Mitochondrial DNA variation in natural populations of Japanese larch (*Larix kaempferi*)

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Abstract

Levels of genetic variation within and among natural populations of Japanese larch [*Larix kaempferi* (Lamb.) Carrière] were evaluated by Restriction Fragment Length Polymorphism (RFLP) analysis of mitochondrial DNA (mtDNA) obtained from 209 individuals representing 14 natural populations distributed across most of the species' natural range in central Japan. Polymorphisms were screened by two RFLP techniques: Southern hybridization of mitochondrial gene probes and Polymerase Chain Reaction (PCR)-RFLP analysis of sequence-tagged sites. However, obvious polymorphism was only detected around the *cox*III gene, following digestion with the *Eco*RI restriction enzyme, in the Southern hybridization analysis. Five haplotypes were identified across the 14 populations, with 11 populations exhibiting little or no genetic variation. Within-population genetic variation was low (H_S = 0.158), while population differentiation was substantial (a standardized genetic differentiation measure, G'_{ST} = 0.581). The northernmost, marginal population on Mt. Manokamidake made a significant contribution to the total detected genetic variation. The mtDNA variation displayed geographic structure despite the restricted natural distribution of Japanese larch. Results of this study should be useful for establishing criteria for the genetic conservation and management of natural populations of the species.

Key words: genetic diversity, genetic structure, Japanese larch, mitochondrial DNA, population differentiation, RFLP.

Introduction

Japanese larch, *Larix kaempferi* (Lamb.) Carrière (Pinaceae), is the only endemic, deciduous conifer species in Japan. The natural distribution of this species is restricted to subalpine and montane areas in central Honshu (HAYASHI, 1960; HORIKAWA, 1972). Natural Japanese larch forests occur as pioneer communities on arid and immature soils in volcanic areas, flood plains and stream banks, and they form secondary communities in climax forests that have previously been clear cut. Japanese larch is often found in open forests, together with other species such as *Salix bakko*, *Abies homolepis*, *A. veitchii* and *Tsuga diversifolia*, and it sometimes forms the timberline at high elevations in the species' range, such as on Mt. Fuji (TATEWAKI et al., 1965; NUMATA, 1974). Since it is one of the most important silvicultural species in Japan, artificial plantations have frequently been established, from Hokkaido to central Honshu, especially since the late 1880s in upper montane zones, both within and outside its natural distribution range (TODA and MIKAMI, 1976).

Japanese larch was successfully transplanted to Europe from the late 19th century and its seed sources were of interest to European foresters. In 1956, worldwide provenance trials of Japanese larch began in Japan, with parallel trials established in ten European countries, and the U.S.A., Canada and New Zealand (TODA and MIKAMI, 1976). In Japan, significant variation has been observed within and among provenances of this species, including variation in some morphological and phenological traits (TODA and MIKAMI, 1976), mechanical and anatomical properties (MIKAMI and NAGASAKA, 1974; TAKATA et al., 1992a, 1992b), and resistances to diseases and low temperature (OKADA et al., 1970; TODA and MIKAMI, 1976). The extent of genetic variation within and among populations of Japanese larch has also been examined, using allozyme genetic markers, and greater genetic variation has been found within than among populations (TOMARU and UCHIDA, 2007).

Studies using various kinds of genetic markers have shown that the distribution of genetic variation in forest tree species differs among genomes (e.g., TOMARU et al., 1998; TANI et al., 2003; GAMACHE et al., 2003; WANG and GE, 2006). Generally, organelle genomes are more highly differentiated among populations than nuclear genomes. This difference is largely attributed to the uni-parental inheritance of organelle genomes and the limited seed dispersal of most tree species (BIRKY et al., 1989; PETIT et al., 1993; ENNOS, 1994). Previous studies on pines (Pinaceae), however, have reported higher levels of genetic differentiation in mitochondrial genomes than in chloroplast genomes, because the former is maternally inherited, while the latter is paternally inherited (WAGNER et al., 1987; NEALE and SEDEROFF, 1989; PETIT et al., 1993). Although the relatively high levels of structural rearrangement and relatively low levels of point mutations exhibited by plant mtDNA compared to cpDNA limit its utility in phylogenetic studies, mtDNA is capable of revealing variation at the inter-specific and population levels (AVISE et al., 1987; PALMER, 1992; KARP et al., 1996), and it has been widely used for studies of genetic variation in species belonging to Pinaceae (STRAUSS et al., 1993; DONG and WAGNER, 1993; LATTA and MITTON, 1997; WU et al., 1998; TSUMURA and SUYAMA, 1998; SINCLAIR et al., 1999).

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In the study presented here we analyzed Restriction Fragment Length Polymorphisms (RFLPs) of mitochondrial DNA (mtDNA) to evaluate the levels of genetic variation within and among natural populations of Japanese larch. We then used the acquired information to make inferences on the factors that may have influenced the detected patterns of genetic variation and the probable phylogeography of the species. Finally, we compared our data with the reported allozyme variation for this species (TOMARU and UCHIDA, 2007) to compare the patterns of genetic variation between mitochondrial and nuclear genomes. We expected that mtDNA variation would show greater geographic influence (and therefore give more geographic information) than did allozyme variation.

Materials and Methods

Geographic distribution of the study species, sample collection, and DNA extraction

Japanese larch trees occur naturally in most of the central Honshu region (*Fig. 1*; HAYASHI, 1960; HORIKAWA, 1972). The northern and eastern geographic limit of the distribution is Mt. Manokamidake in Miyagi Prefecture. The population on Mt. Manokamidake is extremely isolated, and has a very small number of individuals (11 at present; HANDA and YUASA, 2008). The southern limits of this species are Mt. Tenguishi and Mt. Yamazumi at the southern part of the Akashi mountain range in Shizuoka Prefecture. Mt. Haku-san was previously described as the western limit of this species, but no expeditions since 1956 have found any extant population in this location, and the current western limit of Japanese larch is somewhere along the Hida mountain range (TODA and MIKAMI, 1976).

Young needle tissue was collected from 209 trees from 14 natural populations (mean = 14.9 trees per population) distributed across most of the species' natural range (*Fig. 1*, *Table 1*). Needles were not directly collected from the Manokamidake population, but from grafted clones, which were propagated from 15 individuals of the population used for *ex-situ* conservation at the Tohoku Regional Breeding Office (Forest Tree Breeding Center, Forestry and Forest Products Research Institute, Japan). In the natural populations, the needle tissues were collected from randomly selected individuals (regardless of age, height or other morphological characteristics) spaced at least 50 meters apart, to avoid sampling maternal half-sibs. The location and sample size of each population is shown in *Table 1*. Total genomic DNA was extracted from 3 to 5 grams of needles using the method described by TSUMURA et al. (1995).

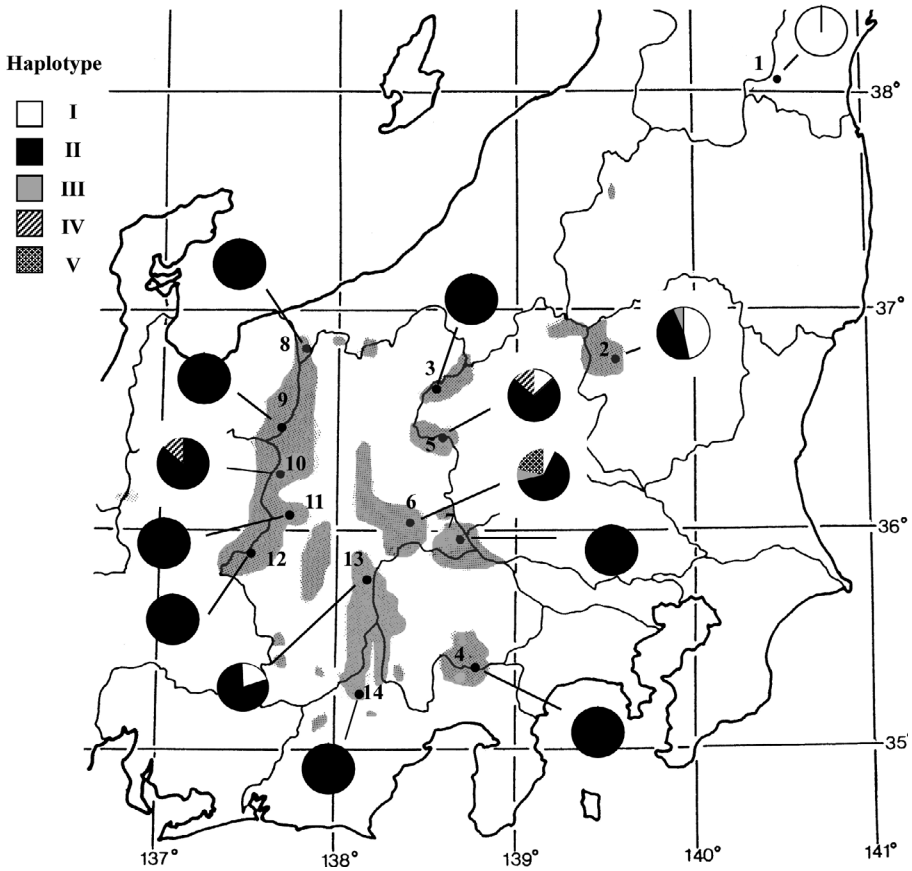

Figure 1. – Natural distribution (shaded areas; HAYASHI, 1960) and frequencies of mtDNA haplotypes (indicated by pie charts) in the 14 natural populations of Japanese larch. Dots indicate the locations of the populations studied and numbers by the dots correspond to the population numbers in *Table 1.*

Table 1. – Locations and sample sizes of the 14 natural populations of Japanese larch.

Population number and name	Locality	Longitude (E)	Latitude (N)	Elevation (m)	Sample size
1 Manokamidake	Mt. Manokamidake, Miyagi	140°30'	38°05'	1500	15
2 Yashubara	Mt. Nantai, Tochigi	139°33'	36°47'	1700	15
3 Manza	Mt. Manza, Gunma	138°30'	36°39'	1700-1800	15
4 Oniwa	Mt. Fuji, Yamanashi	138°43'	35°23'	2350-2500	15
5 Kutsukake	Mt. Asama, Nagano	138°34'	36°24'	1400-1450	15
6 Inago	Mts. Yatsugatake, Nagano	138°24'	36°03'	1750-1800	14
7 Kobushigatake	Mt. Kobushigatake, Nagano	138°43'	35°57'	1500	15
8 Rengedake	Mt. Rengedake, Niigata	137°48'	36°48'	2180	15
9 Lower Takasegawa	Omachi, Nagano	137°41'	36°24'	1380	15
10 Kamikochi	Azumi, Matsumoto, Nagano	137°40'	36°15'	1620	15
11 Hachimori	Mt. Hachimori, Nagano	137°43'	36°04'	1920	15
12 Ontake	Mt. Ontake, Nagano	137°28'	35°55'	1900-2000	15
13 Kai-Komagatake	Mt. Kai-Komagatake, Nagano	138°13'	35°45'	1500	15
14 Daimugen	Mt. Daimugen, Shizuoka	138°10'	35°15'	2100-2200	15

RFLP analysis using Southern hybridization

Three mitochondrial genes (*cox*I, *cox*III and *nad*4) were used as probes for Southern hybridization. The probes were labeled with digoxygenin (DIG) during Polymerase Chain Reaction (PCR) amplification following the manufacturer's protocol (Roche Diagnostics). The primers used to amplify *cox*I and *cox*III were the same as those used by TSUMURA and SUYAMA (1998), while the primer sequences used to amplify *nad*4 were designed based on a complete sequence (see LAMATTINA and GRIENENBERGER, 1991) by K. YOSHIMURA at the Forestry and Forest Products Research Institute, Japan (personal communication). The primer sequences used were 5'-CAAACAGGAACCACCGATTT-3' and 5'-AACCACACGATTATAGCC-3'. Total genomic DNA (approximately 2 to 4 µg) was digested with 18 restriction enzymes (*Apa*I, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hind*III, *Hinf*I, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sty*I, *Xba*I and *Xho*I). Digested fragments were separated by electrophoresis in a 0.7% agarose gel, blotted onto a nylon membrane (Biodyne B, Pall) and hybridized with the three gene probes. Blotting, hybridization, washing, and detection by chemiluminescence were conducted following the protocols prescribed in the Roche Diagnostics's manual for filter hybridization. Preliminary screening of RFLPs was conducted on five individuals from each of the ten selected populations using the 18 restriction enzymes and the three gene probes. Only the gene probe that displayed polymorphism after digestion with a specific restriction enzyme was analyzed in the full survey of all populations.

PCR-RFLP analysis

Eleven mtDNA sequence-tagged sites (STS) – *nad*1 exons B-C, *nad*4 exons 1-2, *nad*4 exons 2-4, *rps*14-cob (DEMESURE et al., 1995), UBC460, C8, B11 (SEMERIKOV et al., 2005), and *mh*02, *mh*09', *mh*27, *mh*50 (JEANDROZ et al., 2002) – were amplified by PCR using the conditions described in the cited studies, except that we used different annealing temperatures from 50 to 62 °C. We successfully amplified products for *nad*1 exons B-C, B11, and *mh*50 at annealing temperature of 50 °C and screened them for PCR-RFLPs using five individuals from each of the 14 populations and ten four-base pair recognition restriction enzymes (*Hinf*I, *Hae*III, *Taq*I, *Rsa*I, *Msp*I, *Nde*II, *Cfo*I, *Alu*I and *Hha*I, and *Sau*3AI).

Statistical analysis

Haplotypes were identified based on distinct restriction fragments. Haplotype frequencies were estimated for each population and means were obtained over all populations. The haplotype frequencies were used to estimate the gene diversity statistics, H_T, H_S, and G_{ST} (NEI, 1973; NEI and TAJIMA, 1981). H_T is the gene diversity in the total population, while H_S is the average gene diversity within populations, and G_{ST} is the measure of gene differentiation among populations. The test for significance of population differentiation was based on 1000 randomizations of haplotypes among populations, where the statistic used was the log-likelihood G (GOUDET et al., 1996). These calculations were performed using the FSTAT software (GOUDET, 2002). Since G_{ST} is dependent on the levels of genetic diversity with-

in populations, its values cannot be simply compared between loci or species if the loci or species to be compared exhibit different levels of genetic diversity (HEDRICK, 2005). Therefore, to compare the level of genetic differentiation among populations revealed by mtDNA RFLP in this study with that detected using allozymes (TOMARU and UCHIDA, 2007), we calculated an alternative measure of genetic differentiation, G'_{ST} (HEDRICK, 2005), which is a standardized version of G_{ST} and has the same range (0-1) for all levels of genetic diversity.

Using the MEGA program (KUMAR et al., 2001), a neighbor-joining (NJ) tree (SAITOU and NEI, 1987) was constructed based on NEI's (1978) unbiased genetic identity to examine the genetic relationships among populations.

Results

Identification of mitochondrial DNA haplotypes

In the preliminary screening of the RFLPs we did not observe obvious polymorphism in the PCR-RFLP analysis, but detected it in the RFLP analysis using Southern hybridization with a combination of *Eco*RI and *cox*III. Five distinct hybridization patterns were detected from *Eco*RI fragments with the *cox*III probes, and were represented as five haplotypes from I to V (*Fig. 2*). Haplotypes I, II, and IV had four distinct restriction fragments, while haplotypes III and V had five distinct fragments. All haplotypes shared three common fragments with lengths of 5.2, 4.3 and 3.9 kb. Haplotypes I, II and IV differed only in their largest fragments, which were 8.7, 20.1 and 24.8 kb long, respectively. In contrast, haplotypes III and V each had two large restriction fragments; 21.5 and 18.8 kb, and 20.1 and 16.8 kb long, respectively.

Geographic distribution of mtDNA haplotypes

Fig. 1 shows the geographic distribution of mtDNA haplotypes across the investigated 14 populations. Mean haplotype frequencies varied from 0.010 (haplotype III) to 0.822 (II). Haplotype I was fixed in the Manokamidake population (population 1), but was also present at varying frequencies in four other populations: Yashubara (2), Kutsukake (5), Inago (6) and Kai-Komagatake (13). Haplotype II was present in 13 populations,

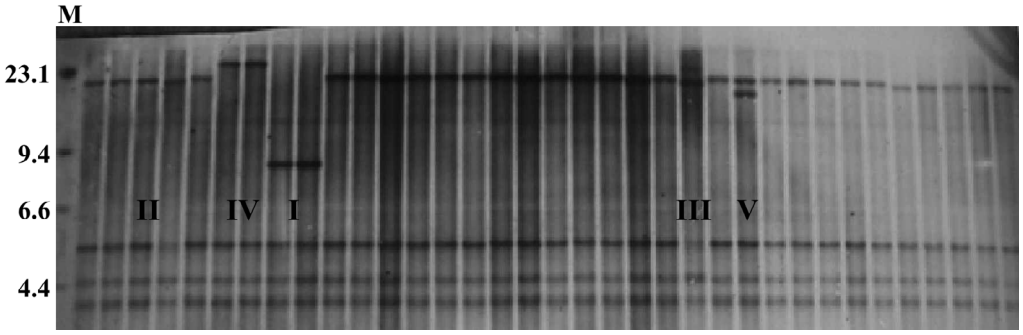

Figure 2. – Southern hybridization patterns of *Eco*RI fragments with the *cox*III probe showing the five haplotypes (I–V). Lane M shows the molecular size marker (*Hind*III-digested lambda phage DNA) and the numbers to the left indicate fragment sizes (kb) of the size marker.

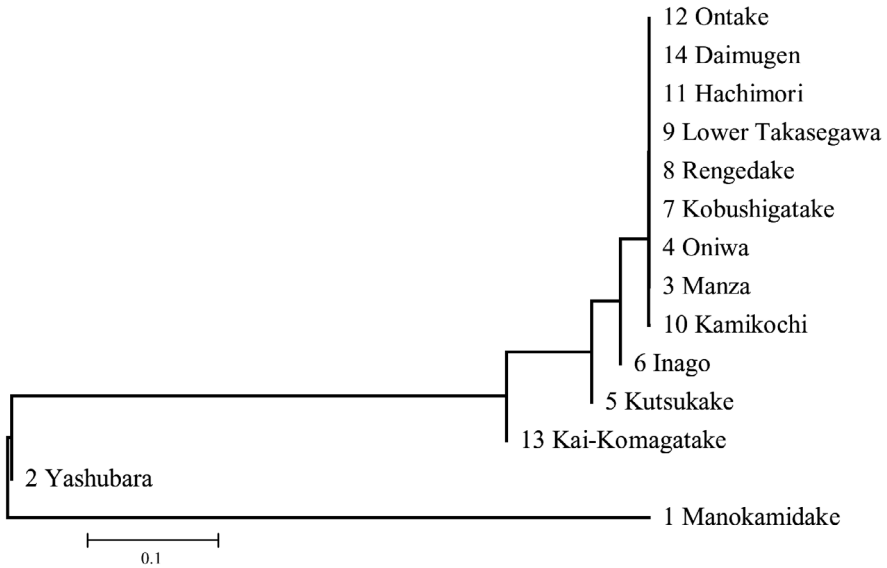

Figure 3. – Neighbor-joining tree derived using pairwise unbiased genetic identities for the 14 natural populations of Japanese larch. The length of the bars is equal to 0.1, estimated by 1 – unbiased genetic identity (0.9).

covering almost the entire species' geographic distribution. It was fixed in eight populations, and present at high frequencies in Yashubara (2), Inago (6), Kutsukake, Kamikochi (10) and Kai-Komagatake (13). Haplotypes III, IV and V were observed, at low frequencies, in only one or two populations: Haplotype III was detected in the Yashubara (2) and Inago (6) populations; Haplotype IV was present in the Kutsukake (5) and Kamikochi (10) populations; and Haplotype V was found exclusively in the Inago population (6), with three other haplotypes.

Of the 14 populations, 11 exhibited little or no genetic variation within populations, while three [Yashuubara (2), Kutsukake (5), and Inago (6)] showed high intrapopulation variation (*Fig. 1*). The gene diversity per population varied from zero for populations having fixed haplotypes, to 0.600 for the Yashubara population (2). Average gene diversity within populations was low (H_S = 0.158), while two measures of genetic differentiation among populations were substantial (G_{ST} = 0.483, G'_{ST} = 0.581; *Table 2*). The genetic differentiation among populations was statistically significant ($P < 0.001$; *Table 2*).

The geographic distribution of mtDNA haplotypes is shown in the NJ tree (*Fig. 3*), which identified the separation of the Manokamidake population (1) from all other populations. Since the tree was constructed using data from a single marker, the clustering of populations is straightforward. The close relationship of the Manokamidake (1) and Yashubara (2) populations (as shown in the tree) was due to the large number of individuals sharing haplotype I in the two populations. This was clearly supported by the pairwise genetic identity (0.738) between the Manokamidake (1) and Yashubara (2) populations, which was highest among all population comparisons (*Table 3*). The other 12 populations, where haplotype II was either fixed or dominant, were contained in one large subgroup.

Discussion

The failure to detect RFLP in any of the investigated mtDNA regions except around the *cox*III gene in this study suggests that there is a limited source of mtDNA variation in Japanese larch, and that this region may be one of the few 'hotspots' of mtDNA variation in the species. However, even with this single polymorphism, high haplotype diversity was detected, as demonstrated by the higher estimates of H_T (0.306) and H_S (0.158) obtained from the mtDNA-RFLP data, as compared to previously published estimates based on nuclear-encoded allozymes (H_T = 0.127 and H_S = 0.120; *Table 2*). The haplotype diversity of mtDNA in the species is likely to be a result of the high levels of structural rearrangement found in plant mtDNA, as previously suggested by many authors (e.g. PALMER, 1992; STRAUSS et al., 1993; DONG and WAGNER, 1993).

The patterns of genetic variation within and among natural populations of Japanese larch detected by the mtDNA-RFLP analysis are in agreement with predictions for maternally inherited markers, and follow the general trends observed in many other species, particularly conifers (DONG and WAGNER, 1993; STRAUSS et al., 1993; LATTA and MITTON, 1997; TSUMURA and SUYAMA et al., 1998; WU et al., 1998; TANI et al. 2003; also see citations in NEWTON et al., 1999 and PETIT et al., 2005). Our estimates of genetic variation within the Japanese larch populations (H_S = 0.158) are close to those obtained in an intensive mtDNA genome survey of California closed cone pines, *Pinus radiata*, *P. muricata* and *P. attenuata* (H_S = 0.22; WU et al. 1998). However, mtDNA variation in Japanese larch is rather characterized by high differentiation among populations, which is contrary to the low differentiation among populations detected in the previous study using nuclear-encoded allozymes of the species (TOMARU and UCHIDA, 2007) (*Table 2*), and in

Table 2. – Mitochondrial DNA variation within and among populations of Japanese larch in comparison to allozyme variation.

Marker	H_T	H_S	G_{ST}	G'_{ST}	P value[a]
Mitochondrial DNA RFLP[b]	0.306	0.158	0.483	0.581	< 0.001
(excluding Manokamidake)	(0.212)	(0.170)	(0.195)	(0.224)	(< 0.001)
Allozyme[c]	0.127	0.120	0.057	0.066	< 0.001
(min.-max.)	(0.000-0.499)	(0.000-0.484)	(0.000-0.136)	(0.000-0.172)	

H_T, the gene diversity in the total population; H_S, the average gene diversity within populations; G_{ST}, measure of genetic differentiation among populations; G'_{ST}, standardized measure of genetic differentiation among populations (HEDRICK, 2005).

[a] Probabilities associated with randomization tests for population differentiation using the log-likelihood G as a statistic (GOUDET et al., 1996).

[b] This study.

[c] Average values calculated from data for seven allozyme loci, with min. – max. values at single loci shown in parentheses (TOMARU and UCHIDA, 2007).

Table 3. – Pairwise unbiased genetic identities for the 14 natural populations of Japanese larch.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 Manokamidake	-													
2 Yashubara	0.738	-												
3 Manza	0.000	0.738	-											
4 Oniwa	0.000	0.738	1.000	-										
5 Kutsukake	0.181	0.868	0.996	0.996	-									
6 Inago	0.108	0.816	0.982	0.982	0.997	-								
7 Kobushigatake	0.000	0.738	1.000	1.000	0.996	0.982	-							
8 Rengedake	0.000	0.738	1.000	1.000	0.996	0.982	1.000	-						
9 Lower Takasegawa	0.000	0.738	1.000	1.000	0.996	0.982	1.000	1.000	-					
10 Kamikochi	0.000	0.737	0.999	0.999	1.000	0.981	0.999	0.999	0.999	-				
11 Hachimori	0.000	0.738	1.000	1.000	0.996	0.982	1.000	1.000	1.000	0.999	-			
12 Ontake	0.000	0.738	1.000	1.000	0.996	0.982	1.000	1.000	1.000	0.999	1.000	-		
13 Kai-Komagatake	0.247	0.910	0.987	0.987	1.000	0.996	0.987	0.987	0.987	0.986	0.987	0.987	-	
14 Daimugen	0.000	0.738	1.000	1.000	0.996	0.982	1.000	1.000	1.000	0.999	1.000	1.000	0.987	-

long-lived woody species (HAMRICK and GODT, 1990). The population differentiation (G'_{ST}) value (0.581) obtained for Japanese larch from the mtDNA-RFLP data was about nine times higher than the corresponding value (0.066) based on allozymes. According to theoretical expectations, the higher population subdivision based on mtDNA, compared with nuclear DNA (nDNA), is due to the maternal inheritance of mtDNA and the low effective number of genes for mtDNA resulting from its haploidy (BIRKY et al., 1989; PETIT et al., 1993). The mitochondrial genome in the genus *Larix* is maternally inherited (DE VERNO et al., 1993). Thus, gene flow is lower for mtDNA than for nDNA because mtDNA is transmitted solely by seed dispersal, while nDNA is transmitted through both seed and pollen dispersal. Since the effective number of genes of mtDNA is lower than that of nDNA, genetic drift has stronger effects on mtDNA than on nDNA. Therefore, the substantial level of population differentiation observed in Japanese larch may be influenced by the strict maternal origin and haploidy of its mitochondrial genome, and reflects limited seed dispersal in natural populations.

Although the estimate of population differentiation for mtDNA in Japanese larch was found to be substantial, it is lower than that in many other forest species (median value for organelle DNA, 0.670; PETIT et al., 2005). However, the low estimate of population differentiation obtained for Japanese larch falls in the observed low G_{ST} range of species distributed in restricted areas (PETIT et al., 2005). The low level of population differentiation in Japanese larch is also demonstrated by the high average pairwise population genetic identity of 0.837, indicating close genetic affinity among populations.

Despite the restricted distribution of Japanese larch, the mtDNA variation exhibits geographic structure across the species' natural range. The wide separation of the Manokamidake population from the central populations as shown in the NJ tree is consistent with the results obtained in a previous analysis using RAPDs (SHIRAISHI et al., 1996). A severe genetic bottleneck, along with the geographic isolation of the Manokamidake population, is suggested to have resulted in the fixation of the reported haplotype now found in this population, which caused its relatively high differentiation from the central populations. This is strongly supported by the remarkably low within-population genetic variation and high genetic divergence from the central populations for the Manokamidake population, revealed by allozyme (TOMARU and UCHIDA, 2007) and nuclear microsatellite (SAN JOSE-MALDIA et al., unpublished) analyses.

In order to demonstrate the contribution of the Manokamidake population to the total genetic variation in Japanese larch, we conducted a separate analysis in which we excluded this population. We found that the estimates of H_T and G'_{ST} decreased by 30% and 60%, respectively (*Table 2*). These findings suggest that the presence of this population is important because it contains a large proportion of the total mtDNA variation in Japanese larch, and that the high conservation priority currently given to this population is warranted. Many studies suggest that peripheral and marginal populations have important potential roles in genetic conservation since they may preserve alleles that are important for adaptation to extreme environmental conditions (e.g., ARNAUD-HAOND et al., 2006). According to MILLAR and LIBBY (1991), an important method for conserving the among-populations component of genetic variation is to protect the most genetically distinct populations. In most cases, peripheral and isolated populations are not only genetically distinct but also morphologically divergent. The distinct traits found in such populations may

be crucial to the species in the face of environmental fluctuations (LESICA and ALLENDORF, 1995). In fact, the morphological traits of cones of individuals belonging to the Manokamidake population are different from those of the central populations (YANO, 1994). Like any marginally peripheral and isolated populations, the Manokamidake population has low genetic variation, but this genetic condition may be historic and not associated with recent population declines (e.g. MATOCQ and VILLABLANCA, 2001). The population may have long endured extreme environmental conditions in the northern limit. Therefore, its persistence may suggest the adaptive potential of the population.

Conclusion

Although there was a low resolution of the phylogeography of Japanese larch due to the limited detected variation in mtDNA, we found that mtDNA variation displayed geographic structure within the species' restricted natural range. The mtDNA variation in this species is consistent with predictions for maternally inherited markers, for which genetic variation among populations is expected to be greater than within-population variation. The results of our study are interesting with respect to the ongoing, large-scale, *in situ* and e*x-situ* conservation programs for natural Japanese larch forests. With the rapid advances of molecular techniques being applied in population genetics, we believe that the results of our study will be useful for the genetic conservation and long term management of this species, when considered in conjunction with genetic information obtained from other highly variable markers (e.g. microsatellites) and potentially adaptive gene markers.

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