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Microsatellite primers for *Halophila ovalis* and cross-amplification in *H. minor* (Hydrocharitaceae)[1]

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- *Premise of the study*: Polymorphic microsatellite primers were developed in the seagrass *Halophila ovalis* to investigate genetic variation.
- *Methods and Results*: Ten polymorphic microsatellite loci were developed in *Halophila ovalis*. The number of alleles per locus ranged from 2 to 12 across 80 *H. ovalis* individuals. These loci were successfully amplified in *H. minor*, and four were monomorphic across 30 individuals.
- *Conclusions*: These results from four *H. ovalis* populations and one *H. minor* population show the broad utility of microsatellite loci in future studies of population genetics. Four distinct alleles were present in *H. minor* but absent in *H. ovalis*, indicating potential divergence between them.

Key words: genetic variation; *Halophila minor*; *Halophila ovalis*; microsatellites; seagrass.

Seagrasses are clonal plants that may form dense beds or patches in the littoral zone of tropical and temperate seas, where they provide habitats and food for organisms and affect sedimentary and biogeochemical processes (Duarte and Chiscano, 1999). The dioecious seagrass *Halophila ovalis* (R. Br.) Hook. f. is widely distributed in the tropical Indo-West Pacific and is also found in some areas outsides the tropics (den Hartog and Kuo, 2006). Previous phylogenetic studies found no or little sequence divergence among morphologically distinct species of *Halophila* (Waycott et al., 2002; Uchimura et al., 2008), and *H. minor* (Zoll.) Hartog and *H. ovalis* were demonstrated to be conspecific (Uchimura et al., 2008). Polymorphic microsatellites are powerful tools for population genetic studies (Liu et al., 2009), providing important information for conspecificity. Here we characterize microsatellite markers for *H. ovalis* and their cross-amplification in *H. minor*. These loci will be employed to assess genetic diversity and differentiation across its distribution range in China.

METHOD AND RESULTS

Genomic DNA of an individual of *Halophila ovalis* was extracted from silica gel-dried leaves using the modified CTAB method (Fan et al., 2004). We used the biotin-streptavidin capture method to develop microsatellite primers (Zane et al., 2002). About 300 ng of genomic DNA was digested with *MseI* restriction enzyme (New England Biolabs, Beverly, MA) and fragments of 200–1000 bp in length were fractionated. We ligated the DNA fragments with an *Mse*I-adaptor pair (Vos et al., 1995). We then used five microliters of a 5× dilution of the adapter-ligated fragments as templates for PCR with *Mse*I-N primer (5′-GATGAGTCCTGAGTAAN-3′) in a volume of 20 μl with the following profile: 3 min denaturation at 95°C; followed by 17 cycles of 30 s denaturation at 94°C, 1 min annealing at 53°C, and 1 min extension at 72°C. For enriching the fragment containing simple sequence repeats, the PCR products were denatured at 95°C for 5 min, and then hybridized with 5′-biotinylated probe $(AG)_{15}$ in 250 μl hybridization solution at 48°C for 2 h. Hybridization products were selectively captured with streptavidin-coated magnetic beads (Promega, Madison, WI). After stringent washing, the captured DNA fragments were eluted in 50 μl of 1×TE. The enriched product was amplified with *Mse*I-N as primers for 30 cycles as described above. After purified with a multifunctional DNA Extraction Kit (Bioteke, Beijing, China), PCR products were ligated into pMD 19-T vector (Takara, Dalian, China), and then transformed into *E. coli* strain DH5α. A total of 770 insert-containing clones were picked out and tested by PCR using $(AG)_{10}$ and $M13^+$/$M13^-$ as primers.

A total of 120 positive clones were selected, purified, and sequenced on an ABI 3730 DNA Sequence Analyzer, and 108 were found to contain simple sequence repeats. Fifty-eight of 108 sequences were discarded because either the repeat was too short or the flanking regions of the repeat sequences were not suitable for designing primers. Finally, 50 primer pairs were designed using program PRIMER3 (Rozen and Skaletsky, 2000). These primers were tested for polymorphism in 23 *H. ovails* individuals drawn randomly from the diverse populations in the southern China coastal seas. PCRs were conducted using a PTC-200 thermal cycler (MJ Research, Waltham, MA) in 20 μL volume containing the following components: 50 ng of genomic DNA, 0.2 mM of each dNTPs, 0.1 μM of each primer, 1×PCR buffer (Mg^{2+} free), 1.5 mM Mg^{2+} and 1 U of Taq DNA polymerase (Sangon, Shanghai, China). Microsatellite loci were amplified under the following conditions: 5 min denaturation at 94°C; 30 or 35 cycles of 30 s at 94°C, 30–60 s at 49–65°C, 30–90 s at 72°C; and a final extension of 72°C for 10 min. Amplified PCR products were resolved on 8% polyacrylamide denaturing gel and visualized by silver staining using pUC19 DNA/*Msp*I (*Hpa*II) (Fermentas, Vilnius, Lithuania) as the ladder. Finally, we obtained 10 polymorphic markers (Table 1).

We further tested the applicability of the 10 loci in 80 individuals of 4 *H. ovalis* populations and 30 individuals of an *H. minor* population. Voucher

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TABLE 1. Characteristics of 10 polymorphic microsatellite loci developed in *Halophila ovalis*. Forward and reverse primers, repeat motif, allele size range, optimal annealing temperature (T_a), and GenBank accession numbers are given.

Locus	Forward primer	Reverse primer	Repeat motif	Allele size range (bp)	T_a(°C)	GenBank accession #
HO2	GAGGTCTTCGTATCGTCTG	GCATCTTGTGGTGGTTCT	$(GAA)_5(GAG)_7$	117-141	61	GQ502725
HO3	CGAGGCTATGTTCCAGAT	CAATGCCCAAGTAGGTGA	$(CT)_{13}$	192-208	57	GQ502724
HO5	GAATGGGAAGGTGAAAGAG	CACGGCACTGTTCATCTAC	$(GA)_{13}$	156-186	60	GQ502726
HO8	ATAACCAAAGCCTCCCAAGC	AAATATCAAACGCCCCTCAC	$(AT)_9(GA)_{10}$	260-296	59	GQ502722
HO20	AGAGGAAAAGAAAAGCGAG	ATGTCACGTGGGACCATAT	$(AG)_{11}$	134-152	62	GQ502729
HO30	CGCCGAAGGAAATGTGGAG	AACCCAACCGATCGACCCT	$(GA)_{10}$	120-128	59	GQ502730
HO31	GGTTGTGCGTGAGGTGAAT	ATACGCAGGTACGCACTCT	$(AG)_6$	215-223	63	GQ502728
HO36	CAACTAACCAAACGAGAAAC	AACCTTGACACCTGCTAATA	$(GA)_{19}$	196-246	49	GQ502723
HO48	ATCGAACCCAATAGACACCAG	CAGGCAACTTAGCAAGAAACT	$(GA)_7GC(GA)_4$	220-240	65	GQ502731
HO51	AGATAAGTTTCACTCCTGTG	ACCAGAACCAATCAAGAT	(GA)19	141-175	46	GU988754

TABLE 2. Number of samples genotyped (n), number of alleles (N_A), observed (H_O) and expected (H_E) heterozygosities for *Halophila ovalis* and *H. minor*. The vouchers of the sampled populations were deposited in the herbarium of East China Normal University, and the accessions were N. N. Xu et al. DJ-3(HsNu), S.Yu et al. XL-4(HsNu), N. N. Xu et al. XW-3(HsNu), N. N. Xu et al. YL-3(HsNu), and N. N. Xu et al. LS-2(HsNu).

	H. ovalis Dongjiao (N19°31′33.6″, E110°51′14.5″)				Xinlong Village (N 19°53′51.3″, E109°29′56.7″)				Xialongwei (N 21°30′2.1″, E109°37′14.9″)				Yingluo Bay (N 21°24′38.2″, E109°45′40.7″)				*H. minor* Liusha Bay (N 21°26′6.0″, E109°57′6.0″)			
Locus	n	N_A	H_O	H_E	n	N_A	H_O	H_E	n	N_A	H_O	H_E	N	N_A	H_O	H_E	n	N_A	H_O	H_E
HO2	20	5	0.700	0.591	20	2	0.800	0.495	20	2	0.300	0.255	20	3	0.500	0.541	14	2	0	0.133
HO3	20	5	0.200	0.719	20	3	0.050	0.141	20	4	0.250	0.516	20	2	0.100	0.180	30	1	0	0
HO5	19	5	0.842	0.723	20	4	0.850	0.749	20	5	0.950	0.666	20	5	0.700	0.734	28	4	0.250	0.328
HO8	20	6	0.650	0.633	19	3	0.632	0.470	20	4	0.700	0.715	19	7	0.158	0.511	27	7	0.444	0.615
HO20	20	6	0.900	0.755	20	6	0.900	0.731	20	4	0.900	0.696	20	2	0.050	0.139	30	1	0	0
HO30	20	2	0.700	0.480	20	2	0.700	0.495	20	2	0.200	0.180	19	2	0	0.100	30	2	0.033	0.033
HO31	20	2	1.000	0.500	20	2	1.000	0.500	20	2	1.000	0.500	20	2	0.450	0.349	30	1	0	0
HO36	19	4	0.895	0.549	19	4	0.474	0.398	20	5	0.700	0.563	13	2	0.154	0.260	24	4	0. 458	0.383
HO48	20	4	1.000	0.588	20	3	0.650	0.491	20	6	0.500	0.506	18	7	0.667	0.784	30	6	0.767	0.676
HO51	17	8	0.882	0.772	18	6	0.944	0.707	20	6	1.000	0.720	15	3	0.133	0.184	29	1	0	0

specimens were deposited in the herbarium of East China Normal University, China. Forward polymorphic primers were labeled with either fluorescent dye 5′HEX, 5′TAMRX, 5′TET, 5′ROX or 5′6-FAM (Sangon). Amplification of each loci was carried out in 10-μl reaction volume for fragment length scoring on ABI 3130 automated sequencer using an internal lane standard (GS500(−250)Liz). Allele binning and calling was performed using software GENEMAPPER 4.0 (Applied Biosystems, Foster City, CA).

Each polymorphic locus had 2–12 alleles per locus and an average of 7.1 across all tested *H. ovalis* individuals (Table 2). The observed and excepted heterozygosities ranged from 0 to 1.000 and from 0.100 to 0.784, respectively, in populations of *H. ovalis* (Table 2) using software TFPGA v1.3 (Miller, 1997). All ten loci were successfully amplified in the *H. minor* population. However, four loci (*HO3*, *HO20*, *HO31,* and *HO51*) were monomorphic, and the six polymorphic loci had 2–7 alleles per locus (Table 2). Among the polymorphic loci, *H. minor* had 3 and 1 distinct alleles at loci *HO8* and *HO48*, respectively, which were absent in *H. ovalis*.

CONCLUSIONS

Microsatellites reported here may be useful for further studies of population genetics, which play a critical role in future protection and maintenance of the seagrasses *H. ovalis* and *H. minor*. Four distinct alleles are present in *H. minor* but absent in *H. ovalis,* indicating potential divergence between them.

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