Plant Diversity 44 (2022) 170–180

Contents lists available at ScienceDirect

Plant Diversity

journal homepage: http://www.keaipublishing.com/en/journals/plant-diversity/
http://journal.kib.ac.cn

Research paper

Phylogenetic relationship, biogeography, and conservation genetics of endangered *Fraxinus chiisanensis* (Oleaceae), endemic to South Korea

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ARTICLE INFO

Article history:
Received 24 January 2021
Received in revised form
3 June 2021
Accepted 13 June 2021
Available online 26 June 2021

Keywords:
East China Sea land bridge
Endemic species
Fraxinus chiisanensis
Korean Peninsula
Genetic diversity
Biogeography

ABSTRACT

Endemic plants are important for understanding phylogenetic relationships, biogeographical history, and genetic variation because of their restricted distribution and their role in conserving biodiversity. Here, we investigated the phylogenetic relationships of the Korean endemic *Fraxinus chiisanensis* by reconstructing the molecular phylogeny of *Fraxinus* based on two nuclear DNA (nrITS and *phantastica*) and two chloroplast DNA (*psbA-trnH* and *rpl32-trnL*) regions. Within our fossil-calibrated phylogenetic framework, we also inferred the biogeographical history of *F. chiisanensis*. To provide a scientific basis for the conservation of *F. chiisanensis*, we determined the levels of genetic diversity and genetic differentiation in this species. Combining information from nuclear and chloroplast DNA sequence data, our molecular phylogenetic analyses identified *F. chiisanensis* as a genetically distinct unit from its sister group, *Fraxinus platypoda* from Japan. Our molecular dating analyses using nuclear and chloroplast DNA data sets show *F. chiisanensis* diverged from its sister *F. platypoda* in the Early or Middle Miocene and differentiated in the Late Miocene on the Korean Peninsula. Our results suggest that the divergence of *F. chiisanensis* was associated with the submergence of the East China Sea land bridge and enhanced monsoons in East Asia. When compared to *F. platypoda*, *F. chiisanensis* exhibits low genetic diversity within populations and high genetic differentiation among populations. These results help us to understand the evolutionary history of *F. chiisanensis* and to develop a conservation strategy for this species.

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1. Introduction

Endemism is an essential factor in studies of the characteristics and origins of a species (Huang et al., 2011; López-Pujol et al., 2011). Phylogenetic and biogeographical studies of endemic plants can also reveal the evolutionary history of species in a specific region (Zhang et al., 2010). However, most endemic species are threatened due to their restricted distribution. To develop a sustainable conservation strategy for protecting endangered endemic species, it is important to understand the phylogenetic relationships and biogeographical history of the species as well as the level of genetic diversity and the genetic differentiation (Ge et al., 2011; Wen et al., 2016).

Fraxinus L. (Oleaceae), commonly known as ash trees, consists of 48 species inhabiting different temperatures in subtropical regions of Europe, East Asia, and North and Central America (Wallander, 2013). This genus originated in North America and later dispersed into East Asia during the Oligocene (Hinsinger et al., 2013). In a recent infrageneric classification of *Fraxinus*, six sections (*Dipetalae*, *Fraxinus*, *Melioides s.l.*, *Ornus*, *Pauciflorae*, and *Sciadanthus*) were recognized based on the position of inflorescence, the presence/absence of a calyx, the number of petals, and the presence/absence of a wing on the leaf petiole (Wallander, 2008). This classification is also supported by the reconstruction of the phylogenetic relationships of the genus using nuclear and chloroplast DNA (cpDNA) sequence data (Hinsinger et al., 2013). *F. chiisanensis* Nakai, in section *Melioides s.l. sensu* Hinsinger et al. (2013), is endemic to South

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Peer review under responsibility of Editorial Office of Plant Diversity.

https://doi.org/10.1016/j.pld.2021.06.004
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Korea, and occurs along the stream valleys and low slopes of mountains in the southern part of the Korean Peninsula (Chang et al., 2002; Chang, 2007; Lee et al., 2009). *F. chiisanensis* is characterized by a lack of petals and the presence of a panicle from the lateral bud of the previous year, a persistent calyx, and a brownish terminal bud (Chang et al., 2002; Chang, 2007). The results of flavonoid analyses indicate that *F. chiisanensis* is a distinct species with a different chemical composition from *Fraxinus chinensis* and *Fraxinus mandshurica* (Min et al., 2001), which were once considered parental species (Chung, 1957; Lee, 1980; Song et al., 1988). Thus, morphological and chemical evidence indicate that *F. chiisanensis* is a distinct species within the genus. However, the sister group of *F. chiisanensis* is controversial. Previous studies that used nuclear and chloroplast DNA sequence data with limited sampling of *F. chiisanensis* from arboretums reported that *F. chiisanensis* is sister to either *F. platypoda* Oliv. or the *Fraxinus cuspidata* Torr.-*Fraxinus spaethiana* Lingelsh. (synonym of *F. platypoda*) clade (Wallander, 2008; Hinsinger et al., 2013; Nesom, 2014). Therefore, the phylogenetic relationships of *F. chiisanensis* need to be evaluated using broader sampling collected from natural populations.

Human-induced habitat loss has decreased population sizes of *Fraxinus chiisanensis*, which has recently been designated an endangered species on the IUCN Red List of Threatened Species (Kim et al., 2016). Most of South Korea's conservation efforts have been solely directed at endangered species whose populations have declined sharply or whose survival is at risk (NIBR, 2012). A preliminary study of *F. chiisanensis* populations using 15 allozyme loci reported that genetic diversity within *F. chiisanensis* is low and genetic differentiation among populations is high (Lee et al., 2009). However, some allozymes deteriorate rapidly after collecting plant leaves in the field and, thus, many loci are not detectable (Wendel and Weeden, 1989). Also, it is important to assess exact levels of genetic variation within and among populations in *F. chiisanensis* via comparison with closely related species (Qiu et al., 2009a, b; Chung et al., 2014).

Two theories have been proposed to explain the migration routes of endemic species in South Korea. One theory posits that a refuge existed mainly in northeastern China and these populations colonized South Korea via the Baek-du-dae-gan, the main Korean mountain range that spans most of the Peninsula from the north to south and acted as a refuge during the Last Glacial Maximum (LGM) (Choi, 1998; Chung et al., 2006, 2017). The second hypothetical route is the East China Sea (ECS) land bridge between East China-South Japan and southern South Korea (Qiu et al., 2009a, b; Qi et al., 2014; Lee et al., 2016). This migration is related to the expansion across the continental shelf that emerged in the ECS as a result of an 85–130-m decrease in the sea level during the LGM (Millien-Parra and Jaeger, 1999). However, these migration routes through the Korean Peninsula were determined using terrestrial herbs and shrubs; few woody plants have been examined. Furthermore, biogeographical study of *F. chiisanensis* has not been conducted.

Most biogeographical studies of plants have been based on cpDNA data, which has revealed genetic variation throughout plant ranges and allowed inferences on evolutionary history (Qiu et al., 2009a; Setoguchi et al., 2006; Sun et al., 2014). However, cpDNA data are unable to provide information about pollen gene flow, a main component forming genetic variation within and among populations (Bai et al., 2010; Liao et al., 2016). One solution to this limitation is to use both nuclear and cpDNA markers. Here, we used two nuclear DNA (nuclear ribosomal internal transcribed spacer region [nrITS]; *phantastica*) and two cpDNA (*psbA-trnH*; *rpl32-trnL* intergenic spacer [IGS]) regions, which have been successfully used in previous molecular studies of *Fraxinus* (Arca et al., 2012; Hinsinger et al., 2013), to investigate the systematics and biogeography of *F. chiisanensis*. Specifically, we aimed to (1) determine the closest relatives of *F. chiisanensis*; (2) infer the divergence events of *F. chiisanensis*; and (3) elucidate the level of genetic diversity and differentiation of this species through comparison with sister species. This study provides a phylogeny, genetic information, and a biogeographical history of *F. chiisanensis*, which together establish a reliable foundation from which to make decisions regarding the conservation of this species.

2. Materials and methods

2.1. Plant materials and sampling strategy

A total of 70 individuals representing five extant populations of *Fraxinus chiisanensis* were collected in South Korea over the entire geographic range of the species. Because the species is rare and endangered, sampling a large number of individuals was not possible in most populations. Thus, fresh leaves of 10–20 individuals were randomly collected from each population, depending on the availability of adult trees and the population sizes of each location. To compare the level of genetic variation in *F. chiisanensis* to that of close relatives, we also sampled 133 individuals from nine populations of *F. platypoda* (4 populations/63 individuals) and *F. chinensis* (5/70) in South Korea and Japan (Table 1). We also included three individuals of *F. platypoda* from China. To assess the phylogenetic relationships of *F. chiisanensis*, we analyzed the *Fraxinus* species in a broad phylogenetic framework. This data set included 46 species representing six sections of *Fraxinus sensu* Wallander (2008) and Hinsinger et al. (2013). For molecular dating analyses, we included 42 species, excluding four from which we could not obtain nuclear and/or cpDNA regions used in this study from NCBI. Based on previous phylogenetic studies (Wallander, 2008; Hinsinger et al., 2013; Kim et al., 2019), we also included three species (*Olea europaea* L., *Osmanthus fragrans* Lour., and *Ligustrum vulgare* L.) of Oleaceae as outgroups. The sampled taxa and GenBank accession numbers of all species are listed in Table S1.

2.2. Laboratory experiments

Total genomic DNA was extracted from silica-dried leaf material or herbarium specimens using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The entire nrITS region (ITS1-5.8S-ITS2) was amplified using primers ITS1 and ITS4 (White et al., 1990). The *phantastica* region was amplified using primers Phan-F-M13 and Phan-R-M13 (Hinsinger et al., 2013). Two cpDNA regions were used: (1) the *rpl32-trnL* IGS was amplified using the primers rpL32-F and trnL (Shaw et al., 2007); and (2) the *psbA-trnH* IGS was amplified with the primers psbAF and trnHR (Sang et al., 1997).

The target regions of DNA were amplified from 200 ng of genomic DNA in a 25 μL mixture with a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA). Thermocycling conditions were as follows: 2 min at 95 °C, 35 cycles of 20 s at 95 °C (denaturation), 40 s at 52 °C (annealing temperature for nrITS and *psbA-trnH*) or 56 °C (for *phantastica* and *rpl32-trnL*), and 1 min 20 s at 72 °C (extension), with a final cycle of 10 min at 72 °C. The amplified DNA samples were purified for sequencing using a PCR purification kit (Qiagen) according to the manufacturer's instructions. All PCR products were directly sequenced using the amplification primers on an ABI3730 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Table 1
Population localities and sample size of *Fraxinus chiisanensis*, *F. platypoda*, and *F. chinensis* surveyed for nrITS, *phantastica*, and two cpDNAs (*psbA-trnH* and *rpl32-trnL*).

Species	Locality	Latitude (N)	Longitude (E)	Abbreviation	*N*
Fraxinus chiisanensis	Mt. Deogyu, Seoljeon-myeon, Muju-gun, Jeollabuk-do, S Korea	35° 52′ 10.7″	127° 46′ 26.8″	CS_DY	10
	Mt. Kagho, Sangchon-myeon, Yeongdong-gun, Chungcheongbuk-do, S Korea	36° 3′ 40.9″	127° 51′ 47.4″	CS_KH	10
	Mt. Minjuji, Yonghwa-myeon, Yeongdong-gun, Chungcheongbuk-do, S Korea	36° 2′ 11.3″	127° 52′ 23.4″	CS_MJ	20
	Mt. Jiri, Macheon-myeon, Hamyang-gun, Gyeongsangnam-do, S Korea	35° 18′ 7.3″	127° 30′ 55.9″	CS_JR	20
	Mt. Baekun, Okryong-myeon, Kwangyang-si, Jeollanam-do, S Korea	35° 6′ 12.5″	127° 39′ 17.0″	CS_BU	10
	Mean				14.0
	Species level				70
F. platypoda	Mt Gouzen, Oita Prefecture, Kyushu, Japan	33° 11′ 40.7″	130° 52′ 50.5″	PL_OI	16
	Ooyamasawa, Saitama Prefecture, Honshu, Japan	35° 58′ 11.9″	138° 45′ 40.8″	PL_SA	16
	Yotagiri Riv. Nagano Prefecture, Honshu, Japan	35° 40′ 44.2″	137° 51′ 18.7″	PL_NA	15
	Mt. Gangaharasuriyama Yamanashi Prefecture, Honshu, Japan	35° 41′ 24.6″	138° 52′ 51.5″	PL_YA	16
	Mean				15.8
	Species level				63
F. chinensis	Mt. Deogyu, Seoljeon-myeon, Muju-gun, Jeollabuk-do, S Korea	35° 53′ 1.9″	127° 46′ 36.4″	CH_DY	10
	Mt. Kagho, Sangchon-myeon, Yeongdong-gun, Chungcheongbuk-do, S Korea	36° 3′ 33.0″	127° 50′ 46.7″	CH_KH	10
	Mt. Minjuji, Yonghwa-myeon, Yeongdong-gun, Chungcheongbuk-do, S Korea	36° 2′ 22.9″	127° 50′ 56.9″	CH_MJ	20
	Mt. Jiri, Macheon-myeon, Hamyang-gun, Gyeongsangnam-do, S Korea	35° 21′ 10.9″	127° 30′ 55.9″	CH_JR	20
	Mt. Baekun, Okryong-myeon, Kwangyang-si, Jeollanam-do, S Korea	35° 5′ 23.8″	127° 35′ 57.2″	CH_BU	10
	Mean				14
	Species level				70

2.3. Phylogenetic analyses

Complementary DNA sequences were assembled for each individual using DNA Baser v.4 (http://www.DnaBaser.com) to evaluate chromatograms for confirmation of base calls and to edit contiguous sequences. A multiple sequence alignment was performed with MAFFT v.6 (Katoh et al., 2019) using the default alignment parameters.

The incongruence length difference (ILD) test was used to assess data congruency between nuclear and cpDNA data sets (Farris et al., 1995) using PAUP* 4.0b10 (Swofford, 2002) and 1000 heuristic search replications. The ILD test indicated significant incongruence ($P = 0.012$) between the two data sets (nuclear and cpDNA). Because of the stochastic manner in which lineages sort during speciation and hybridization (Maddison, 1997; Nakhleh, 2010), it is common that gene trees differ in topology from each other. In the current study, the combined data set was better resolved for the phylogenetic relationship of *Fraxinus* than either individual analysis although some nodes were still weakly supported. Furthermore, supported (bootstrap support value > 80) incongruent clades were not found upon comparison of parsimony bootstrap consensus trees generated from nuclear and cpDNA data sets. Therefore, for our phylogenetic analyses of *Fraxinus*, we chose to combine two data sets with their weakly-incongruent trees, as suggested by Nie et al. (2008) and Kim et al. (2015).

Phylogenetic reconstructions of the combined sequences were performed by maximum parsimony (MP), maximum likelihood (ML), and Bayesian inference (BI) analyses. For MP analyses, all characters and character states were weighted equally and unordered. The most parsimonious trees were searched with a heuristic algorithm comprising tree bisection-reconnection, branch swapping, MULPARS, and the alternative character state in PAUP* 4.0b10. Bootstrap analyses (PBS; 1000 pseudoreplicates) were conducted to examine the relative level of support for individual clades on the cladograms of each search (Felsenstein, 1985).

Before running ML and BI analyses, the models of nucleotide substitution were selected based on the Akaike information criterion (AIC; Akaike, 1974) as implemented in MODELTEST v.3.1 (Posada and Crandall, 1998). The models were as follows: GTR + I + G (nrITS and cpDNA) and HKY + G (*phantastica*). For ML analysis of the combined nuclear and cpDNA data set, the IQ-TREE web server (http://iqtree.cibiv.univie.ac.at) was used. Support value (LBS) of branches was calculated with 1000 replicates of rapid bootstrap (Stamatakis et al., 2008).

BI analysis of the combined data set was conducted using MrBayes v.3.12 (Ronquist et al., 2012). Four chains of the Markov chain Monte Carlo (MCMC) inference were run simultaneously, with sampling every 1000 generations for a total of 20,000,000 generations. The first 5,000,000 generations (i.e., 5000 trees) were treated as the "burn-in" of the chains and were deleted. We plotted the log-likelihood scores of sample points against generation time using Tracer v.1.6 (Rambaut and Drummond, 2014) to ensure that stationarity was attained after the first 5,000,000 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium (Huelsenbeck and Ronquist, 2001). In addition, we used AWTY (Nylander et al., 2008) to compare the split frequencies from the different runs and to plot cumulative split frequencies to ensure that stationarity was reached. A maximum *a posteriori* tree was constructed by summarizing the remaining trees from parallel runs in a majority rule consensus tree, yielding the posterior probability (PP) values for each clade.

2.4. Estimation of divergence time

To estimate the divergence time for crown and stem nodes of *Fraxinus chiisanensis* based on the nuclear (nrITS and *phantastica*) and cpDNA (*psbA-trnH*; *rpl32-trnL* IGS) sequence data, we used BEAST v.1.5.4 (http://beast.bio.ed.ac.uk; Drummond and Rambaut, 2007). The BEAUti interface was employed to generate input files for BEAST, in which the GTR + I + G (for both nuclear and cpDNA) model was applied with a birth-death speciation tree prior and an uncorrelated lognormal molecular clock model. Posterior distributions of parameters were approximated using two independent MCMC analyses of 100,000,000 generations with a 10% burn-in. The results were verified using Tracer v.1.6 to ensure that plots from the two analyses converged on the same area and then combined. The samples from the posterior analysis were summarized on a maximum clade credibility tree, which had the maximum sum of posterior probabilities on its internal nodes, using TreeAnnotator v.1.5.4 (Drummond and Rambaut, 2007) with the posterior probability limit set to 0.5, summarizing the mean node heights. Means and 95% higher posterior densities (HPDs) of age estimates were obtained from the combined outputs using Tracer. The results were visualized using FigTree v.1.4.2 (Rambaut, 2014).

Two calibration points were used to estimate the divergence in *Fraxinus* phylogeny. Fossils of *Fraxinus* members are readily recognizable according to their winged samaras (Hably et al., 2000; Jung and Lee, 2009). The crown age of *Fraxinus* was constrained to be 44 (±3.5) million years ago (mya) with normal distribution based on the samaras of *Fraxinus wilcoxiana* Berry, which are the oldest fossils described from the Middle Eocene of North America (Call and Dilcher, 1992). In addition, the crown age of European *Fraxinus* species was calibrated with a log-normal distribution having an offset of 23 mya, a mean of 0.1, and a standard deviation of 1.0 based on the fossil pollens of *Fraxinus praedicta* Heer, described from the Early Miocene of Europe and related to the extant *Fraxinus angustifolia s.l.* (Palamarev, 1989). Both fossil calibrations were also used to understand the biogeographical history of *Fraxinus* through molecular clock analysis (Hinsinger et al., 2013).

In addition to the above divergence time estimates, the nature of divergence between *F. chiisanensis* and *F. platypoda* was further investigated using individual data sets. This data set comprised all haplotypes of nrITS, *phantastica*, and cpDNA revealed in *F. chiisanensis* and *F. platypoda*, and *F. cuspidata* as outgroup. To estimate nucleotide mutation rates of nrITS, *phantastica*, and cpDNA, we applied the same substitution model, tree prior and prior distribution models, and calibration points. We performed two independent runs with 50,000,000 generations each, with parameters sampled every 1000 generations and a burn-in of 10% were performed using BEAST v.1.5.4. According to the analyses, mutation rates of nrITS (2.2×10^{-3} substitutions/site/mya [s/s/mya]), *phantastica* (5.4×10^{-4} s/s/mya), and cpDNA (8.8×10^{-4} s/s/mya) were obtained for *Fraxinus*. These mutation rates were used to estimate the divergence times between *F. chiisanensis* and *F. platypoda*.

2.5. Genetic variation analyses

Using DnaSP v.6.12 software (Rozas et al., 2017), ribotypes, phantypes, and chlorotypes of *Fraxinus chiisanensis*, *F. platypoda*, and *F. chinensis* were determined based on variations in nrITS, *phantastica*, and cpDNA sequences, respectively, with gaps excluded. All types of diversity (ribotypes [Rd], phantypes [Pd], chlorotypes [Cd]; Nei and Tajima, 1983) and nucleotide diversity per site (Pi; Nei, 1987) within populations and species were determined from the nrITS, *phantastica*, and cpDNA data sets. We also used PERMUT to estimate two parameters of genetic differentiation (*Gst* and *Nst*) within the species (Pons and Petit, 1996). The *Gst* (coefficient of genetic variation over all populations; Nei, 1987) was calculated solely based on haplotype frequencies, whereas *Nst* (the equivalent coefficient, considering sequence similarities between haplotypes) considered the genetic relationships among haplotypes. The values of *Gst* and *Nst* were compared with 1000 permutation tests for the presence of phylogeographical structure. An analysis of molecular variance (AMOVA) was performed with Arlequin v.3.0 (Excoffier et al., 2005) based on the Euclidean distance matrix to determine the partitioning of variation within and between populations. Significant levels of variance components were assessed by 1000 permutations. The evolutionary relationships between sequence types were constructed by coalescent simulation using the median-joining model implemented in NETWORK v.4.5 (http://www.fluxus-engineering.com; Bandelt et al., 1999).

3. Results

3.1. Sequence characteristics and geographical distribution of sequence types

The nrITS data set resulted in 602 bp of aligned sequence from 70 individuals (five populations) of *Fraxinus chiisanensis* with two variable sites. Three ribotypes were distinguished within the nrITS matrix. The *phantastica* data set had 596 bp of aligned sequence with 13 variable sites. Eleven phantypes were identified in *F. chiisanensis*. The combined cpDNA data set resulted in 1397 bp of aligned sequence with six variable sites and four chlorotypes. The combined data set of the four DNA regions was 2595 bp in length and contained 21 nucleotide substitutions. Based on these polymorphic sites, 21 genotypes were identified in *F. chiisanensis* (Table S2). The number of genotypes of *F. chiisanensis* was lower than that of *F. platypoda* (number of genotypes = 49) and *F. chinensis* (26) (Table S3).

The geographic distribution and frequencies of three ribotypes, 11 phantypes, and four chlorotypes in five populations of *Fraxinus chiisanensis* are shown in Table S4. Of these, one ribotype (R3), six phantypes (P4, P6–P8, P10, P11), and one chlorotype (C3) were found in a single population and the remaining types were found in at least two populations. Unlike *F. platypoda* and *F. chinensis* (Tables S5 and S6), no single types, except C1, dominated across the entire distributional range of *F. chiisanensis* (Table S4).

3.2. Phylogenetic analyses

The combined data matrix consisted of 2760 characters (nrITS, 616; *phantastica*, 605; *psbA-trnH*, 561; *rpl32-trnL*, 978). There were 859 (31.1%) variable sites, of which 447 (16.2%) were potentially informative. Maximum parsimony analysis of the 21 *Fraxinus chiisanensis* genotypes with 46 *F. platypoda* genotypes, 26 *F. chinensis* genotypes, and 43 *Fraxinus* species (51 accessions) and three outgroup species of Oleaceae resulted in >10,000 parsimonious trees with a length of 2005 steps, a consistency index of 0.531, and a retention index of 0.871. The topology of the ML and BI trees (not shown) were identical to the MP strict consensus (Fig. 1). The MP tree showed that six sections of *Fraxinus* were reciprocally formed clades. The accessions of *F. chiisanensis* and *F. chinensis* formed clusters according to traditionally recognized species. However, *F. platypoda* accessions from China have a strong affinity to *F. mandshurica* instead of the Japanese accessions. *F. chiisanensis* was found to be a sister of *F. platypoda* from Japan (PBS = 92, LBS = 89, PP = 0.99). Our MP tree showed that *F. cuspidata* and *F. chiisanensis-F. platypoda* are monophyletic and this clade was sister to the remaining of *Melioides s.l.*, but with low support (Fig. 1).

3.3. Molecular dating analyses

The chronogram and results of divergence time estimation for *Fraxinus chiisanensis* and *F. platypoda* are shown in Fig. 2. Based on the nuclear DNA data set, the divergence age between *F. chiisanensis* and its sister *F. platypoda* was estimated at 22.7 mya (95% HPD = 12.1–34.5 mya; node N3), in the Early Miocene. The crown nodes of *F. chiisanensis* and *F. platypoda* were estimated to have occurred at 6.7 mya (95% HPD = 3.0–11.2 mya; node N1) and 6.4 mya (95% HPD = 2.9–10.4 mya; node N2), in the Late Miocene, respectively. Divergence time estimates based on the cpDNA data set were younger or similar as those from the nuclear DNA data set. The split between *F. chiisanensis* and *F. platypoda* was estimated at 12.1 mya (95% HPD = 5.5–19.2 mya; node C3). The crown nodes of *F. chiisanensis* and *F. platypoda* were estimated at 5.5 mya (95% HPD = 1.1–9.0 mya; node C1) and 7.4 mya (95% HPD = 2.7–13.0 mya; node C2), in the Late Miocene, respectively.

Based on the mutation rates, estimates of divergences of *F. chiisanensis* and *F. platypoda* differed between nrITS, *phantastica*, and cpDNA data sets. Mean estimated divergence dates between *F. chiisanensis* and *F. platypoda* in the cpDNA data (9.0 mya [95% HPD = 3.0–15.5 mya]) were younger than those of nrITS (15.9 mya [95% HPD = 7.3–26.1 mya]) and *phantastica* data (20.9 mya [95%

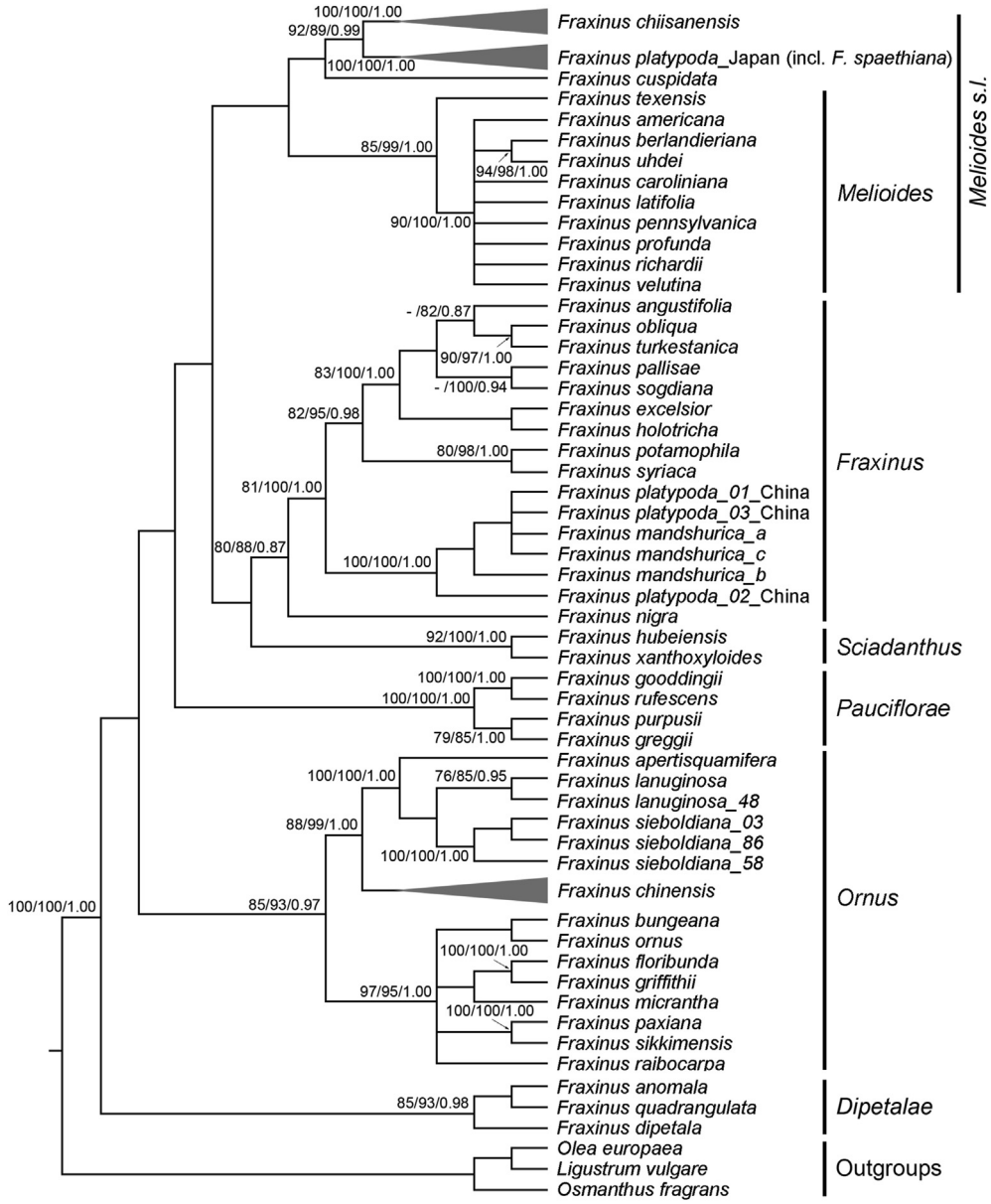

Fig. 1. Strict consensus tree of 10,000 most parsimonious trees from the parsimony analysis of the combined sequence data from the two nDNA (nrITS and *phantastica*) and the two cpDNA (*psbA-trnH* and *rpl32-trnL*) regions. The numbers near nodes indicate corresponding support values (maximum parsimony bootstrap [PBS]/maximum likelihood bootstrap [LBS]/Bayesian posterior probability [PP]); a dash (–) indicates that a node did not receive >75% BP in the maximum parsimony analysis. Sections of *Fraxinus* classification follows Hinsinger et al. (2013).

HPD = 10.5–34.5 mya]). Although the cpDNA estimate was younger than that of the nuclear data set, the HPDs of time estimates on both data sets overlap.

3.4. Genetic variation analyses

Levels of diversity of three sequence types (Rd, Pd, Cd) and nucleotide diversity (*Pi*) were investigated using the nrITS, *phantastica*, and cpDNA regions across all five populations of *Fraxinus chiisanensis* (Table 2). The Rd and Pd showed the highest diversity in the CS_MJ population of *F. chiisanensis*, while the Cd exhibited the relatively high diversity in CS_KH and CS_MJ. At the species level, the Rd and Cd of *F. chiisanensis* (Rd = 0.540, Cd = 0.653) were lower than those of *F. platypoda* (Rd = 0.744, Cd = 0.886), while higher than those of *F. chinensis* (Rd = 0.164, Cd = 0.499). The Pd of *F. chiisanensis* (Pd = 0.867) was higher than that of *F. platypoda* (Pd = 0.531) and *F. chinensis* (Pd = 0.693). The same pattern was also observed in a comparison of the nucleotide diversity of *F. chiisanensis*, *F. platypoda*, and *F. chinensis* (Table 2).

Among five populations of *Fraxinus chiisanensis*, the values of genetic differentiation (*Gst*) were 0.403 for nrITS, 0.279 for *phantastica*, and 0.405 for cpDNA regions (Table S7). In contrast, low genetic differentiation was detected among populations of *F. platypoda* (*Gst* = 0.073 for nrITS, 0.019 for *phantastica*, and 0.042 for cpDNA regions) and *F. chinensis* (0.038 for nrITS, 0.088 for *phantastica*, and 0.099 for cpDNA regions). All three *Fraxinus* species showed significant phylogeographic structures at the species level (*Nst* > *Gst*; all *P* values < 0.01; Table S7).

Analysis of molecular variance (AMOVA) showed significant ($P < 0.0001$) genetic differentiation both among and within

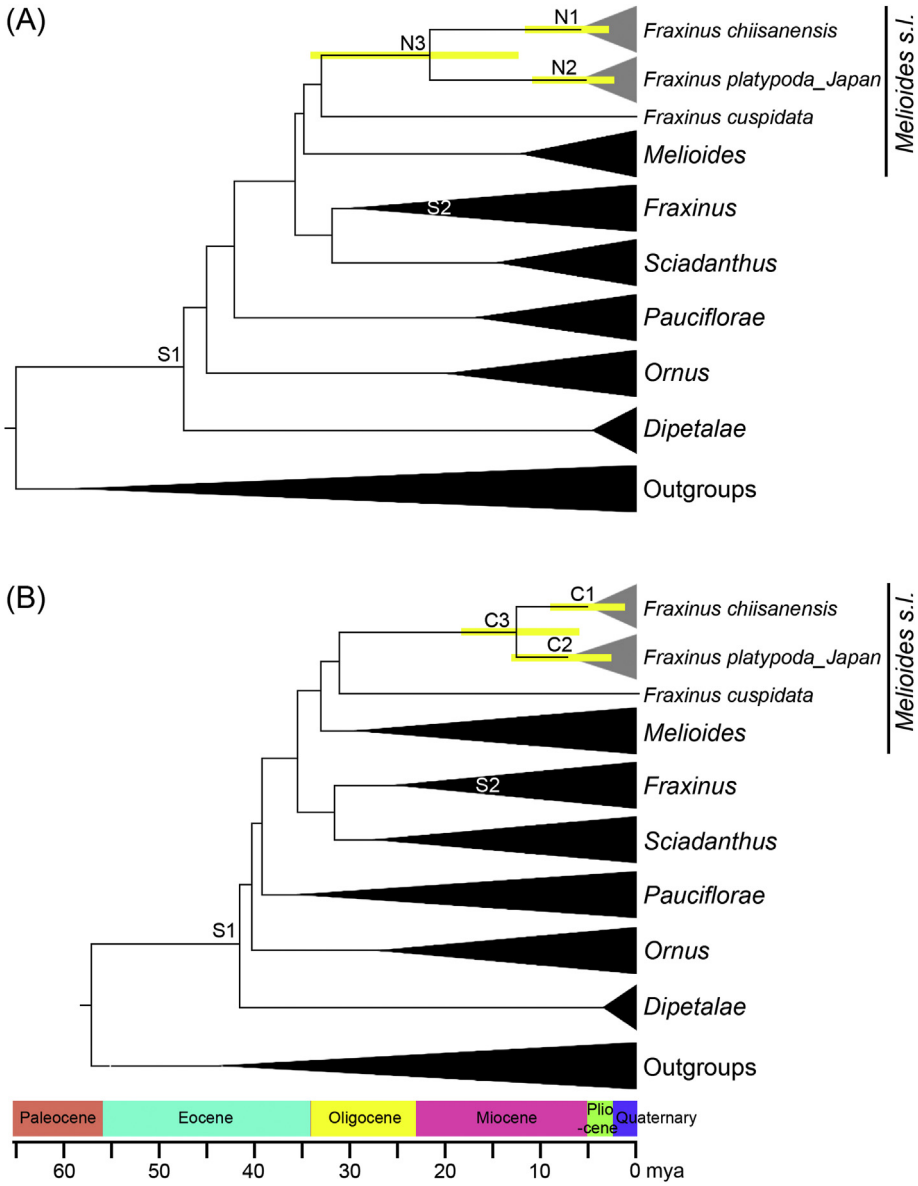

Fig. 2. Chronogram showing divergence times estimated in BEAST based on (A) nuclear and (B) cpDNA sequence data. Yellow bars represent 95% high posterior density for the estimated mean dates. Nodes labeled S1 and S2 are calibration points used in the analysis (for more details, see materials and methods). Nodes labeled N1–N3 and C1–C3 indicate nodes of interest. Sections of *Fraxinus* classification follows Hinsinger et al. (2013).

populations in the three species (Table 3). The AMOVA partitioned 45.9%, 61.5%, and 42.3% of total variation among populations of *F. chiisanensis* based on nrITS, *phantastica*, and cpDNA, respectively and 54.1%, 38.5%, and 57.7% within populations, respectively. In contrast, an examination of the proportion of diversity among and within populations of the other two species indicated that most of the variation (nrITS = 86.8%, *phantastica* = 98.0%, and cpDNA = 99.5% in *F. platypoda*; nrITS = 97.9%, *phantastica* = 93.6%, and cpDNA = 95.2% in *F. chinensis*) occurred within populations, with only <14% occurring among populations (Table 3).

The genetic relationship among the populations of *Fraxinus chiisanensis* was assessed by minimum spanning network analyses based on the sequence variation of the nrITS, *phantastica*, and cpDNA data sets. The reconstructed network of ribotypes and chlorophytes exhibited low resolution in the *F. chiisanensis* populations (Fig. S1). In contrast, in network analysis using phantypes, the five populations of *F. chiisanensis* were largely divided into three groups: (I) a group that includes three phantypes (P1, P2, P11) of the CS_JR, CS_MJ, and CS_DY populations; (II) a group that includes four phantypes (P3, P4, P6, P7) of CS_MJ and CS_KH; and (III) a group that includes four phantypes (P5, P8–P10) of CS_MJ, CS_JR, and CS_BU (Fig. 3). The phantypes of the CS_MJ population showed a relatively broad distribution to be included in all groups. The grouping of the *F. chiisanensis* genotypes is associated with the geographical distance between these populations (Fig. S2).

4. Discussion

4.1. *Phylogenetic relationships of* Fraxinus chiisanensis

Our phylogenetic analyses using the combined data set of two nuclear DNA (nrITS and *phantastica*) regions and two cpDNA (*rpl32-trnL* and *psbA-trnH*) regions support the monophyly of *Fraxinus chiisanensis* (PBS = 100, LBS = 100, PP = 1.00) (Fig. 1). Based

Table 2
Sequence polymorphism and genetic diversity within populations of *Fraxinus chiisanensis*, *F. platypoda*, and *F. chinensis* based on nrITS, *phantastica*, and cpDNA (*psbA-trnH* and *rpl32-trnL*).

Populations	N	nrITS				*phantastica*				cpDNAs			
		S	H	Rd	Pi	S	H	Pd	Pi	S	H	Cd	Pi
Fraxinus chiisanensis													
CS_DY	10	0	1	0.000	0.00	2	2	0.467	1.57	1	2	0.356	0.26
CS_KH	10	0	1	0.000	0.00	3	2	0.356	1.79	1	2	0.467	0.34
CS_MJ	20	2	3	0.616	1.21	11	6	0.847	8.57	5	3	0.426	0.84
CS_JR	20	1	2	0.505	0.86	7	4	0.658	2.61	1	2	0.357	0.24
CS_BU	10	0	1	0.000	0.00	1	2	0.533	0.89	1	2	0.356	0.26
Mean	14	0.6	1.6	0.224	0.41	4.8	3.2	0.572	3.09	1.8	2.2	0.392	0.39
Species level	70	2	3	0.540	1.00	13	11	0.867	8.70	6	4	0.653	0.73
F. platypoda													
PL_OI	16	8	6	0.833	6.01	4	4	0.350	1.02	12	7	0.800	1.75
PL_SA	16	9	6	0.683	4,43	2	3	0.342	0.60	12	8	0.758	1.94
PL_NA	15	6	6	0.800	2.80	5	6	0.705	1.60	11	11	0.933	1.88
PL_YA	16	4	4	0.442	1.17	4	7	0.692	1.72	9	9	0.908	1.79
Mean	16	6.8	5.5	0.690	3.33	3.8	5	0.522	1.24	11	8.8	0.850	1.84
Species level	63	18	17	0.744	4.04	8	13	0.531	1.25	20	26	0.886	1.85
F. chinensis													
CH_DY	10	0	1	0.000	0.00	2	3	0.511	0.93	3	2	0.200	0.46
CH_KH	10	0	1	0.000	0.00	3	3	0.600	1.57	3	3	0.378	0.46
CH_MJ	20	10	3	0.353	2.39	3	4	0.595	1.62	6	4	0.658	0.84
CH_JR	20	1	2	0.100	0.17	4	6	0.684	1.80	8	5	0.368	0.95
CH_BU	10	1	2	0.200	0.34	5	5	0.756	2.20	3	3	0.511	0.66
Mean	14	2.4	1.8	0.131	0.58	3.4	4.2	0.629	1.62	4.6	3.4	0.423	0.67
Species level	70	12	5	0.164	0.86	7	12	0.693	1.76	14	10	0.499	0.78

Note: *N*, number of individuals; *S*, number of polymorphic sites; *H*, number of DNA sequence types; *Rd*, ribotype diversity; *Pd*, phantype diversity; *Cd*, chlorotype diversity; *Pi*, nucleotide diversity ($\times 10^{-3}$). Population abbreviations are given in Table 1.

Table 3
Analysis of molecular variance for populations of *Fraxinus chiisanensis*, *F. platypoda*, and *F. chinensis* based on nrITS, *phantastica*, and cpDNA regions.

Species	Source of variation	*d.f.*	nrITS				*phantastica*				cpDNAs			
			Sum of squares	Variance components		$P^{\dagger}$	Sum of squares	Variance components		$P^{\dagger}$	Sum of squares	Variance components		$P^{\dagger}$
				Absolute	%			Absolute	%			Absolute	%	
Fraxinus chiisanensis	Among populations	4	10.8	0.184	45.9	<0.0001	104.2	1.836	61.5	<0.0001	38.9	0.621	42.3	<0.0001
	Within populations	65	14.1	0.216	54.1	<0.0001	74.7	1.149	38.5	<0.0001	84.6	1.301	57.7	<0.0001
F. platypoda	Among populations	3	11.0	0.165	13.2	<0.0001	1.4	0.007	2.0	<0.0001	4.1	0.006	0.5	<0.0001
	Within populations	59	63.6	1.078	86.8	<0.0001	21.6	0.366	98.0	<0.0001	75.2	1.275	99.5	<0.0001
F. chinensis	Among populations	4	2.6	0.010	2.1	<0.0001	4.2	0.037	6.4	<0.0001	9.1	0.068	4.8	<0.0001
	Within populations	65	32.3	0.496	97.9	<0.0001	35.1	0.539	93.6	<0.0001	88.5	1.362	95.2	<0.0001

Note: *d.f.*, degree of freedom; †Levels of significance are based on 1000 interaction steps.

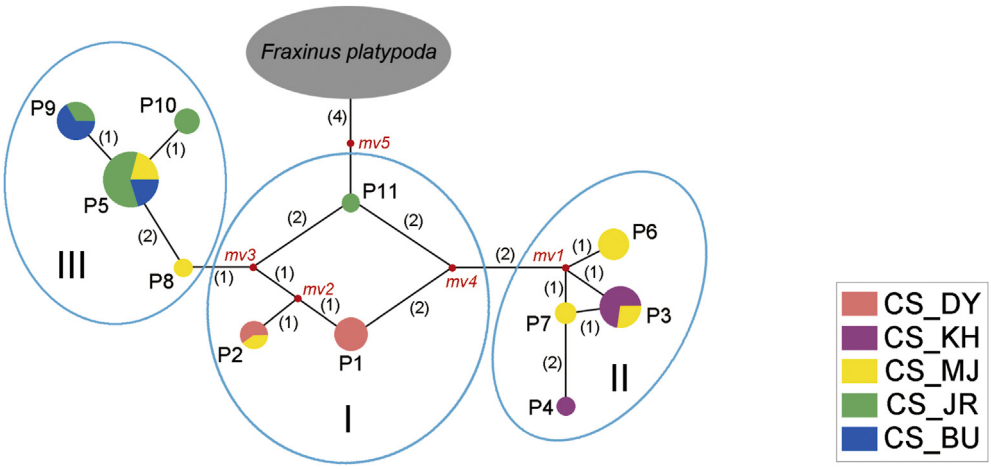

Fig. 3. Minimum spanning network of 11 phantypes in *Fraxinus chiisanensis*. Sizes of colored circles are proportional to genotype frequencies. Median vectors (mv) produced by the network program, representing missing or not sampled genotypes. Population abbreviations are given in Table 1.

on the examination of flowers, apetalous *F. chiisanensis* was believed to be a hybrid between *F. mandshurica* and *F. chinensis* (Chung, 1957; Lee, 1980; Song et al., 1988). However, our molecular phylogenetic analyses do not support the hybrid origin of *F. chiisanensis*. Morphologically, *F. chiisanensis* can be clearly distinguished from *F. mandshurica* and *F. chinensis* by a combination of characters including the presence of a panicle from the leafless lateral bud of previous years, a persistent calyx, and a brownish naked bud (Chang et al., 2002). Thus, morphological and molecular features make *F. chiisanensis* a distinct group within *Fraxinus*.

Within *Fraxinus*, the sister of *F. chiisanensis* is *F. platypoda* from Japan (PBS = 92, LBS = 89, PP = 1.00; Fig. 1). This result is in line with previous studies of the molecular phylogenetic relationships of *Fraxinus* based on limited sampling of these species (Wallander, 2008; Hinsinger et al., 2013). The sister relationship between *F. chiisanensis* and *F. platypoda* is also supported by several morphological characteristics (e.g., apetalous flower, persistent calyx, lateral inflorescence, and samara with a terete seed cavity), although it is difficult to identify morphological synapomorphies for this well-defined clade within *Fraxinus*. The close relationship between *F. chiisanensis* and *F. platypoda* suggests that this species pair should be grouped into the same section in the genus. Since Nakai (1929) suggested that *F. chiisanensis* belonged to the section *Dipetalae*, comprising three *Fraxinus* species from North and Central America, Wallander (2008) proposed that this species should be included in the *Melioides* section based on the lateral inflorescence and samara shape. Later, Hinsinger et al. (2013) also claimed that this species pair should be included in an expanded *Melioides* section based on DNA sequence data. Although we found a close relationship between *F. chiisanensis*-*F. platypoda* and section *Melioides*, the support value for this clade is very low (Fig. 1). The phylogenetic position of *F. chiisanensis*-*F. platypoda* in *Fraxinus* requires further investigation with a more appropriate data set, including a comprehensive sampling of *Melioides s.l.*

The accessions of *Fraxinus platypoda* collected from Japan and China did not form a group according to the taxonomic species (Fig. 1). This disparity between the Japanese and Chinese accessions of *F. platypoda* may have one of two possible explanations. The first is that the Chinese *F. platypoda* was misidentified. This species has been characterized by swollen petiole bases covering the lateral buds (Wallander, 2008). However, this characteristic is also found in other *Fraxinus* species (Wei and Green, 1996). The second explanation is that *F. platypoda* from Japan and China are different species. *F. spaethiana* from Japan is morphologically similar to the Chinese *F. platypoda* and some authors placed the former in synonymy under the latter (e.g., Wei and Green, 1996). However, *F. spaethiana* can be distinguished from the Chinese *F. platypoda* by the absence of densely yellowish villous on the abaxial side of the leaves (Wallander, 2008). According to the phylogenetic species concept, we suggest resurrecting the taxonomic status of *F. spaethiana*.

4.2. *Biogeography of* Fraxinus chiisanensis

Although no studies of East Asian fossils of *Fraxinus* have indicated its biogeographical history in China, Japan, and Korea, there are two hypotheses to explain the speciation and migration on the Korean Peninsula (Bai et al., 2010): (1) A refuge existed mainly in northeastern China and these populations colonized South Korea via the main Korean mountain range (i.e., Baek-du-dae-gan route), which is supported by a study of the widespread, cold-temperate species, *Pinus koraiensis* Sieb. et Zucc. (Kim et al., 2005), *Veratrum album* ssp. *oxysepalum* (Turcz.) Miyabe & Kudo (Kikuchi et al., 2010), and *Lilium cernuum* Kom. (Chung et al., 2017); (2) A refuge existed in southern South Korea from south Japan via the ECS land bridge. This hypothesis is verified by phylogeographic analyses of *Kirengeshoma koreana* Nakai (Qiu et al., 2009b), *Neolitsea sericea* (Blume) Koidz. (Lee et al., 2013), and *Leontopodium japonicum* (Thunb.) Miq. (Lee et al., 2016). Our molecular phylogenetic analyses showed that *F. chiisanensis* populations from South Korea formed a clade with *F. platypoda* from Honshu and Kyushu (Japan), rather than *F. chinensis* and *F. mandshurica*, which mainly occurred in northeastern China (Fig. 1). Based on this phylogenetic relationship and distribution pattern, the ECS land bridge rather than the Baek-du-dae-gan route was the main route for the formation of *F. chiisanensis* in the Korean Peninsula. There are several taxa with divergence patterns similar to *F. chiisanensis* in South Korea (e.g., *K. koreana* [Qiu et al., 2009b] and *Camellia japonica* L. [Ryu et al., 2019]).

The nrITS, *phantastica*, and cpDNA data sets indicate that the sequence types of Korean *Fraxinus chiisanensis* and Japanese *F. platypoda* are both monophyletic lineages (Fig. 3 and S1). The current distribution of *F. chiisanensis* and *F. platypoda* in South Korea and Japan suggest that the geographic separation of a formerly widespread ancestral stock into two isolated groups occurred through submergence of the ECS land bridge. However, this scenario is unlikely considering the divergence time between *F. chiisanensis* and *F. platypoda*. Although divergence time between *F. chiisanensis* and *F. platypoda* differed for the nuclear and cpDNA data sets, the two species were found to have diverged in the Miocene epoch (N3 and C3 in Fig. 2), which is similar to the finding of previous molecular dating analysis of *Fraxinus* (Hinsinger et al., 2013). The divergence time between *F. chiisanensis* and *F. platypoda* is independent of the submergence of the ECS land bridge during the Late Pleistocene. Instead, partial submergence of a large Miocene land bridge in the Chinese, Japanese, and Korean region may have induced the divergence of two *Fraxinus* species in South Korea and Japan (Ota, 1998).

The climate and geological changes that occurred during the Quaternary have profoundly shaped the present distribution and genetic structure of many plant species in warm temperate regions of East Asia. Unlike most plant species in East Asia that diverged in the Quaternary (Li et al., 2008; Qiu et al., 2009a; Chung et al., 2014), our molecular dating analyses based on nuclear and cpDNA data sets suggest that the divergence of *Fraxinus chiisanensis* occurred in the Late Miocene (6.7 mya [N1] and 5.5 mya [C1] in Fig. 2). The existence of *Fraxinus* species on the Korean Peninsula during the Miocene is also supported by fossil evidence (Jung and Lee, 2009), although it is difficult to determine the relationships between these fossil species and extant *F. chiisanensis*. This timing indicates that the divergence within *F. chiisanensis* may have been initiated by the East Asian monsoon, which was enhanced by the continued uplifts of the Himalayas and the Tibetan Plateau (Zheng et al., 2004; Spicer, 2017; Spicer et al., 2020). The enhanced monsoon system was involved in the initial species diversification during the Late Miocene and Pliocene (Ha et al., 2018; Chen et al., 2020; Wang et al., 2020). For example, the divergence of *Forsythia* species in East Asia mainly occurred during the Late Miocene and Pliocene (Ha et al., 2018). The intensification of the East Asian monsoon brought high precipitation in summer during the Late Miocene epoch. As for the relationship between rainfall and diversification of plant species in East Asia, Wang et al. (2011) suggested that annual rainfall is a useful predictor for the species diversification of woody plants. Thus, our results suggest that changing climate conditions and tectonic activities before the Quaternary may have been an important factor in the initial divergence of *F. chiisanensis.*

4.3. *Conservation of* Fraxinus chiisanensis

The nrITS and cpDNA data set analyses of five populations of *Fraxinus chiisanensis* revealed a low degree of sequence type diversity (Rd = 0.540, Cd = 0.653) compared to its sister species, *F.*

platypoda from Japan (Rd = 0.744, Cd = 0.886; Table 2), although the Pd of *F. chiisanensis* showed higher diversity than that of *F. platypoda*. Based on nrITS and cpDNA data sets, the mean value within the population diversity of *F. chiisanensis* (mean Rd = 0.224, Cd = 0.392) was considerably lower than that of *F. platypoda* (mean Rd = 0.690, Cd = 0.850). In general, aside from the population size of a species, the level of genetic diversity in a population is associated with breeding system and evolutionary time (Frankham et al., 2002). Species that are pollinated by the wind and have a long evolutionary history exhibit relatively high genetic diversity. Both *F. chiisanensis* and *F. platypoda* are anemophilous flowers and have a similar time of species diversification (Fig. 2). Therefore, the low genetic diversity detected in *F. chiisanensis* is not related to the breeding system or evolutionary history in the Late Miocene. Instead, the low genetic diversity of *F. chiisanensis* may be associated with human activities in addition to its high genetic differentiation (Table S7). Our finding that *F. chiisanensis* has high population differentiation was reinforced by AMOVA, which showed that a larger proportion of genetic variation exists among *F. chiisanensis* populations than among *F. platypoda* populations (Table 3). Over the last several decades, the extant populations of *F. chiisanensis* have decreased due to human timber collection (Lee et al., 2009). The populations of *F. chiisanensis* are now smaller and more isolated from each other than in the past, especially the DY, KH, and BU populations. Under these conditions, genetic drift may reduce genetic variation in isolated populations. Furthermore, many studies indicate that smaller populations have lower genetic diversity than larger populations due to genetic drift (Frankham, 1996; Fischer et al., 2000).

The main goal of conservation for endemic species is to ensure the existence of a species and to maintain its evolutionary potential by retaining natural levels of genetic diversity (Frankham et al., 2002). In general, there is no alternative to *in situ* conservation for most wild plant species because it continues to expose natural populations to evolutionary processes (Meilleur and Hodgkin, 2004). Therefore, habitat conservation for endangered species, including *Fraxinus chiisanensis*, is critical. However, even in a natural habitat, populations can suffer a rapid loss of genetic diversity that is predicted to have many negative consequences for population viability (Ellstrand and Elam, 1993; Kim et al., 2008a, b). This loss can occur as a result of numerous anthropogenic effects, such as partial habitat destruction, pollution, and climate change. Korean *F. chiisanensis* populations in this study showed lower genetic diversity and higher genetic differentiation than congenic species that occurred in Korea and Japan (Tables 2 and S7). The survival of Korean *F. chiisanensis* is increasingly precarious due to habitat destruction caused by humans. Immediate actions are required to prevent further decreases in genetic variation, a decline in adaptive ability, and an increased risk of species extinction. Therefore, it is important to be aware of *F. chiisanensis* population sizes to implement *in situ* conservation methods.

Ex situ conservation has numerous limitations and is not an alternative to *in situ* conservation (Volis and Blecher, 2010), but it can help to conserve/restore the genetic diversity of an endangered species, especially when accompanied by reintroduction into natural habitats (Menges, 1991; Schwartz et al., 2006). Therefore, *ex situ* conservation oriented towards reintroduction should be applied to Korean *Fraxinus chiisanensis* populations with low genetic diversity within populations undergoing a high level of habitat destruction. *F. chiisanensis* trees mainly reproduce sexually (Wallander, 2008). Therefore, seeds can be harvested and maintained *ex situ*. Because *F. chiisanensis* has low population genetic variation, its total genetic coverage can be reasonably covered by collecting only a few samples per population. The observed high genetic differentiation between Korean populations implies that the extinction of any population will reduce total genetic variability. Thus, every population of *F. chiisanensis* should be considered as a target for *ex situ* conservation. Although translocation can rapidly enlarge the gene flow among populations, it is important to avoid the outbreeding depression that can result from the admixture of individuals from different populations. Each population should be preserved separately in well-designed pools, while individuals from the same population need to be kept in the same pool for genetic enhancement of outcrossing.

Volis and Blecher (2010) proposed 'quasi *in situ* conservation' for endangered plant species, which uses *ex situ* collections in natural or semi-natural environments as a part of a complex *ex situ-in situ* conservation strategy. Moreover, they demonstrate the effectiveness of quasi *in situ* conservation using critically endangered *Iris atrofusca* from Israel (Volis et al., 2009). Thus, to design the most appropriate and effective quasi *in situ* conservation and environmental management strategies for *F. chiisanensis* species, detailed studies of field sampling strategies and reproduction biology should be performed.

Author contributions

J-HK conceived and designed the experiments. CK, D-KK, HS, and J-HK collected plant material. CK performed the experiments and analyzed the data. CK wrote the draft and J-HK revised the draft.

Declaration of competing interest

All authors agreed on the contents of the paper and declared that no competing interests exist.

Acknowledgements

The authors thank Drs. S. Kikuchi, S. Kanetani, and J. Worth (Forestry and Forest Products Research Institute) for help in collecting plant material. This work was supported by grants from the Korea National Arboretum (Grant no. KNA1-1-13,14-1) and Gachon University (Grant no. 2018-0320) to J-H Kim.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pld.2021.06.004.

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