Characterization of 16 Microsatellite Markers for the Oreinotinus Clade of Viburnum (Adoxaceae)

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PRIMER NOTE

CHARACTERIZATION OF 16 MICROSATELLITE MARKERS FOR THE *OREINOTINUS* CLADE OF *VIBURNUM* (ADOXACEAE)[1]

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- *Premise of the study:* Microsatellite loci were isolated from four species of *Viburnum* (Adoxaceae) to study population structure and assess species boundaries among morphologically similar South American *Viburnum* species of the *Oreinotinus* clade.
- *Methods and Results:* Using a microsatellite-enriched library and mining next-generation sequence data, 16 microsatellites were developed. Each locus was tested on two populations of *V. triphyllum* and one population of *V. pichinchense*. For nuclear loci, one to 13 alleles were recovered, expected heterozygosity ranged from 0 to 0.8975, Simpson diversity index ranged from 0.0167 to 1.000, and Shannon diversity index ranged from 0 to 2.3670 in a given population. For the mitochondrial locus, three to six alleles were recovered and unbiased haploid diversity values ranged from 0.756 to 0.853 in a given population.
- *Conclusions:* The 16 microsatellite loci developed for the *Oreinotinus* clade (*Viburnum*, Adoxaceae) will inform investigations of population structure and species boundaries within this group.

Key words: Adoxaceae; genetic diversity; *Viburnum dentatum*; *Viburnum hallii*; *Viburnum pichinchense*; *Viburnum trilobum*; *Viburnum triphyllum*.

Viburnum L. (Adoxaceae) is a clade of approximately 165 species of shrubs and small trees that occur in northern temperate forests, the mountains of Central and South America, and subtropical montane forests of Southeast Asia. The phylogeny of *Viburnum* provides a clear understanding of relationships among major clades (Spriggs et al., 2015). However, evolutionary relationships within *Viburnum* clades that have experienced upward shifts in diversification rates, such as *Oreinodentinus*, are largely unresolved (Spriggs et al., 2015). *Oreinodentinus* is composed of *Oreinotinus* (ca. 32 species in Latin America; Killip and Smith, 1930; Morton, 1933) and *Dentata* (possibly three species native to eastern North America; Spriggs et al., 2015).

Phylogenetic analyses using plastid regions and the nuclear ribosomal internal transcribed spacer (ITS) region have supported the monophyly of *Oreinotinus* but have not fully resolved species relationships within the clade (Spriggs et al., 2015). Furthermore, relationships within the South American *Oreinotinus* clade are best described as a polytomy, and species boundaries are difficult to assess due to morphological similarity and ontogenetic variation. Although species in the South American *Oreinotinus* clade have been delimited based on morphological characters (Killip and Smith, 1930), our field studies suggest an evolutionary investigation will yield different species boundaries. More variable molecular markers are needed for such an analysis. Microsatellite loci (simple sequence repeats [SSRs]) have been developed to distinguish cultivated varieties of *V. dilatatum* Thunb. and closely related species (Dean et al., 2011) that belong to the distantly related *Viburnum* clade, *Succotinus*, of eastern Asia (Spriggs et al., 2015). Development of SSR loci specific to *Oreinotinus* will allow investigation of population dynamics and species boundaries within this group. We describe 16 novel microsatellite markers developed from *V. hallii* (Oerst.) Killip & A. C. Sm. (*Oreinotinus*) and *V. trilobum* Marshall (*Opulus*) and recovered from mining next-generation sequence (NGS) data for *V. dentatum* L. (*Dentata*) and *V. triphyllum* Benth. (*Oreinotinus*).

METHODS AND RESULTS

Construction of a microsatellite-enriched library and mining of NGS data were used to identify candidate loci. *Viburnum hallii* (collected from Ecuador) and *V. trilobum* (collected from Massachusetts, USA; Appendix 1) were used to construct microsatellite libraries (following V. Symonds, personal communication). Total genomic DNA was extracted from silica-dried leaves using a FastDNA kit (MP Biomedicals, Santa Ana, California, USA). DNA was digested using Sau3AI and was visualized using gel electrophoresis. Linkers constructed with SAU-LA and SAU-LB oligos were ligated to the DNA fragments for 16 h at 16°C. A nested PCR was used to verify linker ligation. PCR products

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were hybridized to a mix of $(CA)_n$ and $(GA)_n$ biotinylated probes. DNA fragments containing microsatellites were recovered from the PCR products using Streptavidin MagneSphere Paramagnetic Particles (Promega Corporation, Madison, Wisconsin, USA). SAU-LA primers were used to construct a second strand and repeat-enriched library. These products were cloned using a StrataClone PCR Cloning Kit (Agilent Technologies, Santa Clara, California, USA) and screened using T7 and M13R plasmid primers. Colonies with inserts containing repeat regions (144 selected from *V. hallii* and 144 from *V. trilobum*) were grown in liquid cultures and subjected to rolling circle amplification and sequencing in a single direction at the Interdisciplinary Center for Biotechnology Research at the University of Florida (Gainesville, Florida, USA). Using Primer3 (Rozen and Skaletsky, 1999), we designed primers for 22 fragments containing at least six dinucleotide repeats. PCR amplification of SSRs was as follows: 94°C for 3 min; 40 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 45 s; and a 20-min extension at 72°C. Finally, we retained only those loci with sequences verified through cloning. Six to eight alleles from two different individuals per locus were isolated and sequenced using a StrataClone PCR Cloning Kit (Agilent Technologies) following the manufacturer's protocol with the exception of using half reactions. Sanger sequencing was performed at the DNA Analysis Facility on Science Hill at Yale University (New Haven, Connecticut, USA). Six loci were optimized.

We mined Illumina 100-bp paired-end NGS data from *V. dentatum* (collected from Connecticut, USA) and *V. triphyllum* (collected from Ecuador; SRP041815). First, data were assembled using reference-based assembly to a *Lonicera* L. plastid (M. Moore, personal communication) using the read mapping assembler in Geneious R8 (Biomatters, Auckland, New Zealand). Reads mapped to *Lonicera* were saved and thereafter considered plastid regions. Using the same approach, the unused reads were mapped to *Helianthus* L. mitochondria (NC023337). Mapped reads were saved and thereafter considered mitochondrial regions; unused reads were considered part of the nuclear genome. The plastid, mitochondrial, and nuclear sequence sets were subjected to de novo assembly using Velvet version 1.2.10 (Zerbino and Birney, 2008) within Geneious R8 (Biomatters). Putative SSRs that contained five or more perfect dinucleotide repeats were identified from the assemblies using MSATCOMMANDER (Faircloth, 2008) followed by primer design using Primer3 (Rozen and Skaletsky, 1999). Ten loci were optimized.

All SSR loci were screened in 46 individuals from two populations of *V. triphyllum* ($n = 16$, $n = 17$) and one population of *V. pichinchense* Benth. ($n = 13$). Forward primers were complemented with a fluorescently labeled M13 primer (5′-CACGACGTTGTAAAAC-3′) for fragment detection (Schuelke, 2000). Loci were amplified in 10-μL reaction volumes containing 1 unit of GoTaq polymerase (Promega Corporation), 1× GoTaq Reaction Buffer, 25 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 μM forward primer, 5 μM reverse primer, 8 μM labeled M13 primer, and 1.9–82.9 ng of DNA. Adding 1.5 μg of bovine serum albumin (BSA) improved amplification of some loci (Table 1). PCR amplification conditions were the same as described above. For locus DN16, a fluorescently labeled forward primer (5 μM per reaction) was used instead of the M13 system and amplified as follows: 94°C for 2 min; 30 cycles of 94°C for 20 s, 52°C for 20 s, and 72°C for 40 s; and final extension of 72°C for 5 min. Fragment analysis was performed at the DNA Analysis Facility on Science Hill at Yale University.

Polyploidy has been detected in two of the four *Oreinotinus* taxa that have been subject to chromosome counts (Egolf, 1962; Donoghue, 1982). Phylogenetic studies sampling a low-copy nuclear gene region (*GBSSI*) detected additional duplications in *Viburnum* clades where polyploidy had been confirmed, including *Oreinotinus* (Winkworth and Donoghue, 2004). Furthermore, we recovered three or more alleles per locus from 10% of the individuals sampled. For the purposes of these analyses, we first considered these viburnums to be allotetraploids. For nuclear loci, we calculated expected heterozygosity (H_e) and Shannon diversity index (H') using ATETRA with 100,000 Monte Carlo simulations of possible allele combinations for partial heterozygotes (Van Puyvelde et al., 2010). We then calculated an additional diversity index that assumed autotetraploidy, Simpson diversity index (D), using the polysat package of

Table 1. Characteristics of 16 microsatellite loci developed in *Viburnum triphyllum* and *V. pichinchense.*

Locus[a]	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	GenBank accession no.
H121	F: *ACCCCCTCTCCTTCTCTGTG R: GGAGAGTGTAAGGCTCTACTC	$(CT)_9$	156–174	KX447798
O42	F: *GGCCATTAGAAAAGGTCTTCG R: CCACGCGGGAATAATACG	$(TA)_6(TG)_5(TA)(TG)_5$	183–189	KX447799
O91	F: *CCACAATGGCTTCCTTTGTAA R: CCGAAGAATCCATGTTGGAC	$(AC)_{12}$	200–266	KX447800
O104	F: *GGTAATCGACCACAACACGA R: ATCTCGAGGGAGACTGCAAC	$(AC)_{19}$	168–186	KX447801
O121[b]	F: *CTCTCTCCTGTGTCTACCTTGAGC R: TGGGGGTTGTAATTTCTCCA	$(AG)_{14}$	112–158	KX447802
H81	F: *GGGCCGAGTTCTTTTAAACC R: GAAGCGAAATGCACTCAACA	$(GA)_{16}$	199–233	KX447797
TN2	F: *GGTTGAGTGTTGCAGGAAGG R: CGACCTTTGGCAATGGACTC	$(GT)_5$	236–244	KX447804
TN3	F: *AGTGTGGGTATGAGATGGGC R: ACTTACTCACGCTCCACTCG	$(TA)_5$	138–144	KX447805
DM1[c]	F: *GCCCTATACCCACCCAATTTC R: ATACGAGTCCAAAGGCAGGG	$(AT)_6$	411–427	KX447806
DN10	F: *GTTAGCAATGGGGAGCCAAG R: GAGTCGATCGCTCAAATAGAGG	$(CA)_{10}$	140–150	KX447809
DN13	F: *CAACTTTGGTGGGTTTGAAAGC R: TTCTGCCAATTGATGCATCTTG	$(CT)_9$	222–238	KX447810
DN15	F: *TTCTTTCCCTCCCTCTGCAG R: CAGAGGCTAGGGTTATGGGC	$(TA)_8$	108–134	KX447811
DN16[b]	F: AACTCTCACCGCTCACCATC R: TGGGCTTGAGAGTTCGCTAG	$(AG)_8$	352–380	KX447812
DN18	F: *CACTCCATTGCACCTTCACC R: TGGTAAGGTGGCATATCGGG	$(TA)_8$	240–250	KX447813
DN19[b]	F: *CCTCCAGAGCTTCGCCTC R: TCACCGTAGCTAGAAATGTCAG	$(CT)_8$	449	KX447814
DN22	F: *GTGCCTTAACAGCCCAGAAG R: AGGGCTTGGACTCCGAAATC	$(AG)_7$	373–483	KX447815

[a]Annealing temperature for all loci was 52°C.
[b]Loci amplified with the addition of BSA.
[c]Loci located in the mitochondria.
*Primer preceded by a fluorescently labeled M13 tag (CACGACGTTGTAAAACGAC).

TABLE 2. Genetic properties of the 16 microsatellite loci for three populations of *Viburnum triphyllum* and *V. pichinchense* located in Ecuador.[a]

	V. triphyllum 1 (n = 17)				*V. triphyllum* 2 (n = 16)				*V. pichinchense* (n = 13)			
Locus	*A*	H_e	H'	*D*	*A*	H_e	H'	*D*	*A*	H_e	H'	*D*
H121	7	0.7409	1.5760	0.1471	10	0.8548	2.0705	0.0500	3	0.5207	0.8587	0.4359
O42	3	0.5468	0.8661	0.7794	3	0.5753	0.9327	0.7583	3	0.5822	0.9533	0.6154
O91	12	0.8685	2.2034	0.0441	13	0.8680	2.2660	0.0500	7	0.8046	1.7369	0.1923
O104	6	0.7426	1.5264	0.2564	7	0.7605	1.5844	0.2418	8	0.7942	1.7799	0.1667
O121	9	0.8249	1.9063	0.0500	10	0.8441	2.0591	0.0417	9	0.8540	2.0497	0.0513
H81	9	0.7807	1.8124	0.0809	12	0.8975	2.3670	0.0167	5	0.6945	1.3326	0.2179
TN2	2	0.0564	0.1293	0.8824	4	0.3200	0.6361	0.4250	2	0.0737	0.1599	0.8462
TN3	3	0.3806	0.6779	0.5956	3	0.1732	0.3653	0.7583	2	0.2604	0.4293	0.7179
DN10	1	0.0000	0.0000	1.0000	1	0.0000	0.0000	1.0000	3	0.5694	0.9596	0.3788
DN13	6	0.6753	1.2996	0.4191	4	0.6165	1.1566	0.2500	3	0.5124	0.8017	0.5897
DN15	3	0.3156	0.5841	0.5074	4	0.4120	0.7990	0.2833	2	0.0730	0.1586	0.8462
DN16	6	0.7696	1.5824	0.1544	6	0.7824	1.6406	0.1500	6	0.7796	1.6249	0.1282
DN18	1	0.0000	0.0000	1.0000	1	0.0000	0.0000	1.0000	1	0.0000	0.0000	1.0000
DN19	1	0.0000	0.0000	1.0000	1	0.0000	0.0000	1.0000	1	0.0000	0.0000	1.0000
DN22	3	0.5030	0.7768	0.5956	1	0.0000	0.0000	1.0000	3	0.5736	0.9675	0.2821
DM1[b]	6	0.8250[c]	—	—	6	0.8530[c]	—	—	3	0.7560[c]	—	—

Note: *A* = number of alleles sampled; *D* = Simpson diversity index; H' = Shannon diversity index; H_e = expected heterozygosity; n = number of individuals sampled.

[a] Refer to Appendix 1 for voucher and locality information.

[b] Mitochondrial locus.

[c] Unbiased haplotype diversity reported instead of expected heterozygosity.

R (Clark and Jasieniuk, 2011). For organellar loci, unbiased haploid diversity was calculated using GenAlEx (Peakall and Smouse, 2006, 2012). Rare alleles were grouped together as one haplotype.

Statistics per locus are in Table 2. Among nuclear loci, the number of alleles per locus per population varied from one to 13 alleles, H_e from 0 to 0.8975, H' from 0 to 2.3670, and *D* from 0.0167 to 1.0000. For organellar loci, three to six alleles per locus per population were detected, and unbiased haploid diversity ranged from 0.756 to 0.853.

CONCLUSIONS

The 16 microsatellite loci developed for the South American *V. triphyllum* and *V. pichinchense* are variable and will be informative in studies of population dynamics and species boundaries among species of the *Oreinotinus* clade.

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Appendix 1. Locality and voucher information for all samples in this study.[a]

Species	Voucher information	Latitude	Longitude	Locality
V. dentatum L.[b]	*W. L. Clement and M. J. Donoghue 244*	—	—	Marsh Botanic Gardens, Yale University, New Haven, Connecticut, USA
V. hallii (Oerst.) Killip & A. C. Sm.[b]	*P. W. Sweeney et al. 1827*	–0.16587	–78.29389	Imbabura, Ecuador
V. pichinchense Benth.	*P. W. Sweeney et al. 1808*	–0.23202	–78.63820	Pichincha, Ecuador
	P. W. Sweeney et al. 1809	–0.22412	–78.64078	Pichincha, Ecuador
	P. W. Sweeney et al. 1810	–0.22412	–78.64078	Pichincha, Ecuador
	P. W. Sweeney et al. 1811	–0.22836	–78.63997	Pichincha, Ecuador
	P. W. Sweeney et al. 1812	–0.22836	–78.63997	Pichincha, Ecuador
	P. W. Sweeney et al. 1813	–0.22412	–78.64078	Pichincha, Ecuador
	P. W. Sweeney et al. 1814	–0.22412	–78.64078	Pichincha, Ecuador
	P. W. Sweeney et al. 1815	–0.22944	–78.63964	Pichincha, Ecuador
	P. W. Sweeney et al. 1816	–0.23202	–78.63820	Pichincha, Ecuador
	P. W. Sweeney et al. 1817	–0.23202	–78.63820	Pichincha, Ecuador
	P. W. Sweeney et al. 1818	–0.23111	–78.63658	Pichincha, Ecuador
	P. W. Sweeney et al. 1819	–0.23547	–78.63181	Pichincha, Ecuador
	P. W. Sweeney et al. 1820	–0.23575	–78.63097	Pichincha, Ecuador
V. trilobum Marshall[b]	*M. J. Donoghue and R. C. Winkworth 2*	—	—	Arnold Arboretum, Boston, Massachusetts, USA
V. triphyllum Benth.[b]	*P. W. Sweeney et al. 1783*	–3.58931	–79.18901	Loja, Ecuador
V. triphyllum Population 1	*P. W. Sweeney et al. 1747*	–4.09720	–79.95067	Loja, Ecuador
	P. W. Sweeney et al. 1748	–4.09720	–79.95067	Loja, Ecuador
	P. W. Sweeney et al. 1749	–4.09720	–79.95067	Loja, Ecuador
	P. W. Sweeney et al. 1750	–4.09720	–79.95067	Loja, Ecuador
	P. W. Sweeney et al. 1751	–4.09720	–79.95067	Loja, Ecuador
	P. W. Sweeney et al. 1752	–4.09216	–79.95525	Loja, Ecuador
	P. W. Sweeney et al. 1753	–4.09216	–79.95525	Loja, Ecuador
	P. W. Sweeney et al. 1754	–4.09216	–79.95525	Loja, Ecuador
	P. W. Sweeney et al. 1755	–4.09216	–79.95525	Loja, Ecuador
	P. W. Sweeney et al. 1756	–4.09488	–79.94771	Loja, Ecuador
	P. W. Sweeney et al. 1757	–4.09442	–79.94802	Loja, Ecuador
	P. W. Sweeney et al. 1758	–4.09442	–79.94802	Loja, Ecuador
	P. W. Sweeney et al. 1759	–4.09124	–79.95113	Loja, Ecuador
	P. W. Sweeney et al. 1760	–4.09124	–79.95113	Loja, Ecuador
	P. W. Sweeney et al. 1761	–4.09124	–79.95113	Loja, Ecuador
	P. W. Sweeney et al. 1762	–4.09124	–79.95113	Loja, Ecuador
	P. W. Sweeney et al. 1763	–4.09199	–79.94384	Loja, Ecuador
V. triphyllum Population 2	*P. W. Sweeney et al. 1680*	–3.99517	–79.26857	Loja, Ecuador
	P. W. Sweeney et al. 1681	–3.99517	–79.26857	Loja, Ecuador
	P. W. Sweeney et al. 1682	–3.99502	–79.26685	Loja, Ecuador
	P. W. Sweeney et al. 1683	–3.99650	–79.26112	Loja, Ecuador
	P. W. Sweeney et al. 1685	–3.99992	–79.25980	Loja, Ecuador
	P. W. Sweeney et al. 1686	–4.00323	–79.25863	Loja, Ecuador
	P. W. Sweeney et al. 1687	–4.00567	–79.25797	Loja, Ecuador
	P. W. Sweeney et al. 1688	–4.00710	–79.25788	Loja, Ecuador
	P. W. Sweeney et al. 1689	–4.00292	–79.25720	Loja, Ecuador
	P. W. Sweeney et al. 1690	–4.00292	–79.25720	Loja, Ecuador
	P. W. Sweeney et al. 1691	–4.00292	–79.25720	Loja, Ecuador
	P. W. Sweeney et al. 1692	–4.00292	–79.25720	Loja, Ecuador
	P. W. Sweeney et al. 1693	–4.00292	–79.25720	Loja, Ecuador
	P. W. Sweeney et al. 1694	–4.00292	–79.25720	Loja, Ecuador
	P. W. Sweeney et al. 1695	–4.00172	–79.25698	Loja, Ecuador
	P. W. Sweeney et al. 1696	–4.00208	–79.25540	Loja, Ecuador

[a] Voucher specimens are deposited at the Yale University Herbarium (YU), New Haven, Connecticut, USA.
[b] Specimens used in marker development.