Molecular Markers Within and Beyond *Viburnum*: A Single Set of SSRs for an Immense Genus

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Abstract

Comprised of more than 160 species, *Viburnum* is the largest genus in the Viburnaceae (formerly Adoxaceae) alongside *Adoxa* and *Sambucus*. The native range of *Viburnum* species spans much of the Northern Hemisphere and extends into the mountains of South America and Southeastern Asia. Their wide geographic range has made *Viburnum* particularly interesting to phylogeographers and phylogeneticists. This diverse genus is also horticulturally valuable with varying traits such as fragrance, fruit color, and bud and inflorescence form. There is sufficient morphological diversity in the genus for there to be more than 70 species and intraspecific hybrids in cultivation, and in 2017, viburnums generated $23.2 million in wholesale and retail sales in the U.S. alone. Viburnums' horticultural value is in large part due to their ornamental qualities varying throughout the year, but also lasting much of the year. Typically, molecular markers are developed only for single species when breeding and assessing genetic diversity. This is despite many markers displaying cross-transferability to other closely related species and genera. Microsatellite markers, also known as simple sequence repeats (SSRs), were previously developed from three of the four large clades of *Viburnum* (*Valvatotinus, Laminotinus,* and *Porphyrotinus*). For markers to span the entire genus, genomic SSRs (gSSRs) were developed from *V. farreri,* which is a member of the East Asian center of diversity, the fourth large clade (*Crenotinus*), and a popular ornamental in the United States. The four sets of markers (a total of 49) were then tested on 46 *Viburnum* species and five closely related species in the rest of the Viburnaceae and the Caprifoliaceae. The markers developed here in conjunction with previous markers eliminate the need to develop markers for each species in this massive genus and closely related taxa. These SSRs are suitable and ready to use for breeding, exploring genetic diversity, and delimiting species and cultivars in *Viburnum* and possibly select species in the rest of the Viburnaceae and the Caprifoliaceae.

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Introduction

Viburnum L. is a genus of evergreen and deciduous trees and shrubs classified in the Viburnaceae (formerly Adoxaceae and Caprifoliaceae; Dipsacales). Morphologically, *Viburnum* species are consistent with opposite (or rarely whorled) leaves and overall flower and fruit construction (Donoghue, 1983). However, there is considerable diversity in endocarp shape, fruit color, and leaf and bud morphology (Winkworth and Donoghue, 2005). The geographic range spans temperate and subtropical regions of the Northern Hemisphere and extends into the mountains of Southeast Asia and South America. Centers of diversity are located in eastern Asia and the mountains of Central America (Donoghue, 1983).

The somatic chromosome numbers of *Viburnum* species range from 18 to 72 (Egolf, 1962; Zhang et al., 2016), and therefore could range in ploidy level from diploid to octaploid. Not all species have been studied equally, but the majority of the species are $2n=2x=18$ (Egolf, 1962; Winkworth and Donoghue, 2004; Zhang et al., 2016). Studied *Viburnum* genome sizes are classified as small to intermediate when compared to other plants and have 2C values ranging from 4.29 to 24.23 Gbp (Olszewska and Osiecka, 1984; Bai et al., 2012; Pustahija et al., 2013; Zhang et al., 2013; Moeglein et al., 2020). However, the genome sizes are larger than other woody genera, such as *Cornus* with 2C values ranging from 1.89 to 6.66 Gbp (Zonneveld et al., 2005; Siljak-Yakovlev et al., 2010; Bai et al., 2012; Cao et al., 2014; Fridley and Craddock, 2015) and *Pyrus* ranging from 1.13 to 1.27 Gbp (Dickson et al., 1992; Jedrzejczyk and Sliwinska, 2010; Pustahija et al., 2013).

Throughout the genus *Viburnum,* there are many desirable ornamental traits, including fragrant flowers, attractive foliage, colorful fruits, and vibrant fall colors. As a result, the genus overall has year-round ornamental interest. Viburnums range in size from 1 to 9 meters, in scent from unpleasant to sweet, in fall leaf color from glossy red to purple, and in flower color from white to pink (Kluepfel et al., 2021). Additionally, they are moderate- to fast-growing plants that can grow up to approximately half a meter per year (Kluepfel et al., 2021). There are more than 70 species and interspecific hybrids in cultivation (Dirr, 2007) that generated $23.2 million in wholesale and retail sales in 2017 alone (USDA-NASS, 2020).

Traditional breeding methods only can produce hybrids with closely related species, however, crosses with more distantly related species have also been completed with embryo rescue techniques (Hoch et al., 1995). A linkage map was subsequently constructed from the hybrids produced with embryo rescue, and SSRs could be added to produce a more robust map for future breeding efforts (Al-Niemi et al., 2011). There are hybrids such as *V.* ×*burkwoodii* and *V.* ×‘NCVXI’ already on the market that are salt-tolerant, making them ideal ornamentals for arid regions that use reclaimed water for irrigation (Chen et al., 2020; Sun et al., 2020). Species also have varying levels of resistance to pathogens such as Verticillium wilt and leaf spot causing fungi (Al-Niemi et al., 2011). Viburnums are also impacted by common ornamental diseases and pests such as powdery and downy mildews, aphids, spider mites, and scales (Williamson, 2021).

Viburnum species have been used for many purposes outside of landscaping as well. The shoots of various *Viburnum* species have been used to make arrow shafts for at least 5,000 years, and arrow shafts constructed from *V. lantana* were found alongside Otzi the Iceman (Wierer et al., 2018). Species, such as *V. opulus* (cranberry bush), have historically been used in food and pharmaceuticals and specifically for the treatment of heart disease, coughs, colds, digestive troubles, and bleeding (Česonienė et al., 2010). Studies investigating the biological and antioxidant compounds in *Viburnum* species have centered around *V. opulus* genotypes (e.g., Kraujalytė et al., 2013; Kajszczak et al., 2020), but other species have also been investigated including *V. foetens* (Bibi et al., 2010) and *V. macrocephalum* (Shao et al., 2019). As a more comprehensive approach, the association between molecular markers, antimicrobial effects, and anthocyanin and phenolic compound levels were evaluated for *V. opulus* cultivars, *V. sargentii*, and *V. trilobum* (Paulauskas et al., 2015).

The taxonomy of *Viburnum* and the remainder of Dipsacales has been debated and modified for more than 200 years. Linnaeus originally erected *Viburnum* (1799), and the genus was revisited by Ørsted (1860), who identified the principle variable characters within the group. Morton (1933) proposed 10 sections just for the Central American species. Rehder (1940) recognized nine sections for Asian species and did not address species outside of this region. Hara (1983) revisited the entire genus and recognized 10 sections, which included the nine Rehder suggested and one for the Central American species. *Viburnum* was generally considered monophyletic since 1948 (Wilkinson, 1948), but its position within the Dipsacales was not certain. *Viburnum* was historically classified in the Caprifoliaceae alongside *Weigela* Thunb. and *Lonicera* L. *Viburnum* was later moved to the Adoxaceae with *Adoxa* L., *Tetradoxa* C.Y. Wu*, Sinadoxa* C.Y.Wu, Z.L.Wu, & R.F.Huang*,* and *Sambucus* L. based on nuclear gene sequences and morphological characteristics (Donoghue et al., 1992; Donoghue et al., 2001). *Tetradoxa* and *Sinadoxa* are now synonyms of *Adoxa* (Flora of China Editorial, 2011), and the family name has been changed to the Viburnaceae (Ulloa Ulloa et al., 2018), but includes the genera that were traditionally classified in the Adoxaceae.

Since the 1983 study (Hara, 1983), the sections and subsections of *Viburnum* have been reconsidered but mostly only as separate entities (Winkworth and Donoghue, 2005). Relationships within and between geographic regions were rarely considered until 2004 when the chloroplast *trn*K intron and nuclear ribosomal ITS (Donoghue et al., 2004) and duplicated nuclear gene *GBSSI* regions (Winkworth and Donoghue, 2004) were sequenced. These two datasets were then combined and subsequently analyzed together to further clarify phylogenetic relationships within the genus (Winkworth and Donoghue, 2005). The result of these studies was 12 well-supported species groups. Not all species were represented in their datasets, however, they expected unsampled taxa to fall into their 12 groups. Despite these conclusions, they also determined increased sampling was needed to resolve relationships at the base of the tree. Resolution of the section *Megalotinus* was needed the most. *Megalotinus* was determined to be polyphyletic and was subsequently dissolved into *Punctata, Lutescentia, Sambucina,* and *Coriacea* (Clement and Donoghue, 2011). The chloroplast genes *mat*K and *rbc*L along with *trn*H-*psb*A and nuclear ITS were then used as barcodes to delineate *Viburnum* species, but there was limited success due to low sequence variability among closely related species (Clement and Donoghue, 2012). Next, the sampling was expanded to the entire chloroplast genome of 22 species, resulting in the 16 subclades and 13 larger clades recognized today (Clement et al., 2014). Despite the ever-increasing sampling and data, there is still uncertainty in the relationship among all the clades and subclades.

Studies before 2017 (Eaton et al., 2017), only used chloroplast regions and nuclear ITS to discern phylogenetic, biogeographic, and evolutionary relationships and progressions. Since 2017, restriction-site associated DNA sequencing (RAD-seq) has increasingly been applied to those biological questions. To date, there are 118 *Viburnum* species and nine other species in the Viburnaceae with RAD-seq datasets. These datasets have been combined with chloroplast gene and nuclear ITS sequences in the most recent study concerned with *Viburnum* phylogenetics and biogeography (Landis et al., 2020). Despite this massive amount of data, most species are only represented once in the RAD-seq datasets and there are still unresolved taxonomic issues that need to be addressed at the population level. One notable exception was when RAD-seq was applied to *V. nudum* complex (Spriggs et al., 2019a). Multiple accessions of species were sequenced and three separate lineages in North America were discerned, therefore supporting three species. This study only addressed three out of the more than 160 species in *Viburnum* overall, and they advocated for population-level studies to investigate the presence of cryptic species.

Molecular markers consisting of microsatellites are commonly employed to investigate genetic diversity in plants at the population level. Microsatellites, also known as simple sequence repeats (SSRs), are repetitive tracts in the DNA that occur ubiquitously throughout the genome. The tandemly repeating motifs can range in size from one to five (mono- to penta-nucleotide repeats) (Powell et al., 1996). Dinucleotide repeats are the most common length and $[AT]_n$ is the most common sequence (e.g., Nowicki et al., 2019a; Lin et al., 2020; Hamm et al., 2021). These sections of the genome can have substantially higher mutation rates than other areas of the genome due to polymerase slippage during replication (Ellegren, 2004). These repetitive regions with high genetic diversity are flanked by more conserved regions, which allows for primers to be designed to target the regions of the genome with SSRs (Gupta and Varshney, 2000). The primers can then be used in polymerase chain reactions (PCR) and sized or sequenced to determine the allele(s) present in an individual. The codominant, reproducible, and highly variable nature of these markers make them ideal candidates for evaluating genetic diversity, determining population structure, identifying cultivars/species, and assisting in breeding efforts.

Historically, SSRs were isolated from microsatellite libraries made from genomic DNA. SSRs are now mostly mined from expressed-sequence-tag datasets (eSSRs or EST-SSRs) or genomic sequencing datasets (gSSRs). Typically, eSSRs display greater transferability than gSSRs due to the conserved nature of expressed regions of the genome. Also as a consequence of the conserved nature of these regions, eSSRs typically display lower diversity and number of alleles than gSSRs (Tabbasam et al., 2014; Nowicki et al., 2020). Despite these trends, studies have also concluded there is not a significant overall difference in the transferability or ability to detect diversity between the two types of markers (e.g., Mnejja et al., 2010). The conserved nature of the flanking regions enables one set of molecular markers to be transferred to closely related species. Cross-transferable markers allow for fewer to be developed and for inferences to be drawn across species boundaries. Additionally, the cross-species transferability of countless other eSSR and gSSR markers have been demonstrated in *Cornus* (dogwood) (Wadl et al., 2010), *Foeniculum vulgare* (fennel) (Aiello et al., 2020), *Prunus* (stonefruit) (Mnejja et al., 2010), and *Cercis* (redbud) (Wadl et al., 2012). Overall, SSR markers in eudicots have been found to have an average of cross-species amplification rates of almost 80% within genera and almost 60% among genera (Barbara et al., 2007). Polymorphic amplification rates are lower with success rates of almost 60% within genera and 10% among genera (Barbara et al., 2007). SSR loci from *Cornus* species had amplification success ranging from 7 to 100% within the genus (Wadl et al., 2010). Whereas loci from *Cercis* species had amplification success ranging from 25% to 100% (Wadl et al., 2012). SSR marker sets from various *Prunus* species had amplification rates within genus from 64 to 100% (Mnejja et al., 2010).

SSR markers were developed for three of the four major clades of *Viburnum* including *Laminotinus*, *Porphyrotinus*, and *Valvatotinus*. The markers were developed in *V. dilatatum* (*Succotinus* subclade; *Laminotinus* clade; Dean et al., 2011), *V. rufidulum* (*Lentago* subclade; *Valvatotinus* clade; Dean et al., 2015), and *V. triphyllum* and *V. pichinchense* (*Oreinotinus* subclade; *Porphyrotinus* clade; Barish et al., 2016). The cross-species amplification of some markers was tested in a limited capacity in their initial development, and a preliminary analysis of the *V. dilatatum* and *V. rufidulum* markers was conducted as part of Dean's thesis (Dean, 2014). Additionally, some of the markers developed from *V. dilatatum* were used in *V. opulus*, *V. trilobum*, and *V. sargentii*, (Paulauskas et al., 2015). However no large-scale analysis has been completed on the markers already developed, and no markers have been developed for the *Crenotinus* clade.

Chapter 1: Development and Characterization of 15 Novel Genomic SSRs for *Viburnum farreri*

This chapter is a reformatted version of a published manuscript with the same name in the journal Plants in 2021. The authors are Trinity P. Hamm, Marcin Nowicki, Sarah L. Boggess, William E. Klingeman, Denita Hadziabdic, Matthew L. Huff, Margaret E. Staton, and Robert N. Trigiano.

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My principal contributions were: conceptualization, software, validation, formal analysis, investigation, resources, data curation, original draft preparation, visualization, and project administration.

Abstract

The *Viburnum* genus is of particular interest to horticulturalists, phylogeneticists, and biogeographers. Despite its popularity, there are few existing molecular markers to investigate genetic diversity in this large genus, which includes over 160 species. There are also few polymorphic molecular tools that can delineate closely related species within the genus. *Viburnum farreri*, a member of the *Solenotinus* subclade and one of the centers of diversity for *Viburnum*, was selected for DNA sequencing and development of genomic simple sequence repeats (gSSRs). In this study, 15 polymorphic gSSRs were developed and characterized for a collection of 19 *V. farreri* samples. Number of alleles per locus ranged from two- to- eight and nine loci had four or more alleles. Observed heterozygosity ranged from 0 to 0.84 and expected heterozygosity ranged from 0.10 to 0.80 for the 15 loci. Shannon diversity index values across these loci ranged from 0.21 to 1.62. The markers developed in this study add to the existing molecular toolkit for the genus and will be used in future studies investigating cross-transferability, genetic variation, and species and cultivar delimitation in the *Viburnum* genus and closely allied genera in the Adoxaceae and Caprifoliaceae.

Introduction

The genus *Viburnum* L. (Adoxaceae, formally classified in Caprifoliaceae (Ii, 2003)) includes about 163 species (Landis et al., 2020) that are native to temperate and subtropical regions of the Northern Hemisphere and extend into the mountains of South America and Asia (Hoch et al., 1995; Winkworth and Donoghue, 2005). Major centers of species diversity occur in eastern Asia and Latin America (Rehder, 1908; Morton, 1933; Donoghue, 1983; Hara, 1983). Species of *Viburnum* range from shrubs to small trees, and there are more than 70 species and interspecific hybrids in cultivation (Dirr, 2007). In 2017, Viburnums generated USD23.2 million in wholesale and retail sales in the U.S. alone (USDA-NASS, 2020). In addition to horticultural value, the *Viburnum* genus also serves as a suitable model for studying phylogeography and evolution (Winkworth and Donoghue, 2005; Clement and Donoghue, 2011; Spriggs et al., 2015; Lens et al., 2016; Edwards et al., 2017; Landis et al., 2020). Much progress has been made in the phylogenetic classification of this genus (Donoghue et al., 2004; Winkworth and Donoghue, 2004; Winkworth and Donoghue, 2005; Clement and Donoghue, 2012; Eaton et al., 2017; Choi et al., 2018) ranging from moving *Viburnum* and related genera from Caprifoliaceae to Adoxaceae (Donoghue et al., 1992) to providing formal phylogenetic definitions for 30 clades and subclades within *Viburnum* (Clement et al., 2014).

Viburnum farreri Stearn, fragrant viburnum, is classified within the clade *Crenotinus* and the subclade *Solenotinus* (Clement et al., 2014) and is native to the East Asian center of diversity. This China-native species produces paniculate inflorescences with opposite branches (Donoghue, 1980), making cultivars such as ‘Album’, ‘Nanum’, and ‘Candidissimum’ popular ornamental specimens. *Viburnum farreri*, historically known as *V. fragrans*, was first introduced to European gardens in 1911 (Stearn, 1966). Despite a rich history of cultivation, molecular tools are not available for determining genetic diversity and population structure of this species or other species in the *Solenotinus* subclade. Development of resources that could be used to delineate species and investigate genetic diversity within *Viburnum* would assist breeding programs and help resolve phylogenetic topology at low taxonomic levels. Attempts have been made to use barcode sequences to distinguish species, but due to inadequate sequence diversity, this method had limited applicability in differentiating species within subclades (Clement and Donoghue, 2012). Recent literature has discussed the need to revisit the taxonomy of several *Viburnum* subclades, including *Solenotinus*, to more definitively draw conclusions on the evolutionary history of the genus (e.g., Spriggs et al., 2015; Spriggs et al., 2019a; Landis et al., 2020).

Microsatellites, also known as simple sequence repeats (SSRs), are tandem repeats located throughout most eukaryotic genomes, which can be composed of mono-, di- to penta-nucleotide motifs (Powell et al., 1996). They are a class of neutral markers that are co-dominant in nature. SSRs are particularly suited to study closely related individuals and species due in part to their highly polymorphic nature, caused by polymerase slippage during DNA replication (Ellegren, 2004). Flanking regions of these repetitive motifs are mostly conserved, which allows for designed primers to target amplification of the SSR loci (Gupta and Varshney, 2000). The resulting polymerase chain reaction (PCR) products can then be sized to determine the polymorphisms and therefore, alleles of specific loci. As a result of these properties, SSRs are frequently used to investigate genetic diversity (e.g., Edwards et al., 2020; Ony et al., 2020) and delimit species and subspecies (e.g., Nowicki et al., 2019a; Luber et al., 2020).

Historically, SSR markers have been developed by constructing microsatellite libraries. SSRs are now commonly discovered by mining next generation sequencing (NGS) data. Genomic SSR markers (gSSRs) are developed from genomic sequences, whereas expressed sequence tag SSR markers (EST-SSRs or eSSRs) are developed from RNA sequencing data. gSSR typically exhibit more alleles and are more informative for genotyping and estimating genetic diversity compared to eSSRs (Tabbasam et al., 2014; Nowicki et al., 2020). SSR markers in general can cross-transfer to closely related species and genera to yield informative products (Peakall et al., 1998). eSSRs commonly display greater cross-transferability than gSSRs because they are more likely to be within functional gene sequences and therefore are typically more conserved (Ellis and Burke, 2007; Ouyang et al., 2018). eSSRs and gSSRs have both transferred to closely related species within and outside of their genus, including well-studied economically important agronomic crops (e.g., Peakall et al., 1998; Arnold et al., 2002) as well as a number of woody ornamental species including *Cercis canadensis* (Wadl et al., 2012), *Cornus* species (Wadl et al., 2010), and *Fothergilla* species (Hatmaker et al., 2015).

gSSRs have been developed for four of the ~163 species of *Viburnum* including *V. dilatatum* (Dean et al., 2011), a member of the *Succotinus* subclade, *V. rufidulum* (Dean et al., 2015), a member of the *Lentago* subclade, and *V. triphyllum* and *V. pichinchense* (Barish et al., 2016), which are members of the *Oreinotinus* subclade (Clement et al., 2014). These gSSRs were developed for species/cultivar identification as well as investigation of population genetic diversity. They represent species in three of the larger clades (*Laminotinus, Valvatotinus*, and *Porphyrotinus*, respectively), leaving only *Crenotinus* unrepresented. A preliminary study on the cross-transferability of the markers developed for *V. dilatatum* (Dean et al., 2011) and *V. rufidulum* (Dean et al., 2015) was conducted and demonstrated wide, but not complete transferability of these gSSRs (Dean, 2014). Inclusion of markers for *V. farreri,* a member of the *Crenotinus* clade and the *Solenotinus* subclade, would provide more extensive coverage of species across the entire *Viburnum* genus.

The objectives of this study were to develop additional gSSRs from de novo assembled genomic Illumina sequencing data of *V. farreri* 'Nanum' and apply them to estimate the genetic diversity of the species. The markers described herein from *V. farreri* in the *Solenotinus* subclade make the overall set of developed markers for the genus more complete and will allow study of cross-transferability to all subclades as described by Clement et al. (Clement et al., 2014) for use in downstream studies of the *Viburnum* genus and other closely related genera.

Materials and Methods

Plant Materials and gDNA extraction

Samples of *V. farreri* were obtained from the Morton Arboretum (MA), Mt. Airy Arboretum, Arnold Arboretum (AA), University of Washington Botanical Garden (UWBG), U.S. National Arboretum (USNA), and U.S. National Arboretum Herbarium (NA) (Table 1.1; note all tables and figures are located in the appendix). Nineteen of the 22 samples were of garden origin (Table 1.1). Living specimens sampled from arboreta were originally collected and planted between 1940 and 2020, and herbarium specimens were collected between 1938 and 2007.

Leaves from arboreta samples were dried before DNA extraction, except for the *V. farreri* 'Nanum' (collected from the Mountain Hort. Crops Res. & Ext. Center at North Carolina State University) sample, which was flash frozen and used for Illumina MiSeq sequencing. DNA was sequenced in order to maintain a similar discovery method to all other SSRs designed for *Viburnum*. All samples were frozen in liquid nitrogen and then homogenized once, or twice if needed, using a Beadmill 24 (Fishers Scientific, Pittsburgh, Pennsylvania, U.S.). Genomic DNA (gDNA) was isolated from the 'Nanum' sample with a CTAB method (Diversity Arrays Technology, 2019). gDNA was isolated from all other samples using an Omega E.Z.N.A. Plant DNA Kit (Omega Bio-tek Inc., Norcross, Georgia, U.S.). The manufacturer's protocol was followed except that 2% polyvinylpyrrolidone (PVP) (Fisher BioReagents, Waltham, Massachusetts, U.S.) was added to the P1 Buffer and the incubation time at 65°C was increased from 10 min to 30 min. Quality of the isolated gDNA was assessed with a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.). If gDNA isolated from samples with the E.Z.N.A. kit was unsuccessful in downstream applications, gDNA was re-isolated from those samples using the CTAB method (Kubisiak et al., 2012).

gSSR Development and Screening

gDNA extracted from the 'Nanum' sample was submitted for Illumina MiSeq 600v3 (paired-end 2 × 300 bp) sequencing [Oklahoma Medical Research Foundation (OMRF), Oklahoma City, Oklahoma, U.S.]. The miSeq raw reads are available at NCBI Bioproject PRJNA706016. Read quality was assessed with FastQC version 0.11.7 (Andrews, 2010) before and after trimming and quality filtering with Trimmomatic version 0.39 (Bolger et al., 2014). The minimum read length kept was set to 36 base pairs (bp). Reads were only retained if the mean quality score was ≥ 30. Reads were trimmed of adapter sequences and ends were trimmed until a minimum q-score of 30 was reached. The reads were assembled using Assembly By Short Sequences (ABySS) version 2.1.4 (Simpson et al., 2009). Default settings were used outside of the k-mer size, which was set to 64. DustMasker version 2.10.0 (Morgulis et al., 2006) was used to mask low-complexity DNA sequences before mining for SSRs. Finally, the masked file and assembled scaffolds were inputted into a custom Perl script (Staton and Ficklin, 2018) to identify SSR regions and develop primers with Primer3 version 2.5.0 (Untergasser et al., 2012). This script searched for di-, tri-, and tetra-nucleotide repeats with designed primers that would result in a product size between 100 and 400 bp.

Twenty di-, 15 tri-, and 15 tetra-nucleotide primer pairs (total of 50) were selected randomly from outputs of the Perl script for screening Primers were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, U.S.). PCR was conducted with all 50 primer pairs on three randomly selected *V. farreri* gDNA samples. The reaction volume was 10 µL and included 5 µL of 2× AccuStart II PCR SuperMix (Quantabio, Qiagen Beverly, Inc., Beverly, Massachusetts, U.S.), 3 µL autoclaved water, 1 µL of mixture of 5 µM forward and reverse primers, and 1 µL of 2 ng/µL of gDNA. The PCR thermal profile included 3 min of initial denaturation at 94°C, 10 touchdown (Don et al., 1991) cycles (94°C for 40s, 63°C -0.5°C/cycle for 40s, and 72°C for 30s), 30 cycles (94°C for 40s, 58°C for 40s, and 72°C for 30s), and a final extension of 4 min. The PCR products were visualized with capillary electrophoresis (QIAxcel Advanced Electrophoresis System; Qiagen) and analyzed using a 25 to 500 bp DNA size marker and an internal 15/600 bp alignment marker. Of the 50 primer pairs evaluated, 17 had well-defined peaks and were polymorphic; other primer pairs can be investigated in future studies. These 17 primer pairs were used to amplify DNA from the 22 samples. Two *Viburnum* samples and two primer pairs were eliminated from the study because of low and/or inconsistent amplification. The allele sizes were determined using QIAxcel ScreenGel version 1.6.0.10. Raw allele sizes were then statistically binned into allelic classes with FlexiBin (an Excel macro; Amos et al., 2007).

Estimation of Diversity Indices

All data analyses were performed in R version 4.0.3 (R Core Team, 2020). The binned dataset was first clone corrected with *poppr* version 2.8.6 (Kamvar et al., 2014). The two USNA samples were genetically identical at the investigated loci, so one of these samples was eliminated from further consideration, resulting in a 19-sample dataset with unique multi-locus genotypes. The 19-sample dataset was then used to calculate various population diversity indices, including the following: number of alleles, percent missing data, Shannon's diversity index, expected and observed heterozygosity. Indices were calculated using *poppr* and *hierfstat* version 0.5-7 (Goudet, 2005). The package *poppr* was also used in calculations to test if pairs of loci were in linkage disequilibrium. Calculations were performed with the standardized index of association ($\bar{r}_d$), which takes the number of loci used into account as opposed to the index of association (I_A) (Agapow and Burt, 2001).

Results and Discussion

gSSR Development

A total of 14,541,259 assembled scaffolds of *V. farreri* 'Nanum' were analyzed for gSSRs with a minimum of six repeats for di- and tri-nucleotide motifs and a minimum of four repeats for tetra-nucleotide motifs. gSSRs were located in 390,541 of the scaffolds with a total of 424,029 SSRs identified and included the following: 301,148 di-, 37,171 tri-, and 36,696 tetra-nucleotide repeats (Figure 1.1). $[AT]_n$ was the most (179,546 SSRs) commonly found motif. These results are similar to other gSSR studies developed from NGS data, including $[AT]_n$ as the most common di-nucleotide repeat and $[GC]_n$ being the least common (e.g., Nowicki et al., 2019b; Lin et al., 2020). Primer pairs were developed for 101,174 SSRs including 83,687 di-, 7,996 tri-, and 9,491 tetra-nucleotide repeats. Primers were not developed for any of the 49,014 identified compound SSRs (defined as SSRs separated by less than 15 bp). Fifty primer pairs were selected for the initial screening, and 15 were informative for our *V. farreri* collection.

gSSR Characteristics and Diversity Indices

Sixty-seven alleles were detected with the 15 gSSRs and loci yielded two- to- eight alleles per locus with an average of 4.47 alleles per locus (Table 1.2). Selected, polymorphic gSSRs present promising resources that can be used to assess genetic diversity within larger datasets. These gSSRs could also potentially be used to identify cultivars because they were able to capture genetic variability among the studied cultivars. The percent of missing data per locus ranged from 0 to 21 with only 3.9% missing data in the entire dataset. The only missing data were with samples from herbaria that were collected between 1966 and 1987. Therefore, the missing data could be due to low quality DNA and not mutations in the primer regions. Furthermore and more notably, nine out of the 15 gSSRs were not missing any data. The observed heterozygosity (H_o=0.23) varied greatly from the expected heterozygosity (H_e=0.60), and this result may be explained by the cultivated origin of most of the samples. The Shannon Diversity Index was very low (1.10) and indicated low allele species richness/evenness, which could be caused by the limited number of samples used in this dataset. These initial values of basic diversity measures are included as an illustration and possible point of reference for future in-depth studies of this (or related) species.

Linkage disequilibrium among loci was investigated using the standardized index of association ($\bar{r}_d$), which accounts for the number of loci sampled (Agapow and Burt, 2001). The pairwise comparison between loci revealed a range of $\bar{r}_d$ from -0.18 to 0.80 (Figure 1.2), but the only loci with a high pairwise $\bar{r}_d$ were VF20_37 and VF20_44. Therefore, VF20_37 and

VF20_44 were in linkage disequilibrium. Most loci used in this study were well-dispersed throughout the *V. farreri* genome because most pairs of loci did not have a high $\bar{r}_d$. The linkage disequilibrium observed between VF20_37 and VF20_44 can be caused by numerous factors including, but not limited to, physical proximity. Other factors that could have contributed are population differentiation, asexual reproduction, and natural selection (Agapow and Burt, 2001). It is crucial to demonstrate the SSRs are generally lacking any possible pairwise linkage. Indeed, were more, or stronger linkages detected, those would render our markers useless, as this would skew any diversity measures relying on markers undergoing independent inheritance. As no genomes are available for Dipsacales, we are unable to verify whether the single pair of SSRs showing LD is indeed in physical proximity to one another.

The studied dataset was small and does not consist of individuals within the same population. All accessions were obtained from arboreta and herbaria, and all individuals except one were cultivated and/or of unknown origin. Recent wild-collections of *V. farreri* are very scant and made obtaining native samples and samples in general difficult. Many cultivated plants are the product of non-random mating and clonal reproduction, which may have contributed to the two markers being in linkage disequilibrium. Consequently, the VF20_37 and VF20_44 gSSRs may not be physically close or linked to each other in the genome. More samples would be needed to investigate the linkage disequilibrium further. Although two of the 15 gSSRs developed were associated, this will not diminish the utility of this marker set as a resource for studying genetic diversity in the species.

Previously, gSSRs have been used successfully in cross-transferability studies with ornamental plant genera including *Cercis* (redbud) (Wadl et al., 2012) and *Cornus* (dogwood) (Wadl et al., 2010). gSSRs were also developed for safflower with similar methods to our study, and those markers also displayed cross-transferability (Ambreen et al., 2015). A preliminary study of cross-transferability of *Viburnum* species gSSRs (developed in Dean et al., 2011; Dean et al., 2015) was completed and indicated wide-transferability was possible (Dean, 2014). Therefore, the markers developed in this study should have some cross-transfer success with other *Viburnum* species and help fill in missing data from gaps left by the previously developed markers. In future studies, we plan to explicitly evaluate the cross-transferability of these markers to other *Viburnum* species, other species in the Adoxaceae, as well as related genera in the Caprifoliaceae, such as *Lonicera* and *Weigela*.

Conclusions

The 15 gSSR markers developed from *V. farreri* in this study are likely distributed throughout the genome, are polymorphic, and thus informative, and useful for estimating genetic diversity. The polymorphic loci will be beneficial in more advanced studies of *V. farreri* and informative in cross-transfer studies involving many *Viburnum* species across all clades as well as closely related genera.

Appendix: Tables and Figures

Table 1.1. Twenty-two *Viburnum farreri* specimens

Species/cultivars analyzed	**Specimen origin/accession number**[a]	**Provenance**	**Year collected**
V. farreri	MtA 785	no record	no record
V. farreri	UWBG 1190-49	Garden Origin	no record
V. farreri[b]	NA 0111167	Garden Origin	1938
V. farreri[b]	NA 0111164	Garden Origin	1941
V. farreri	MA 314-49*2	Garden Origin	1949
V. farreri	NA 0111168	Garden Origin	1966
V. farreri	NA 0111169	Garden Origin	1966
V. farreri	MA 533-70*2	Garden Origin	1970
V. farreri	MA 398-83*1	Garden Origin	1983
V. farreri	NA 0111163	Garden Origin	1985
V. farreri	USNA 59728-H	Garden Origin	1987
V. farreri	USNA 59728-J	Garden Origin	1987
V. farreri	NA 0111166	Garden Origin	1987
V. farreri	MA 915-2005*2	Known wild origin	2005
V. farreri	NA 0052257	Garden Origin	2007
V. farreri ‘Album’	MA 1036-40*1	Garden Origin	1940
V. farreri ‘Candidissimum’	UWBG 1052-52	Garden Origin	no record
V. farreri ‘Candidissimum’	MtA 200704033 5664	no record	2007
V. farreri ‘KLMW’	MA 120-2012*1	Garden Origin	2012
V. farreri ‘Nanum’	MA 252-2002*1	Garden Origin	2002
V. farreri ‘Nanum’	AA 293-2003*C	Garden Origin	2003
V. farreri ‘Nanum’[c]	NCSU 2020-063	Garden Origin	2020

[a] Sample sources: MA = The Morton Arboretum, Lisle, IL; MtA = Mt. Airy Arboretum, Cincinnati, OH; AA = Arnold Arboretum, Boston, MA; NA = U.S. National Arboretum Herbarium, Washington, D.C.; UWBG = University of Washington Botanical Garden, Seattle, WA; USNA = U.S. National Arboretum, Washington, D.C.

[b] Samples were excluded from analysis due to low amplification rates.

[c] Sample used for NGS sequencing and gSSR development.

Table 1.2. Characteristics of 15 gSSR loci developed from *Viburnum farreri*

Locus	GenBank #	Primer sequences	Repeat motif	Allele size range (bp)	N	Missing (%)	H’	H_o	H_e
VF20_01	MW326735	F: ACGATAAATGTGTATGCTCGC R: AACCCGGGAAGAAAGGTTACC	$[AT]_6$	203-205	2	0.00	0.66	0.00	0.48
VF20_02	MW326736	F: GAACCCTTTGAACACATGGCC R: CCAAGAAGCTTCGAAACTAGTTCC	$[AC]_{13}$	280-300	6	0.00	1.61	0.16	0.80
VF20_03	MW326737	F: AGCAATGTTCTAGGTCAGGGC R: CGATTTGCCCTAATCTTAGCGC	$[GA]_6$	177-197	7	0.00	1.24	0.26	0.62
VF20_05	MW326738	F: TGAAATGCAGACTGAAACGC R: GTTTGGTTCACGTCTGGTTGG	$[AT]_7$	290-315	6	0.00	1.43	0.00	0.72
VF20_14	MW326739	F: GGTTCACTGTTCATATGAATGATGC R: ATAAAGAAGTGCCACCCGTCC	$[TC]_7$	218-245	6	5.26	1.51	0.11	0.75
VF20_17	MW326740	F: GATGGTGCCAACTGATGAAGC R: GACTTCTAGGAGGTTGGTGCC	$[AT]_{12}$	366-385	8	5.26	1.62	0.83	0.74
VF20_20	MW326741	F: AATGCTCAAATTGCTTACGC R: TCTTAGAGCCTTGGATACTCCG	$[TA]_9$	116-130	5	0.00	1.47	0.42	0.76
VF20_21	MW326742	F: TAGATGCCTTGTTGTTGTTGC R: CAAACGTGATTGCTGGATGGG	$[TAT]_7$	176-196	6	0.00	1.33	0.37	0.67
VF20_22	MW326743	F: TCAATCAGAGCCTTGTTTGTGC R: ATTGTTTGTTGCAGCTTTGGC	$[GTA]_6$	117-119	2	0.00	0.58	0.00	0.40
VF20_24	MW326744	F: GGAGGAGATATGAGTGGGTTGG R: AGATGATGATGATGAGTGTACC	$[TAT]_6$	358-392	6	15.79	1.20	0.12	0.59
VF20_37	MW326745	F: GTTGACAGCGTTATGAAATTGG R: CCATAACCTAGGATCCTTGAGC	$[AAAT]_4$	390-395	2	5.26	0.69	0.11	0.51
VF20_41	MW326746	F: TCAGGTTGGCTCATGATACCG R: ATGGAACCACTACAACCAACC	$[TCCC]_4$	391-394	2	21.05	0.69	0.00	0.51
VF20_43	MW326747	F: TTCACGGTGAGTCAAGGAACC R: ATTGAAATGCAAGGGTCGACC	$[TTTA]_5$	284-314	3	5.26	0.85	0.11	0.55
VF20_44	MW326748	F: ATTTGACAACAACCCTACGCG R: GGCATGAGTAGGATGAAATGTTGG	$[TCTT]_4$	363-376	4	0.00	1.37	0.84	0.76
VF20_46	MW326749	F: ACATGCTTTGCACATGAAGGG R: AACAACCCGAACCTGACTTGC	$[TTTA]_4$	150-182	2	0.00	0.21	0.11	0.10
mean					4.47	3.86	1.10	0.23	0.60

N = number of alleles; Missing (%) = percent of individuals primers did not amplify on; H’ = Shannon’s diversity index; He = expected heterozygosity; Ho = observed heterozygosity

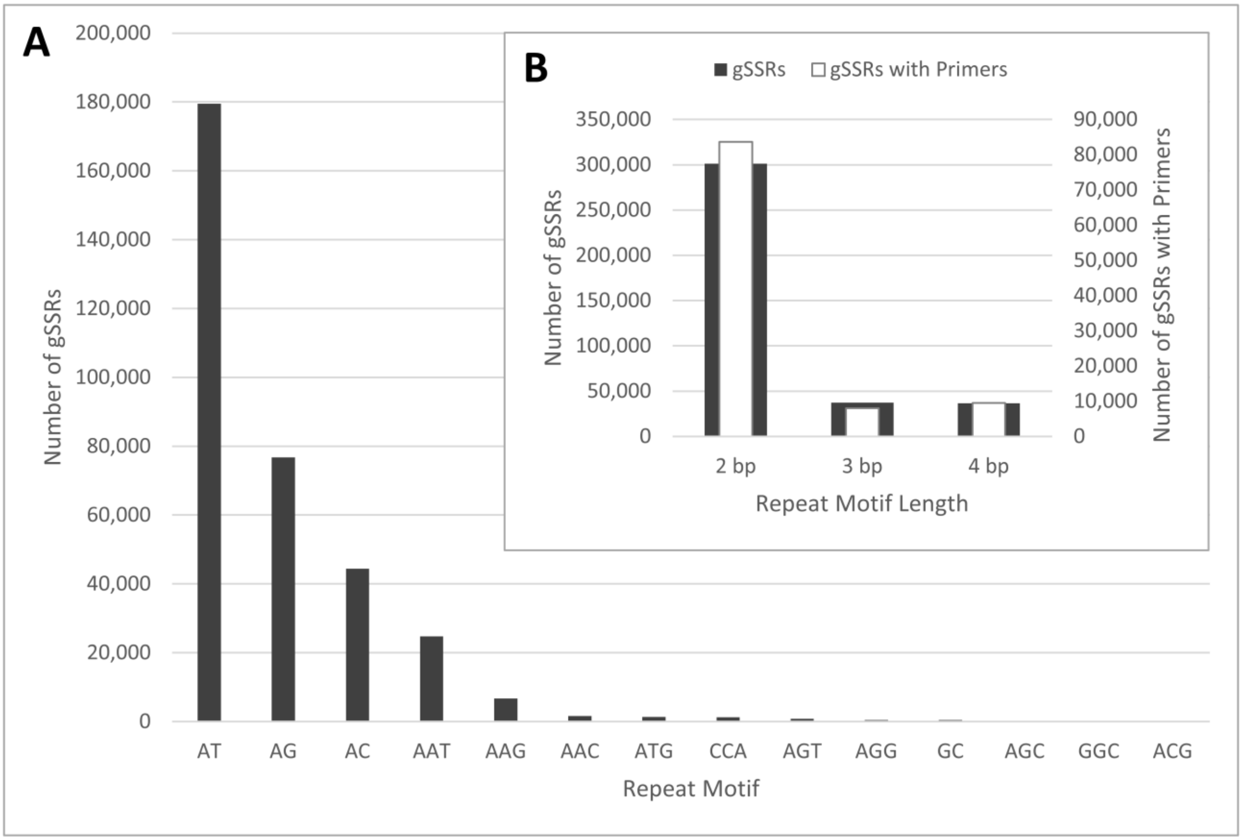

Figure 1.1. Genomic simple sequence repeats (gSSRs) discovered in the de novo assembled genome of Viburnum farreri ‘Nanum’. Overall number of gSSRs identified with our algorithm are in grey, based on repeat motif (A) and repeat motif length (B). Note specific repeat motif frequencies for tetra-nucleotide repeats were not calculated and therefore not included in A. The number of gSSRs with primers designed for the locus based on repeat motif length are depicted in white and on the secondary axis (B). bp = base pairs.

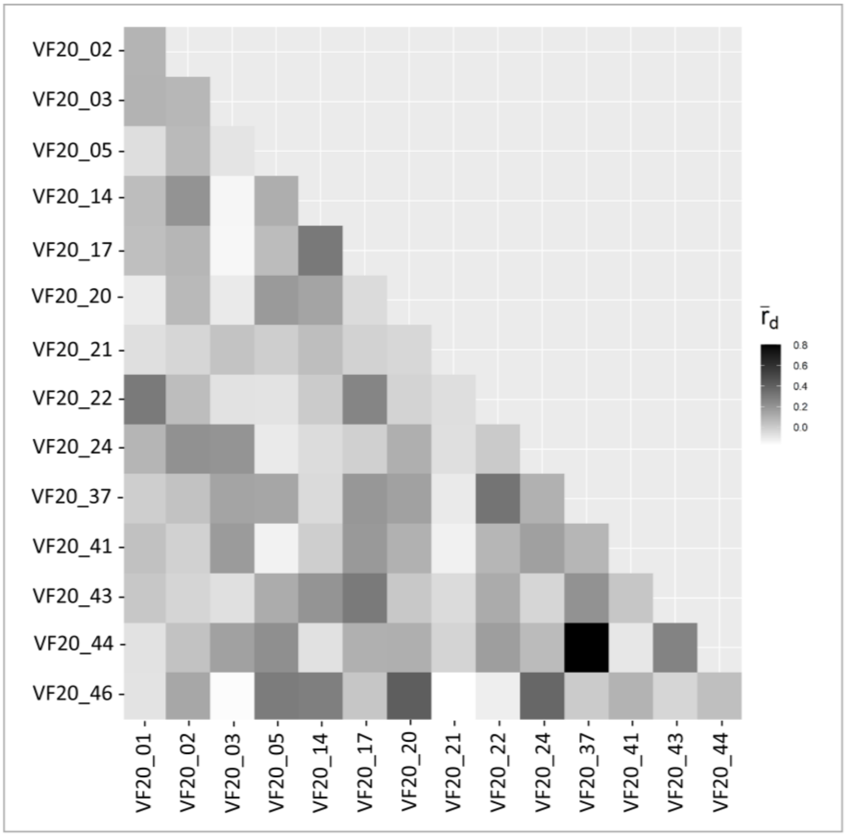

Figure 1.2. Linkage disequilibrium of the developed genomic simple sequence repeats (gSSRs). Pairwise $\bar{r}_d$ was calculated and displayed in a heatmap to infer if any loci were associated. The darker the square, the more of an association there is between the two loci. The lighter the square, the less of an association.

Chapter 2: Cross-Species Amplification of 49 Simple Sequence Repeat Markers Across *Viburnum* and Closely Related Taxa

Abstract

Approximately 160 species are classified within the *Viburnum* genus with many cultivated for horticultural purposes. The distribution of the genus is dispersed in temperate and subtropical regions of the Northern Hemisphere. *Viburnum* is also a useful model for studying evolutionary history and inferring how species expanded into their current distributions. Simple sequence repeat (SSR) markers were previously developed for five *Viburnum* species including *V. dilatatum* (*Succotinus* subclade; *Laminotinus* clade), *V. farreri* (*Solenotinus* subclade; *Crenotinus* clade), *V. rufidulum* (*Lentago* subclade; *Valvatotinus* clade), and *V. triphyllum* and *V. pichinchense* (*Oreinotinus* subclade; *Porphyrotinus* clade). The cross-species amplification of some markers was evaluated in a limited capacity, but there has not been any published wide-scale evaluation for all 49 markers. In this study, 49 markers were evaluated for the cross-species amplification to 206 samples from 46 *Viburnum* species and five additional species in the Viburnaceae and Caprifoliaceae. A subset of 14 potentially comprehensive markers for the species classified in Viburnaceae was identified and the ability to detect polymorphisms in species outside of their subclade was also demonstrated. The 49 markers had overall amplification success in 53% of the samples and 58% success within the *Viburnum* genus and 11% outside the genus. The comprehensive marker set amplified loci in 75% of all samples tested, 81% of *Viburnum* samples, and 15% outside of the genus. This set of markers can be applied to study genetic diversity and populations of most *Viburnum* species and close allies.

Introduction

Viburnum L. was formally classified within the Caprifoliaceae (honeysuckle family) alongside *Lonicera* L. and *Weigela* Thunb. This large genus, comprised of approximately 160 species (Landis et al., 2020), is now classified in the Viburnaceae (formerly Adoxaceae; Ulloa Ulloa et al., 2018) with *Adoxa* L. and *Sambucus* L. Species within *Viburnum* are native to temperate and subtropical regions of the Northern Hemisphere, and the range extends into the Southern Hemisphere in the mountainous regions of Southeastern Asia and South America. The somatic chromosome numbers of *Viburnum* species range from 18 to 72 (Egolf, 1962; Zhang et al., 2016), and therefore could range in ploidy level from diploid to octaploid. Not all species have been studied equally, but the majority of the species are 2n=2x=18 (Egolf, 1962; Winkworth and Donoghue, 2004; Zhang et al., 2016). Studied *Viburnum* genome sizes are classified as small to intermediate when compared to plants overall and have 2C values ranging from 4.29 to 24.23 Gbp (Olszewska and Osiecka, 1984; Bai et al., 2012; Pustahija et al., 2013; Zhang et al., 2013; Moeglein et al., 2020). However, the genome sizes are larger than other woody genera, such as *Cornus* with 2C values ranging from 1.89 to 6.66 Gbp (Zonneveld et al., 2005; Siljak-Yakovlev et al., 2010; Bai et al., 2012; Cao et al., 2014; Fridley and Craddock, 2015) and *Pyrus* ranging from 1.13 to 1.27 Gbp (Dickson et al., 1992; Jedrzejczyk and Sliwinska, 2010; Pustahija et al., 2013). The genus *Viburnum* includes many species of shrubs to small trees with year-round ornamental qualities, which are grown for their fragrant flowers and attractive foliage. There are 70 species and interspecific hybrids in cultivation (Dirr, 2007) that generated $23.2 million in wholesale and retail sales in 2017 (USDA-NASS, 2020). This well-sampled, geographically widespread, and abundant genus also provides a model for addressing evolution, biogeography, phylogenetics, and ecology-related questions, and has been the topic of many investigations (e.g., Winkworth and Donoghue, 2005; Clement et al., 2014; Spriggs et al., 2015; Spriggs et al., 2019b; Landis et al., 2020).

Much progress has been made in the phylogenetic classification of this genus (Donoghue et al., 2004; Winkworth and Donoghue, 2004; Winkworth and Donoghue, 2005; Clement and Donoghue, 2012; Eaton et al., 2017; Choi et al., 2018), but knowledge gaps persist in the taxonomy that if resolved, would provide better insight into the aforementioned topics. DNA barcoding was implemented but had a low success rate due to low sequence variability (Clement and Donoghue, 2012). Restriction-site-associated DNA sequencing (RADseq) was applied to the *V. nudum* species complex in North America and was successful in identifying three independent lineages in support of three separate species (Spriggs et al., 2019a). However, this next generation sequencing (NGS) approach may not be feasible for every species in this genus because of the relatively large costs associated with NGS in addition to the relatively large genome sizes of some *Viburnum* spp.

Four sets of genomic microsatellite markers, also known as simple sequence repeat (SSR) markers, were developed from species within the four major clades of *Viburnum* including *Laminotinus* (*Succotinus* subclade; Dean et al., 2011), *Valvatotinus* (*Lentago* subclade; Dean et al., 2015), *Porphyrotinus* (*Oreinotinus* subclade; Barish et al., 2016), and *Crenotinus* (*Solenotinus* subclade; Hamm et al., 2021) (phylogenetic tree shown in Figure 2.1; all tables and figures are located in the appendix). SSRs consist of short nucleotide motifs that are tandemly repeated. The nucleotide motifs can be between one and five basepairs (bp) long and are ubiquitous across the genome (Powell et al., 1996). These regions of the genome can accumulate mutations faster than others due to polymerase slippage during DNA replication and are flanked by relatively conserved genetic sequences, thus allowing for the design of polymerase chain reaction (PCR) primers (Peakall et al., 1998; Gupta and Varshney, 2000).

The resulting amplicons can then be sized to determine the alleles in an individual. The co-dominant nature, hypervariability, reproducibility, and PCR applicability make these genetic markers a popular option for population analyses (e.g., Ony et al., 2020), species and cultivar delimitation (e.g., Manechini et al., 2018), and breeding (e.g., Scariot et al., 2006). Furthermore, SSR markers often transfer to closely related species and genera, and only require small amounts of low-quality DNA for successful amplification. A cost analysis was performed in 2020 on SSR markers used in conjunction with the QIAxcel capillary electrophoresis system and determined the cost per sample per locus to be less than $12 (Stackhouse et al., 2021), making this a very affordable option for large-scale use. The combination of high polymorphism, low cost, and low-quality DNA requirements make SSRs an optimal tool for resolving *Viburnum* phylogeny at low taxonomic levels.

Using SSR markers developed in other species to study related species is common practice and has been demonstrated in various ornamental taxa such as *Cornus* (dogwood; Wadl et al., 2010) and *Cercis* (redbud; Wadl et al., 2012) as well as food crops such as *Glycine* (soybean; Peakall et al., 1998), *Prunus* (stonefruit; Mnejja et al., 2010), and *Foeniculum vulgare* (fennel; Aiello et al., 2020). A meta-analysis was completed on studies reporting cross-species amplification success in plants, fungi, and animals, and determined for eudicots overall. There was an amplification rate of almost 80% within genera and almost 60% among genera with polymorphic markers having a success rate of almost 60% within genera and 10% among genera (Barbara et al., 2007). The cross-species amplification of some of the *Viburnum* markers was displayed in their initial development publications, and a preliminary analysis was performed as part of Dean's thesis (Dean, 2014). Additionally, the markers developed from *V. dilatatum* were used in *V. opulus, V. trilobum,* and *V. sargentii* (Paulauskas et al., 2015), but no large-scale analysis has been published with any of the four sets.

The development of unique markers for each of the 163 species would be cost- and time-prohibitive. Therefore, the overarching goal of the study was to develop a set of comprehensive markers that could conceivably apply to studies of genetic diversity, population genetics, and potentially phylogenetic of all the species within the Viburnaceae and some allied genera in the Caprifoliaceae. The specific aims to accomplish this goal were the following: 1) To evaluate the cross-species amplification of the 49 previously published markers within the genus; 2) To compile a set of comprehensive markers for the genus, and 3) To demonstrate the ability of the comprehensive marker set to detect polymorphisms in species outside of the subclades from which they were developed.

Materials and Methods

Plant Materials and gDNA extraction

Leaf samples were collected from many arboreta and herbaria across the country (Table 2.1) except for the few species found locally in Eastern Tennessee. The goal was to obtain three different samples of each species and three species from each of the 16 subclades, following the classification scheme proposed in Clement et al. (2014) (i.e., *Lentago*, *Punctata, Euviburnum, Pseudotinus, Urceolata, Solenotinus, Lutescentia, Tinus, Sambucina, Coriacea, Succotinus, Lobata, Opulus, Mollotinus, Dentata,* and *Oreinotinus).* For the purposes of this study, those 16 subclades will be referred to as subclades and *Valvatotinus*, *Crenotinus*, *Porphyrotinus*, and *Laminotinus* will be referred to as clades, despite there being higher classifications than those four. For additional information about the formal phylogenetic definitions, see Clement et al. (2014). Due to limited sampling of some subclades, it was not possible to meet the sampling goal for every subclade, but 46 *Viburnum* species and five closely related species in the Viburnaceae and the Caprifoliaceae were obtained for a total of 206 samples (Table 2.1). Viburnaceae and Caprifoliaceae are both classified within Dipsacales, making Caprifoliaceae species ideal candidates for outgroup samples. For visualization of relatedness, the phylogenetic tree from Landis et al. (2020) was trimmed to only include the *Viburnum* species represented in this study (Figure 2.1).

Genomic DNA (gDNA) isolations followed the same protocol as Hamm et al. (2021). Leaf samples were frozen in liquid nitrogen before homogenization using a Beadmill 24 (Fishers Scientific, Pittsburgh, PA, U.S.). All leaf samples from arboreta were dried between pieces of newspaper before freezing. gDNA was extracted from leaf samples using the Omega E.Z.N.A. Plant DNA Kit (Omega Bio-tek Inc., Norcross, GA, U.S.), following the manufacturer's protocol except for adding 2% mass/volume polyvinylpyrrolidone (PVP) (Fisher BioReagents, Waltham, Massachusetts, U.S.) to the P1 Buffer and the incubation time at 65°C was increased from 10 min to 30 min. DNA from herbaria samples dating back to 1932 were successfully extracted with this kit. A CTAB protocol (Diversity Arrays Technology, 2019) was used with a few herbaria samples because of the paucity of leaf material. The quality of the extracted gDNA was assessed with a NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, U.S.).

SSR primers and genotyping conditions

A total of 49 primer pairs from four previous studies were used that were developed from the four major clades of *Viburnum*. Eleven markers were developed from *V. dilatatum* in

Laminotinus (Dean et al., 2011), seven from *V. rufidulum* in *Valvatotinus* (Dean et al., 2015), 16 from *V. triphyllum* and *V. pichinchense* in *Porphyrotinus* (Barish et al., 2016), and 15 from *V. farreri* in *Crenotinus* (Hamm et al., 2021) (Table 2.2). All SSR markers were single locus and polymorphic in the species from which they were obtained and included simple-perfect and compound-imperfect motifs. For simplicity, the markers developed from *V. triphyllum* and *V. pichinchense* were renamed Vore01-16 (Vore = *Viburnum Oreinotinus* subclade) in the same order found in the published table (original names in parentheses in Table 2.2; Barish et al., 2016). PCR was conducted with all 49 primers and 206 samples. A reaction volume of 10 µL, consisting of 5 µL of 2x Accustart II PCR SuperMix (Quantabio, Qiagen Beverly, Inc., Beverly, MA, U.S.), 3 µL autoclaved water, 1 µL of a mixture of 5 µM forward and primers, and 1 µL of 2 ng/µL gDNA was used. A single PCR thermal profile was used with all samples and markers and is as follows: 3 min of initial denaturation at 94°C, 10 touchdown (Don et al., 1991) cycles (94°C for 40s, 60°C -0.5°C/cycle for 40s, and 72°C for 45s) and 30 cycles (94°C for 40s, 55°C for 40s, 72°C for 45s), and a final extension at 72°C for 4 min. This thermal profile was selected as a compromise among all of the varying protocols from the initial development publications. PCR products were visualized with capillary electrophoresis (QIAxcel Advanced Electrophoresis System; Qiagen) and analyzed using a 25 to 500 basepair (bp) DNA size marker and an internal 15/600 bp alignment marker. Due to the wide variety of species and varying genetic distances among the samples, four positive controls were included on every 96-well PCR plate. The positive control samples were all from freshly collected leaves of species that were used in marker development (*V. dentatum, V. dilatatum, V. farreri*, and *V. rufidulum)*. A negative control of sterile water was also included on every plate. Allele sizes were determined using QIAxcel ScreenGel version 1.6.0.10.

Scoring of Amplification

Only peaks greater than 0.1 Relative Fluorescent Units (RFUs) were considered amplified alleles. Any amplicon greater than 600 bp was not sized because it was outside the 600 bp maximum of the alignment marker. A reaction was considered within the expected bp range and a successful cross-amplification if the amplicon size was within approximately 50 bp of the expected allele size from the original characterized species. Most of the strong peaks with limited noise were within 50 bp of the expected range, so 50 bp outside of the expected range was selected as the cutoff. If an amplicon was outside of that range, it was recorded as an * in the spreadsheet.

Proof of Concept -- Comprehensive Marker Set

Once cross-amplification was completed with all 49 markers, a comprehensive marker set was identified. The 14 markers identified displayed high amplification rates across most subclades. To prove these markers can characterize any species in the genus, a subset of the data was analyzed more closely. More than three leaf samples were obtained for *V. carlesii*, *V. opulus*, *V. plicatum*, and *V. tinus*, which are all members of subclades where markers were not developed. The amplification rates and observed heterozygosity were then calculated for each of the 14 comprehensive markers in the four species.

Results

Overall Dataset

Overall, out of a total of 10,094 potential reactions, 6,261 (62%) resulted in any amplification, and 5,368 (53%) produced amplicons within the expected size. Reactions with any amplification include ones that did not produce any amplicons close to the expected range, but did produce amplicons outside the range and/or greater than 600 bp. Amplicons outside of the expected range were assumed to be from non-target loci. Therefore, 893 reactions (6,261 any amplification – 5,368 expected size amplifications) only produced amplicons from non-target loci. Eleven percent of the reactions (1,097) produced any amplicons outside of the expected bp range. Seven percent or 682 of the reactions produced any amplicons greater than 600 bp. Reactions with “any amplification” (i.e., within the expected size, outside the expected range, and/or greater than 600 bp) were included in Table 2.2, and the rest of the percentages only include amplicons that were within the expected bp range. The full datasets with allele sizes are in an attached document (allele_size_tables.xlsx), with a tab for each of the four marker sets. The overall amplification rate for the markers in *Viburnum* was 58% and 11% in samples outside of the *Viburnum* genus.

The “any amplification” percentage and percentage of amplifications within the expected size were calculated for each marker with the lowest being 2% with Vf20_43 and the highest being 92% with Vore07 (Table 2.2). The marker set with the overall highest amplification was *Oreinotinus* (68%) and the lowest amplification percentage was *V. farreri* (40%). The *V. dilatatum* (a member *Succotinus* subclade and *Laminotinus* clade) markers amplified the most loci in *Succotinus* (95%), *Coriacea* (*Laminotinus*; 83%), and *Lobata* (*Laminotinus*; 82%; Table 2.3). The *V. farreri* (*Solenotinus* subclade and *Crenotinus* clade) markers amplified the most loci in *Solenotinus* (68%), *Lutescentia* (*Crenotinus*; 66%), and *Dentata* (*Porphyrotinus*; 54%). The

Oreinotinus subclade (*Porphyrotinus*) markers amplified the most loci in *Succotinus* (*Laminotinus*; 98%), *Dentata* (*Porphyrotinus*; 97%), and *Opulus* (not classified in a clade; 95%). The *V. rufidulum* (*Lentago* subclade and *Valvatotinus* clade) markers amplified the most loci in *Lentago* (93%), *Euviburnum* (*Valvatotinus*; 61%), and *Opulus* (not classified; 60%). The frequency of amplification per marker per clade was also calculated (Figure 2.2). The markers developed for the *Oreinotinus* subclade amplified loci in species the most uniformly across all clades.

Proof of Concept -- Comprehensive SSR Marker Set

Fourteen markers were selected as a starting point to characterize *Viburnum* species, regardless of clade. These markers included VD003, VD004, VD016, Vf20_02, Vf20_14, Vf20_22, Vore02, Vore07, Vore12, Vore14, Vore15, VR004, VR005, VR011, which displayed high amplification rates across most clades (Figure 2.3). This set of markers successfully amplified 75% of all samples, including 81% of *Viburnum* species samples and 15% of species not classified in the *Viburnum* genus. This comprehensive marker set amplified alleles well and detected heterozygosity in the four species selected for “proof of concept” (Table 2.4). The observed heterozygosity ranged from 0 to 1 with an average of 0.31 in *V. carlesii*, 0.44 in *V. opulus*, 0.27 in *V. plicatum*, and 0.41 in *V. tinus*. The amplification rates and observed heterozygosity were not as high in the species outside of *Viburnum*, from which amplification success was 16% in the rest of the Viburnaceae and 14% in Caprifoliaceae. The average observed heterozygosity was 0.17 for the species of the Viburnaceae and 0.02 for species in the Caprifoliaceae.

Discussion

Amplification within expected vs. outside size range

The transferability of SSR markers between individuals, species, and genera is dependent upon the conservation of the primer sites. Despite primer sequences being around 20 bp in length, they can bind to locations in the genome other than the target locus, causing spurious banding. This is especially prevalent in cross-amplification studies. Anything greater than 50 bp outside of the published expected range was considered to be a different locus than the target one, which happened in markers across all four sets and were not congregated to a set from a single source. This 50 bp cutoff was selected because the majority of strong peaks with little to no spurious banding occurred within 50 bp of the expected size range. Consequently, some of the amplicons outside of the 50 bp cutoff could be from the target loci.

Sequencing the amplicons would resolve this uncertainty, but sequencing was outside the scope of this thesis. However, it is highly recommended in cross-species studies due to the potential of homoplasy (Peakall et al., 1998; Wadl et al., 2010).

Future research with these markers should involve sequencing amplicons from species of distantly related *Viburnum* subclades from the marker source species to confirm the expected locus was targeted. Additionally, amplicons that were greater than 50 bp outside of the expected range should be sequenced to confirm whether they contain the expected SSR. This could be especially helpful for potentially using these markers in the Caprifoliaceae and the rest of the Viburnaceae. Many markers produced clean, strong bands in samples from outside of *Viburnum*, but most amplicons were too far outside of the expected size range to assert they were the correct locus without sequencing. After sequencing, more of the markers could potentially be used for a larger variety of species. Additionally, increasing the annealing temperature in the PCR protocol could help eliminate amplifying non-target loci.

Overall amplification

The transferability of SSR markers throughout plant species overall is lower than in animals (Barbara et al., 2007). On average the cross-amplification success of markers in eudicots is 71%, with amplification success within genera almost 80% and among genera almost 60% (Barbara et al., 2007). A cross-species amplification study in *Prunus* determined their markers had cross-species amplification of 84% within the genus and 38% outside *Prunus* (Mnejja et al., 2010). Despite the comparable size of the *Prunus* genus, with about 200 species (Potter, 2011), *Viburnum* markers displayed much lower cross-species amplification with 58% within genus and 11% outside of the genus. In the *Prunus* study, the marker group's performance in different *Prunus* crops ranged from 64% to 100%, whereas amplification success in this study ranged from 7% (VR in *Oreinotinus*) to 98% (Vore in *Succotinus*).

The overall trends of marker amplification success aligned with the subclades the markers were developed from. VD markers amplified the highest percentage of target loci in the *Laminotinus* and *Porphyrotinus* clades. Vf markers amplified the most in the *Crenotinus* clade and *Pseudotinus* subclade (not classified). VR markers amplified the most in *Valvatotinus* and *Crenotinus.* Additionally, VR markers amplified the highest percentage of target loci in the Viburnaceae and Caprifoliaceae out of the four marker sets. Vore markers had high amplification success in most clades, which is likely due to the SSRs initially being identified in four different species and subsequently developed in two species (Barish et al., 2016). This process was effectively selecting for markers that displayed cross-species amplification from the beginning. Despite this, the performance of Vore markers in the *Oreinotinus* subclade was poor compared to most other subclades. The leaf samples for the *Oreinotinus* subclade were from herbaria and collected between 1935 and 1998, therefore the sample age is likely a contributing factor to DNA degradation and the resulting low amplification success. The older sample age also likely explains the relatively low amplification success of all 49 makers in the *Oreinotinus* subclade.

The Vore markers that amplified particularly poorly in *Oreinotinus* were not initially discovered in species in the *Oreinotinus* subclade (Barish et al., 2016). Vore05 was originally from *V. trilobum* (*Opulus* subclade) and had 0% amplification in the *Oreinotinus* subclade. The Vore16 locus was isolated from *V. dentatum* (*Dentata* subclade) and only had 17% amplification in the *Oreinotinus* subclade. It should be noted Vore09 was mined from mitochondrial NGS data of *V. dentatum* and despite the marker amplifying well and being polymorphic, all samples were inherently homozygous at this locus and special consideration should be taken before use (Barish et al., 2016).

Phylogenetic and Morphological Classification Comparisons

The phylogenetic position of lone species (i.e., *V. clemensiae* and *V. amplificatum*) and subclades (i.e., *Pseudotinus,* and *Urceolata*) have been changed as additional data has accrued. *Viburnum clemensiae* has been placed as sister to the rest of *Viburnum* (Clement et al., 2014; Spriggs et al., 2015; Eaton et al., 2017), but recently has been considered sister to a clade containing *Crenotinus, Valvatotinus,* and subclades *Pseudotinus* and *Urceolata* (Landis et al., 2020). Vf markers had the highest amplification rate (47%), and *V. farreri* and *V. clemensiae* are also two of the few species that have panicle-like inflorescence. However, Vf and VD markers worked almost equally (47% and 45% respectively) in the *V. clemensiae* samples. This could indicate that *V. clemensiae* would be better suited as sister to all *Viburnum* species and not just a fraction of the species.

Pseudotinus has been classified as a polytomy with *Valvatotinus* and rest of *Viburnum* (*Pluriviburnum*; Clement et al., 2014), sister to *Valvatotinus* (Spriggs et al., 2015), and sister to *Urceolata* (Eaton et al., 2017; Landis et al., 2020). Out of the morphological features of buds, leaf margin, inflorescence architecture, and extrafloral nectaries, this subclade shares the most features in common with *V. rufidulum* in *Valvatotinus* (Clement et al., 2014). However, VR markers displayed the lowest amount of amplification to this subclade. The results of this study therefore better support the placement of *Pseudotinus* as sister to *Urceolata* rather than sister to *Valvatotinus*.

Urceolata has been classified as sister to *Amplicrenotinus* (*Crenotinus* + *V. amplificatum*; Clement et al., 2014; Spriggs et al., 2015) and sister to *Pseudotinus* (Eaton et al., 2017; Landis et al., 2020). Vf (*Crenotinus*) markers exhibited the lowest amplification success in *Urceolata*, which supports not classifying *Urceolata* as sister to *Amplicrenotinus*. *Urceolata* does not share many morphological features with any of the species markers were developed from, but interestingly it shares the least morphological features with *V. dilatatum* (Clement et al., 2014). The only feature they share is the umbel-like inflorescence, which is present in the majority of *Viburnum* species. Despite this, VD markers had the highest amplification percentage at 39%.

Proof of Concept -- Comprehensive SSR Marker Set

The primary goal of this study was to evaluate the cross-species amplification success of the 49 developed markers to guide future research and classification in *Viburnum*. Depending on the application, some markers will have higher amplification rates and polymorphisms than others, but as an initial go-to set, the following 14 markers are recommended: VD003, VD004, VD016, Vf20_02, Vf20_14, Vf20_22, Vore02, Vore07, Vore12, Vore14, Vore15, VR004, VR005, and VR011. If a higher annealing temperature is used, VR028 would be an ideal marker to either add to the set or replace VR005. VR005 was excluded from the recommended set because 21% of the reactions had numerous spurious bands. If investigating a species outside of *Viburnum*, markers outside of this comprehensive set would likely have higher amplification success. Adding markers with higher amplification success outside of *Viburnum* such as Vf20_21, Vore04, Vore10, VR028, and VR043 to markers VD016, Vf20_22, Vore07, Vore12, and Vore14 within the comprehensive set would likely yield better results for studies concerning species placed outside of *Viburnum*.

This comprehensive marker set provides coverage of all subclades and the proof-of-concept species. Similar to the overall study, this subset of the data also demonstrates that the more distance between the sample species and species the marker was developed from, the less conserved the primer site. *Viburnum carlesii* is a member of the *Euviburnum* subclade and *Valvatotinus* clade which helps to explain Vore12 (from *Porphyrotinus* clade) having low amplification rates. *Viburnum opulus* is a member of the *Opulus* subclade, which is not classified in one of the four clades but is closest related to the *Laminotinus* clade (*V. dilatatum*). Although most markers in the comprehensive set amplified loci from the majority of the samples, the principle of evolutionary distant species having less conserved primer sites helps explain why the VR markers had lower amplification rates than others. *Viburnum tinus* is a member of the *Tinus* subclade, which is also not classified in a clade. *Tinus* is closest related to the

Porphyrotinus and *Laminotinus* clades and more genetically distant from *V. rufidulum*, which is likely the cause of two of the VR markers having very low amplification rates.

Despite these comprehensive markers displaying wide cross-species amplification, they still also have relatively high observed heterozygosity rates. Once sequencing is performed on select amplicons, these comprehensive SSR markers will be ready for use within and outside *Viburnum*. Future applications for these markers could include characterization and population-level studies for any species within or closely related to *Viburnum* as well as adding them to established linkage maps (Al-Niemi et al., 2011) for future breeding efforts.

Appendix: Tables and Figures

Table 2.1. Classification and sources of plant materials used in this study.

Subclade	Species	Cultivar or variety (if applicable)	Location	Accession #	Year
Lobata	*acerifolium*		The Morton Arboretum	234-82	1982
			The Morton Arboretum	702-2000*1	2000
			U.S. National Arboretum	82648	2013
Sambucina	*beccarii*		Yale Peabody Museum	YU.100207	2011
			Yale Peabody Museum	YU.100211	2011
			Yale Peabody Museum	YU.100220	2011
Succotinus	*betulifolium*		The Morton Arboretum	855-2005*2	2005
			U.S. National Arboretum	67777-J	1996
			Herbarium of the Arnold Arboretum	275755	2007
Mollotinus	*bracteatum*		Spring Grove Cemetery and Arboretum	141	N/A
			Arnold Arboretum	229-98*A	1998
			Arnold Arboretum	1067-87*A	1987
Lantana	*carlesii*	‘Cayuga'	Spring Grove Cemetery and Arboretum	NG	N/A
			Spring Grove Cemetery and Arboretum	87	N/A
			The Morton Arboretum	532-62*1	1962
		var. bitchiuense	Mt. Airy Arboretum	201404053 6249	2014
			Mt. Airy Arboretum	1952 766	1952
		‘Cayuga'	Mt. Airy Arboretum	199206157 3582	1992
		‘Summer Hill'	Mt. Airy Arboretum	201004035 5944	2010
			The Morton Arboretum	720-46*1	1946
			The Morton Arboretum	912-73*1	1973
		‘Prairie Rose'	JC Raulston Arboretum	160904	2016
			U.S. National Arboretum	61-H	1938
			U.S. National Arboretum	78299	2009
		‘Diana'	U.S. National Arboretum	79946-H	2011
		‘Compactum'	U.S. National Arboretum	76904	2007
Lentago	*cassinoides*		The Morton Arboretum	224-94*3	1994
		‘J.N. Select'	The Morton Arboretum	535-2010*1	2010
		‘Lil Ditty'	Mt. Airy Arboretum	201803022 6522	2018
			Morris Arboretum	2000-337*B	2000
	clemensiae		Herbarium of the Arnold Arboretum	No. 30356	1932
			Herbarium of the Arnold Arboretum	No. 31500 = 31491	1933

Table 2.1. continued

Subclade	Species	Cultivar or variety (if applicable)	Location	Accession #	Year
Coriacea	*coriaceum*		U.S. National Arboretum Herbarium	NA0026928	1978
			University of Tennessee Herbarium	N/A	1983
			Carnegie Museum of Natural History Herbarium	CM256182	1966
Orienotinus	*costaricanum*		U.S. National Arboretum Herbarium	NA 0133740	1974
			U.S. National Arboretum Herbarium	NA 0133741	1976
			U.S. National Arboretum Herbarium	NA 0133742	1972
Coriacea	*cylindricum*		Arnold Arboretum	747-2016*D	2016
			University of Washington Botanical Garden	118-09	N/A
			Morris Arboretum	2016-214*#	2016
Tinus	*davidii*		University of Washington Botanical Garden	324-71	N/A
			U.S. National Arboretum Herbarium	NA0107012	1937
			U.S. National Arboretum Herbarium	NA0107011	1937
Oreinotinus	*dentatum*		The Morton Arboretum	119-98*1	1998
			Spring Grove Cemetery and Arboretum	7A	N/A
		‘Blue Muffin'	Mt. Airy Arboretum	200312112 5471	2003
Succotinus	*dilatatum*		The Morton Arboretum	49-86*2	1986
			Spring Grove Cemetery and Arboretum	MB	N/A
			Mt. Airy Arboretum	1938 805	1938
Mollotinus	*ellipticum*		The Morton Arboretum	33-60*1	1960
			University of Washington Botanical Garden	527-61	N/A
			Gray Herbarium	386453	1981
Solenotinus	*farreri*		The Morton Arboretum	398-83*1	1983
		‘Nanum’	Arnold Arboretum	293-2003*C	2003
			U.S. National Arboretum Herbarium	NA0111163	1985
Pseudotinus	*furcatum*		Arnold Arboretum	11-98*A	1998
			JC Raulston Arboretum	110961	2013
			University of Tennessee Herbarium	N/A	1990
Oreinotinus	*hartwegii*		U.S. National Arboretum Herbarium	NA0133731	1995
			U.S. National Arboretum Herbarium	NA0133732	1998
			U.S. National Arboretum Herbarium	NA0133733	1989

Table 2.1. continued

Subclade	Species	Cultivar or variety (if applicable)	Location	Accession #	Year
Sambucina	*inopinatum*		U.S. National Arboretum Herbarium	NA0133730	1976
Lobata	*kansuense*		Herbarium of the Arnold Arboretum	No. 27348	1997
			Herbarium of the Arnold Arboretum	289806	2005
			Herbarium of the Arnold Arboretum	No. 27417	1997
Pseudotinus	*lantanoides*	aka alnifolium	Arnold Arboretum	438-2011*B	2011
		aka alnifolium	Arnold Arboretum	681-2016*B	2016
			Herbarium of the Arnold Arboretum	721669	1983
			Herbarium of the Arnold Arboretum	960443	2015
Punctata	*lepidotulum*		U.S. National Arboretum Herbarium	NA0107013	1934
			U.S. National Arboretum Herbarium	NA0107014	1934
Lutescentia	*lutescens*		Yale Peabody Museum	YU.100177	2011
			Yale Peabody Museum	YU.100180	2011
			Yale Peabody Museum	YU.100205	2014
Lantana	*macrocephalum*		Spring Grove Cemetery and Arboretum	ARCH	N/A
			Mt. Airy Arboretum	1950 794	1950
			U.S. National Arboretum	67343-HJ	1984
			U.S. National Arboretum	49597-H	1980
Mollotinus	*molle*		The Morton Arboretum	488-85*1	1985
			The Morton Arboretum	596-2001*1	2001
			U.S. National Arboretum Herbarium	NA0115499	2012
			U.S. National Arboretum Herbarium	NA0088717	2010
			U.S. National Arboretum Herbarium	NA0088713	2010
Solenotinus	*odoratissimum*		University of Washington Botanical Garden	1404-56	N/A
			Yale Peabody Museum	YU.100880	2013
		var. awabuki 'Chindo'	National Arboretum	56506H	1985
			U.S. National Arboretum Herbarium	NA0107026	1971
			U.S. National Arboretum Herbarium	NA0107025	1965
			U.S. National Arboretum Herbarium	NA0107024	1966

Table 2.1. continued

Subclade	Species	Cultivar or variety (if applicable)	Location	Accession #	Year
Opulus	*opulus*	var. americanum 'Compactum'	The Morton Arboretum	374-76*1	1976
		var. opulus	The Morton Arboretum	163-99*2	2002
		‘Notcutt'	Spring Grove Cemetery and Arboretum	27A	N/A
		‘Roseum'	Spring Grove Cemetery and Arboretum	80	N/A
		‘Nanum'	Mt. Airy Arboretum	199206148 3573	1992
		‘Notcutts'	Mt. Airy Arboretum	195604 759	1956
			University of Washington Botanical Garden	376-89-A	N/A
			University of Washington Botanical Garden	952-41	N/A
			University of Washington Botanical Garden	X-137	N/A
		‘Xanthocarpum'	University of Washington Botanical Garden	95-46	N/A
		‘Roseum'	Morris Arboretum	2009-047*A	2009
		‘Aureum'	Morris Arboretum	1983-028*B	1983
		‘Roseum'	JC Raulston Arboretum	970288	1997
		var. caluescens	JC Raulston Arboretum	N/A	N/A
			U.S. National Arboretum	74961-H	2012
		var. americanum	U.S. National Arboretum	41717	1977
		var. opulus	U.S. National Arboretum	66971	1974
			U.S. National Arboretum	73649	2003
Lobata	*orientale*		The Morton Arboretum	235-72*1	1972
			JC Raulston Arboretum	80255	2012
			U.S. National Arboretum Herbarium	NA0107015	1964
Lutescentia	*plicatum*	‘Lanarth'	The Morton Arboretum	338-2006*1	2006
			The Morton Arboretum	370-76*1	1976
		‘Mariesii'	Spring Grove Cemetery and Arboretum	126	N/A
		‘Pink Beauty'	Spring Grove Cemetery and Arboretum	65	N/A
		‘Popcorn'	Spring Grove Cemetery and Arboretum	121	N/A
		‘Shasta'	Spring Grove Cemetery and Arboretum	55	N/A
			Spring Grove Cemetery and Arboretum	53	N/A
			Spring Grove Cemetery and Arboretum	SGFH	N/A
		f. tomentosum 'Summer Snowflake'	Mt. Airy Arboretum	201108058 6056	2011

Table 2.1. continued

Subclade	**Species**	**Cultivar or variety (if applicable)**	**Location**	**Accession #**	**Year**
Lutescentia	*plicatum*	‘Newzam' Newport TM	Mt. Airy Arboretum	198205 719	1982
		‘Pink Sensation'	Mt. Airy Arboretum	201304021 6148	2013
			Mt. Airy Arboretum	194011 729	1940
		f. tomentosum 'Fireworks'	Mt. Airy Arboretum	200010057 5244	2000
		f. tomentosum 'Mariesii'	Mt. Airy Arboretum	1957 792	1957
		‘Mary Milton'	JC Raulston Arboretum	30523	2005
Tinus	*propinquum*		University of Washington Botanical Garden	136-67-A	N/A
			JC Raulston Arboretum	N/A	N/A
			U.S. National Arboretum	49604-J	1980
Lentago	*prunifolium*		The Morton Arboretum	532-81*5	1981
			Spring Grove Cemetery and Arboretum	143	N/A
			Mt. Airy Arboretum	764	1938
Punctata	*punctatum*		Yale Peabody Museum	YU.100198	2011
			Yale Peabody Museum	YU.100202	2011
			Yale Peabody Museum	YU.101617	2015
Oreinotinus	*recognitum*		Arnold Arboretum	633-93*A	1993
			Arnold Arboretum	39-68*C	1968
			Arnold Arboretum	19-60*A	1960
Lentago	*rufidulum*		The Morton Arboretum	698-2000*1	2000
			Eliza Gate Park Collected by Dr. Klingeman	N/A	2020
			Eliza Gate Park Collected by Dr. Klingeman	N/A	2020
Sambucina	*sambucinum*	var. tomentosum	A.C. Moore Herbarium	USCH00680 27	2013
Opulus	*sargentii*		The Morton Arboretum	174-2003*1	2003
			The Morton Arboretum	363-81*7	1981
			The Morton Arboretum	361-93*1	1993
Lantana	*schensianum*		The Morton Arboretum	310-2002*1	2002
			The Morton Arboretum	277-2002*2	2002
			The Morton Arboretum	431-84*1	1984
Succotinus	*setigerum*		Spring Grove Cemetery and Arboretum	136	N/A
			JC Raulston Arboretum	N/A	N/A
			University of Tennessee Herbarium	8516	1986
Solenotinus	*sieboldii*	‘Seneca'	Spring Grove Cemetery and Arboretum	134	N/A
			Mt. Airy Arboretum	779	1966

Table 2.1. continued

Subclade	Species	Cultivar or variety (if applicable)	Location	Accession #	Year
Solenotinus	*sieboldii*		University of Washington Botanical Garden	117-95-A	N/A
Pseudotinus	*sympodiale*		Yale Peabody Museum	YU.100868	2013
			Yale Peabody Museum	YU.101545	2015
			U.S. National Arboretum Herbarium	NA0019385	1980
			U.S. National Arboretum Herbarium	NA0020487	1987
			U.S. National Arboretum Herbarium	NA0030171	1995
Urceolata	*taiwanianum*		U.S. National Arboretum Herbarium	NA0107016	1977
			U.S. National Arboretum Herbarium	NA0107017	1971
			U.S. National Arboretum Herbarium	NA0044607	1964
			U.S. National Arboretum Herbarium	NA0018904	1978
Sambucina	*ternatum*		U.S. National Arboretum Herbarium	NA0133729	1986
Tinus	*tinus*	‘Lucidum'	University of Washington Botanical Garden	250-41	N/A
		‘Pink Prelude'	University of Washington Botanical Garden	423-90	N/A
		‘Purpureum'	University of Washington Botanical Garden	50-10	N/A
		‘Spring Bouquet'	University of Washington Botanical Garden	229-89	N/A
		‘Anui'	JC Raulston Arboretum	N/A	N/A
			Carnegie Museum of Natural History Herbarium	CM317215	1981
			Carnegie Museum of Natural History Herbarium	CM317130	1985
			A.C. Moore Herbarium	USCH0001011	1999
			A.C. Moore Herbarium	USCH0001012	1993
Oreinotinus	*tinoides*		U.S. National Arboretum Herbarium	NA0133734	1938
			U.S. National Arboretum Herbarium	NA0133735	1972
			U.S. National Arboretum Herbarium	NA0133736	1978
Opulus	*trilobum*	‘Wentworth'	Spring Grove Cemetery and Arboretum	77	N/A
		‘Compactum'	Spring Grove Cemetery and Arboretum	126	N/A
			Gray Herbarium	691486	1986
Oreinotinus	*triphyllum*		U.S. National Arboretum Herbarium	NA0133737	1976
			U.S. National Arboretum Herbarium	NA0133738	1971

Table 2.1. continued

Subclade	Species	Cultivar or variety (if applicable)	Location	Accession #	Year
Oreinotinus	*triphyllum*		U.S. National Arboretum Herbarium	NA0133739	1935
Urceolata	*urceolatum*		U.S. National Arboretum Herbarium	NA0107019	1980
			U.S. National Arboretum Herbarium	NA0107018	1980
			Herbarium of the Arnold Arboretum	No. 1664	1992
			Herbarium of the Arnold Arboretum	No. 22673	1980
	Species unknown		U.S. National Arboretum	69941-009	1999
Species outside of *Viburnum* genus					
Genus	Species				
***Adoxa* (Viburnaceae)**	*moschatellina*		U.S. National Arboretum Herbarium	NA0107020	1975
			U.S. National Arboretum Herbarium	NA0107021	1970
			U.S. National Arboretum Herbarium	NA0107022	1953
			U.S. National Arboretum Herbarium	NA0107023	1981
			U.S. National Arboretum Herbarium	NA0107034	1936
			U.S. National Arboretum Herbarium	NA0107033	1962
			University of Tennessee Herbarium	TENN-V-0207068	1940
			University of Tennessee Herbarium	8526	1967
***Sambucus* (Viburnaceae)**	*nigra*		Morris Arboretum	2017-164*B	2017
			Gray Herbarium	2183123	1980
			Herbarium of the Arnold Arboretum	2183118	1950
***Sambucus* (Viburnaceae)**	*canadensis*		Morris Arboretum	2015-234*A	2015
			Morris Arboretum	2013-064*A	2013
			Morris Arboretum	2015-235*D	2015
***Lonicera* (Caprifoliaceae)**	sp.		Ten Mile Creek Greenway	RNT collected	2020
			along Northshore Lyon's crossing	RNT collected	2020
			Lakeshore Park	TPH collected	2020
			Lakeshore Park	TPH collected	2020
			Lakeshore Park	TPH collected	2020
***Weigela* (Caprifoliaceae)**		‘Spilled Wine’	Pope’s Greenhouse	RNT collected	2021

Table 2.2. Characteristics of 49 SSR markers and their amplification percentages in all 206 *Viburnum* and closely related species samples.

Locus	GenBank #	Primer sequences (5’-3’)	Repeat motif	Allele size range (bp)	Any amp[a] (%)	Expected amp[b] (%)
VD[c]003	HQ997898	F: TGGCTCAGATGCATTGAAGAATAG R: GCTGCATGCATCTTCAAATAGG	$[CA]_{12}$	105-143	79	78
VD004	HQ997899	F: GCTGCATGCATCTTCAAATAGG R: ATATCTCGAGGGAGACTGCAACAG	$[AC]_{16}$	108–156	85	83
VD005	HQ997900	F: TTTTAAAACTTTGCACCCTTGCAC R: AGAATAAAGTCCAGCTCCCTGACC	$[CA]_{7}$	115–178	86	74
VD006	HQ997901	F: ATAACCATATGCGTGTGTATGTTGG R: GACGTTGCAGGAGCTTCTTATCTC	$[GT]_{8}$	137–141	8	7
VD009	HQ997902	F: GTTTGGGACATGTTCAGTTCTTCC R: AATGTCAGCAAATCAAAATCCAAAC	$[TG]_{12}$	116–163	53	53
VD012	HQ997903	F: TCGACTCTACATTCACTACCCTCC R: CATACGGGTATACGCACACATGC	$[AC]_{16}$	128–174	44	17
VD014	HQ997904	F: GCAAACCAAACCACACAAACAC R: ATCTAGGTCGGCTGCTACTGATTG	$[CT]_{6}[CA]_{7}$	142–204	75	69
VD016	HQ997905	F: TACCCCTCACAAACACAAACACTG R: AACAATAATGGTGTGGGGTGTTG	$[AC]_{12}$	71–129	94	82
VD017	HQ997906	F: ACCAACCCAATTGCTCAATATCAC R: GGTTGTCCGCCAGAAGTAGTAGTG	$[AC]_{6}$	165–170	38	37
VD018	HQ997907	F: CTTGCTCGATTTCCCTTATTTGTC R: ATCTCAAGCAAGTCTCACTCCCTC	$[CA]_{16}$	93–112	64	61
VD019	HQ997908	F: AAAGTTGCAAATTACACGCTGATG R: TACCTCCAATTTCACGGTTCTCTC	$[TG]_{16}$	125–167	71	65
Vf20[d] _01	MW326735	F: ACGATAAATGTGTATGCTCGC R: AACCCGGGAAGAAAGGTTACC	$[AT]_{6}$	203-205	72	72
Vf20_02	MW326736	F: GAACCCTTTGAACACATGGCC R: CCAAGAAGCTTCGAAACTAGTTCC	$[AC]_{13}$	280-300	84	78
Vf20_03	MW326737	F: AGCAATGTTCTAGGTCAGGGC R: CGATTTGCCCTAATCTTAGCGC	$[GA]_{6}$	177-197	22	5
Vf20_05	MW326738	F: TGAAATGCAGACTGAAACGC R: GTTTGGTTCACGTCTGGTTGG	$[AT]_{7}$	290-315	64	58

Table 2.2. continued

Vf20_14	MW326739	F: GGTTCACTGTTCATATGAATGATGC R: ATAAAGAAGTGCCACCCGTCC	$[TC]_7$	218-245	81	75
Vf20_17	MW326740	F: GATGGTGCCAACTGATGAAGC R: GACTTCTAGGAGGTTGGTGCC	$[AT]_{12}$	366-385	72	27
Vf20_20	MW326741	F: AATGCTCAAATTGCTTACGC R: TCTTAGAGCCTTGGATACTCCG	$[TA]_9$	116-130	34	18
Vf20_21	MW326742	F: TAGATGCCTTGTTGTTGTTGC R: CAAACGTGATTGCTGGATGGG	$[TAT]_7$	176-196	31	10
Vf20_22	MW326743	F: TCAATCAGAGCCTTGTTTGTGC R: ATTGTTTGTTGCAGCTTTGGC	$[GTA]_6$	117-119	88	83
Vf20_24	MW326744	F: GGAGGAGATATGAGTGGGTTGG R: AGATGATGATGATGAGTGTACC	$[TAT]_6$	358-392	21	13
Vf20_37	MW326745	F: GTTGACAGCGTTATGAAATTGG R: CCATAACCTAGGATCCTTGAGC	$[AAAT]_4$	390-395	24	23
Vf20_41	MW326746	F: TCAGGTTGGCTCATGATACCG R: ATGGAACCACTACAACCAACC	$[TCCC]_4$	391-394	11	11
Vf20_43	MW326747	F: TTCACGGTGAGTCAAGGAACC R: ATTGAAATGCAAGGGTCGACC	$[TTTA]_5$	284-314	4	2
Vf20_44	MW326748	F: ATTTGACAACAACCCTACGCG R: GGCATGAGTAGGATGAAATGTTGG	$[TCTT]_4$	363-376	64	63
Vf20_46	MW326749	F: ACATGCTTTGCACATGAAGGG R: AACAACCCGAACCTGACTTGC	$[TTTA]_4$	150-182	77	69
Vore[e]01 (H121)[f]	KX447798	F: ACCCCCTCTCCTTCTCTGTG R: GGAGAGTGTAAGGCTCTACTC	$[CT]_9$	156-174	71	54
Vore02 (O42)	KX447799	F: GGCCATTAGAAAAGGTCTTCG R: CCACGCGGGAATAATACG	$[TA]_6[TG]_5[TA][TG]_5$	183–189	83	83
Vore03 (O91)	KX447800	F: CCACAATGGCTTCCTTTGTAA R: CCGAAGAATCCATGTTGGAC	$[AC]_{12}$	200–266	39	38
Vore04 (O104)	KX447801	F: GGTAATCGACCACAACACGA R: ATCTCGAGGGAGACTGCAAC	$[AC]_{19}$	168–186	82	78
Vore05 (O121)	KX447802	F: CTCTCTCCTGTGTCTACCTTGAGC R: TGGGGGTTGTAATTTCTCCA	$[AG]_{14}$	112–158	65	64
Vore06 (H81)	KX447797	F: GGGCCGAGTTCTTTTAAACC R: GAAGCGAAATGCACTCAACA	$[GA]_{16}$	199–233	76	76
Vore07	KX447804	F: GGTTGAGTGTTGCAGGAAGG	$[GT]_5$	236–244	93	92

Table 2.2. continued

(TN2)		R: CGACCTTTGGCAATGGACTC				
Vore08	KX447805	F: AGTGTGGGTATGAGATGGGC	$[TA]_5$	138–144	77	41
(TN3)		R: ACTTACTCACGCTCCACTCG				
Vore09	KX447806	F: GCCCTATACCCACCCAATTTC	$[AT]_6$	411–427	84	84
(DM1)		R: ATACGAGTCCAAAGGCAGGG				
Vore10	KX447809	F: GTTAGCAATGGGGAGCCAAG	$[CA]_{10}$	140–150	83	69
(DN10)		R: GAGTCGATCGCTCAAATAGAGG				
Vore11	KX447810	F: CAACTTTGGTGGGTTTGAAAGC	$[CT]_9$	222–238	80	80
(DN13)		R: TTCTGCCAATTGATGCATCTTG				
Vore12	KX447811	F: TTCTTTCCCTCCCTCTGCAG	$[TA]_8$	108–134	80	68
(DN15)		R: CAGAGGCTAGGGTTATGGGC				
Vore13	KX447812	F: AACTCTCACCGCTCACCATC	$[AG]_8$	352–380	72	39
(DN16)		R: TGGGCTTGAGAGTTCGCTAG				
Vore14	KX447813	F: CACTCCATTGCACCTTCACC	$[TA]_8$	240–250	83	83
(DN18)		R: TGGTAAGGTGGCATATCGGG				
Vore15	KX447814	F: CCTCCAGAGCTTCGCCTC	$[CT]_8$	449	85	81
(DN19)		R: TCACCGTAGCTAGAAATGTCAG				
Vore16	KX447815	F: GTGCCTTAACAGCCCAGAAG	$[AG]_7$	373–483	62	53
(DN22)		R: AGGGCTTGGACTCCGAAATC				
VR^{g}004	KC236414	F: CTACCGCACATATGCACCTAC	$[TG]_6\ldots[TG]_7$	210–223	56	41
		R: TACAGATCGGGAAGGTGTAAGG				
VR005	KC236415	F: TGCTTCCATCTCTTTTCTCTCC	$[TG]_{12}\ldots[TG]_6$	113–182	56	40
		R:GTGTGTGGCTGTGTTTGTACG				
VR008	KC236416	F: CCAAGCCCCATTTTATAAATACC	$[GT]_{15}$	93–123	25	25
		R: CATTCTGCCATTTTATTGAGTCC				
VR011	KC236417	F: GCATATGTGCACACACGAGAG	$[CA]_{14}$	162–245	80	79
		R: TATGGATTGGGAAGGTGTTAGG				
VR012	KC236418	F: CAAAGTGGGCAGAGAAGTAGC	$[TA]_7$	216–247	50	23
		R: CATTGTAGAGCACCACAAATTCC				
VR028	KC236419	F: GCTCGTAGCAGGGGTGTGTAT	$[CA]_5\ldots[AC]_8$	116–185	79	52
		R: CACGCACACTGCACACACT				
VR043	KC236420	F: TATGTGAGGATGAAGGTGATGG	$[CT]_6\ldots[TC]_5\ldots[TC]_5$	213–276	33	33
		R: TTACATTCTGGCAGTAGCAACC				
Overall					62	53

[a] Includes samples that only produced amplicons greater than 50 bp outside of the published expected range or greater than 600 bp

[b] Only includes samples that produced amplicons within 50 bp of the published expect range

[c] *Viburnum dilatatum* SSR markers were reported in Dean et al. (2011)

[d] *Viburnum farreri* SSR markers were reported in Hamm et al. (2021)

[e] *Oreinotinus* SSR markers were reported in Barish et al. (2016)

[f] Names in parentheses are original names of SSR markers from Barish et al. (2016)

[g] *Viburnum rufidulum* SSR markers were reported in (Dean et al., 2015)

Table 2.3. Amplification percentages per SSR marker set and subclade

Clade	**Subclade**	**VD N=11 (%)**	**Vf N=15 (%)**	**Vore N=16 (%)**	**VR N=7 (%)**
Valvatotinus					
	Lentago	60	42	68	93
	Punctata	49	33	50	57
	Euviburnum	57	32	66	61
Crenotinus					
	Solenotinus	38	68	60	58
	Lutescentia	44	66	73	41
Laminotinus					
	Succotinus	95	46	98	33
	Lobata	82	46	82	40
	Coriacea	83	46	74	31
	Sambucina	68	32	77	17
Porphyrotinus					
	Mollotinus	70	44	94	47
	Dentata	74	54	97	57
	Oreinotinus	48	17	57	7
N/A					
	Pseudotinus	45	47	70	39
	Urceolata	39	17	20	27
	Opulus	73	49	95	60
	Tinus	81	40	79	21
	V. clemensiae	45	47	28	7
	Adoxaceae	8	8	12	17
	Caprifoliaceae	9	8	13	17

N= number of markers in the set

Table 2.4. Comprehensive 14 SSR marker set amplification percentages and observed heterozygosity in *Viburnum carlesii* (*Euviburnum* subclade), *V. opulus* (*Opulus* subclade), *V. plicatum* (*Lutescentia* subclade), and *V. tinus* (*Tinus* subclade)

Locus	***V. carlesii* (*Euviburnum*) N=14**		***V. opulus* (*Opulus*) N=18**		***V. plicatum* (*Lutescentia*) N=15**		***V. tinus* (*Tinus*) N=9**		***Viburnaceae* N=14**		***Caprifoliaceae* N=6**	
	Amp^{b}	**H_o^c**	**Amp**	**H_o**	**Amp**	**H_o**	**Amp**	**H_o**	**Amp**	**H_o**	**Amp**	**H_o**
VD003	100%	0.14	94%	0.00	100%	0.60	100%	0.56	0%	0.00	0%	0.00
VD004	79%	0.09	94%	0.82	100%	0.00	100%	0.56	0%	0.00	0%	0.00
VD016	100%	0.07	94%	0.00	67%	0.20	100%	0.89	64%	0.89	67%	0.00
Vf20_02	100%	0.50	94%	0.12	100%	0.47	100%	0.78	7%	0.00	0%	0.00
Vf20_14	100%	0.14	94%	0.00	100%	0.00	100%	0.00	7%	0.00	17%	0.00
Vf20_22	93%	0.92	94%	0.24	100%	1.00	100%	0.78	43%	0.17	0%	0.00
Vore02	100%	0.00	94%	1.00	93%	0.29	100%	0.00	7%	0.00	0%	0.00
Vore07	100%	1.00	100%	1.00	100%	0.07	100%	1.00	36%	0.40	50%	0.33
Vore12	29%	0.25	89%	0.00	93%	0.00	100%	0.00	7%	1.00	33%	0.00
Vore14	100%	0.00	94%	0.82	100%	0.00	100%	0.00	7%	0.00	17%	0.00
Vore15	100%	0.43	94%	0.88	100%	0.67	100%	0.89	14%	0.00	0%	0.00
VR004	100%	0.14	67%	0.00	100%	0.47	0%	0.00	0%	0.00	0%	0.00
VR005	29%	0.50	78%	0.21	20%	0.00	33%	0.00	0%	0.00	17%	0.00
VR011	100%	0.21	94%	1.00	100%	0.00	100%	0.33	29%	0.00	0%	0.00
Mean	88%	0.31	91%	0.44	91%	0.27	88%	0.41	16%	0.18	14%	0.02

N= number of individuals sampled; Amp = percent amplification around the expected bp size; H_o = Observed heterozygosity

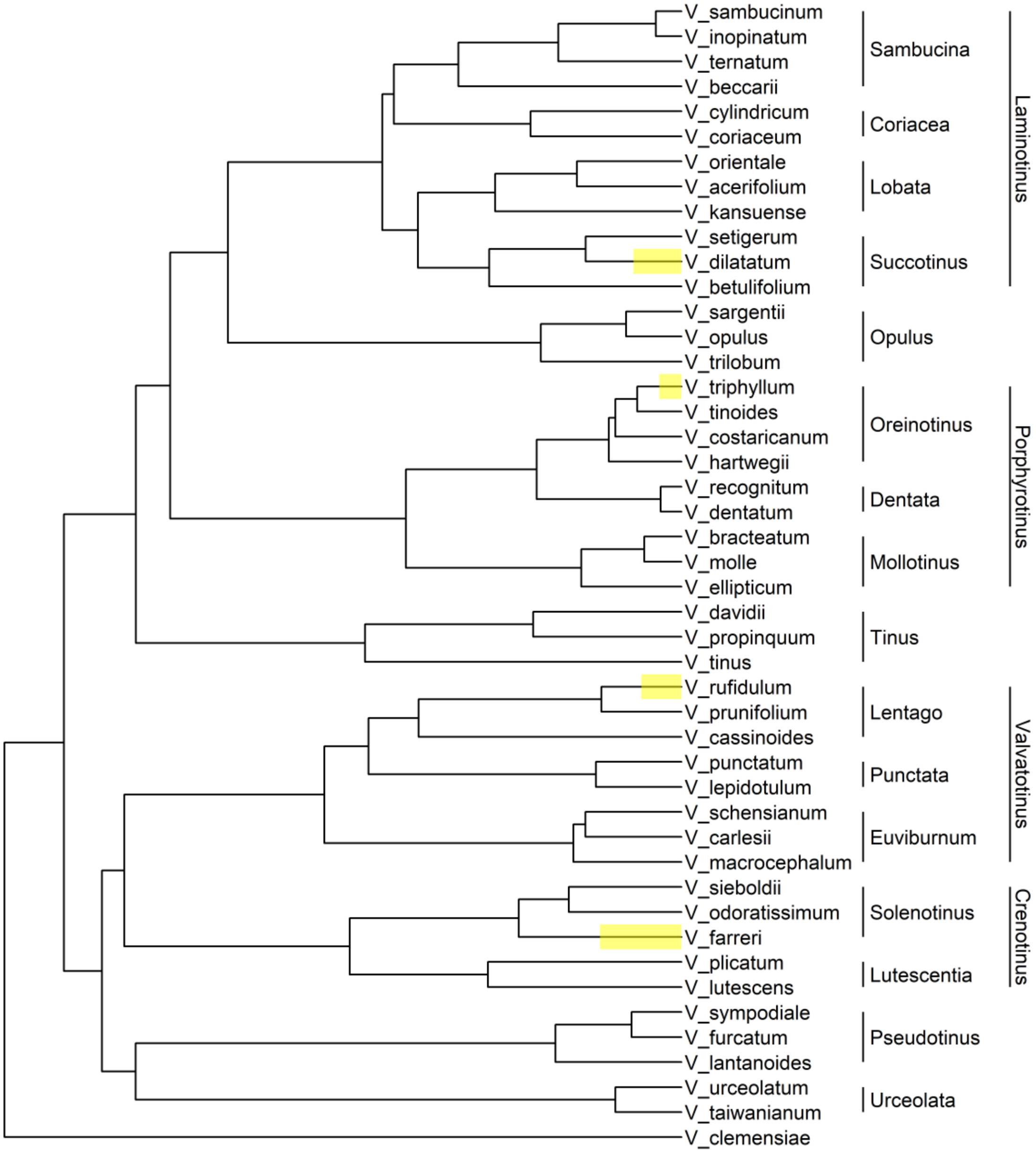

Figure 2.1. Phylogenetic tree of *Vibunum* species included in this study. This tree is a trimmed version of the one published in Landis et al. (2020) based on RAD-seq data. Highlighted nodes are species in which markers were developed. Subclades are horizontal and clades are vertical.

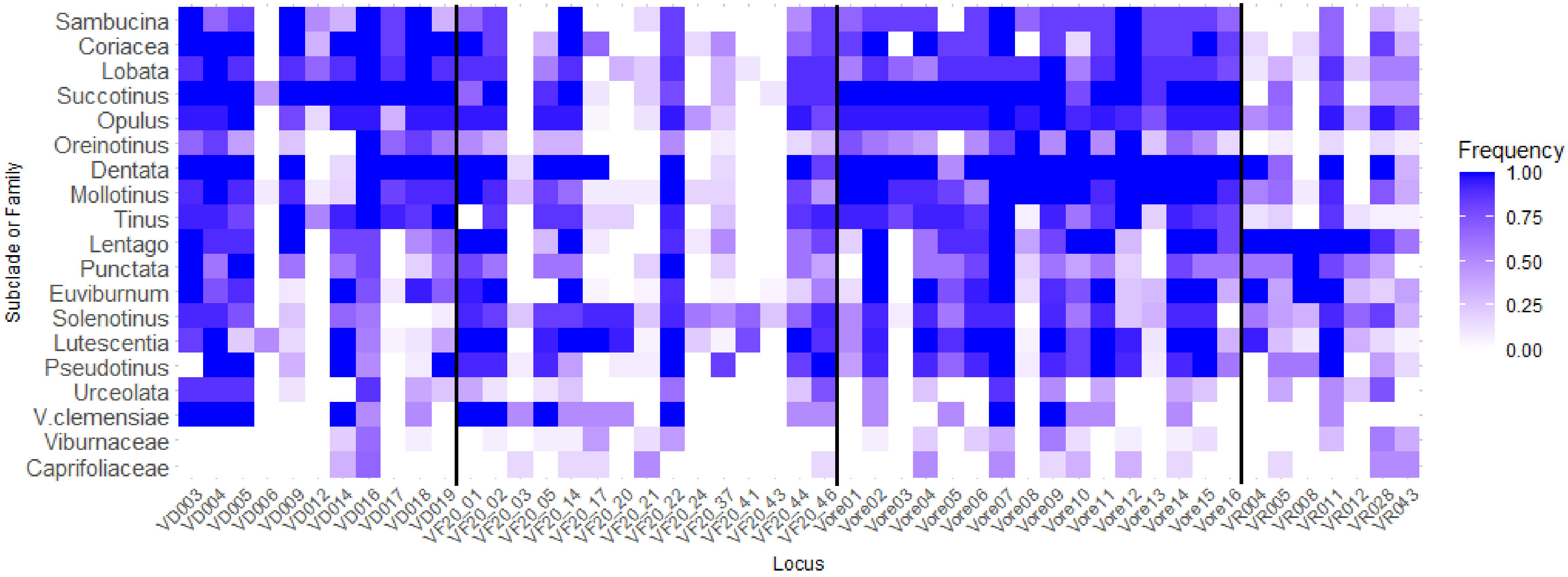

Figure 2.2. Heatmap of frequency of amplification per subclade or family for each individual of the 49 markers (loci). Black lines mark the separation of marker set groups. The darker the square, the higher the amplification frequency of that marker for the sample group.

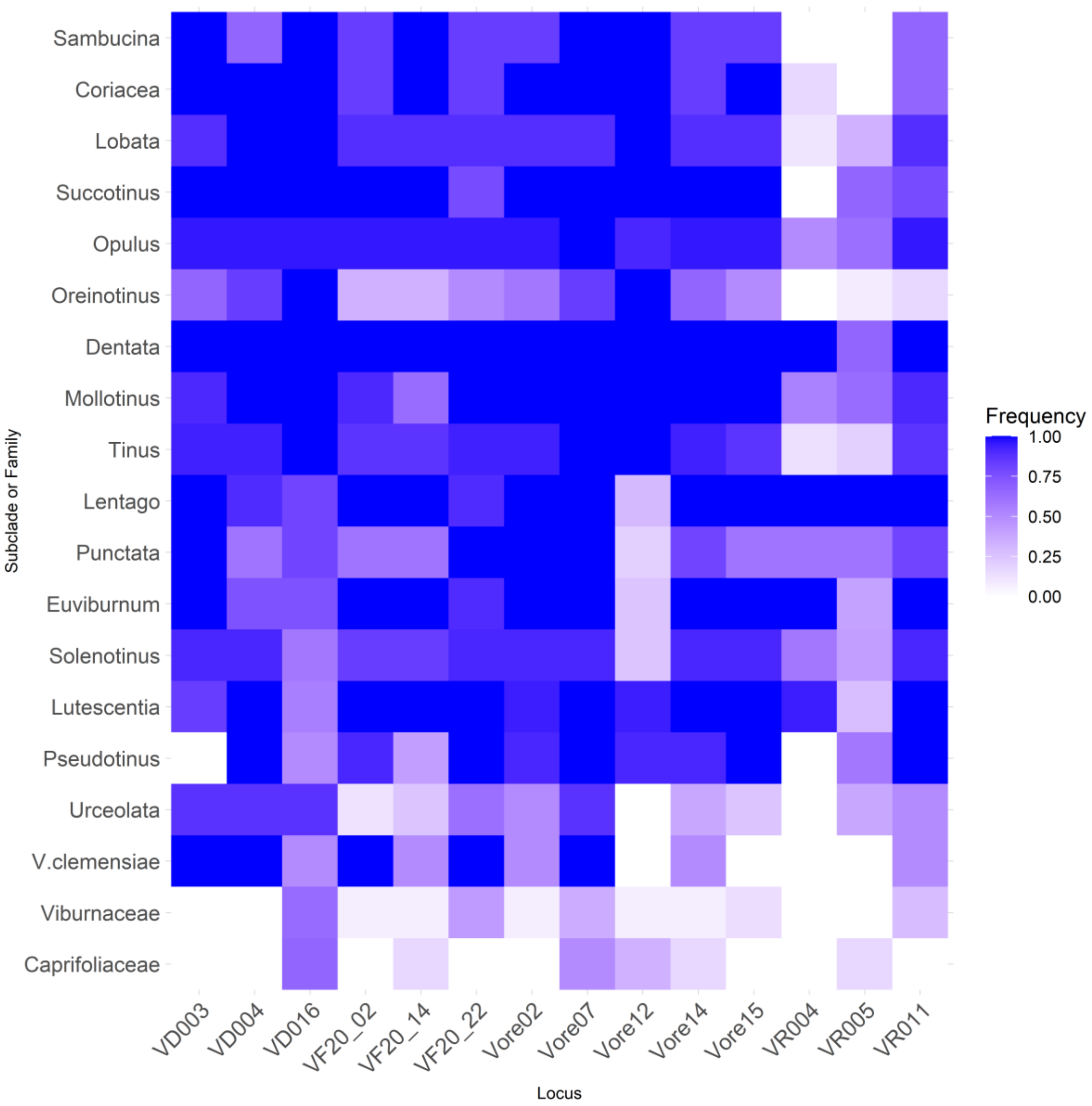

Figure 2.3. Heatmap of 14 comprehensive SSR markers on all 206 samples broken down by subclade or family. The darker the square, the greater the amplification frequency was for that group of samples.

Conclusions

Despite the popularity of *Viburnum* species as ornamentals and the genus overall as a model for evolutionary biology, biogeography, and phylogenetic studies, few molecular markers exist for exploring genetic diversity in the *Viburnum* genus and closely related taxa (Dean et al., 2011; Dean et al., 2015; Barish et al., 2016; Hamm et al., 2021). The cross-species amplification and therefore overall utility of the few already developed SSR markers was also not investigated. This study aimed to develop new markers for the *Solenotinus* subclade in the *Crenotinus* clade and subsequently test the cross-amplification of all four sets of developed SSR markers on a subset of species representing all 16 subclades.

SSR markers were successfully developed from *V. farreri* in the *Solenotinus* subclade and *Crenotinus* clade. The 15 markers were polymorphic with observed heterozygosity ranging from 0 to 0.84 and the number of alleles per locus ranging from two to eight in just 19 samples of *V. farreri.* Shannon diversity index values ranged from 0.21 to 1.62. The markers were also likely well distributed across the genome, with only one pair of markers having a pairwise $\bar{r}_d$ greater than 0.5. Because of the information provided by the diversity indices, these gSSR markers could be used successfully to investigate genetic diversity and population studies.

The 15 gSSR markers developed from *V. farreri* were combined with 11 *V. dilatatum*, seven *V. rufidulum,* and 16 *V. triphyllum,* and *V. pichinchense* gSSRs to test the cross-species amplification of the combined 49 gSSR marker set. Overall, 5,368 out of the potential 10,094 reactions (53%) produced amplicons close the published expected bp range, with a 58% amplification rate within the *Viburnum* genus and 11% outside of *Viburnum.* A subset of 14 markers was identified as comprehensive markers to use as a starting point in future studies. Depending on the target species, other markers can be substituted in as needed, but the markers VD003, VD004, VD016, Vf20_02, Vf20_14, Vf20_22, Vore02, Vore07, Vore12, Vore14, Vore15, VR004, VR005, and VR011 serve as an initial go-to set with wide cross-species amplification and the ability to capture heterozygosity in subclades they were not developed from. With limited sequencing to confirm the presence or absence of the SSR motifs, these markers are ready for use in genetic diversity studies, differentiating species/cultivars, and assisting in breeding efforts.

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Vita

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