



Sea, wind, or bird: Origin of *Fagus multinervis* (Fagaceae) inferred from chloroplast DNA sequences

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엽록체 염기서열을 통한 너도밤나무(너도밤나무과)의 기원 추론

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ABSTRACT: To elucidate the origin and patterns of establishment of insular plants on Ulleungdo Island, maternally inherited chloroplast DNA, which is useful for tracing seed movements, was used. *Fagus multinervis*, an endemic species that dominated broadleaf deciduous forests on Ulleungdo Island, is an excellent model for such a study. To understand the diversity and spatial distribution of the chloroplast haplotypes of *F. multinervis*, nucleotide sequences of the *psbA-trnH* region were determined from 144 individuals sampled throughout the island. Results of a phylogenetic analysis of the region with close relatives of *F. multinervis* suggest that *F. multinervis* is sister to a clade of *F. japonica* and *F. engleriana*. No haplotype variation was found within *F. multinervis*. This remarkably low cpDNA haplotype diversity is in contrast to the findings of previous allozyme studies of *F. multinervis* populations that showed high genetic diversity on Ulleungdo Island. Repeated colonization during the early stage of establishment via birds that migrated from a source area where the *F. multinervis* cpDNA haplotype was geographically structured may have resulted in the observed pattern of haplotype diversity. Alternatively, long-distance dispersal of seeds of the progenitor of *F. multinervis* via birds or typhoons to Ulleungdo may have been a single event, whereas the immigration of pollen from the mainland likely occurred frequently. Comparative phylogeographic studies of other species endemic to Ulleungdo Island and their close relatives on the neighboring mainland are necessary for a more complete understanding of the evolution of the island's native species.

Keywords: *Fagus multinervis*, chloroplast DNA, haplotype, Ulleungdo Island

적 요: 울릉도의 낙엽활엽수림에 우점하고 있는 너도밤나무를 대상으로 엽록체 반수체형(haplotype)의 다양성과 공간적 분포를 파악하기 위해 울릉도 전역에서 채집한 총 144개체로부터 *psbA-trnH* 구간의 염기서열을 결정하였다. 너도밤나무의 근연종을 포함하여 계통분석을 수행한 결과, 너도밤나무의 엽록체 반수체형은 일본산 *F. japonica*와 중국산 *F. engleriana* 분계조와 자매관계를 이루는 것으로 나타났다. 또한, 분석한 모든 너도밤나무의 개체들은 동일한 염기서열을 갖고 있는 것으로 나타나, 엽록체 반수체형의 다양성은 매우 낮은 것으로 판명되었다. 이러한 결과는 동위효소 분석에 근거한 유전자 다양성이 매우 높다는 기존 연구 결과와 대비되는 것으로서, 너도밤나무는 핵 유전자의 다양성은 높으나 엽록체 유전자의 다양성은 낮은 것으로 판단되며, 이것은 두 가지 가설로 설명할 수 있다. 하나는 너도밤나무의 조상이 울릉도로 이주하여 정착할 초기 단계에서 엽록체 반수체형이 지역적인 구조를 갖는 조상 모집단으로부터 종자가 지속적으로 유입된 결과로 해석할 수 있다. 다른 하나는 육지의 조상 모집단으로부터 새 또는 태풍에 의해 소수의 종자가 유입되어

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정착한 후, 바람에 의해 조상 모집단의 화분이 지속적으로 유입된 결과인 것으로 추론할 수 있다. 울릉도 내의 다양한 고유 자생식물의 기원을 규명하는 데 있어서 모계유전을 함으로 인해 종자의 이동을 추적할 수 있는 엽록체 DNA에 근거한 비교계통지리학적 연구가 필요한 것으로 사료된다.

주요어: 너도밤나무, 엽록체 DNA, 반수체형, 울릉도

Ulleungdo Island is an oceanic island located in the East Sea, 130 km east of the eastern coast of the Korean peninsula and 280 km northwest from the western coast of Japan. The island is very small, with a size of about 73 km². The island originated from the seafloor as a result of volcanic activity and emerged above sea level about 1.8 million years ago (Kim, 1985). Since the island was formed, it has been isolated from the neighboring mainland even during periods of glaciation (Kim et al., 2000). Seonginbong Peak (elevation 984 m), the island's highest peak, is located at the center of the island and comprises five major ridges with very steep slopes. With the influence of the maritime environment, broadleaf evergreen plants occupy the lowland of the island, whereas broadleaf deciduous forests are well developed in mountainous areas above 300 m. A recent survey of island flora shows that there are 484 native vascular plants belonging to 116 families, 40 of which are endemic to the island (Sun et al., 2014).

The number of endemic taxa on Ulleungdo Island is very high given its small area. Each of the endemic taxa is known to have evolved via anagenetic speciation, in which a progenitor population dispersed from the neighboring mainland and evolved into a new species (Sun and Stuessy, 1998). Anagenetic speciation presumes a single introduction of the ancestral population to the island. Isolation plays an important role in the process, as frequent immigration and establishment of the progenitor species would prevent an insular population from splitting into a separate lineage. Pfosser et al. (2002), in a study of two *Acer* species endemic to Ulleungdo, described two contrasting cases: *A. takesimensis* Nakai, which descended from a single introduction, and *A. okamotoanum* Nakai, which was derived from progenitors that migrated to the island multiple times. Although many systematic studies have investigated diverse groups of endemic species on Ulleungdo Island (Park et al., 1993; Sun and Stuessy, 1998; Weiss et al., 2002; Woo et al., 2002; Ohkawa et al., 2006; Pfosser et al., 2006; Oh et al., 2010; Shin et al., 2014), it remains unclear whether many of these species were derived from single introductions or from multiple introductions.

Chloroplast DNA is inherited by the ovulate parent in most angiosperms (Corriveau and Coleman, 1998) including Fagaceae (Dumolin et al. 1995); thus, it has been a useful tool for tracing seed dispersal (Dumolin et al., 1995; Hamilton

1999; Petit et al., 2002; Cannon and Manos, 2003; McLachlan et al., 2005). Investigation of chloroplast haplotype diversity and spatial distribution of endemic species on Ulleungdo Island would provide insight into the history of seed dispersal from mainland. One endemic species of Ulleungdo Island, *Fagus multinervis* Nakai, provides an excellent subject for the study of haplotype diversity. Plants of the species are an important component of the island's forest ecosystem, as they form nearly pure stands in the deciduous forests. High genetic diversity within the population and high effective population size have been reported for the species based on allozyme data (Chung et al., 1998; Ohkawa et al., 2006).

In this study, nucleotide sequences of the *psbA-trnH* region of the chloroplast genome from 144 individuals of *F. multinervis* were determined to elucidate the diversity and spatial distribution of chloroplast haplotypes in this species and to provide insight into the origin of the species.

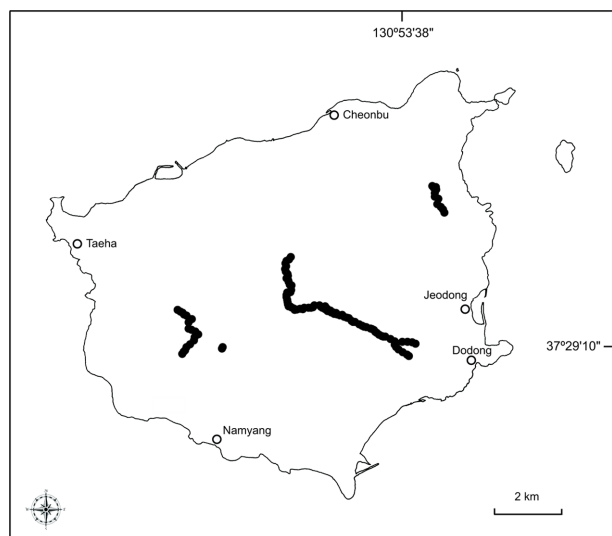
Materials and Methods

Plant materials were sampled throughout Ulleungdo Island, as plants of *F. multinervis* dominate the deciduous forest. Leaves were collected from 144 fully mature trees in four subpopulations (Table 1; Fig. 1). Eighty-five plants were sampled from the south-facing slope along the Seonginbong trail, and 26 plants were sampled from its north-facing slope. Nineteen samples were collected in Taeharyung and Namyang, representing the western subpopulation, and 14 in Baekwoon-dong, representing the eastern subpopulation. Voucher information is provided in Table 1. Species closely related to *F. multinervis*, namely *F. crenata* Blume, *F. engleriana* Seemen ex Diels, and *F. japonica* Maxim., were also included (Table 1).

The *psbA-trnH* region was examined because it was shown to be the most variable of the four regions of chloroplast DNA (*trnK-matK*, *trnL-trnF*, *psbA-trnH*, and *atpB-rbcL*) studied in eight *Fagus* species in a preliminary analysis (Oh et al., unpublished data). Total DNA was isolated from leaves, which had been dried in silica gel in the field, using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA). The *psbA-trnH* region was amplified via polymerase chain reaction (PCR) using WizPure Taq DNA polymerase (Wizbio Solutions, Seongnam, Korea) in 25- μ L reactions under the following

Table 1. Collection locality and voucher information. All voucher specimens are deposited at Daejeon University Herbarium (TUT).

Species	Locality	Elevation (m)	Voucher	Number of individuals
<i>Fagus multinervis</i>	Seonginbong trail beginning from the KBS station and Daewon temple to Seonginbong Peak	350–950	<i>Oh 6160–Oh 6164, Oh 6181–Oh 6207, Oh 6209–Oh 6221, Oh 6223–Oh 6250, Oh 6252–Oh 6255, Oh 6266–Oh 6268, Oh 6270–Oh 6274</i>	85
	Seonginbong trail beginning from Seonginbong Peak to Nari-dong	450–950	<i>Oh 6275–Oh 6300</i>	26
	Taeharyung and Namyang	350–450	<i>Oh 6120F, Oh 6124F–Oh 6127F, Oh 6128, Oh 6130, Oh 6303–Oh 6305, Oh 6134–Oh 6138, Oh 6140, Oh 6306–Oh 6308</i>	19
	Baekwoon-dong, Ulleung trail	350–400	<i>Oh 6145–Oh 6158</i>	14
<i>F. crenata</i>	Japan, cultivated in Arnold Arboretum, #AA 24098*B	unknown	<i>Oh 5446</i>	1
<i>F. engleriana</i>	China, Hubei province, Hsing-shan Hsien, cultivated in Arnold Arboretum, #AA 7416*A	unknown	<i>Oh 5454</i>	1
<i>F. japonica</i>	Japan, cultivated in Arnold Arboretum, #AA 51-2005*C	unknown	<i>Oh 5449</i>	1

**Fig. 1.** Location of collection sites on Ulleungdo Island, Korea.

conditions: initial denaturation at 95°C for 3 min, 35 cycles of 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 7 min. The primers *psbA* (5-GTTATGCATGAACGTAATGCTC-3) and *trnH* (5-CGCGCATGGTGGATTCACAATCC-3), both published by Sang et al. (1997), were used for amplification and sequencing of the *psbA-trnH* region. PCR products were examined in 1% agarose gels, purified, and directly sequenced in SolGent (Daejeon, Korea) using the sequencing primers. Sequences were edited in Sequencher version 5.0 (Gene Codes Corporation, Ann

Arbor, MI, USA) and aligned using MUSCLE (Edgar, 2004).

Phylogenetic analysis was conducted using the maximum parsimony (MP) method. All characters were treated as unordered, and weighted equally in the MP analyses in PAUP* (Swofford, 2002). Gaps resulting from multiple alignment of indels were scored as separate characters. An exhaustive search was used to find the MP tree for the data. The bootstrap analysis (Felsenstein, 1985) with 500 pseudoreplicates was conducted with simple sequence addition and TBR branch swapping.

Results and Discussion

The length of the amplified *psbA-trnH* region ranged from 429 bp in *F. japonica* to 435 bp in *F. multinervis*. The length of the region in *F. crenata* and *F. engleriana* was 430 bp and 434 bp, respectively. The nucleotide sequences were deposited in GenBank (KT382182–KT382185). The final alignment resulted in 441 sites, including four scored indel characters (Fig. 2). There were eight variable sites, including four indels. Two of the eight variable sites were parsimoniously informative, of which only one site was derived from base substitution.

All 144 individuals of *F. multinervis* sampled on Ulleungdo Island had the same haplotype (Fig. 2). *Fagus multinervis* has a unique haplotype supported by three changes, one from a base substitution and two from indels (Figs. 2, 3). *Fagus multinervis*, *F. japonica*, and *F. engleriana* are members of

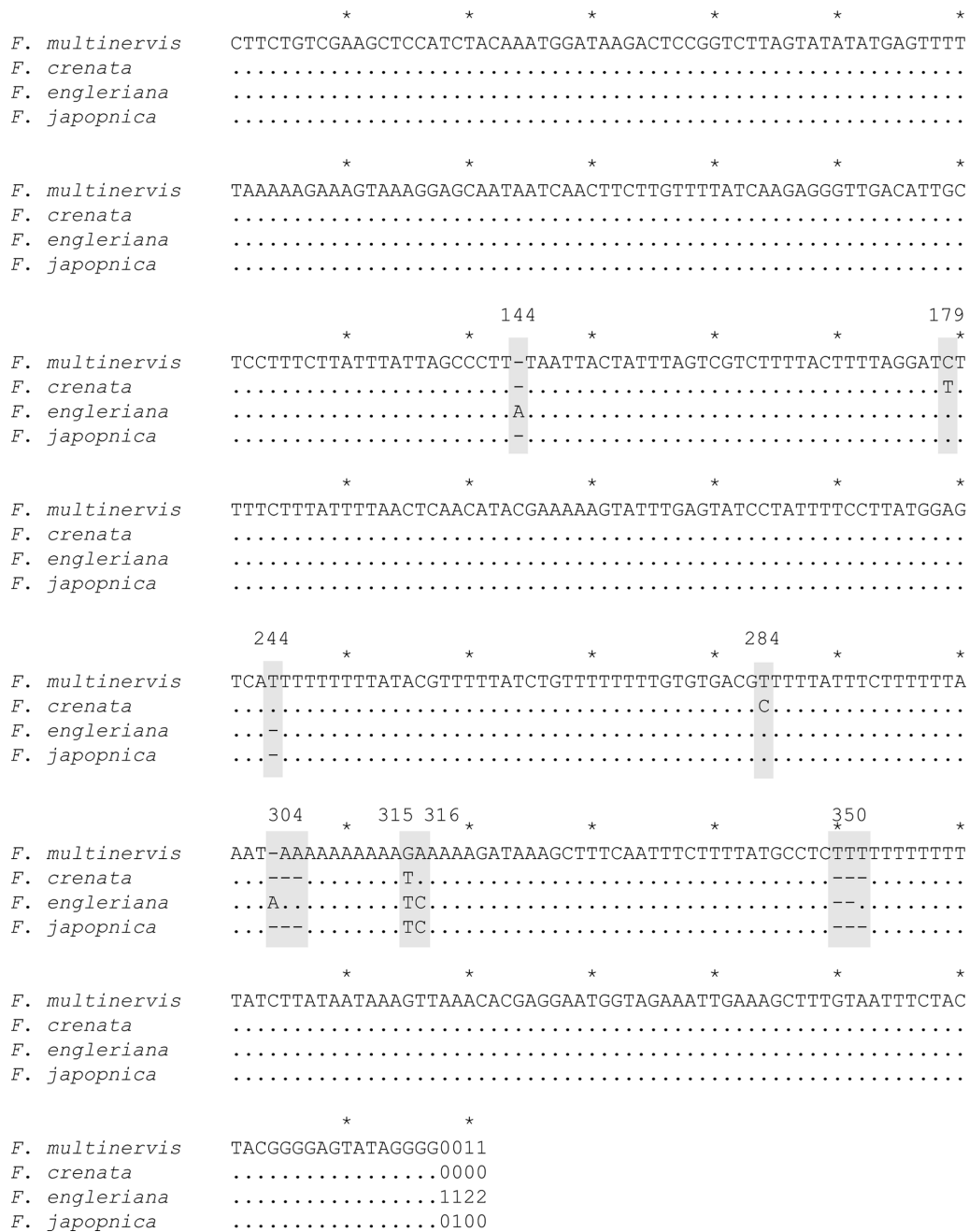


Fig. 2. Alignment of the *psbA-trnH* region. Variable regions are shaded with the position number above. A dot within the alignment indicates the same nucleotide as in the first taxon. Every tenth position in the alignment is indicated by an asterisk. Four indel-characters are added at the end of the alignment.

subg. *Engleriana* (Shen, 1992), and previous molecular studies strongly supported the monophyly of the subgenus (Denk et al., 2005). Thus, the tree was rooted by *F. crenata*.

Phylogenetic analysis of the *psbA-trnH* data resulted in a single MP tree, with the length of 10, CI of 1.0, and RI of 1.0. The MP tree of subg. *Engleriana* shows that *F. multinervis* is sister to *F. engleriana* and *F. japonica* (Fig. 3), suggesting that the insular species should have been derived from the

common ancestor that gave rise to *F. multinervis* and an ancestor of *F. engleriana* and *F. japonica*. Although no *Fagus* populations are present on the Korean Peninsula, fossil records indicated that the beech dominated temperate deciduous forests on the southeastern coast of Korea (Paik et al., 2012).

Results of this study indicate that haplotype diversity in *F. multinervis* is very low in comparison with other species of *Fagus*. In *F. crenata*, nine haplotypes were reported in 488

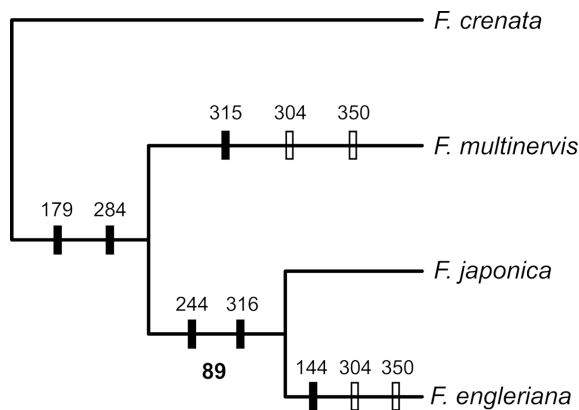


Fig. 3. The single most parsimonious tree of *F. multinervis* and its closely related species. Variable characters are indicated with boxes with the corresponding position or character number in alignment. A closed box represents a character that has a unique change throughout the tree. An open box indicates a character that has changes to different character states on multiple branches. Bootstrap values are indicated below a branch.

individuals by using the *trnT-trnF* region (Okaura and Harada, 2002). In another study that used *trnL-trnF* and the entire *trnK* intron (including *matK*), 13 haplotypes were found in 109 individuals of *F. crenata* (Fujii et al., 2002). In North American *F. grandifolia*, 17 haplotypes were found in 122 trees on the basis of three non-coding regions, *trnL-trnF*, *trnK-matK*, and *atpB-rbcL* (McLachlan et al., 2005). In a study of Greek populations of *F. sylvatica*, 13 haplotypes were found in 40 populations by using cpDNA microsatellite data (Hatziskakis et al., 2009). Chloroplast regions used in the previous studies, such as *trnK-matK*, *trnL-trnF*, *psbA-trnH*, and *atpB-rbcL*, were screened in a preliminary analysis for this study, and a single haplotype was found in *F. multinervis*. A genomic approach using a large number of samples may be necessary to completely understand the haplotype diversity of *F. multinervis*.

A single introduction of the seed of the progenitor of *F. multinervis* to the island, followed by its subsequent isolation, would have resulted in low haplotype diversity in *F. multinervis*. This is probably the simplest explanation for the observed results, as frequent immigration of progenitors and their establishment on the island during the early stage of evolution of *F. multinervis* would have led to increased haplotype diversity. In addition, a low rate of molecular evolution may be associated with the low haplotype diversity. Rates of evolution of the chloroplast genome in tree species that have long generation times, such as *F. multinervis*, are lower than the rates in herbaceous species that have short generation times (Smith and Donoghue, 2008).

By contrast, high genetic diversity within a population of *F.*

multinervis was reported on the basis of allozyme analyses (Chung et al., 1998; Ohkawa et al., 2006). The mean number of alleles per locus (*A*) and the mean number of alleles per polymorphic locus (*Ap*) were as high as in other species of *Fagus*, such as *F. sylvatica*, *F. grandifolia*, *F. japonica*, and *F. crenata* (Ohkawa et al., 2006). The numbers of observed and expected heterozygosities were comparable to those in *F. japonica* and much higher than those detected in other species (Ohkawa et al., 2006). *Fagus japonica* is a species endemic to Japan and distributed on the Pacific side of northern Honshu, Shikoku, and Kyushu, whereas another Japanese endemic species, *F. crenata*, shows a wide distribution range from southern Hokkaido to Kyushu. *Fagus grandifolia* and *F. sylvatica* are distributed widely in eastern North America and throughout Europe, respectively. Ulleungdo Island is very small when compared with the large distributional range of other species. If *F. multinervis* was derived from a single ancestor and isolated from its progenitor, we would expect low diversity in the allozyme as well as in chloroplast data because of the founder effect. In a survey of allozyme variation in six native species on Ulleungdo Island (Chung et al., 1998), a low level of variation was found in *Anemone maxima* Nakai, *Campanula takesimana* Nakai, *Tiarella polyphylla* D. Don, and *Thymus magnus* (Nakai) Nakai, and a moderately high level of diversity was detected in *F. multinervis* and *Pinus parviflora* Siebold & Zucc.

Why does *F. multinervis* have a high genetic diversity in the nuclear gene pool but a very low diversity in its chloroplast haplotype? Dispersal syndromes and population structure of its progenitor on the mainland may have resulted in the pattern of diversification on Ulleungdo Island. Seeds of progenitors of native species of Ulleungdo could have been dispersed by sea current, wind, or birds from the neighboring mainland. Dispersal via a floating mass such as a broken tree trunk may have been possible, as the North Korean Cold Current and the East Korea Warm Current flow around Ulleungdo Island. However, the nut of *F. multinervis* is relatively large and its surfaces are smooth, and it probably would not have survived long-distance dispersal via sea currents.

The Westerlies and typhoons that blow over Ulleungdo Island may play an important role in the dispersal of propagules from the mainland to Ulleungdo Island. Beech nuts are too heavy to be transported by the Westerlies. Typhoons accompanied by strong winds, however, may have transported the nut from the mainland to Ulleungdo Island, as typhoons often move through the East Sea in August and September, the fruiting season of *F. multinervis*. Thus, nuts of *F. multinervis* may have been dispersed via typhoons. However, the

probability of wind dispersal and establishment of beech nuts would be low, as it has to be assumed that a beech nut needs to be air-borne for more than several hours to reach the small island.

Dispersal of nuts via birds may also have resulted in the colonization of propagules from the mainland. It is likely that nuts of the progenitor of *F. multinervis* were biotically dispersed. Although bird species that disperse the nuts of *F. multinervis* on Ulleungdo Island are unknown, it has been widely documented that beech nuts of the North American *F. grandifolia* are dispersed by blue jays (Johnson and Adkisson, 1985) and those of the European *F. sylvatica* are carried by European jays (Perea et al., 2007). Beech forests usually form a pure stand (Hokusima et al., 2013), but chloroplast haplotype diversity within a population is generally low. Chloroplast haplotype is geographically structured in many species of *Fagus* and other species of Fagaceae (Whittemore and Schaal, 1991; Fujii et al., 2002; Okaura and Harada, 2002; Petit et al., 2002; Kano et al., 2004), and very few haplotypes or even a fixed haplotype is found in one population. If the nuts of the progenitor of *F. multinervis* were introduced frequently to Ulleungdo Island by birds that had actively harvested the nuts, a large number of seeds may have been brought to the island over time before the extinction of the progenitor on the mainland. Repeated colonization of seeds from the same progenitor population in which chloroplast DNA is geographically structured may have resulted in the diversification pattern found on Ulleungdo Island.

Alternatively, the pattern of genetic diversity of *F. multinervis* may have been associated with independent dispersal of seeds and pollen. In this scenario, long-distance dispersal of the seed of the progenitor of *F. multinervis* from the mainland via birds or typhoons to Ulleungdo Island is a rare event that resulted in the low chloroplast haplotype diversity observed in the current study. However, immigration of pollen from the mainland may have frequently occurred, which would have resulted in increasing genetic diversity in the nuclear genome. Pollination occurs in April to May, and the Westerlies may have played a role in the transport of pollen. As discussed above, fossil record indicates that beech forests were developed on the eastern coast of the Korean Peninsula until the vegetation went extinct because of volcanic activity (Paik et al., 2010; 2012). It may be possible that these populations may have been progenitors of Ulleungdo beech.

In conclusion, the patterns of genetic diversity of *F. multinervis* that show a relatively high allozyme diversity with a very low chloroplast haplotype diversity may be explained by frequent seed dispersal from geographically structured

progenitors on the mainland or by differences in the dispersal rates of seeds and pollen from source areas. Detailed comparative phylogeographic studies of other species endemic to Ulleungdo Island and their close relatives on the neighboring mainland will be necessary for a more complete understanding of the evolution of the island's native species.

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