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Viburnum phylogeny: evidence from the duplicated nuclear gene GBSSI

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Abstract

DNA sequencing studies of the granule-bound starch synthase gene (*GBSSI*) indicate the presence of two loci in *Viburnum*. Gene trees from separate and combined phylogenetic analyses of the *GBSSI* paralogues are generally congruent with each other and with trees from previous analyses, especially those of Donoghue et al. [Syst. Bot. 29 (2004) 188] based on nuclear ribosomal ITS and chloroplast *trnK* intron DNA sequences. Specifically, our *GBSSI* trees confirm (i) the monophyly of some and non-monophyly of other traditionally recognized taxonomic sections, (ii) the presence of three major supra-sectional lineages within *Viburnum*, and (iii) the resolution of many species relationships within the section-level clades. Analyses of *GBSSI* also provide greater resolution of relationships within the largest supra-sectional lineage. Relationships at the base of the *Viburnum* phylogeny remain uncertain; in particular, the position of the root, relationships among the supra-sectional clades, and the exact placement of several smaller groups (e.g., *Viburnum clemensiae, Viburnum urceolatum*, and section *Pseudotinus*). In two lineages each *GBSSI* paralogue is represented by two distinct sequences. The presence of additional copies appears to be correlated with polyploidy in these clades. Placement of the homoeologues in our gene trees suggests the possibility of a hybrid origin for these polyploids.

Keywords: GBSSI; Phylogeny; Polyploidy; Viburnum; waxy

1. Introduction

Previous phylogenetic analyses have clearly established the placement of *Viburnum* within the Dipsacales (Backlund and Bremer, 1997; Backlund and Donoghue, 1996; Bell et al., 2001; Donoghue, 1983b; Donoghue et al., 1992, 2001, 2003; Judd et al., 1994; Pyck et al., 1999). Specifically, *Viburnum* appears to be the sister group of *Sambucus* plus *Adoxa* and its relatives. In the phylogenetic taxonomy proposed by Donoghue et al. (2001) these lineages constitute the Adoxaceae, the sister group of a redefined Caprifoliaceae that includes Morinaceae, Valerianaceae, and Dipsacaceae.

Viburnum, with approximately 175 species of shrubs and small trees, is the largest clade within Adoxaceae. Although most widely distributed in the Northern Hemisphere, with major centers of species diversity in eastern Asia and Latin America (Donoghue, 1983a; Hara, 1983; Morton, 1933; Rehder, 1908), Viburnum also extends into the Southern Hemisphere in the mountains of South-East Asia (Kern, 1951) and South America (Killip and Smith, 1931). Oersted (1861) split Viburnum into a number of separate genera. However, phylogenetic studies and several unique morphological features support the monophyly of the group. Perhaps the most striking morphological characteristic of Viburnum is the pattern of carpel abortion (Donoghue, 1983b; Wilkinson, 1948). Specifically, development of the single-seeded drupe fruit from the inferior tri-carpellate ovary involves abortion of two carpels; the single

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remaining ovule is pushed across the ovary into a previously sterilized carpel. Within *Viburnum*, flower and fruit morphology is generally conserved, but there is remarkable variation in features such as endocarp shape, fruit color, leaf morphology (e.g., tri-lobed or simple), bud morphology (e.g., the number and arrangement of scales), and inflorescence form (e.g., panicle- or umbellike). These characters, among others, have been used to diagnose the 10 section-level taxa commonly recognized within *Viburnum*.

Although the monophyly and phylogenetic placement of Viburnum have been clearly established, relationships within the group have received less attention. Previous studies supported the monophyly of most of the traditionally recognized sections, but failed to confidently resolve relationships among these lineages (e.g., Donoghue, 1983a). However, a recent investigation by Donoghue et al. (2004) has dramatically improved our understanding of Viburnum phylogeny. Their analyses of nuclear ribosomal ITS (nrITS) and chloroplast trnK intron sequences confirmed many of the earlier results and, more importantly, provided a generally well-supported hypothesis of relationships among the sections. Specifically, Donoghue et al. (2004) identified three major supra-sectional groups. The largest of these lineages consisted of four subclades. Two corresponding directly to the traditional sections Opulus (circum-boreal) and Tinus (Eurasia); one containing section Oreinotinus (Latin America) plus the purple-fruited New World members of section Odontotinus; and one consisting of the predominantly red-fruited Old World Odontotinus species, the purple-fruited New World species Viburnum acerifolium, and Viburnum cylindricum of section Megalotinus (Asia). The non-monophyly of Odontotinus was anticipated based on previous analyses of morphology (Donoghue, 1983a) and unpublished molecular data (Donoghue and Baldwin, 1993; Donoghue and Sytsma, 1993; see also Baldwin et al., 1995). The second suprasectional lineage included Viburnum plicatum of section Tomentosa (Asia) and a clade containing most representatives of section Solenotinus (Asia). Traditionally included within Solenotinus, the rare Bornean species Viburnum clemensiae was instead placed sister to the remainder of Viburnum in the analyses of Donoghue et al. (2004). This result was unexpected given that V. clemensiae produces paniculate inflorescences characteristic of Solenotinus. In the third supra-sectional lineage, a clade corresponding to section Pseudotinus (North America and Asia) was sister to one containing section Lentago (North America) and a core group of species from section Viburnum (Eurasia). Traditionally placed in section Viburnum, the Taiwanese-Japanese species Viburnum urceolatum was instead allied with the Solenotinus-Tomentosa clade. This novel result was not entirely surprising given the unusual combination of morphological features in V. urceolatum (e.g., sympodial growth pattern;

tubular, often red-colored corollas). Although the Donoghue et al. (2004) study clarified many aspects of *Viburnum* phylogeny, their findings need to be tested and several key issues remain unresolved: (i) relationships among the supra-sectional lineages, (ii) relationships among sub-clades within the larger supra-sectional grouping, and (iii) the position of the root.

Molecular phylogenetic studies in plants have relied heavily on nrITS (Baldwin et al., 1995) and various chloroplast loci (e.g., Chase et al., 1993; Johnson and Soltis, 1995; Taberlet et al., 1991). However, in many cases these markers have provided only limited resolution and support for relationships. Increasingly, DNA sequences from low-copy number nuclear genes (e.g., adh, Sang et al., 1997b; phytochromes, Mathews and Donoghue, 2000; ncpGS, Emshwiller and Doyle, 1999; and nia, Howarth and Baum, 2002) are being included in phylogenetic studies. Obviously there are many such loci, each one potentially useful at several taxonomic levels. Specifically, evolutionarily conservative exons are expected to be applicable to higher-level problems whereas faster-evolving introns are more likely to be useful at the species level and below (Sang, 2002). The bi-parental inheritance and limited intergenomic concerted evolution exhibited by low-copy nuclear genes makes them particularly useful for studying the origins of hybrid and polyploid lineages. Although investigations of nrITS variation within individuals and populations (e.g., Campbell et al., 1997; Fuertes Aguilar and Nieto Feliner, 2003; Fuertes Aguilar et al., 1999), and conflict between phylogenies derived from chloroplast and nuclear genes (e.g., Sang et al., 1997a; Soltis and Kuzoff, 1995), have provided insights into reticulate evolution, there are situations in which these approaches are of limited use (Barrier et al., 1999; Wendel et al., 1995). Low-copy nuclear genes may also provide opportunities to address difficult phylogenetic problems. For example, in cases where appropriate outgroups are uncertain or unavailable, duplications of such loci may help to root the tree (Donoghue and Mathews, 1998; Mathews and Donoghue, 1999; Sang, 2002). Although potentially valuable for phylogenetic studies, low-copy number loci pose significant practical problems, including marker selection, primer design, and the need to clone amplification products. The complex evolutionary dynamics of these loci, which can include duplications, deletions, and lineage sorting, may also cause problems for phylogenetic analysis (Sang, 2002).

One low-copy nuclear locus previously used in phylogenetic analyses is the granule-bound starch synthase gene (*GBSSI* or *waxy*, so-called for its mutant phenotype in maize). The gene has been reported to be single-copy in diploid grasses (Clark et al., 1991; Wang et al., 1990) and several dicot lineages (Mérida et al., 1990; van der Leij et al., 1991; Wang et al., 1999). Mason-Gamer et al. (1998) were the first to investigate

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the phylogenetic utility of the gene. In their study GBSSI proved phylogenetically informative at various levels within Poaceae, exon sequences resolved higher-level relationships whereas introns differentiated closely related species. More recently, Mason-Gamer (2001) and Ingram and Doyle (2003) have used GBSSI sequences to provide evidence for the origin of the allopolyploids in North American Elymus and African Eragrostis, respectively. A single copy of GBSSI has also been assumed in phylogenetic studies of Liquidambar (Li and Donoghue, 1999), Ipomea (Miller et al., 1999), Triosteum (Gould and Donoghue, 2000), and Solanum section Lycopersicon (Peralta and Spooner, 2001). In contrast, Evans et al. (2000) documented the existence of two paralogous GBSSI loci in Rosaceae and Rhamnaceae. Analyzed in combination, these loci proved useful for resolving higher-level relationships within Rosaceae and, in particular, for understanding the origin of Maloideae (Evans et al., 2000; Evans and Campbell, 2002). GBSSI has also been used to clarify relationships between more closely related lineages in Rosaceae. Based on analyses of one GBSSI paralogue, Smedmark et al. (2003) suggested that several members of Geinae originated through allopolyploidy.

The aim of the present study was to examine phylogenetic relationships within *Viburnum* using *GBSSI* gene sequences. Using a comparable sample of taxa, we hoped to test the phylogenetic hypotheses of Donoghue et al. (2004) and to gain insights into relationships left unresolved by that analysis.

2. Materials and methods

2.1. Plant material

We obtained DNA sequences from 41 Viburnum species representing all of the traditionally recognized sections and major species groups. For rooting purposes, we also obtained sequences from Sambucus canadensis. This sample of species matches that of Donoghue et al. (2004) with three exceptions. We omitted one outgroup, Sambucus racemosa, one accession of the widespread Asian species, Viburnum sargentii, and Viburnum rhytidophyllum (section Viburnum), as our ongoing studies suggest that our current accession of this species may be a horticultural hybrid. Voucher information for each accession, and GenBank numbers for the corresponding sequences, are given in Appendix A.

2.2. DNA extraction, amplification, and sequencing

Genomic DNA was isolated from silica dried leaf tissue or herbarium samples using either a modified cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990), or the Qiagen DNeasy Plant Kit (La Jolla, CA).

Preliminary investigations of *GBSSI* used the primers GBSSF2 and GBSSR2 (Li and Donoghue, 1999) to amplify the region spanning exons 9–12 (Fig. 1). Characterization of this fragment suggested the presence of two paralogues in *Viburnum*; indeed, our ongoing studies suggest that the gene is duplicated throughout Dipsacales and perhaps more widely (see Section 4). Using our preliminary data, along with sequences available from GenBank, we designed a set of oligonucleotide primers to both extend the region amplified and to target the copies individually (Table 1; Fig. 1). We refer to the *Viburnum* copies as *WAXY1* and *WAXY2* throughout this paper.

For this study we characterized the region between exons 8 and 12 for both *GBSSI* paralogues (Fig. 1). PCR amplifications were carried out in $25\,\mu$ L reaction volumes, containing 1× PCR buffer (Perkin–Elmer), 6.25 mM MgCl₂, 625 μ M each dNTP (Invitrogen), 5% bovine serum albumin (v/v; New England Biolabs), 10 pM each amplification primer, 1 U AmpliTaq DNA polymerase (5 U/ μ L; Perkin–Elmer), and 10–100 ng of total cellular DNA. Typically the copy-specific primer combinations WAX81F/WAX121R and WAX82F/



Fig. 1. Schematic diagram of the *GBSSI* gene showing approximate positions of oligonucleotide primers and amplified region (modified from van der Leij et al., 1991). The line indicates intron sequences, boxes are exons (black, untranslated exon; shaded and numbered, 13 translated exons). The entire diagram corresponds to approximately 4.66kb in potato. Translational start (ATG), stop codon (TAA), and polyadenylation sites (poly(A)) are noted. The PCR amplified fragment obtained in this study is indicated by the thickened line below the drawing. Arrows above exons mark approximate locations and directions for the primers of Li and Donoghue (1999); below the exons are primers used in this study (A, WAX81F or WAX82F; B, WAX121R or WAX122R; C, WAX91F or WAX92F; D, WAX101R or WAX102R).

Table	1
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Oligonucleotide primers used in PCR amplification and sequencing of *waxy*

Locus ampified	Primer name	Oligonucleotide sequence
WAXYI	WAX81F WAX91F WAX101RB WAX121R	5'-caatctacctgatcaatataag-3' 5'-gatattacaaccgtaagatcc-3' 5'-gggatggcagcagctaga-3' 5'-acaataggcacctgttcca-3'
WAXY2	WAX82F WAX92F WAX102R WAX122R	5'-caateteeetgaegagtta-3' 5'-ttaaetgaeaaataeaeagge-3' 5'-tgagrgatmgettetaeaagg-3' 5'-taageattgaaetgaaageaa-3'

WAX122R were used in amplifications. However, when necessary these were combined with either the copy-specific internal primers or those of Li and Donoghue (1999) to amplify two overlapping fragments. Thermocycling conditions for PCR were: initial denaturation at 98 °C for 3 min, 35–40 cycles of 1 min at 95 °C (denature), 1 min at 50 °C (annealing), and 2 min at 72 °C (extension), with a final incubation at 72 °C for 5 min. Amplification products were purified using the QIAquick PCR Purification Kit (Qiagen). Automated sequencing reactions used the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and were analyzed on either an ABI 377 automated DNA sequencer, ABI 3100 Genetic Analyzer, or MJ Research BaseStation 51 Fragment Analyzer.

For certain accessions we either consistently amplified multiple PCR fragments or attempts at direct sequencing failed. In such cases amplification products were cloned using a TOPO TA Cloning Kit (Invitrogen). Between 10 and 50 colonies from each cloning reaction were screened by direct PCR. Screening used 20 µL reactions containing $1 \times Q$ solution (Qiagen), $1 \times PCR$ buffer (Qiagen), 500 µM each dNTP (Invitrogen), 10 pM M13 forward primer, 10pM M13 reverse primer, and 0.75U DNA polymerase (5U/µL; Qiagen). Thermocycling conditions were: cell lysis at 98 °C for 10 min, then 25 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C (extension), with a final incubation at 72°C for 5min. For each accession 5–12 positive clones were purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced as above using both M13 universal primers and internal primers as appropriate.

2.3. Selection of cloned sequences

For accessions that required cloning, preliminary multiple sequence alignments of clones were prepared using ClustalX (Thompson et al., 1994), then visually inspected, and adjusted for minor improvement. Alignments for individual accessions were of two distinct types, (i) all sequences were highly similar, differing by only one or a few nucleotides, or (ii) two or more sequence types were distinguished by numerous nucleotide and/or length differences. For alignments consisting of similar sequences, a single clone (where possible exhibiting the consensus sequence) was selected to represent the accession in subsequent analyses. Alignments containing divergent sequences were analyzed with SplitsTree 3.2 (Huson, 1998) prior to selecting clones for final analyses. The ability of phylogenetic network methods, such as those implemented in SplitsTree 3.2, to graphically represent sequence incompatibilities make them powerful tools for exploratory data analyses. Here we use this approach to identify potential PCR recombinants, a task that can be difficult when reading directly from sequence alignments or using bifurcating trees. Based on this initial screening, clones representing each of the distinct sequence types were included in our final alignments.

2.4. Sequence alignments

We prepared several data matrices for phylogenetic analysis. In each case multiple sequence alignments for full analyses were prepared using ClustalX, with the resulting matrices inspected visually and modified of minor improvement. Previous phylogenetic studies (e.g., Donoghue et al., 2004; Eriksson and Donoghue, 1997) have indicated that although Sambucus is the closest extant relative of *Viburnum* there is considerable molecular distance between them. To investigate the impact of including Sambucus sequences on resolution and support for ingroup relationships we compiled two alignments for each of the GBSSI paralogues, one containing only ingroup sequences and the other including Sambucus. We also constructed a pair of combined data sets, one with and one without Sambucus, by concatenating the individual matrices such that species (or topologically congruent homoeologues) were terminals. Prior to phylogenetic analysis of these combined data sets we tested for incongruence using the partition homogeneity test as implemented in PAUP*4.0b10 (Swofford, 2002).

2.5. Phylogenetic analyses

Phylogenetic trees were inferred using both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria as implemented in PAUP*4.0b10. Heuristic MP analyses used "tree-bisection-reconnection" (TBR) branch swapping, zero-length branches collapsed, and all characters equally weighted. Analyses were repeated 100 times with RANDOM ADDITION. Parsimony bootstrap analyses used 1000 replicates with nearest neighbor interchange (NNI) branch swapping. The best-fitting model of sequence evolution for ML analyses was determined using a series of likelihood ratio tests as implemented in PORN* (Bell, 2001). Subsequent heuristic ML tree searches used the most appropriate model (with parameters simultaneously estimated via ML), TBR branch swapping, and collapsed zero-length branches. Maximum likelihood analyses were repeated 100 times with RANDOM ADDITION. Bootstrap analyses used 1000 replicates with NNI branch swapping; parameters values for these tests were estimated from the optimal ML tree.

3. Results

3.1. Aligned DNA sequences

Within *Viburnum* the high degree of similarity among DNA sequences for each of the *GBSSI* paralogues

 Table 2

 Statistics from the aligned data matrices for the ingroup taxa

	WAXY1	WAXY2
No. of sequences	47	44
Sequence length (bp)	959-1310	861-1240
Aligned length (bp)	1339	1286
No. of inferred indels	29	23
No. of excluded sites	431	501
No. of constant sites	691	538
No. of varied sites	217	247
No. of parsimony informative sites	105	113
%GC content range (all sites)	38.2-39.8	37.5-39.5
%GC content mean (all sites)	38.9	38.5
%GC content range (varied sites only)	38.2-40.6	49.8-56.3
%GC content mean (varied sites only)	40.6	53.4

resulted in data matrices containing little alignment ambiguity. For WAXY1 exon sequences differed only by nucleotide substitutions whereas both point and length mutations occurred within introns. In contrast, both nucleotide substitutions and length mutations were found in WAXY2 exons. Specifically, exon sequences from V. urceolatum, all members of Pseudotinus, the A homoeologue from section Oreinotinus and the D homoeologue from Viburnum erubescens and Viburnum sieboldii contained indels. In contrast to our Viburnum-only alignments, matrices that included the Sambucus WAXYI sequence contained considerable uncertainty within intron regions and for the most part these regions could not be aligned unambiguously. We therefore excluded most of the intron sequences from these analyses; the exception being areas immediately flanking the exons that were easily aligned.

To investigate the effects of inferred indels on our reconstructions we performed a series of preliminary phylogenetic analyses in which (i) indels were coded as missing data or (ii) gapped position were excluded. Generally, these two approaches produced highly similar results. However, we used matrices in which gapped and ambiguous positions were excluded in further analyses, as in a few cases indel boundaries could not be unambiguously aligned. Statistics on these aligned data matrices are presented in Table 2; data matrices and resulting trees are available in TreeBASE (study Accession No. S1070, matrix Accession Nos. M1824–M1826).

3.2. Analyses of WAXY1 sequences

Preliminary data analyses indicated that two distinct forms of the *WAXY1* paralogue are present in *V. erubescens* of section *Solenotinus*, all representatives of section *Oreinotinus* and *Viburnum dentatum* (New World *Odontotinus*). Both forms were included in our phylogenetic analyses.

For the *Viburnum*-only data set the MP search recovered 721 equally parsimonious trees, each 307 steps long with a consistency index (CI) of 0.798 (without autapo-

morphies, CI=0.672) and a retention index (RI) of 0.891. Although these trees were similar in many respects, several areas were unresolved. In particular, (i) relationships at the base of the tree, (ii) the monophyly of section *Pseudotinus*, (iii) relationships within section Viburnum and the Old World Odontotinus, and (iv) relationships within the clade containing Viburnum ellipticum, Viburnum molle, Viburnum rafinesquianum and one of the homoeologues from Oreinotinus and V. dentatum. The ML search used a HKY+G substitution model and resulted in а single topology $(-\ln L = 3397.40930;$ Fig. 2) that was highly similar to the topologies recovered using parsimony. Both parsimony and ML bootstrap analyses provided moderate to strong support for many of the major clades within Viburnum.

The distinct *WAXY1* sequence types from *V. dentatum* and section *Oreinotinus* did not form a monophyletic group in either the MP or ML trees. Instead these sequences formed two clades; one united with *V. ellipticum*, *V. molle*, and *V. rafinesquianum* of the New World *Odontotinus* (homoeologue *A*) and the other sister to this entire group (homoeologue *B*). Likewise, the two sequence types found for *V. erubescens* were placed separately on the *WAXY1* trees. One form (homoeologue *C*) fell within a clade containing several other members of section *Solenotinus*. The second form (homoeologue *D*) was united with a sequence from *Viburnum odoratissimum* (section *Solenotinus*) and together these were sister to *Viburnum plicatum* (section *Tomentosa*).

Alignments of WAXY1 sequences from Viburnum also contained informative indels. Particularly striking was a 316 nt insertion within intron 8, which appears to be unique to three species of Old World Odontotinus (V. dilatatum, V. erosum, and Viburnum melanocarpum). Several indels characterized the supra-sectional or section-level clades; specifically, the Lentago–Viburnum lineage, section Lentago (including Viburnum nudum), section Opulus, section Pseudotinus, and homoeologue B from Oreinotinus and V. dentatum. Indels also supported a relationship between Viburnum cinnamomifolium and Viburnum davidii, and one between Viburnum edule and Viburnum trilobum.

Inclusion of the *Sambucus WAXY1* sequence weakly places the root of the *Viburnum* phylogeny between *V. clemensiae* and all other viburnums (52% bootstrap support). Given this result, and the same finding by Donoghue et al. (2004) we rooted our ingroup-only trees (e.g., Fig. 2) along the *V. clemensiae* branch.

3.3. Analyses of WAXY2 sequences

Analyses of DNA sequences from cloned amplification products indicated the presence of two distinct forms of *WAXY2* in *V. erubescens*, *V. odoratissimum*, and *V. sieboldii* from section *Solenotinus*, as well as in



Fig. 2. Single optimal maximum likelihood tree obtained from the analysis of *Viburnum WAXY1* sequences, showing bootstrap values over 50% (1000 replicates). Thickened lines and bold font names mark lineages that appear twice in the gene tree; letters following names are used to differentiate homoeologues. Names of traditionally recognized sections are noted at the right.

all representatives of section *Oreinotinus*. Both forms were included in phylogenetic analyses.

Maximum parsimony analysis of Viburnum WAXY2 sequences resulted in 552 optimal trees, each 329 steps long with CI = 0.842(without autapomorphies, CI=0.723), and RI=0.900. As in *WAXY1* analyses, the MP trees were very similar to one another but again several areas were unresolved. Specifically, (i) relationships at the base of the tree, (ii) relationships among the sections Megalotinus, Odontotinus, and Oreinotinus, (iii) the monophyly of the clade containing sections Lentago and Viburnum, and (iv) relationships within sections Viburnum and Pseudotinus, as well as within the Old World Odontotinus clade. A HKY+G substitution model was used for the ML search and this resulted in a single optimal topology $(-\ln L = 3278.18862; \text{ Fig. 3}).$ This tree is very similar to those based on parsimony analyses of WAXY2. Furthermore, although somewhat less well resolved it is also generally consistent with the gene trees recovered from analyses of WAXY1. The highly similar placement of duplicated sequences from section Oreinotinus and three members of section Solenotinus was of particular interest. As in WAXY1 trees, one form of WAXY2 (the A homoeologue) from Oreinotinus was closely linked to V. ellipticum, V. molle, and V. rafinesquianum (New World Odontotinus) and the other (the B homoeologue) is sister to that clade. The placement of duplicated WAXY2 sequences from V. erubescens, V. odoratissimum, and V. sieboldii is consistent with results from our analyses of WAXY1 sequences. Specifically, one copy (the C homoeologue) forms a clade with members of Solenotinus for which only one copy is found, whereas the other sequences (the Dhomoeologue) form a clade sister to V. plicatum. Bootstrap tests of WAXY2 also provide moderate to strong support for many relationships within Viburnum.

Fig. 3. Single optimal maximum likelihood tree obtained from the analysis of *Viburnum WAXY2* sequences, showing bootstrap values over 50% (1000 replicates). Thickened lines and bold font names indicate lineages that appear twice in the gene tree; letters following names are used to differentiate homoeologues. Names of traditionally recognized sections are noted at the right.

Several clades recovered in trees based on WAXY2 nucleotide substitutions were also supported by inferred indels. Several higher-level clades were supported by indels; specifically section *Pseudotinus*, the *Lentago–Viburnum* lineage, and the Old World *Odontotinus–cylindricum* clade. Indels also support relationships between smaller groupings, (i) *Viburnum prunifolium* and *Viburnum rufidulum*, (ii) *Viburnum cordifolium* and *Viburnum furcatum*, (iii) the *Solenotinus D* homoeologue clade and, (iv) a clade containing the *B* homoeologue from *Viburnum hartwegii*, *Viburnum jucundum*, and *Viburnum triphyllum* (note the *V. hartwegii* sequence appears to contain a deletion within this large insertion).

We were not able to obtain *WAXY2* sequences from *Sambucus canadensis*. Therefore our *WAXY2* gene trees (e.g., Fig. 3) are rooted along the *V. clemensiae* branch

based on analyses of *WAXY1*, the combined *GBSS1* data set (see below), and the root position obtained by Donoghue et al. (2004).

3.4. Combined analyses

As noted in preceding sections, WAXY1 and WAXY2 are represented by two distinct sequences in the Oreinotinus-dentatum clade and several members of section Solenotinus. To incorporate these additional copies in combined analyses we concatenated the duplicated genes based on their topological position in the separate analyses described above. For example, for each representative of the Oreinotinus-dentatum group we combined the A homoeologues from WAXY1 and WAXY2; that is, those Oreinotinus-dentatum sequences associated with V. ellipticum, V. molle, and V. rafinesquianum. Similarly, we combined the *B* homoeologues; that is, those Oreinotinus-dentatum sequences that form the sister group of the clade containing V. ellipticum, V. molle, and V. rafinesquianum and the A homoeologue sequences. Duplicated sequences from section Solenotinus were combined in the same fashion. For several taxa we were unable to obtain sequences for both GBSSI copies. Specifically, we lack WAXY1 sequences that correspond to the C homoeologue from V. odoratissimum and the D homoeologue from V. sieboldii; we lack WAXY2 sequences for V. edule, Sambucus canadensis, all members of section *Tinus*, and sequences corresponding to the Bhomoeologue from V. dentatum. In these cases failure of our PCR-based approach may reflect silencing and degradation of the locus (i.e., a loss), or alternatively sequence modifications that simply prevent amplification rather than indicate loss of the locus (e.g., nucleotide substitutions at priming sites, or large length mutations within introns). For our combined analyses these absences were coded as missing data and this accounted for approximately 7% of the cells in the combined data matrix.

For the ILD test we only included taxa or homoeologues that were represented by both paralogues (i.e., WAXYI and WAXY2) in the combined data set. The ILD test indicated that the two data sets were not in significant conflict (P=0.38). Parsimony analyses of the full data set resulted in 243 trees of 640 steps, with CI=0.816 (without autapomorphies, CI=0.690) and RI=0.891. These trees differ in two respects. First, relationships at the base of the phylogeny are unstable. Specifically, they differ in their resolution of relationships between V. clemensiae, V. urceolatum, section Pseudotinus, and the three supra-sectional lineages identified by Donoghue et al. (2004). They also differ in the placement of V. nudum relative to the core clades of sections

Fig. 4. Single optimal maximum likelihood tree obtained from the combined analysis of *Viburnum WAXY1* and *WAXY2* sequences, showing bootstrap values over 50% (1000 replicates). Thickened lines and bold font names indicate lineages that appear twice in the gene tree; letters following names are used to differentiate homoeologues. Names of traditionally recognized sections are noted at the right.

Lentago and Viburnum. In these trees V. nudum is placed sister to either the remainder of Lentago, to section Viburnum, or to a clade containing both of these lineages. The ML search used a HKY+G substitution model and recovered a single optimal topology $(-\ln L =$ 6788.20418; Fig. 4) that was highly similar to those based on parsimony. In general, this tree combines the well-supported clades from separate analyses of the two data sets. It also contains less ambiguity than the trees from separate analyses, suggesting that the two data sets are largely complimentary. For example, the WAXY1 analyses do not clearly resolve the position of section Opulus, whereas WAXY2 trees strongly support this group as sister to the large clade containing Megalotinus, Odontotinus, Oreinotinus, and Tinus. Not surprisingly, this result is also recovered in the combined analysis. Similarly, analyses of the combined data set indicate a close relationship between V. furcatum and Viburnum lantanoides-a result suggested by WAXY1 but left unresolved by WAXY2. In addition to generally improved resolution, the combined analyses also provide increased bootstrap support for many clades.

As in the individual analyses, our combined trees support the separate placement of duplicated GBSSI copies from V. dentatum, section Oreinotinus, and several members of section Solenotinus. In the case of V. dentatum and section Oreinotinus, the A homoeologues form a clade (62% bootstrap support) that is united with V. ellipticum, V. molle, and V. rafinesquianum (100% bootstrap support); the B homoeologues form a strongly supported (97% bootstrap value) sister group to this clade. For section Solenotinus, the C homoeologues from V. erubescens, V. odoratissimum, and V. sieboldii are associated with members of Solenotinus that are represented only once (i.e., V. farreri and V. suspensum), forming a clade with 99% bootstrap support. Sister to this is a strongly supported group (99% bootstrap support) consisting of V. plicatum and a clade containing the D homoeologues from V. erubescens, V. odoratissimum, and V. sieboldii.

Inclusion of the *Sambucus WAXY1* sequence in combined analyses (we did not recover the *WAXY2* paralogue) placed the root of the tree between *V*. *clemensiae* and the remainder of *Viburnum* (less than 50% bootstrap support). Given this results, and similar findings in other analyses (e.g., *WAXY1* and Donoghue et al., 2004) we rooted our ingroup-only trees from combined analyses (e.g., Fig. 4) using *V. clemensiae*.

4. Discussion

4.1. An ancient duplication of GBSSI

Our analyses suggest that two main copies of the GBSSI gene (i.e., WAXYI and WAXY2) are present in

most Viburnum species. Based on this observation it seems likely that the gene was duplicated prior to the origin of Viburnum; perhaps well before, given the level of sequence divergence between the paralogues. Although the exact timing remains uncertain, preliminary results from broader analyses also favor an ancient duplication of GBSSI. Specifically, our initial studies have recovered two paralogues from other members of the Dipsacales (e.g., Diervilla, Heptacodium, Triosteum, Lonicera, Leycesteria, and Dipelta) and from the Asterales (e.g., Scaevola), suggesting that this duplication may predate the origin of the Campanulid clade (RCW, D. Howarth, and MJD, unpub.). Indeed it is possible that this duplication occurred prior to the divergence of asterids and rosids. Preliminary phylogenetic analyses that used a duplicate gene rooting approach and included representatives of the GBSSI paralogues from asterids and those from the Rosaceae (Evans et al., 2000) could not exclude the possibility that the gene copies stem from the same duplication event. More extensive analyses may help to clarify the timing and mechanism of duplication.

4.2. Evolutionary dynamics of the GBSSI loci

Duplicated gene loci have several potential evolutionary fates. One possibility is that both copies retain their original function. In such cases expression of one or both copies may be modified so that both are required to maintain normal function (Cronn et al., 1999; Force et al., 1999). Alternatively, gene duplication may result in relaxed selection, allowing the development of a novel function, or gene inactivation (Wendel, 2000). The maintenance of additional gene copies may also promote complex patterns of molecular evolution (e.g., interlocus recombination, gene conversion, and concerted evolution).

Our sequencing of Viburnum GBSSI paralogues suggests that WAXY1 and WAXY2 may have, at least in certain lineages, differing evolutionary trajectories. In our WAXY1 data set exon sequences differed only by nucleotide substitutions; there were no stop codons and exon-intron boundary sequences were conserved. These and similar observations from preliminary sequencing of a larger portion of the WAXY1 paralogue (spanning exons 2–12) suggest that this paralogue is functional in most cases. In contrast WAXY2 appears to have been pseudogenized in several lineages. Specifically, sequences from V. urceolatum, all members of Pseudotinus, the Oreinotinus A homoeologue, and the D homoeologue from V. erubescens and V. sieboldii, contain length mutations that introduce multiple stop codons or disrupt the exon-intron boundaries. Further, the failure to recover WAXY2 sequences from V. edule, Sambucus canadensis, all members of section Tinus, and the V. dentatum B homoeologue may reflect the formation of pseudogenes in these lineages. Although we cannot discount the possibility that the apparently functional *WAXY2* loci have been inactivated by epigenetic mechanisms, preliminary sequences for several taxa suggest that stop codons do not occur elsewhere in the coding regions of these loci.

4.3. Gene trees

Gene trees from the separate and combined analyses of the *GBSSI* paralogues are generally congruent with one another and with those reported by Donoghue et al. (2004) based on analyses of the *trnK* intron and nrITS. Furthermore, these relationships are often well supported. In the few cases of apparent conflict between data sets, the relationships suggested are poorly supported by one or both data sets. In the following sections we describe the key features of our *GBSSI* trees and compare these results with those of Donoghue et al. (2004). These results are summarized in Fig. 5.

4.3.1. Monophyly of the traditional sections

Consistent with Donoghue et al. (2004) our GBSSI gene trees suggest the monophyly of four traditionally recognized sections—Opulus, Oreinotinus, Pseudotinus, and Tinus. Support for the monophyly of Opulus and Tinus is strong in all analyses (76–100% bootstrap support). For *Pseudotinus* monophyly is well supported by analyses of WAXY2 and the combined data set (both 100% bootstrap support), as well as by four inferred indels (including two in the WAXY1 alignment). However, this clade was only weakly supported in our WAXY1 analyses; the clade was present in the optimal ML tree, but received less than 50% support in bootstrap analyses. There was also only weak support for the monophyly of Oreinotinus in some analyses. Specifically, for both WAXY1 homoeologues Viburnum stenocalyx formed a basal polytomy with V. dentatum; this arrangement was also found for the *B* homoeologue in the combined analysis. However, we suggest that these results are consistent with the monophyly of Oreinotinus as recovered in analyses of WAXY2 (66 and 99% bootstrap support for the A and B homoeologues, respectively) and for the A homoeologue in the combined tree (93% bootstrap support).

For sections Lentago, Viburnum, and Solenotinus only a single species falls outside of a core clade. In the case of section Lentago, four of the five representatives form a strongly supported clade (100, 89, and 100% bootstrap support in WAXY1, WAXY2, and combined analyses, respectively). However, as in nrITS and trnK intron analyses, the placement of V. nudum remains uncertain. In WAXY1 ML analyses this species is placed sister to the remainder of Lentago with 58% bootstrap support. This position is also supported by an indel in the WAXY1 alignment. However, in WAXY2 and combined analyses V. nudum is weakly supported as sister to a clade containing the core species of sections Lentago and Viburnum (less than 50% bootstrap support in both cases). Consistent with Donoghue et al. (2004) V. urceolatum is well separated from the core of section Viburnum in our analyses. Although relationships at the base of the GBSSI trees are uncertain, our gene trees suggest this species is more closely related to a clade containing the core Solenotinus plus section Tomentosa. The nonmonophyly of Solenotinus is also consistent with the results of Donoghue et al. (2004). As in that study, V. clemensiae is separated from the core of Solenotinus; when Sambucus is included V. clemensiae is weakly supported as sister to the remainder of Viburnum (52% bootstrap support in WAXY1).

The non-monophyly of section *Odontotinus* has been clearly demonstrated in previous analyses (e.g., Donoghue, 1983a; Donoghue and Baldwin, 1993; Donoghue and Sytsma, 1993; Donoghue et al., 2004). As in these studies, our *GBSSI* trees indicate the division of this large group into two distinct lineages, a predominantly red-fruited Asian clade and a purple-fruited North American clade, which also includes the Latin American section *Oreinotinus*. In the case of the red-fruited Asian clade, *WAXY1* and combined analyses suggest that this group is monophyletic (55 and 60% bootstrap support, respectively) and sister to *V. cylindricum* of section *Megalotinus*. In our *WAXY2* trees, however, *V. cylindricum* forms a polytomy with two Old World *Odontotinus* subclades.

Our analyses include only a single representative, V. cylindricum, of the predominantly south east Asian section Megalotinus. The inclusion of additional species will be necessary to test the monophyly of this morphologically diverse group and to firmly establish the position of Megalotinus relative to Old World Odontotinus. Likewise, the Asian section Tomentosa is represented in our analyses by a single species, V. plicatum. Section Tomentosa is often considered mono-specific, although some authors recognize a second, morphologically very similar species, Viburnum hanceanum (e.g., Hara, 1983).

4.3.2. Relationships among sections

The largest supra-sectional clade identified by Donoghue et al. (2004) contained sections *Megalotinus*, *Odontotinus*, *Opulus*, *Oreinotinus* and *Tinus*. This grouping is also recovered in our analyses of *GBSSI* (66, 79, and 94% bootstrap support in *WAXY1*, *WAXY2*, and combined analyses, respectively). Perhaps more importantly, our *GBSSI* analyses provide greater resolution and support for relationships among the main subclades than in previous analyses. In the *WAXY2* and combined analyses section *Opulus* is well supported as sister to the remaining lineages (100 and 94% bootstrap support, respectively). Although this relationship was also recovered in the combined analyses of Donoghue et al. (2004) it received less than 50% bootstrap support. The remaining subclades fall into two distinct lineages. One of these links

Fig. 5. (A) A comparison of the maximum likelihood topologies of combined analyses from Donoghue et al., 2004 (left) and this study (right). Only relationships with greater that 50% bootstrap support are shown, other resolution is collapsed. Thickened lines and bold font indicate duplicated *GBSSI* gene lineages; for taxa that appear twice in the combined *WAXY1/WAXY2* tree we have matched the homoeologue that is topologically most congruent with the corresponding taxa in the Donoghue et al. (2004) tree. Bootstrap values above 50% are indicated. (B) A potential *Viburnum* species tree derived from the combined *WAXY1/WAXY2* analysis. Only relationships with greater that 50% bootstrap support are shown, other resolution is collapsed; the *GBSSI* homoeologue that is topologically most congruent with the corresponding taxa in the Donoghue et al. (2004) tree is retained, but species relationships within these two clades reflect evidence from both homoeologues. Dashed lines with arrow-heads represent hypothesized hybridization events giving rise to the allopolyploid lineages (see text).

V. cyindricum (Megalotinus) with the Old World Odontotinus plus the North American purple-fruited species, V. acerifolium. This association received 100% bootstrap support in the WAXY1 and combined analyses; although it appears in the ML topology for WAXY2, bootstrap support was less than 50%. Within this clade, GBSSI analyses suggest a sister relationship between V. cylindricum and the Old World Odontotinus-acerifolium clade (bootstrap support of 55 and 61% in WAXY1 and combined analyses, respectively). However, as noted above, further sampling of *Megalotinus* is required to clarify the relationships between these two groups. Within the second major subclade, which contains section Tinus, the New World Odontotinus, and the Latin American Oreinotinus, relationships are somewhat obscured by the presence of additional duplications (see Section 4.4) and the apparent absence of WAXY2 in section Tinus. However, based on analyses of WAXY1 and the combined data set it appears that *Tinus* is sister to a New World Odontotinus-Oreinotinus clade (81 and 76% bootstrap support, respectively).

The direct link between V. plicatum (section Tomentosa) and the core of Solenotinus (i.e., with the exception of V. clemensiae) reported by Donoghue et al. (2004) is also supported by analyses of GBSSI. Bootstrap analyses of the WAXY2 and combined data sets provide strong support (96 and 99% bootstrap value, respectively), whereas WAXY1 offered only weak support (bootstrap support of 55%) for this relationship.

The Lentago-Pseudotinus-Viburnum clade identified by Donoghue et al. (2004) was not recovered in our analyses of GBSSI paralogues. As in the earlier study, our gene trees provide support for a lineage containing the core groups of sections Lentago and Viburnum (91, 53, and 91% bootstrap support in WAXY1, WAXY2, and combined analyses, respectively). However, our GBSSI trees do not support a direct link between this clade and section Pseudotinus. Instead, analyses of WAXYI and the combined data set suggest that Pseudotinus is sister to the clade containing Solenotinus, Tomentosa, and V. urceolatum. This arrangement was not well supported (less than 55% in both cases) and so the exact relationships of Pseudotinus remain uncertain.

Although three major lineages are now clearly identified within Viburnum, relationships among these clades, and the positions of V. clemensiae, V. urceolatum, and section Pseudotinus remain uncertain. As noted above, gene trees for WAXY1 and the combined data set suggest a clade containing V. urceolatum, section Pseudotinus, and the Solenotinus-Tomentosa clade. However, this grouping is only weakly supported (53% bootstrap support in combined analyses). The position of the root also remains uncertain. Consistent with Donoghue et al. (2004), inclusion of an outgroup species suggests that the root lies between V. clemensiae and the remainder of Viburnum. However, this placement received only 52% bootstrap support in analyses of *WAXY1*, and less the 50% in combined analyses. Preliminary experiments with duplicate gene rooting also provide some support for *V. clemensiae* being sister to the rest of *Viburnum*. In such analyses, *V. clemensiae* was typically placed at the base of the *WAXY1* clade, although this relationship was not well supported; there was generally no resolution of the root position in the *WAXY2* clade. Resolving the position of the root clearly requires more data.

4.3.3. Relationships within sections

As in Donoghue et al. (2004), relationships within the section-level clades remain somewhat uncertain due to limited sampling. Nevertheless, within each of the section-level clades there are important results that we highlight with reference to earlier studies.

In the Old World Odontotinus clade most relationships suggested by GBSSI are not well supported. However, our combined analyses do suggest relationships that are generally consistent with those from Donoghue et al. (2004). One interesting finding is strong support for a link between the North American species, V. acerifolium, and the Chinese species, V. kansuense, in WAXY2 and combined analyses (92 and 89% bootstrap support, respectively). Donoghue et al. (2004) also recovered this relationship, which directly links species with tri-lobed leaves. It will be especially interesting to include V. orientale (Caucusus mountains), which has tri-lobed leaves that are very similar to those of V. acerifolium. Similarly, the Chinese species V. foetidum should also be included, as its leaves also sometimes produce rounded lobes that resemble those of V. kansuense.

For section *Tinus* analyses of the *WAXY1* paralogue, both alone and within the combined data set (we failed to recover *WAXY2* sequences), indicate the same relationships as trees based on nrITS and the *trnK* intron sequences. Specifically, the two Asian species, *V. cinnamomifolium* and *V. davidii*, form a clade (89 and 86% bootstrap support in *WAXY1* and combined analyses, respectively) that is sister to the European species, *V. tinus*. Although this congruence is promising, increased sampling is required to critically evaluate the biogeographic history of the group.

Within the core of section Lentago our GBSSI analyses unite the Mexican species, V. elatum, with V. prunifolium and V. rufidulum of eastern North America, to the exclusion of another eastern North American species, V. lentago. This finding is congruent with the nrITS trees of Donoghue et al. (2004) but inconsistent with their chloroplast trees. Donoghue et al. (2004) suggested that conflict between nuclear and chloroplast markers may support the hypothesis that V. prunifolium originated as a hybrid between V. rufidulum and V. lentago. Our results are also consistent with this interpretation, although the position of the Mexican V. elatum remains to be accounted for. Additional chloroplast sequences will be especially useful in future studies of this problem.

The Donoghue et al. (2004) analyses provided strong support for two lineages within the New World Odontotinus-Oreinotinus clade. Although our GBSSI gene trees support a similar result the situation is complicated by additional gene duplications (Fig 5.). In both studies an Oreinotinus-dentatum clade is sister to one containing V. ellipticum, V. rafinesquianum, and V. molle. However, in our GBSSI analyses a second clade of Oreinotinusdentatum sequences is nested within the ellipticum-rafinesquianum-molle lineage (discussed further in Section 4.4). Previous studies have not resolved relationships with Oreinotinus, but in our combined analysis both homoeologues suggest identical relationships. Specifically, V. hartwegii and V. triphyllum are sister to V. jucundum, and this clade is sister to V. stenocalyx. This arrangement makes biogeographic sense as it separates the three species that occur south of the Isthmus of Tehuantepec from V. stenocalyx, which grows in the volcanic belt of central Mexico.

The presence of duplicate sequences also complicates comparisons within the *Solenotinus–Tomentosa* clade. However, in both Donoghue et al. (2004) and our analyses *V. odoratissimum* and *V. sieboldii* are strongly linked.

For sections Opulus and Pseudotinus the results suggested by GBSSI are incongruent with those of Donoghue et al. (2004). In the case of Opulus, combined analyses of nrITS and trnK strongly support a direct link between the widespread Asian species, V. sargentii, and the North American V. trilobum. However, our analyses of WAXY1 and the combined data set (we did not recover WAXY2 for V. edule) suggest a closer relationship between V. trilobum and the other North American species, V. edule (67 and 64% bootstrap support in WAXY1 and combined analyses, respectively; plus an indel in WAXY1). For Pseudotinus, Donoghue et al. (2004) found the Japanese species, V. furcatum, and V. cordifolium of the Himalayas to be sister. In contrast, GBSSI analyses strongly support a link between V. furcatum and V. lantanoides of eastern North America, to the exclusion of V. cordifolium (99 and 98% bootstrap support in WAXYI and combine analyses, respectively). Resolution of relationships within these two groups will require further sampling and additional markers, but in both cases the results will have important implications for biogeography and character evolution (e.g., the evolution of enlarged sterile flowers at the margins of the inflorescences).

4.4. Additional copies of WAXY1 and WAXY2 result from polyploidy

Our analyses indicate that some members of the New World *Odontotinus–Oreinotinus* clade and of section *Solenotinus* contain an additional copy of each *GBSSI* paralogue. Within the New World Odontotinus-Oreinotinus lineage two distinct sequence types were recovered from V. dentatum and all representatives of section Oreinotinus. In our combined analysis these sequences types form separate clades; one (homoeologue A) is united with V. ellipticum, V. rafinesquianum, and V. molle and the other (homoeologue B) is sister to this entire grouping. Within section Solenotinus V. erubescens, V. odoratissimum, and V. sieboldii also appear twice on our combined tree. For each of these species one copy (homoeologue C) is directly linked with V. plicatum of section Tomentosa; sequences from the other copy (homoeologue D) are included within a clade that is sister to the V. plicatum-homoeologue C lineage, and which also contains Solenotinus species that occur once in the gene trees.

Chromosome counts are available for many Viburnum species. To date the majority of species studied are x=9; the exception is section Solenotinus where x=8 is reported (Donoghue, 1982; Egolf, 1956). Chromosome counts have also identified a number of polyploid lineages within Viburnum. Indeed several of the species that have additional GBSSI copies are reported to be polyploids. Within Solenotinus Egolf (1956) reported polyploidy in V. erubescens (2n=32), V. odoratissimum (2n=32, 40), and V. sieboldii (2n=16, 32). Similarly, all counts for the V. dentatum species complex (2n=36, 72) indicate polyploidy (Egolf, 1956). There are few chromosome counts for members of section Oreinotinus. Egolf (1956) reported 2n=18 for a V. hartwegii specimen of unknown provenance, whereas Donoghue (1982) counted 2n=36 from Viburnum lautum and Viburnum blandum of southern Mexico. Clearly, further karyological studies are needed within Oreinotinus, but the presence of additional GBSSI copies supports the view that polyploidy may characterize the entire group, including V. hartwegii. Outside of Solenotinus and the Oreinotinus-dentatum clade polyploidy is very limited. Only V. tinus (2n=36, 72; Tinus), V. rafinesquianum (2n=36; New World Odontotinus), V. molle (2n=36,72; New World Odontotinus), V. setigerum (2n=18, 36;Old World *Odontotinus*) and *V. hanceanum* (2n = 72;Tomentosa) are reported to be polyploid. All other members of these clades that have been studied are 2n = 18, except for V. plicatum (Tomentosa) which is reported to be 2n=16, 18 (Egolf, 1956; Janaki Ammal, 1953; see Donoghue et al., 2004; for discussion of this polymorphism). We included three of these polyploids in our analyses (i.e., V. molle, V. rafinesquianum, and V. tinus). In contrast to polyploids from Solenotinus and the Oreinotinus-dentatum clade we identified just a single form of each GBSSI paralogue from these accessions. Despite the additional step of cloning PCR products for WAXY1 from V. molle and both forms from V. rafinesquianum we did not identify a divergent second homoeologue. One potential explanation is that these polyploids arose independently, either by autopolyploidy or following hybridization between closely related lineages. In such cases the homoeologues may not have distinct sequences. Alternatively, it is possible that despite efforts to identify further copies, we have simply failed to recover the additional homoeologue; perhaps in these lineages this copy was rapidly silenced and is no longer detected by our PCR-based approach.

The placement of duplicated sequences within gene trees can provide insights into the origin of polyploidy lineages (e.g., Barrier et al., 1999; Doyle et al., 2003; Ge et al., 1999; Mason-Gamer, 2001; Small et al., 1999). For example, characterizing homoeologues from an allopolyploid may help identify the closest extant relatives of the original genome donors (Cronn et al., 1999; Doyle et al., 2003; Sang, 2002). Indeed our gene trees suggest that allopolyploidy may provide an explanation for the additional *GBSSI* copies found in some members of Solenotinus and in the Oreinotinus-dentatum clade. In the case of *Solenotinus*, our trees suggest that the polyploids may have arisen following hybridization between early members of sections Solenotinus and Tomentosa. However, we remain cautious as this hypothesis is somewhat complicated by the placement of V. erubescens. For both WAXY1 and WAXY2 the D homoeologue of V. erubescens is more closely related to sequences from two diploid species, V. suspensum and V. farreri, than it is to sequences from the polyploids, V. odoratissimum and V. sieboldii (as expected if the polyploids were derived from a single event). Possible explanations for this observation include: (i) independent allopolyploidization events involving different diploid members of Solenotinus, (ii) introgressive hybridization between diploids and polyploids, (iii) incorrect gene trees owing to limited data or complex patterns of molecular evolution (discussed further below). For polyploids of the Oreinotinus-dentatum lineage GBSSI gene trees suggest that a diploid ancestor of the *ellipticum-rafinesquianum-molle* group may have donated one of the parental genomes. However, as the other homoeologue is not directly associated with a diploid lineage the second putative ancestor remains uncertain. Although it is possible that we have simply failed to sample an appropriate diploid, this seems unlikely given our current understanding of the phylogeny. Perhaps a more likely explanation is that the ancestral diploid lineage is extinct and this sequence type now occurs only in the polyploid lineage. As describe above, hypotheses on the origin of polyploidy in the Oreinotinus-dentatum clade are complicated by the occurrence of related polyploids that appear only once in gene trees (i.e., V. molle, V. rafinesquianum, and V. tinus).

Although allopolyploidy provides an appealing explanation for the placement of additional copies of *WAXY1* and *WAXY2* in our gene trees, there are other possibilities. One potential alternative, just mentioned, is

that polyploidization occurred earlier in the phylogeny, and one of the homologues was lost (or we failed to detect it) in related species represented only once in our gene trees. For example, in the New World Odontotinus-Oreinotinus clade we might suggest polyploidization following divergence of Tinus and failure to recover the B homoeologue from V. ellipticum, V. rafinesquianum, and V. molle. However, such scenarios also require the presumably very rare subsequent loss of an entire set of chromosomes to re-establish diploidy (see Ramsey and Schemske, 2002; Stebbins, 1980). Another alternative explanation is that differences in the rate or pattern of molecular evolution between the homoeologues accounts for their phylogenetic position. The placement of gene lineages duplicated by polyploidy may be complicated by factors such as the amount of divergence between homoeologues at the time of polyploidization, the nature of chromosome pairing in the polyploid derivative, the action of copy correction mechanisms or concerted evolution, and differential selection on homoeologues. It is conceivable that differences in the molecular evolution of the duplicates, perhaps associated with functional shifts (e.g., see Mathews et al., 2003), could lead to errors in the placement of homoeologues on gene trees and therefore to misidentification of their parentage. Clearly, data from additional nuclear loci will be required to confirm the origins of polyploids in Viburnum. However, the GBSSI gene trees provide initial insights into the complexity of these situations.

4.5. Future directions

To a large extent phylogenetic analyses of morphology (Donoghue, 1983a), chloroplast restriction site data (Donoghue and Sytsma, 1993), and DNA sequences (Donoghue and Baldwin, 1993; Donoghue et al., 2004; this study) provide a consistent picture of relationships within Viburnum. These studies, in broad terms, agree on the monophyly and non-monophyly of the traditionally recognized taxonomic sections, on relationships among several sections, and on species relationships within several section-level clades. However, our understanding of relationships at the very base of the Viburnum phylogeny and those within several section-level clades remains limited. Resolving relationships at the base of the tree is critical to our understanding of the evolutionary history of the group. However, given the evidently great genetic distance between Viburnum and its closest living relatives this problem will be particularly difficult to resolve, requiring much more data. Although our understanding of basal relationships is still limited our studies do provide a context within which the evolution of smaller groups can be addressed.

Relationships among many of the section-level clades appear to be well established. However, we are still uncertain about the relationship between the Old World *Odontotinus* and the morphologically diverse section *Megalotinus*. Resolving this will require the inclusion of sequences from additional *Megalotinus* species and probably the use of additional markers. Similarly, more data are needed to resolve relationships within several section-level clades and species complexes. Of particular interest will be (i) testing the origins of polyploid species within the New World *Odontotinus–Oreinotinus* clade and section *Solenotinus*, (ii) establishing species relationships within sections *Opulus* and *Pseudotinus*, and (iii)

Appendix A

Accession details for sampled taxa

more detailed studies within section *Lentago* to address the possibility of homoploid hybrid speciation.

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Faxon	Section	Location	Voucher	GenBank Accession No.	
				WAXY1	WAXY2
Viburnum					
V. acerifolium L.	OD	Arn. Arb. 1505-67 (Eastern North America)	D & W 27	AY596829	AY596876
V. carlesii Hemsley	V	Cult. Arn. Arb. 17981-2-A (Japan, Korea)	D & W 24	AY596830	AY596877
V. cinnamomifolium Rehder	TI	Cult. Univ. of Washington Arb. 1392-56 (China)	R. Olmstead 2002-120 (WTU)	AY596831	
V. clemensiae Kern	S	Mt. Kinabalu, Malaysia	J. Beaman 11781 (K)	AY596832	AY596878
V. cordifolium Wallich ex DC.	РТ	Sichuan Province, China	D. Boufford et al. 27388 (A)	AY596833	AY596879
V. cylindricum Ham. ex D. Don	М	Yunnan Province, China	D. Boufford et al. 29342 (A)	AY596834	AY596880
V. davidii Franchet	TI	Cult. vic. Corvalis, OR, USA (China)	Donoghue, voucher lacking	AY596835	
V. dentatum L.	OD	Cult. Arn. Arb.5070-1-A (Eastern North America)	D & W 33	A, AY596862 B, AY596867	AY596905
V. dilatatum Thunb.	OD	Cult. Arn. Arb.138-52-A (China, Japan, Korea)	D & W 19	AY596836	AY596881
V. edule (Michaux) Raf.	OP	Northern Wisconsin, USA	W. Alverson, voucher lacking	AY596837	
V. elatum Bentham	L	Chiapas, Mexico	Donoghue 472 (A, YU)	AY596838	AY596882
V. ellipticum Hooker	OD	vic. Corvalis, OR, USA	Donoghue, voucher lacking	AY596839	AY596883
V. erosum Thunb.	OD	Cult. Arn. Arb.78-90-A (China, Japan, Korea)	D & W 16	AY596840	AY596884
V. erubescens Wallich ex DC.	S	Sichuan Province, China	D. Boufford et al. 27190 (A)	C, AY596872 D, AY596874	C, AY596914 D, AY596917
V. farreri Stearn	S	Cult. Arn. Arb. 656-89-A (China)	D & W 18	AY596841	AY596885
V. furcatum Blume	PT	Kyoto Prefecture, Japan	Tsugaru & Takahashi 19958 (MO)	AY596842	AY596886
V. hartwegii Bentham	OR	Chiapas, Mexico	Donoghue 486 (A, YU)	A. AY596863	A. AY596906
		(I m)		B. AY596868	B. AY596910
V. japonicum (Thunb.) Sprengel	OD	Cult. US. Nat. Arb. 56545 (China)	Donoghue, voucher lacking	AY596843	AY596887
V. jucundum Morton	OR	Chiapas, Mexico	Donoghue 244 (A, YU)	A, AY596864 B AY596869	A, AY596907 B AY596911
V kansuense Batalin	OD	Sichuan Province China	D Boufford et al 27416 (A)	AY 596844	AY 596888
V. lantana L.	V	Cult. Arn. Arb. 1089-60-A	D & W 26	AY596845	AY596889
V. lantanoides Michaux	РТ	Cult. Arn. Arb.155-97-B (Eastern North America)	D & W 2	AY596846	AY596890
V. lentago L.	L	Cult. Arn. Arb. 18021-A (Eastern North America)	D & W 21	AY596847	AY596891
V. lobophyllum Graebn.	OD	Cult. Arn. Arb.1875-80-A	D & W 25	AY596848	AY596892
V. melanocarpum Hsu in Chen et al.	OD	Cult. Arn. Arb.386-81-D (China)	D & W 12	AY596849	AY596893
V. molle Michaux.	OD	Cult. Arn. Arb.18294-A (Eastern North America)	D & W 5	AY596850	AY596894

(continued on next page)

Гахоп	Section	Location	Voucher	GenBank Accession No.	
				WAXY1	WAXY2
V. nudum L.	L	Cult. US. Nat. Arb. 32198-C (Eastern North America)	Donoghue, voucher lacking	AY596851	AY596895
V. odoratissimum Ker Gawler	S	Cult. Univ. of Washington Arb. 1404-56 (Japan, Korea, Taiwan, Phillipines)	R. Olmstead 2002-118 (WTU)	AY596873	C, AY596915 D, AY596918
V. plicatum var. tomentosum Thunb.(Miquel)	ТО	Cult. Arn. Arb. 72-98-A (China, Japan, Taiwan)	D & W 10	AY596852	AY596896
V. prunifolium L.	L	Cult. Arn. Arb. 22586-A (Eastern North America)	D & W 13	AY596853	AY596897
V. rafinesquianum Schultes	OD	Cult. Arn. Arb.17974-A (Eastern North America)	D & W 4	AY596854	AY596898
V. rufidulum Raf.	L	Cult. Arn. Arb. 21418-A (Eastern North America)	D & W 14	AY596855	AY596899
V. sargentii Koehne	OP	Cult. Arn. Arb.398-68-B (China, Japan, Korea, Siberia)	D & W 17	AY596856	AY596900
V. sieboldii Miq.	S	Cult. Arn. Arb. 616-6-A (Japan)	D & W 3	AY596875	C, AY596916 D, AY596919
V. stenocalyx Oersted (Hemsley)	OR	DF, Mexico	Donoghue 127 (A, YU)	A, AY596865 B, AY596870	A, AY596908 B, AY596912
V. suspensum Lindley	S	Cult. Santa Barbara, CA (Japan)	D & W 36	AY596857	AY596901
V. tinus L.	TI	Cult. Santa Barbara, CA (Europe)	D & W 35	AY596858	
V. trilobum Marshall	OP	Cult. Arn. Arb.724-68-E (North America)	D & W 22	AY596859	AY596902
V. triphyllum Benth.	OR	Loja, Ecuador	C. Bell EC-026 (YU)	A, AY596866 B, AY596871	A, AY596909 B, AY596913
V. urceolatum Sieb. & Zucc.	V	Cult. US. Nat. Arb. 40200 (China, Japan, Taiwan)	Donoghue, voucher lacking	AY596860	AY596903
V. utile Hemsley	V	Cult. US. Nat. Arb. (China)	Egolf 2336-E	AY596861	AY596904
Outgroup					
Sambucus canadensis L.		Cult. Marsh Bot. Gard., Yale Univ. (North America, Mexico, Central America)	D & W 37	AY598465	

Appendix A (continued)

Abbreviations for traditionally recognized sections are: L, *Lentago*; M, *Megalotinus*; OD, *Odontotinus*; OP, *Opulus*; OR, *Oreinotinus*; PT, *Pseudotinus*; S, *Solenotinus*; TI, *Tinus*; TO, *Tomentosa*; V, *Viburnum*. All Donoghue and Winkworth (D & W) collections are deposited at Yale University (YU) and the Arnold Arboretum at Harvard University (A); locations of remaining voucher specimens are noted. Generalized geographic ranges for specimens obtained from cultivated plants noted in parentheses. Where divergent homoeologues have been recovered these forms are denoted as in text.

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