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Full Length Research Paper

Genetic diversity of an endangered species, *Fokienia hodginsii* (Cupressaceae)

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***Fokienia hodginsii* (Cupressaceae) is distributed in montane evergreen forests in North and Central Vietnam and extends to southeastern China at 900 m above sea level. The species has been threatened in its area of distribution in recent years because of habitat destruction and over-exploitation. The genetic variation of *F. hodginsii* in Vietnam was investigated on the basis of eight ISSR markers. Three hundred and twenty-two sampled trees from eight populations in six provinces of Lao Cai, Ha Giang, Son La, Hoa Binh, Thanh Hoa and Nghe An, were used as material for the study. The ISSR data showed low genetic variability at both population and species level, with an average of 0.0701 and 0.1145, respectively. Genetic differentiation among populations was high (Gst = 0.2771), indicating limited gene flow (Nm = 1.3046). However, the implication of the results from this study which is to conserve genetic resources of this species was discussed.**

Key words: *Fokienia hodginsii,* ISSR markers, genetic diversity, species conservation.

INTRODUCTION

In recent years, there is a dramatic increase in the number of endangered species and hence extinction can no longer be regarded as natural. The endangerment of species usually occurs owing to the destructive interventions that human activities cause on natural resources. The consequences of deforestation and over-exploitation of biological resources lead to the loss of biodiversity. The degradation of forests has increasingly eliminated the habitats of species. Forest populations are being reduced in size and they become fragmented due to the fact that many species are in danger of extinction. Due to fluctuations in the number of individuals, through random demographic and environmental forces, such small populations faced an increased probability of extinction. Small and isolated populations often lead to a reduction in gene flow, in that they increased random genetic drift and inbreeding (Barrett and Kohn, 1991). Consequently, there will be a reduction in genetic diversity, which results in reduced fitness and increased susceptibility to environmental stochasticity (van Treuren et al., 1993).

Fokienia hodginsii (Cupressaceae) is a widely distributed conifer in Vietnam. It occurs in montane evergreen forests on granite or limestone derived soils in provinces of Ha Giang, Lao Cai, Dien Bien, Yen Bai, Son La, Phu Tho, Hoa Binh, Thanh Hoa, Nghe An, Ha Tinh, Thua Thien Hue, Kon Tum, Gia Lai, Dac Lac, Lam Dong, Ninh Thuan and Khanh Hoa, extending to Southeastern China and Laos (Luu and Thomas, 2000). It is also found in the forest plantations of Lao Cai and Nghe An in 1990s and cultivated in private gardens at villages of Tay Son (Nghe An) and Thai An (Ha Giang). *F. hodginsii* prefers the high humidity ranging from 81% in December to 86% in September (Van et al., 2000), high precipitation from 1268 to 3000 mm, and a mean annual temperature from 15.4 to 23.6°C. *F. hodginsii* is

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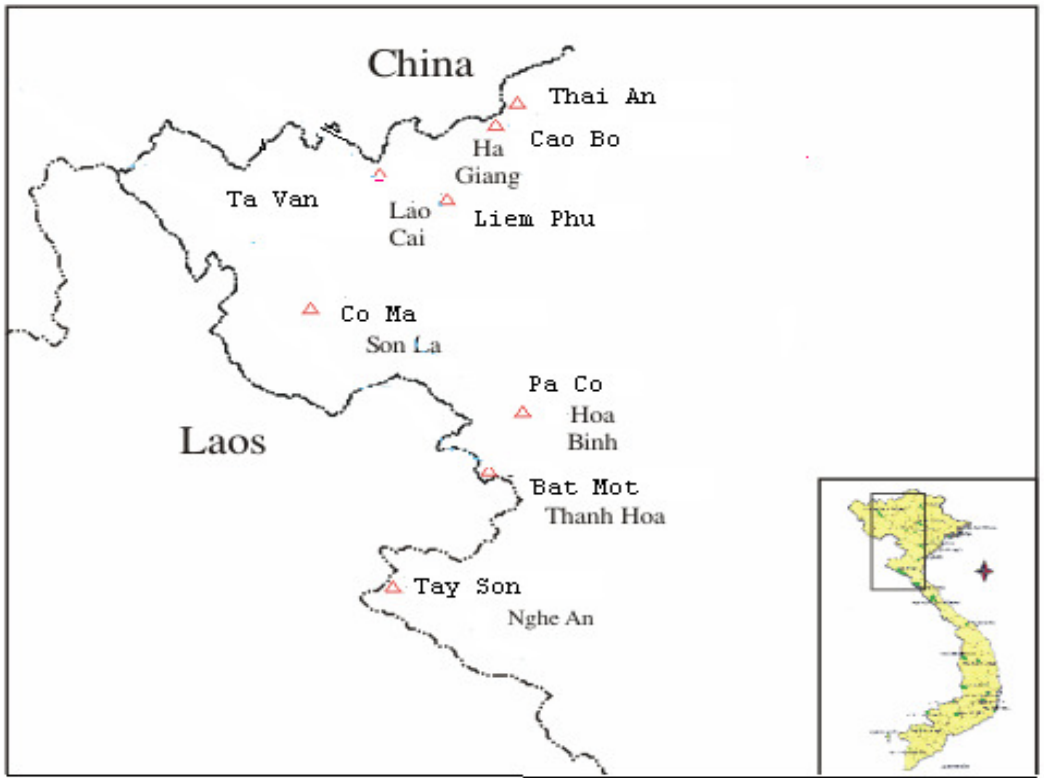

Figure 1. Map showing the studying sites of *Fokienia hodginsii.*

over-exploited by local people and forestry enterprises, due to the fact that human activities have degraded or completely destroyed its habitat. Thus, this species is recorded in many patches of secondary forests including protected areas. These include the vegetation of bare limestone faces on steeps and ridges of mountains at Cat Cat, Sin Chai and Ta Van (Sa Pa), Liem Phu (Van Ban), Pa Co (Mai Chau) and Tay Son (Ky Son), where forests have been completely destroyed. The abundant species in the vegetation are shrubs and ferns. The vegetation of the remaining habitats of *F. hodginsii* includes the little disturbed forests and secondary forests. The nature of the terrain is very complex and is located on mountain slopes above 20 to 30°, and sometimes on steep slopes at about 60 to 70°.

The studies conducted on population genetics of various conifers showed low levels of genetic differentiation among populations (Ledig et al., 2001; Epperson and Chung, 2001; Shea and Furnier, 2002; Jorgensen et al., 2002; Lee et al., 2002). In *Abies sibirica,* Larionova et al. (2007) found a low level of genetic variation, and that of genetic differentiation among populations were revealed in Middle Siberia. In another species (*Abies* species), Aquirre-Planter et al. (2000) demonstrated the low genetic variation within populations and high genetic differentiation among populations from Southern Mexico and Guatemala in comparison to most coniferous species reported.

At present, there is very little information on ecology parameters of *F. hodginsii*, especially the lack of data on the genetic variability of this species. It is therefore difficult to develop the conservation strategies of *F. hodginsii.* The objective of this study was to investigate the genetic variation of *F. hodginsii* using inter-simple sequence repeat markers (ISSRs) and to provide technical assistance to Protection Forestry Department on issues related to the conservation of this conifer in Vietnam.

MATERIALS AND METHODS

Plant materials

The research was carried out in eight sites, two each in Nghe An and Ha Giang, and one each in Son La, Hoa Binh, Thanh Hoa and Nghe An (Figure 1 and Table 1). At Ta Van and Bat Mot, the original vegetations were lightly disturbed. Its structure is complex and it includes three strata. The canopy comprises the tallest trees and is usually discontinuous. However, they may grow as high as35 to 40 m with 0.5 to 3 m in breast height diameter (dbh). Besides species in Fagaceae, the canopy is also dominated by species in Altigiaceae, Elaeocarpaceae, Lauraceae and Dipterocarpaceae. Some conifers appeared scattered in this layer. The understorey is composed of trees that are fairly close together to form a continuous layer, with high number of species. It is made up of young trees of the canopy and species in Theaceae,

Table 1. Collection localities of *Fokienia hodginsii* for ISSR analysis.

Population	Samples size	Collection locality	Altitude (m)	Latitude	Longitude
Ta Van	50	Ta Van, Sapa, Lao Cai	1500	22°12'N	103°05'E
Liem Phu	97	Liem Phu, Van Ban, Lao Cai	1650	21°50'N	104°18'E
Cao Bo	40	Cao Bo, Vi Xuyen, Ha Giang	1740	22°08'N	104°47'E
Thai An	41	Thai An, Quan Ba, Ha Giang	1770	22°01'N	105°07'E
Co Ma	35	Co Ma, Thuan Chau, Son La	1250	21º20'N	103º37'E
Pa Co	15	Pa Co, Mai Chau, Hoa Binh	1000	21°30'N	104°52'E
Bat Mot	21	Bat Mot, Thuong Xuan, Thanh Hoa	1345	20°00'N	104°57'E
Tay Son	23	Tay Son, Ky Son, Nghe An	1900	19°22'N	104°06'E

Table 2. List of primers used for ISSR amplification (Y: C or T).

Primer code	Primer sequences (5' to 3')	Annealing temperature (°C)
UBC810	GAG AGA GAG AGA GAG AT	40
UBC811	GAG AGA GAG AGA GAG AC	40
UBC815	CTC TCT CTC TCT CTC TG	40
UBC835	AGA GAG AGA GAG AGA GYC	45
UBC836	AGA GAG AGA GAG AGA GYA	40
UBC840	GAG AGA GAG AGA GAG AYT	45
UBC841	GAG AGA GAG AGA GAG AYC	45
UBC857	ACA CAC ACA CAC ACA CYG	46

Rosaceae and Euphorbiaceae. The ground layer is more complex with species in Rubiaceae, Poaceae, Acanthaceae and Zingiberaceae. The original vegetations at Tay Son have been greatly degraded by human activities such as over-exploitation for commerce, fuel wood collection and building, and create development of a light-demanding species. However, three strata also characterize this vegetation structure. The vegetation with drained soil, tall canopies up to 25 to 30 m, wet and warm summer, dry and cool winter were observed at these sites. The composition and structure of the vegetation were determined by levels of disturbance. Dominant species were *Pometia pinnata* (Sapindaceae), *Wrightia tonkinensis* (Apocynaceae), *Lithocarpus corneus* (Fagaceae), in Dipterocarpaceae, Lauraceae, Fabaceae and *Neohouzeana spp*. (Poaceae), and light abundant favourable species. Shrubs include species in Araceae, Zingiberaceae and Rubiaceae. These altered the spatial distribution and age class structure of trees in these sites. *F. hodginsii* were found on bare limestone faces on ridges and steeps of mountains at all study areas with small population sizes (<50 individuals per population).

In this study, 322 sample individuals were used from 8 known populations. The collected samples were wrapped by marked aluminum paper and placed in liquid nitrogen. They were transferred to the Laboratory of Molecular Biology, Institute of Ecology and Biological Resources, and subsequently stored at -76°C until it was ready for use in DNA extraction. The samples were identified on the basis of past taxonomic treatments of collected specimens from these populations.

DNA extraction

Genomic DNA was extracted from young leaves using the modified CTAB method by Xavier and Karine (2000). The concentration of total DNA was determined using a fluorometer and diluted to 10 ng/µl.

DNA amplification for ISSR

Polymerase chain reaction (PCR) was carried out in 25 µl solution consisting of 2.5 µl reaction buffer, 2.5 µl $MgCl_2$, 2 µl dNTP, 0.1 µl of primer, 1.25 units Tag DNA polymerase (Invitrogen) and 1.5 µl of template DNA. A total of eight ISSR primers were used in this study (Table 2). The reaction mixture was subjected to amplification in the Gene Amp PCR System 2400, under the following thermal cycler: an initial denaturing step at 94°C for 4 min, followed by 35 cycles consisting of 1 min at 94°C, 30 s annealing temperature for each primer (Table 2) and 1 min extension at 72°C, and 10 min at 72°C for a final cycle to complete the extension of any remaining products before holding the samples at 4°C until they were analyzed. The amplification products were separated by electrophoresis on 7.5% polyacrylamide gels in 1 x TAE buffer, and then stained by ethidium bromide for 10 min. The banding patterns were visualized under UV light and photographed using a MEGA 8.4 Panasonic camera. 100 bp ladder was used as DNA standard (Invitrogen).

Data analysis

ISSR bands were scored as present (1) or absent (0). The binary data were analysed by PopGene v.1.31 (Yeh and Boyle, 1999) to estimate the genetic diversity parameters: the effective number of alleles per locus (Ae), the proportion of polymorphic loci (P), the Nei's (1973) gene diversity (H), and the Shannon's index (I).

Genetic diversities within and among the populations were

Table 3. Primers, number of fragments scored, number of polymorphic bands and percentage polymorphism from amplification profiles of 182 individuals of *Fokienia hodginsii* generated using eight ISSR markers.

Primer repeat	Code	Number of fragments scored	Number of polymorphism
$(GA)_8T$	UBC810	20	20
$(GA)_8C$	UBC811	18	18
$(CT)_8G$	UBC815	20	18
$(AG)_8YC$	UBC835	22	22
$(AG)_8YA$	UBC836	20	20
$(GA)_8YT$	UBC840	19	19
$(GA)_8YC$	UBC841	24	24
$(AC)_8YG$	UBC857	27	27
All		170	168

Table 4. Genetic diversity in eight *Fokienia hodginsii* populations.

Populations	N	Ae	P	H (s.d.)	I (s.d.)
Ta Van	19	1.094 (0.209)	31.76	0.0635 (0.1236)	0.1053 (0.1881)
Liem Phu	51	1.089 (0.182)	46.47	0.0640 (0.1116)	0.1129 (0.722)
Cao Bo	31	1.109 (0.250)	28.24	0.0669 (0.1411)	0.1053 (0.2070)
Thai An	32	1.111 (0.247)	29.41	0.0692 (0.1397)	0.1100 (0.2066)
Tay Son	22	1.117 (0.239)	40.00	0.0760 (0.1371)	0.1254 (0.2030)
Bat Mot	12	1.104 (0.230)	29.41	0.0671 (0.1319)	0.1089 (0.1978)
Co Ma	19	1.107 (0.214)	32.94	0.0716 (0.1303)	0.1174 (0.1988)
Pa Co	13	1.133 (0.269)	32.35	0.0828 (0.1499)	0.1309 (0.2206)
Mean	-	1.108	33.82	0.0701	0.1145
All	200	1.195 (0.247)		0.0926 (0.1380)	0.1765 (0.1938)

N: The mean number of individuals sampled, Ae: The effective number of alleles per locus, P: The proportion of polymorphic loci, H: Nei's (1973) genetic diversity and I: Shannon's information index.

analyzed for each polymorphic locus using Nei's (1987) genetic diversity statistics: the total genetic diversity (Ht), the genetic diversity within populations (Hs), and the coefficient of genetic diversity (Gst). The genetic differentiation among populations was estimated from allele frequencies using Nei's (1972) genetic distance and identified for all pairs of populations. UPGMA cluster analysis of genetic distances was generated to examine the genetic associations among populations or among individuals within populations using Nei's (1972) genetic distance.

The gene flow between populations (Nm) was also determined using Gst value: Nm = 0.5 (1-Gst)/ Gst.

A binary data matrix of the present and absent banks was generated from the eight primer markers from 8 populations. The distance matrix was generated from the data with the AMOVA-PREP 1.01 (Miller, 1998). The matrix was analyzed in an analysis of molecular variance (AMOVA) using the program ARLEQUIN 3.1 (Excoffrier et al., 2005).

RESULTS

The eight ISSR primers produced a total of 170 bands across all 322 individuals of eight *F. hodginsii* populations (Table 3). The proportion of polymorphic bands was 98.82% (168 bands); whereas the mean number of polymorphic bands per primer was 21. The maximum number of bands were yielded by the repeat $(AC)_8YG$ with 27 bands, while the minimum number of bands were yielded by $(GA)_8YT$ with 18 bands.

Genetic diversities are shown in Table 4. At the population level, the P value ranged from 29.41 (Bat Mot) to 46.47% (Liem Phu), with an average of 33.82%. H ranged from 0.0635 (HLS) to 0.0828 (PC), with an average of 0.0701, and I ranged from 0.1053 (Ta Van and Cao Bo) to 0.1309 (Pa Co), with an average of 0.1145 (Table 3). At species level, these values were P = 98.82%, H = 0.0926 and I = 0.1765.

As shown in Table 5, the total genetic diversity (Ht) among all the populations of *F. hodginsii* was found to be 0.097, whereas the genetic diversity within populations (Hs) averaged 0.0701. The coefficient of genetic differentiation (Gst) was found at 0.2771. The gene flow (Nm) calculated among all the populations of *F. hodginsii* was high (1.3046). However, all genetic identities obtained in comparisons of the eight *F. hodginsii* populations exceeded 0.9. Their genetic identity averaged 0.9677, ranging from 0.9538 (Cao Bo and Co

Table 5. Nei's (1987) genetic diversity within and among populations of some species of conifers.

Species	N	Hs	Ht	Gst	Nm	Reference
Fokienia hodginsii	200	0.0701 (0.0041)	0.0970 (0.0101)	0.2771	1.3046	This study
Pseudotsuga menziesii	-	0.1546	0.1594	0.0260	-	Yeh and O'Malley (1980)
Picea sitchensis	-	0.147	0.159	0.079	-	Yeh and El-Kassaby (1979)
Pinus longaeva	-	0.465	0.484	0.038	-	Hichert and Hamrick (1983)
Pinus rigida	-	0.147	0.152	0.03	-	Guries and Ledig (1982)

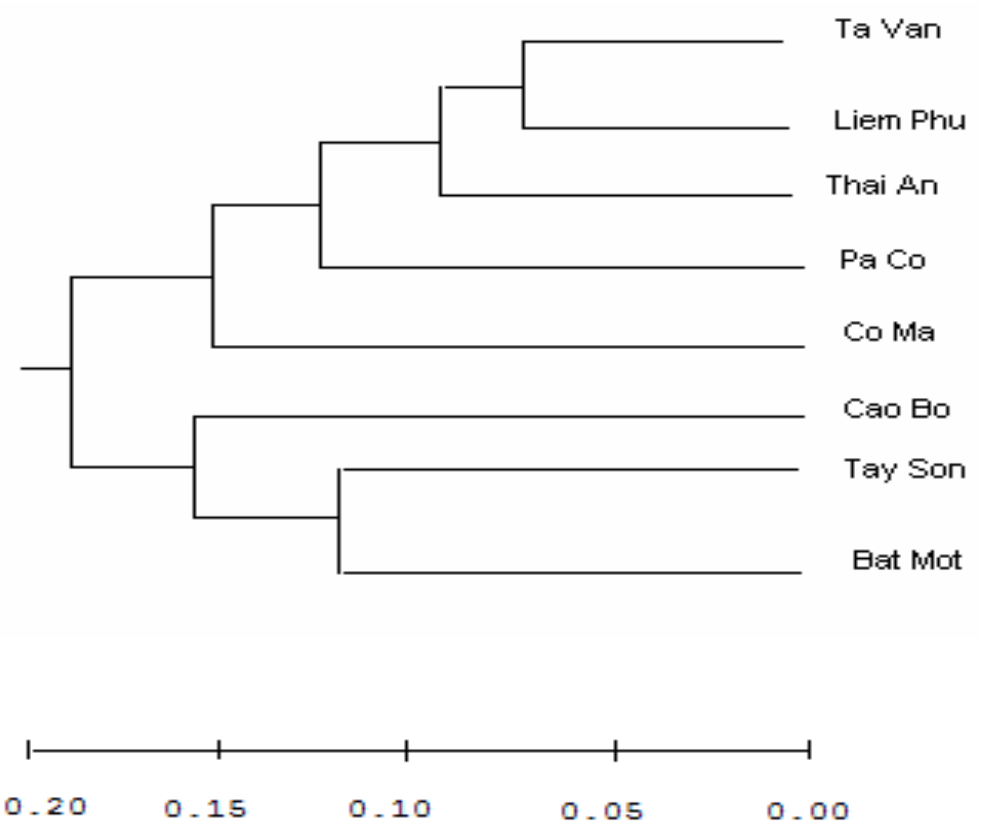

Figure 2. UPGMA dendrogram based on Nei's (1972) genetic distance among the eight *Fokienia hodginsii* populations.

Ma) to 0.9855 (Liem Phu and Hoang Lien), and the mean genetic distance between them was 0.0336, ranging from 0.0146 (Hoang Lien and Liem Phu) to 0.0473 (Co Ma and Cao Bo).

The clustering of *F. hodginsii* populations using the UPGMA dendrogram was based on pairwise genetic distances, which showed that the two groups were separated clearly at population level. The most geographically close populations were clustered together. One group included five populations in North Vietnam (Ta Van, Liem Phu, Thai An, Pa Co and Co Ma) and two populations (Ta Van and Liem Phu) clustered together in Hoang Lien Son National Park, with lower genetic diversity (Table 3). The second group included the three remaining populations (Cao Bo, Tay Son and Bat Mot). Cao Bo in North Vietnam was separated clearly from two geographically close populations (Tay Son and Bat Mot) in Central Vietnam, with lower proportion of polymorphic loci (28.24%), genetic diversity (0.0669) and Shannon's index (0.1053) (Figure 2).

The analysis of molecular variance (Table 6) revealed that the genetic variation among populations within groups and populations was 2.7 and 96.52%, respectively. Pairwise comparisons of populations based on the Fst values indicated that the highest value was found between the Ta Van and Tay Son populations

Table 6. Analysis of molecular variance in *Fokienia hodginsii* from eight populations.

Source of variation	d.f.	Sum of squares	Variance component	% Total variation	P-value
Among 4 population groups (Ta Van-Liem Phu, Cao Bo-Thai An, Tay Son-Bat Mot, Co Ma-Pa Co)	3	7.714	0.0038	0.78	<0.05
Among populations within groups	4	6.488	0.0133	2.7	<0.05
Within populations	767	360.824	0.4748	96.58	<0.05

(Fst = 0.0528) and the lowest was found between the Bat Mot and Co Ma populations (Fst = 0.0137). However, the significant differentiation was found between populations at 0.05 level.

DISCUSSION

ISSR markers were used to assess the genetic diversity measures within and among populations of *F. hodginsii* in this study. Both population and species levels have lower values of genetic diversities than the other coniferous species. High values of genetic variability were reported for populations of many conifers such as *Pinus strobus* (P = 47.8% and H = 0.195) (Rajora et al., 1998), *Pinus pinceana* (P = 56.5% and H = 0.174) (Ledig et al., 2001), and *Pinus brutia* (P = 68% and H = 0.271) (Korol et al., 2002). Low genetic variabilities have also been found in some conifers possessing restricted occurrences such as: *Abies sibirica*, where P = 20% and H = 0.0642 (Larionova et al., 2007); *Abies flinckii*, where P = 30.2% and H = 0.113; *Abies guatemalensis*, where P = 20% and H = 0.069; *Abies hickeli*, where P = 28.2% and H = 0.1; *Abies religiosa*, where P = 31.8% and H = 0.108 (Aquirre-Planter et al., 2000); *Abies lasiocarpa*, where P = 43.4% and H = 0.124 (Shea, 1990); and *Picea breweriana*, where P = 44.2% and H = 0.129 (Ledig et al., 2005). In other studies, high levels of genetic variability within and among conifers were also obtained for *Picea sitchensis* (Hs = 0.147 and Ht = 0.159) (Yeh and El-Kassaby, 1979), *Pinus longaeva* (Hs = 0.465 and Ht = 0.484) (Hichert and Hamrick, 1983), and *Pinus rigida* (Hs = 0.147 and Ht = 0.152) (Guries and Ledig, 1982). Our results confirm the suggestion that the genetic structure of the natural populations of *F. hodginsii* is strongly affected by small population sizes. The number of observed individuals was small and it varied considerably with about 50 individuals at Cao Bo and 150 at Thai An secondary forests, and below 100 at Bat Mot and Ta Van little disturbed forests, inside the National Park of Xuan Lien and Hoang Lien Son, respectively. Such small populations are the occurrence of inbreeding and the effect of genetic drift (Barrett and Kohn, 1991; Ellstrand and Elam, 1993). The current distribution of *F. hodginsii* was strongly influenced by fragmented habitats. The species occupies the forests at 1300 to 1900 m elevation. The forests have been greatly fragmented by human activities and they form small forest patches. A few natural populations of *F. hodginsii* remain in such small patches. The forests were destroyed and converted into other landscape unsuitable for *F. hodginsii* survival. In addition, the low level of genetic variability in this species might be caused by founder effects associated with the altered climatic conditions. The logging activity and the associated creation of gaps caused a change in the original vegetation structure. There was variation in the spatial distribution, age class structure of trees and the invasion of exotic species. However, *F. hodginsii* was distributed by a characteristic terrain and climate. Therefore, the species was exposed to geographic isolation.

A limited genetic variability within populations also indicated considerable levels of differentiation among populations. Estimates of the Gst value for *F. hodginsii* populations showed high amounts of genetic differentiation (Gst = 0.2771). This value was clearly higher than those reported in other coniferous species, such as *Pseudotsuga menziesii* (Gst = 0.026) (Yeh and O'Malley, 1980), *P. longaeva* (Gst = 0.038) (Hichert and Hamrick, 1983), *Pinus sibirica* (Gst = 0.041) (Goncharenko et al., 1993), *Pinus monophylla* (Gst = 0.033) (Hamrick et al., 1994), *Pinus albicaulis* (Gst = 0.034) (Jorgensen and Hamrick, 1997), and *Pinus flexilis* Gst = 0.101 (Jorgensen et al., 2002). In other cases, the Gst value detected was higher for *Pinus attennuata* (Gst = 0.24), *Pinus muricata* (Gst = 0.29) (Wu et al., 1999) and *Picea asperata* (Gst = 0.34) (Xue et al., 2005). The result confirmed the assumption that genetic drift increased genetic differentiation among populations (Ellstrand and Elam, 1993). It was observed that the high differentiation could be a consequence of limited gene flow (Nm = 1.3046), due to the fact that fragmented habitat caused gene flow barriers and decreased migration among populations for *F. hodginsii*. However, founder effects might contribute to the high level of genetic differentiation among the populations.

In conclusion, *F. hodginsii* maintained low level of genetic variability and high level of genetic population

differentiation, which are the results of human interference. *F. hodginsii* habitat has been degraded and fragmented, and only a few natural populations survived. Based on a conservation point of view, effective management strategies for *F. hodginsii* should include both *in-situ* and *ex-situ* activities. Establishment of seed orchards from all the populations should secure genetic sources of this species. Nonetheless, monitoring of the genetic variability in planted populations is important to ensure that the high level of genetic diversity is maintained.

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