# GENETIC DIFFERENTIATION OF ORGANELLE DNA POLYMORPHISMS IN SAGHALIN FIR FROM HOKKAIDO

#### Eiji Hayashi<sup>1</sup>, Masatoshi Ubukata, Kazuya Iizuka & Naoei Itahana

Hokkaido Regional Breeding Office, Forest Tree Breeding Center, Ebetsu, Hokkaido 069-0836, Japan <sup>1)</sup> Present address: Forest Tree Breeding Center, Ibaraki 319-1301, Japan; phone: +81-293-32-7000, fax: +81-293-32-7306, e-mail: ehayas@nftbc.affrc.go.jp

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# ABSTRACT

The investigation of genetic differentiation among Saghalin fir (*Abies sachalinensis* Mast.) populations was carried out with genetic markers of contrasting mode of inheritance. The expected mode of inheritance for the two organelle genomes was also confirmed. Restriction fragment length polymorphisms (RFLPs) were analyzed among 264 individuals, including plus trees, from 19 natural populations. Total genomic DNAs was digested with two restriction endonucleases (*Bam*HI and *Eco*RI) and hybridized with a cytochrome oxidase subunit II (*cox*II) probe from mitochondrial (mt) DNA of sugar beet and an ATPase  $\alpha$ -subunit (*atp*A) probe from chloroplast (cp) DNA of Saghalin fir. Three variants were observed in *Bam*HI / *cox*II, and two in *Eco*RI / *atp*A. Nei's gene diversity within populations ( $H_e$ ) was 0.232 and 0.001 respectively. Genetic differentiation is higher when using a maternally inherited mtDNA marker ( $G_{ST} = 0.715$ ), than using a paternally inherited cpDNA marker ( $G_{ST} = 0.003$ ). Especially southwestern populations have genetically different from the rest. The high differentiation of *cox*II gene might be the result of high evolutionary rate and low migration rate of mitochondrial genome. These results provide information for elucidating phylogeography and consequently for an application to conservation purpose of this fir.

Key words: genetic differentiation, RFLP, organelle DNAs, maternal inheritance, paternal inheritance, *Abies* sachalinensis

# **INTRODUCTION**

Saghalin fir (*Abies sachalinensis* Mast.) is widely distributed throughout the islands of Hokkaido and Saghalin and the Southern Kurile Islands as a dominant tree species of four forest zones: the cool-temperate forests, the pan-mixed forests, the subalpine forests, and alpine forests. In the cool-temperate forests, it is associated with the broad-leaved trees represented by the beech (*Fagus crenata*) and in the subalpine forests, with *Picea jezoensis* and *Picea glehnii*. Sometimes it also forms pure forests (JAPAN FOREST TECHNICAL ASSOCIATION 1966). This fir is one of the most important forest trees in Hokkaido prefecture as an ornamental tree, a source of building materials and as a genetic resource.

Evidence concerning the mode of inheritance of chloroplast (cp) DNA has been found in progeny from intra- and inter- specific crosses for Douglas fir (NEALE et al. 1986), Pinus (WAGNER et al. 1987; NEALE & SEDEROFF 1989), Larix (STINE et al. 1989, SZMIDT et al. 1987), Picea (SZMIDT et al. 1988; SUTTON et al. 1991), Abies (ZIEGENHAGEN et al. 1995) and Chamaecyparis (KONDO et al. 1998). Generally, cpDNA seems to be paternally inherited in the family Pinaceae. Hence, cpDNA gene flow is considered to occur through pollen and seed dispersal in these species. Since mitochondrial (mt) DNA of Pinaceae species is predominantly maternally inherited (NEALE & SEDEROFF 1989; DEVERNO et al. 1993; SUTTON et al. 1991), its migration depends only on seed dispersal. Plant mtDNA is subject to rapid structural evolution (SEDEROFF 1987; PALMER 1992). The mtDNA variation, caused by its relatively frequent genome rearrangements, can be easily observed by restriction fragment pattern analysis. Therefore, strong population differentiation, resulting from the maternal inheritance of mtDNA and restricted seed dispersal, have been reported (DONG & WAGNER 1993; STRAUSS et al. 1993; TSUMURA & SUYAMA 1998). Confirmation of the mode of inheritance of organelle DNAs is an important step for further study of the population genetics of Saghalin fir.

Previous studies of the fir have revealed genetic variation in morphological and phenological traits of cone, seed and, seedling (HATAKEYAMA 1981). Seedling characters, resistance to snow-damage, freezing resistance, and winter desiccation-resistance of the species have also been well documented (OKADA *et al.* 1973; OKADA 1983; EIGA 1984). Allozyme variation

among natural populations in relation to environmental gradients in Hokkaido has been detected in a recent study (NAGASAKA et al. 1997). Moreover, a recent phylogenetic study of Abies species, in which restriction fragment length polymorphisms (RFLPs) of mtDNA were detected by coxI and coxIII gene probes, showed within-population variation of two eastern populations of Saghalin fir (TSUMURA & SUYAMA 1998). No variation of mtDNA within populations was detected in one northern and two western populations sampled. Although that study showed diversity within and among five natural populations of Saghalin fir, more information concerning diversity among plus trees, and differentiation of populations from which plus trees were collected, is needed to design conservation programs. This paper reports the diversity of organelle DNAs by use of RFLPs, and confirmation of the mode of inheritance for the two organelle genomes.

### MATERIALS AND METHODS

#### **Plant materials**

Figure 1 shows the location of the populations studied. In the first sample collection, flashing buds from 210 trees representing 19 natural populations were sampled at the gene conservation plantation of plus tree in Hokkaido Regional Breeding Office, west of Ebetsu City, Hokkaido, North Japan (50 m asl.; 43°04'N, 141°31'E). To supplement these samples, a second collection was made from 54 individuals representing three natural populations (in Iwanai, Kucchan, and Gamushi). The results of RFLP analysis from the



**Figure 1.** Sampled locations (circles) of Saghalin fir. White circles represent populations, in which plus trees were collected. Black circle represents gene conservation plantation in Hokkaido Breeding Office. Key to location abbreviations: Nk, Nakatonbetsu; Kt, Kotanbetsu; Ash, Asahi; Sr, Saroma; Nm, Nemuro; Ak, Akkeshi; Asy, Asyoro; Rk, Rikubetsu; Shr, Shiranuka; Shn, Shintoku; Oy, oyubari; Kn, Kanayama; Fr, Furenai; Ur, Urakawa, Tm, Tomakomai; Jy Jyozankei; Iw, Iwanai; Kc, Kucchan; Gm, Gamushi.

supplemental collections were combined with the data from the plus trees. A third sample collection was made at an operational progeny test plantation consisting of intra-specific crosses produced by controlled pollination. From each reciprocal tree family for which parents showed polymorphisms in *Bam*HI / *cox*II or *Eco*RI / *atp*A enzyme-probe analyses, flashing buds of fourteen individuals were collected. These samples were used in the study of inheritance of organelle DNA.

#### **DNA** analysis

For total DNA extraction, 1 g of each sampled bud was used following a modified CTAB procedure (MURRAY & TOMPSON 1980). The bud was ground in liquid nitrogen using a mortar and pestle. Total DNA was extracted from the powdered bud by a one hour digestion at 65 °C in 4 ml of CTAB buffer, 100 mM Tris-HCl (pH.8.0), 2% CTAB, 2% PVP, 0.1% mercaptoethanol, 1.4 M NaCl, and 20 mM EDTA. The digestion was followed by two chloroform-isoamyl alcohol (CIA, 24:1) extractions. The crude DNAs were precipitated at room temperature by adding 3 M sodium acetate (pH.5.2) to a final concentration of 0.3 M, and isopropanol (1:1 with the CIA extract). Pellets were recovered by centrifugation at 4 °C for 20 min. at 16,000 g, and were resuspended in 2 ml of TE. The solution was incubated with RNase (1mg·ml<sup>-1</sup>) at 37 °C for 1 hour. Two buffered phenol treatments and one CIA extraction followed the RNase treatment. Purified DNA was reprecipitated, followed by centrifugation as above, dried in vacuo, and resuspended in 1 ml of TE.

Five  $\mu$ g of total DNA were digested with four restriction endonucleases, *Bam*HI, *Eco*RI, *Dra*I, and *Hind*III following the manufacturer's recommendations. Digested DNAs were electrophoretically separated on 0.8% agarose gels. DNA samples were transferred to nylon membranes and bound by baking. Blotted restriction fragments were visualized by enhanced chemiluminescence (ECL), following southern hybridization (SOUTHERN 1975). An ECL Gene Detection System (Amersham Life Science) was used for probe labelling and signal detection, following the protocol of the manufacturer.

The cpDNA probe used in this work was amplified by polymerase chain reaction from total DNA of Gamushi 106, a Saghalin fir plus tree, using the following primers:

#### 5'-AGACGGAAATACCCACATTA-3' and

5'-TCAAGTAGGAGATGGCATTG-3', designed from the *atp*A sequence of *Pinus thunbergii*.

The mtDNA probes were amplified from a pUC vector containing a cytochrome oxidase subunit I [*cox*I, 1.6 kbp *Eco*RI fragment (SENDA *et al.* 1991)], a

cytochrome oxidase subunit II [coxII, 0.4 kbp SaII-HindIII fragment (SENDA *et al.* 1991)] and a NADH dehydrogenase (nad9, KUBO *et al.* 1993) sequence from sugar beet (*Beta vulgaris* L.), a ATPase  $\alpha$ -subunit gene [atpA, 1.4 kbp EcoRI-HindIII fragment (SENDA *et al.* 1993)] and a ATPase subunit 9 gene [atp9, 1.3 kbp HindIII-BamHI fragment (MORIKAMI & NAKAMURA 1987)] from pea (*Pisum sativum*) using the following primers:

5'-CGACGTTGTAAAACGACGGCCAGT-3' and 5'-GGAAACAGCTATGACCATGATTAC-3'. Then the amplified probe DNAs were purified by phenol treatment and CIA extraction. Purified probe DNAs were precipitated by adding polyethylene glycol solution [13% (v/w), 1.6 M NaCl] of equal volume to the CIA extract, followed by centrifugation as above, and dried *in vacuo*.

In this paper, the *cox*II probe from sugar beet mtDNA and the *atp*A probe from Saghalin fir cpDNA are frequently abbreviated to '*cox*II (mt)' and '*atp*A (cp)' respectively.

## **Population genetic Analysis**

To assess the statistical significance of the difference in variant frequencies (observed in the coxII gene analysis) between 4 populations locate on the southwestern peninsula and other populations, Fisher's exact test of independence (SOKAL & ROHLF 1981) was conducted. Gene diversity statistics (NEI 1978) were calculated using the derived variant frequencies. Total gene diversity  $(H_{\tau})$  consists of the genetic variation within  $(H_s)$  and among  $(H_e)$  populations.  $G_{ST}$ , the coefficient of gene differentiation among populations was calculated using the equations  $G_{ST} = H_e / H_T$ ,  $H_e = H_T - H_S$ . These estimates were compared with those of PONS & PETIT (1995). Using the FSTAT program (GOUDET 1995), the fixation index  $\theta$  (WRIGHT 1965) was also estimated, together with its standard deviation, by the method of WEIR (1990).

#### RESULTS

#### Inheritance of organelle DNAs

In order to select proper enzyme/probe combinations for use in more detailed analyses, the preliminary survey was carried out on randomly selected 30 individuals. Two enzyme-probe combinations [BamHI / coxII(mt) and EcoRI / atpA (cp)] which gave clear polymorphisms were selected. When the other enzyme-probe combinations were used, the monomorphic patterns were detected frequently. Instead, since the signals were sensitive to the extent of the stringency for membrane washing after hybridization, it was difficult to obtain polymorphisms reproducibly. In order to identify appropriate lines to study organelle DNA inheritance, RFLPs of parental trees used in crosses were investigated with both of these enzyme-probe combinations. Iwanai 106 and Gamushi 106 differed from each other in fragment variant, and when 14 trees of each reciprocal cross between these two parental trees were analyzed by *Bam*HI / *cox*II (mt), all progeny showed restriction fragments identical to the maternal tree (Fig. 2a). When analyzed by *Eco*RI / *atp*A (cp), however, all progeny showed restriction fragments identical to the paternal tree (Fig. 2b).

#### Diversity within and between populations

Table 1 shows restriction fragment variant frequencies of organelle DNAs in natural populations. The detection of just one fragment by each probe-enzyme combination per sample suggests that probe-homologous sequences are present in single copies. Three variants were detected in the BamHI / coxII (mt) analysis, and two in the EcoRI / atpA (cp) tests. The BamHI / coxII combination detected, predominantly, a fragment of 11.7 kb in samples from northern and eastern populations. The most prevalent fragment detected in two southwestern populations was 9.1 kb long, and samples from Asyoro and Shintoku populations had a rare 7.7 kb variant, distinguishing them from all other populations. No within-population variation was observed in three northern populations (Nakatonbetsu, Kotanbetsu, and Asahi), three eastern populations (Nemuro, Akkeshi, and Rikubetsu), one central population (Kanayama), and three southern populations (Furenai, Urakawa, and Tomakomai). Fisher's exact test of independence shows that the difference in variant frequencies between 4 populations (Jyozankei, Iwanai, Kucchan, and Gamushi) which locate on the southwestern peninsula and other populations is statistically significant (p < p0.02). In the EcoRI / atpA (cp) analysis, two variants were detected. All population showed intrapopulation variation. Nei's gene diversity values (NEI 1978) for organelle DNAs are shown in Table 2. Polymorphism of coxII ( $H_e = 0.232$ ) was greater than that of atpA ( $H_e$ = 0.001). Gene differentiation coefficients among populations  $(G_{ST})$  is higher when using a maternally inherited marker, coxII. The estimates of PONS & PETIT (1995) were quite close to those of NEI.

#### DISCUSSION

#### **Organelle DNA diversity and differenciation**

In this investigation heterologous coxII probes derived



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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31

**Figure 2.** Inheritance of organelle DNAs in Saghalin fir. (a) Maternal inheritance of mitochondrial DNA restriction fragments in progeny from reciprocal crosses of Saghalin fir. *Bam*HI digests of parents and progeny hybridized with cytochrome oxidase subunit II mitochondrial DNA probe. (b) Paternal inheritance of chloroplast DNA restriction fragments in progeny from the reciprocal crosses of Saghalin fir. *Eco*RI digests of parents and progeny hybridized with the ATPase  $\alpha$  subunit chloroplast DNA probe. Lane 1, Lambda *Hind* III DNA size markers; Lanes 2 and 3, parental trees of the crosses, Iwanai 106 and gamushi 106, respectively; Lanes 4–17, progeny of the cross Iwanai 106 (female) × Gamushi 106 (male); Lanes 18–31, progeny of the cross Gamushi 106 (female).

from sugar beet were used in hybridization with mtDNA from Saghalin fir. RFLP analysis using a homologous probe from Saghalin fir would presumably give more reliable results than the same analysis using heterologous probes. However, the heterologous probes were believed to be adequate for this analysis because previous work has shown that mtDNA probes derived from angiosperm species hybridize with mtDNA of gymnosperm species (NEALE & SEDEROFF 1989; SUTTON *et al.* 1991; WAGNER *et al.* 1991; DEVERNO *et al.* 1993; ). Furthermore, the rate of nucleotide substitution in coding sequences of plant mtDNA is extremely low (SEDEROFF 1987; PALMER 1992).

The estimated genetic diversity  $(H_T)$  of *cox*II in Saghalin fir (Table 2) is greater than that of *Pinus banksiana*, but less than that of *P. contorta* and *P. muricata* (DONG & WAGNER 1993; STRAUSS *et al.* 1993). The

genetic diversity of *P. contorta* and *P. banksiana* is correlated with dispersal of the populations, the isolation of refugia during the glacial period, and subsequent migration of the species (DONG & WAGNER 1993).

We note that the apparent differentiation of the *cox*II gene among populations is due solely to the polymorphism in southwestern populations, since Fisher's exact test showed significant differences in variant frequencies between southwestern populations and the other populations. This result is not consistent with previous reports. TSUMURA & SUYAMA (1998) detected gene diversity only in central and eastern populations. The estimated diversity of mtDNA in this study was greater than that of the reported for this fir ( $H_T$  = 0.292). The contradictions might be due to the following reasons. Firstly, small number of populations studied by TSUMURA & SUYAMA is important. For an accurate measure-

Populations	N -	BamHI/coxII (mt)			<i>Eco</i> RI/ <i>atp</i> A (cp)	
		11.7 (kb)	9.1	7.7	6.6	4.9
Nakatonbetsu	5	1.00	0.00	0.00	0.40	0.60
Kotanbetsu	14	1.00	0.00	0.00	0.79	0.21
Asahi	11	1.00	0.00	0.00	0.45	0.55
Nemuro	7	1.00	0.00	0.00	0.57	0.43
Akkeshi	14	1.00	0.00	0.00	0.64	0.36
Rikubetsu	17	1.00	0.00	0.00	0.53	0.47
Saroma	20	0.85	0.15	0.00	0.65	0.35
Shiranuka	17	0.94	0.06	0.00	0.53	0.47
Asyoro	20	0.95	0.00	0.05	0.40	0.60
Shintoku	18	0.94	0.00	0.06	0.67	0.33
Kanayama	10	1.00	0.00	0.00	0.50	0.50
Furenai	9	1.00	0.00	0.00	0.78	0.22
Urakawa	6	1.00	0.00	0.00	0.33	0.67
Tomakomai	8	1.00	0.00	0.00	0.50	0.50
Oyubari	13	0.92	0.08	0.00	0.54	0.46
Jyozankei	6	0.83	0.17	0.00	0.50	0.50
Iwanai	15	0.93	0.07	0.00	0.53	0.47
Kucchan	20	0.15	0.85	0.00	0.55	0.45
Gamushi	34	0.21	0.79	0.00	0.59	0.41
Total	264	0.80	0.19	0.01	0.56	0.44

Table 1. Frequencies of restriction fragment phenotypes of organelle DNAs from 19 populations of Saghalin fir.

ment of the subdivision of the diversity at a single locus, sampling many populations rather than many individuals per population is necessary (PONS & PETIT 1995). Secondly, differences in location of the genes studied on the mitochondrial genome might be a significant factor. The rapid structural transformation and slow rate of base sequence evolution of the mitochondrial genome have already been discussed (SEDE-ROFF 1987; PALMER 1992). Thirdly, there may be significant differences inherent to the gene studied. Plant coxII genes have an interesting feature: sugar beet (Senda et al. 1991), petunia (PRUITT & HANSON 1989), carrot (TURANO et al. 1987), and most monocotolydenous species analyzed so far (including Maize, Fox & LEAVER 1981; wheat, BONEN et al. 1984; and rice, KAO et al. 1984) have introns in their coxII genes. In contrast, Oenothera, pea and soybean (studied by HIESEL & BRENNICKE 1983; MOON et al. 1985; and GRABAU et al. 1988, respectively) do not contain introns. The probe used in this study is located at one of the two exons that occur adjacent to the intron. Although it is not clear that Saghalin fir has an intron or not in the coxII gene, it is possible that the large insertion in the intron may cause at least some of the intraspecific variation in RFLP variants.

Both Saghalin fir and *A. veitchii* are included in the same taxonomic section (FARJON 1990; YAMAZAKI 1995). TSUMURA & SUYAMA (1998) also indicated that

these species have a genetically close relationship, sharing the same major RFLP-based mtDNA haplotype, and rbcL sequence in their cpDNA. Moreover, A. veitchii had little intra-specific variation in mtDNA. In this report, we also detected variation of mtDNA in Saghalin fir by use of RFLP. The mtDNA variation and sequence results might reflect its distribution during the last glacial period, and the relationship between these species. There may have been refugia of this species in northeast Hokkaido during the last glacial age (OKADA 1983). In that case, the western populations might have lost their mtDNA variation during their migration westward after the last glacial period. Further investigation, covering the natural distribution of the species more completely, might elucidate more thoroughly the inter- and intraspecific phylogeography.

# Maternal inheritance of mtDNA and paternal inheritance of cpDNA

Although transmission of mitochondrial DNA in angiosperms is predominantly maternal, ultrastructural studies by electron microscope have shown that different species of gymnosperms have differing modes of transmission. Fertilization mechanisms and male gamete characteristics consistent with paternal transmission of organelles, both chloroplasts and mitochondria, are found in Cupressaceae and Taxodiaceae, but

Markers	$H_S$ a	$H_T$	$G_{ST}$	$\theta$ (s.d.) <sup>b</sup>
<i>cox</i> II (mitochondria)	0.092 (0.096)	0.324 (0.331)	0.715 (0.710)	0.609*** (0.016)
<i>atp</i> A (chloroplast)	0.491 (0.517)	0.493 (0.493)	0.003 (-0.049)	-0.028*** (0.017)

Table 2.	Population	genetic para	meters of RFLI	<b>P</b> markers with	contrasting mo	des of inheritance.
		0				

<sup>a)</sup> PONS & PETIT (1995) estimates were shown in parentheses for  $H_{s}$ ,  $H_{T}$  and  $G_{sT}$ .

<sup>b)</sup>  $\theta$  is Wright's fixation index (WRIGHT 1965; WEIR 1990). Test of significance of  $\theta$  by permuting variants within the total population with 1000 permutations \*\*\* p < 0.001.

maternal mitochondrial inheritance and paternal chloroplast inheritance in progeny have been demonstrated in Pinaceae, Cephalotaxaceae, Podocarpaceae, and Taxaceae (CHESNOY & THOMAS 1971). RFLP analysis has supported the results of these ultrastructural studies (NEALE et al. 1986; SZMIDT et al. 1987; NEALE & SEDEROFF 1989; STINE et al. 1989; NEALE et al. 1991; SUTTON et al. 1991; WAGNER et al. 1991; DEVERNO et al. 1993; ZIEGENHAGEN et al. 1995; KONDO et al. 1998). In the study presented here, the RFLP technique was used to monitor inheritance of organelle DNAs in reciprocal crosses of Saghalin fir. The maternal inheritance of the coxII sequence (Fig. 2a) suggests that mtDNA is maternally inherited in Saghalin fir, as well as in other Pinaceae species. In contrast to the maternal inheritance of mtDNA, the mode of inheritance of chloroplast DNA seems to be paternal (Fig. 2b). ENNOS (1994) defined the method in which estimate of relative rate of pollen and migration among population was caluculated using  $F_{ST}$ . The derived value caluculated from frequencies of variant 11.7 kb mitochondria variant and 6.6 kb chloroplast variant was 11.96. This value was supports that pollen migration rate are greater than seed migration rate in Saghalin fir. Consequently, the different rate of gene flow through seed and pollen dispersal reflected in the differentiation of each genome (Table 2). Average heterozygosity ( $F_{IT} = 0.105$ ), corresponding to genetic diversity, has been estimated by isozyme analysis of this fir (NAGASAKA et al. 1997). The difference in genetic diversities between mtDNA and genomic DNA might be due to the frequent recombination and rearrangements characteristic of the mitochondrial genome (SEDEROFF 1987; PALMER 1992) and the higher rate of migration of nuclear genome.

In this inheritance study, however, progenies of two crosses were analyzed. The number of offsprings examined of each cross was 14. Because only a limited number of crossed and offsprings were analyzed, paternal leakage of mtDNA and maternal leakage of cpDNA can not be excluded. Paternal mtDNA leakage in *Pinus* have reported (WAGNER *et al.* 1991). A simple binomial model of organelle inheritance to determine the power of the experiment to distinguish between the hypothesis of strict maternal inheritance and the hypothesis involving the presence of both organelle types within a progeny has been presented (MILLIGAN 1992). Even if no progeny containing paternally derived organelles are found in 14 offsprings as this study, it is impossible to assure that the degree of paternal transmission is not more than 28% (P < 0.01). The possibility of biparental transmission cause recombination among different organelle genotypes, and may affect the patterns of diversity observed in organelle DNAs (CHAPMAN *et al.* 1982; BIRKY *et al.* 1989; BIRKY 1991). Therefore, inferences concerning the genetic parameters presented in this paper must be approached with care.

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