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Genetic variation within a population and seed characteristics of *Abies nephrolepis* in Mt. Hambaeksan

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Abstract

Abies nephrolepis (Trautv. ex Maxim.) Maxim. is a subalpine conifer species and its southernmost natural populations are distributed in South Korea. Conservation actions should be considered as population decline is expected due to climate change. For ex-situ conservation of *A. nephrolepis*, securing enough genetic diversity and quantity of seeds is important. There were several studies on the weak genetic variation among populations of *A. nephrolepis* in South Korea, but there were few studies on genetic variation within a population. This study aimed to provide a reference for ex-situ conservation of an *A. nephrolepis* population in South Korea. Genetic variations and seed characteristics were investigated in an *A. nephrolepis* population at Mt. Hambaeksan, Gangwon-do, South Korea. The population of *A. nephorlepis* in Mt. Hambaeksan had the observed heterozygosity of 0.809 and the expected heterozygosity of 0.820. Spatial autocorrelation analysis revealed that the individuals had a positive genetic relationship within a 30 m distance, which was interpreted as a minimal sampling distance. The bigger trees had a stronger spatial genetic structure than the smaller trees. The inferred number of clusters (K) within a population did not converge: STRUCTURE inferred K=1 while GENELAND inferred K=2, but principal coordinates analysis (PCoA) supported K=1. Sampling simulation found that at least 20 individuals need to be sampled to secure the genetic diversity in a population. The seeds of *A. nephrolepis* population in Mt. Hambaeksan had similar characteristics to other populations in South Korea, with a germination percentage of 32.2%. However, there was a lower purity,

probably caused by the higher temperature and less precipitation in the sampling year. The correlation analysis showed that the seed weight could be the most effective indicator of seed quality. The mother trees that were genetically closer to overall individuals had poorer seed quality, but it was insignificant. This study provided some strategies that contribute to the ex-situ conservation of *A. nephrolepis.* Further studies on the mating system and gene dispersal are needed to improve the understanding of the genetic structure of an *A. nephrolepis* population.

Keywords: subalpine conifer, spatial genetic structure, microsatellite marker, seed production, ex-situ conservation
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1. Introduction

1.1. Study background

Abies nephrolepis (Trautv. ex Maxim.) Maxim. is naturally distributed in the subalpine or boreal forests of eastern Russia, northeast China, and the Korean peninsula (Zhang et al., 2013; Farjon and Filler, 2013). The habitats have cool, moist summers and cold winters with sufficient snow (Zhang et al., 2013; Farjon, 2017). It was assessed as Least Concern (LC) in 2013 according to IUCN Redlist (Zhang et al., 2013). However, recently, populations in South Korea are declining because of climate change (Korea Forest Service, 2021). The populations in South Korea are especially vulnerable and declining faster because they are the southern limit populations of *A. nephrolepis*. It was expected that the potential habitats of *A. nephrolepis* on the Korean peninsula will decrease to 36.4% by 2070–2099, and most regions in South Korea were expected to be inhabitable (Yun et al., 2018).

A. nephrolepis is one of the seven endangered subalpine conifers selected by the government of South Korea (Korea Forest Service, 2021). According to the Korea Forest Service (KFS), *A. nephrolepis* had a decline rate of 31% in the first monitoring implemented in 2019–2020. KFS also estimated that the higher temperature and droughts are the causes of this decline. KFS aims to solve this problem using nature–based solutions and scientific research. One strategy to reach the goal is to build up the foundation for conservation. This strategy includes prioritization based on genetic diversity and the establishment of seed protocols.

There were several studies on the genetic diversity of *A. nephrolepis* populations in Korea. Genetic variation can be studied on two levels: genetic variation among populations or within a population. Most studies on populations of *A. nephrolepis* in South Korea were about the genetic variation among populations (Woo et al., 2008; Hong et al., 2011; Seo and Lim, 2022). These studies revealed less than 5% of the total diversity was from the among-population variation. Considering the low differentiation among populations in South Korea, more studies on the within-population variation are necessary. There was one study about the genetic structure within a population of *A. nephrolepis* in Mt. Odaesan, Korea (Lee et al., 2008), but this study has a limitation in using isozyme markers that have low resolution. Until now, there is no study on the within-population variation in *A. nephrolepis* using microsatellite or more polymorphic markers.

For the restoration and conservation of tree species, seeds are the primary source of propagation (Brown and Hardner, 2000). Especially for species that do not propagate asexually, seed collection becomes more necessary. Most tree species, including *A. nephrolepis,* rarely propagate asexually in nature (Heybroek, 1984). The genus *Abies* has several characteristics that make effective seed collections more important. First, *Abies* is typically a poor seed producer. Various species in *Abies* showed high proportions of empty seeds, and low germination percentages that rarely exceed 50% (Bonner and Karrfalt, 2008). Second, *Abies* reaches reproductive maturity at 20 years on average (Bonner and Karrfalt, 2008). For *A. nephrolepis*, the generation length is 50 years (Zhang and Rushforth, 2013). For effective seed collection, understanding the seed characteristics of target populations is a prerequisite.

1.2. Objectives of this research

The goal of this study was to produce a reference for the effective ex-situ conservation *of A. nephrolepis*. For this, this study had two major objectives. The first was to understand the within-population variation of *A. nephrolepis* in Mt. Hambaeksan through genetic diversity indices and spatial genetic structure. The second was to identify the characteristics of seed production of *A. nephrolepis* population in Mt. Hambaeksan.

2. Literature Review

2.1. *Abies nephrolepis*

Abies nephrolepis is a wind-pollinating conifer belonging to the section *Balsamea* of *Abies* (Farjon, 2017). It can be characterized by its dark green notched leaves with white stomatiferous lines on the lower surface (Dirr, 2009). *A. nephrolepis* is closely related *to Abies koreana* Wilson (Yang et al., 2015). A key morphological trait to distinguish these species is the bract of the cone, but in South Korea, it is hard to classify them because of the natural hybridization and the overlay of habitats and morphological characters (Chang et al., 1997; Yang et al., 2015). These unclear boundaries caused long controversies over the classification of these two species. However, several studies have found *A. nephrolepis* is closer to other species rather than *A. koreana* (Xiang et al., 2009; Xiang et al., 2018). The most recent study, regarding the classification of 52 species in *Abies,* showed that *A. nephrolepis* was closer to *Abies sachalinensis* (F. Schmidt) Masters (Xiang et al., 2018). *A. sachalinensis* also has a neighboring distribution with *A. nephrolepis* and can hybridize with *A. nephrolepis* (Semerikova et al., 2011; Farjon and Filer, 2013) (Figure 1). However, a previous study found that they were distinguished through chloroplast simple sequence repeats (cpSSRs) and amplified fragment length polymorphisms (AFLPs) (Semerikova et al., 2011).

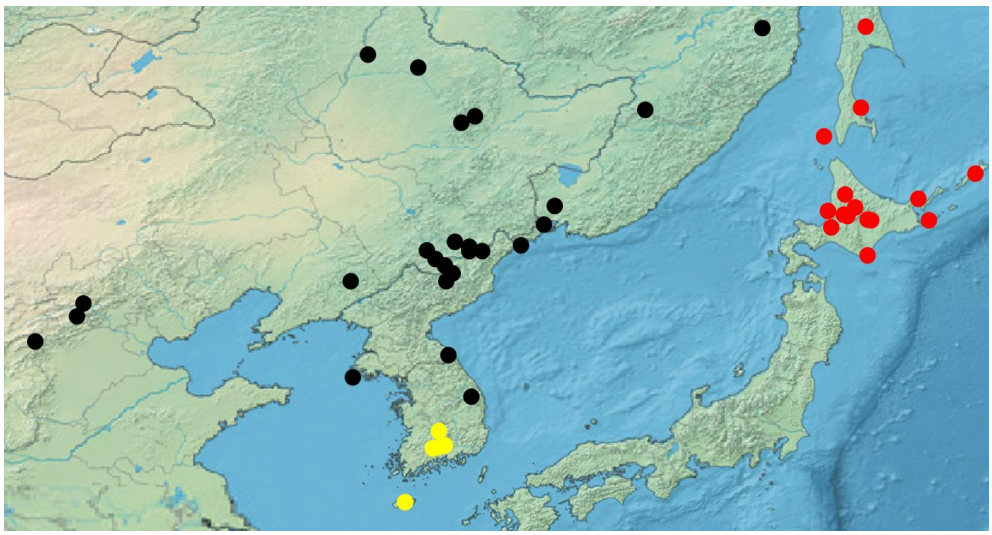

Figure 1. Distribution map of three *Abies* species in East Asia (Farjon and Filer, 2013). Red dots are the occurrences of *Abies sachalinensis,* yellow dots are those of *Abies koreana,* and black dots are those of *Abies nephrolepis.*

There were several studies on the ecological characteristics of *A. nephrolepis* in South Korea. Kim et al. (2019) studied the seedling dynamics of *A. nephrolepis* in Mt. Seoraksan. They found that rock exposure, open canopy, and species richness were positively related to seedling density. In contrast, herbaceous cover and basal area were negatively related. Lee et al. (2020) used landscape indices to analyze the habitat characteristics of *A. nephrolepis* in Seoraksan National Park. Shin and Oh (2022) analyzed the vegetative characteristics of *A. nephrolepis* in the southern region of Gangwon-do and classified the stand structure into four different communities. The sustainability of the habitats was investigated with species distribution models (SDMs) (Lim et al., 2018; Yun et al., 2018; Lee et al., 2020). They estimated that subalpine species, including *A. nephrolepis,* will have a significant loss of habitable areas.

Population genetics of *A. nephrolepis* were also studied in South Korea. They were mostly about the genetic differentiation among populations. A study with inter-simple sequence repeat (ISSR) markers found that only 4-8% of the genetic variation came from the variation among populations (Woo et al., 2008). Similarly, studies with simple sequence repeat (SSR) markers found minor variation among populations: 2-3.9% (Hong et al., 2011; Seo and Lim, 2022). These studies concluded that, in South Korea, populations *of A. nephrolepis* had a low level of differentiation and only a few populations would be enough for conservation. There was one study that dealt with the genetic variation in *A. nephrolepis* within a population (Lee et al., 2008). In this study, there was an absence in spatial genetic structure. However, the authors discussed that genetic markers with higher resolution should be applied for better understanding.

2.2. Spatial genetic structure within population

In forest trees, 90% of the total genetic diversity is often found within a population (White et al., 2007). Spatial genetic structure (SGS) provides important information on the genetic variation within a population. SGS implies the spatial distribution of genotypes. If a population has developed SGS, it has a non-random distribution of genotypes (White et al., 2007). Studying SGS is getting more essential because spatial structure influences genetic parameters such as mating system, selection, and genetic drift (Epperson, 1989). SGS can be applied to studies regarding the variations among populations. Therefore, when studies are about SGS within a population, it is often called fine-scale spatial genetic structure (FSGS). Various factors create SGS, such as limited gene dispersal, historical disturbance, and microenvironmental heterogeneity that leads to micro-selection. Among these, limited gene dispersal is a predominant determinant of SGS in plants (Pandey et al., 2011; Yao et al., 2011).

There are three major methods to evaluate SGS (White et al., 2007). Plotting the genetic cluster is the first method. The second is to examine the genetic differentiation among clusters using genetic diversity indices. The last but commonly applied method is to quantify the similarity among individuals and compare it with the random arrangement.

Bayesian clustering models are a prominent tool to infer SGS. These models describe multi-locus genotypes as clines or clusters. Bayesian clustering models can be divided into two types: spatially explicit or non-explicit models (Frantz et al., 2009). A representative program of the non-spatial method is STRUCTURE

(Pritchard et al., 2000). It only uses multi-locus genotype data to infer the genetic structure. While STRUCTURE has the highest popularity in population studies, spatially explicit Bayesian clustering algorithms are getting more attention with the development of landscape genetics. The examples are GENELAND (Guillot et al., 2005), TESS (Chen et al., 2007), and BAPS5 (Corander et al., 2008). All Bayesian clustering models have different algorithms and assumptions, therefore may not converge. Among them, STRUCTURE and GENELAND similarly assume Hardy-Weinberg and linkage equilibrium within a cluster. Several studies are using these two methods together because GENELAND inferred comparable results to STRUCTURE (Frantz et al., 2009). Lately, more studies are using both spatial and non-spatial models together.

Spatial autocorrelation analysis describes how genotypes are interdependent through the space, and has been applied to various studies (Hardy and Vekemans, 1999). There are various parameters applied to spatial autocorrelation analysis. Epperson (1995) applied Moran's I to spatial autocorrelation analysis. Loiselle et al. (1995) developed a pairwise kinship coefficient. Smouse and Peakall (1999) developed a spatial autocorrelation method based on the covariance of the genetic distance of multi-allele and multi-locus data. Because Moran's I had a vulnerability to sampling bias, the method of Smouse and Peakall (1999) had arisen as an alternative (Hardy and Vekemans, 1999). Other than these, various parameters were developed to estimate the pairwise relatedness between individuals and applied to spatial autocorrelation analysis. The results of spatial autocorrelation analysis are often visualized by correlograms. Correlograms depict the change in parameters according to geographic distance. Here, parameters represent pairwise genetic

relatedness or distance between individuals.

To estimate SGS, Cavers et al. (2005) recommended sampling 100 individuals minimum for 10 microsatellite markers. They also noted that, if more than 5 polymorphic loci are guaranteed, increasing sampling size is more effective than increasing the number of markers. When SGS is weak, it is especially efficient to increase the sampling size rather than the marker number (Vekemans and Hardy, 2004). The age structure of the population affects SGS because it is related to self-thinning, succession, and so forth. (Cavers et al., 2005). Investigating the differences in SGS through generations can offer information about historical and demographic factors within the population.

2.3. Ex-situ conservation

Ex-situ conservation was defined as the "conservation of components of biological diversity outside their natural habitat" by Convention on Biological Diversity (CBD) (1992). Ex-situ conservation has a critical role in providing materials for restoration and preventing extinction, therefore, it gets highly important when additional threats can lead the species to extinction vortex (IUCN/SSC, 2014). More species are being led to endangerment because of increased threats, such as habitat loss, climate change, and pollution. *A. nephrolepis* in South Korea is one of those. It is getting more vulnerable to additional threats because of the decline caused by climate change.

The components of biological diversity include germplasm, such as seeds and pollens, cuttings, grafts, or anything that can be a source of propagation. Among them, seeds are one of the most important materials because they occupy less volume and stay viable for a long period (Brown and Hardner, 2000). Seed quality is a major factor that affects the sampling strategy for ex-situ conservation (Brown and Hardner, 2000). The important indicators of seed quality include purity, germination percentage, and viability. While the germination percentage is more easily tested, some researchers argue that viability is more important because a lack of knowledge on dormancy and germination can produce imprecise information (Godefroid et al., 2009). Establishing a good sampling scheme is especially important for the seeds of *Abies.* It is not only because of the poor seed quality but also to avoid inbreeding depression. An increase in homozygosity can cause embryo death in the genus *Abies*, which leads to more empty seeds and lower viability (Williams, 2009).

The materials for ex-situ conservation need to be collected from distanced trees because it helps avoid inbreeding and encompass the genetic variation that target populations have (Brown and Marshall, 1995). Typically, the goal of ex-situ gene conservation is to capture at least one copy of 95% of the common alleles that occur in target populations at frequencies over 0.05 (Brown and Marshall, 1995; White et al., 2007). It is impractical to set the goal to include whole alleles because it requires a much larger sampling size. The sampling size to reach this goal differs by species because of the distinct characteristics of each species, including the mating and pollination systems (White et al., 2007). To establish an appropriate sampling strategy for the target species, it is required to understand the pattern and extent of the genetic variation among and within its populations.

For the investigation of genetic diversity, microsatellite markers are commonly used for tree species. Microsatellites, also known as simple sequence repeats (SSRs), are codominant, highly reproducible, and polymorphic markers (Bagnoli et al., 2011). Even after the emergence of single nucleotide polymorphism (SNP) markers, microsatellite markers are being commonly applied to trees because they are much more cost-effective and offer a similar level of accuracy in several assessments (Glaubitz and Moran, 2000; Bagnoli et al., 2011). Therefore, microsatellite markers are assessed as useful markers to estimate the relationship or relatedness among individuals of unknown ancestry (Glaubitz and Moran, 2000). However, microsatellite markers have a problem with null alleles, which produce genotyping errors. Even at low frequencies, null alleles have an enormous impact on estimation, but omitting the loci with null alleles can reduce inferential power (Wagner et al., 2006).

To handle this problem, correcting null alleles was suggested as a solution (Wagner et al., 2006). Several programs offer calculations and adjustments of null alleles (Van Oosterhout et al., 2004; Wagner et al., 2006).

There were various studies using microsatellite markers to investigate the populations of *Abies* species. Microsatellite markers for *Abies* species have already been developed, including specific markers for *A. nephrolepis* and *A. koreana* (Hansen et al., 2005; Cremer et al., 2006; Lian et al., 2007; Postolache et al., 2013; Hong et al., 2016).

There are several sampling strategies that are commonly recommended for conservations and genetic diversity studies. For ex-situ conservation, it was recommended to collect seeds from 10 populations and 25-30 individuals in each population (Hoban and Schlarbaum, 2014). In population genetics, it was recommended to sample 25-30 individuals for a reliable estimation of allele frequency and heterozygosity (Hale et al., 2012). They noted that sampling over 30 individuals produced fewer benefits compared to its cost.

3. Materials and Methods

3.1. Study site

This study was conducted at Mt. Hambaeksan, a mountain on the border of Taebaek-si and Gohan-ri, Gangwon-do. It is the sixth-highest mountain in South Korea with an altitude of 1,573 m. A transmitting station and a military camp are located on the top of the mountain. With the road leading up to the top, it is open for trekking and easily accessible to tourists. The mountain belongs to the temperate middle province among the floristic zones of the Korean peninsula (Choi et al., 2015). According to the nearest weather station in Taebaek, the climate normal (1991-2020) showed an average annual temperature of 9.0℃, precipitation of 1,308.0 mm, relative humidity of 66.1%, and wind speed of 1.4 m/s. In 2021, the sampling year, the average annual temperature was 9.5℃ with precipitation of 1207.4 mm, relative humidity of 69.1%, and wind speed of 1.4 m/s (Korea Meteorological Administration, 2022). In a previous research studying phylogeography of *A. nephrolepis* and *A. koreana* in Korea, a natural population at Mt. Hambaeksan was the southernmost population with a dominant mitochondrial DNA haplotype of *A. nephrolepis* (Yang et al., 2015). Considering these, Mt. Hambaeksan has necessities for conservation studies.

The study area was about 5 ha in the northern aspect near the top of the mountain (latitude: 37.161661°N-37.163287°N, longitude: 128.918825°E -128.923118°E, altitude: 1482.8 -1544.4 m) (Figure 2). *A. nephrolepis* was one of the most dominant tree species in this area. Other tree species commonly found in the area include *Taxus*

cuspidata Siebold et Zucc., *Sorbus commixta* Hedl., and *Acer komarovii* Pojarkova. Shrubs or vines including *Tripterygium regelii* Sprague et Takeda, *Rhododendron schlippenbachii* Maxim., *Rhododendron brachycarpum* D. Don ex G. Don, and *Thuja koraiensis* Nakai. were also common. Especially, *T. regelli* and *T. koraiensis* were mostly found as colonies over the study area.

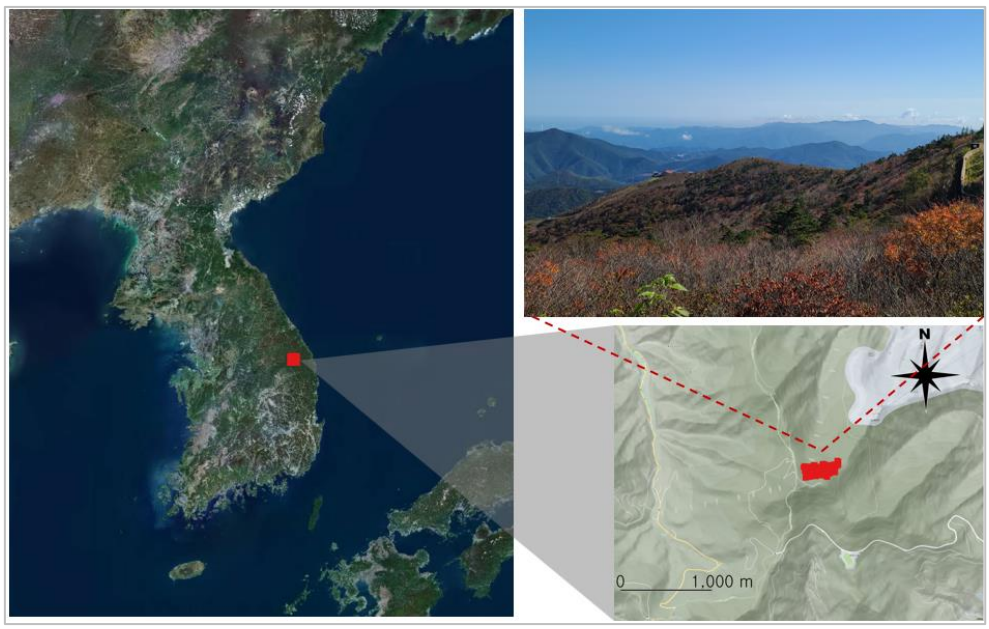

Figure 2. Location of the study plot at Mt. Hambaeksan.

3.2. Within-population genetic variation

3.2.1. Sampling

In this study, all mature flowering individuals were sampled. A mature flowering tree was defined as a tree with cones or strobili in the sampling year. A total of 165 individuals were investigated (Figure 3). Fresh needles were collected for DNA extraction. Height and diameter at breast height (DBH) were measured. Locations of each individual were recorded in coordinates within a 3-4 m error range using Garmin GPSMAP 64s (Garmin, Olathe, KS, US) and drawn on the map using QGIS (QGIS Development Team, 2009). Collected leaves were stored in a -80℃ deep freezer (CLN-71UWM, Ilwon Freezer, Namyangju, Gyeonggi, Korea) after being transported to the laboratory.

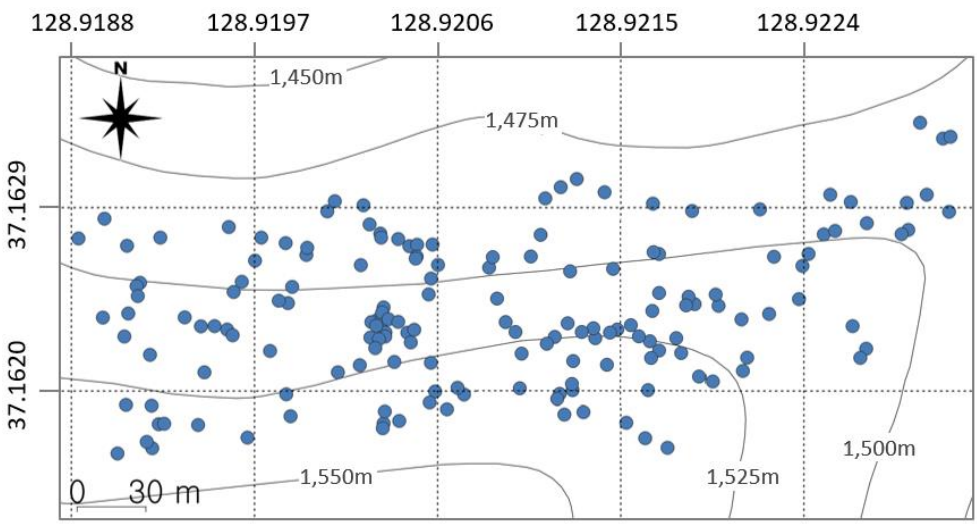

Figure 3. Locations of sampled individuals at Mt. Hambaeksan.

3.2.2. Genetic diversity

3.2.2.1. DNA extraction

About 50 mg of needles were put into a 2 mL tube (Eppendorf, Hamburg, Germany) with 2 mm and 4 mm stainless beads. Tubes with samples were deep-frozen by liquid nitrogen or stored in a -80℃ deep freezer (CLN-71UWM, Ilwon Freezer, Namyangju, Gyeonggi, Korea) for at least 6 hours. Needles in the tube were ground twice by TissueLyser Ⅱ (QIAGEN, Hilden, Germany) at the frequency of 25-30/s for 60-90 seconds. For DNA extraction, QIAGEN DNeasy Plant mini kit (QIAGEN, Hilden, Germany) and GeneAll Exgene Plant SV mini (GeneAll, Seoul, Korea) were used, following each protocol with slight modifications. The modifications included increasing the time of 65℃ incubation and ice incubation to 30 minutes. The concentration and quality of the extracted DNA were measured with NanoVue Plus (Biochrom, Holliston, MA, US). Total DNA was diluted to 5 μg/μL for the next steps.

3.2.2.2. PCR amplification and genotyping

Microsatellite markers developed for *A. nephrolepis*, *A. koreana* (Hong et al., 2016), and *A. sachalinensis* (Lian et al., 2007) were used in this study. Ten microsatellite markers were tested (Table 1).

Table 1. Characteristics of 10 microsatellite loci used in the study

Locus	Primer sequences (5' to 3')	Repeat motif	Ta (℃)	GenBank Accession No.
AK87	F: GCAGCCTTATCTTCATTTTGTC R: CACTTGAGCCACACTTGAACTA	$(TG)_N$	58	KP289899
AK171	F: GGCATTTGAACACTTACACTGA R: AGATTTTTGTTGGAATCTGCAC	$(TG)_N$	58	KP869867
AK173	F: GAGACTAGCATATACACCATCGG R: AAGGGAATACACTCAGTCGAGA	$(CA)_N$	58	KP289900
AK176	F: TTACACCGTTAAAAAGGGAATG R: CTCATGATGTGTAGCCATTTGT	$(TG)_N$	58	KP869869
AK240	F: AGAGAAGGGTCGAGGAATTATC R: GAAAGTAGCAAGTGTAACTTATGC	$(CA)_N$	58	KP869872
AK246	F: TAGATTGGCATATTGGACATCA R: ATAGGTTGTTGAGCTGGATGTT	$(TG)_N$	58	KP869873
AK247	F: GGATGGTGCTTTGTTGATATTT R: AAATGGTTTGAGCAACATTCTT	$(TG)_N$	58	KP869874
AK252	F: TGCATGTTGTTAGTTGGTAAGG R: TCTAGGTGGAGCAACAAGAGAT	$(TG)_N$	58	KP869875
As13	F: ATGCAAGCAACCATCGATATG R: GTTTCTTCCATAGAACACCTC	$(TG)_N$	55	AB290134
As20	F: TCTTGCAACGAGGGGATCCATAACCTG R: CTAAGCATTGAGCCACATAATTC	$(TG)_N$	55	AB290136

Ta; annealing temperature

PCR amplifications were performed with a total volume of 12 μL containing 20 ng DNA, 1 X A-Star *Taq* Reaction Buffer (BioFact, Daejeon, Korea), 0.2 mM dNTP Mix (BioFact, Daejeon, Korea), 0.5 U A-Star *Taq* DNA Polymerase (BioFact, Daejeon, Korea), 0.04 μM HEX/FAM M-13 primer, and 0.2 μM of each reverse and forward primer. PCR programs were performed as described in each reference. For AK87, AK171, AK 173, AK176, AK240, AK246, AK247, and AK252 (Hong et al., 2016), the PCR program comprised 10 minutes of pre-denaturation at 95℃, 35 cycles of 30 seconds of denaturation at 95℃, 30 seconds of annealing at 58℃, and 30 seconds of extension at 72℃, and final-extension at 72℃ for 7 minutes. For As13 and As20 (Lian et al., 2007), the PCR program comprised 9

minutes of pre-denaturation at 94℃, 40 cycles of 30 seconds of denaturation at 94℃, 30 seconds of annealing at 55℃, and 1 minute of extension at 72℃, and final-extension at 72℃ for 5 minutes. PCR was conducted using Bioer LifePro Thermal Cycler (Hangzhou Bioer Technology, Hangzhou, Zhejiang, China).

Fragment analysis was performed with PCR products by the National Instrumentation Center for Environmental Management (NICEM) in Seoul National University, with GeneScan 500 ROX size standard (Applied Biosystems, Waltham, MA, US) and ABI 3730 XL DNA Analyzer (Applied Biosystems, Waltham, MA, US). Genotyping was conducted using Microsatellite 1.4.7 plugin of Geneious Prime 2022.1.1 (Kearse et al., 2012). Large allele dropout, stutter allele, and null allele were checked and adjusted using MICRO-CHECKER (Van Oosterhout et al., 2004) with the Oosterhout algorithm, 1,000 permutations.

3.2.2.3. Genetic diversity index

Genetic diversity indices were calculated using GenAlEx 6.503 (Peakall and Smouse, 2012). The number of alleles (A), number of effective alleles (A_E), observed heterozygosity (H_O), expected heterozygosity (H_E), and fixation index(F) were calculated.

3.2.3. Spatial genetic structure

As spatial genetic structure (SGS) can differ through life-history stages, this study only sampled adult trees. However, this study checked whether SGS differs by diameter class within the same life-history stage. To group the individuals in two different diameter class, median (15.3 cm) of the DBH was used as a criterion to make similar

sample size for each group. Individuals bigger than the median were grouped as the bigger trees (BT; 84 individuals with an average of 19.2 cm), and the others were grouped as the smaller trees (ST; 81 individuals with an average of 12.4 cm).

3.2.3.1. Spatial autocorrelation

The correlation coefficient (r) of Smouse and Peakall (1999) was used to analyze the spatial autocorrelation. It quantifies spatial autocorrelation among individuals using the correlation coefficient between spatial distance and covariance of genetic distance.

The genetic distance at a locus can be calculated as squared distance based on the genotypes. The multi-locus distance can be calculated by adding the genetic distances across loci. Below is the computation for the covariance of genetic distance.

$$c_{ij} = \frac{\left[-d_{ij}^2 + \frac{\sum_{j=1}^{N} d_{ij}^2 + \sum_{i=1}^{N} d_{ij}^2}{\mathrm{N}} - \frac{\sum_{i \neq j}^{N} d_{ij}^2}{N^2}\right]}{2}$$

∵ d_{ij}^2=squared genetic distance between i^{th} and j^{th} individuals,

N=population number.

The correlation coefficient (r) can be calculated from the covariance of genetic distance and the spatial distance. The computation is as below.

$$r^{(h)} = \frac{\sum_{i \neq j}^{N} x_{ij}^{(h)} c_{ij}}{\sum_{i=1}^{N} x_{ii}^{(h)} c_{ii}}$$

∵ x_{ij}=spatial distance between i^{th} and j^{th} individuals (h steps apart)

The correlation coefficient was calculated using GenAlEx 6.503 (Peakall and Smouse, 2012). The analysis was performed on whole individuals, then also by diameter class: BT and ST, using even distance classes (10 m, 20 m, 40 m). Correlograms were plotted at the end point of the distance interval. Statistical significance of R values and correlograms was determined using 999 permutations and 1,000 bootstraps.

3.2.3.2. *Sp* statistic

Sp statistic, developed by Vekemans and Hardy (2004), is another index to quantify the extent of SGS. *Sp* statistic enables the comparison of SGS between different populations and species because it is insensitive to the sampling scheme. *Sp* statistic estimates SGS with regression slope of kinship coefficient against spatial distance. The kinship coefficient (F_{ij}) is defined as the probability that a random gene from *i* is identical to a random gene from *j*. With the kinship coefficient, the computation of *Sp* statistics is as below.

$$Sp = -b_{-log} / (1 - F_1)$$

∵ b_{-log} =slope of the linear regression of kinship coefficient on ln(distance), F_1=average kinship coefficient over the first distance class

SPAGeDi 1.5 (Hardy and Vekemans, 2002) was used to calculate the *Sp* statistic. F_{ij} of Loiselle et al. (1995) was used with an even distance class (20 m). *Sp statistic* was calculated for whole individuals, then by diameter class: BT and ST. Statistical significance of b_{-log} and F_1 was determined using 10,000

permutations.

3.2.3.3. Genetic clustering

To identify the genetic clusters within a population, two different Bayesian clustering methods were used: GENELAND (Guillot et al., 2005a) and STRUCTURE (Pritchard et al., 2000). Both methods use multi-locus genotype data to describe genetic structure, but the most distinctive characteristic of GENELAND is that it also includes geographic coordinates to infer the number of populations and the spatial location of genetic discontinuities (Guillot et al., 2005a). Spatial consideration of genetic discontinuities is getting more important as it helps to identify the abiotic and biotic factors related to SGS (Guillot et al., 2005b).

GENELAND 4.9.2 was performed in R 4.2.0 environment (R Core Team, 2022). Twenty independent Markov chain Monte Carlo (MCMC) runs were performed with 500,000 iterations and 500 thinning intervals. Spatial model and null allele model were assumed under the possibility of having 1-10 populations (K). Correlated frequency model was selected because it is more appropriate for the detection of subtle structures with a low differentiation (Guillot, 2008). Among the 20 independent runs, a run with the highest log posterior probability was selected for further analysis. There was a burn-in of 200 saved iterations.

STRUCTURE 2.3.4. was run with 20 independent simulations for each value of K (1-10). The length of the burn-in was set to 100,000, and 100,000 MCMC iterations were run after the burn-in period. Admixture model and correlated frequency model were assumed. With the results, major modes on each K were obtained using CLUMPAK (Kopelman et al., 2015). To determine the optimal

number of K, Pritchard's method (Pritchard et al., 2000) and Evanno's ΔK method (Evanno et al., 2005) were both considered. STRUCTURE HARVESTER (Earl and vonHoldt, 2012) was used to calculate and visualize the results of the two methods.

If clusters were found in any program, F_{ST} between the clusters was calculated using the analysis of molecular variance (AMOVA) in GenAlEx 6.503 (Peakall and Smouse, 2012). Statistical significance was determined using 999 permutations. Principal coordinate analysis (PCoA) was performed as an additional tool to validate the K value. PCoA was also conducted in GenAlEx 6.503 (Peakall and Smouse, 2012).

3.2.4. Sampling simulation study

The goal of ex-situ conservation is to secure over 95% of common alleles that appear at over 0.05 frequency in the populations. A sampling simulation study was performed to find the sampling size that reaches this goal. The simulation only considered common alleles, as noted above. The sampling simulation study was carried out using Python 3.10.6 (Van Rossum and Drake, 2009). Individuals were randomly sampled with a sampling size of 5-50 and checked whether they confirmed the goal. The random sampling was permuted 1,000 times at each sampling size.

3.3. Seed characteristics and germination

3.3.1. Cone sampling and analysis

In this study, cones were sampled from 31 mother trees (Figure 4). The average neighboring distance of the mother trees was about 35 m. For all sampled mother trees, heights, DBH, and cone abundance were investigated. At least three cones were sampled from each mother tree and a total of 141 cones were collected in September 2021. Collected cones were separated by their mother tree and transported to the laboratory.

Three undamaged cones of each mother tree were selected and measured for their width and length. They were dried both indoors and outdoors until the scales were naturally separated from the cone axis. The drying process was continued for 1–2 weeks, depending on the status of each cone. The dried cones were measured for their weight. Seeds were extracted from the dried cones and cleaned.

After the cleaning, seed potential, percent developed seeds, percent damaged seeds, purity, and 100-seed weight were investigated. Cone analysis was carried out following the guidebook and the woody plant seed manual from the United States Department of Agriculture (USDA) Forest Service (Bramlett et al., 1977; Bonner and Karrfalt, 2008), and the guidelines of the Korea National Forest Seed and Variety Center (2019). Classified seeds were kept in a 3℃ refrigerator (SR-S45BI, LG Electronics, Seoul, Korea).

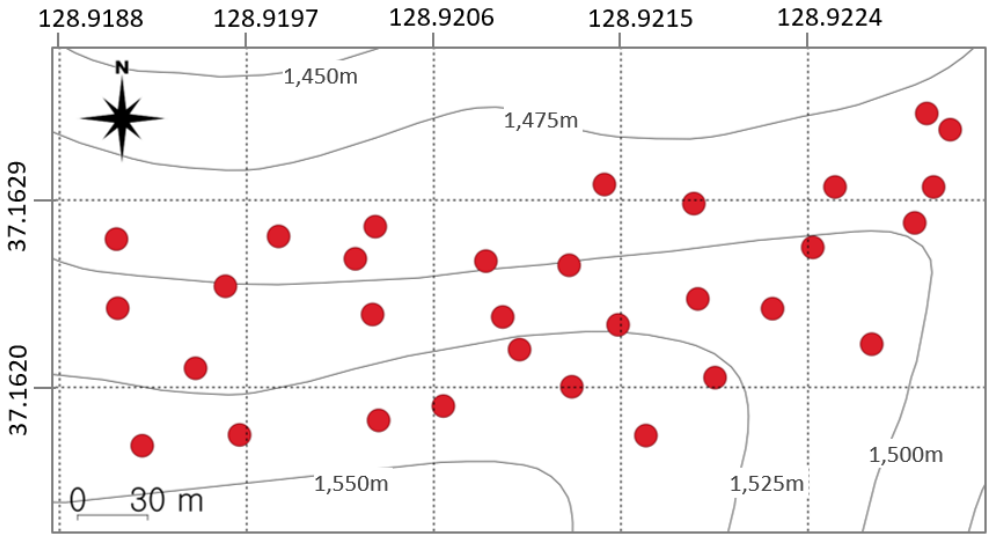

Figure 4. Locations of sampled mother trees at Mt. Hambaeksan.

3.3.2. Germination test

All pure seeds (13,166 seeds) from the three cones of each mother tree were used. Pre-germination chilling (pre-chilling) was conducted for 90 days in a 3℃ refrigerator (SR-S45BI, LG Electronics, Seoul, Korea) from December 2021 to February 2022. The seeds were placed on 90 mm Petri dishes (Hyundai Micro, Anseong, Gyeonggi, Korea) with two layers of No.2 filter paper (Hyundai Micro, Anseong, Gyeonggi, Korea). Deionized water was regularly provided into the dish.

A germination test was conducted in March 2022, with all pure seeds that were alive (12,994 seeds) after pre-chilling. The seeds were placed on two layers of No.2 filter paper (Hyundai Micro, Anseong, Gyeonggi, Korea) in a Petri dish (Hyundai Micro, Anseong, Gyeonggi, Korea) (Figure 5-a). The germination test was carried out for 28 days in a growth chamber (BF-600THG, BioFree, Seoul,

Korea) (Figure 5-b). The growth chamber was maintained at 20℃ temperature and 50% of humidity. Eleven hours of dark and 13 hours of light were provided every day. Seeds were counted as germinated when a radicle or cotyledon was developed over 1 mm (Figure 5-c). After the test, germination percentage, mean germination time, and germination energy was calculated (Table 2). The germination energy was based on day 6, which was the day when the most seeds germinated. Seeds that failed to germinate were cut to verify the existence of the embryo and investigate viability (Demir et al., 2008; Godefroid et al., 2009; Domin et al., 2019).

Sixteen seedlings of each mother tree were transplanted to the soil and tested for survivorship (Figure 5-d). Thirty mother trees were used except one mother tree that lacked germinated seedlings. A total of 480 seedlings were transplanted into pots. Each pot had a 3 cm width and 5 cm depth. The soil was sterilized with an autoclave (BF-60AC, Biofree, Seoul, Korea) before the transplantation. The seedlings were stored together in the laboratory or greenhouse under the condition of 25-30℃ temperature. The survivorship was counted at 2 months and 6 months after the transplantation. Seedlings were considered dead when the whole leaves turned brown.

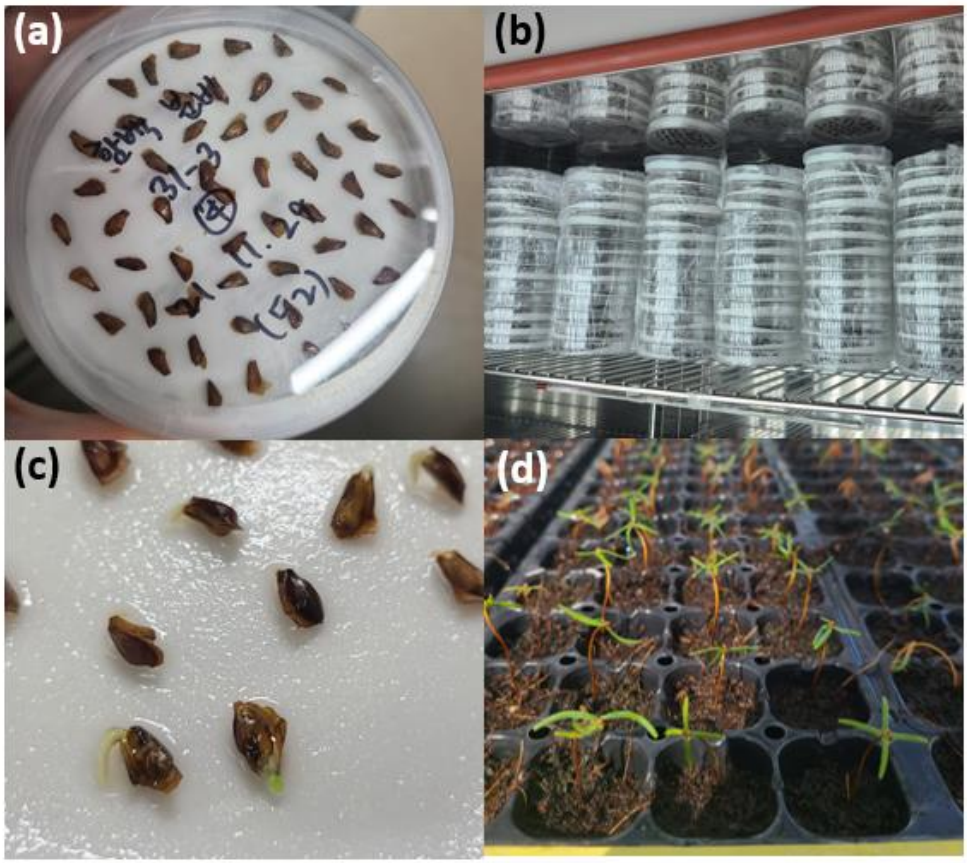

Figure 5. Pictures of the germination test and transplantation in the study. (a) seeds placed on a Petri dish, (b) the growth chamber, (c) germinated seeds, and (d) transplanted seedlings.

Table 2. Formulae for the analysis of cone and seed characteristics

Indicator	Computation
SP	$2 \times$ Fertile scales
P-DS	$\frac{Total\ developed\ seeds}{Seed\ potential} \times 100$
P-DM	$\frac{Damaged\ seeds}{Total\ developed\ seeds} \times 100$
Purity	$\frac{Weight\ of\ pure\ seeds\ (g)}{Weight\ of\ total\ developed\ seeds\ (g)} \times 100$
GP	$\frac{Germinated\ seeds}{Tested\ seeds} \times 100$
MGT	$\frac{\sum nD}{\sum n}$
GE	$\frac{Germinated\ seeds\ until\ Day\ 6}{Tested\ seeds} \times 100$
Viability	$\frac{Viable\ Seeds}{Tested\ Seeds} \times 100$

SP; seed potential, P−DS; percent developed seeds, P−DM; percent damaged seeds, GP; Germination percentage, MGT; mean germination time, D; days from the beginning of the germination test, n; number of seeds newly germinated at time D, GE; germination energy

3.3.3. Statistical analysis

With data from the cone analysis and germination test, statistical analysis was performed in R 4.2.0 (R Core Team, 2022). Skewed data sets were normalized by log, cube, square, or square root transformation. Pearson's correlation test was conducted to identify the relationship between the traits of mother trees and seeds.

Statistical analysis was also conducted between seed

characteristics and genetic data of each mother tree. To check the relationship between genetic relatedness and seed quality, Pearson's correlation test was conducted. Seed quality was quantified using purity and viability. To quantify genetic relatedness, the genetic distance by Smouse and Peakall (1999) and the distance of spatial autocorrelation were used. The genetic distance to potential pollen donors was summed on each mother tree. The number of trees within the distance of genetic relatedness was also counted on each mother tree. Correlation analysis was performed to check whether mother trees with more relatives had lower seed qualities.

4. Results

4.1. Within-population genetic variation

4.1.1. Selection of microsatellite markers

Among the 10 microsatellite markers used in this study, nine markers were successfully amplified, and one (AK240) failed. Among the nine markers, two markers, AK171 and AK252, were estimated to have null alleles (Table 3), but the estimated null allele frequencies of both markers were not over 0.2.

All amplified loci had over three effective alleles, therefore considered polymorphic. All individuals were distinguished by their genotypes using any combination of three markers.

Table 3. Characteristics of nine amplified microsatellite markers in the population of *A. nephrolepis* at Mt. Hambaeksan

Locus	Null allele frequency	Size range(bp)	A	A_E
AK87	-0.018	280-324	21	10.789
AK171	0.175	220-252	15	3.613
AK173	0.016	176-216	18	7.511
AK176	-0.020	328-354	7	4.583
AK246	0.022	166-176	6	3.885
AK247	0.037	184-206	12	9.159
AK252	0.138	305-327	11	5.320
As13	-0.028	244-272	12	5.978
As20	-0.028	192-236	20	5.617

A; number of alleles, A_E; number of effective alleles

4.1.2. Genetic diversity

The genetic diversity indices of *A. nephrolepis* in Mt. Hambaeksan were calculated (Table 4). The number of alleles (A) was 13.556 and the number of effective alleles (A_E) was 6.273. A high level of heterozygosity was found, with the observed heterozygosity (H_O) of 0.809 and the expected heterozygosity (H_E) of 0.820. A low level of inbreeding was observed. The fixation index (F), which is also known as the inbreeding coefficient, was 0.015. The genetic diversity indices calculated by the diameter class had similar values.

Table 4. Genetic diversity indices estimated in the population of *A. nephrolepis* at Mt. Hambaeksan over nine microsatellite loci.

		A	A_E	H_O	H_E	F
All individuals	Mean	13.556	6.273	0.809	0.820	0.015
	SE	1.788	0.811	0.031	0.021	0.022
Bigger trees	Mean	12.333	6.204	0.789	0.814	0.036
	SE	1.624	0.813	0.046	0.026	0.034
Smaller trees	Mean	13.000	6.202	0.828	0.819	−0.011
	SE	1.700	0.784	0.024	0.020	0.023

A; number of alleles, A_E; number of effective alleles, H_O; observed heterozygosity, H_E; expected heterozygosity, F; fixation index, SE; standard error

4.1.3. Spatial genetic structure

4.1.3.1. Spatial autocorrelation

The results of spatial autocorrelation analysis with all individuals are shown in Figure 6. Significantly positive spatial genetic structure

(SGS) was found up to 30 m. Individuals were distributed in genetic randomness from the distance interval of 30–40 m. Until 400 m, which was near the farthest distance between the trees, individuals did not show significant SGS.

When spatial autocorrelation analysis was applied by the diameter class, two groups had different results. The bigger trees (BT) showed a similar result to that of all individuals. The bigger trees had significantly related individuals until 30 m and distributed in genetic randomness from the distance interval of 30–40 m (Figure 7–a). In contrast, the smaller trees (ST) did not show significant SGS at all distance classes (Figure 7–b).

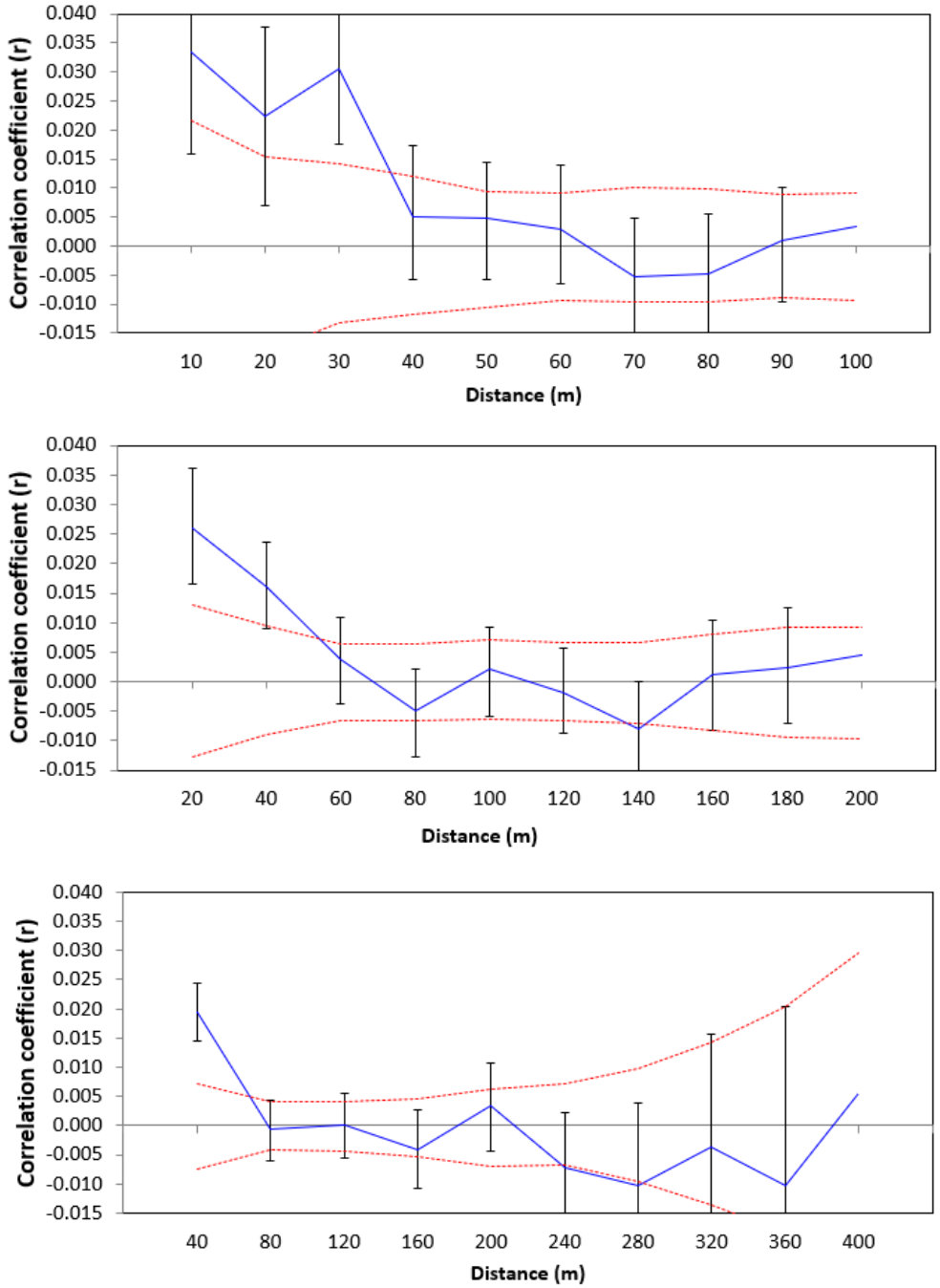

Figure 6. Correlograms obtained in the population of *A. nephrolepis* at Mt. Hambaeksan, in three distance intervals (10 m, 20 m, and 40 m). The blue line indicates values of r with black error bars of 95% confidence interval. The red dotted line indicates the upper and lower limits of the null hypothesis with a 95% confidence interval.

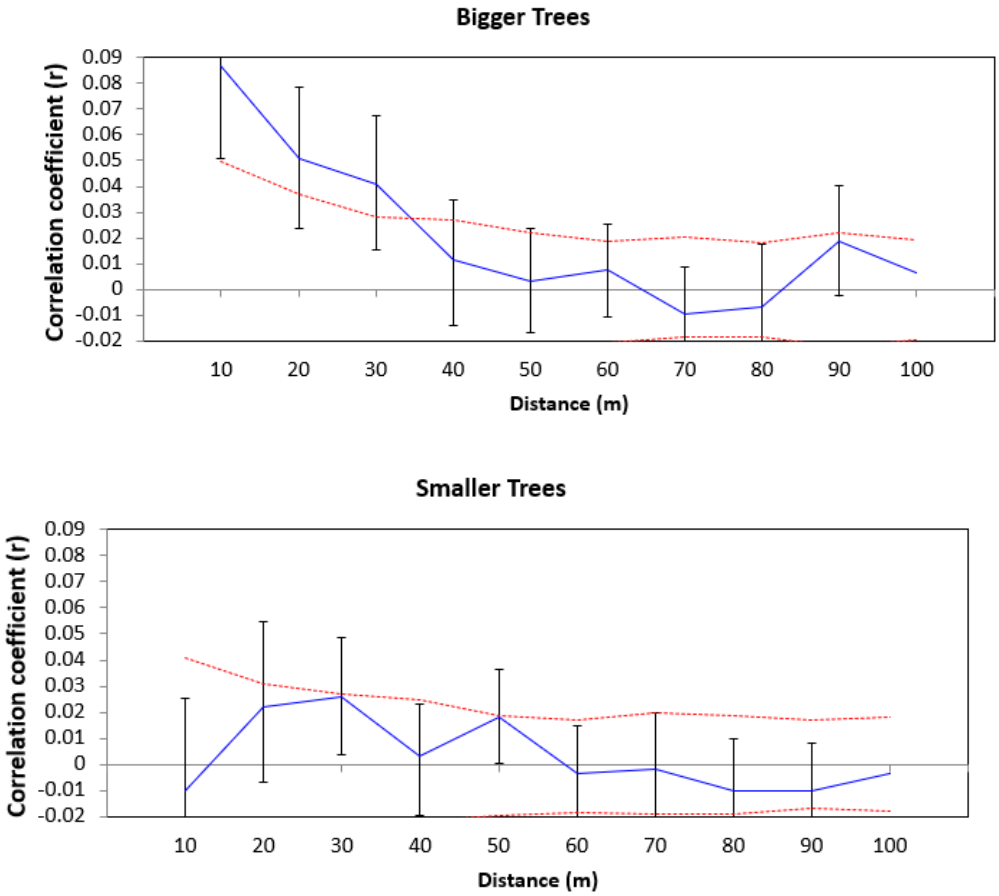

Figure 7. Correlograms obtained by the diameter class in the population of *A. nephrolepis* at Mt. Hambaeksan. The blue line indicates values of r with black error bars of 95% confidence interval. The red dotted line indicates the upper and lower limits of the null hypothesis with a 95% confidence interval.

4.1.3.2. *Sp* statistic

The *Sp* value obtained from all individuals was 0.0036 (Table 5). When the values were calculated by the diameter group, the bigger trees had 0.0081 of *Sp*, which was higher than the value obtained from all individuals. However, the smaller trees had a lower value of *Sp,* 0.0021, and it was not statistically significant. It indicated that the bigger trees had a greater level of SGS. It differed from the expectation that smaller trees might develop stronger SGS due to a lack of self-thinning in younger generations. Overall, SGS that was found in all individuals could have been resulted from the SGS in the bigger trees.

Table 5. *Sp* statistics estimated in the population of *A. nephrolepis* at Mt. Hambaeksan

	F_1	$b-log$	Sp
All individuals	0.0103***	−0.0036***	0.0036
Bigger trees	0.0270***	−0.0079***	0.0081
Smaller trees	0.0030	−0.0021	0.0021

*** $p < 0.001$

When the *Sp* value was compared to the average of outcrossing or wind-dispersed species (Table 6), SGS in the population of *A. nephrolepis* at Mt. Hambaeksan was relatively weak.

Table 6. *Sp* statistics estimated in previous studies

Species	Materials	*Sp*	References
A. sachalinensis	Seedlings	0.0044	Lian et al., 2008
	Saplings	0.0040	
	Juveniles	0.0036	
	Adult	0.0033	
A. alba		−0.001 to 0.014	Paluch et al., 2019; Major et al., 2021
Wind−dispersed species		0.0064	Vekemans and Hardy, 2004
Outcrossing species		0.0126	Vekemans and Hardy, 2004

4.1.3.3. Genetic clustering

There were differences in the optimal number of clusters estimated from the two Bayesian clustering models, GENELAND and STRUCTURE.

In 20 independent runs in GENELAND, all runs produced consistent results, K=2. With a run having the highest posterior probability, the clusters were drawn on the map as (b) and (c) in Figure 8. One cluster (GL1, green area in Figure 8−c) was described in U−shape, and the other one (GL2, gray area in Figure 8−c) clustered all the other individuals in three different areas. With the two clusters obtained by GENELAND, AMOVA was performed. The two clusters had an F_{ST} of 0.009 (p=0.001). However, PCoA failed to demonstrate a clear clustering that coincides with the GENELAND result (Figure 9).

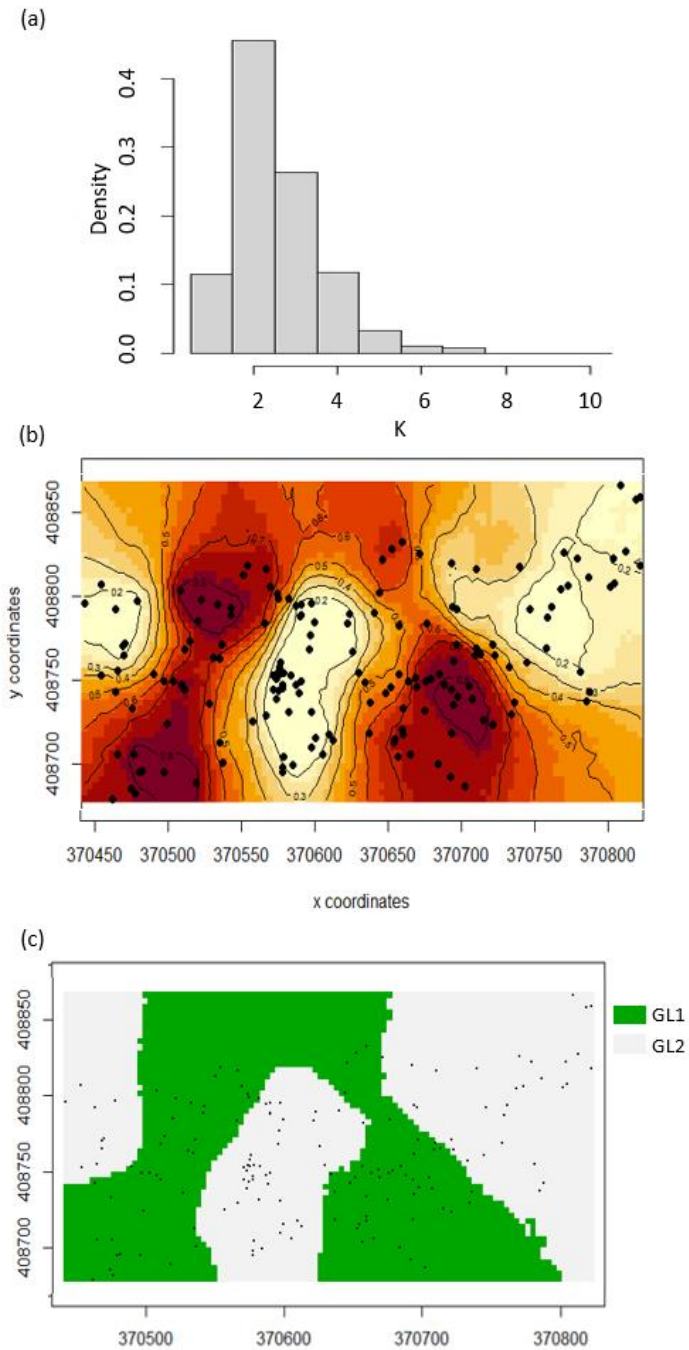

Figure 8. Results of GENELAND in the population of *A. nephrolepis* at Mt. Hambaeksan. (a) the number of clusters along the chain, (b) map of posterior probability to belong to GL1, and (c) map of cluster membership. X and Y coordinates are in UTM coordinates.

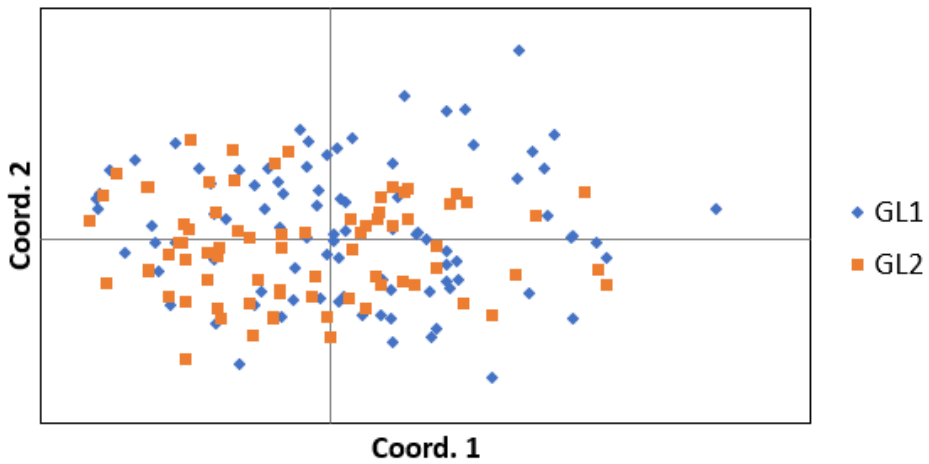

Figure 9. A result of principal coordinate analysis on GENELAND clusters estimated in the population of *A. nephrolepis* at Mt. Hambaeksan. Coord. means coordinate.

The best K inferred from STRUCTURE was different depending on the methods to detect the K. Pritchard's method presented K=1 as the best K (Figure 10-a). However, Evanno's ΔK method presented K=2 as the best K (Figure 10-b). Although Evanno's ΔK method was developed later to improve the detection of the number of clusters, it cannot calculate the value at K=1. Because of the inability to identify K=1, Evanno's ΔK method selects K=2 more frequently (Cullingham et al., 2020). Some researchers also evaluated that Evanno's ΔK method only brings little improvement compared to Pritchard's method (Guillot et al., 2009). Furthermore, PCoA did not demonstrate a clear clustering that coincides with the result of K=2 (Figure 10-c). Therefore, in this study, K=1 by Pirthcard's method was chosen as the optimal number of clusters produced from STRUCTURE. In other words, STRUCTURE did not detect genetic substructure in the *A. nephrolepis* population at Mt. Hambaeksan, unlike the result of GENELAND.

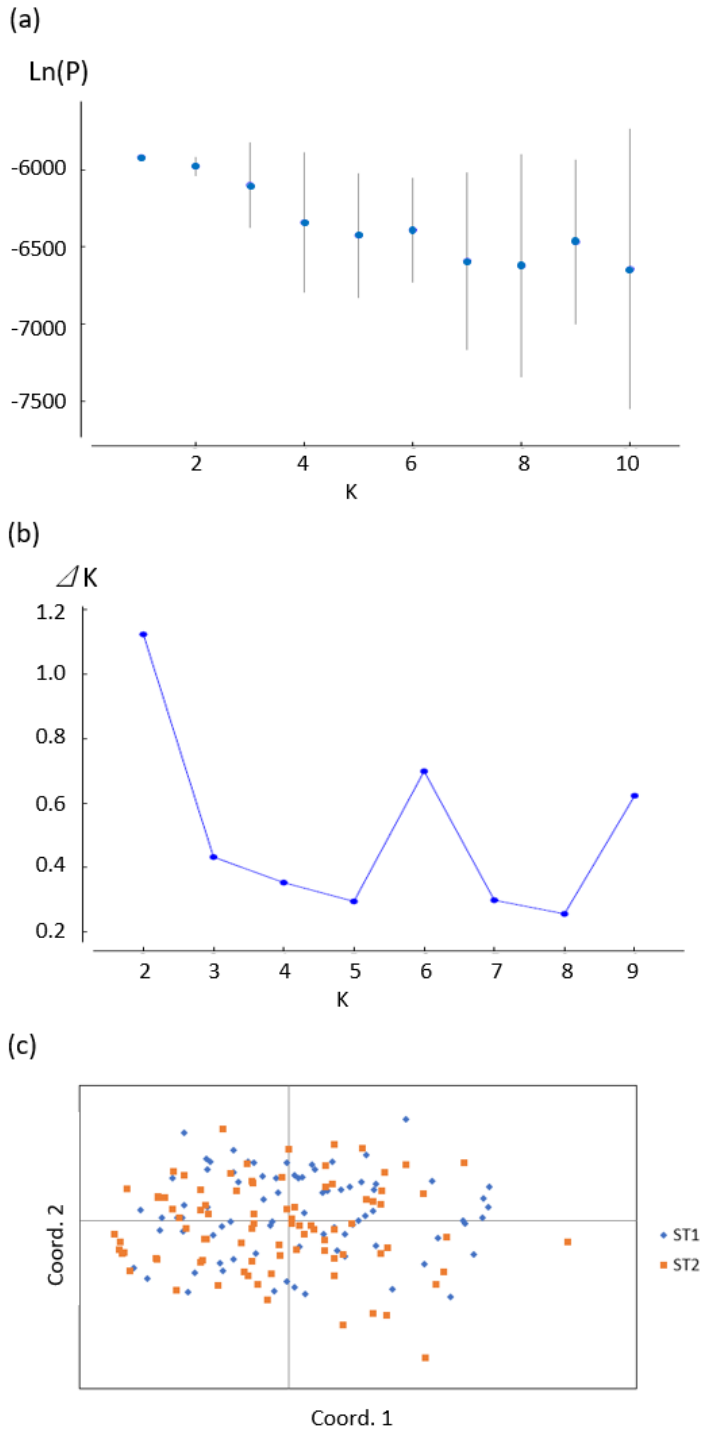

Figure 10. Detection of the optimal number of clusters in the population of *A. nephrolepis* at Mt. Hambaeksan by STRUCTURE. (a) Pritchard's method, (b) Evanno's ΔK method, and (c) results of principal coordinate analysis. Coord. means coordinate.

4.1.4. Sampling simulation study

According to the simulation study, sampling 15 individuals captured 95.9% of the common alleles on average. If the variance is considered, sampling 20 individuals ensured capturing over 95% of the common alleles (Figure 11-a). However, sampling 20 individuals only showed a 31.1% of success rate (Figure 11-b). Here, the success rate is the number of times among iterations that sampled over 95% of common alleles at every locus. At least 25 individuals were needed to exceed 50%. Sampling 30 individuals had approximately 80% of success rate (79.9%), 35 individuals had an 89.4% of success rate, and 40 individuals were required to have over 90 or 95% of success (95.8%) (Figure 11-b).

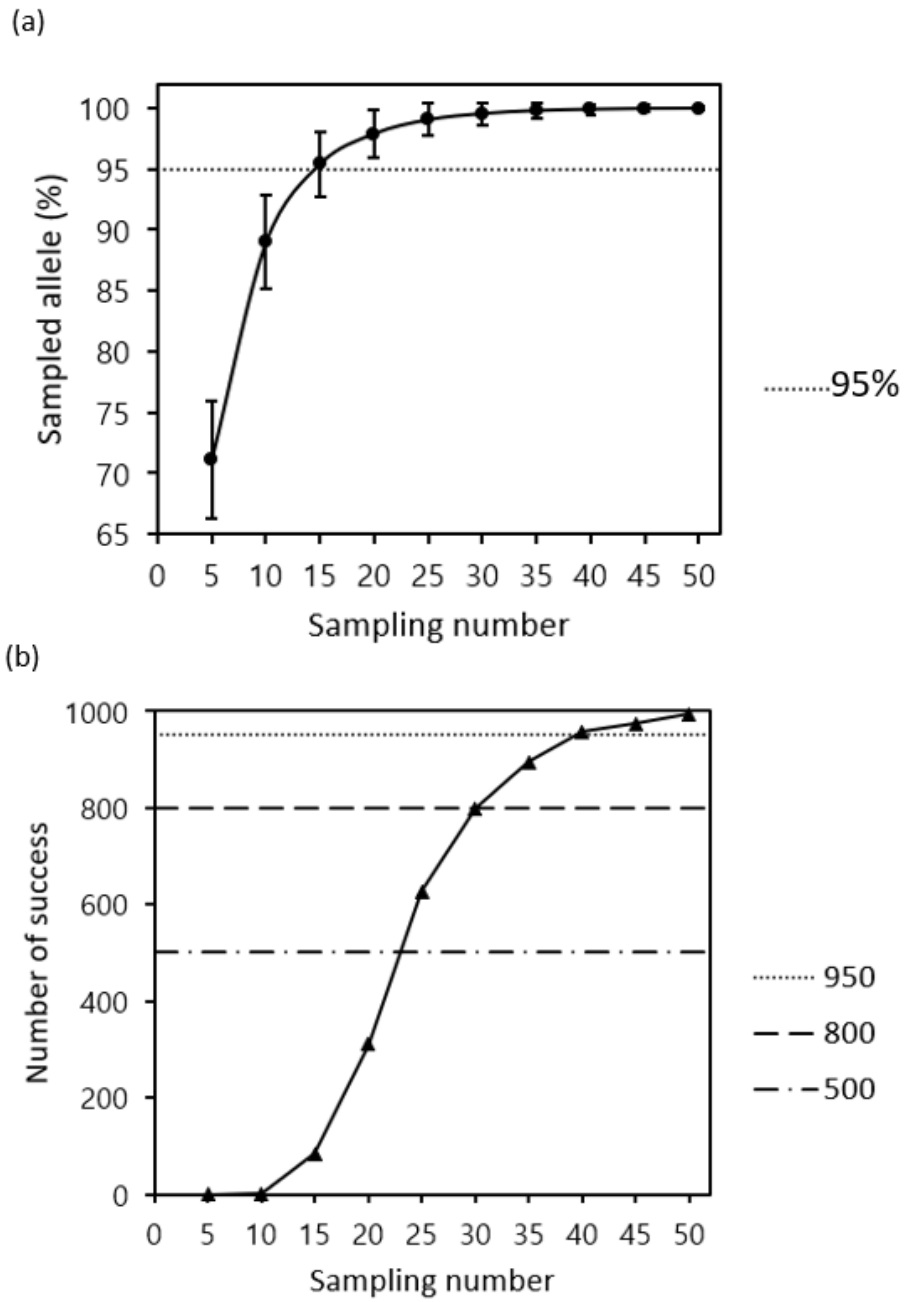

Figure 11. Results of sampling simulation in the population of *A. nephrolepis* at Mt. Hambaeksan. (a) 'Sampled allele (%)' indicates the average percentage of sampled common alleles over loci, error bars indicate standard deviations, and (b) 'Number of success' represents the number of times that reached the goal at every locus.

4.2. Seed characteristics and germination

4.2.1. Seed characteristics

4.2.1.1. Phenotypes of mother trees

The mother trees, together, had all common alleles at every locus. On average, the mother trees had a diameter at breast height (DBH) of 14.55 cm (±3.86 cm) which was a little smaller than the average obtained from all trees (15.75±4.23 cm) but within the error range. The smallest tree that produced cones had a DBH of 5.2 cm. The average height of the mother trees was 6.5 m with a standard deviation of 1.4 m. It was taller than the average of all trees (5.3±1.4 m). The smallest tree that produced cones had a height of 2.4 m.

The cone abundance, which is the number of cones produced in a tree, was 55.77 (±67.36) on average. As shown in Figure 12, the variance in cone abundance was huge. The least productive tree only produced five cones, while the most productive one produced about 270 cones. Overall, the top 20% of the most productive mother trees produced 61% of the total cones, and 81% of the cones were produced by the top 42% of the most productive mother trees.

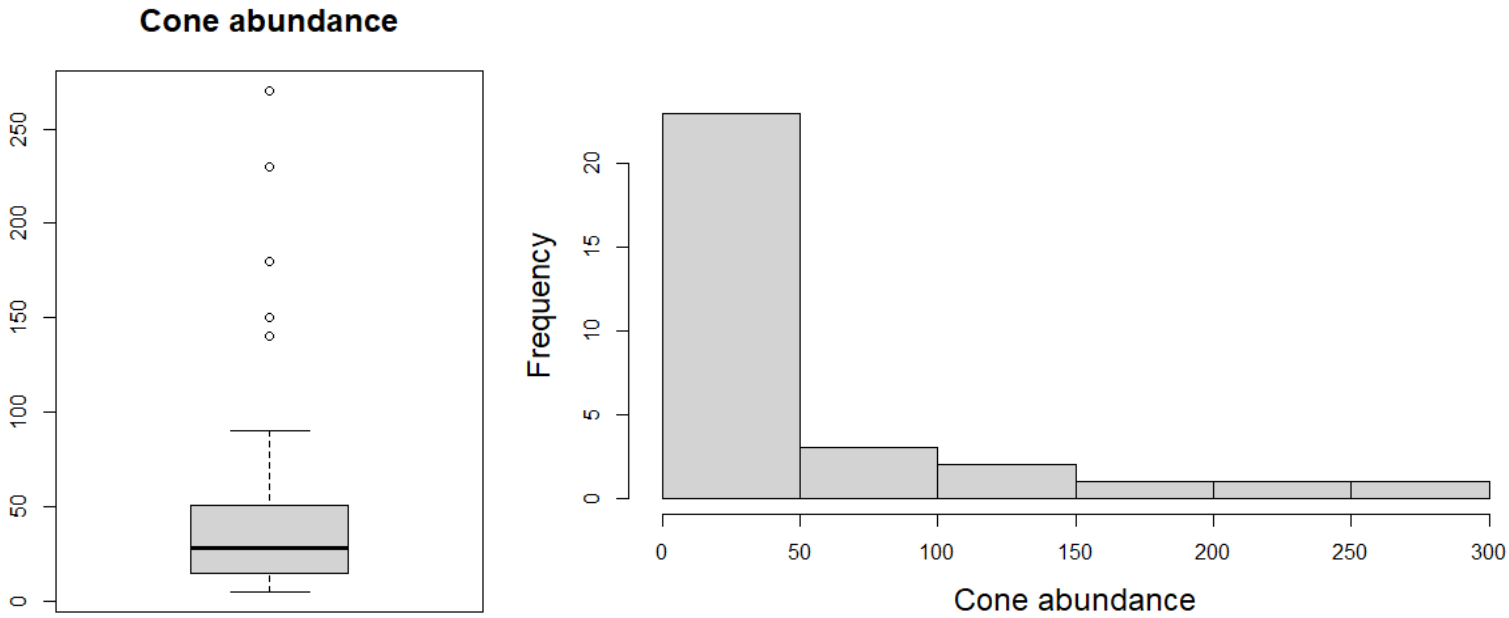

Figure 12. Boxplot and histogram of cone abundance of the mother trees in the population of *A. nephrolepis* at Mt. Hambaeksan.

4.2.2.2. Cone analysis

The results of the cone analysis are shown in Table 7. The cone and seed characteristics of sampled mother trees were similar to a previous study that analyzed 5 different populations in South Korea (Song et al., 2008). The cone length was slightly smaller than the average range of the previous study, while the cone width was relatively bigger within the average range. The seed weight was about the middle of the average range of the previous study. However, the purity was about 10% lower than the forest seed standard quality investigated by the Forestry Research Institute (Kim and Yoon, 1994), which is now known as the National Institute of Forest Science.

Table 7. Cone and seed characteristics in the population of *A. nephrolepis* at Mt. Hambaeksan

	Ref	CL (mm)	CW (mm)	DW (g)	W100 (g)	SP	DS	Purity (%)	P−DS (%)	P−DM (%)
Mean (±sd)	This study	55.35 (±6.90)	23.40 (±1.66)	6.578 (±1.888)	0.958 (±0.184)	278.4 (±43.9)	259.3 (±44.7)	70.2 (±6.6)	93.0 (±4.7)	10.4 (±11.4)
Range (min −max)	This study	36.95 −71.83	19.68 −26.23	3.433 −11.392	0.504 −1.433	174 −378	158 −365	48.7 −81.2	73.1 −100.0	1.1 −62.9
Average range of other pops	Song et al., 2008	55.6 −64.5	20.8 −23.4	–	0.7 −1.1	–	–	–	–	–
Standard quality	Kim and Yoon, 1994	–	–	–	0.93	–	–	82	–	–

Ref; Reference, CL; cone length, CW; cone width, DW; dry weight of cone, W100; 100−seed weight, SP; seed potential, DS; number of developed seeds, P−DS; percent developed seeds, P−DM; percent damaged seeds, sd; standard deviation, min; minimum, max; maximum, pops; populations

4.2.2. Germination test

Benefiting from the long pre-chilling period, seeds germinated early (Figure 13). A seed was first germinated only a single day after the germination test began. The mean germination time was 7.4 days and the germination energy on day 6 was 15.4 %. In the end, the germination percentage was 32.2% and the viability was 34.2% (Table 8). There was only a little gap between the germination percentage and viability. There was a high variance in the germination percentage and viability among the dishes. It might be because the dishes contained seeds from different mother trees and cones.

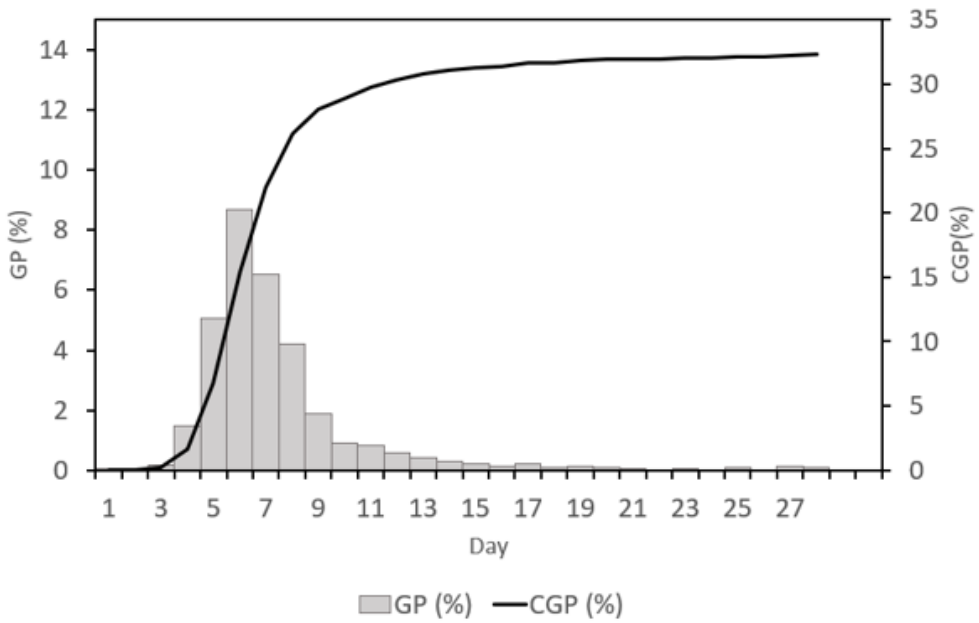

Figure 13. Germination curve of *A. nephrolepis* seeds from Mt. Hambaeksan. GP means germination percentage each day. CGP means cumulative germination percentage.

Table 8. Germination characteristics in the population of *A. nephrolepis* at Mt. Hambaeksan

	Mean (±sd)	Range (min−max)
GP (%)	32.2 (±15.1)	3.9−72.5
Viability (%)	34.2 (±15.8)	3.9−73.5

GP; Germination percentage, sd; standard deviation, min; minimum, max; maximum

Survivorship was calculated at 2 and 6 months after the transplantation. The survivorship after 2 months was 77.5% and the survivorship after 6 months dropped to 64.2%.

4.2.3. Statistical analysis

Correlation analysis was first conducted on the phenotypes of mother trees. It was found that the DBH and cone abundance had a positive relationship, with a correlation coefficient of r=0.61 (Figure 14).

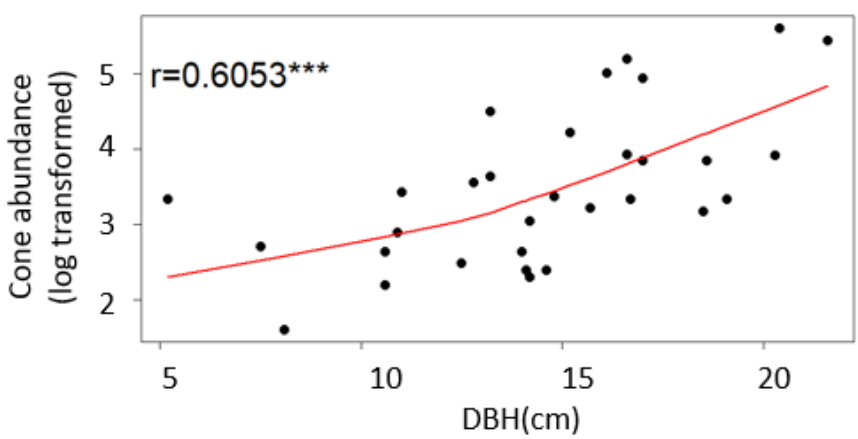

Figure 14. Correlation between DBH and cone abundance in the population of *A. nephrolepis* at Mt. Hambaeksan. *** $p < 0.001$

The results of the correlation analysis on cone characteristics are shown in Figure 15. The cone length, cone width, dry weight of a cone, 100-seed weight, and seed potential had positive relationships with each other. All of them also had a positive relationship with purity. Among them, dry weight of a cone had the strongest correlation with purity. However, for the germination percentage, only 100-seed weight had a significant relationship.

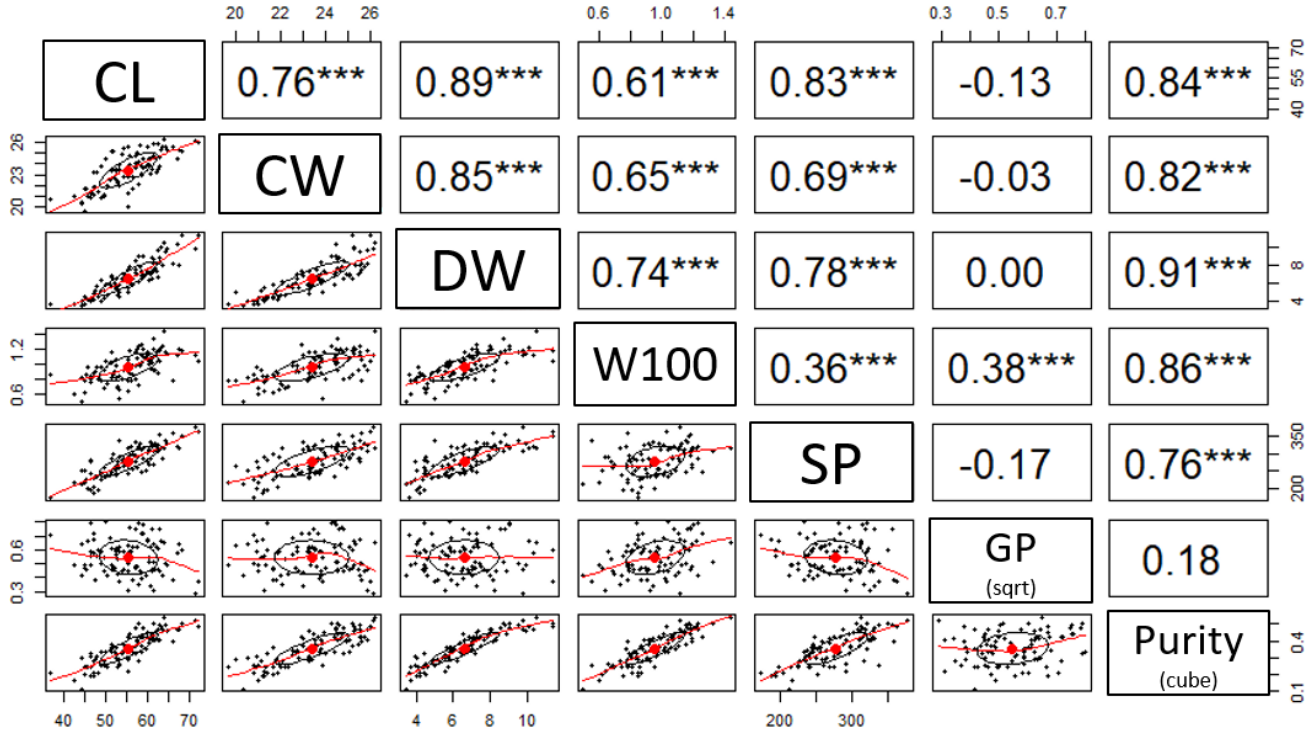

Figure 15. Results of correlation analysis on cone characteristics in the population of *A. nephrolepis* at Mt. Hambaeksan. CL, CW, DW, W100, SP, and GP each represent cone length, cone width, dry weight, 100-seed weight, seed potential, and germination percentage. (sqrt) and (cube) each indicate there was a square root and cube transformation. *** $p < 0.001$

There were differences in the seed quality by mother tree, and some mother trees had huge variations among cones within themselves (Figure 16). Correlation analysis failed to discover a significant relationship between genetic relatedness and seed quality. Neither genetic distance nor the number of trees within the distance of spatial autocorrelation (30 m) was not significantly related to the seed quality. However, there was a weak tendency that cannot be ignored (Figure 17).

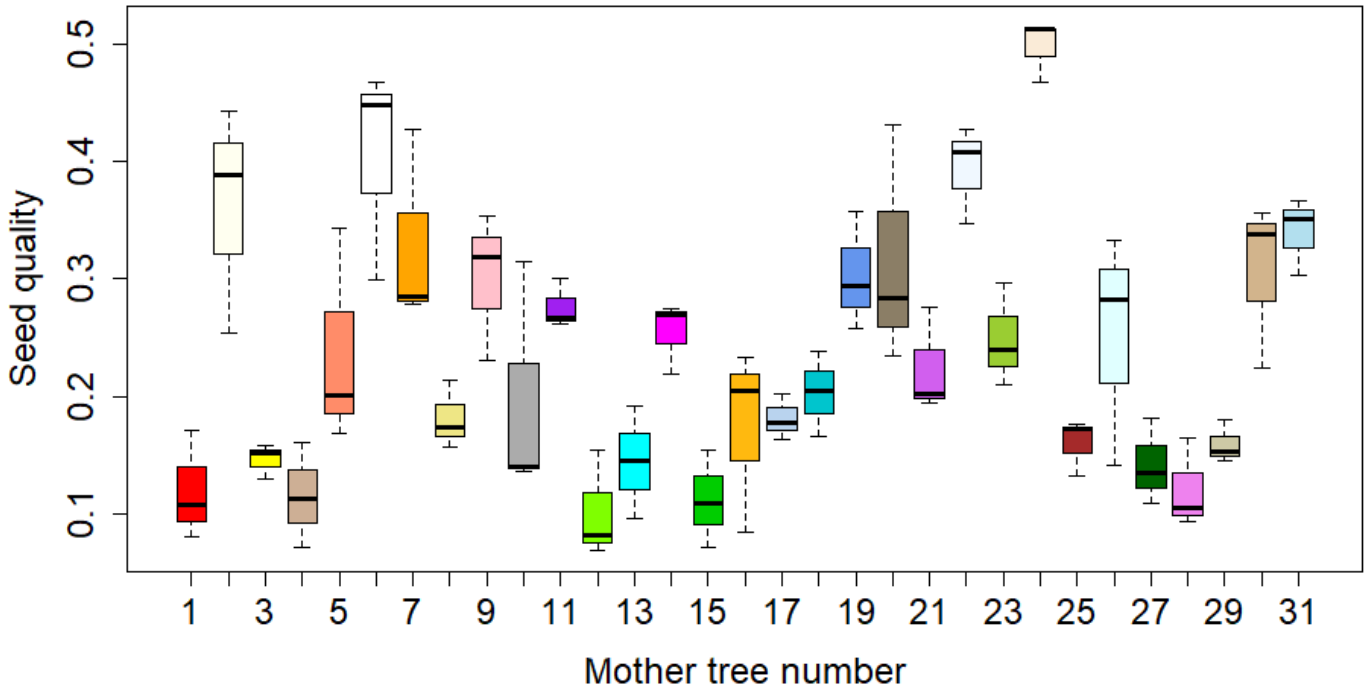

Figure 16. Boxplot of the seed quality by mother tree in the population of *A. nephrolepis* at Mt. Hambaeksan. Seed quality here was obtained by multiplying the purity and viability.

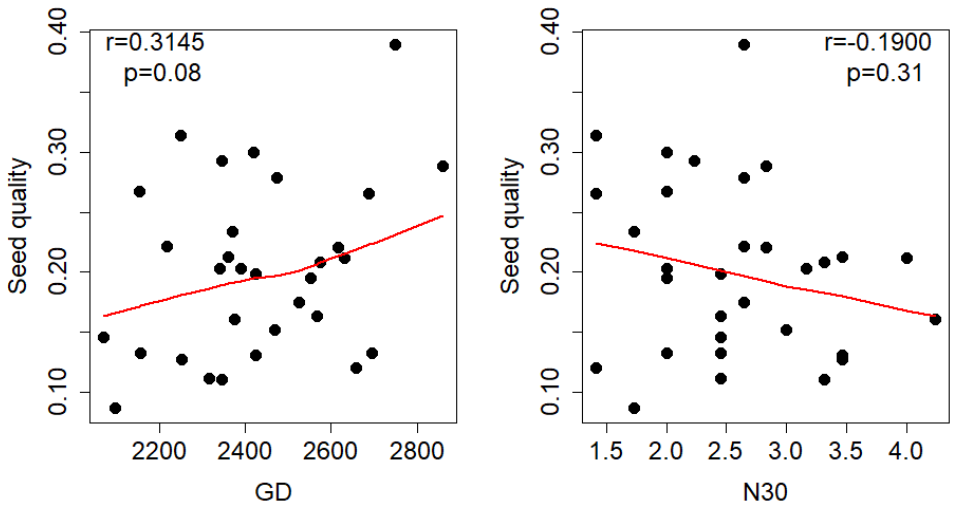

Figure 17. Scatterplot between the genetic distance and seed quality in the population of *A. nephrolepis* at Mt. Hambaeksan. Seed quality here is calculated by multiplying viability (after square root transformation) and purity (after cube transformation). GD stands for the sum of genetic distance to other trees. N30 means the number of trees within 30 m (after square root transformation).

5. Discussions

5.1. Spatial genetic structure

Genetic diversity indices showed a high level of genetic diversity in Mt. Hambaeksan. The heterozygosity was higher than in most studies on *A. nephrolepis* in South Korea (Woo et al., 2008; Hong et al., 2011). However, it might be caused by the difference in markers. The other studies used markers that lacked polymorphism, which can result in low heterozygosity. When the result was compared to the study that used markers with a similar level of polymorphism (Seo and Lim, 2022), other populations in South Korea had a similar level of heterozygosity. The inbreeding coefficient (F) was low (F=0.015), and it was also similar to the previous study by Seo and Lim (2022). A low level of inbreeding coefficient was expectable because *A. nephrolepis* is an outcrossing species.

The spatial autocorrelation analysis and Sp statistics revealed that spatial genetic structure (SGS) existed within Mt. Hambaeksan. The individuals were genetically related until 30 m, and random after that. Although it is not directly comparable, it was similar to the results of other species in the genus *Abies*. *A. koreana* in Mt. Hallasan showed genetic randomness when the individuals were farther than 10–35 m (Chae et al., 2021). For other *Abies* species in different regions, *Abies balsamea* (L.) Mill. had 10–70 m of SGS (Shea and Furnier, 2002), *Aies alba* Mill. had 17–121 m (47 m on average) of SGS (Major et al., 2021), and *A. sachalinensis* had the SGS of 60 m in juveniles (Lian et al., 2008). The Sp values, which is a more comparable indicator of SGS, also showed that *A. nephrolepis* in Mt,

Hambaeksan had similar extent of SGS to other species in *Abies* (Table 6).

When the spatial autocorrelation analysis and *Sp* statistics were applied by diameter group, it revealed that the bigger trees had a stronger structure than the smaller trees. While there was no significant SGS among the smaller trees, the bigger trees had a stronger SGS with *Sp*=0.0081. This was away from the expectation that small trees would have stronger SGS because of a lack of thinning after the half-sib structure of recruits near the maternal source. However, considering only adult trees were used in this study, the impact of thinning might have already disappeared. Several studies also stressed that younger generations of outcrossing species can have a weaker SGS (Latouche-Halle et al., 2003; Jacquemyn et al., 2006; Jones and Hubbell, 2006). According to these studies, an increase in genetic structure towards older life-history stages can result from local selection, historical events, and demographic factors.

I examined each factor to find the reasons for weakened genetic structure in the smaller diameter class in this study. First, microhabitat selection of related individuals can cause stronger SGS in older trees. In this study, the bigger trees had a higher fixation index. This leaves the possibility of microhabitat selection. However, in this case, microhabitat selection should have happened during the adult stage when *A. nephrolepis* was old enough. This contradicts the common belief that adult trees have adapted to their given microhabitat. Second, demographic factors, like overlapping generations, can weaken the structure. After random dispersion, if young generations join the upper canopy beside their relatives, it results in a stronger structure in the adult stage. In this study,

however, both diameter classes were at the adult stage, having similar diameter class intervals. Therefore, both groups could have overlapping generations. Finally, historical events such as the founder effect, bottleneck, or non-steady state source-sink dynamics can cause increased structure in adult trees. Active gene dispersal after the establishment of a few founders can weaken the structure towards younger individuals. A major proof of bottleneck or founder events is a reduced number of alleles. In this study, the bigger trees had a higher fixation index and lower heterozygosity, but they did not have a significantly reduced number of alleles. Therefore, it is hard to conclude the cause of this structure yet, and it leaves more to be studied. Moreover, although diameter at breast height (DBH) is commonly used as an indirect indicator of age, it is hard to compare the diameter class directly to the age structure. Among the sampled individuals in this study, cores were extracted from the two trees in different diameter classes (one had a DBH of 15.2 cm and the other one had a DBH of 21.6 cm), but they were estimated to have similar ages. To study the change in SGS through generations, the age of each individual should be investigated.

The genetic clustering study with two Bayesian clustering models resulted in different numbers of clusters within the population. While STRUCTURE did not find evidence of clustering within the population, GENELAND found two clusters. There are several studies reviewing Bayesian clustering models (Latch et al., 2006; Guillot, 2008). These studies reported that a low level of genetic differentiation can increase the error rate in the Bayesian clustering model. For this reason, applying multiple Bayesian models with other independent inference methods, such as principal component analysis (PCA) or principal coordinate analysis (PCoA), is necessary (François and

Durand, 2010).

In this study, PCoA failed to validate the clusters suggested in GENELAND and did not suggest any clustering. Although there were two clusters found using GENELAND, they had a very low level of differentiation, F_{ST}=0.009. This could increase the error rate of Bayesian clustering models. If the result of GENELAND can be explained with abiotic barriers, it becomes more plausible. In this study area, the cluster in the middle, the GL2 area inside the U-shaped zone (Figure 8-c), had some margins that are related to the area where barriers exist. The barriers include stony areas or shrub colonies that inhibit the regeneration of *A. nephrolepis*. However, it only explains a few margins of the clusters, and there is no apparent abiotic barrier that can explain the overall structure suggested in GENELAND. Therefore, in this study area, there is a higher possibility that genetic clusters had not developed.

A simulation study showed that sampling 20 individuals can capture more than 95% of the common alleles on average. However, when the goal was to capture over 95% of the common alleles at every locus, more individuals were needed. This difference was caused because there were differences in the number of alleles among the loci. This made some loci easily reach the goal, while some other loci are much harder to reach the same goal. Much more individuals will be required to include the rare alleles in the populations. Although this study only considered common alleles, rare alleles can contain some important information about the population.

5.2. Seed production

The average DBH of the mother trees was 14.55 cm with 6.5 m height, and the smallest tree that produced cones had 5.2 cm DBH and 2.4 m height. Considering the generation length of *A. nephrolepis*, this indicates a slow growth. In a previous study on the growth of *A. nephrolepis* seedlings at Mt. Gyebangsan, the author estimated that it would have taken 43 years to reach 2 m height on average, and 22 years for fast-growing individuals (Choi, 2021).

The seeds and cones of *A. nephrolepis* in Mt. Hambaeksan mostly had similar characteristics to other populations. However, the purity was approximately 10% lower than the standard quality. Considering the standard quality was published in 1994, changes in climate conditions could have resulted in poorer purity. There were several studies on the relationship between climate change and seed quality. They found that the increased temperature and moisture stress can reduce the seed yield and quality, causing shortened duration of stigma receptivity (Singh et al., 2013). Temperature irregularity affects flowering timing, which cause asynchronous flowering within a population (Maity and Pramanik, 2013). The sampling year, 2021, had a higher temperature and less precipitation compared to the normal years, 1991-2020. The average annual temperature was about 0.5℃ higher, and annual precipitation was about 100 mm less. This might have affected pollen production or pollination, which can negatively impact the pure seed production. Especially, the genus *Abies* lacks pollination drop and uses rainwater as a replacement (Owens et al., 1998). Therefore, decreased precipitation could have decreased pollination success and led to fewer pure seeds.

In this study, the germination percentage and viability only showed

a little gap. It could be because of the successful germination and dormancy breaking of the pure seeds. However, as many seeds were damaged during the long pre-chilling period, the viability after the germination test can differ from the viability before any treatment. Therefore, non-destructive methods such as X-rays are necessary. The viability calculated before any treatment will offer better information because it can also be applied to other dormancy-breaking methods or direct germination. The survivorship after transplantation was over 60%. However, real survivorship can be lower because only seedlings that successfully developed cotyledons were transplanted and grew in ideal conditions. Testing germination and growth directly in the field will offer a better understanding of the survivorship.

The damaged seeds from Mt. Hambaeksan were mostly fungal damaged. Not only fungal-damaged seeds, but fungal growth also constantly appeared. Therefore, an appropriate post-collection treatment seems important to improve seed quality. There was no insect damage found in the cones collected from Mt. Hambaeksan. However, insect damage on *Abies* exists in South Korea. Kim et al. (2020) studied cone insects of *A. koreana* in Mt. Hallasan. They found that 70.5% of the cones were damaged on average, and the most common insect was *Dioryctria abietella* (Denis & Schiffermüller). Also, in the seed orchard of *A. nephrolepis,* insect damage affected seed production (Kim et al., 2021). These studies were conducted in sites at lower altitudes or latitudes compared to Mt. Hambaeksan. Considering climate change and the high mobility of insects, regular monitoring is important to prevent the outbreak.

The correlation analysis between seed characteristics found that more cones existed in trees that have bigger DBH. This result

corresponds to the previous research. For some species in Pinaceae, older trees produced more cones while younger trees produced more pollens (Williams, 2009). The amount of pollen of *A. nephrolepis* was not measured in this study, therefore, further studies might offer interesting information about the relationship between age and strobili production. There was a positive relationship between seed weight and seed quality. With this result, prioritizing heavier seeds in propagation seems efficient. For sampling, direct evaluation of seed weight is impossible because it needs post-collection treatment such as drying or cleaning. Cone weight can be a good alternative because it had the strongest correlation with the seed weight and also had a positive relationship with the purity.

The correlation analysis failed to find a significant relationship between genetic relatedness and seed quality. As the genus *Abies* has a post-zygotic barrier and lacks pre-zygotic barriers, it was expected that mother trees having more genetically close pollen trees would have poorer seed quality than the opposite. The insignificance could be because of the sufficient gene exchange among mother trees. Because *A. nephrolepis* is a predominantly outcrossing wind-dispersed conifer, occupying the upper canopy in the study plot allows active pollen dispersal. However, the statistical insignificance was not obvious and a weak tendency was found, so it leaves more to be studied.

5.3. Strategy for ex-situ conservation

For an efficient ex-situ conservation, genetic structure and seed characteristics must be considered. With this study, several sampling and management strategies for *A. nephrolepis* in Mt. Hambaeksan could be established.

For the ex-situ conservation of *A. nephrolepis* in Mt. Hambaeksan, materials need to be collected from distant individuals that are at least 30 m away. To encompass sufficient genetic diversity, at least 20 individuals have to be sampled. However, if it is important to secure the common alleles at every locus, more individuals need to be sampled. Keeping sampling individuals distant, over 30 m away, will help reduce the optimal number of samples. In this study, 31 mother trees, which were 35 m apart from each other on average, included all common alleles. Therefore, sampling 20-30 individuals with a 30 m distance will secure sufficient genetic diversity.

Some strategies for seed collection could also be established based on this study. Although trees with bigger DBH might have more cones in a tree, it does not ensure seed quality or genetic diversity. Therefore, cones must be collected from various distant trees regardless of size. Within a tree, collecting heavier cones would be effective, as heavier cones have a higher probability of good quality. After the collection of cones, proper post-collection storage is important. Cones should not be stored in moist conditions to prevent fungal growth and damage. After seeds are separated from the cones, heavier seeds should be chosen as they have a higher possibility to germinate.

For further propagation, seeds need to be transplanted within a few days or weeks after the germination. This is because the

seedlings that grew on the Petri dish for a longer period showed drastically lowered survivorship after the transplantation. Even before the transplantation, seedling deaths were commonly found in the Petri dish. Finally, if only a few germinated seedlings need to be selected for propagation, it is better to avoid the seeds whose germination had started with cotyledon. In the germination test, few seeds germinated with cotyledon, while most seeds germinated with radicle. Those seedlings that germinated with cotyledons did not survive or grow well.

6. Conclusions

This study aimed to produce a reference for efficient ex-situ conservation of *A. nephrolepis*. The genetic variation and seed characteristics in a population of *A. nephrolepis* at Mt. Hambaeksan were investigated.

A. nephrolepis in Mt. Hambaeksan had developed a spatial genetic structure (SGS). The individuals had a positive genetic relationship until 30 m. The extent of SGS was weak, and there was no cluster found within the population. Sampling 20-30 individuals was enough to secure genetic diversity. The cones and seeds had similar characteristics to other populations in South Korea. There was a decrease in purity, probably caused by higher temperature and less precipitation in the sampling year. Correlation analysis revealed that the diameter at breast height (DBH) was positively related to the cone abundance, and the seed weight was the most effective indicator of the seed quality. The mother trees that were genetically closer to other individuals seemed to have poorer seed quality, but it was insignificant.

This study was the first to investigate SGS and seed production together in a population of *A. nephrolepis* in South Korea. With the results, it was possible to design an efficient sampling scheme and a few guides for propagation. Together, this study contributes to the establishment of an effective ex-situ conservation of *A. nephrolepis* in Mt. Hambaeksan. It can also be a reference for further studies on the within-population variation in *A. nephrolepis*.

Further studies need to be continued for a better understanding of *A. nephrolepis* in Mt. Hambaeksan. Demographic and historic factors

need to be studied deeper to investigate the change in SGS through generations. Further studies on the mating systems and pollen dispersal are also necessary. Seed damage is another part to investigate. Fungi, which might be the major cause of the seed damage in this study, need to be identified to reduce unnecessary loss of seeds.

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초 록

분비나무 *Abies nephrolepis* (Trautv. ex Maxim.) Maxim.는 아고산 침엽수종의 하나로 남방한계 집단이 국내에 분포해 있다. 현재 분비나무는 기후변화 취약종으로 쇠퇴가 진행되고 있어 보전의 중요성이 증가하고 있으며, 현지외 보전을 위해서는 유전다양성과 종자에 대한 이해가 필수적이다. 그 동안 분비나무 집단에 대해서는 집단 간 변이를 확인하는 연구가 주로 진행되어 왔으며, 집단 간 분화가 작음을 확인하였다. 그러나 분비나무의 집단 내 변이에 대한 연구는 거의 진행된 바가 없다. 본 연구는 분비나무의 현지외 보전 전략 수립에 필요한 정보를 제공하는 것을 목표로 하였다. 이를 위해 함백산 분비나무의 집단 내 유전구조와 종자 특성을 파악하고자 하였다. 결과적으로 함백산 분비나무의 이형접합도 관측치는 0.809, 이형접합도 기대치는 0.820로 나타났다. 공간적 자기상관성 분석 결과, 분비나무 개체는 30 m 이내에서 서로 유전적으로 연관되어 있음이 확인되었다. 이는 곧 샘플링에 있어 개체 간 거리가 30 m 이상 간격이 유지되어야 함을 의미한다. 직경급 간 공간적 유전구조의 정도를 비교한 결과, 직경이 큰 나무들에서 상대적으로 강한 구조를 가지는 것으로 확인되었다. 집단 내 하부 집단의 수(K)를 계산한 결과, STRUCTURE와 GENELAND는 각각 K=1, K=2로 차이를 보였다. 그러나 주좌표 분석 결과, 특별한 하부 구조가 발견되지 않아 K=1의 결과가 지지되었다. 샘플링 시뮬레이션 결과, 충분한 유전변이를 확보하기 위해서는 적어도 20 개체 이상이 채집되어야 하는 것으로 나타났다. 함백산 분비나무의 종자는 기존 연구의 다른 집단과 유사한 특성을 보였으며, 32.2%의 발아율을 보였다. 하지만 순량율의 경우 분비나무 임목종자표준품질에 비해 다소 낮은 수치를 보였다. 이는 채집 연도가 평년에 비해 기온이 더 높았고 강수량이 더 적었던 것과 관련되었을 것으로 추정되었다. 상관분석 결과, 종자 무게가 종자

품질의 가장 효과적인 지표로 사용될 수 있을 것으로 보였다. 다른 개체들과 유전적 거리가 비교적 가까운 모수에서 종자 품질이 떨어지는 경향이 존재하였으나 통계적으로 유의하지는 않았다. 본 연구는 분비나무의 공간적 유전구조와 종자생산을 함께 고려함으로써 현지외 보전에 적용가능한 정보를 제공하였다. 추후 유전자 분산 및 교배양식에 대한 연구를 통해 분비나무의 유전구조에 대한 이해를 높일 수 있을 것으로 기대된다.

주요어: 기후변화 취약종, 공간적 유전구조, 마이크로새틀라이트 마커, 종자 생산, 현지외 보전
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