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 sup-
ported 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 bound-
aries 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. dila-
tatum 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 dynam-
ics 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 Bentham. (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 then used in a Stratagene PCR Cloning Kit (Agilent Technologies, Santa Clara, California, USA) and screened using T7 and M13 plasmid primers. Colonies with inserts containing repeat regions (144 selected from V. hallii and 144 from V. trifolium) were saved and thereafter considered mitochondrial regions; unused reads were mapped to *Helianthus* were saved and thereafter considered plastid regions. Using the same approach, *Lonicera* in Geneious R8 (Biomatters, Auckland, New Zealand). Reads mapped to plastid (M. Moore, personal communication) using the read mapping assembler First, data were assembled using reference-based assembly to a *L.* *Lonicera* (collected from Ecuador; SRP041815). from Connecticut, USA) and *V. triphyllum* from USA). Six loci were optimized.

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

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | GenBank accession no. |
|-------|--------------------------|--------------|------------------------|-----------------------|
| H121  | *ACCCCTCCTCCCTCTCTTCTTG*  | (CT)_b       | 156–174                | KX447798              |
|       | GAGGAGGCTTAAGGGGTCCTCCT  |              |                        |                       |
| O24   | *GCCCATAGGAAAAAGGCTTCTTG* | (TA)_b(TA)(TG)_b | 183–189                | KX447799              |
|       | CACCCGGGAATAATACG         |              |                        |                       |
| O91   | *CCCAATGGGCTTCTTGGTAA*    | (AC)_12      | 200–266                | KX447800              |
|       | CGGAAAGATCCATGGTGAGC      |              |                        |                       |
| O104  | *GTTTCAGCCTACACACAG*      | (AC)_10      | 168–186                | KX447801              |
|       | ATCTCGAGGGAGCTCCACAC      |              |                        |                       |
| O121_p| *CTCTCTCCTGCGCTACGTAGAC*  | (AG)_14      | 112–158                | KX447802              |
|       | TGCGGCTTTATCTCCTCCA       |              |                        |                       |
| H81   | *GGGCGAGGTCCTTTAAAAAC*    | (GA)_16      | 199–233                | KX447797              |
|       | GAGGCTAAGCTCCCTGCAACACCA  |              |                        |                       |
| TN2   | *GTTGTTGGTTGACAGGAGG*     | (GT)_5       | 236–244                | KX447804              |
|       | GCACCTTGGCAATGGGACTC      |              |                        |                       |
| TN3   | *AGTGTTGGTATGAGTAGGCG*    | (TA)_3       | 138–144                | KX447805              |
|       | ACTCTACTGACCTCCACTCTG     |              |                        |                       |
| DMI_p | *GCCTCATATAACCCCAATTTCT*  | (AT)_6       | 411–427                | KX447806              |
|       | ATAAGGCTGCAAAGCGCAG       |              |                        |                       |
| DN10  | *GTTCAGCGAAAGGGCGCAACG*   | (CA)_10      | 140–150                | KX447809              |
|       | GATTCGACATGCTCCTAAAGGAG   |              |                        |                       |
| DN13  | *CAAGCTTGAGCTGAGTTAGGACG* | (CT)_b       | 222–238                | KX447810              |
|       | TCTGACCATAAGTGATGACCTTG   |              |                        |                       |
| DN15  | *TTTTTCTCCCTCCCTCTCAG*    | (TA)_4       | 108–134                | KX447811              |
|       | CAGACGCTAGGGTATAGGCG      |              |                        |                       |
| DN16_p| *AACCTCCACCGGCTCCACATC*   | (AG)_5       | 352–380                | KX447812              |
|       | TGCGTGAAGGAGTCTGCTAG      |              |                        |                       |
| DN18  | *CAGGTCCGGCTTCCACAC*      | (TA)_5       | 200–240                | KX447813              |
|       | TGCTAGGTGGTTATGATGCGG     |              |                        |                       |
| DN19_p| *CCTCCAGGCTTCCCTCCTC*    | (CT)_7       | 449                    | KX447814              |
|       | TCACCCTAGCTAAAGGTCTG      |              |                        |                       |
| DN22  | *GGTCCCTTAAACCGCCAAGG*    | (AG)_7       | 373–483                | KX447815              |
|       | AGGGGTGGACTCCGAAATCT      |              |                        |                       |

*Annealing temperature for all loci was 52°C.

*Loci amplified with the addition of BSA.

*Loci located in the mitochondria.

*Primer preceded by a fluorescently labeled M13 tag (CAGCACGTGGTAAACCGAC).
Table 2. Genetic properties of the 16 microsatellite loci for three populations of *Viburnum triphyllum* and *V. pichinchense* located in Ecuador.\(^a\)

| 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 | 2.0204 | 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 |
| 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 |
| DMI\(^b\)| 6 | 0.8250| —— | —— | 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.

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R (Clark and Jasieniuk, 2011). For organellar loci, unbiased haplotype 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 haplotype 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 | -0.22412 | -78.64078 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1809 | -0.22412 | -78.64078 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1810 | -0.22836 | -78.63997 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1811 | -0.22836 | -78.63997 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1812 | -0.22412 | -78.64078 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1813 | -0.22412 | -78.64078 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1814 | -0.22944 | -78.63964 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1815 | -0.23202 | -78.63820 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1816 | -0.23202 | -78.63820 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1817 | -0.23111 | -78.63658 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1818 | -0.23547 | -78.63181 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | P. W. Sweeney et al. 1819 | -0.23575 | -78.63097 | Pichincha, Ecuador |
| *V. pichinchense Benth.* | 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 | -79.5067 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1748 | -4.09720 | -79.5067 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1749 | -4.09720 | -79.5067 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1750 | -4.09720 | -79.5067 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1751 | -4.09720 | -79.5067 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1752 | -4.09216 | -79.5525 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1753 | -4.09216 | -79.5525 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1754 | -4.09216 | -79.5525 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1755 | -4.09216 | -79.5525 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1756 | -4.09488 | -79.94771 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1757 | -4.09442 | -79.94802 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1758 | -4.09442 | -79.94802 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1759 | -4.09124 | -79.95113 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1760 | -4.09124 | -79.95113 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1761 | -4.09124 | -79.95113 | Loja, Ecuador |
| *V. triphyllum Population 1* | P. W. Sweeney et al. 1762 | -4.09124 | -79.95113 | Loja, Ecuador |
| *V. triphyllum Population 1* | 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 |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1681 | -3.99517 | -79.26857 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1682 | -3.99502 | -79.26685 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1683 | -3.99650 | -79.26112 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1685 | -3.99992 | -79.25980 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1686 | -4.00323 | -79.25863 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1687 | -4.00567 | -79.25797 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1688 | -4.00710 | -79.25788 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1689 | -4.00292 | -79.25720 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1690 | -4.00292 | -79.25720 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1691 | -4.00292 | -79.25720 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1692 | -4.00292 | -79.25720 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1693 | -4.00292 | -79.25720 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1694 | -4.00292 | -79.25720 | Loja, Ecuador |
| *V. triphyllum Population 2* | P. W. Sweeney et al. 1695 | -4.00172 | -79.25698 | Loja, Ecuador |
| *V. triphyllum Population 2* | 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.