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Development of 11 polymorphic microsatellite markers in a macrophyte of conservation concern, *Vallisneria americana* Michaux (Hydrocharitaceae)

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Abstract

***Vallisneria americana* Michaux (wild celery) is currently a target of submersed aquatic vegetation restoration efforts in the Chesapeake Bay watershed. To aid these efforts, we have developed 11 polymorphic microsatellite markers to assess the distribution and degree of genetic diversity in both restored and naturally occurring populations in the Chesapeake Bay. In 59 individuals from two populations, we detected two to 10 total alleles per locus. Observed heterozygosity ranged from 0.125 to 0.929, and two loci exhibited significant deviations from Hardy–Weinberg equilibrium in at least one of the populations assayed.**

Keywords: Chesapeake Bay, conservation genetics, submersed aquatic vegetation (SAV), wild celery

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Vallisneria americana Michaux (wild celery) is a dioecious, clonal macrophyte native to eastern North American freshwater and oligohaline habitats (Wilder 1974; Korschgen & Green 1988; Catling *et al*. 1994). Where it occurs, *V. americana* is a dominant member of freshwater submersed aquatic vegetation (SAV) communities. As a member of these communities, *V. americana* provides habitat for many fish and aquatic invertebrates (Rozas & Minello 2006), furnishes migratory waterfowl with considerable food resources (Korschgen & Green 1988), and improves water quality by stabilizing sediments (Madsen *et al*. 2001) and buffering nutrient levels (Moore 2004). Severe declines of SAV in the Chesapeake Bay have paralleled declines in the health and economic productivity of this renowned estuary (Kemp *et al*. 1983; Orth & Moore 1983, 1984; Boesch *et al*. 2001), prompting a regional consensus to restore SAV populations to historic levels (Chesapeake Executive Council 2000). However, progress has been constrained by an insufficient understanding of environmental and biological variables that determine the restoration potential of each species. Currently uncompleted, an assessment of the genetic diversity of natural populations and restoration stock is essential to aid large-scale restoration effort, as genetic diversity has been linked to plant fitness and restoration potential (Williams 2001). We are therefore investigating the distribution and degree of genetic variation in nbsp;*V. americana* from both current restoration stocks and naturally occurring populations in the Chesapeake Bay. Below is a description of our development of 11 microsatellite markers for use in this characterization.

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Using purified DNA from an individual collected at Weir Cove in the Gunpowder River (Maryland, USA), Genetic Identification Services (GIS) created DNA libraries enriched for ATG, AAC, AAG and CAG nucleotide repeats as described by Jones *et al*. (2002). Transformed clones were selected from blue-white screening media, grown overnight in Luria-Bertani broth, and then used directly as template for polymerase chain reaction (PCR). Plasmid DNA was amplified with universal M13 primers and purified (QIAGEN QIAquick PCR Purification Kit and Millipore MultiScreen MAFB NOB Plates) before sequencing. Ninety-five plasmids containing inserts were sequenced by GIS on an ABI PRISM 377 DNA Sequencer with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences). We sequenced an additional 379 clones on an ABI 3730xl DNA Analyser using the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems). Sequence traces were trimmed, aligned and visually inspected with CodonCode Aligner (http://www.codoncode.com/aligner/). After screening out highly similar sequences using a local database BLAST search (Altschul *et al*. 1997), 203 primer pairs were designed using DesignerPCR version 1.03 (Research Genetics, Inc.) (52 pairs from GIS) and Primer 3 (Rozen & Skaletsky 2000).

Leaf tissue was collected from two naturally occurring *V. americana* populations in the northern stem of the Chesapeake Bay, one population in the Gunpowder River (Dundee Creek, $n = 30$) and another at the confluence of the Back and Middle rivers (Rocky Point-Hawks Cove, $n = 29$). Genomic DNA was isolated and purified using either of the following two methods: (i) hand-grinding ~100 mg of frozen leaf tissue under liquid nitrogen in conjunction with a DNeasy Plant Mini Kit (QIAGEN), or (ii) disrupting ~20 mg of frozen leaf tissue in a QIAGEN TissueLyser and completing the purification on a BioSprint 96 workstation (QIAGEN) with BioSprint 96 DNA Plant Kits (QIAGEN). Purified stock DNA aliquots were diluted 1 part to 5 parts nano-pure H_2O to their final working concentrations.

Amplification was examined using at least two individuals representing separate populations. Primer sets with robust and specific amplification were further assayed for fragment length polymorphism across a minimum of eight individuals. PCR was performed on an MJ Research PTC-200 Peltier Thermal Cycler using proprietary reagents in the Top*Taq* DNA Polymerase Kit (QIAGEN). Standard conditions were as follows: 1.0 μL DNA template, 0.3 μM each primer, 0.05 U/μL Top*Taq* DNA Polymerase, 175 μM each dNTP, 1× Coral Load (for direct loading of product to electrophoresis gels; excluded in fluorescent-labelled reactions), 1× PCR buffer, 2.5 mM MgCl, and nano-pure H_2O to a final volume of 10 μL. We used a touchdown programme (designed from preliminary reactions) with the following parameters: 94 °C, 3 min; [94 °C, 40 s; 65 °C, 40 s (–0.3 °C/cycle); 72 °C, 30 s] × 30 cycles; (94 °C, 40 s; 55 °C, 40 s; 72 °C, 30 s) × 5 cycles; 72 °C, 12 min. Amplicons were visualized on 1.5% agarose gels stained with ethidium bromide. Fragment length polymorphism was detected on an ABI 3730xl DNA Analyser with GeneScan 500 ROX or 500 LIZ Size Standard (Applied Biosystems) after tagging the PCR product with fluorescent-labelled forward primers (Applied Biosystems). Peak data were analysed using GeneMapper software version 3.7 (Applied Biosystems), and all allele calls were visually inspected. To eliminate or reduce the signal of confounding nonspecific amplicons, some loci required reaction optimization, including varying magnesium ion concentration and using additives (Table 1). Dimethyl sulfoxide (5%) and 1× Q-solution (QIAGEN) were independently added to increase specificity. Because we found

Table 1 Characteristics of 11 *Vallisneria americana* microsatellite loci and primers (data pooled from two populations unless noted otherwise; Dundee Creek (Pop1) $n = 30$, Rocky Point-Hawks Cove (Pop2) $n = 29$)

Locus	Repeat motif	Primer sequence (5′–3′)	Nonstandard PCR condition(s)	*N* (Pop1, Pop2)	Size range (bp)	*A*	**Pop1** H_O H_E	**Pop2** H_O H_E	GenBank Accession no.
Vaam_AAG002	$(GAA)_7$	F: 6-FAM-TTGTGTTGAAGGAACCGAATGA	1× Q-solution	30,29	319–325	3	0.567	0.517	EU938635
		R: GTAGGAGGAAGTGGTATGGGAA					0.467	0.469	
Vaam_ATG002	$(TCA)_{11}$	F: NED-CAACTCCACACACACAGAAGTA	5% DMSO	30,29	141–166	7	0.700	0.759	EU938638
		R: TGTTTGATCGGCCTAGATGATG					0.833	0.791	
Vaam_AAG004	$(CTT)_8$	F: NED-TGCTCAGGAAGAGTTCAGTCTA	1× Q-solution	30,28	376–397	6	0.633	0.536	EU938636
		R: CCCCAAGCAGAAACAGTAAGAT					0.612	0.577	
Vaam_M13	$(GAA)_9$	F: NED-CTCAGCATCAGCAACACC	5% DMSO,	29,29	263–283	8	0.690	0.655	EU938633
		R: ACACGCTCCTCCACTACG	3mM Mg^{2+}				0.774	0.731	
Vaam_M16	$(TTG)_6$	F: HEX-AAATGCTGGAAGGAGACTATC	1× Q-solution	30,29	187–190	2	0.167	0.448	EU938637
		R: TTCTGGAATCTTAGTTTGGAAC					0.155	0.390	
Vaam_M49	$(CTT)_2T(CTT)_{16}$	F: PET-AAGGAGATGAAACAAAGATGG	Standard	30,29	165–198	9	0.700	0.724	EU938634
		R: GCCCAAATAAAATGAAGAGAG					0.775	0.772	
Vaam_AAG_X012	$(AAG)_9$	F: PET-CCAAATGGGCTGAATGTTGATG	Standard	30,29	202–214	5	0.633	0.517	FJ417084
		R: TGAAATAACGACCTCACTTGGG					0.528	0.543	
Vaam_AAG_X013	$(TCC)_5(TTC)_2$ $TCC(TTC)_7$	F: VIC-TTATTCCTTCGACCTTCGTTCC	5% DMSO	24,26	216–249	5†	0.125*	0.269*	FJ417085
		R: AATGCTCCATAACCAGACAAGG					0.439	0.686	
Vaam_ATG_X030	$(CAT)_8$	F: 6-FAM-CTCCTTCCTATATGCTTCTGCG	5% DMSO	30,29	153–162	3	0.333	0.586	FJ417088
		R: CATGCTCCCATGTCTTACCTAC					0.344	0.515	
Vaam_AAG_X051	$(AAG)_{12}$	F: VIC-TTCTGGAAGTTAGTAATGACC	1× Q-solution	30,28	172–199	10	0.800	0.929	FJ417086
		R: GCATTGAGGAGATTGAAGGATT					0.859	0.876	
Vaam_AAG_X071	$(AGA)_{13}$	F: 6-FAM-GGAAATTCGAGTAGCTGTACCA	1× Q-solution	30,29	224–245	8	0.533*	0.793	FJ417087
		R: GTTCTAGCGAAATCGGTTGTTG					0.718	0.807	

Nonstandard PCR condition(s), additions or changes to the standard reaction conditions described in the text; DMSO, dimethyl sulfoxide; Q-solution is provided in the QIAGEN Top*Taq* DNA Polymerase Kit; *N*, number of individuals successfully genotyped; *A*, total number of alleles observed; H_O, observed heterozygosity; H_E, expected heterozygosity; *value differs significantly from expectation under Hardy–Weinberg equilibrium ($P_{Bonferroni} < 0.0045$); †potential null alleles were detected at this locus.

these additives to have locus-specific effects on amplification consistency and allelic amplification bias (i.e. null alleles), we assayed each locus across a subset of individuals and compared the profile of the standard condition with those of each additive to determine the optimal chemistry.

We found 11 microsatellite loci to be both robust and polymorphic, and with these markers we successfully detected 637 of the 649 (98%) possible genotypes ($n = 59$). Allelic diversity and heterozygosity levels were estimated in Arlequin version 3.11 (Excoffier *et al*. 2005). Total alleles per locus ranged from two to 10 (average of six). Expected and observed heterozygosities ranged from 0.155 to 0.876 and from 0.125 to 0.929, respectively. Two loci (Table 1) exhibited significant deviation from Hardy–Weinberg equilibrium, following correction for multiple comparisons ($P_{\text{Bonferroni}} < 0.0045$). Significant linkage disequilibrium was detected in seven of 55 possible locus pairs before correction ($P < 0.05$); after correction however ($P_{\text{Bonferroni}} < 0.0009$), no significant linkage disequilibrium was detected, according to FSTAT version 2.9.3 (Goudet 2001). Evidence of a possible null allele at the locus Vaam_AAG_X013 was detected by Micro-Checker 2.2.3 (van Oosterhout *et al*. 2004). We anticipate that the use of these variable loci to quantify genetic variation will provide data for more informed management decisions and allow for an examination of the effect genetic diversity on fitness.

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