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Selection of Restoration Material for *Abies koreana* Based on Its Genetic Diversity on Mt. Hallasan

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Abstract: The restoration of damaged or disrupted forests with genetically appropriate restoration planting material that can adapt to future environmental conditions will ensure the conservation of forest genetic resources. *Abies koreana* is endemic to the Republic of Korea, with declining populations under current environmental changes. In this study, we examined the genetic diversity of its largest population growing on Mt. Hallasan to determine the sampling size of planting material from the population that will ensure 95% coverage of alleles in the population. We evaluated the genetic diversity and spatial genetic structure of three subpopulations of *A. koreana* on Mt. Hallasan. A total of 456 samples were evaluated using 10 microsatellites. The observed heterozygosity and expected heterozygosity were 0.538 and 0.614 at the population level, respectively. The differences among the subpopulations accounted for 4% of the total variance. Intervals between individuals of the sample to be extracted were based on the two-target distance (5 and 10 m) inferred from the spatial genetic structure. Through random sampling methods considering the target distance, we showed that genetic diversity can be captured by obtaining at least 35 individuals in the population of *A. koreana* on Mt. Hallasan.

Keywords: *Abies koreana*; sampling strategy; genetic diversity; restoration material; Mt. Hallasan

Citation: Chae, S.-B.; Lim, H.-I.; Kim, Y.-Y. Selection of Restoration Material for *Abies koreana* Based on Its Genetic Diversity on Mt. Hallasan. *Forests* **2022**, *13*, 24. https://doi.org/10.3390/f13010024

Received: 13 October 2021
Accepted: 21 December 2021
Published: 24 December 2021

1. Introduction

Approaches to restore damaged or disrupted forests should utilize genetically appropriate restoration material that can adapt to future environmental conditions and ensure the sustainable use and conservation of forest genetic resources [1]. The introduction of exotic species alters the existing unique genetic traits of native species, resulting in genetic pollution. Introgression may lead to the loss of native species identity through hybridization, which is exacerbated by the smaller number of populations [2]. To prevent this, it is important to use plant material native to the habitat to be restored [3]. Therefore, the selection of the restoration material should be based on the unique genetic characteristics of native habitats to ensure sustainable conservation of the genetic resources, by improving plant adaptability to changes in the environment [4]. Genetic diversity is a crucial factor for species survival and adaptation, and changes in population genetic diversity, estimated using genetic statistics, are a predictor of population resilience to environmental changes [5,6].

Abies koreana E.H. Wilson is endemic to the Republic of Korea, where it is mainly distributed on Mt. Jirisan, Mt. Hallasan, and Mt. Deogyusan [7]. It is a representative coniferous species that is commonly distributed in the sub-alpine region above 1300 m altitude [8]. The species has conservation value and is recognized as vulnerable to climate change. In 1998, it was designated as "Near Threatened" by the IUCN Red List rating and upgraded to "Endangered" in 2011 [9]. Mt. Hallasan is a distribution area with a single community of *A. koreana*, covering the largest area [10].

Mt. Hallasan was proclaimed a natural reserve in 1966 and was designated as Hallasan National Park in 1970, a UNESCO Biosphere Reserve in 2002, and a World Natural Heritage

Site in 2007 [11–14]. Thus, the area is of high conservation value. However, owing to growth decline, climate change, and other factors, the distribution of *A. koreana* on Mt. Hallasan has declined by 15.2% in 10 years since 2006; according to a survey conducted in 2017–2018, the incidence of dead trees was 28.2% [8,15]. Additionally, it has become difficult to regenerate the natural *A. koreana* forest because of damage to pinecones caused by insect pests and climate change in the habitat [16,17]. Therefore, appropriate restoration and conservation measures are urgently needed to preserve and maintain the stability of the declining *A. koreana* population on Mt. Hallasan.

Genetic variation in a population can be explained by various parameters, including allele and genotype frequency, gene diversity, heterozygosity level, and disequilibrium coefficient [18]. Marshall and Brown (1975) argued that a key indicator of optimal sampling is the number of such alleles that each population possesses, that is, the number of alleles that attain appreciable frequencies in only one population or in a few adjacent populations. The goal is to include more than 95% of the alleles that occur in the target population with a frequency greater than 0.05 [19]. However, sampling to detect alleles without a sampling strategy may waste more resources and overlap genetically similar individuals as the number of unnecessary samples increases. Therefore, extraction of genetically similar material should be avoided. For optimal sampling, spatial genetic structural analysis is used to understand the distribution of genetically similar specimens and determine the appropriate sampling distance [20–22]. The sample size is then inferred, and it includes more than 95% of the common alleles occurring in the target population with a frequency greater than 0.05.

The purpose of this study was to present a sampling strategy for the conservation and restoration of genetic resources of *A. koreana* population on Mt. Hallasan. First, we evaluated the genetic diversity of *A. koreana* on Mt. Hallasan and conducted structure analysis to see if there is any signature of population divergence. Second, we determined the spatial genetic structure of each population. Third, after random sampling based on the spatial genetic structure of each population, the number of specimens containing 95% or more of the common alleles that occurred *A. koreana* population on Mt. Hallasan at a frequency greater than 0.05 were selected. Finally, we suggested the minimum number of individuals that should be sampled to obtain optimal restoration material for the *A. koreana* population on Mt. Hallasan.

2. Materials and Methods

2.1. Study Sites

Needle leaves were collected from three subpopulations, from Yeongsil, Bangaeoreum, and Jindallaebat, which are representative distribution areas for *A. koreana* on Mt. Hallasan (Table 1, Figure 1). Mt. Hallasan has a representative large-scale population of Korean fir, with a total area of 757 ha [8]. The mean age of *A. koreana* in Yeongsil, Bangaeoreum, and Jindallaebat is estimated to be 73, 58, and 70 years, respectively [16]. Overall, it is one population, but because the species mainly grows in the sub-alpine area of the mountain, the main distribution area appears discontinuous. Therefore, the population was divided into three sub-groups based on the distribution and region (west, east, and south) and referred to as subpopulations. For each subpopulation, all mature trees (diameter at breast height, DBH $\geq$6 cm) distributed within the study sites were selected. The height and DBH of each individual were measured, and the average values are presented in Table 1. For spatial structure analysis, the position of each individual was determined using a GPS (GPS map60CSx; Garmin, Schaffhausen, Switzerland).

Table 1. Location of sampled *Abies koreana* subpopulations.

No.	Subpopulation	Sample Size	Height (m)	DBH (cm)	Geographic Location		
					Latitude	Longitude	Altitude (m)
1	Bangaeoreum	128	3.9	17.4	126°31.0′	33°21.3′	1610–1646
2	Yeongsil	152	3.6	17.3	126°30.5′	33°21.6′	1652–1663
3	Jindallaebat (Azalea Field)	176	5.0	22.7	126°33.3′	33°22.1′	1493–1551

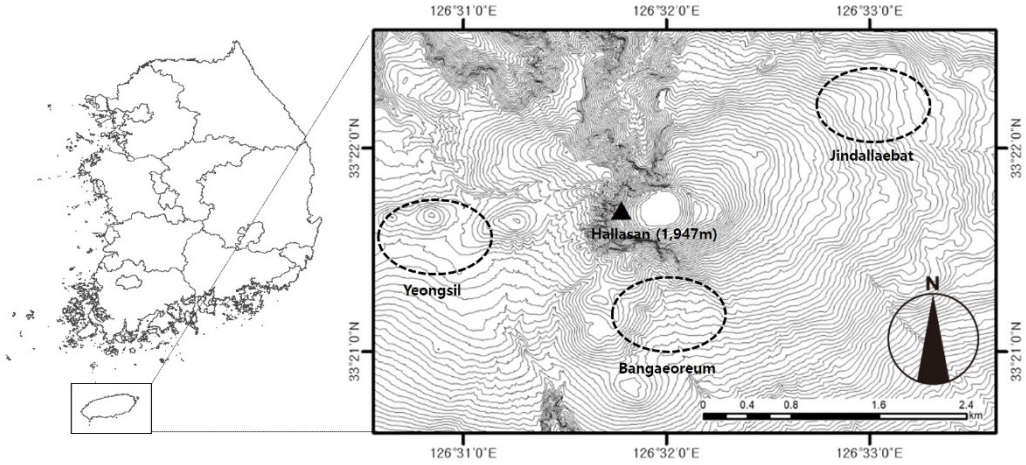

Figure 1. Location of *Abies koreana* on Mt. Hallasan.

2.2. DNA Extraction, Amplification, and Sizing

The total genomic DNA was extracted from fresh needle leaves of mature trees using a Plasmid SV mini kit (GeneAll Biotechnology, Seoul, Korea), and its concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Ten nuclear microsatellite (nuclear simple-sequence repeat, nSSR) markers developed for different *Abies* species [23–25] were selected for the present study (Table 2). The polymerase chain reaction (PCR) amplification was performed with 15 µL reaction mixtures containing 20 ng of template DNA, 1× reaction buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.04 µM FAM-labeled M13 (−19) sequencing primer, 0.2 µM primer mix, and 0.5 U Taq DNA polymerase (Biofact, Daejeon, Korea). The PCR conditions included denaturation at 94 °C for 5 min; 10 cycles at 94 °C for 60 s, 58–63 °C for 30–60 s, and 72 °C for 60 s; 25 cycles at 94 °C for 30 s, 51–58 °C for 30–60 s, and 72 °C for 60 s; and a final extension at 72 °C for 10 min. The PCR products were separated on an ABI 3730 xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Foster City, CA, USA), and the genotypes were determined using Gene Mapper v5.0 (Applied Biosystems, Thermo Fisher Scientific, USA). All loci were checked for the occurrence of null alleles, large allele dropout, and stutter bands using MICRO-CHECKER v2.2.3 software [26].

2.3. Genetic Data Analysis

Analysis of molecular variance (AMOVA) and analyses of genetic diversity indices, namely the number of alleles (A), number of effective alleles (A_e), expected heterozygosity (H_e), observed heterozygosity (H_o), and fixation index (F_{IS}), were performed to determine the differences in genetic variation between subpopulations. The Nei's genetic distance and pairwise F_{ST} value among the subpopulations were calculated. To visualize the genetic distance among the subpopulations, the principal co-ordinates analysis (PCoA) was performed using Nei's genetic distance. All the analyses were performed for each population using GenAlEx v.6.5 [27]. The significance level of deviation of F_{IS} from zero was determined using the FSTAT v2.9.4 software with 1000 permutations [28]. The genetic

structure of the subpopulations was identified using the Bayesian clustering method with the STRUCTURE v2.3.4 software [29]. Additionally, a pre-specified number of gene clusters (K), from 1 to 6, was assumed, in which the simulations were run 40 times per K value. All runs involved 100,000 Markov chain Monte Carlo samplings with a burn-in period of 100,000 iterations. Finally, the optimal K value was estimated by calculating ΔK, where the K values were calculated according to the method of Evanno et al. [30] based on the mean log probability and the standard deviation of the data using the STRUCTURE HARVESTER program [31]. Moreover, CLUMP [32] and DISTRUCT [33] were used to align 40 replicates and display the results separately.

Table 2. Characteristics of microsatellite markers used in the study.

No.	Primer	Primer (5′–3′)	Repeat motif	Reference
1	Aag02	F: TATTCCTCCACTTGGGTGCT R: GGTGGAGATCCGTATGCAAT	$(GA)_{13}$	[23] (*Abies alba*)
2	Aat04	F: CCATGTATGGTGCTCCTCCT R: CCTTCATTGCAGAAAAGCAA	$(CAG)_{11}$	[23] (*A. alba*)
3	Aat05	F: AGCATCCACATTCCGTAACC R: AGTTGACCGTTGGAGAGCAG	$(GCA)_{7}$	[23] (*A. alba*)
4	Aat12	F: ATCCATATCTCCTGCCTTGC R: CTTTCCAGGTGATCTGATTGC	$(AG)_{12}$	[23] (*A. alba*)
5	Aat15	F: AGGAGGAGGTTCAGCATGTC R: CTTGCTCTCTGACCCAGTTG	$(AGA)_{8}$	[23] (*A. alba*)
6	SF83	F: AGCAGCATAACCAAGGGTCAA R: TCTGAATTTCTAAAGGCGGC	$(CTT)_3 \ldots (GCC)_5$	[23] (*A. alba*)
7	NFF07	F: CCCAAACTGGAAGATTGGAC R: ATCGCCATCCATCATCAGA	(GA)33	[24] (*A. nordmanniana*)
8	NFF15	F: CGCCTCCCTCCATTACTTC R: TCGTCTAGAGAGGCGAAATTCT	$(AC)_{15}$	[24] (*A. nordmanniana*)
9	C49	F: GACGAAGATCAGTACAAGGCACGA R: GCGATCCTTCAATTTGTCCTTCTC	$(AGGAGA)_{7}$	[25] (*A. firma*)
10	C28104	F: CGAGGAAGAAGCCAAGTTATCAGG R: CACAGTTAAAAAGGCGGCCTACAG	$(ATA)_{5}$	[25] (*A. firma*)

2.4. Spatial Genetic Structure Analysis

GeneAlEx v.6.5 [27] was used to determine the spatial genetic structure. Spatial autocorrelation analysis was performed using the location of the individual and the genetic distance of Smouse and Peakall [21]; Distance evaluation (distance class) was performed at 10 intervals of 5 m, and 999 permutations were performed for each evaluation. The 95% confidence interval was calculated, and its significance was verified [21].

2.5. Comparison between Sampling Strategies

Intervals between individuals of the samples to be extracted were based on two target distances (5 and 10 m) inferred from the spatial genetic structure. We randomly selected up to 40 samples in units of 5 from each subpopulation, and samples were randomly selected from the same units of all subpopulations. According to each of the 25 sampling strategies (8 units × 3 subpopulations + 1 population), 10 subsets were generated using the Python script. Additionally, the number of alleles on Mt. Hallasan with a frequency ≥0.05 was compared according to the sample size extracted based on the sampling strategy. Mean comparison was conducted using Duncan's test at $p \leq 0.05$ with R [34].

3. Results

3.1. Genetic Diversity

As MICRO-CHECKER results revealed no evidence of genotyping error due to stuttering or large allelic dropout. The mean frequency of null alleles across loci ranged from 0.005 to 0.085. The average genetic diversity of the population on Mt. Hallasan is presented

in Table 3. The mean number of alleles (A), effective alleles (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e), and fixation index (F_{IS}) were 8.0, 4.2, 0.538, 0.614, and 0.083, respectively (Table 3). The Jindallebat subpopulation showed the highest genetic diversity (A = 7.5, A = 4.3, H_o = 0.589, and H_e = 0.619) and the lowest fixation index (0.015). However, the Bangaeoreum subpopulation showed the lowest genetic diversity (A = 6.8, A = 3.8, H_o = 0.480, and H_e = 0.591) and the highest value of fixation index (0.136).

Table 3. Genetic diversity of *Abies koreana* subpopulations.

Subpopulation		A	A_e	H_o	H_e	F_{IS}
Bangaeoreum	Mean	6.8	3.8	0.480	0.591	0.136 ***
	SE	1.8	1.0	0.066	0.078	0.076
Yeongsil	Mean	7.0	3.6	0.527	0.595	0.071 ***
	SE	1.7	0.7	0.062	0.076	0.058
Jindalleabat (Azalea Field)	Mean	7.5	4.3	0.589	0.619	0.015 ***
	SE	2.0	1.1	0.076	0.083	0.065
Mt. Hallasan	Mean	8.0	4.2	0.538	0.614	0.083 ***
	SE	2.1	1.0	0.065	0.079	0.059

A: number of alleles; A_e: number of effective alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; F_{IS}: fixation index; ***: Significant deviation from Hardy–Weinberg equilibrium ($p < 0.001$); SE, standard error.

According to the AMOVA, the differences among the subpopulations accounted for only 4% ($p < 0.01$) of the total variance, and the variability within subpopulations accounted for 96% of the variance (Table 4). The genetic differentiation among subpopulations, as measured using the F_{ST} value, was 0.041.

Table 4. Analysis of molecular variance.

Source	Degrees of Freedom	Sum of Squares	Mean Squares	Estimated Variation	Percent of Variation	p
Among subpopulations	2	100.133	50.066	0.287	4%	0.001
Within subpopulations	453	3099.492	6.842	6.842	96%	-
Total	455	3199.625		7.129	100%	-

Based on the Nei's genetic distance, the genetic distance between subpopulations was 0.031–0.050 (Table 5, Figure 2). The pairwise F_{ST} estimated among subpopulations was 0.010–0.016 (Table 5).

From the STRUCTURE analysis, the value of ΔK was the highest at K = 2, based on the theory suggested by Evanno et al. [30] (Figure 3b). When K was set at 2, the proportion of the two clusters was visualized in all populations (Figure 3c).

Table 5. Nei's genetic distance below the diagonal and pairwise F_{ST} values above the diagonal among the three subpopulations of *Abies koreana*.

Subpopulation	Bangaeoreum	Yeongsil	Jindalleabat
Bangaeoreum	-	0.016 ***	0.010 ***
Yeongsil	0.050	-	0.013 ***
Jindalleabat	0.031	0.046	-

***: $p < 0.001$.

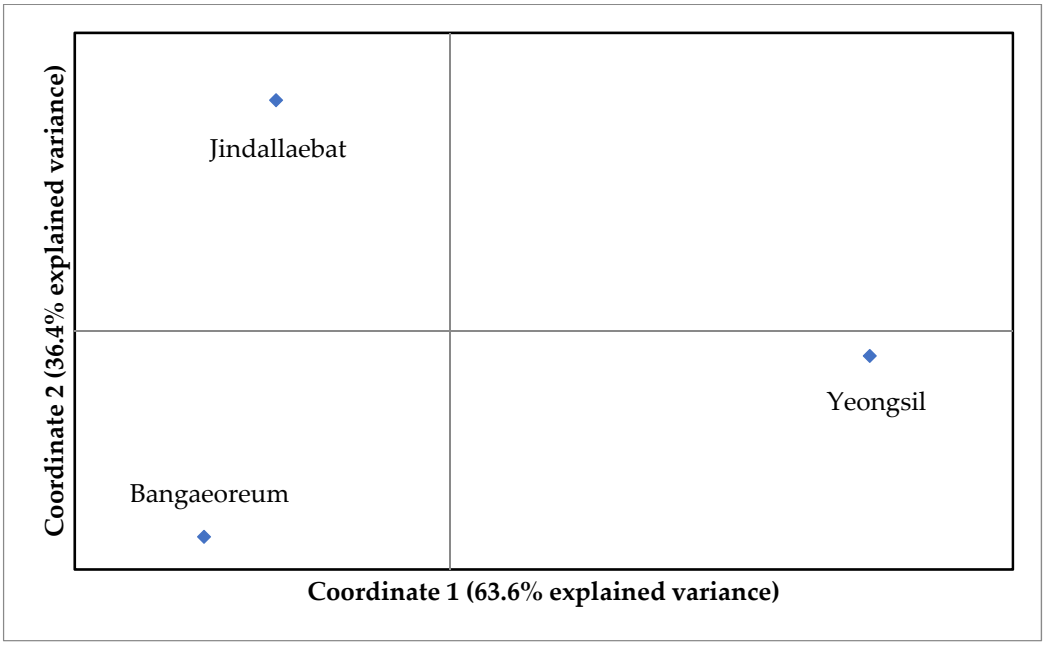

Figure 2. Principal co-ordinate analysis of the genetic distances among the three subpopulations of *Abies koreana* on Mt. Hallasan.

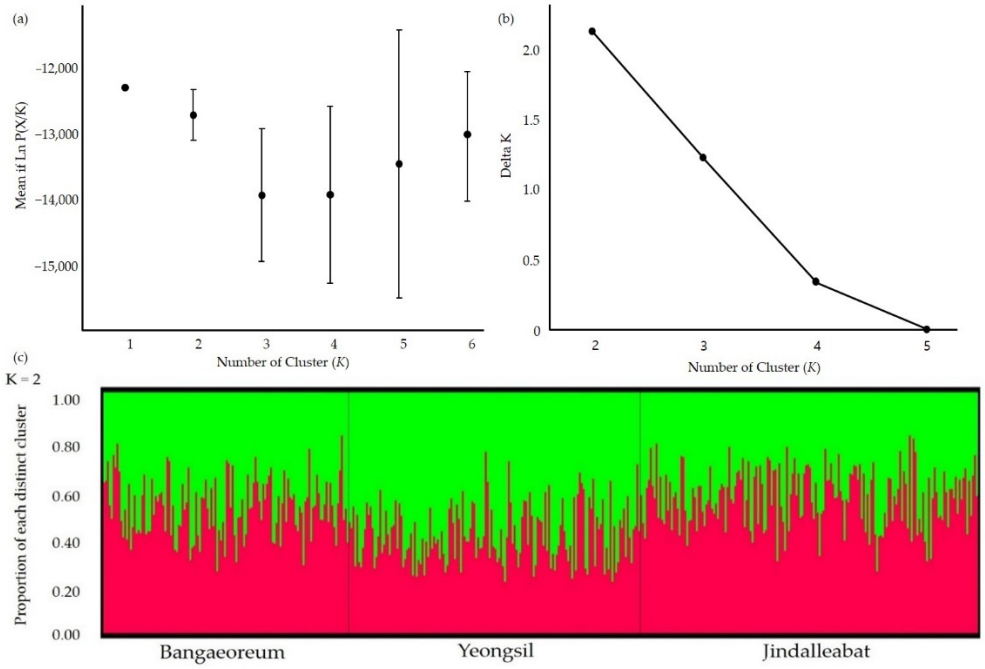

Figure 3. Results of Bayesian clustering analysis using STRUCTURE. (**a**) The mean log-likelihood value (±standard deviation) of the data was based on 40 repetitions for each K value. (**b**) The delta K value was changed with each K value. (**c**) The proportion of cluster membership at the population level in the three subpopulations of *Abies koreana*, assuming K = 2. The horizontal bars denote the samples; the green and red shades represent the genetic clusters.

3.2. Spatial Genetic Structure

The spatial autocorrelation analysis of the genetic variation of an individual revealed that within the Bangaeoreum subpopulation, individuals distributed within a distance of 5 m were genetically similar and those within the range from 5 to 25 m were randomly dis-

tributed. Individuals in the range of 25–30 m were genetically distinct, and those distributed within >30 m were randomly distributed. In the Yeongsil subpopulation, genetic similarity was observed among individuals within 10 m distance, and this subpopulation was randomly distributed within >10 m. Genetic similarity in the Jindalleabat subpopulation was observed among individuals within approximately 10 m distance, and it was randomly distributed within 10–15 m, 20–30 m, and ≥35 m. Genetically different individuals were found within 15–20 m and 30–35 m (Figure 4).

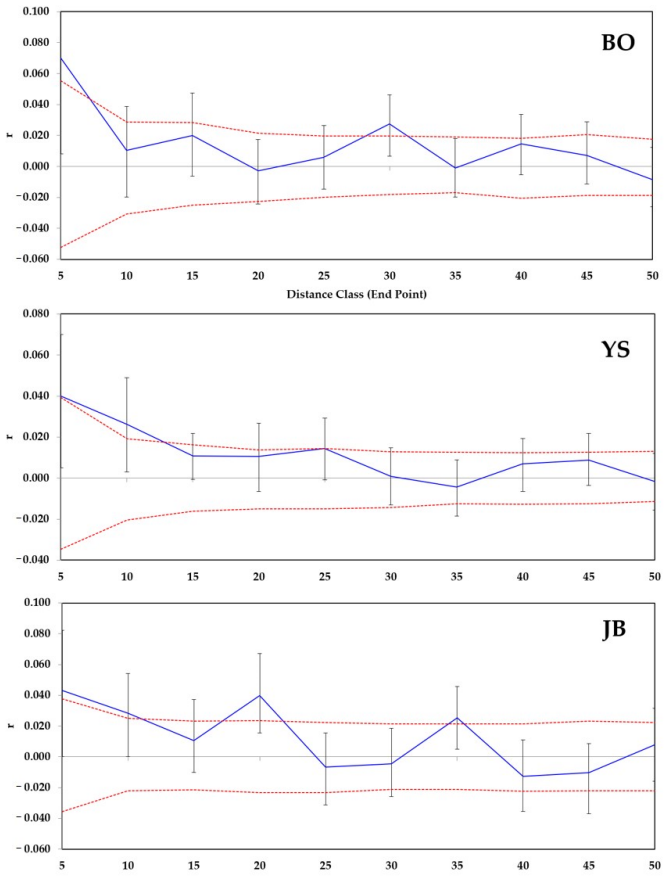

Figure 4. Distogram for *Abies koreana* based on Tanimoto genetic distance using 10 nuclear simple-sequence repeat (nSSR) markers. The r value is an autocorrelation coefficient. The solid line represents the correlation between genetic distance and geographic distance. The dotted lines are 95% confidence intervals. BO, Bangaeorum; YS, Yeongsil; JB, Jindallaebat.

3.3. Sample Size

The number of individuals randomly sampled at a distance from each subpopulation containing alleles with a frequency ≥0.05 is listed in Table 6. Sampling of 20 and 35 individuals from each subpopulation led to the detection of 95% and 100% of all alleles, respectively. At the population level, 100% of the alleles were recorded by sampling 30 individuals, 10 from each subpopulation.

Table 6. Comparison of allele numbers with frequency ≥0.05 in *Abies koreana* population on Mt. Hallasan according to the sample size extracted based on the sampling strategy. Values with different letters are significantly different according to Duncan's test at $p \leq 0.05$.

Subpopulation	**No. of Samples**							
	5	**10**	**15**	**20**	**25**	**30**	**35**	**40**
Bangaeoreum (%)	70 ± 3.9^{e}	89 ± 3.0^{d}	93 ± 5.3^{c}	95 ± 3.6^{bc}	98 ± 1.6^{ab}	99 ± 1.2^{a}	100^{a}	100^{a}
Yeongsil (%)	73 ± 4.6^{d}	86 ± 5.6^{c}	93 ± 5.5^{b}	97 ± 2.8^{a}	99 ± 2.8^{a}	99 ± 1.2^{a}	100^{a}	100^{a}
Jindalleabat (%)	75 ± 3.6^{e}	90 ± 4.0^{d}	95 ± 4.4^{c}	97 ± 2.1^{bc}	98 ± 2.1^{ab}	100^{a}	100^{a}	100^{a}

Population	**No. of Samples**														
	9	**15**	**24**	**30**	**39**	**45**	**60**	**69**	**75**	**84**	**90**	**99**	**105**	**114**	**120**
Mt. Hallasan (%)	87 ± 6.0^{c}	95 ± 1.6^{b}	98 ± 2.8^{ab}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}	100^{a}

4. Discussion

The genetic diversity of *A. koreana* on Mt. Hallasan (H_e = 0.614) was lower than that of the other large populations on Mt. Jirisan and Mt. Deogyusan (H_e = 0.672, 0.655) [35,36] and similar to that of a small population on Mt. Geumwonsan (H_e = 0.612) [35]. Mt. Jirisan and Mt. Deogyusan are located in the Korean peninsula, and the population on Mt. Geumwonsan comprises a small group of less than 20 individuals with a low genetic diversity. Individual movement on Mt. Hallasan, which is an island, is limited, and as its population diversity is lower than that of other populations of similar size, it is necessary to prioritize the conservation of the large populations during restoration.

The AMOVA results showed that the degree of differentiation among the subgroups of Mt. Hallasan was significantly higher than that in the population Mt. Jirisan (0.4%) [34]. However, low value in pairwise F_{ST} and Nei's genetic distance, indicates that there is no differentiation between subpopulations. Additionally, according to the STRUCTURE results, there was no strong evidence of differentiation between subpopulations. The two clusters seem to indicate some mixing of genotypes, but genetic variation is equally distributed in all three subpopulations. Although the number of clusters, K = 2, had the highest log probability based on the data, it was not significantly more supported than K = 1 based on the theory suggested by Pritchard et al. [30] (Figure 3a). Moreover, it is a common and well-known bias of STRUCTURE to show K = 2, even without population subdivision [37,38]. These results evidently indicate that there is no difference between subpopulations of *A. koreana* in Mt. Hallasan. Therefore, it is thought that the optimal sampling method for restoration of *A. koreana* population on Mt. Hallasan is to extract samples from all locations, regardless of subpopulations.

The minimum distance between individuals with random distribution on Mt. Hallasan population was 5 m in the Bangaeorum subpopulations and 10 m in the Yeongsil and Jindallaebat subpopulations. The topography and average height of individual trees contributed to the differences in each subgroup. In the Bangaeorum subpopulation, the topography affected the scattering distance of the seeds into the valley and prevented their spread over a wide range. The tree height and topography in the Yeongsil subpopulation were similar to those in the Bangaeorum subpopulation. However, the trees in the Jindallaebat subpopulation were 5 m taller than those in the other two subpopulations, and this increased the scattering distance of the seeds, and thereby the seed distribution.

Sample size, which is the number of randomly sampled trees in each subpopulation, will ensure the detection of all common alleles (frequency of $\geq$0.05) of each subpopulation. A sample size of 20 individuals was sufficient to identify 95% of the alleles, and extraction from 35 individuals covered 100% of the alleles. Additionally, 100% allele coverage was possible by 30 individuals, 10 from each subpopulation. Similar to the previous results, which demonstrated that there was little differentiation between subpopulations in the Mt. Hallasan, the results of the sample size simulations showed that genetic diversity could be captured by samples of more than 35 individuals, regardless of locations. Therefore, as an optimal sampling strategy for restoration material, collecting at least 35 samples regardless of subpopulations can capture the genetic diversity of the *A. koreana* in Mt. Hallasan.

5. Conclusions

Abies koreana, endemic to the Republic of Korea, is distributed in sub-alpine regions with declining populations under current environmental changes. Appropriate restoration and conservation measures are urgently needed to conserve the stability of the declining *A. koreana* population on Mt. Hallasan. In this study, we estimated the genetic diversity, genetic differentiation, and spatial genetic structure of *A. koreana* population on Mt. Hallasan. Based on the results, we proposed sampling 35 individuals, as an optimal sampling strategy to capture the genetic diversity of *A. koreana* on Mt. Hallasan. Future analysis including crossbreeding rate, effective population size, and seed dispersal patterns would be helpful to estimate the effect of the mating system on genetic variation in the progeny of the

analyzed populations. Our study thus provides insights for the development of strategies to restore and conserve this endangered species in future climate change scenarios.

Author Contributions: Experimental design, S.-B.C., H.-I.L. and Y.-Y.K.; investigation, S.-B.C. and H.-I.L.; formal analysis, S.-B.C.; project administration, Y.-Y.K.; supervision, Y.-Y.K.; writing—original draft, S.-B.C.; writing—review and editing, S.-B.C. and H.-I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Institute of Forest Science of the Republic of Korea (grant number: "FG0802-2018-01-2021").

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: We would like to acknowledge all the people who helped us with the sampling.

Conflicts of Interest: The authors declare no conflict of interest.

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