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Chloroplast Noncoding DNA Sequences Reveal Genetic Distinction and Diversity between Wild and Cultivated *Prunus yedoensis*

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Abstract. **Cultivated flowering cherries (*Prunus* subgenus *Cerasus*), which are one of the most popular ornamental trees around the world, have been developed through artificial hybridizations among wild flowering cherries. Among the hundreds of cultivars of flowering cherries, *Prunus* ×*yedoensis* 'Somei-yoshino' is the most common and widespread. However, its origin and genetic relationship to wild *P. yedoensis*, naturally occurring on Jeju Island, South Korea, have long been debated. We used sequence polymorphisms in eight chloroplast DNA (cpDNA) noncoding regions to distinguish wild and cultivated flowering cherries among 104 individuals (55 accessions). We were able to distinguish two distinct groups, one corresponding to wild *P. yedoensis* collections from Jeju Island and the other collections of cultivated *P.* ×*yedoensis* from Korea, Japan, and the United States. The chlorotype diversity of wild *P. yedoensis* in Jeju Island and cultivated *P.* ×*yedoensis* collections in the United States was quite high, suggesting multiple natural hybrid origins and long history of cultivation from different original sources, respectively.**

Flowering cherries are one of the most popular ornamental trees with high horticultural value. They are widely planted for street, commercial, and residential landscapes, and cherry blossoms herald spring's arrival every year. Among the hundreds of cultivars of flowering cherries, 'Somei-yoshino' is the most common and widespread flowering cherry in East Asia (Korea and Japan) and the United States (Bailey and Bailey, 1976; Cheng et al., 2000; Iketani et al., 2007). It was first documented as "Yoshino cherry" in a survey of Ueno Park, Tokyo, Japan. In 1900, it was renamed "Somei-yoshino cherry" to distinguish it from the mountain cherries of Yoshino, Nara Prefecture, Japan (Kuitert, 1999). Its scientific name was initially given as *Prunus yedoensis* (Matsumura, 1901), but later the name *P.* ×*yedoensis* 'Somei-yoshino' was proposed based on the artificial hybrid origin and clonality as confirmed by molecular markers (Iketani et al., 2006, 2007; Innan et al., 1995; Kato et al., 2012). To avoid confusion on the common names of numerous cultivars and their scientific names throughout this paper, we used the term "cultivated *P.* ×*yedoensis*" to refer collectively various cultivars of *P.* ×*yedoensis* and their derivatives, whereas *P.* ×*yedoensis* 'Somei-yoshino' refers to one specific cultivar, Somei-yoshino.

In 1912, 3020 japanese flowering cherry trees (1800 cultivated *P.* ×*yedoensis*, simply known as "Somei-Yoshino," and 1220 comprising 11 cultivars of *P. serrulata*) were planted around the Tidal Basin in Washington, DC, as a gift from Japan. Over time, the trees were replaced primarily by cultivated *P.* ×*yedoensis* cherry trees grown in American nurseries. In 1965, another 3800 cultivated *P.* ×*yedoensis* cherry trees grown in the United States but donated from Japan were planted (Jefferson and Fusonie, 1977). Now, Potomac Park (Washington, DC) draws hundreds of thousands of visitors to view the cherry blossoms every spring. In Korea, cultivated *P.* ×*yedoensis* ("Wang-beot-na-mu" in Korean, but without known genealogical records) were extensively planted during the Japanese occupation, and they remain one of the most favorite trees planted along roadsides and in residential landscapes.

In spite of its horticultural importance, the botanical origin of *P.* ×*yedoensis* 'Somei-yoshino', especially relative to wild *P. yedoensis* found on Jeju Island, Korea, has long been controversial and enigmatic. It was originally described based on a cultivated cherry of unknown origin planted abundantly in Tokyo and Yokohama, but never found in the wild in Japan (Wilson, 1916). Currently, its origin is considered as a hybrid between *P. spachiana* f. *ascendens* (=*P. subhirtella* var. *ascendens* = *P. pendula*) and *P. speciosa* (=*P. serrulata* var. *lannesiana*) as proposed by Wilson (1916). The hybrid origin hypothesis of *P.* ×*yedoensis* 'Somei-yoshino' was supported by the crossing experiments (Takenaka, 1963). Several additional studies conducted to determine the parentage of the hybrid

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indicated that *P. spachiana* f. *ascendens* was the female parent and *P. speciosa* (=oshima cherry) was the pollen donor (Innan et al., 1995; Kaneko et al., 1986; Kato et al., 2014; Ohta et al., 2006, 2007). Nakamura et al. (2015) further suggested that *P. ×yedoensis* 'Somei-yoshino' originated by the artificial hybridization of cultivars derived from *P. spachiana* f. *ascendens* (i.e., Komatsuotome) and *P. speciosa.* In addition to *P. ×yedoensis* 'Somei-yoshino', numerous other cultivars of *P. ×yedoensis* have been developed (e.g., 'Amagi-yoshino', 'Mikado-yoshino', 'Funabara-yoshino', 'Mishima-zakura', 'Sotorihime', etc.) and their genealogical origins and cultivar names are likely to be complex and often difficult to be traced back because of long history of cultivation in Japan (Iketani et al., 2007). Kato et al. (2014) attempted to trace the origins of japanese flowering cherry cultivars, including *P. ×yedoensis* cultivars and 'Somei-yoshino', based on nuclear simple sequence repeat (SSR) markers.

Unlike the lack of natural stands of cultivated *P. ×yedoensis* in Japan, populations of wild *P. yedoensis* have been reported to be native in Korea (Jung and Oh, 2005; Kim, 1998; Kim et al., 1998; Park et al., 1984). In 1908, it was first collected by Emile Taquet, missionary and botanical collector, from Mt. Halla, Jeju Island, Korea, and reported by Koehne (1912) under the name of *P. yedoensis* var. *nudiflora*. Later, it was redesignated *P. yedoensis* (Nakai, 1916)*;* consequently, it shares the same name of *P. yedoensis* with cultivated *P. ×yedoensis* without additional formal taxonomic treatments. Recently, Cho et al. (2014) provided convincing evidence of the natural hybrid origin of wild *P. yedoensis* from two sympatric lineages on Jeju Island. *P. spachiana* f. *ascendens* was identified as the maternal parent based on cpDNA phylogeny, but the paternal parent was not precisely determined from the other lineage of *P. serrulata* and *P. sargentii* complex. The molecular evidence for hybrid origin was further corroborated by several intermediate or unique morphological traits expected for hybrid origin species (Rieseberg, 1995; Rieseberg and Ellstrand, 1993). The same study documented the multiple bidirectional hybrid origins of wild *P. yedoensis* on Jeju Island.

Both wild *P. yedoensis* and cultivated *P. ×yedoensis* including 'Somei-yoshino' share the same maternal parent in common; however, the paternal contribution is most likely different from each other because the purported pollen donor to the origin of cultivated *P. ×yedoensis*; i.e., *P. speciosa* (oshima cherry), is not native to Jeju Island, Korea. It is indigenous on Izu Oshima and on all the other neighboring Izu Islands, and southern Izu Peninsula and the coastal region of the Boso Peninsula in Japan (Iwatsuki et al., 1995; Kuitert, 1999). Although the previous studies (Cho et al., 2014; Innan et al., 1995; Kaneko et al., 1986; Kato et al., 2014; Nakamura et al., 2015; Ohta et al., 2006, 2007) are suggestive of independent hybrid origins between wild *P. yedoensis* and cultivated *P. ×yedoensis*, they exhibit very similar morphological features. Kim et al. (1998) reported that uniform reproductive trait variations (i.e., flowers, fruit, and inflorescences) in cultivated *P. ×yedoensis* were within the wide range of wild *P. yedoensis* found on Jeju Island. Several molecular phylogenetic analyses were also conducted to decipher the relationship between them, but these studies have limited value in determining the precise genetic relationship because of limited sampling and lack of reproducibility of experiments (Jung and Oh, 2005; Jung et al., 1998), phenotypic band pattern similarities (Jung et al., 1997; Roh et al., 2007), and insufficient conclusion validity (Roh et al., 2007).

Presently, we aimed to determine the genetic relationship and degree of diversity between wild *P. yedoensis* and cultivated *P. ×yedoensis* (including 'Somei-yoshino') cherry accessions based on maternally inherited cpDNA. We extensively sampled flowering cherry trees of cultivated *P. ×yedoensis* (both known and unknown genealogical origins) for the landscape from Korea, Japan, and the United States, as well as wild collections of *P. yedoensis* on Jeju Island, Korea. We provided further evidence that wild *P. yedoensis* native to Jeju Island, Korea, is a distinct taxonomic entity from typical cultivated *P. ×yedoensis* including 'Somei-yoshino'.

Materials and Methods

Plant materials. We sampled 104 individuals representing nine species of flowering cherries and two outgroup species [*P. dulcis* and *P. persica*] from subgenus *Amygdalus* of *Prunus* (Table 1). Our samples included eight taxa (wild *P. yedoensis*, cultivated *P. ×yedoensis* including 'Somei-yoshino', *P. spachiana* f. *ascendens*, *P. sargentii*, *P. serrulata* var. *spontanea*, *P. serrulata* var. *pubescens*, *P. speciosa*, and *P. takesimensis*) from section *Pseudocerasus*, *P. maximowiczii* from section *Phyllomahaleb*, and *P. mahaleb* from section *Mahaleb*. The samples of wild *P. yedoensis*, which included two national monument sites (Bonggaedong and Shinyeri located on Mt. Halla), were collected mostly from undisturbed natural forest surrounding Mt. Halla in Jeju Island, South Korea. Their identities were confirmed by C-S. Kim, the leading expert on genus *Prunus* in South Korea. The samples of cultivated *P. ×yedoensis* represent ornamental and landscape plants that were collected from South Korea, Japan, and the United States. Specifically, 43 individuals of cultivated *P. ×yedoensis* were collected from the areas of Jeju Island, Seoul, and Jinhae in South Korea. In the United States, 17 individuals were collected from Potomac Park, three individuals from American University, and two individuals (the clones of trees planted in 1912 by First Lady Helen Taft and Viscountess Chinda, wife of the Japanese Ambassador) from the U.S. National Arboretum in Washington, DC. Although these landscape plants were known primarily as *P. ×yedoensis* 'Somei-yoshino', we recognized them as cultivated *P. ×yedoensis*, given their unknown precise genealogical records. Finally, two individuals were collected from old trees planted in the late 19th century at the Koishikawa Botanical Gardens (University of Tokyo, Tokyo, Japan). These two trees are the same individuals studied by Iketani et al. (2007) and represent typical *P. ×yedoensis* 'Somei-yoshino', which is a clone from a single tree. The cultivated *P. ×yedoensis* including 'Somei-yoshino' accessions from East Asia (South Korea/Japan) and the United States were determined by M-S. Cho and E.J. Cheong/M.J. Pooler of the U.S. National Arboretum, respectively. *P. spachiana* f. *ascendens*, the maternal contributor in the hybrid origins of both wild *P. yedoensis* and cultivated *P. ×yedoensis* including 'Somei-yoshino', were collected from Jeju Island, South Korea, and Japan. The samples of *P. speciosa*, which is considered a paternal contributor of the cultivated *P. ×yedoensis*, were from the cultivated trees on Jeju Island, Korea. Voucher specimens collected from Korea were deposited at Ha Eun Herbarium of Sungkyunkwan University in Suwon, South Korea (Table 1).

DNA isolation, polymerase chain reaction (PCR) amplification, and sequencing. Silica gel–dried leaves collected from natural populations were used as sources of DNA,

Table 1. The *Prunus* species sampled in this study to determine phylogenetic relationships among species of flowering cherries. Voucher specimens are deposited in the Ha Eun Herbarium, Sungkyunkwan University (Suwon, South Korea) for accessions with specimen numbers marked. The accessions without specimen numbers were not vouchered.

Species	Pool no.	Samples (no.)	Sample name	Location	Voucher specimen
Prunus dulcis		1	Pd	USDA-ARS (Washington, DC)	
Prunus persica		1	Pp	USDA, ARS (Washington, DC)	
Prunus mahaleb		1	Pm	USDA, ARS (Washington, DC)	
Prunus sargentii		1	Ps	Jeju Island, South Korea	
Prunus serrulata var. *spontanea*		2	Pse1, 3	Jeju Island, South Korea	
Prunus speciosa (cultivated)		2	Psp1, 2	Jeju Island, South Korea	
Prunus maximowiczii		1	MX876	Bangseongyo, Jeju Island, South Korea	SKK Cho et al., 110503163
Prunus serrulata var. *pubescens*		1	PB10	Dooryunsan, South Korea	SKK Cho et al., 120417137
Prunus takesimensis		1	TA	Seonginbong, Ulleung Island, South Korea	SKK Cho et al., 140424001
Prunus spachiana f. *ascendens*		1	PE809	Mysterious Road, Jeju Island, South Korea	SKK Cho et al., 110412126
		1	PE857	Youngsil, Jeju Island, South Korea	SKK Cho et al., 110502134
		1	PE832	Kwaneumsa, Jeju Island, South Korea	SKK Cho et al., 110419133
		1	PE817	Pyoseonri, Jeju Island, South Korea	SKK Cho et al., 110414128
		1	PE821	Bonggae-dong, Jeju Island, South Korea	SKK Cho et al., 110414130
		1	PP382	Sendai, Japan	SKK Cho et al., 130422034
		1	PP377	Sendai, Japan	SKK Cho et al., 130422006
Prunus yedoensis wild on Jeju Island		1	Jeju01	Kwaneumsa, Jeju Island, South Korea	
		1	Jeju02	Kwaneumsa, Jeju Island, South Korea	
		1	Jeju03	Yoisol, Jeju Island, South Korea	
		1	Jeju04	Eorimok, Jeju Island, South Korea	
		1	Jeju05	Bonggaedong#2, Jeju Island, South Korea	
		1	Jeju06	Bonggaedong#1, Jeju Island, South Korea	
		1	Jeju07	Shinyeri, Jeju Island, South Korea	
		1	Jeju08	Haryeri, Jeju Island, South Korea	
		1	Jeju09	Haryeri, Jeju Island, South Korea	
		1	Jeju10	Sancheondan, Jeju Island, South Korea	
		1	YE3-A	Haryeri, Jeju Island, South Korea	SKK Cho et al., 110413101
		1	YE3-1	Haryeri, Jeju Island, South Korea	SKK Cho et al., 110413100
		1	YE92	Daepodong, Jeju Island, South Korea	SKK Cho et al., 120510104
		1	YE814	Wimiri, Jeju Island, South Korea	SKK Cho et al., 110413105
		1	YE833	Kwaneumsa, Jeju Island, South Korea	SKK Cho et al., 110419108
		1	YE872	Seongpangyo, Jeju Island, South Korea	SKK Cho et al., 110503110
		1	YE873	Kwaneumsa, Jeju Island, South Korea	SKK Cho et al., 110503111
		1	YE875	Tamra Education Center, Jeju Island, South Korea	SKK Cho et al., 110503112
		1	YE879	Eorimok, Jeju Island, South Korea	SKK Cho et al., 110503114
Cultivated *P.* ×*yedoensis* landscape from Korea	Jeju11	5	Py11, 12, 13, 14, 15	Jeju University, Jeju Island, South Korea	
	Jeju12	2	Py16, 17	Sancheondan, Jeju Island, South Korea	
	Jeju13	3	Py18, 19, 20	Hyondonro, Jeju Island, South Korea	
	Jinhae1	4	Jinhae1, 5, 20, 25	Jinhae, South Korea	

Continued next page

Table 1. Continued.

Species	Pool no.	Samples (no.)	Sample name	Location	Voucher specimen
	Jinhae2	8	Jinhae42, 44, 45, 46, 47, 48, 49, 50	Jinhae, South Korea	
	Jinhae3	4	Jinhae53, 63, 64, 68	Jangboksan, Jinhae, South Korea	
	Seoul1	12	Seoul3, 6, 7, 11, 13, 15, 22, 23, 30, 35, 36, 37	Seoul, South Korea	
	Seoul2	5	Seoul58, 59, 60, 64, 65	Seoul, South Korea	
Cultivated *P.* ×*yedoensis* landscape from the United States.	USAU	3	USAU1, 2, 3	American University (Washington, DC)[z]	
	USNA	2	USNA1 (Chinda), 2 (Taft)	U.S. National Arboretum (Washington, DC)[y]	
	USNM1	4	NM1, 2, 6, 7	National Park Service (NPS) golf course (Washington, DC)[x]	
	USNM2	2	NM16, 19	NPS, Inlet Bridge (Washington, DC)[w]	
	USNM3	3	NM23, 26, 30	NPS, Tidal Basin (Washington, DC)[w]	
	USNM4	3	NM44, 45, 47	NPS, Kutz Bridge (Washington, DC)[v]	
	USNM5	4	NM49, 51, 52, 54	NPS, Near Sylvan Theater (Washington, DC)[u]	
	USNM6	1	NM55	NPS, near Jefferson Memorial (Washington, DC)[u]	
P. ×*yedoensis* 'Somei-yoshino', Landscape from Japan		1	YEKO5041	Koishikawa Botanical Gardens, University of Tokyo, Tokyo, Japan	
		1	YEKO4981	Koishikawa Botanical Gardens, University of Tokyo, Tokyo, Japan	

[z]Planted in 1943; Original source of accessions from American University was known as grown in a commercial nursery in the United States.
[y]Accessions from National Arboretum are clonally propagated from the trees planted in 1912 by First Lady Helen Taft and Viscountess Chinda of Japan.
[x]Unknown source and planting year (U.S. Department of the Interior, National Park Service, n.d.).
[w]Shipped from Japan in 1912 (U.S. Department of the Interior, National Park Service, n.d.).
[v]Recent planting, around 2002 (U.S. Department of the Interior, National Park Service, n.d.).
[u]Recent planting ≈1986–88 (U.S. Department of the Interior, National Park Service, n.d.).

and total genomic DNA was extracted using the DNeasy plant mini kit (Qiagen, Valencia, CA). For molecular markers, we selected five cpDNA intergenic spacer regions (*trn*L-*trn*F, *atp*B-*rbc*L, *atp*F-*atp*H, *trn*H-*psb*A, and *trn*S-*trn*G) and three coding regions (*rpl*16, *rpo*B, and *rpo*C1). The primer pairs used for PCR amplification are specified in Table 2. PCR amplification was performed on the thermal cycler program as follows: 2 min of initial denaturation at 95 °C, 35 cycles with 20 s denaturation at 95 °C, 40 s annealing at 52 °C, and 1 min extension at 72 °C, with a final extension of 5 min at 72 °C. All PCR products were purified with the Inclone Gel and PCR purification Kit (InClone Biotech Co., Seoul, Korea). Direct sequencing reactions were carried out for the purified PCR products using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) at Geno Tech Corp. (Daejeon, Korea). Sequence fragments were assembled and edited using Sequencher (version 4.7; GeneCodes, Ann Arbor, MI). The same ID number was given to the individuals collected from the same locality with identical sequences, resulting in total 55 accessions (Table 1).

Phylogenetic analysis. Various tree building methods [maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses] were used based on eight concatenated cpDNA regions to determine overall phylogenetic relationships among accessions (104 individuals, 55 accessions). Gaps in the cpDNA noncoding regions were treated as missing data and were excluded or coded as simple binary characters (Simmons and Ochoterena, 2000) using SeqState 1.4.1 software (Muller, 2005). Fitch parsimony (Fitch, 1971) implemented in PAUP version 4.0 (Swofford, 2002) was used for MP analysis. ML analysis was conducted using RaxmlGUI version 1.5 (Silvestro and Michalak, 2012). BI analysis was done using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). Bootstrap support (BS) for the robustness of groups was calculated by bootstrap analysis with 1000 bootstrap replicates (Felsenstein, 1985). To further determine the genetic relationship between wild *P. yedoensis* and cultivated *P.* ×*yedoensis*, a cpDNA haplotype network was constructed using TCS version 1.21 (Clement et al., 2000) including wild *P. yedoensis* and cultivated *P.* ×*yedoensis* and their maternal parent *P. spachiana* f. *ascendens* (93 individuals, 44 accessions). Two data matrices for phylogenetic and haplotype network analysis are provided in Supplemental Data 1 and 2, respectively.

Results

Phylogeny based on cpDNA sequences. We merged the identical cpDNA sequences from 104 individuals and identified 55 accessions as operational taxonomic units. A total of 4566 aligned characters were used for the phylogenetic analysis: 793 sites for *atp*B-*rbc*L, 578 sites for *atp*F-*atp*H, 298 sites for *rpl*16, 452 sites for *rpo*B, 588 sites for *rpo*C1, 419 sites for *trn*H-*psb*A, 933 sites for *trn*L-*trn*F, and 442 sites for *trn*S-*trn*G, with 63 binary coded indel characters. ML analysis revealed a nearly identical tree to the 50% majority-rule consensus tree of MP analysis. The BI tree (not shown) was identical to one of the MP trees. No major topological differences were found when the gaps were treated as either missing data or coded as binary characters. But, the resolutions and nodal branch support within each of the two major lineages were slightly increased when the gaps were coded. Thus, we present the gaps coded ML (Fig. 1).

The phylogenetic tree based on eight concatenated cpDNA regions recognized two major lineages within flowering cherries with *P. mahaleb* being sister to these two major lineages. The first lineage (Clade 1) [58% ML BS, 1.00 BI posterior probability (PP)] included the majority of wild *P. yedoensis*, cultivated *P.* ×*yedoensis* including 'Somei-yoshino', and *P. spachiana* f. *ascendens* accessions, whereas the second lineage (Clade 2) (93% ML BS, 1.00 BI PP) included all the remaining species of *P. serrulata*, *P. sargentii* and closely related species, and *P. maximowiczii*. Several exceptional accessions of wild *P. yedoensis* (3-1 and 875) and *P. spachiana* f. *ascendens* (817 and 821) were also included in the second lineage (Clade 2). Clade 1 was highly unresolved with very low bootstrap values, but two distinct groups were identified. One group included wild *P. yedoensis* and *P. spachiana* f. *ascendens* from Jeju Island. The other group included primarily cultivated *P.* ×*yedoensis* including 'Somei-yoshino'. Within each group, four exceptional accessions were found; two wild accessions of *P. yedoensis* (833 and Jeju02) were grouped in otherwise typically cultivated group, whereas two accessions of cultivated *P.* ×*yedoensis* (USNM3 and Jeju12) were placed in otherwise typically wild group. Despite these exceptions, the two groups of wild *P. yedoensis* and cultivated *P.* ×*yedoensis* could be distinguished from each other based on 2-bp substitutions in the *trn*S-G and *rpl*16 regions.

Chloroplast haplotype diversity and network relationships. The haplotype network constructed using TCS (Fig. 2) exhibited the same relationships among haplotypes as the cpDNA phylogeny (Fig. 1). The network comprised of 11 haplotypes and the members of each haplotype are presented in Table 3. Haplotype H1 included the accessions of *P. spachiana* f. *ascendens* from Jeju Island (South Korea) and Japan, wild *P. yedoensis* from Jeju, and cultivated *P.* ×*yedoensis* from the United States. Two haplotypes from Jeju Island, H2 and H3,

Table 2. The sequence information of primers used for polymerase chain reaction and DNA sequencing to determine phylogenetic relationships between wild and cultivated flowering cherries.

Chloroplast DNA region	Forward primer name and sequence	Reverse primer name and sequence	Reference
*atp*B-*rbc*L	atpB-1: ACATCKARTACKGGACCAATAA	rbcL-1: ACACCAGCTTTRAATCCAA	Chiang et al. (1998)
*atp*F-H	atpF: ACTCGCACACACTCCCTTTCC	atpH: GCTTTTATGGAAGCTTTAACAAT	CBOL Plant Working Group (2009)
*rpl*16	rpl16f: TTGCTTCGGTAGATATGCTCTTC	rpl16r: CAAAGACCCCTTTCCTTTGT	Roh et al. (2007)
*rpo*B	rpoB1f: AAGTGCATTGTTGGAACTGG	rpoB4r: GATCCCAGCATCACAATTCC	Chase et al. (2007)
*rpo*C1	rpoC1f: GTGGATACACTTCTTGATAATGG	rpoC4r: CCATAAGCATATCTTGAGTTGG	Chase et al. (2007)
*trn*H-*psb*A	trnH^{GUG}: CGCGCATGGTGGATTCACAATCC	psbA: GTTATGCATGAACGTAATGCTC	Shaw and Small (2004)
*trn*L-F	trn-c: CGAAATCGGTAGACGCTACG	trn-f: ATTTGAACTGGTGACACGAG	Taberlet et al. (1991)
*trn*S-*trn*G	trnS^{UUC}: AGATAGGGATTCGAACCCTCGGT	trnG2S: TTTTACCACTAAACTATACCCGC	Shaw et al. (2005)

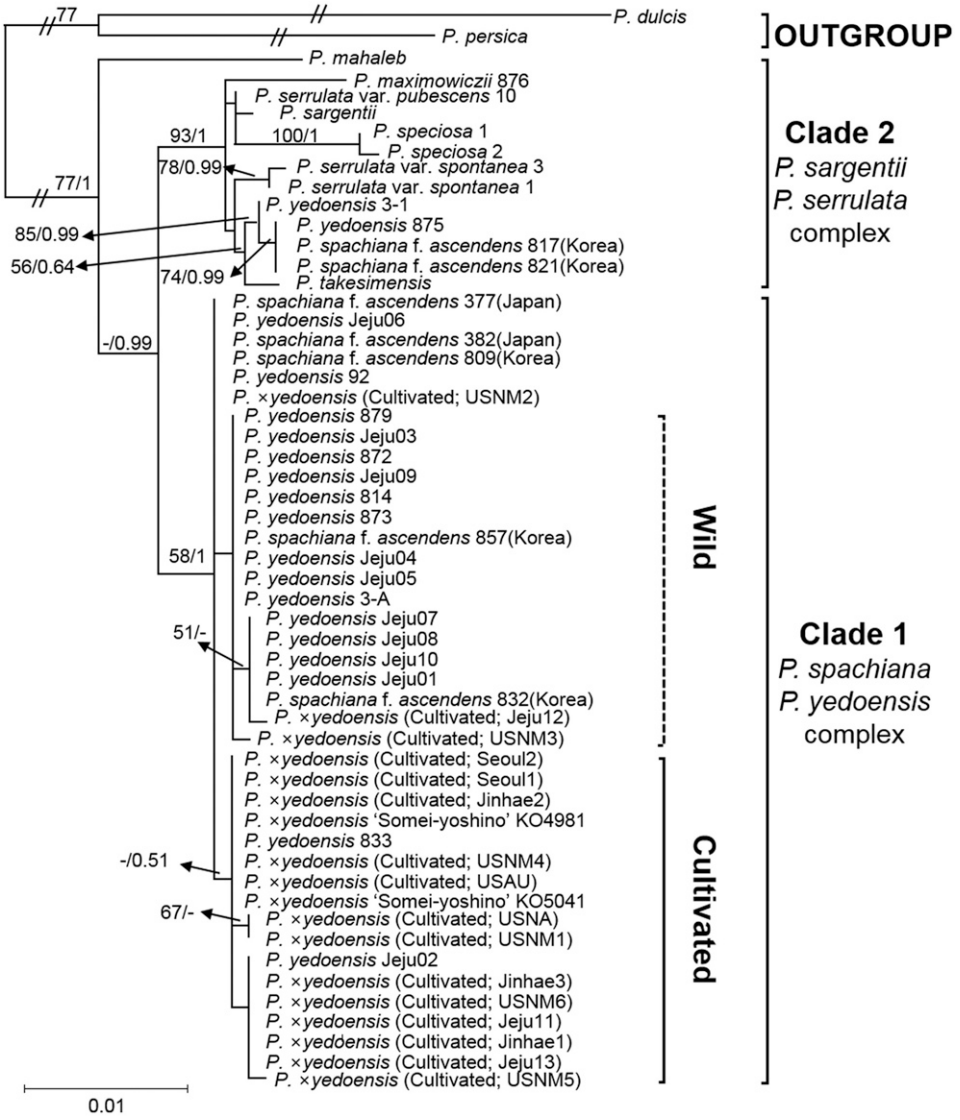

Fig. 1. RAxML tree based on eight concatenated coding and noncoding regions of chloroplast DNA showing phylogenetic relationships among accessions of wild and cultivated flowering cherries. Numbers above branches represent bootstrap support (BS) percentages based on maximum likelihood analysis and posterior probability (PP) values in Bayesian inference analysis ("-" symbol indicates <50% BS and PP).

occurred exclusively in wild *P. yedoensis* and its maternal contributor, *P. spachiana* f. *ascendens*. Haplotype H4 containing the exceptional accession of cultivated *P.* ×*yedoensis* of Jeju12 was connected to H3. Cultivated *P.* ×*yedoensis* included five haplotypes (H5, H6, H7, H8, and H9), excluding H4 haplotype (see more discussion later). All accessions of cultivated *P.* ×*yedoensis* collected from Japan and Korea belonged to H6 and H7, whereas the U.S. accessions included over six haplotypes. Haplotype H5 containing one U.S. accession (USNM3) was connected to H2 (wild type closely related to H1) and H6 (cultivar type closely related to H1). Haplotype H8 of one U.S. accession (USNM5) was also connected to H7 (Korean and U.S. accessions) and H9 (U.S. accessions). The haplotype H6 included the typical *P.* ×*yedoensis* 'Somei-yoshino' planted in the late 19th century at the Koishikawa Botanical Gardens (University of Tokyo, Japan), majority of landscaping cultivated *P.* ×*yedoensis* trees from Korea (Seoul and Jinhae), and some accessions from the United States. Two haplotypes, H10 and H11, which comprised wild *P. yedoensis* and *P. spachiana* f. *ascendens* from Jeju Island, were distantly related to the main haplotype network by eight inferred or missing haplotypes. These two haplotypes represent a reverse direction of hybridization in the origin of wild *P. yedoensis* from Jeju Island; i.e., *P. serrulata*/*P. sargentii* as maternal and *P. spachiana* f. *ascendens* as paternal parent. The wild haplotypes (H1, H2, H3, and H4) were separated from the primarily cultivated haplotypes (H5 through H9) based on *rpl*16 (A/T position at 1592) and *trn*S-G (A/T position at 4114) (Fig. 2).

A total of five (six including possibly misidentified individual of haplotype H4) cpDNA haplotypes were found in wild *P. yedoensis* in Jeju Island, reflecting the chloroplast diversity of *P. spachiana* f. *ascendens*, which served as its maternal parent. Two accessions of wild *P. yedoensis* (92 and Jeju06) had haplotype H1 together with the putative maternal parent species of *P. spachiana* f. *ascendens* (809) from Jeju Island. Most accessions of wild *P. yedoensis* (except two accessions collected around Kwaneum Temple) had haplotype H2 and H3, derived from the H1 and H2 haplotype, respectively. Haplotype H4, derived from H3, contains one accession of cultivated *P.* ×*yedoensis* Jeju12, which was presumably wild *P. yedoensis*, but possibly misidentified as cultivated *P.* ×*yedoensis* (see more discussion later). The H2, H3, and H4 haplotypes found in wild *P. yedoensis* were interconnected closely and were derived from the H1 haplotype. However, they were separated from the haplotypes of typical *P.* ×*yedoensis* 'Somei-yoshino' from the Koishikawa Botanical Gardens, Japan (H6), and its derived haplotype H7 from Korea (Jinhae and Jeju Island). The spatial distribution of wild *P. yedoensis* haplotypes reflected their genealogical relationships in the network. The haplotype H1 and H2 included accessions scattered all around Mt. Halla, whereas two derived haplotypes from H2, i.e., H3 and H4, occurred in geographical proximity, the north and southeast facing slopes of Mt. Halla. The haplotype H3 included three accessions (wild *P. yedoensis* Jeju01, Jeju10, *P. spachiana* f. *ascendens* 832) collected from the northern slope and two accessions (*P. yedoensis* Jeju07 and Jeju08) from the southeastern slope. Haplotype H4 also included one accession (presumably wild *P. yedoensis* Jeju12) from the northern slope of the mountain.

Discussion

Because of the nearly indistinguishable morphological similarities between wild *P. yedoensis* on Jeju Island, Korea, and cultivated *P.* ×*yedoensis*, heated debate over their taxonomic identities and origins has continued over several decades. Roh et al. (2007) initially suggested that wild *P. yedoensis* from Jeju Island is sufficiently different from the cultivated *P.* ×*yedoensis* (recognized as yoshino cherry hybrids primarily sampled from the U.S. National Arboretum and the National Park Service, and included other cultivars, such as 'Akebono', 'Amagi Yoshino', and 'Mikado Yoshino') based on the haplotype of *rpl*16 and *trn*L-F and inter simple sequence repeat (ISSR) markers. However, their conclusions were poorly verified by two cpDNA sequences (i.e., *rpl*16 gene and *trn*L-F intergenic spacer). In that study, two haplotypes (TA and AA) were identified within wild *P. yedoensis* sampled from Jeju Island. One of two wild *P. yedoensis* haplotypes (AA) was shared by majority of cultivated *P.* ×*yedoensis* hybrids from Japan and the United States. The other haplotype (TA) was also shared by cultivated *P.* ×*yedoensis* (the cultivar Akebono). Based on these haplotype patterns, it may well be concluded that wild *P. yedoensis* and cultivated *P.* ×*yedoensis* are taxonomically identical. However, the authors did not make this point. Surprisingly in

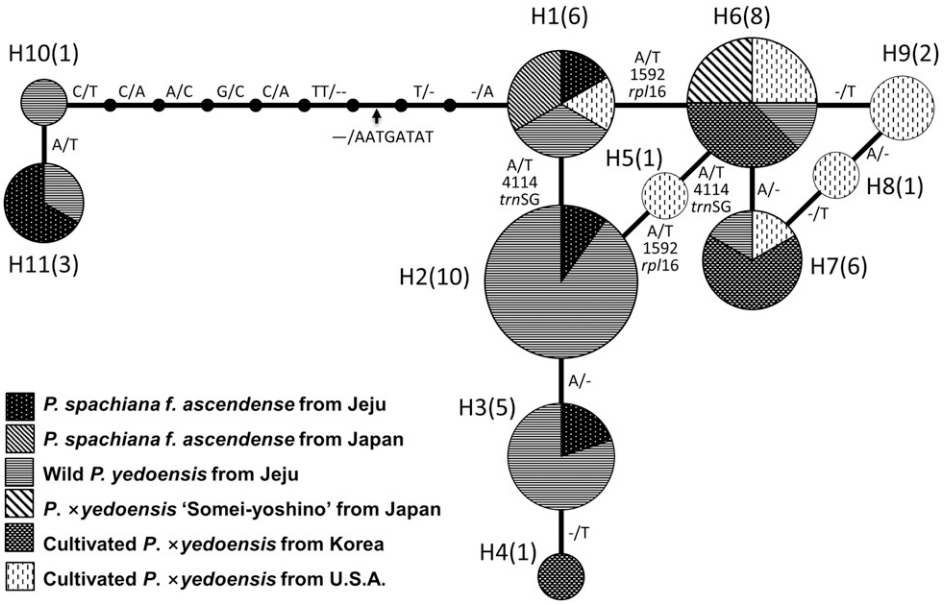

Fig. 2. A chloroplast haplotype network of wild *P. yedoensis*, cultivated *P.* ×*yedoensis* including 'Somei-yoshino' from the United States, Japan, and South Korea; and *P. spachiana* f. *ascendens* from South Korea (Jeju Island) and Japan. Each haplotype is represented by a circle, where the proportion and taxon of individuals possessing each haplotype are illustrated. Size of circles is proportional to the number of accessions marked with each haplotype number in parenthesis.

Table 3. Chloroplast DNA (cpDNA) haplotypes found among 44 accessions (93 individuals) of wild *Prunus yedoensis*, cultivated *P.* ×*yedoensis* including 'Somei-yoshino', and *P. spachiana* f. *ascendens*. Each haplotype includes accessions with identical sequences across all cpDNA regions. The individuals collected from the same location were pooled together and treated as one accession in case they reveal the same sequences. The number of individuals pooled under same accession is marked in parentheses.

Haplotype	Accessions (no.)	Accessions
H1	6	Wild *P. yedoensis* 92 (1), Jeju06 (1)
		Cultivated *P.* ×*yedoensis* USNM2 (2) (United States)
		P. spachiana f. *ascendens* 809 (1) from Jeju Island (Korea),
		P. spachiana f. *ascendens* 377 (1), 382 (1) (Japan)
H2	10	Wild *P. yedoensis* 3-A (1), 814 (1), 872 (1), 873 (1), 879 (1), Jeju03 (1), Jeju04 (1), Jeju05 (1), Jeju09 (1)
		P. spachiana f. *ascendens* 857 (1) from Jeju Island (Korea)
H3	5	Wild *P. yedoensis* Jeju01 (1), Jeju07 (1), Jeju08 (1), Jeju10 (1)
		P. spachiana f. *ascendens* 832 (1) from Jeju Island (Korea)
H4	1	Cultivated *P.* ×*yedoensis* Jeju12 (2) (Korea)
H5	1	Cultivated *P.* ×*yedoensis* USNM3 (3) (United States)
H6	8	Wild *P. yedoensis* 833 (1)
		P. ×*yedoensis* 'Somei-yoshino' cherry KO4981 (1), KO5041 (1) (Japan)
		Cultivated *P.* ×*yedoensis* Jinhae2 (8), Seoul1 (12), Seoul2 (5) (Korea)
		Cultivated *P.* ×*yedoensis* USAU (3), USNM4 (3) (United States)
H7	6	Wild *P. yedoensis* Jeju02 (1)
		Cultivated *P.* ×*yedoensis* Jeju11 (5), Jeju13 (3), Jinhae1 (4), Jinhae3 (4) (Korea)
		Cultivated *P.* ×*yedoensis* USNM6 (1) (United States)
H8	1	Cultivated *P.* ×*yedoensis* USNM5 (4) (United States)
H9	2	Cultivated *P.* ×*yedoensis* USNA (2), USNM1 (4) (United States)
H10	1	Wild *P. yedoensis* 3-1 (1)
H11	3	Wild *P. yedoensis* 875 (1)
		P. spachiana f. *ascendens* 817 (1), 821 (1) from Jeju Island (Korea)

that study, the *trn*L-F haplotype of *P. subhirtella* f. *ascendens* (=*P. spachiana* f. *ascendens*) sampled from Korea did not match with any haplotypes of cultivated *P.* ×*yedoensis* or wild *P. yedoensis* (Roh et al., 2007). This was very unusual because *P. subhirtella* f. *ascendens* is the maternal contributor in the hybrid origins of both taxa. Furthermore, their phenogram of ISSR markers showed that 11 accessions of cultivated *P.* ×*yedoensis* were distantly related to wild *P. yedoensis*, whereas the other four accessions of cultivated *P.* ×*yedoensis* were closely related to wild accessions of *P. yedoensis* and *P. subhirtella* f. *ascendens* sampled from Japan.

In this study, the genetic relationship revealed by cpDNA phylogeny and haplotype network has provided some resolution to the controversy over taxonomy and origin of *P. yedoensis* and cultivated *P.* ×*yedoensis*. We surveyed the variation in cpDNA sequences among accessions of *P. spachiana* f. *ascendens* sampled from both regions of Korea and Japan, in an effort to discern possible independent hybrid origins between them. The present data strongly support that *P. spachiana* f. *ascendens* was the maternal parent in the hybrid origins of both taxa, given the maternal inheritance of chloroplast genome in Rosaceae (Brettin et al., 2000; Kaneko et al., 1986; Matsumoto et al., 1997; Raspé, 2001). All the accessions of wild *P. yedoensis* and cultivated *P.* ×*yedoensis* (except two accessions, wild *P. yedoensis* YE3-1 and YE875) shared the most recent common ancestor with *P. spachiana* f. *ascendens* in Clade 1 (Fig. 1). Within this clade, both taxa were separated into wild or cultivated groups.

One novel finding of this study is that wild *P. yedoensis* and cultivated *P.* ×*yedoensis* could be distinguished based on the contribution of maternal lineages. Clade 1 (Fig. 1) included several accessions with no sequence variation: *P. spachiana* f. *ascendens* from Japan (PE377 and PE382) and Jeju Island (PE809), wild *P. yedoensis* from Jeju (YE92 and Jeju06) and cultivated *P.* ×*yedoensis* from the United States (USNM2). For *P. spachiana* f. *ascendens*, it is expected that some cpDNA sequences occur exclusively within each region (Japan and Jeju Island), whereas others are shared between two regions. Given this expectation, our current results can be explained as follows. Individuals sharing the same cpDNA sequences between Japan and Jeju Island most likely contributed as a maternal donor for the origin of wild *P. yedoensis* and cultivated *P.* ×*yedoensis* in both regions. The remaining accessions of wild *P. yedoensis* and cultivated *P.* ×*yedoensis* in Clade 1 (Fig. 1) display marked sequence differences between wild or cultivated groups. The distinction of wild *P. yedoensis* group from the cultivated one is clear, even though two exceptional accessions in each group, two cultivated *P.* ×*yedoensis* (Jeju12 and USNM3) were nested within the wild group, and two wild *P. yedoensis* (YE833 and Jeju02) were in cultivated group. *P. yedoensis* Jeju12 collected in the forest not far from a roadside in Sancheondan (the site of an ancient altar) was originally acquired as cultivated *P.* ×*yedoensis*, as they are usually planted along the streets and roads, whereas most wild *P. yedoensis* are found in undisturbed natural habitats in Mt. Halla. Presumably wild *P. yedoensis* Jeju12 might be misidentified as cultivated one due in part to obscure collection site.

Within the group of cultivated *P.* ×*yedoensis*, two accessions of wild *P. yedoensis* (YE833 and Jeju02) were collected from the natural forest around Kwaneum Temple in Mt. Halla. Given this unexpected placement of these two wild accessions in the cultivated clade, we suggest the possibility that cultivated flowering cherries (i.e., cultivated *P.* ×*yedoensis*) escaped and spread out to natural habitats in the forest surrounding Kwaneum Temple and adjacent areas, creating mixed stands of both wild and cultivated flowering cherries. There is a possibility that we incorrectly identified these individuals as wild ones rather than cultivated ones. This possibility seems plausible as we confirmed that two other accessions (i.e., Jeju01 and 873) collected from the same area around Kwaneum Temple were wild ones (Fig. 1). However, the possibility that these two individuals are truly wild ones could not be completely ruled out. This is because one of two individuals (i.e., Jeju 02) appears to be quite old, with an estimated age of 140 years, which precedes the cultivation history of cultivated *P.* ×*yedoensis* in Korea. In addition to the dichotomously branching events assumed by phylogenetic tree, the haplotype network (Fig. 2) also reveals the clear distinction among haplotypes between wild *P. yedoensis* and cultivated *P.* ×*yedoensis* with the same caveat that *P. yedoensis* 833 and Jeju02 and *P. yedoensis* Jeju12 were misidentified as wild and cultivated individuals, respectively.

The high variability of haplotypes across 93 individuals (44 accessions) of wild *P. yedoensis*, cultivated *P.* ×*yedoensis* and *P. spachiana* f. *ascendens* (H1 through H11) reflects the high level of genetic diversity in the populations of flowering cherries. Especially, the high level of haplotype diversity in ornamental flowering cherries of cultivated *P.* ×*yedoensis* was likely contributed by centuries of propagation and cultivation, which led to the selection of hundreds of genotypes with diverse origin and ornamental traits (Flower Association of Japan, 1982; Kato et al., 2014). Moreover, extensive plantings for ''cherry viewing'' resulted in the naturalization of species and selections beyond their native ranges and spontaneous hybridizations between native and cultivated species or cultivars (Jefferson and Wain, 1984). The U.S. collections (six haplotypes) exhibited relatively high diversity compared with those from Korea (two haplotypes) and Japan (one haplotype of *P.* ×*yedoensis* 'Somei-yoshino') in the haplotype network. The higher diversity of U.S. collections (22 individuals) was likely caused by replacement trees from diverse sources of plant materials including U.S. commercial nurseries and international sources, as older trees planted around the Tidal Basin are generally similar, although not identical, to each other (Pooler, 1999). The older trees planted around the Tidal Basin, which were shipped from Japan in 1912, either share the same haplotype (H1) as wild *P. yedoensis* in Jeju Island (USNM2) or possess unique cultivar haplotype (H5) (USNM3). Even those older trees with the same shipment year possess different haplotypes, suggesting different genealogical origins. In addition, the earlier shipped accessions have different haplotypes from those of recently planted trees in Washington, DC, further increasing the haplotype diversity of U.S. collections. Given lack of accurate documentation on genealogical records of flowering cherries in the United States and the presence of diverse chloroplast haplotypes in addition to typical *P.* ×*yedoensis* 'Somei-yoshino' cherry (H6), it is plausible that some cultivated flowering cherries are not the same typical clonal type of *P.* ×*yedoensis* 'Somei-yoshino' in Japan and Korea, but they belong to different cultivar groups or the offspring (derivative) of 'Somei-yoshino'. It is required to determine the precise genealogical relationships among flowering cherry cultivars in the United States.

In conclusion, the genetic analyses recognized two distinct groups of wild *P. yedoensis* and cultivated *P.* ×*yedoensis* based on cpDNA sequences. Our current results also confirmed that *P. spachiana* f. *ascendens* served as maternal parent separately for the origin of wild *P. yedoensis* in Jeju Island, Korea, and cultivated forms of *P.* ×*yedoensis* in Japan. The paternal parents for both taxonomic groups are yet to be determined, and their independent origins require future confirmation. Ornamental flowering cherries of cultivated *P.* ×*yedoensis,* specifically collected from the United States exhibited markedly genetic diversity, most likely caused by repeated selections through centuries of propagation and cultivation from uncertain sources. The cpDNA diversity in natural populations of wild *P. yedoensis* on Jeju Island, Korea, is purported as the consequence of recurrent spontaneous bidirectional hybridizations in the wild.

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