Phylogenetic analysis of *Viburnum* (Adoxaceae) in Korea using DNA sequences

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ABSTRACT: The nucleotide sequences of the chloroplast *rbcL*, *matK*, and *psbA-trnH* and nuclear internal transcribed spacer (ITS) regions were determined from all species of *Viburnum* in Korea with multiple accessions to reconstruct the phylogeny and to evaluate the utility of the DNA sequences as DNA barcodes. The results of phylogenetic analyses of the cpDNA and ITS data are consistent with the findings of previous studies of *Viburnum*. Four morphologically closely related species, *V. dilatatum*, *V. erosum*, *V. japonicum*, and *V. wrightii*, were included in a strongly supported sister clade of *V. koreanum* and *V. opulus*. *Viburnum odoratissimum* is suggested to be sister to the *V. dilatatum/V. koreanum* clade in the cpDNA data, while *V. odoratissimum* is a sister to *V. furcatum* in the ITS data. *Viburnum burejaeticum* and *V. carlesii* are strongly supported as monophyletic. Our analyses of DNA barcode regions from multiple accessions of the species of *Viburnum* in Korea confirm that six out of ten species in Korea can be discriminated at the species level. The *V. dilatatum* complex can be separated from the remaining species according to molecular data, but the resolution power to differentiate a species within the complex is weak. This study suggests that regional DNA barcodes are useful for molecular species identification in the case of *Viburnum* when flowering or fruiting materials are not available.

Keywords: DNA barcode, morphology, relationship, species identification, *Viburnum*

The genus *Viburnum* L. consists of 150–200 species of small trees and shrubs widely distributed in temperate and subtropical regions of Asia, Europe, North Africa, and the Americas with high species diversity in eastern Asia and Latin America (Rehder, 1908; Donoghue, 1983; Hara, 1983; Donoghue et al., 2004). *Viburnum* is characterized within Adoxaceae by having simple leaves and single-stoned drupes (Donoghue et al., 2003). Plants of *Viburnum* have been widely utilized as ornamentals due to their showy flowers and fragrantcy, such as *V. opulus* L., and *V. carlesii* Hemsl. and as a medicine source, such as *V. tinus* L. and *V. erosum* Thunb. (Mabberley, 1997; Ahn, 1998; Kwon et al., 2003).

The phylogeny of *Viburnum* has been extensively studied using various DNA regions in both nuclear and chloroplast genomes (Donoghue et al., 2004; Winkworth and Donoghue 2004, 2005; Clement and Donoghue 2011, 2012; Clement et al., 2014). These studies have improved our understanding of

the phylogeny, the evolution of morphological characters and the biogeography of *Viburnum*. Most traditionally recognized sections within *Viburnum* (Hara, 1983) were supported as monophyletic (Donoghue et al., 2004; Winkworth and Donoghue 2004, 2005; Clement and Donoghue 2011, 2012; Clement et al., 2014). Clement et al. (2014) proposed a phylogenetic classification (Cantino et al., 2007) based on strongly supported and congruent clades among different data sets of chloroplast DNA.

In Korea, ten species of *Viburnum* belonging to five sections (Hara, 1983) or clades (Clement et al., 2014) are distributed (Hong and Im, 2003; Kim, 2007) (Table 1). Previous phylogenetic studies using chloroplast DNA regions attempted to resolve higher level relationships in *Viburnum* and included most of the species, but only one accession per species was analyzed (Clement and Donoghue 2012; Clement et al., 2014). Phylogenetic studies using nuclear genomes, such as internal

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Table 1. List of species included in this study with higher classification categories and information of the sources of materials. Sectional classification of *Viburnum* follows Hara (1983) and clade name Clement et al. (2014). CB, Chungcheongbuk-do; GB, Gyeongsangbuk-do; GG, Gyeonggi-do; GW, Gangwon-do; JB, Jeollabuk-do; JJ, Jeju-do; JN, Jeollanam-do.

Species	Sect.	Clade	Locality and accession number
<i>V. burejaeticum</i> Regel & Herder	<i>Viburnum</i>	<i>Euviburnum</i>	GW, Pyeongchang-gun, 7129 GW, Pyeongchang-gun, 7194 GW, Pyeongchang-gun, 356575 China, Jilin, Antu, 289365
<i>V. carlesii</i> Hemsl.	<i>Viburnum</i>	<i>Euviburnum</i>	GW, Hongcheon-gun, 277454 GW, Yanggu-gun, 154489 GG, Incheon-si, Ongjin-gun, 110788 GB, Andong-si, 121542 GB, Ulleungdo Island, 7274
<i>V. dilatatum</i> Thunb.	<i>Odontotinus</i>	<i>Succotinus</i>	GB, Seongju-gun, 445136 JB, Wanju-gun, 7214 JN, Yeongam-gun, 448592 JJ, Jeju-si, Gujwa-eup, 512282 Japan, Honshu, Aomori Prefecture, J9050 Japan, Kyushu, Saga Prefecture, Kyushu-003
<i>V. erosum</i> Thunb. var. <i>erosum</i>	<i>Odontotinus</i>	<i>Succotinus</i>	GB, Gunsan-si, 7219 GB, Namwon-si, 278061 GB, Pohang-si, 277686 JB, Imsil-gun, 127413 JN, Hongdo Island, 7224 JJ, Seogwipo-si, Namwon-eup, 257106
<i>V. furcatum</i> Blume ex Maxim.	<i>Pseudotinus</i>	<i>Pseudotinus</i>	JJ, Jeju-si, Aewol-eup, 291710 JJ, Jeju-si, Aewol-eup, 329347 JJ, Jeju-si, Aewol-eup, 386202 JJ, Seogwipo-si, Hawon-dong, 7203 Japan, Hokkaido, Sapporo Prefecture, 7464
<i>V. japonicum</i> (Thunb.) C. K. Spreng.	<i>Odontotinus</i>	<i>Succotinus</i>	JN, Gageodo Island, 7216 JN, Gageodo Island, 289119 JN, Gageodo Island, 337388 JN, Gageodo Island, 473764 Japan, Kyushu, Nagasaki, Kyushu-043
<i>V. koreanum</i> Nakai	<i>Opulus</i>	<i>Opulus</i>	GW, Inje-gun, 7294
<i>V. odoratissimum</i> Ker Gawl. var. <i>awabuki</i> (K. Koch) Zabel ex Rümpler	<i>Solenotinus</i>	<i>Solenotinus</i>	JJ, Jeju-si, Gujwa-eup, 393880 JJ, Seogwipo-si, Andeok-myeon, 434902 JJ, Seogwipo-si, Jungmun-dong, 7317 JJ, Seogwipo-si, Namwon-eup, 418149 JJ, Seogwipo-si, Seongsan-eup, 7321
<i>V. opulus</i> L. var. <i>calvescens</i> (Rehder) H. Hara	<i>Opulus</i>	<i>Opulus</i>	GW, Cheorwon-gun, 7275 GW, Inje-gun, 252268 GW, Yanggu-gun, 154759 GW, Yeongwol-gun, 235185 GG, Incheon-si, 7273 GG, Yeoncheon-gun, 7276 CB, Danyang-gun, 253062
<i>V. wrightii</i> Miq.	<i>Odontotinus</i>	<i>Succotinus</i>	GW, Hoengseong-gun, 198135 GW, Inge-gun, 163578 GW, Inge-gun, 458890 GW, Pyeongchang-gun, 197686 GB, Ulleungdo Island, 293906 JB, Jeongeup-si, J600415 JJ, Jeju-si, Aewol-eup, J50374 JJ, Jeju-si, Gujwa-eup, 7539

transcribed spacer (ITS) regions (Donoghue et al., 2004) and *GBSSI* (Winkworth and Donoghue 2004), included some of the species, though the phylogenetic relationships among the species, especially those in the same clade, have remained unclear. In particular, *V. burejaeticum* Regel & Herder, *V. wrightii* Miq., and *V. koreanum* Nakai were not included in the phylogenetic analyses using nuclear DNA regions (Donoghue et al., 2004; Winkworth and Donoghue 2004).

The utility of DNA barcoding is well established in variety of plant taxa (Hebert et al., 2003; Kress et al., 2005; Lahaye et al., 2008; CBOL Plant Working Group, 2009; Xiang et al., 2011; Kim et al., 2014). The performance of DNA barcoding in *Viburnum* with *rbcL*, *matK*, *rpl32-trnL*, *psbA-trnH*, *trnK*, and ITS regions from 112 species showed very low levels of species resolution among closely related species. The establishment of a regional barcode database with multiple accessions encompassing the distributional range in Korea and an assessment of the levels of variation in *Viburnum* are necessary to make the molecular species identification tool useful (Kim et al., 2014; Youm et al., 2016).

This study aims to determine the DNA sequences of *rbcL*, *matK*, *psbA-trnH*, and ITS regions from multiple accessions of all species of *Viburnum* in Korea to assess the level of differentiation of the species and to evaluate the resolution power of DNA barcodes to identify a species. In addition, we provide a phylogeny of *Viburnum* with Korean species, onto which some important morphological characters are mapped.

Materials and Methods

Taxon and gene sampling

Ten taxa distributed in Korea were included in this study (Table 1, Appendix 1). *Viburnum erosum* var. *taquetii* (H. Lév) Rehder was not included due to a lack of appropriate samples. Taxonomic identity of this taxon should be evaluated further, as a diagnostic character of *V. erosum* var. *taquetii* appears to be plastic. Four to eight accessions per taxon were analyzed, except for *V. koreanum*, whose distribution is limited at the high elevation of Mt. Seoraksan in Korea (Table 1). *Viburnum clemensiae* Kern was included as an outgroup based on previous studies (Winkworth and Donoghue, 2004; Clement and Donoghue, 2012; Clement et al., 2014).

Dried leaves of herbarium specimens or fresh leaves collected in the wild were used to isolate total DNA with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Three cpDNA regions, in this case the *rbcL*, *matK*, and *psbA-trnH* regions and nuclear ribosomal ITS regions were amplified via polymerase chain reactions (PCR). Primers for the cpDNA

regions from previous studies were used (Soltis et al., 1992; Sang et al., 1997; Cuénoud et al., 2002; Kress and Erickson, 2007; Kim et al., 2014). The ITS regions were amplified using the primers *its6* and *its9* (Youm et al., 2016). For each PCR reaction, 1 µL of total DNA was included in a 20 µL reaction mixture with Solg EF-Taq DNA polymerase (Solgent, Daejeon, Korea). Amplification of the target regions was conducted with a Veriti thermal cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95°C for 2 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; followed by a final extension at 72°C for 5 min. PCR products were examined on a 1% agarose gel in 1× TBE buffer, after which they were purified and sent to Solgent or Bionics (Seoul, Korea) for sequencing. Sequencing reaction used the PCR primers with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

For certain accessions of *V. erosum*, *V. japonicum* C. K. Spreng., and *V. wrightii*, direct sequencing results produced stuttering of the base readings and revealed the presence of multiple superimposed peaks. PCR products of those accessions were cloned using the T-Blunt PCR cloning kit (Solgent). Potentially transformed white colonies were incubated in Terrific Broth (Sambrook et al., 1989) at 37°C overnight, and plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). Two to four transformed clones per accession were sequenced with M13R and T7 primers. Sequences were determined using a 3730xl DNA analyzer (Applied Biosystems). Sequences were edited in Sequencher version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA), aligned manually.

Data analysis

Multiple sequences were aligned using MUSCLE (Edgar, 2004) and were adjusted manually as needed. The three chloroplast regions were concatenated and analyzed simultaneously. The ITS data were analyzed separately and in combination with the cpDNA data. With regard to the accessions for which ITS sequences were determined by cloning, one sequence per accession was randomly selected and included in the combined data. *Viburnum clemensiae* was used as an outgroup. Phylogenetic analyses were conducted using the maximum parsimony (MP) criterion in PAUP* (Swofford, 2002) and Bayesian method (Huelsenbeck and Ronquist, 2001). All characters were treated as unordered and were weighted equally in the MP analyses. Gaps resulting from multiple alignments of indels were treated as missing. Heuristic searches were used with 100 replicates of random sequence additions with tree bisection-reconnection branch

swapping, with all of the best trees saved at each step (MulTrees). Bootstrap analyses (Felsenstein, 1985) of 500 pseudoreplicates were conducted for cpDNA and the combined data with heuristic searches and a simple sequence addition in PAUP* to evaluate the support for each clade. “Fast bootstrap” with 50,000 pseudoreplicates was used for the ITS data.

Bayesian phylogenetic analyses were performed with the program MrBayes version 3.2.5 (Huelsenbeck and Ronquist, 2001). A Markov chain Monte Carlo algorithm was employed for 1,000,000 generations, sampling trees every 100 generations, with four independent chains running simultaneously. Priors were set based on the Akaike information criterion using Modeltest (Posada and Crandall, 1998). The first 2,000 trees (200,000 generations) were discarded as “burn-in,” and the remaining trees for which the log-likelihood values had reached a plateau were imported in PAUP* to calculate the posterior probability of each clade.

We examined key morphological characteristics using the materials collected in field and herbarium specimens deposited in KB (the herbarium of the National Institute of Biological Resources). Voucher specimens for the cross-section of stone to investigate presence of grooves of the stone surfaces and their numbers are provided in Appendix 2.

Results

The lengths of the *rbcL*, *matK*, and *psbA-trnH* regions were 565, 814, and 383–505, respectively. The final alignment of the cpDNA data includes 1,884 sites, among which 51 were parsimoniously informative (2.7%). The ITS data consist of 636 sites, of which 85 were parsimoniously informative (13.4%). The alignment of the combined data of the Korean *Viburnum* included 51 sequences with 2,517 sites, among which 134 were parsimoniously informative (5.3%). The

summary of data and MP analysis were provided in Table 2. Best-fitting model for molecular evolution was selected as the TIMeF model with equal rate for all three data sets.

Phylogenetic analysis of the cpDNA data resolved well-supported clades of species (Fig. 1). Four accessions of *V. burejaeticum* were supported as monophyletic, and those of *V. carlesii* Hemsl. formed a clade with *V. burejaeticum*. Monophyly of *V. carlesii* was not supported in the cpDNA data. Very low levels of variation among *V. dilatatum* Thunb., *V. erosum*, *V. japonicum*, and *V. wrightii* (hereafter the *V. dilatatum* complex) were found. The cpDNA data failed to differentiate the species in the *V. dilatatum* complex (Fig. 1). Most accessions of *V. erosum* and *V. dilatatum*, three of nine accessions of *V. wrightii*, and all accessions of *V. japonicum* showed the same sequences. Five accessions of *V. wrightii* and one of *V. dilatatum* were resolved as a sister to the remaining accessions of the species complex. DNA sequences from plants collected in Japan (*V. japonicum* Kyushu 043 and *V. wrightii* Kyushu 003) were identical to those plants in Korea. *Viburnum koreanum* was supported as a sister to *V. opulus* var. *calvescens* (Rehder) H. Hara. Accessions of *V. odoratissimum* var. *awabuki* (K. Koch) Zabel ex Rümpler and *V. furcatum* formed separate clades supporting the monophyly of each species (Fig. 1). *Viburnum odoratissimum* var. *awabuki* was a sister to the clade of the *V. dilatatum* complex and *V. koreanum* + *V. opulus* (Fig. 1). These species were resolved as a sister clade to the *V. burejaeticum* and *V. carlesii* clade. In our analysis of the cpDNA data, *V. furcatum* was supported as a sister to all of the Korean species (Fig. 1). One accession of *V. furcatum* collected in Hokkaido, Japan was differentiated from the Korean accessions.

The ITS data also showed similar patterns in the cpDNA data (Fig. 2). Noteworthy differences were that *V. furcatum* was a sister to *V. odoratissimum* var. *awabuki*, albeit low support values, and that the accessions of *V. carlesii* were strongly supported as monophyletic. All sequences of *V.*

Table 2. Summary statistics of cpDNA, ITS, and combined data as used in our phylogenetic study of *Viburnum*.

	cpDNA	ITS	Combined
Number of sequences	53	63	51
Number of sites	1,884	636	2,517
Number of variable sites	81	115	185
Number of parsimony-informative sites	51	85	134
Number of MP trees	4	2,075	2,231
Tree length	90	187	272
Consistency index (CI), excluding autapomorphy	0.8833	0.6115	0.7333
Retention index (RI)	0.9843	0.9398	0.9424

MP, maximum parsimony; ITS, internal transcribed spacer.

Fig. 1. One of four maximum parsimony trees from a phylogenetic analysis of the concatenated data of *rbcL*, *matK*, and *psbA-trnH* regions from *Viburnum* in Korea. Bootstrap values greater than 50% are indicated above the corresponding branch and posterior probability in Bayesian analysis was below. The accession number follows the species name to distinguish each individual. *Viburnum clemensiae* was used as an outgroup.

Fig. 2. One of 2,075 maximum parsimony trees from a phylogenetic analysis of the internal transcribed spacer regions from *Viburnum* in Korea. Dotted lines represent branches collapsed in the strict consensus tree. Bootstrap values greater than 50% are indicated above the corresponding branch and posterior probability in Bayesian analysis was below. Accession numbers are indicated following the species name to distinguish each individual. In some accessions of *V. erosum* and *V. japonicum*, the sequences were determined by cloning. The clone number follows the accession number. *Viburnum clemensiae* was used as an outgroup.

Fig. 3. Strict consensus tree of 2,231 maximum parsimony trees from a phylogenetic analysis of the combined cpDNA and internal transcribed spacer (ITS) data from *Viburnum* in Korea. Bootstrap values greater than 50% are indicated above the corresponding branch and posterior probability in Bayesian analysis was below. The accession number follows the species name to distinguish each individual. For cloned ITS sequences, one randomly selected clone per accession was included in the combined data. *Viburnum clemensiae* was used as an outgroup.

japonicum except for accession 7216 formed a cohesive unit, a sister to some of the accessions of *V. erosum* (Fig. 2). A phylogenetic analysis of the combined data was consistent with the ITS tree (Fig. 3).

The species resolution power, by which each species was differentiated in the phylogenetic tree, was calculated and found to be 60% in separate analyses of the cpDNA and ITS data and in the combined analysis. *Viburnum burejaeticum*, *V. carlesii*, *V. koreanum*, *V. opulus* var. *calvescens*, *V. odoratissimum* var. *awabuki*, and *V. furcatum* were distinguished by any of the sequence data. However, the DNA barcode sequences did not differentiate the four species of the *V. dilatatum* complex.

Discussion

The results of our phylogenetic analysis of three chloroplast regions (Fig. 1) are consistent with the findings of previous studies (Donoghue et al., 2004; Winkworth and Donoghue 2004, 2005; Clement and Donoghue 2011, 2012; Clement et al., 2014). Classification of *Viburnum* using phylogenetic nomenclature (Clement et al., 2014) was based on a phylogenetic analysis of the chloroplast genome, which recognized four major clades, *V. clemensiae*, *Valvatotinus*, *Pseudotinus*, and *Pluriviburnum*. Our analysis of the cpDNA data supports these clades (Fig. 1). *Viburnum burejaeticum* and *V. carlesii* classified in sect. *Viburnum* (Hara, 1983) are recognized in *Euviburnum* in the phylogenetic nomenclature (Clement et al., 2014). *Euviburnum* along with *Lentago* and *Punctata* belong to a more inclusive clade of *Valvatotinus*. *Viburnum furcatum* is a member of *Pseudotinus*. *Pluriviburnum* is a large clade containing most of the diversity of *Viburnum* (Clement et al., 2014). The Korean taxa, specifically the *V. dilatatum* complex, *V. koreanum*, and *V. opulus* var. *calvescens*, belong to *Pluriviburnum*. The phylogenetic relationships in our analysis of the cpDNA data suggested that *Valvatotinus* is more closely related to *Pluriviburnum* than it is to *Pseudotinus* with a low bootstrap support value (Fig. 1). *Valvatotinus* was suggested as a sister clade of *Pseudotinus* in previous studies (Clement et al., 2014). However, support values for a close relationship between *Valvatotinus* and *Pseudotinus* were low (Clement et al., 2014).

A phylogenetic analysis of the ITS regions placed *Viburnum odoratissimum* var. *awabuki* as a sister to *V. furcatum* with relatively high bootstrap support (Fig. 2). The results of a combined analysis of the cpDNA and ITS regions are consistent with the ITS data (Fig. 3), suggesting that phylogenetic signals from the ITS regions are stronger than

cpDNA. Previous analysis of the ITS region (Donoghue et al., 2004) and nuclear *GBSSI* (Winkworth and Donoghue, 2004) also showed a close relationship between *Pseudotinus* and *Solenotinus*, to which *Viburnum odoratissimum* belongs.

Viburnum furcatum is characterized by having a pair of caducous bud scales resulting in naked buds at a later stage (from late autumn to spring), umbellate inflorescences with marginal sterile flowers, and one deep ventral groove in the stones (Fig. 4). Our DNA sequence data indicate that the species can be clearly discriminated from other species (Figs. 1, 2). It is distributed from South Sakhalin, Japan and to Korea and then to Taiwan (Hara, 1983; Kim, 2007). Although it is common in Japan, its distribution in Korea is restricted, occurring at rather high elevations on Mt. Hallasan and on Ulleungdo Island. A sporadic occurrence of the species at Mt. Jabyeongsan in Gangwon province in Korea was reported (Son et al., 2008). There is little molecular variation within the species (Figs. 1, 2). The ITS regions were identical across the Korean accessions examined in this study. ITS sequence from the plant from Hokkaido (accession number 7464) had unique insertion. The Japanese accession was distinguished from the plants on the Jejudo Island in the cpDNA data (Fig. 1), suggesting that there may have been haplotype diversity with the species. We did not sample plants from Ulleungdo Island or Mt. Jabyeongsan in this study. The cpDNA regions and perhaps more variable regions will be useful to infer the origins of the populations.

Viburnum odoratissimum var. *awabuki* is found on Jejudo Island in Korea and in Japan, Taiwan, and the Philippines. This species differs from others by having a basic chromosome number of $x = 8$, which is rare in *Viburnum* (Zhang et al., 2016). Most other species of *Viburnum* have a basic chromosome number of $x = 9$ (Egolf, 1962). The species is easily recognized by having subentire leaves and paniculate inflorescences borne on evergreen trees (Fig. 4). Our DNA barcodes clearly differentiate the species (Figs. 1, 2).

Viburnum burejaeticum is distributed at high latitudes in Korea, the Russian Far East, and northeastern China. We included samples from Gangwon-do Province in Korea and Jilin in China (Table 1), and there was no intra-specific variation in both the cpDNA and ITS regions (Figs. 1, 2). This species is morphologically distinguished by naked buds and exerted stamens from rotate corolla and is often confused with *V. dilatatum* when flowers are unavailable. However, *V. burejaeticum* does not have an extrafloral nectary, whereas *V. dilatatum* has an extrafloral nectary on the abaxial surface of the leaves (Fig. 4). *Viburnum carlesii* was considered to be closely related to *V. burejaeticum* by having naked buds and

Fig. 4. Summary phylogeny of *Viburnum* in Korea with a comparison of key morphological characteristics. Character A, presence or absence of bud scales; B, habit of plants; C, leaf margin; D, distribution of extrafloral nectary; E, inflorescence type; F, fruit color in maturity, G, number of grooves on the dorsal surface and ventral surface of the stone. An exemplar cross-section of the stone is provided following the character G to show the dorsal and ventral groove, if present. An asterisk is used to indicate that plants in Hokkaido lack a petiolar extrafloral nectary.

stones with two dorsal and three ventral shallow grooves (Hara, 1983). The two species represent the *Euviburnum* clade (Clement et al., 2014). Our molecular data show that the two species can be discriminated (Figs. 1, 2). *Viburnum carlesii* can be easily distinguished from *V. carlesii* by having hypocrateriform corollas in which stamens are included.

Viburnum koreanum and *V. opulus* are members of the sect. *Opulus* (Hara, 1983). Our data with multiple accessions from different populations of *V. opulus* in Korea resolved the two species as distinct (Figs. 1, 2). The presence of an extrafloral nectary on the upper part of the petiole is a synapomorphy for *Opulus* (Clement et al., 2014). All materials examined here showed these characteristics. *Viburnum koreanum*, distributed in northern and central Korea and in Manchuria and Hokkaido is rare throughout the range, while *V. opulus* is common. All specimens labelled as *V. koreanum* deposited in KB were incorrectly identified; they were *V. opulus*. *Viburnum koreanum* can be distinguished from *V. opulus* by having sharply serrate

leaves and smaller inflorescences lacking marginal sterile flowers (Hara, 1983). Hara (1983) reported that plants from Hokkaido lack a petiolar extrafloral nectary. This can be explained as a secondary loss of the trait within a species.

The *V. dilatatum* complex, which consists of four closely related species, is characterized by free bud scales and serrate leaves with pinnate veins and extrafloral nectaries at proximal regions on the abaxial surface. The complex formed a highly supported clade in both cpDNA and ITS trees (Figs. 1, 2). Relationships among the species within the complex are unresolved, however. The DNA barcodes examined in this study could not differentiate the species. This suggests that it is difficult to infer a species boundary from the sequence data in the *V. dilatatum* complex. A previous cpDNA analysis with 113 species of *Viburnum* found that *V. dilatatum* is a sister to a clade that includes *V. erosum*, *V. wrightii*, and *V. japonicum* with low support values (Clement et al., 2014). Our cpDNA data show that some accessions of *V. wrightii* are differentiated

from the remaining three species (Fig. 1). However, other accessions of *V. wrightii* possess a haplotype identical to those of the other species. Likewise, four of six accessions of *V. erosum* formed with a distinct clade in the ITS data but the remaining two accessions were nested within the *V. dilatatum* clade (Fig. 2). All accessions of *V. dilatatum* except for J9050 formed a cohesive unit particularly in the ITS data (Fig. 2). The outlier accession J9050 was placed outside of the *V. dilatatum* clade and had the same cpDNA haplotype as *V. wrightii*. Incomplete lineage sorting may have played a role in the patterns observed in the DNA barcodes in the *V. dilatatum* complex.

The species in the complex can be differentiated by morphological characteristics. *Viburnum japonicum* is easily distinguished from other Korean species in the *V. dilatatum* complex by having glabrous evergreen leaves. This species is distributed in warm areas of southern Japan from Honshu, Kyushu to the Ryukyu Islands. The distribution range has recently been expanded to include Gageodo Island in southwestern Korea (Hong and Im, 2003). *Viburnum erosum* is distributed in southern Korea and offshore islands in western Korea; western Japan; and southern China. It can be distinguished by having stipules. None of the other species of *Viburnum* in Korea have stipules. Pubescence on branchlets has been used to distinguish the four species (Kim, 2007). Branchlets of *V. japonicum* are glabrous, and those of *V. wrightii* are usually glabrous or occasionally pubescent with long simple hairs. Branchlets of *V. erosum* have stellate hairs, whereas those of *V. dilatatum* are densely pubescent with stellate and simple hairs.

Our analyses of DNA barcode regions from multiple accessions of the species of *Viburnum* in Korea confirm that six out of ten species in Korea can be discriminated at the species level. The *V. dilatatum* complex can be separated from the remaining species according to molecular data, but the resolution power to differentiate a species within the complex is absent in our data. This study suggests that regional DNA barcodes are useful for molecular species identification for *Viburnum* when flowering or fruiting materials are not available.

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Conflict of Interest

Authors declare that there are no conflicts of interest.

Literature Cited

- Ahn, D. K. 1998. Illustrated Book of Korean Medicinal Herbs. Kyohaksa, Seoul, 854 pp.
- Cantino, P. D., J. A. Doyle, S. W. Graham, W. S. Judd, R. G. Olmstead, D. E. Soltis, P. S. Soltis and M. J. Donoghue. 2007. Towards a phylogenetic nomenclature of Tracheophyta. *Taxon* 56: 822–846.
- CBOL Plant Working Group. 2009. A DNA barcode for land plants. *Proceedings of the National Academy of Sciences of the United States of America* 106: 12794–12797.
- Clement, W. L., M. Arakaki, P. W. Sweeney, E. J. Edwards and M. J. Donoghue. 2014. A chloroplast tree for *Viburnum* (Adoxaceae) and its implications for phylogenetic classification and character evolution. *American Journal of Botany* 101: 1029–1049.
- Clement, W. L. and M. J. Donoghue. 2011. Dissolution of *Viburnum* section *Megalotinus* (Adoxaceae) of Southeast Asia and its implications for morphological evolution and biogeography. *International Journal of Plant Sciences* 172: 559–573.
- Clement, W. L. and M. J. Donoghue. 2012. Barcoding success as a function of phylogenetic relatedness in *Viburnum*, a clade of woody angiosperms. *BMC Evolutionary Biology* 12: 73.
- Cuénoud, P., V. Savolainen, L. W. Chatrou, M. Powell, R. J. Grayer and M. W. Chase. 2002. Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid *rbcL*, *atpB*, and *matK* DNA sequences. *American Journal of Botany* 89: 132–144.
- Donoghue, M. J. 1983. The phylogenetic relationships of *Viburnum*. *Advances in Cladistics* 2: 143–166.
- Donoghue, M. J., B. G. Baldwin, J. Li and R. C. Winkworth. 2004. *Viburnum* phylogeny based on chloroplast *trnK* intron and nuclear ribosomal ITS DNA sequences. *Systematic Botany* 29: 188–198.
- Donoghue, M. J., C. D. Bell and R. C. Winkworth. 2003. The evolution of reproductive characters in Dipsacales. *International Journal of Plant and Science* 164: S453–S464.
- Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.
- Egolf, D. R. 1962. A cytological study of the genus *Viburnum*.

- Journal of the Arnold Arboretum 43: 132–172.
- Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.
- Hara, H. 1983. A Revision of Caprifoliaceae of Japan with Reference to Allied Plants in Other Districts and the Adoxaceae. Academia Scientific Books, Tokyo, 336 pp.
- Hebert, P. D. N., A. Cywinska, S. L. Ball and J. R. deWaard. 2003. Biological identifications through DNA barcodes. *Proceedings of the Royal Society B: Biological Sciences* 270: 313–321.
- Hong, H.-H. and H.-T. Im. 2003. *Viburnum japonicum* (Caprifoliaceae): an unrecorded species in Korea. *Korean Journal of Plant Taxonomy* 33: 271–277.
- Huelsenbeck, J. P. and F. Ronquist. 2001. MrBayes: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755.
- Kim, H. M., S.-H. Oh, G. S. Bhandari, C.-S. Kim and C.-W. Park. 2014. DNA barcoding of Orchidaceae in Korea. *Molecular Ecology Resources* 14: 499–507.
- Kim, T. J. 2007. Viburnaceae Raf. In *The Genera of Vascular Plants of Korea*. Park C.-W. (ed.), Academy Publishing Co., Seoul. Pp. 944–946.
- Kress, W. J. and D. L. Erickson. 2007. A two-locus global DNA barcode for land plants: the coding *rbcL* gene complements the non-coding *trnH-psbA* spacer region. *PLoS ONE* 2: e508.
- Kress, W. J., K. J. Wurdack, E. A. Zimmer, L. A. Weigt and D. H. Janzen. 2005. Use of DNA barcodes to identify flowering plants. *Proceedings of the National Academy of Sciences of the United States of America* 102: 8369–8374.
- Kwon, M. K., H. E. Lee, J. Y. Park and Y. S. Hahn. 2003. Antimicrobial activities of berry extract of domestic plants on 4 kinds of pathogenic microorganism. *Journal of the East Asian Society of Dietary Life* 13: 433–438.
- Lahaye, R. M. van der Bank, D. Bogarin, J. Warner, F. Pupulin, G. Gigot, O. Maurin, S. Duthoit, T. G. Barraclough and V. Savolainen. 2008. DNA barcoding the floras of biodiversity hotspots. *Proceedings of the National Academy of Sciences of the United States of America* 105: 2923–2928.
- Mabberley, D. J. 1997. *The Plant-Book*. 2nd ed. Cambridge University Press, Cambridge, 680 pp.
- Posada, D. and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics* 14: 817–818.
- Rehder, A. 1908. The Viburnums of eastern Asia. In *Trees and Shrubs*. Vol. II, Part II. Sargent, C. S. (ed.), Houghton Mifflin, Boston, MA. Pp. 105–116.
- Sambrook, J., E. F. Fritsch and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Press, New York, 2100 pp.
- Sang, T., D. Crawford and T. Stuessy. 1997. Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84: 1120–1136.
- Soltis, P. S., D. E. Soltis and C. J. Smiley. 1992. An *rbcL* sequence from a Miocene *Taxodium* (bald cypress). *Proceedings of the National Academy of Sciences of the United States of America* 89: 449–451.
- Son, H.-D., D.-H. Kim and H.-T. Im. 2008. A short record for the distribution of 4 rare plants. *Korean Journal of Plant Taxonomy* 38: 565–571.
- Swofford, D. L. 2002. PAUP* Phylogenetic analysis using parsimony (* and other methods), version 4.0. Sinauer Associates, Sunderland, MA.
- Winkworth, R. C. and M. J. Donoghue. 2004. *Viburnum* phylogeny: evidence from the duplicated nuclear gene *GBSSI*. *Molecular Phylogenetics and Evolution* 33: 109–126.
- Winkworth, R. C. and M. J. Donoghue. 2005. *Viburnum* phylogeny based on combined molecular data: implications for taxonomy and biogeography. *American Journal of Botany* 92: 653–666.
- Xiang, X.-G., H. Hu, W. Wang and X.-H. Jin. 2011. DNA barcoding of the recently evolved genus *Holcoglossum* (Orchidaceae: Aeridinae): a test of DNA barcode candidates. *Molecular Ecology Resources* 11: 1012–1021.
- Youm, J. W., S.-W. Han, S. W. Seo, C. U. Lim and S.-H. Oh. 2016. DNA barcoding of Schisandraceae in Korea. *Korean Journal of Plant Taxonomy* 46: 273–282.
- Zhang, N., W. Sun and J. Yang. 2016. Chromosome counts and karyotype analysis of *Viburnum* taxa (Adoxaceae). *Caryologia* 69: 12–19.

Appendix 1. List of samples included in phylogenetic study.

- V. burejaeticum*: KOREA. Gangwon-do: Pyeongchang-gun, 4 Sep 2016, Suh H. J. 7129 (TUT); 5 May 2017, Lee D.H. 7194; 8 May 2012, Kim J. H. et al. KIMJH12048 (KB 356575).
- CHINA. Jilin: Antu, 4 Jun 2011, Kim J. S. BDS72 (KB 289365).
- V. carlesii*: KOREA. Gangwon-do: Hongcheon-gun, 6 May 2004, Yoo K. O. et al. 1306 (KB 277454); Yanggu-gun, 1 May 2008, Yoo K. O. VP_KB_387162_0143 (KB 154489). Gyeonggi-do: Incheon-si, Ongjin-gun, 14 Apr 2007, Hyun J. O. 2007018 (KB 110788). Gyeongsangbuk-do: Andong-si, 24 Apr 2007, Jung K. Y. ANH en070424 003 (KB 121542); Ulleung-gun, Ulleungdo

Island, 29 May 2017, *Ahn J. G.* 7274 (TUT).

V. dilatatum: KOREA. Gyeongsangbuk-do: Seongju-gun, 26 May 2012, *Kang S. H.* 358021 (KB 445136). Jeollabuk-do: Wanju-gun, 14 May 2017, *Yun N. R.* 7214 (TUT). Jeollanam-do: Yeongam-gun, 19 Oct 2013, *Kim M. H.* seed3482 (KB 448592). Jeju-do: Jeju-si, Gujwa-eup, 2 Sep 2014, *Moon M. O.* 5806 (KB 512282).

JAPAN. Honshu: Aomori Pref., 4 Aug 2009, *Sun B. Y. et al.* J9050 (KB).

V. erosum var. *erosum*: KOREA. Gyeongsangbuk-do: Gunsan-si, 20 May 2017, *Hwang S. H.* 7219 (TUT); Pohang-si, 27 Jul 2005, *Kim Y. D. et al.* 05171 (KB 277686). Jeollabuk-do: Namwon-si, 2006, *Baek W. K. s. n.* (KB 278061); Imsil-gun, 5 May 2007, *Sun B. Y.* 1029 (KB 127413). Jeollanam-do: Sinan-gun, Hongdo Island, 20 May 2017, *Oh S. H. et al.* 7224 (TUT). Jeju-do: Seogwipo-si, Namwon-eup, 15 Jun 2009, *Kang C. M. et al.* 27315 (KB 257106).

V. furcatum: KOREA. Jeju-do: Jeju-si, Aewol-eup, 27 May 2010, *Moon M. O.* 339 (KB 291710); 16 Jun 2010, *Kim C. H. et al.* 50400 (KB 329347); 20 Jul 2011, *Ko S. C. et al.* HNHM 20010656 (KB 386202); Seogwipo-si, Hawon-dong, 15 May 2017, *Oh S. H. et al.* 7203 (TUT).

JAPAN. Hokkaido: Sapporo Pref., 27 Aug 2017, *Oh S. H. et al.* 7464 (TUT).

V. japonicum: KOREA. Jeollanam-do: Sinan-gun, Gageodo Island, 17 May 2017, *Lee D. H.* 7216 (TUT); 22 May 2011, *Nam K. H. et al.* Gageo_211 (KB 289119); 2 May 2009, *Hyun J. O. et al. s. n.* (KB 337388); 24 Oct 2013, *Choi H. G. et al.* 754 (KB 473764).

JAPAN. Kyushu: Nagasaki Pref., 21 Aug 2018, *Hwang S. H. et al.* Kyushu-043 (TUT).

V. koreanum: KOREA. Gangwon-do: Inge-gun, 3 Jun 2017, *Lee D. H.* 7294 (TUT).

V. odoratissimum var. *awabuki*: KOREA. Jeju-do: Jeju-si, Gujwa-eup, 5 May 2011, *Choi B. H. et al.* 336083006 (KB 393880); Seogwipo-si, Andeok-myeon, 21 Mar 2012, *Kang S. H. s. n.* (KB 434902); Seogwipo-si, Jungmun-dong, 12 Jun 2017, *Choi Y. G.* 7317 (TUT); Seogwipo-si, Namwon-eup, 5 Sep 2012, *Kang C. M. et al.* 36118 (KB 418149); Seogwipo-si, Seongsan-eup, 13 Jun 2017, *Choi Y. G.* 7321 (TUT).

V. opulus var. *calvescens*: KOREA. Gangwon-do: Cherwon-gun, 29 May 05 2017, *Hwang S. H.* 7275 (TUT); Inge-gun, 30 Jun 2009, *Ko S. C.* HNHM_0339 (KB 252268); Yanggu-gun, 23 Jun 2008, *Yoo K. O. et al. s. n.* (KB 154759); Yeongwol-gun, 26 May 2010, *Nam K. H. et al.* SHY291 (KB 235185). Gyeonggi-do: Incheon-si, 29 May 2017, *Oh S. H. et al.* 7273 (TUT); Yeoncheon-gun, 30 May 2017, *Hwang S. H.* 7276 (TUT). Chungcheongbuk-do: Danyang-gun, 4 Aug 2009, *Ko S. C. et al.* HNHM_1135 (KB 253062).

V. wrightii: KOREA. Gangwon-do: Hoengseong-gun, 3 Jun 2008, *Ko S. C.* 20080009 (KB 198135); Inge-gun, 5 Sep 2009, *Kim H. W. et al.* 20080936 (KB 163578); Inge-gun, 16 Sep 2013, *Han B. W. et al.* NAPI S2013086 (KB 458890); Pyeongchang-gun, 30 Jun 2008, *Jung G. Y.* ANH en080630 104 (KB 197686). Gyeongsangbuk-do: Ulleung-gun, Ulleungdo Island, 16 Sep 2005, *Hyun J. O. et al.* NAPI 20101239 (KB 293906). Jeollabuk-do: Jeongeup-si, 15 May 2011, *Kim C. H. et al.* J600415 (KB). Jeju-do: Jeju-si, Aewol-eup, 16 Jun 2010, *Kim C. H. et al.* J50374 (KB); Gujwa-eup, 4 May 2018, *Oh S. H. et al.* 7539 (TUT).

JAPAN. Kyushu: Saga Pref., 20 Aug 2018, *Hwang S. H. et al.* Kyushu-003 (TUT).

Appendix 2. List of samples used in anatomical examination of the stones.

V. burejaeticum: KOREA. Gangwon-do: Pyeongchang-gun, 4 Sep 2016, *Suh H. J.* 7129 (TUT).

V. carlesii: KOREA. Gyeonggi-do: Incheon-si, Ongjin-gun, Deokjeokdo Island, 17 Jun 2009, *Hyun J. O. et al.* NAPI 20100398 (KB 292954).

V. dilatatum: KOREA. Jeju-do: Seogwipo-si, Namwon-eup, 17 Sep 2017, *Oh S. H.* 7507 (TUT).

V. erosum var. *erosum*: KOREA. Jeju-do: Seogwipo-si, Namwon-eup, 15 Jun 2009, *Kang C. M. et al.* 27315 (KB 257106).

V. furcatum: KOREA. Jeju-do: Seogwipo-si, Saegdal-dong, 15 Aug 2017, *Hwang S. H. et al.* 7403 (TUT).

V. japonicum: KOREA. Jeollanam-do: Sinan-gun, Gageodo Island, 25 Oct 2013, *Choi H. K. et al.* 893 (KB 474443).

V. koreanum: CHINA. Jilin: Fusong, 5 Aug 2018, *Suh H. J.* 7590 (TUT).

V. odoratissimum var. *awabuki*: KOREA. Jeju-do: Jeju-si, Daepo-dong, 15 Aug 2017, *Hwang S. H. et al.* 7405 (TUT).

V. opulus var. *calvescens*: KOREA. Gangwon-do: Yanggu-gun, 23 Jun 2008, *Yoo K. O. et al. s. n.* (KB 154759).

V. wrightii: KOREA. Gangwon-do: Inge-gun, 16 Sep 2013, *Han B. W. et al.* NAPI S2013086 (KB 458890).