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Viburnum Phylogeny Based on Chloroplast *trnK* Intron and Nuclear Ribosomal ITS DNA Sequences

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ABSTRACT. Phylogenetic analyses of chloroplast *trnK* intron and nuclear ribosomal ITS DNA sequences yield significant improvements in our understanding of relationships, character evolution, and biogeography in *Viburnum* (Adoxaceae). We confirm that most of the ten traditionally recognized sections are monophyletic. The most striking exception is *Odontotinus*, which is divided into: (i) a purple-fruited New World clade within which the Latin American section *Oreinotinus* is nested, and (ii) an Old World, mostly red-fruited clade that is closely related to *V. cylindricum* (section *Megalotinus*) and the New World, purple-fruited *V. acerifolium*. We identify three major supra-sectional groupings: (i) a clade consisting of the *Odontotinus-Oreinotinus-Megalotinus* complex, the circum-boreal section *Opulus*, and the Eurasian section *Tinus*, (ii) a clade containing the Old World section *Viburnum*, the New World section *Lentago*, and, with less support, section *Pseudotinus*, and (iii) a clade containing the Asian sections *Tomentosa* and *Solenotinus*. Two species are not clearly allied to any of these supra-sectional clades: *V. urceolatum*, a Taiwanese/Japanese species traditionally placed in section *Viburnum*, and *V. clemensiae*, a Bornean species previously assigned to section *Solenotinus*. The placement of the root also remains uncertain, but probably does not fall within any of the three major supra-sectional clades. Knowledge of relationships within sections is useful in clarifying historical biogeography and the evolution of sterile flowers and fruit color. The one case of conflict between datasets highlights a possible instance of homoploid hybrid speciation.

Viburnum contains approximately 175 species of shrubs and small trees distributed around the Northern Hemisphere (Oersted 1861; Rehder 1908; Hara 1983; Donoghue 1983a), with significant extensions into the mountains of southeast Asia (Kern 1951) and Latin America (Killip and Smith 1931; Morton 1933; Donoghue 1982). Although rather uniform in flower and fruit morphology, *Viburnum* is well known for striking variation in several features, including the presence or absence of naked buds and of sterile flowers around the margins of the inflorescences. Based on these and other characters (e.g., endocarp shape, inflorescence form, and leaf morphology) *Viburnum* has been subdivided by several authors, most commonly into ten taxa formally recognized as sections (Table 1; Oersted 1861; Hara 1983).

Previous analyses have strongly supported both the monophyly of *Viburnum* (Wilkinson 1948; Donoghue 1983b; Donoghue and Friedman 1988; Benko-Iseppon and Morawetz 2000) and its placement within the Dipsacales (Donoghue 1983b; Donoghue et al. 1992; Judd et al. 1994; Backlund and Donoghue 1996; Backlund and Bremer 1997; Pyck et al. 1999; Donoghue et al. 2001; Bell et al. 2001). However, phylogenetic relationships within *Viburnum* have received much less attention. An analysis of morphological characters by Donoghue (1983a) found nine of the ten traditionally recognized sections to be monophyletic. The exception was section *Odontotinus* that contained a clade corresponding to the Latin American section *Oreinotinus*. Subsequently, two unpublished molecular phylogenetic studies (Donoghue and Sytsma 1993; Donoghue and Baldwin 1993; summarized in Baldwin et al. 1995) confirmed many aspects of the morphological results, but called other findings into question. In particular, the molecular studies indicated that the phylogenetic arrangement within section *Odontotinus* was more complicated than suggested by Donoghue (1983a).

Although these earlier studies were useful in evaluating the monophyly of the sections, they did little to clarify relationships among and within the sections, or to establish the position of the root within *Viburnum*. Consequently, our understanding of biogeography and character evolution in the group has remained rather rudimentary. The aim of the present study was to assemble two directly comparable molecular datasets — one nuclear and one chloroplast—in the hope that together these would improve our knowledge of the evolution and biogeography of *Viburnum*.

MATERIALS AND METHODS

We obtained nuclear ribosomal ITS and chloroplast *trnK* DNA sequences from 43 accessions of *Viburnum* (42 species and two specimens of the widespread *V. sargentii*), representing all of the sections and major species complexes, as well as from two species of *Sambucus* for rooting purposes. Tissue samples were either silica gel preserved field collections or were obtained from herbarium specimens. Appendix 1 provides voucher information for each accession and GenBank numbers for the sequences.

DNA Extraction, Amplification, and Sequencing. Genomic DNA was extracted from dried tissue samples using either a cetyltrimethylammonium bromide (CTAB) protocol modified from Doyle and Doyle (1990), or the Qiagen DNeasy Plant Kit (La Jolla, CA).

The 5′ portion of the *trnK* intron was amplified in reaction vol-

TABLE 1. Traditionally recognized sections of *Viburnum* (e.g. Hara 1983), with the approximate number of species and generalized geographic range for each.

Section *Lentago*—7 spp. Eastern North America, except *V. elatum* in Mexico.
Section *Megalotinus*—18 spp. Southeast Asia, extending west to India and south to Indonesia.
Section *Odontotinus*—45 spp. Temperate Asia and Eastern North America, except *V. orientale* in the Caucasus Mountains.
Section *Opulus*—5 spp. Circumboreal.
Section *Oreinotinus*—42 spp. Mexico, Caribbean, Central and South America.
Section *Pseudotinus*—4 spp. Asia, except *V. lantanoides* in Eastern North America.
Section *Solenotinus*—25 spp. Asia, extending west to India and south to Indonesia.
Section *Tinus*—9 spp. Asia, except *V. tinus* in Europe.
Section *Tomentosa*—2 spp. China, Japan.
Section *Viburnum*—18 spp. Asia, except *V. lantana* in Europe.

umes of 25 μL containing 1× PCR buffer (Perkin-Elmer), 6.25 mM $MgCl_2$, 625 μM each dNTP (Invitrogen), 5% bovine serum albumin (v/v; New England Biolabs), 10 pM each amplification primer, 1 U AmpliTaq DNA polymerase (5 U/μL; Perkin-Elmer) and 10–100 ng of total cellular DNA. Typically the oligonucleotide primers trnK-11 and matK510R (Young et al. 1999) were used in amplifications. However, when necessary these were combined with the internal primers VIBmatK1F (5′-TATATTAGTGCCTGATACGGG-3′) and VIBmatK1R (5′-GATCTATCTAGCTCTAAATATC-3′) to amplify the region in two overlapping sections. Thermocycling conditions for PCR were: initial denaturation at 98°C for 3 minutes, 35 cycles of 1 minute at 95°C (denature), 1 minute at 50°C (annealing), and 2 minutes at 72°C (extension), with a final incubation at 72°C for 5 minutes. The ITS locus was amplified in 20 μL reactions containing 1× Q solution (Qiagen), 1× PCR buffer (Qiagen), 500 μM each dNTP (Invitrogen), 10 pM primer ITS-I (Urbatsch et al. 2000), 10 pM primer ITS4 (White et al. 1990), 1 U DNA polymerase (5 U/μL; Qiagen), and 10–100 ng of total cellular DNA. The PCR profile was similar to that described for the *trnK* intron but included a 54°C annealing temperature and a 1 minute extension time.

Amplification products for both loci were purified using the QIAquick PCR Purification Kit (Qiagen). Automated sequencing of PCR fragments was performed using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit and analyzed using either an ABI Prism 377 automated DNA sequencer or MJ Research BaseStation 51. DNA sequencing of the *trnK* intron used the four oligonucleotide primers described above; for the ITS locus the amplification primers and, when necessary, the primers ITS2 and ITS3 (White et al. 1990) were used in sequencing.

Initial results from the ITS locus suggested the presence of multiple sequence types in some accessions. In these cases PCR fragments were cloned using a TOPO TA Cloning Kit (Invitrogen). Between 10 and 15 colonies from each cloning reaction were screened by direct PCR. Screening used reaction mixtures as described for the ITS locus and the M13 primers. Thermocycling conditions were: cell lysis at 98°C for 10 minutes, then 25 cycles of 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C (extension), with a final incubation at 72°C for 5 minutes. For each accession four or five positive clones were purified using the QIAprep Spin Miniprep Kit (Qiagen) and sequenced as above using the M13 primers.

Alignments and Phylogenetic Analyses. We obtained preliminary multiple alignments of DNA sequences using the progressive alignment procedure implemented in ClustalX (Thompson et al. 1994). These alignments were then visually inspected and adjusted for minor improvement. Prior to phylogenetic analyses all ambiguous and gapped positions were excluded from the data matrix.

For the *trnK* intron and ITS data sets phylogenetic trees were inferred using both maximum parsimony (MP) and maximum likelihood (ML) optimality criteria as implemented in PAUP*4.0b10 (Swofford 2001). MP analyses used heuristic searches with ''tree-bisection-reconnection'' (TBR) branch swapping, zero-length branches collapsed, and all characters equally weighted. Analyses were repeated 100 times with RANDOM ADDITION. To determine the best-fitting model of sequence evolution for ML analyses, a hierarchical series of likelihood ratio tests was performed on the MP tree topologies using the program PORN* (Bell 2001). Subsequent heuristic ML tree searches used the most appropriate model (with parameters simultaneously estimated via maximum likelihood), TBR branch swapping, and collapsed zero-length branches. Analyses were repeated 100 times with RANDOM ADDITION. Bootstrap tests used 1000 replicates with nearest neighbor interchange (NNI) branch swapping; parameters for these tests were fixed to values estimated on the optimal maximum likelihood tree.

We tested for incongruence between the two datasets using the partition homogeneity test as implemented in PAUP*4.0b10. Phylogenetic analyses of the combined data set were conducted as described for the individual data sets.

RESULTS

Aligned DNA Sequences. Statistics for the aligned data matrices are presented in Table 2. The data matrix for our chloroplast marker contained little alignment ambiguity as the DNA sequences were highly similar. In contrast, alignment of several regions within the ITS

TABLE 2. Statistics from the aligned data matrices. For the ITS data set it is difficult to confidently infer the exact number of indels due to uncertainty in the alignment of several regions.

	ITS	*trnK* intron
No. of taxa sequenced	45	45
Sequence length (bp)	607–619	1137–1146
Aligned length (bp)	641	1147
No. of indels	see table caption	4
No. of excluded sites	85	16
No. of constant sites	410	1010
No. of varied sites	146	121
No. of parsimony informative sites	108	75
%GC content range (all sites)	57.9–64.2	32.0–33.1
%GC content mean (all sites)	61.6	32.6
%GC content range (varied sites only)	47.3–71.2	42.1–52.1
%GC content mean (varied sites only)	61.3	47.5

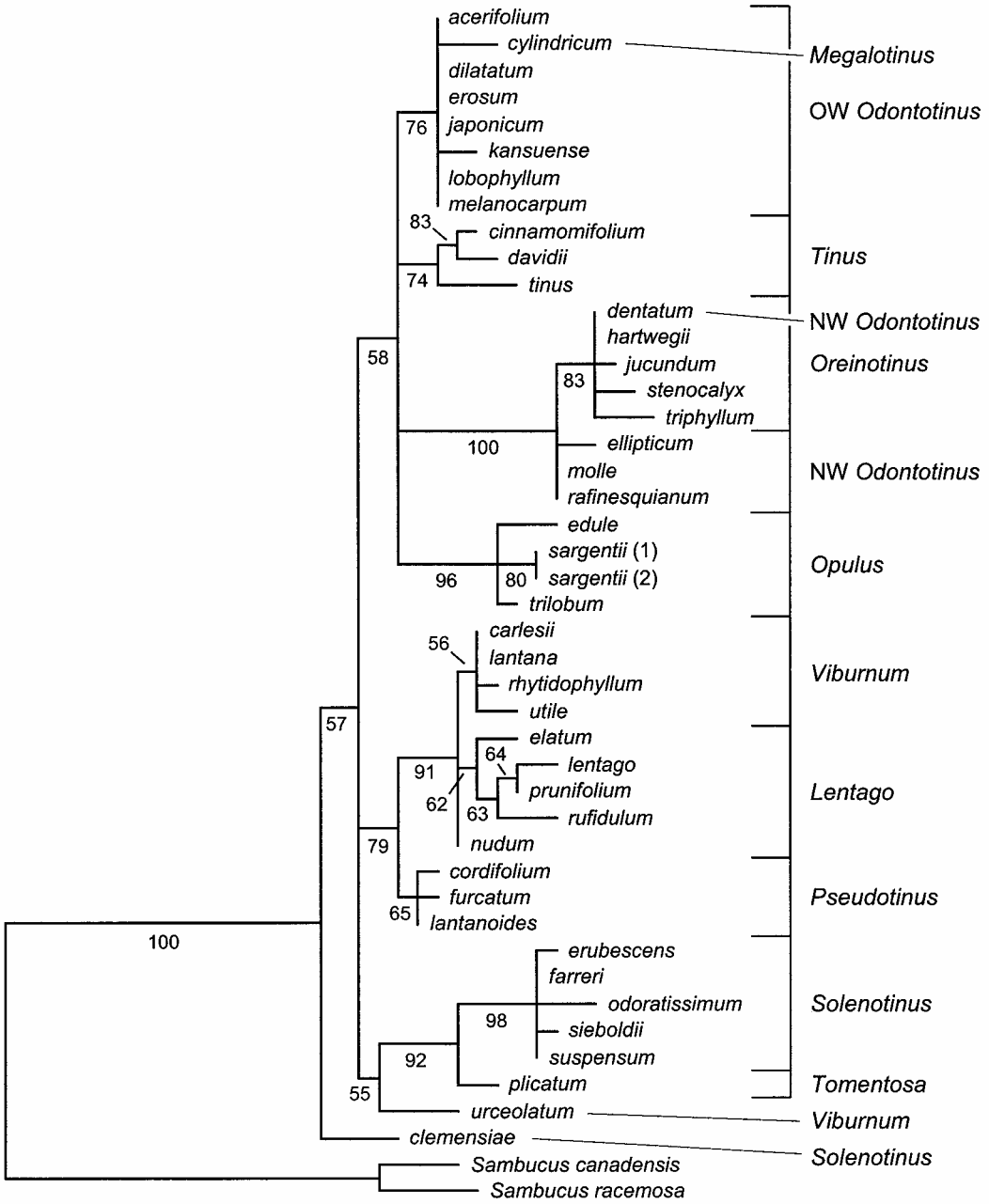

FIG. 1. Single optimal maximum likelihood tree obtained from the analysis of *trnK* sequences, showing bootstrap values over 50% (1000 replicates). Names of the traditional sections of *Viburnum* are given on the right.

data set was confounded by the presence of overlapping but non-identical indels. These regions, as well as all other gapped or ambiguous positions, were excluded from the analyzed data matrices. Consequently, there were no missing cells in our data sets. Nucleotide sequences are deposited in GENBANK (see Appendix 1 for accession numbers); aligned data matrices and trees are available in TreeBASE (study accession number S998, matrix accession numbers M1677—M1679).

Analyses of the* trnK *Intron Sequences. MP analyses resulted in four equally parsimonious topologies, each 140 steps long with a consistency index (CI) of 0.907 and a retention index (RI) of 0.955. These trees differed only in the placement of two species; *V. odoratissimum* and *V. cylindricum* were placed either within or as basal branches of their respective clades. The ML search used a GTR+G+I substitution model and resulted in a single tree (–lnL=2552.8024; Fig. 1) iden-

tical in topology to the strict consensus of the four MP trees. Furthermore, bootstrap support for clades identified in these two analyses was similar, providing moderate (i.e. greater than 65% bootstrap support) to strong support (i.e. >80% bootstrap support) for many of the major clades within *Viburnum*.

Two parsimony informative indel regions excluded from the analyses are compatible with topologies inferred from nucleotide substitutions. Specifically, members of section *Pseudotinus* (e.g., *V. cordifolium*, *V. furcatum*, and *V. lantanoides*) share a 9-nucleotide deletion (positions 552–560), and the two outgroup taxa differ from all *Viburnum* species by a 5-nucleotide indel (positions 606–610). The two remaining indels were single nucleotide deletions; one unique to *V. davidii* (position 465 in the aligned data matrix) and the other to *Sambucus racemosa* (position 377).

Analyses of the ITS Sequences. Initial DNA sequencing suggested that several *Viburnum* species might contain multiple ITS sequence types. We cloned PCR fragments for these species, and obtained sequences from four or five clones. Preliminary analyses of these data indicated that in each case clones from a given accession formed a clade in the ITS phylogeny. Therefore, the sequence of a single clone was used to represent each of these species in the data matrix.

MP searches recovered 240 optimal trees, each 297 steps long with CI=0.586 and RI=0.785. Although the consensus of these topologies (Fig. 2) provides limited resolution and support for higher-level relationships within *Viburnum*, ITS sequences do provide support for the relationships between species within sections. The ML analysis, which used a GTR+G substitution model, again recovered a single tree (–lnL= 2576.17788). However, as in the MP results many of the higher-level relationships suggested by this tree were not well supported in bootstrap analyses.

Combined Analyses. The partition homogeneity test indicated that the two datasets did not conflict significantly (P=0.10). MP analyses of the combined data set recovered 72 trees of 446 steps, with CI=0.675 and RI=0.832. Using a GTR+G+I substitution model the ML search recovered a single optimal topology (–lnL=5165.09407). Generally, this tree simply combines the well-supported clades identified in the *trnK* and ITS analyses; however, in most cases bootstrap values for these clades are higher. Two exceptions are discussed in detail below: the relationship between *Pseudotinus* and the *Viburnum-Lentago* clade and relationships among the species of section *Lentago*.

Discussion

Considered individually, neither the *trnK* nor ITS data sets confidently resolved relationships both among and within the major *Viburnum* clades. Generally, the *trnK* data provided support for the previously recognized sections and their relationships to one another, while the ITS region was more informative about relationships among species within the sections. This difference is evident in comparing the tree topologies in Figs. 1 and 2; the *trnK* tree is well-resolved towards the base and poorly resolved distally, whereas the reverse is true in the ITS tree. This shape contrast is paralleled by differences in the alignability of the sequences (*trnK* being easier to align than ITS) and consistency indices (*trnK* with less homoplasy than ITS). As emphasized below, these observations are consistent with closer inspection of the bootstrap values and the apparent complementarity of the datasets when combined.

Monophyly of the Traditional Sections. Where our taxon sampling allowed a proper test (we included only one species each from sections *Tomentosa* and *Megalotinus*) most of the sections were found to be monophyletic, or non-monophyly was only weakly supported. Analysis of the chloroplast data set alone provided support for the monophyly of sections *Pseudotinus* (65% bootstrap support), *Opulus* (96%), and *Tinus* (74%). In the combined analysis, bootstrap support for each of these groups was higher than in either individual data set. Sections *Lentago* and *Oreinotinus* are paraphyletic in the optimal ML tree inferred from the combined data set. However, in both cases the result was only weakly supported (53% and 61% bootstrap support, respectively) and in analyses of the individual data sets the arrangements were unresolved. For the Latin American section *Oreinotinus*, a relationship with the eastern North American species, *V. dentatum*, is well supported. However, additional taxon sampling is required to resolve whether one or more North American species are sister to, or nested within, the Latin American species. In the case of section *Lentago*, *V. nudum* is clearly distinct from the remaining species, but again more data are needed to establish the exact phylogenetic arrangement.

The three remaining sections—*Odontotinus*, *Viburnum* and *Solenotinus*—are clearly not monophyletic. For section *Odontotinus* this result was anticipated based on earlier analyses (e.g., Donoghue 1983a; Baldwin et al. 1995). In agreement with previous studies we found that *Odontotinus* was divided into two distinct clades—one containing Old World, red-fruited species and the other New World taxa with purple fruits. Our results are also consistent with the earlier studies in suggesting that (i) the Latin American species (section *Oreinotinus*) are nested within the New World *Odontotinus* clade, and (ii) *V. acerifolium*, a purple-fruited New World species, is more closely related to the Old World red-fruited group. However, our analyses also suggest a previously unrecognized relationship between Old World *Odontotinus* and *V. cylindricum*, our sole representative of the primarily southeast-Asian section *Me-*

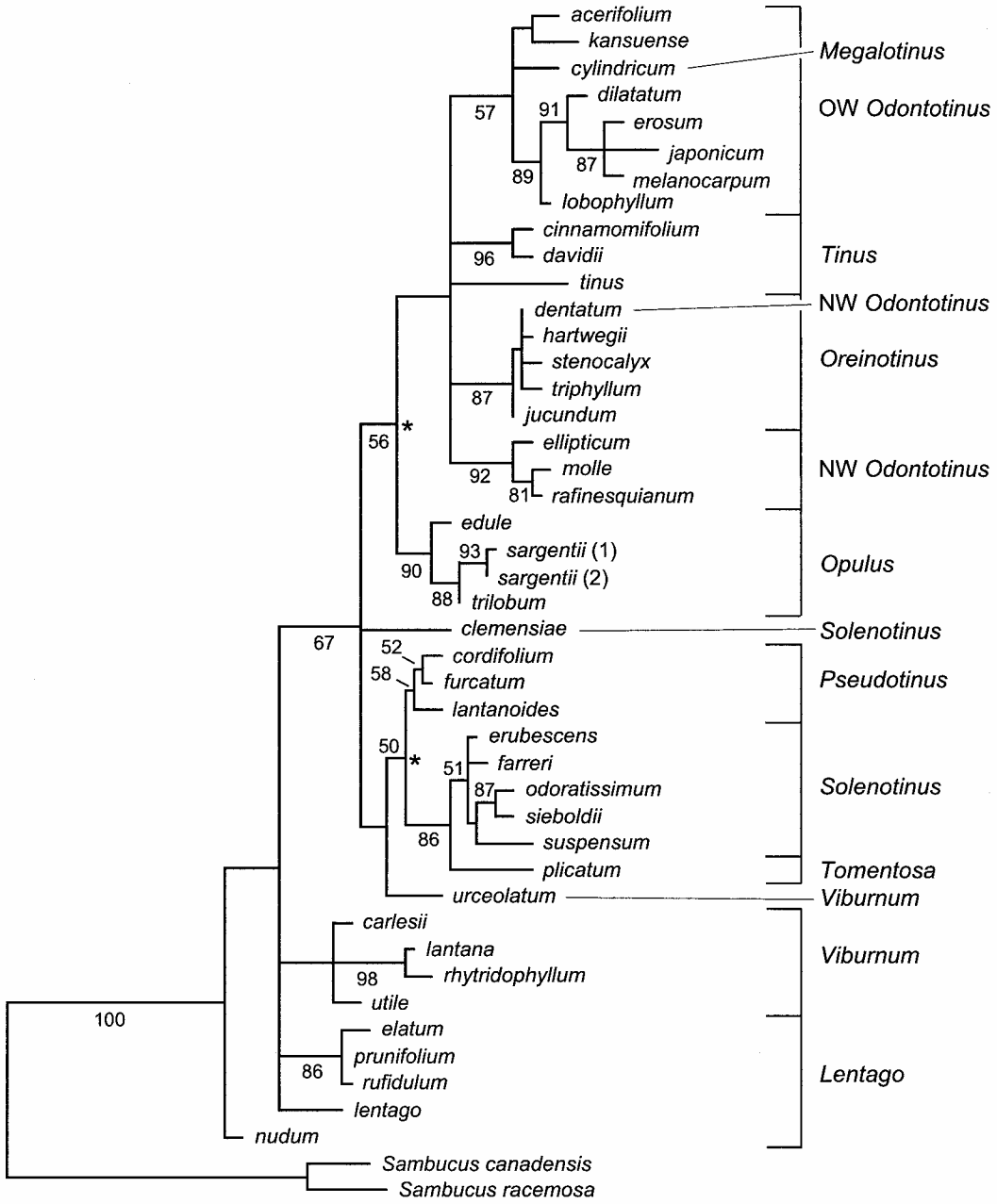

FIG. 2. Strict consensus of 240 maximum parsimony trees obtained from the analysis of ITS sequences (branch lengths under ACCTRAN), showing bootstrap values over 50% (1000 replicates). Nodes marked with asterisks collapse in the majority rule consensus of the maximum likelihood bootstrap trees. Names of the traditional sections of *Viburnum* are given on the right.

galotinus. Additional data are needed to establish whether this result reflects the relationships of *Megalotinus* as a whole, which is notably heterogeneous with respect to leaf, corolla, stamen, and fruit characters (Kern 1951; Hara 1983).

Our analyses indicate that section *Viburnum* is not monophyletic. In our chloroplast and the combined trees four of the five representatives formed a clade (56% and 78% bootstrap support, respectively) that was strongly linked with section *Lentago* (91% and 94% bootstrap support in the chloroplast and combined analyses, respectively). The exception, *V. urceolatum*, was separated from the remainder of the section, instead being weakly linked with the *Tomentosa-Solenotinus* clade (55% bootstrap support in *trnK* analyses). Although *V. urceolatum* has traditionally been placed in

section *Viburnum* based on the presence of naked buds and stellate hairs, molecular evidence for its exclusion from this section is not surprising given its otherwise strange combination of morphological characters. In particular, the flowers of *Viburnum urceolatum* are highly unusual within *Viburnum* as a whole (Hara 1983): the tubular corolla has short erect lobes (as in some species of section *Megalotinus*; e.g., *V. cylindricum*) and is often red in color; the usually bright white stamens are exerted from the corolla. *V. urceolatum* also has a distinctive growth form, producing sympodial plagiotropic axes that resemble those found in section *Pseudotinus* (Donoghue 1981, 1982). Although *V. urceolatum* is clearly not directly linked to the core of section *Viburnum*, additional data are needed to establish its relationships more precisely.

The non-monophyly of section *Solenotinus* was not expected. This group has primarily been distinguished by inflorescence structure; in contrast to the umbel-like inflorescences of all other *Viburnum* species, members of section *Solenotinus* produce distinctive panicle-like inflorescences. These species may also differ from other viburnums in having chromosome numbers of $x=8$ (as apposed to $x=9$ elsewhere in *Viburnum*; Egolf, 1956, 1962) and in lacking glycosides (Norn 1978). However, this character needs further investigation as the original observations relied on only a small sample of species. We sampled six of the ca. 25 species in this group; of these, five formed a well-supported clade (91% bootstrap support in the combined analysis). The one exception is *V. clemensiae* from Mt. Kinabalu in northwestern Borneo (Kern 1951; Beaman et al. 2001), which despite its paniculate inflorescences does not appear closely related to the remaining *Solenotinus*. Although its exact placement within *Viburnum* remains uncertain, this result has implications for morphological evolution in the group. Most importantly, the distribution of paniculate inflorescences becomes disjunct on the tree, implying that this character evolved independently in *V. clemensiae* and the *Solenotinus* clade. This assumes that umbel-like inflorescences are ancestral in *Viburnum*, a view supported by comparison to *Sambucus* where such inflorescences are also inferred to be ancestral (Eriksson & Donoghue 1997). Further, the thick, entire leaves cited by Kern (1951) as evidence of a close relationship between *V. clemensiae* and *V. odoratissimum* (which is nested within core *Solenotinus*) would best be interpreted as having evolved independently in response to similar ecological circumstances; both species occur at lower elevations and in more subtropical forests than do most *Viburnum* species.

Relationships Among the Sections. Although some uncertainties remain, we find support for three supra-sectional clades. It appears that much of this resolution is derived from the *trnK* data set, which even when analyzed alone showed support for each of these three clades. In contrast, relationships among the sections were not well resolved in the ITS analyses. Despite their differing levels of resolution, increased bootstrap support for two of the three supra-sectional clades in the combined analyses implies that the nuclear and chloroplast datasets are largely complementary.

The largest clade recovered in all of our analyses contained the *Odontotinus-Oreinotinus-Megalotinus* grouping discussed above, along with sections *Opulus* and *Tinus*. This clade is poorly supported in analyses of the individual datasets (58% and 56% bootstrap support for *trnK* and ITS analyses, respectively) but more strongly supported in the combined analysis (86% bootstrap value). Such a grouping has not previously been recognized, but in retrospect may be diagnosed by several morphological features. These include the presence of two or more pairs of bud scales (except in *V. cylindricum* and other species of *Megalotinus*, which typically have one pair of scales), leaf margins with prominent teeth in most species, and the tendency for the first two secondary veins to be clustered near the base of the leaf blade. Given this last character it may not be surprising that within this clade we find the evolution of strongly tri-nerved leaves (e.g., *V. cinnamomifolium* and *V. davidii* in section *Tinus*) and of tri-lobed leaves (e.g., *V. acerifolium* and *V. kansuense* within Old World *Odontotinus*, and probably independently in section *Opulus*).

Strongly supported in all three analyses (92%, 86%, and 99% bootstrap support in *trnK,* ITS, and combined analyses, respectively) is a clade containing the core of *Solenotinus*, with paniculate inflorescences, and *V. plicatum* of section *Tomentosa*, with umbel-like inflorescences. Despite this difference, we note that members of the *Solenotinus-Tomentosa* clade share characteristically rounded teeth on the leaf margins (Donoghue and Levin 1986; except where teeth are absent, as in *V. odoratissimum*) and may also be united by chromosome number. In contrast to most other viburnums, where $x=9$ ($2n=18$, 27, 36, or 72), all *Solenotinus* species for which chromosome counts are available have $x=8$ ($2n=16$ or 32, or 40 in some individuals of *V. odoratissimum*). Interestingly, for *V. plicatum* there are reputable reports of both $2n=16$ and $2n=18$ (Janaki Ammal 1953; Egolf 1956, 1962). Egolf (1956) documented that $2n=16$ is the predominant chromosome number in *V. plicatum*, and the only one found in most of the varieties and forms of this species. Furthermore, he noted that only $2n=16$ plants produced abundant fruit. On this basis we suspect that the loss of a chromosome pair marks the entire *Solenotinus-Tomentosa* clade, and that a pair of metacentric chromosomes was later added in some *V. plicatum* lineages.

Our *trnK* and combined analyses also indicate strong support (91% and 94%, respectively) for a clade containing the New World section *Lentago* and the predominantly Asian section *Viburnum* (except *V. urceo-*

latum). Furthermore, in the *trnK* analyses there was support for this clade being sister to section *Pseudotinus* (79% bootstrap value). Consistent with its generally limited resolution of higher-level relationships, this result was not obtained in analyses of the ITS data alone. Although the optimal tree from our ML analyses of the combined data set does indicate a direct relationship between the *Lentago-Viburnum* clade and *Pseudotinus*, this clade received less than 50% support in the bootstrap analyses (Fig. 3). This was the only suprasectional relationship to receive less support in combined analyses than in analyses of the *trnK* data alone, indicating a degree of conflict between the chloroplast and ITS data concerning the position of section *Pseudotinus*.

In retrospect, there may be a number of morphological features that also unite sections *Lentago*, *Viburnum* (except *V. urceolatum*), and *Pseudotinus*. First, in all of these groups the inflorescence is substantially preformed at the end of the growing season, and therefore over-winters with the flowers in a well-developed state. This is obvious in species with naked buds, where the well-formed inflorescence, densely covered by stellate hairs, is exposed throughout the winter. Although the bud scales in section *Lentago* obscure this feature, the well-developed inflorescence is evident as a distinct bulge within the bud (Donoghue 1981). Second, the vernation of the foliage leaves within the resting buds (ptyxis) is similar in these three sections. In these groups the young leaf blades are involute and nonoverlapping where they come into contact. In contrast, elsewhere in *Viburnum* (as in section *Odontotinus* and related lineages) the young leaves are conduplicate and often plicate, with the blades variously overlapping one another in bud. This contrast was carefully documented by Cross (1937, 1938) in his studies of *V. rufidulum* (section *Lentago*) and *V. opulus* (section *Opulus*); additional studies are needed to critically evaluate the distribution of this feature within and among the major *Viburnum* lineages. Third, members of these three sections may share a peculiar pollen exine morphology (Donoghue 1985). In most viburnums the exine is semitectate and reticulate, but in sections *Viburnum* and *Lentago* it is intectate and more or less regularly retipilate to pilate, and the pilae are scabrate (pollen type IC of Donoghue 1985). Previously, this was interpreted as convergence (supported in part by a difference in grain shape; Donoghue 1985), but our molecular results suggest that these traits may instead be homologous. Interestingly, a similar condition is seen in *V. cordifolium* of section *Pseudotinus*, although in this species the pilae do not appear to be scabrate. Finally, it seems likely that the multicellular, peltate scales found on the leaves and elsewhere in section *Lentago* were derived from the stellate trichomes characteristic of species with naked buds.

Within this clade the sister group relationship between sections *Viburnum* and *Lentago*, as opposed to one between *Viburnum* and *Pseudotinus*, is surprising from the standpoint of the evolution of naked buds. Most viburnums have two or more pairs of bud scales. All members of sections *Viburnum* and *Pseudotinus* (as well as *V. urceolatum*, whose relationships remain unclear; see above) have naked buds and in section *Lentago* only a single pair of bud scales is produced. Therefore, the relationships inferred among these sections imply homoplasy in this character. That is, within this clade naked buds either evolved twice independently, or they evolved only once followed by the re-evolution of bud scales in section *Lentago*. The re-evolution of bud scales is not an altogether unlikely scenario when differences in bud scale development are considered. Cross (1937, 1938) carried out detailed studies of bud scale development in *V. opulus* and *V. rufidulum*. *Viburnum opulus* probably exemplifies the standard situation in *Viburnum*, where two (or more) pairs of bud scales are produced, and commitment to a distinct cataphyll developmental pathway is evident at a very early stage in bud scale ontogeny (Cross 1938). In marked contrast, the development of bud scales in *V. rufidulum* resembles that of normal foliage leaves, the main difference being the timing of growth in different zones. Specifically, the bud scale appears directly comparable to the petiole of a leaf; growth of the blade portion is suppressed during scale development but it often expands somewhat when growth resumes in the spring (Cross 1937).

Rooting the* Viburnum *Phylogeny. The exact position of the root within *Viburnum* remains unresolved in these analyses. However, it seems unlikely that the root lies within any of the sections or supra-sectional clades we have identified, but instead falls somewhere between them. One very intriguing possibility is that the root lies between *V. clemensiae* (traditionally of section *Solenotinus*, but see above) and the remainder of *Viburnum*. This placement received 57% bootstrap support in our *trnK* analyses (Fig. 1), and was also recovered in the optimal ML topology for the combined data set (Fig. 3; although bootstrap support was less than 50%). Additional data are needed to evaluate this and other possible placements of the root.

The substantial divergence between *Viburnum* and its nearest relatives presents a continuing problem. This is particularly obvious in our ITS alignments, but large numbers of nucleotide substitutions are inferred between *Sambucus* and *Viburnum* in all of our analyses. A distant relationship between *Viburnum* and the remainder of the Adoxaceae is also evident in broader analyses of Adoxaceae (Eriksson and Donoghue 1997) and the Dipsacales (e.g., Donoghue et al. 2001; Bell et al. 2001). Our ongoing studies of the nuclear locus *waxy* (Winkworth and Donoghue 2002; unpubl. data),

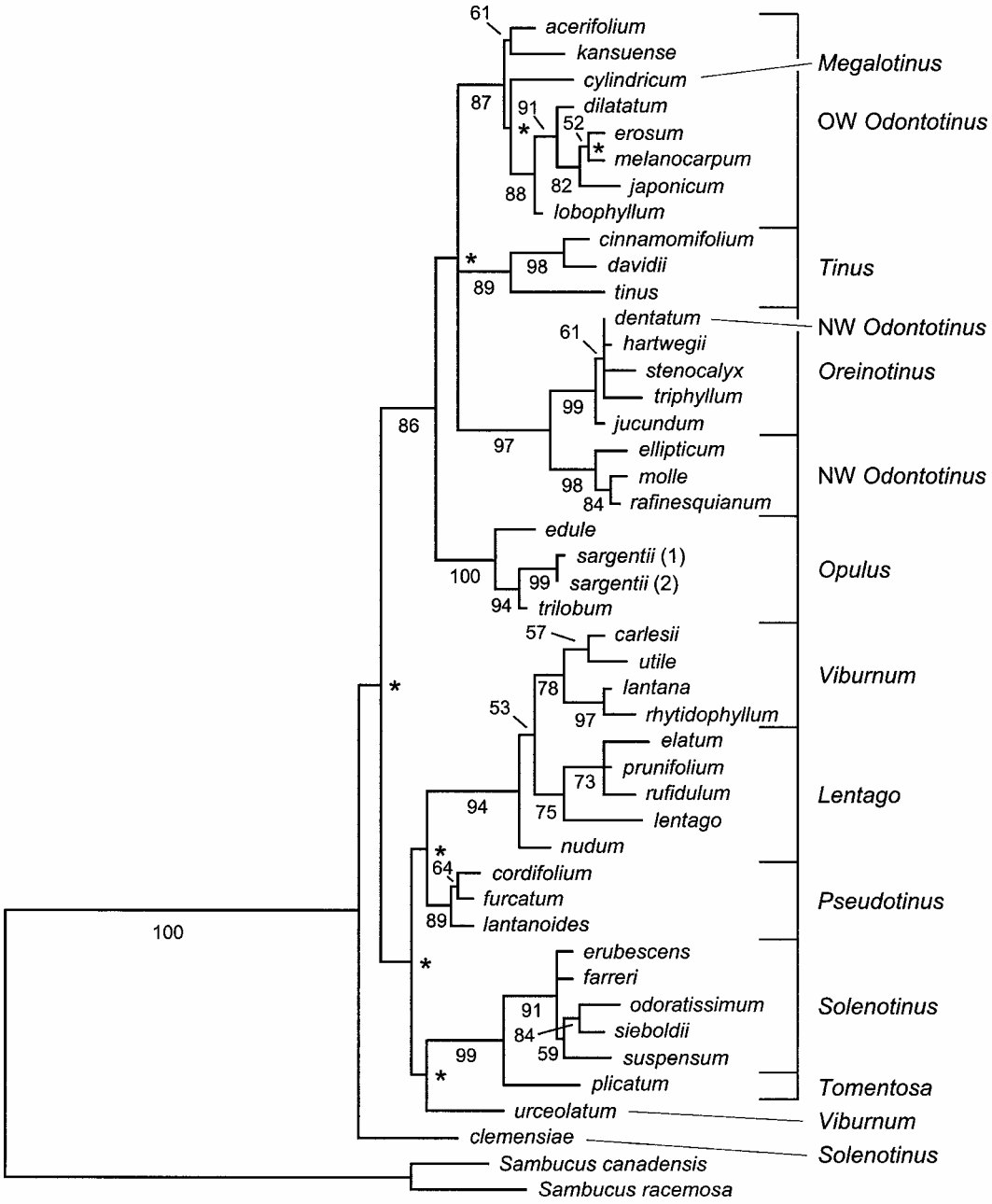

FIG. 3. Single optimal maximum likelihood tree obtained from the combined data set, showing bootstrap values over 50% (1000 replicates). Nodes marked with asterisks collapse in the majority rule consensus of the maximum parsimony bootstrap trees. Names to the right refer to traditionally recognized sections. Names of the traditional sections of *Viburnum* are given on the right.

which we have found to be duplicated in *Viburnum* and its relatives, may ultimately provide an alternative means of establishing the position of the root (cf. Sang et al. 1997; Mathews and Donoghue 2000).

Relationships within Sections. In addition to clarifying phylogenetic relationships among major clades, our results provide some resolution within these groups. At this level the phylogenetic signal stems primarily from the ITS data set. However, the data sets appear to complement one another, as the inferred relationships often show increased bootstrap support in the combined analyses. Here we briefly highlight the most strongly supported and evolutionarily significant of these results.

Within section *Opulus* the ITS and combined analyses indicate that the North American *V. edule*, which lacks sterile marginal flowers, is sister to a strongly supported clade consisting of *V. trilobum* and *V. sargentii* (and presumably also the European *V. opulus*), all of which produce sterile marginal flowers. Within the latter clade our data separate the North American *V. trilobum* from two accessions of the widespread Asian species *V. sargentii*. Within section *Pseudotinus* the evolution of sterile marginal flowers may be more complicated. Although the relationships among *V. furcatum* and *V. lantanoides*, both with sterile marginal flowers, and *V. cordifolium*, which lacks such flowers, are not well resolved by *trnK* or ITS data alone, our combined analyses seem to support a direct link between *V. furcatum* and *V. cordifolium* (64% bootstrap value). Taken at face value this implies that either sterile flowers have evolved twice, or they originated once and were then lost in *V. cordifolium*. Geographically this result unites two Asian species (the Japanese *V. furcatum* and Himalayan *V. cordifolium*) to the exclusion of *V. lantanoides* from eastern North America.

Several other results are of biogeographic interest. *Viburnum lantana*, the sole European representative of the otherwise Asian section *Viburnum*, is closely associated with the Asian *V. rhytidophyllum* (97% bootstrap support in combined ML analyses). We note that these two species can be readily hybridized, and were the parents of a widely planted horticultural form, *V.* × *rhytidophylloides* (Egolf 1956). In any case, this result is consistent with an Asian radiation of section *Viburnum* and its subsequent spread to Europe. In contrast, *V. tinus*, the sole European member of section *Tinus*, is sister to a pair of Asian species, *V. cinnamomifolium* and *V. davidii*. However, in this case a proper test of the geographic origin of the group awaits the addition of other Asian species, such as *V. propinquum*, which are morphologically more similar to *V. tinus*. Within the New World *Odontotinus* clade, we find support for the *V. rafinesquianum* species complex, which includes *V. rafinesquianum*, *V. ellipticum*, and *V. molle* (Donoghue 1982). *Viburnum ellipticum*, the only species native to the west coast of North America (extending south into Northern California), appears to be the sister group of the two eastern North American species.

As noted above, the Old World and New World *Odontotinus* clades generally differ with respect to fruit color—Old World species being predominantly red-fruited and New World species purple-fruited. Within the Old World *Odontotinus* clade, however, several results suggest the independent evolution of purple fruits. The position of the purple-fruited *V. acerifolium*, linked in our combined analyses at the base of this clade with the red-fruited *V. kansuense*, implies an independent origin of purple fruits. This is especially interesting since *V. acerifolium* is a New World species. A second fruit color shift is implied by the position of *V. melanocarpum*, which, as the name implies, also has dark purple fruits. Additional shifts may be required, depending on the final placement of *V. cylindricum* and other *Megalotinus* species, which also generally produce purple fruits at maturity.

The only conflicting relationships suggested by our chloroplast and nuclear data sets are within section *Lentago*. In ITS and combined analyses *V. prunifolium*, *V. rufidulum*, and the only Mexican representative of this otherwise eastern North American group, *V. elatum*, formed a well supported clade (86% and 73% bootstrap support in ITS and combined analyses, respectively). In contrast, *trnK* analyses unite the three eastern North American species—*V. lentago*, *V. prunifolium*, and *V. rufidulum*—to the exclusion of *V. elatum* (63% bootstrap support). Furthermore, the *trnK* analyses suggest a sister group relationship between *V. lentago* and *V. prunifolium*, with 64% bootstrap support. The different relationships inferred from these two data sets may support the hypothesis that *V. prunifolium* originated following hybridization between *V. rufidulum* and *V. lentago* (Brumbaugh and Guard 1956; Rader 1976). Several other observations are compatible with a hybridization scenario. The distribution of *V. prunifolium* lies between those of its putative parents (although there is significant overlap in both directions). Specifically, *V. prunifolium* is distributed across the middle of the eastern United States, with the range of *V. lentago* extending further to the north and that of *V. rufidulum* extending further south. Furthermore, successful hybrids are readily obtained between *V. lentago* and *V. prunifolium* (Egolf 1956); indeed, this is the presumed origin of the horticultural form *V.* × *jackii* (Rehder 1920). Attempts to hybridize these species in other combinations have not been reported, but we assume that these would also be successful. Although introgression in the wild has been suggested (Brumbaugh and Guard 1956), we note that natural hybridization may be limited by differences in flowering times (Rader 1976; Donoghue 1980). In addition, since neither of the DNA sequences obtained for *V. prunifolium* is identical to the corresponding sequence in the putative parents, any such hybridization must either have predated sequence divergence or unsampled variation exists within the parental species. The possibility of homoploid hybrid speciation in section *Lentago* warrants further investigation.

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APPENDIX 1. Accession details and GenBank numbers for sampled *Viburnum* (traditionally recognized section in parentheses) and outgroup species. All Donoghue & Winkworth (D & W) collections are deposited at Yale University (YU) and the Arnold Arboretum at Harvard University (A); locations of remaining voucher specimens are as noted. A generalized geographic range is given in parentheses for specimens obtained from cultivated plants.

V. acerifolium L. (sect. *Odontotinus*)—Cult. Arn. Arb. 1505-67 (Eastern North America), *D & W 27* (ITS AY265114, *trnK* AY265160). ***V. carlesii*** Hemsley (sect. *Viburnum*)—Cult. Arn. Arb. 17981-2-A (Japan, Korea), *D & W 24* (ITS AY265115, *trnK* AY265161). ***V. cinnamomifolium*** Rehder (sect. *Tinus*)—Cult. Univ. of Washington Arb. 1392-56 (China), *R. Olmstead 2002-120 (WTU)* (ITS AY265116, *trnK* AY265162). ***V. clemensiae*** Kern (sect. *Solenotinus*)—Mt. Kinabalu, Malaysia, *J. Beaman 11781 (K)* (ITS AY265117, *trnK* AY265163). ***V. cordifolium*** Wallich ex DC. (sect. *Pseudotinus*)—Sichuan Province, China, *D. Boufford et al. 27388 (A)* (ITS AY265118, *trnK* AY265164). ***V. cylindricum*** Ham. ex D. Don (sect. *Megalotinus*)—Yunnan Province, China, *D. Boufford et al. 29342 (A)* (ITS AY265119, *trnK* AY265165). ***V. davidii*** Franchet (sect. *Tinus*)—Cult. vic. Corvallis, OR, USA (China), *Donoghue, voucher lacking* (ITS AY265120, *trnK* AY265166). ***V. dentatum*** L. (sect. *Odontotinus*)—Cult. Arn. Arb. 5070-1-A (Eastern North America), *D & W 33* (ITS AY265121, *trnK* AY265167). ***V. dilatatum*** Thunb. (sect. *Odontotinus*)—Cult. Arn. Arb. 138-52-A (China, Japan, Korea), *D & W 19* (ITS AY265122, *trnK* AY265168). ***V. edule*** (Michaux) Raf. (sect. *Opulus*)—Northern Wisconsin, USA, *W. Alverson, voucher lacking* (ITS AY265123, *trnK* AY265169). ***V. elatum*** Bentham (sect. *Lentago*)—Chiapas, Mexico, *Donoghue 472 (A, YU)* (ITS AY265124, *trnK* AY265170). ***V. ellipticum*** Hooker (sect. *Odontotinus*)—vic. Corvallis, OR, USA, *Donoghue, voucher lacking* (ITS AY265125, *trnK* AY265171). ***V. erosum*** Thunb. (sect. *Odontotinus*)—Cult. Arn. Arb. 78-90-A (China, Japan, Korea), *D & W 16* (ITS AY265126, *trnK* AY265172). ***V. erubescens*** Wallich ex DC. (sect. *Solenotinus*)—Sichuan Province, China, *D. Boufford et al. 27190 (A)* (ITS AY265127, *trnK* AY265173). ***V. farreri*** Stearn (sect. *Solenotinus*)—Cult. Arn. Arb. 656-89-A (China), *D & W 18* (ITS AY265128, *trnK* AY265174). ***V. furcatum*** Blume (sect. *Pseudotinus*)—Kyoto Prefecture, Japan, *Tsugaru & Takahashi 19958 (MO)* (ITS AY265129, *trnK* AY265175). ***V. hartwegii*** Bentham (sect. *Oreinotinus*)—Chiapas, Mexico, *Donoghue 486 (A, YU)* (ITS AY265130, *trnK* AY265176). ***V. japonicum*** (Thunb.) Sprengel (sect. *Odontotinus*)—Cult. US. Nat. Arb. 56545 (China), *Donoghue, voucher lacking* (ITS AY265131, *trnK* AY265177). ***V. jucundum*** Morton (sect. *Oreinotinus*)—Chiapas, Mexico, *Donoghue 244 (A, YU)* (ITS AY265132, *trnK* AY265178). ***V. kansuense*** Batalin (sect. *Odontotinus*)—Sichuan Province, China, *D. Boufford et al. 27416 (A)* (ITS AY265133, *trnK* AY265179). ***V. lantana*** L. (sect. *Viburnum*)—Cult. Arn. Arb. 1089-60-A (Europe), *D & W 26* (ITS AY265134, *trnK* AY265180). ***V. lantanoides*** Michaux (sect. *Pseudotinus*)—Cult. Arn. Arb. 155-97-B (Eastern North America), *D & W 2* (ITS AY265135, *trnK* AY265181). ***V. lentago*** L. (sect. *Lentago*)—Cult. Arn. Arb. 18021-A (Eastern North America), *D & W 21* (ITS AY265136, *trnK* AY265182). ***V. lobophyllum*** Graebn. (sect. *Odontotinus*)—Cult. Arn. Arb. 1875-80-A (China), *D & W 25* (ITS AY265137, *trnK* AY265183). ***V. melanocarpum*** Hsu in Chen et al. (sect. *Odontotinus*)—Cult. Arn. Arb. 386-81-D (China), *D & W 12* (ITS AY265138, *trnK* AY265184). ***V. molle*** Michaux (sect. *Odontotinus*)—Cult. Arn. Arb. 18294-A (Eastern North America), *D & W 5* (ITS AY265139, *trnK* AY265185). ***V. mudum*** L. (sect. *Lentago*)—Cult. US. Nat. Arb. 32198-C (Eastern North America), *Donoghue, voucher lacking* (ITS AY265140, *trnK* AY265186). ***V. odoratissimum*** Ker Gawler (sect. *Solenotinus*)—Cult. Univ. of Washington Arb. 1404-56 (Japan, Korea, Taiwan, Philippines), *R. Olmstead 2002-118 (WTU)* (ITS AY265141, *trnK* AY265187). ***V. plicatum*** var. ***tomentosum*** Thunb.(Miquel) (sect. *Tomentosa*)—Cult. Arn. Arb. 72-98-A (China, Japan, Taiwan), *D & W 10* (ITS AY265143, *trnK* AY265189). ***V. prunifolium*** L. (sect. *Lentago*)—Cult. Arn. Arb. 22586-A (Eastern North America), *D & W 13* (ITS AY265144, *trnK* AY265190). ***V. rafinesquianum*** Schultes (sect. *Odontotinus*)—Cult. Arn. Arb. 17974-A (Eastern North America), *D & W 4* (ITS AY265145, *trnK* AY265191). ***V. rhytidophyllum*** Hemsley (sect. *Viburnum*)—Cult. Arn. Arb. 133-67-A (China), *D & W 8* (ITS AY265146, *trnK* AY265192). ***V. rufidulum*** Raf. (sect. *Lentago*)—Cult. Arn. Arb. 21418-A (Eastern North America), *D & W 14* (ITS AY265147, *trnK* AY265193). ***V. sargentii*** Koehne (sect. *Opulus*)—**1**: Cult. Arn. Arb. 398-68-B (China, Japan, Korea, Siberia), *D & W 17* (ITS AY265148, *trnK* AY265194); **2**: Cult. Arn. Arb. 719-88-C, *D & W 32* (ITS AY265142, *trnK* AY265188). ***V. sieboldii*** Miq. (sect. *Solenotinus*)—Cult. Arn. Arb. 616-6-A (Japan), *D & W 3* (ITS AY265149, *trnK* AY265195). ***V. stenocalyx*** (Oersted) Hemsley (sect. *Oreinotinus*)—DF, Mexico, *Donoghue 127 (A, YU)* (ITS AY265150, *trnK* AY265196). ***V. suspensum*** Lindley (sect. *Solenotinus*)—Cult. Santa Barbara, CA (Japan), *D & W 36* (ITS AY265151, *trnK* AY265197). ***V. tinus*** L. (sect. *Tinus*)—Cult. Santa Barbara, CA (Europe), *D & W 35* (ITS AY265152, *trnK* AY265198). ***V. trilobum*** Marshall (sect. *Opulus*)—Cult. Arn. Arb. 724-68-E (North America), *D & W 22* (ITS AY265153, *trnK* AY265199). ***V. triphyllum*** Benth. (sect. *Oreinotinus*)—Loja, Ecuador, *C. Bell EC-026 (YU)* (ITS AY265154, *trnK* AY265200). ***V. urceolatum*** Sieb. & Zucc. (sect. *Viburnum*)—Cult. US. Nat. Arb. 40200 (China, Japan, Taiwan), *Donoghue, voucher lacking* (ITS AY265155, *trnK* AY265201). ***V. utile*** Hemsley (sect. *Viburnum*)—Cult. US. Nat. Arb. (China), *Egolf 2336-E* (ITS AY265156, *trnK* AY265202).

Outgroups

Sambucus canadensis L.—Cult. Marsh Bot. Gard., Yale Univ. (North America, Mexico, Central America), *D & W 37* (ITS AY265157, *trnK* AY265203). ***Sambucus racemosa*** L.—vic. Stockholm, Sweden (circumboreal), *T. Eriksson s.n. (S)* (ITS AY265158, *trnK* AY265204).