Genetic Considerations for Selecting the Seedlings to Restore a Small Population of *Abies koreana*, an Endangered Fir Species that is Endemic to Korea

Hyo-In Lim[1*], Seung-Beom Chae[1], Kyung-Tae Kim[2]

1Forest Bioinformation Division, National Institute of Forest Science, Suwon, Republic of Korea; 2Gyeongsangnam-do Forest Environment Research Institute, Jinju, Republic of Korea

ABSTRACT

Korean fir (*Abies koreana* E.H. Wilson), which is a Korean endemic species that has been designated as an endangered species by the International Union for Conservation of Nature, has been declining by approximately 33% in its natural habitat. There are three large and six small populations with a relatively large and small number of individuals, respectively. The Korean fir population in Mt. Geumwonsan is known to have only 20 adult trees and approximately 23 seedlings. It was also observed that the seed production was extremely poor, while the genetic diversity (He=0.612) is lower than that of other large populations in Korea, with a very high fixation index of the seedlings (F=0.318). Therefore, there is a high risk of local extinction due to the inbreeding effect with the limited number of trees and a great need to implement restoration projects immediately to reduce the risk. Selecting the restoration materials for the small population needs to consider the genetic diversity and uniqueness of the natural population while enhancing their adaptability and resilience against environmental change. Hence, to restock the Korean firs into the Geumwonsan population, we evaluated the genetic similarity between the populations in Korea and suggested a guideline to select the appropriate materials for restoration.

Keywords: Korean fir; Endangered species; Small population; Restoration; Genetic considerations

INTRODUCTION

A decline in the natural habitats due to climate change is a global issue. Especially, *Abies* species in sub-alpine regions are reported to be vulnerable to global warming, with the recent worldwide decline. The causes of the decline in *Abies* species in sub-alpine regions are reported due to increased winter temperatures and competition with broadleaf tree species [1-3]. Simply regarding the decline of natural habitats as a decrease in population size, in terms of number of individuals or total area in a given area or region, can lead to worse results with regards to successful sexual reproduction. This would be required for the sustainable regeneration of the population as well as for the maintenance of the genetic diversity to adapt to environmental change [4,5]. In order to mitigate habitat loss, restoration in the form of augmentation or the reestablishment of the population is needed and has been particularly emphasized in the "Global Plan of Action for the Conservation, Sustainable Use and Development of Forest Genetic Resources", which was developed by the Commission on Genetic Resources for Food and Agriculture [6].

Recently a renewed restoration method, i.e., the "genetic restoration", has received great attention, mainly because it re-establishes the declined population with careful consideration of the genetic concerns arising from previous restoration efforts, which include whether the population has a distinct genetic structure from other local populations and how much would the introduction of new genes and genotypes out of the local origin affect the genetic structure of the existing one [7]. A few thematic studies have suggested a guideline for the genetic restoration of declined forest ecosystems [8]. In Korea, a recent demographic research reported that approximately 25% of the natural habitats of the sub-alpine coniferous forests have declined during the past 20 years [9]. Moreover, the Korea Forest Service have established an integrated action plan in 2016to manage the decline in the natural habitats, which was called "A Countermeasure on Conservation and Restoration of Endangered Sub-Alpine Conifer species"

and had four strategies: (Strategy I) surveying the current status of the distribution and growth, (Strategy II) *in situ* and *ex situ* conservation, (Strategy III) genetic and ecological restoration as well as (Strategy IV) in-depth research and cooperation. Hence, the National Institute of Forest Science has taken charge of the research and development of the techniques related to the plan, while the Division of Forest Bioinformation has performed many tasks, such as the evaluation of genetic diversity, establishment of *in/ex situ* conservation, and genetic restoration.

The Korean fir (*Abies koreana* E.H. Wilson) is an endemic and sub-alpine conifer in the country and is also designated as an endangered species (EN) from the International Union for Conservation of Nature [10]. It is naturally distributed in a limited number of populations, that is, a total of nine populations: three large populations in Mt. Jiri, Mt. Deogyu, and Mt. Halla with a relatively large number of adult individuals and six small populations with a few individuals. A recent survey reported that about 33% of the natural habitats of the species have declined during the past 20 years [11], in which the population of Mt. Geumwonsan has become extremely small with very few individuals (only 20 adult trees and approximately 23 seedlings). Furthermore, the population showed very poor seed production in a previous survey. It is reported that genetic diversity is relatively high of three large populations of Korean fir [12,13]. However no genetic analysis was conducted on small populations, including the Mt. Geumwonsan population. Therefore, an immediate and proper genetic restoration to mitigate the ongoing decline progress is required. For the first step in this study to perform the genetic restoration of Mt. Geumwonsan, we evaluated the genetic diversity of the population using nuclear microsatellite markers and examined the genetic similarity between the populations to determine the candidate population that would be used as for further restoration.

MATERIALS AND METHODS

Sample collection and DNA extraction

A total of 96 fresh leaf samples were collected from three large populations (Mt. Jirisan, Mt. Deogyusan, and Mt. Hallasan). Individuals within each population were separated by at least 20 m to minimize the collection of genetically close samples. A total of 43 samples were collected from the Mt. Geomwonsan population, which is located in Wicheon-myeon, Geochang-gun, and Gyeongsangnam-do (Figure 1). The location information of the individual trees that were collected was obtained from using the GPS system (GPS etrex HCx, Garmin Ltd.,USA). The total genomic DNA was extracted from the fresh seedlings using a Plasmid SV mini kit (GeneAll Biotechnology, Seoul, Korea), while the total DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA).

Microsatellite analysis

A total of ten nuclear microsatellite (nSSR) markers were developed in different *Abies* species [14-16] and were selected for the present study (Table 1). The PCR amplification was performed in 15 µL reaction mixtures containing 20 ng of the template DNA, 1x of the reaction buffer, 2.5 mM MgCl$_2$, 0.2 mM dNTPs, 0.04 µM of the M13(-19) sequencing primer that was labeled with FAM, 0.2 µM primer mix, and 0.5 U of the Taq DNA polymerase (Biofact, Daejeon, Korea). The PCR conditions included denaturing at 94°C for 5 min; ten 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, USA), while the genotypes were determined using Gene Mapper v5.0 (Applied Biosystems, Thermo Fisher Scientific, USA).

Data analysis

The number of alleles (*A*), the observed heterozygosity (*H*$_O$), the expected heterozygosity (*H*$_E$), and the inbreeding coefficient (*F*) were assimilated from all markers and the pairwise values of Nei's genetic distance among the populations were calculated. In order to visualize the genetic distances among the populations, the principal co-ordinates analysis (PCoA) was performed using Nei's genetic distance, which were performed using GenAlEx v6.41 [17]. The value of the genetic differentiation between the populations (pairwise *Fst*) was examined using FSTAT [18], while the genetic structure of the populations was identified through the Bayesian clustering method in the STRUCTURE v2.3.4 software [19]. In addition, a pre-specified number of gene clusters (K) from 1 to 4 was assumed, in which the simulations were run 20 times per K value. All runs involved 30,000 Markov chain Monte Carlo samplings with a burn-in period of 30,000 iterations. Finally, the optimal K
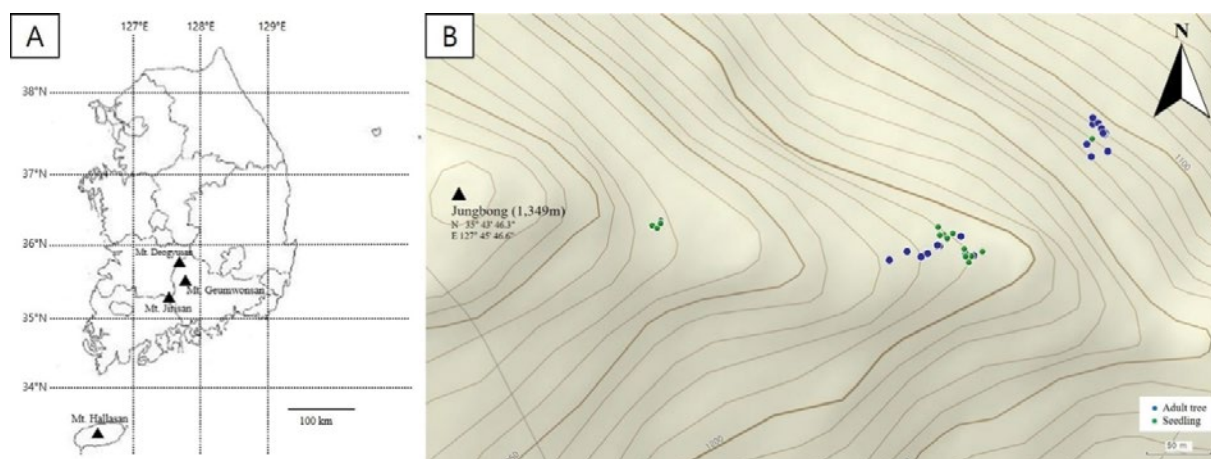

Figure 1: (A) The location of the four Korean fir populations that were examined in this study. (B) The location of the Korean fir samples at Mt. Geumwonsan in South Korea. The blue and green dots represent the adult trees and seedlings, respectively.

Table 1: Characteristics of the ten Microsatellite markers that were used in *Abies koreana*. F and R refer to forward and reverse primers, respectively.

	Primer	GenBank Accession No.	Repeat motif	Primer (5′-3′)	Reference (Species)
1	SF83	DQ218459	$(CTT)_3...(GCC)_5$	F: AGCAGCATAACCAAGGGTCAA R: TCTGAATTTCTAAAGGCGGC	[15] (*A. alba*)
2	Aat04	KF304597	$(CAG)_{11}$	F: CCATGTATGGTGCTCCTCCT R: CCTTCATTGCAGAAAAGCAA	[15] (*A. alba*)
3	Aat05	KF304598	$(GCA)_7$	F: AGCATCCACATTCCGTAACC R: AGTTGACCGTTGGAGAGCAG	[15] (*A. alba*)
4	Aat12	KF304605	$(AG)_{12}$	F: ATCCATATCTCCTGCCTTGC R: CTTTCCAGGTGATCTGATTGC	[15] (*A. alba*)
5	Aat15	KF304608	$(AGA)_8$	F: AGGAGGAGGTTCAGCATGTC R: CTTGCTCTCTGACCCAGTTG	[15] (*A. alba*)
6	Aag02	KF304593	$(GA)_{13}$	F: TATTCCTCCACTTGGGTGCT R: GGTGGAGATCCGTATGCAAT	[15] (*A. alba*)
7	NFF07	AY966495	$(GA)_{33}$	F: CCCAAACTGGAAGATTGGAC R: ATCGCCATCCATCATCAGA	[14] (*A. nordmandiana*)
8	NFH15	AY966492	$(AC)_{15}$	F: CGCCTCCCTCCATTACTTC R: TCGTCTAGAGAGGCGAAATTCT	[14] (*A. nordmandiana*)
9	C49	FX334333	$(AGGAGA)_7$	F: GACGAAGATCAGTACAAGGCACGA R: GCGATCCTTCAATTTGTCCTTCTC	[16] (*A. firma*)
10	C28104	FX334339	$(ATA)_5$	F: CGAGGAAGAAGCCAAGTTATCAGG R: CACAGTTAAAAAGGCGGCCTACAG	[16] (*A. firma*)

Table 2: The genetic diversity of the *A. koreana* populations.

Population	N	A	A_e	H_o	H_e	F
Mt. Jirisan	2	7.6	4.8	0.618	0.656	0.031
Mt. Hallasan	32	6.0	3.8	0.546	0.586	0.044
Mt. Deogyusan	32	7.0	4.4	0.579	0.655	0.104
Mt. Geumwonsan (Adult trees)	23	6.3	4.0	0.568	0.612	0.049
Mt. Geumwonsan (Seedlings)	20	5.4	3.5	0.385	0.592	0.318

*N: Number of samples, A: Number of alleles, A_e: Number of effective alleles, H_o: Observed heterozygosity, H_e: Expected heterozygosity, F: Fixation index

Table 3. A matrix of the pairwise Nei genetic distance (below the diagonal) relative to the pairwise *Fst* (above the diagonal) among the four populations of *Abies koreana*.

Population	Mt. Geumwonsan	Mt. Jirisan	Mt. Hallasan	Mt. Deogyusan
Mt. Geumwonsan	-	0.027*	0.093*	0.018*
Mt. Jirisan	0.087	-	0.047*	0.005
Mt. Hallasan	0.205	0.113	-	0.041*
Mt. Deogyusan	0.072	0.046	0.102	-

value was estimated by calculating ΔK, where the K values were calculated according to the method of Evanno et al. [19] that was based on the mean log probability and the standard deviation of the data using the STRUCTURE HARVESTER program [20]. Moreover, the software CLUMP and DISTRUCT were used to align 20 replicates and display the results separately [21,22].

RESULTS AND DISCUSSION

Genetic diversity

The genetic diversity of most of the distribution areas of the *A. koreana* populations was between 0.586 and 0.656, which is relatively high (Table 2). Among the three populations, the highest level of genetic diversity was observed in the Mt. Jirisan population (A_e=4.8, H_e=0.656). The Mt. Jirisan population has maintained a relatively high level of genetic diversity, which suggested that a

large number of trees and a large area of distribution have affected the diversity [23]. Furthermore, the Mt. Hallasan population had the lowest genetic diversity (A_e=3.8, H_e=0.586) due to its restricted geographic distribution. Therefore, it is necessary to select the most suitable restoration materials to maintain the maximum genetic diversity of the present populations. In addition, the Mt. Geumwonsan population is composed of approximately 20 individuals and has a lower genetic diversity than other large groups [24]. In particular, the fixation index of the natural seedlings is very high (0.318). Therefore, there is a high risk of local extinction in the long term due to the effect of inbreeding depression and the limited number of trees.

Genetic similarity

The Mt. Geumwonsan population was similar to both the Mt. Jirisan and Mt. Deogyusan populations, which are distributed
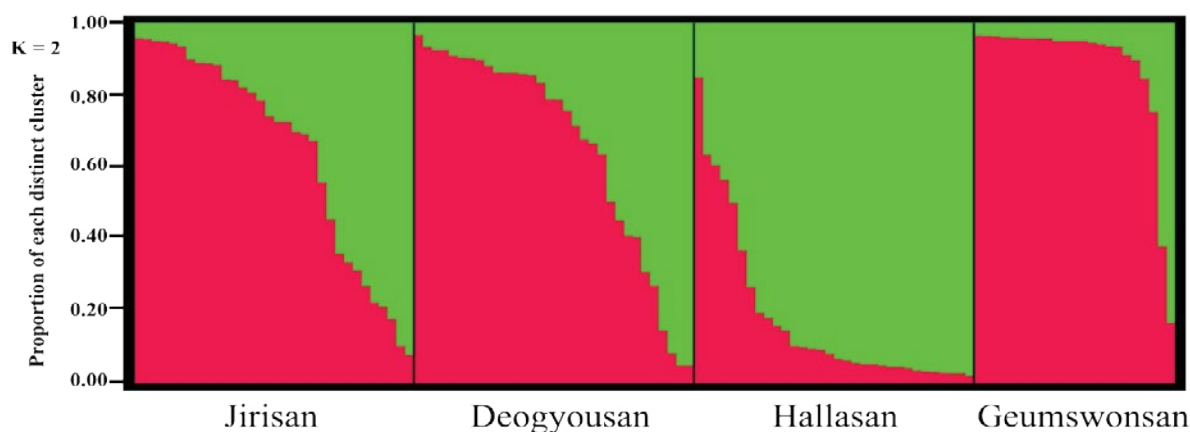
Figure 2: Results of the Bayesian clustering analysis that was conducted on the four populations of *Abies koreana* at K=2 using the STRUCTURE software [19]. The horizontal bars denote the samples, while the green and red color represent the genetic clusters.
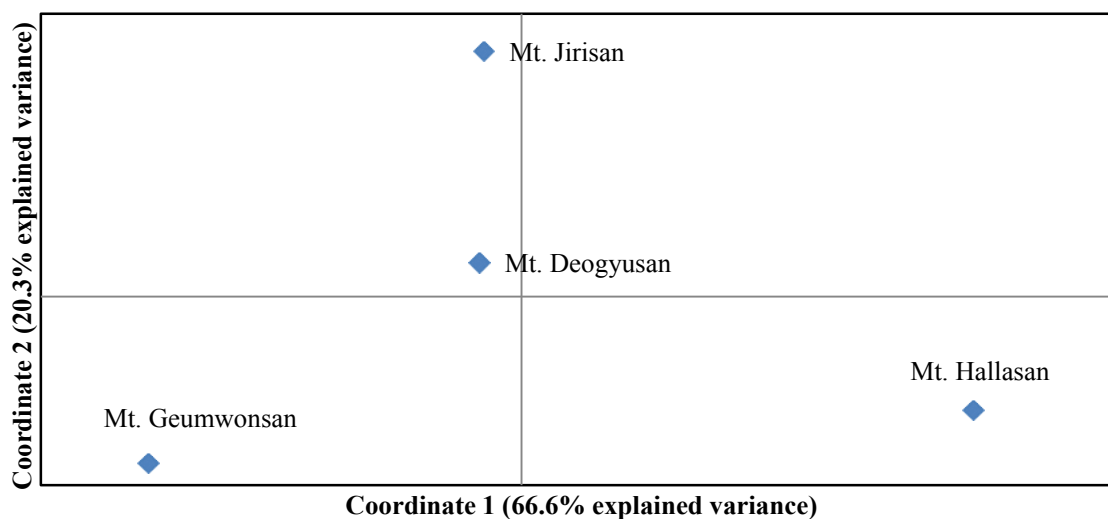
Principal Coordinates Analysis

Figure 3: Principal coordinates analysis of the genetic distances within the four populations of *A. koreana*. Coordinate 3 (not shown) explained an additional 13.1% of the variation.

inland of the Korean peninsula based on the Nei genetic distance (Table 3 and Figure 2). According to the Bayesian clustering analysis, the value of ΔK was the highest at K=2, which was based on the theory suggested by Evanno et al. [19]. Thus, we visualized the assignment probabilities from the two clusters per individual (Figure 3). However, the Mt. Hallasan population, located on Jeju Island, was found to be the most genetically distant group of the Mt. Geumwonsan population. It is estimated that the geographically close inland groups had a relatively stable gene flow until recently. Therefore, in order to maintain the Mt. Geumwonsan population, it is recommended to use seedlings that were propagated from genetically similar populations, such as the Mt. Jirisan and Mt. Deogyusan populations.

CONCLUSION

Based on the results of this study, it can be concluded that the Mt. Geumwonsan populations have a high risk of local extinction. To enhance the genetic diversity of the Geumwonsan population, it is recommended to restock the seedlings that were propagated from

a genetically similar population, such as the Mt. Jirisan and Mt. Deogyusan populations. In addition, to preserve the unique genetic variations of the Geumwonsan population, *ex situ* preservation through asexual propagation is necessary.

REFERENCES

1. Kong WS, Kim K, Lee S, Park H, Cho SH. Distribution of high mountain plants and species vulnerability against climate change. Environ Impact Assess Rev. 2014;23(2):119-136.

2. Koo KA, Park WK, Kong WS. Dendrochronological analysis of *Abies koreana* W. at Mt. Halla, Korea: effects of climate change on the growths. Korean J Ecol. 2001;24(5):281-288.

3. Tanaka N, Nakao K, Tsuyama I, Higa M, Nakazono E, Matsui T. Predicting the impact of climate change on potential habitats of fir (Abies) species in Japan and on the East Asian continent. Procedia Environ Sci. 2012;13:455-466.

4. Auls SU, Nowak C, Balint M, Pfenninger M. The impact of global climate change on genetic diversity within populations and species. Mol Ecol. 2013;22(4):925-946.

5. Hylander K, Ehrlén J. The mechanisms causing extinction debts. Trends Ecol. Evol. 2013;28(6):341-346.

6. Commission on Genetic Resources for Food and Agriculture. Global plan of action for the conservation, sustainable use and development of forest genetic resources. Commission on Genetic Resources for Food and Agriculture, FAO. 2014.

7. Mckay JK, Christian CE, Harrison S ,Rice K J. "How local is local"-A review of practical conceptual issues in the genetics of restoration. Restor Ecol. 2005;13(3):432-440.

8. Thomas E, Jalonen R, Loo J, Boshier D, Gallo L, Cavers S, et al. Genetic considerations in ecosystem restoration using native tree species. For Ecol Manag. 2014;333:66-75.

9. Kim ES, Lee JS, Park GE, Lim J H. Change of subalpine coniferous forest area over the last 20 years. J Korean Soc For Sci. 2019;108(1):10-20.

10. Kim YS, Chang CS, Kim CS, Gardner M. *Abies koreana*. The IUCN Red List of Threatened Species. 2011: e.T31244A9618913.

11. Lim JH, Kim ES, Park GE, Kim YS, Jang KC, Han JG, et al. Status and Conservation of Endangered Sub-Alpine Conifer species in Korea. National Institute of Forest Science. 2019.

12. Ahn JY, Lim HI, Ha HW, Han J, Han SH. Genetic variation of Korean fir sub-populations in Mt. Jiri for the restoration of genetic diversity. J Korean For Soc. 2017;106(4):417-

13. Kwak MH, Hong JK, Park JH, Lee BY, Suh MH, Kim CS. Genetic assessment of *Abies koreana* (Pinaceae) the endangered Korean fir and conservation implications. Conserv Genet. 2017;18(5):1165-1176.

14. Hansen OK, Vendramin G, Sebastiani F, Edwards KJ. Development of microsatellite markers in *Abies nordmanniana* (Stev.) Spach and cross-species amplification in the Abies genus. Mol Ecol. 2005;5:784-787.

15. Postolache D, Leonarduzzi C, Piotti A, Spanu I, Roig A, Fady B, et al. Transcriptome versus genomic microsatellite markers: Highly informative multiplexes for genotyping Abies alba Mill. and congeneric species. Plant Mol Biol Rep. 2014;32:750-760.

16. Uchiyama K, Fujii S, Ishizuka W, Goto S, Tsumura Y. Development of 32 EST-SSR markers for Abies firma (Pinaceae) and their transferability to related species. Appl Plant Sci. 2013;1(2):1200464.

17. Peakall ROD, Smouse PE. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol Ecol. 2006;6:288-295.

18. Goudet J. "FSTAT (version 2.9. 4), a program (for Windows 95 and above) to estimate and test population genetics parameters." Department of Ecology & Evolution, Lausanne University, Switzerland 53. 2003.

19. Evanno G, Regnaut S, Goudet J. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol. 2005;14(8):2611-2620.

20. Earl DA, Von-Holdt BM. Structure Harvester: a website and program for visualizing structure output and implementing the Evanno method. Conservation Genet Resour. 2012; 4(2):359-361.

21. Jakobsson M, Rosenberg, NA. CLUMPP: A cluster matching and permutation program for dealing with label switching and multimodality in analysis of population structure. Bioinformatics. 2007;23(14):1801-1806.

22. Rosenberg NA. DISTRUCT: A program for the graphical display of population structure. Mol Ecol. 2004;4(1):137-138.

23. Allendorf FW, Luikart G, Aitken SN. Conservation and the genetics of populations' second edition (Oxford: Wiley-Blackwell). 2013;6:96-116.

24. Pritchard JK, Stephens M, Donnelly P. Inference of population structure using multilocus genotype data. Genetics. 2000;155(2):945-959.