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PRIMER NOTE

Isolation and characterization of a set of microsatellite loci in the submerged macrophyte, *Vallisneria spinulosa* Yan (Hydrocharitaceae)

LEI CHEN,*† DONALD H. LES,‡ LIMING XU,* XIAOHONG YAO,*† MING KANG* and HONGWEN HUANG*

**Wuhan Botanical Garden/Wuhan Institute of Botany, The Chinese Academy of Sciences, Wuhan, Hubei 430074, China, †Graduate School of the Chinese Academy of Sciences, Beijing 100039, China, ‡Department of Ecology and Evolutionary Biology, University of Connecticut, U-3043, Storrs, Connecticut, USA*

Abstract

Twenty-six microsatellites were isolated and characterized from an AAG-enriched genomic library of *Vallisneria spinulosa*, a dominant submerged macrophyte in the middle-lower reaches of the Yangtze River. Microsatellite polymorphism was evaluated in a random population and 11 microsatellite loci were found polymorphic with 2–18 alleles per locus and observed heterozygosity ranging from 0.281 to 1.000. These polymorphic markers were expected to be valuable for population genetic and demographic analyses of *V. spinulosa*.

Keywords: aquatic macrophyte, clonal diversity, population dynamics, population structure, shallow lake, Yangtze River

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Thousands of shallow lakes are located in the middle-lower reaches of the Yangtze River and are of ecological and economical importance to wetland ecosystem and water resource management in the region. Most areas of these shallow lakes are capable of sustaining a rich variety of submerged macrophytes. *Vallisneria spinulosa* Yan (Hydrocharitaceae) is one of the dominant submerged species in these lakes, which is of ecological importance as structuring components in these shallow lakes (Sun 1992). *V. spinulosa* is capable of both sexual and asexual reproduction. In our previous study, allozyme markers have been used to assess clonal and genetic diversity as well as population genetic structure for *V. spinulosa*. But they lacked sufficient resolution to investigate the population dynamics of *V. spinulosa* (Chen *et al*. submitted). Markers that are more polymorphic are required to infer the historical and contemporary genetic connections for this keystone species in these hydrologically continuous but geographically isolated lakes in the scale of whole region. Here, we report the isolation and characterization of a set of polymorphic microsatellite loci from genome of *V. spinulosa* in order to further investigate population genetic structure and gene flow of *V. spinulosa* in these shallow lakes.

Correspondence: Hongwen Huang, Fax: 0086-27-87510331; E-mail: hongwen@wbgcas.cn

Genomic DNA was extracted from leaf tissue of *V. spinulosa* using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle & Doyle 1987). Microsatellite markers were developed for *V. spinulosa* using a combined approach of protocols as described by Zane *et al*. (2002) and by Hammond *et al*. (1998) with some minor modifications. The DNA was digested with restriction enzyme *Sau*3AI (BioLabs), and fragments ranging from 300 to 1000 bp were ligated into *Sau*3AI linkers (SAULA: 5′-GCGGTACCCGGGAAGCTTGG-3′; and SAULB: 5′-GATCCCAAGCTTCCCGGGTACCGC-3′). The digestion-ligation mixture was then diluted (1:10) and amplified using the *Sau*3AI linker-SAULA. The polymerase chain reaction (PCR) was carried out with an initial extension for 5 min, followed by 28 cycles of 94 °C for 30 s, 67 °C for 30 s, 72 °C for 60 s followed by a final extension step for 10 min at 72 °C. The enrichment for microsatellites was conducted essentially as described in detail by Chen *et al*. (2006). The DNA fragments enriched for microsatellite were amplified with an initial denaturation at 94 °C for 3 min, followed by 24 cycles at 94 °C for 1 min, 68 °C for 1 min, 72 °C for 2 min followed by a final extension step for 10 min at 72 °C using SAULA. PCR products were purified

Table 1 Primer sequences and characteristics of 11 polymorphic microsatellite loci in *Vallisneria spinulosa*

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Allele size range (bp)	N_A	H_O H_E	GenBank Accession no.
VS01	$(AAG)_{13}$	F: TTCCATTTTCCCTTTTGCAC	57	141–162	8	0.594	DQ513266
		R: GCTCGATGTTCCCTTGAGTC				0.764	
VS03	$(AAG)_{23}$	F: GGTGAGTTGCAATCCTTGGT	59	153–257	18	0.906	DQ513267
		R: CCAATTGAGCTTCTGGCAAT				0.930	
VS05	$(TTC)_{14}CAC(TTC)_{11}$	F: ACAAGGGCAAGGAAAAGGTT	57	164–173	3	0.281	DQ513268
		R: AACCCCCATCATCCTCTCTC				0.301	
VS06	$(TTC)_{13}$	F: TTCTGACCACAGGATTTCCA	59	144–186	10	0.750	DQ513258
		R: TCTTACCTCCATCCCTTCCA				0.864	
VS08	$(AAG)_{17}$	F: TCTTACCTCCATCCCTTCCA	61	156–209	10	0.281*	DQ513259
		R: CTCGATTTTGTACCGGTGGT				0.842	
VS10	$(CTT)_{10}$	F: TCATCAGCCATGAAGCAAAC	57	207–231	4	0.375	DQ513260
		R: TCGAAGAAGCACTGACGTGT				0.531	
VS11	$(TTC)_{17}$	F: ATTGGCCATGAAGCACACTT	58	253–269	3	0.500	DQ513261
		R: TGCTTCCAATGCAACAAAAG				0.412	
VS20	$(AAC)_8$	F: CAGACCGACCATGTCAACTG	61	215–230	6	0.813	DQ513262
		R: CCACCTAAGTGTGGGAAAGG				0.800	
VS24	$(GT)_{12}$	F: GTGGACGGTTGTGGTGAAG	60	222–237	2	0.469	DQ513263
		R: CGTGGCAGTCAAAGAGTCAA				0.365	
VS28	$(CA)_9(CG)_8$	F: CAATCTGAGCCTCCACACCT	60	183–185	2	1.000	DQ513264
		R: CCGAGTAGCAGTGTGTAATGC				0.508	
VS29	$(AC)_9$	F: AGGCGAGCACCTGAGATAAG	61	193–197	3	0.906*	DQ513265
		R: CTATGGGCTTCTTGCTGTCC				0.536	

T_a, PCR annealing temperature; N_A, number of alleles; H_O, observed heterozygosity; H_E, expected heterozygosity.
*indicates the observed heterozygosity is significantly different from the expected heterozygosity under Hardy–Weinberg equilibrium ($P < 0.01$).

using E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek). The purified fragments were ligated into the pMD18-T plasmid vector (TaKaRa) and transformed into DH5á competent cells (Invitrogen). Recombinant clones were detected by PCR amplification using M13 forward and reverse primers. Ninety-eight clones with foreign inserts were sequenced by a commercial laboratory (Sunbiotech) using the M13 forward primer and an ABI 377XL DNA sequencer. A total of 46 sequences containing microsatellites were obtained and then subjected to primer designing using PRIMER 3 web interface program (Rozen & Skaletsky 2000). Twenty-nine primer pairs were used for further analyses.

All of the 29 pairs of simple sequence repeat (SSR) primers were tested using 32 *V. spinulosa* individuals randomly sampled from Huanggai Lake in the middle reach of the Yangtze River. PCR amplifications were performed in a final volume of 10 μL containing 50 ng of genomic DNA, 1 × *Taq* buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM each primer, and 0.5 U of *Taq* polymerase (Fermentas). The amplification profiles included an initial denaturing at 94 °C for 5 min, followed by 35 cycles of 50 s at 94 °C, 50 s at 57–61 °C depending on the primer pair (Table 1) and 90 s at 72 °C, followed by a final extension step for 10 min at 72 °C. The gel electrophoresis of PCR products and the visualization by silver staining were carried out as described in Zhen *et al*. (2004). The DNA fragments were sized using a standard 25-bp DNA ladder (Promega).

Of the 29 primer pairs tested, 26 successfully amplified target regions, while 11 revealed polymorphism. These 11 microsatellite loci were sufficiently polymorphic to allow identification of genets. All the 32 individuals tested were revealed as different clones based on these 11 polymorphic loci, suggesting a high clonal diversity in *V. spinulosa*. The number of alleles per locus, observed and expected heterozygosities as well as Hardy–Weinberg expectations and genotypic disequilibrium were estimated using FSTAT version 2.9.3 (Goudet 2001). The number of alleles per locus was 2–18 with an average value of 6.3. The observed and expected heterozygosities ranged from 0.281 to 1.000, and from 0.301 to 0.930, respectively. Deviations between expected and observed heterozygosities were statistically not significant for most loci, except for VS08 and VS29 ($P < 0.01$, after Bonferroni's correction) (Table 1). There was no evidence of linkage disequilibrium (LD) found in all 66 locus pairs but one. Significant LD was only detected between VS05 and VS24 ($P < 0.01$, after Bonferroni's correction). The results proved that most of the loci could be useful for standard population genetic studies.

These loci presented here are the first set of microsatellite markers for *V. spinulosa* and also for *Vallisneria* genus. All

these loci are currently being used for inferring demographic process of *V. spinulosa* in typical shallow lakes in the middle-lower reaches of the Yangtze River, which will provide additional insight and help to develop strategies for the conservation and management of aquatic plant communities in this important freshwater ecosystem.

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