F: GCCAAAGTGGTGGTGAA A R: GAAATGTTGATGGATGA C | (TATC)₆ | 228–236 | 54 | 3 | KP756928 |
| Zj008 | F: ACTTCCCACAACATGGATC A R: GCCCCCTCCTCCTCCTCCA C | (TATC)₆ | 148–160 | 56 | 4 | KP756929 |
| Zj018 | F: CTTAGGGTGTCGCCACTCT A R: CCAAAATACCTCTGTCCTCTC C | (TAGA)₄ | 230–250 | 56 | 4 | KP985542 |
| Zj023 | F: TTTTGCAAGTGGTGTTT A R: CTGTCATGGTGTCAGACGG C | (CTAT)₆ | 329 | 50 | 1 | KP985543 |
| Zj025 | F: GCCGACCTCCTGCACGCTC A R: CTTTCCTCTCCGCCAGCT A | (TATG)₆ | 360–396 | 54 | 5 | KP756930 |
| Zj026 | F: CCTCCTAGCAACTCATC A R: CAAACTCTAAAGCCCAAC C | (TAGA)₆ | 104–116 | 58 | 4 | KP756931 |
| Zj028 | F: CTTCTCTCTCTGGCCAGT A R: TCCAAAAACACCAGCACT A | (TCTA)₆ | 314–350 | 59 | 5 | KP756932 |
| Zj030 | F: GAGGTGATCAAGGAAACCCCA A R: CATAAAGAGGAGCACGACT A | (ATAG)₆ | 272–324 | 54 | 11 | KP756933 |
| Zj033 | F: ACAGGACTAACAGGAGAAGC A R: GTGACAGAGATGGTGGGC A | (ATAG)₂ | 226 | 54 | 1 | KP985545 |
| Zj041 | F: GGGAAACAAAAACACCATC A R: AATGGAACAAACCCACGC A | (TATC)₂ | 144 | 58 | 1 | KP985548 |
| Zj042 | F: CAAACCCTGCACCAAAAC A R: TAGGACCTAAGGCAAACCC A | (TAGA)₆ | 157–169 | 50 | 4 | KP756934 |
| Zj011 | F: ATCCAGGTGGTCTCACCT A R: ATATCTACACCCGCTCTCC A | (TATC)₇ | 336–348 | 56 | 4 | KP985541 |
| Zj029 | F: CCTCCTACAACATACACCACCA A R: GGGAGAGAATAGACCGGAA A | (ATCT)₂ | 149–157 | 58 | 3 | KP985544 |
| Zj036 | F: TTTTCTAAAGGCTTTACCAAA A R: TCACTCTATTTTATATACACCT A | (ATAG)₂ | 290–294 | 56 | 2 | KP985546 |
| Zj037 | F: CCCTCCTCTCGTGTCTTCT A R: TGGCTCTATGGTCTCTCC A | (TAGA)₆ | 382–460 | 52 | 6 | KP985547 |

Note: A = number of alleles observed; T° = annealing temperature of each primer pair.

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

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

| Locus | HQ (N = 16) | FC (N = 16) |
|-------|-------------|-------------|
|       | n  A  Hₑ  Hₛ  PIC  Pᵇ | n  A  Hₑ  Hₛ  PIC  Pᵇ |
| 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  0.0000 |
| Zj018 | 11  4  0.710  0.546  0.623  0.1129 | 16  1  0.000  0.000  0.000  0.0000 |
| 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  2  0.772  0.500  0.710  0.0389 | 16  2  0.585  0.625  0.532  0.9540 |
| Zj029 | 11  3  0.550  0.455  0.466  0.6040 | 16  1  0.000  0.000  0.000  0.0000 |
| 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  0.0000 |
| 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; Hₑ = expected heterozygosity; Hₛ = observed heterozygosity; n = number of individuals genotyped; N = number of individuals in the population sampled; PIC = polymorphic information content.

aLocality and voucher information for the sampled populations are available in Appendix 1.

bP values for deviation from Hardy–Weinberg equilibrium.

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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   |