RESTRICTION SITE POLYMORPHISM IN CHLOROPLAST DNA OF SILVER FIR (*ABIES ALBA* MILL.)

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ABSTRACT

Restriction analyses were performed in two PCR-amplified cpDNA regions of silver fir (*Abies alba* Mill.) from a broad range of European provenances. A total of 42 restriction sites could be detected using 10 restriction endonucleases. A single restriction site polymorphism appeared when a cpDNA region between the *trnS* and *psbC* genes was restricted by *Apa* I and *Hae* III endonucleases. Two distinct variants were detected and confirmed to be paternally inherited. Both variants are present in almost all of the investigated provenances. Preliminary studies of frequency distributions reveal a possible geographical cline: One variant predominantly occurs in the west and south-west, the other one in the east and south-east of the natural range of silver fir.

Key-words: *Abies alba* Mill., cpDNA, PCR, RFLP, intraspecific variation, paternal inheritance

INTRODUCTION

In general, chloroplast DNA (cpDNA) is known to be slow in sequence evolution (PALMER 1987; CLEGG 1991). Hence, in forest trees cpDNA variation is commonly investigated among species, including closely related ones. Results are interpreted in terms of phylogeny and introgression (SZMIDT 1991; STRAUSS *et al.* 1992; WAGNER 1992). In the genus *Abies,* KORMUŤÁK *et al.* (1993) carried out RFLP analyses based on Southern hybridisations. Using a chloroplast *psbA* probe, they detected polymorphism among twelve *Abies* species.

Recently much attention has been drawn to intraspecific variation of chloroplast DNA. CpDNA polymorphisms have been reported for various conifer species (see references in WAGNER 1992; HONG *et al.* 1993; DONG & WAGNER 1994; TSUMURA *et al.* 1994).

The detection of intraspecific cpDNA polymorphisms is expected to provide a powerful methodological basis for ecological genetic investigations in forest trees. Inherited in a predominantly maternal mode in angiosperms and in a predominantly paternal mode in gymnosperms, cpDNA polymorphisms should allow the tracing of either maternal or paternal gene flow among populations. Thus they are helpful for many topics in the evolution of a species, including its glacial and postglacial history. Recent investigations on cpDNA variation in European oak species revealed the benefit of such markers (KREMER & PETIT 1993).

Abies alba is a broad-ranged European forest tree species that is supposed to be autochthonous in most stands (see figure 1). Based on isozyme gene markers, KONNERT & BERGMANN (1995) examined the biogeography of the species. Our investigations aimed at studying the biogeography of silver fir on the cpDNA level. Needles and seeds were investigated from *Abies alba* individuals sampled from a broad range of provenances. Two cpDNA regions of a total of 3,130 bp in length were amplified using universal pairs of primers and subsequently subjected to restriction analysis. Detection of polymorphism was followed by inheritance analysis.

MATERIAL

Screening of cpDNA for detection of polymorphism was performed on the following material basis (for origin of material, see figure 1).

1) Pre-screening: A set of 10 restriction endonucleases was tested on only 5 but geographically broad-ranged individuals.
2) Main screening: If polymorphism was detected, the material basis was enlarged with respect to both number of provenances and number of individuals per provenance.
3) Inheritance analysis: Controlled intra- and interspecific crosses were available for inheritance analysis.

ad 1) Pre-screening was done with needles of test individuals from 5 provenances: Poland (P) and Ore

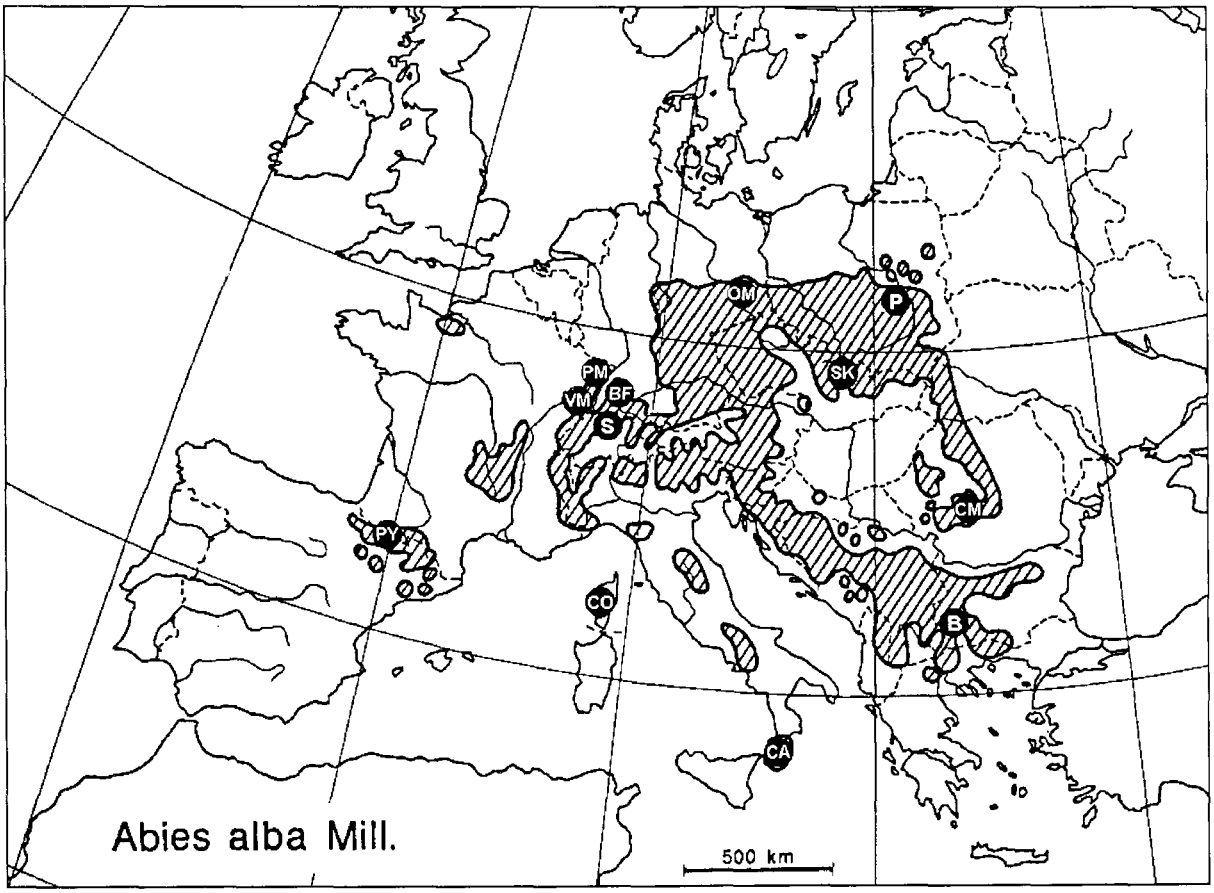

Figure 1 Natural range of silver fir (*Abies alba* Mill.) according to MEUSEL (1965) and origin of samples. *P (Poland), OM (Ore Mountains) S (Switzerland), CO (Corsica), CA (Calabria)*, B (Bulgaria), SK (Slovakia), CM (Carpathian Mountains), PM (Palatinate Mountains), BF (Black Forest), VM (Vosges Mountains), PY (Pyrenees). Provenances in italics were involved in the pre-screening.

Mountains (OM) from the east, Switzerland (S) as a central region, and two southern European regions, Corsica (CO) and Calabria (CA). All but the Ore Mountains individuals were sampled from a provenance trial of the institute.

ad 2) Main screening comprised the above 5 and 7 additional provenances, with 10 individuals each, from Bulgaria (B), Slovakia (SK), Romanian Carpathian Mountains (CM), Palatinate Mountains (PM), Black Forest (BF), Vosges Mountains (VM) and southern Pyrenees (PY). Needle material was collected in natural stands, in most cases from more than one population. If only one population was involved, sampled individuals were separated by a minimum distance of 50–100 m. From the southern Pyrenees a mixed seed sample of 100 seeds from numerous populations was also available.

ad 3) For inheritance analysis, needle material was available from the offspring of six controlled intraspecific crosses and four controlled interspecific crosses with *A. alba* as mother tree: *A. alba* x *A. cephalonica, A. nordmanniana, A. numidica* and *A. pinsapo.*

METHODS

DNA-Extraction

Genomic DNA was extracted according to ZIEGENHAGEN *et al.* (1993). DNA concentration was measured by means of a fluorometer (Hoefer Scientific Instruments, San Francisco). Two needles per individual were extracted and yielded an average of 1µg DNA. For seeds, the mini-preparation yielded 3µg per embryo.

Amplification

Primers

Two pairs of universal primers (A and B) were chosen for amplifying two cpDNA regions of silver fir. Primer pair A was designed by DEMESURE *et al.* (1995). This pair represents sequences from flanking regions of the *trnS* gene [tRNA – Ser (UGA)] and adjacent *psbC* gene

(ps II 44kd). It amplifies the region in between and provides a DNA fragment referred to here as fragment A.Primer A_1: 5′ GGT TCG AAT CCC TCT CTC TC 3′; primer A_2: 5′ GGT CGT GAC CAA GAA ACC AC 3′.

Primer pair B was designed by TABERLET *et al.* (1991). This primer pair represents sequences from flanking regions of the *trnT* gene (UGU) and *trnF* gene (GAA). It amplifies the region in between and provides a DNA fragment referred to here as fragment B. Primer B_1: 5′ CAT TAC AAA TGC GAT GCT CT 3′; primer B_2: 5′ ATT TGA ACT GGT GAC ACG AG 3′. Primers were synthesised by Pharmacia Biotech Europe GmbH, Freiburg.

PCR Conditions

PCR was run in a pre-heated DNA Thermal Cycler (Perkin-Elmer & Co GmbH, Überlingen) with the following cycles and temperatures (DEMESURE *et al.* 1995): 94 °C for 4 min, followed by 35 cycles of 93 °C for 1 min, 57 °C for 1 min and 72 °C for 2 min. Last strand elongation (72 °C) was allowed an additional 10 min. Amplification was performed in 25 µl volume of total reaction mixture. Reaction mixture was composed according to DEMESURE *et al.* (1995) as follows: 20 ng template DNA, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH8, 2mM $MgCl_2$, 0.001% W_1 (Gibco BRL, Life Technologies GmbH, Eggenstein), 10mM mercaptoethanol, 4.4 µg·ml^{-1} bovine serum albumin, 100 µM of each four dNTP, 1 unit of Taq polymerase (Gibco BRL, Life Technologies GmbH, Eggenstein) and 0.54 µM of each primer of the relevant pair. Reaction mixtures were kept on ice and covered with 25 µl of mineral oil.

Restriction analyses

Digestion

PCR products (16 µl) were digested by *Alu* I, *Bam* H I, *Cfo* I, *Hae* III, *Hinf* I, *Nde* II, *Pvu* II, *Rsa* I, *Sma* I and *Taq* I. Each digestion was done in 20 µl of total volume, including 2 µl (20–22 units) of each endonuclease and 2 µl of each relevant endonuclease buffer. Digestion was performed overnight at the temperatures recommended by the enzyme manufacturer Boehringer, Mannheim. After digestion, all 20 µl of digestion volume were loaded onto the gel.

Visualisation of DNA fragments

Undigested PCR products as well as restriction fragments were separated by electrophoresis in 1.5% (w/v) agarose gel, run in tris-borate buffer at 13 V·cm^{-1} for 3 hours.

DNA fragments were visualised by UV fluorescence after staining with ethidium bromide (0.25 µg·ml^{-1} staining solution). The banding patterns were scanned from Polaroid negatives (type 55). Scanning was performed by a personal densitometer (Molecular Dynamics GmbH, Krefeld) and fragment lengths were assessed by FragmeNT Analysis, version 1.1 (Molecular Dynamics GmbH, Krefeld).

Reproducibility

Reproducibility of amplification was tested for each primer pair by 10 PCR routines for each of the prescreened individuals. Both fragments A and B were identically reproduced in all individuals.

Reproducibility of restriction patterns was tested by two to six repeated restrictions per individual for all restriction enzymes used. This test resulted in absolutely reproducible patterns.

In addition, 5 individuals were tested for possible intra-individual variation with regard to the one discovered cpDNA polymorphism. For this, separate analysis of DNA extracted from needles taken from different positions within the same tree was performed. *Hae* III restriction patterns were absolutely stable within the individuals, suggesting that there is no intra-individual variation at this polymorphic restriction site.

Statistics

Frequencies of cpDNA variants were compared between provenances and tested for homogeneity by means of a 2-tailed Fisher's Exact Test (SACHS 1984).

RESULTS

PCR products

Both pairs of universal primers amplified DNA from *A. alba*. The PCR product of primer pair A is a DNA fragment A of ca. 1,605 bp. As far as could be judged from the agarose gels, fragment lengths did not differ between individuals. PCR with primer pair B resulted in amplification of a DNA fragment B with a length of ca. 1,525 bp. For fragment B also, no difference in fragment length between individuals could be observed.

Restriction analyses of PCR products A and B

1. Pre-screening of test individuals from 5 provenances

Table 1 summarises the results of pre-screening. As far as could be judged from the agarose gel, restriction analyses of the two cpDNA regions generated a total of

Table 1 Number of restriction fragments detected by 10 restriction endonucleases in PCR products fragment A and fragment B of test individuals from 5 provenances

	Poland **P**		Ore Mountains **OM**		Switzerland **S**		Corsica **CO**		Calabria **CA**	
Fragment	A	B	A	B	A	B	A	B	A	B
Restriction endonuclease										
Alu I	7	4	7	4	7	4	7	4	7	4
Bam H I	2	0	2	0	2	0	2	0	2	0
Cfo I	0	2	0	2	0	2	0	2	0	2
***Hae* III**	**3**	**0**	**4**	**0**	**4**	**0**	**4**	**0**	**3**	**0**
Hinf I	6	5	6	5	6	5	6	5	6	5
Nde II	5	6	5	6	5	6	5	6	5	6
Pvu II	2	0	2	0	2	0	2	0	2	0
Rsa I	3	0	3	0	3	0	3	0	3	0
Sma I	0	0	0	0	0	0	0	0	0	0
Taq I	4	5	4	5	4	5	4	5	4	5

Table 2 Number of individuals showing each cytotype after *Hae* III restriction of fragment A in 12 provenances with 10 individuals each

	Poland **P**	Ore Mts. **OM**	Switzerland **S**	Corsica **CO**	Calabria **CA**	Bulgaria **B**
Cytotype I	4	6	5	4	4	10
Cytotype II	6	4	5	6	6	0
	Slovakia **SK**	Carpathians **CM**	Palat. Mts. **PM**	Black Forest **BF**	Vosges Mts. **VM**	Pyrenees **PY**
Cytotype I	8	7	1	5	0	0
Cytotype II	2	3	9	5	10	10

55 restriction fragments, 33 for fragment A and 22 for fragment B. These data correspond to 42 restriction sites within a total of 3,130 bp (sum of the lengths of fragments A and B). One single restriction site polymorphism could be detected. This occurs in fragment A after restriction by *Hae* III (see table 1). Whereas fragment A of the test individuals from Poland and Calabria is characterised by three *Hae* III restriction fragments, there are four restriction fragments in the test individuals from the Ore Mountains, Switzerland and Corsica.

This result was the starting point for further investigations to first characterise the restriction site polymorphism and then to analyse the variation on the intra- and inter-provenance level.

2. Screening of 10 individuals from each of 12 provenances

Hae III restriction analyses on fragment A within the enlarged sample size did not reveal any additional restriction site variant. The two variants, visualised in figure 2, are referred to here as cytotype I with three *Hae* III restriction fragments (two fragments of 740 bp, one of 110 bp) and as cytotype II with four *Hae* III restriction fragments (740 bp, 555 bp, 230 bp, 110 bp). Table 2 presents the results of *Hae* III restriction analyses, indicating the number of individuals carrying either cytotype I or II.

Table 2 demonstrates that the detected two cpDNA cytotypes are common and wide-spread in silver fir.

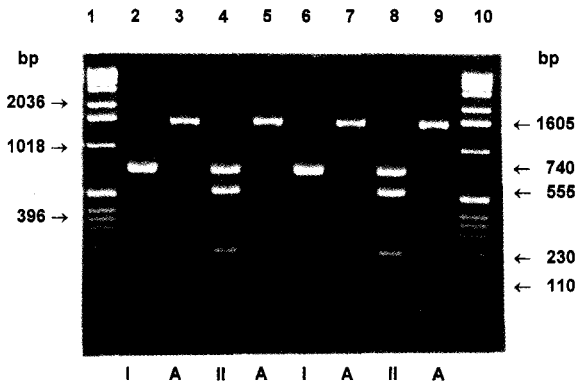

Figure 2 Amplification products A and the two *Hae* III restriction fragment patterns (Cytotype I and II). Lanes 3, 5, 7, 9: Unrestricted amplification products, fragment A (1,605 bp); lanes 2 and 6: Cytotype I (three restriction fragments: two of 740 bp, one of 110 bp), represented by two individuals from Bulgaria; lanes 4 and 8: Cytotype II (four restriction fragments: 740 bp, 555 bp, 230 bp, 110 bp), represented by two individuals from the Pyrenees; lanes 1 and 10: molecular size standard, 1 KB Ladder (Gibco BRL, Inc.). Lengths of fragment A and of *Hae* III restriction fragments in the right margin.

Within a sample of only ten individuals it was possible to detect the presence of both cytotypes in 9 of the 12 investigated provenances. These nine range from eastern over middle to southern European localities. There are only three provenances, Bulgaria in the very south-east, Vosges Mountains in the west and the Pyrenees in the uppermost south-west of natural range where on the basis of this sample size only one cytotype occurs (see shaded columns in table 2).

Hae III restriction site mapping

Figure 2 demonstrates the two different *Hae* III restriction fragment patterns (cytotypes I and II) and the results of fragment length analysis. Lengths are means of data from 15 different gels where each cytotype was repeatedly represented.

Hae III cytotype I is characterised by three restriction fragments. Two of them are of identical length (740 bp). The third small fragment of about 110 bp occurs at the bottom of the gels and often looks a little bit smeary. The existence of two fragments of about 740 bp was experimentally proved. A new primer was designed from the completely sequenced cpDNA of *Pinus thunbergii* [DDBJ Database, accession no. D17510 (WAKASUGI *et al.* 1994)]. The sequence of the primer is 5′ AGC ACT AAA GCT TGG ACA GG 3′. It is located at about 300 bp from the 3′ end of the cpDNA sequence that is homologous to primer A_1 (see above). Together with primer A_2 (see above), the new primer amplifies a fragment that is about 300 bp smaller than the original fragment A. The smaller fragment was expected to retain the decisive *Hae* III restriction site. Figure 2a shows the undigested fragment as well as the *Hae* III restriction patterns representing *Hae* III cytotype I. The figure clearly reveals the above postulated existence of three restriction fragments for cytotype I. *Hae* III cytotype II is characterised by four restriction fragments, 740 bp, 555 bp, 230 bp and 110 bp. It shares two fragments, 740 bp and 110 bp with cytotype I.

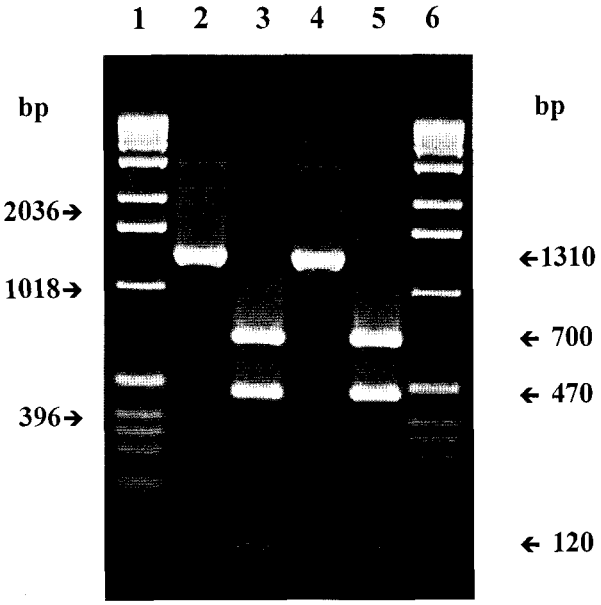

Figure 2a *Hae* III cytotype I after restriction of the "shortened" cpDNA fragment A. Lanes 2 and 4: Unrestricted "shortened" fragment (1,310 bp); lanes 3 and 5: Cytotype I (three restriction fragments: 700 bp, 470 bp, 120 bp), represented by two individuals from Bulgaria; lanes 1 and 6: molecular size standard, 1 KB Ladder, Gibco BRL, Inc.). Lengths of the unrestricted fragments and of the restriction fragments in the right margin.

The mutation which caused the occurrence of either cytotype I or II thus appears to be based on a loss or gain of a *Hae* III restriction site within a section of fragment A which is about 740 bp in length. The sum of the 555 bp and 230 bp fragment in cytotype II exceeds the length of the respective fragment of 740 bp cytotype I. Thus insertion/deletion as a type of mutation will have to be discussed.

Hae III (GG!CC) can be regarded as the 4 bp isoschizomeric restriction endonuclease to the 6 bp endonuclease *Apa* I (GGG!CCC). Comparison of restriction analyses by these two enzymes was a matter of interest. Thus, a set of 50 individuals among which both *Hae* III to *Apa* I restriction. For all 50 individuals there is analogous occurrence of the two different cytotypes I and II for both restriction endonucleases. *Hae* III and *Apa* I

conifer cpDNA, different degrees of intraspecific variation have been discussed. Detected variation ranges from only a few variants in a species even up to intra-individual variation (WAGNER *et al.* 1987; GOVINDARAJU & WAGNER 1988; GOVARINDAJU *et al.* 1989; ALI *et al.* 1991; HONG *et al.* 1993; DONG & WAGNER 1994; TSUMURA *et al.* 1994). In our case the two detected variants seem to be based on a "highly conserved" mutation. This is concluded from the nearly overall occurrence of both cytotypes in the natural range of silver fir. The persistence of both types in a broad range of localities might be explained by long-range pollen transport. The two exceptions, populations of Bulgaria with only cytotype I and Vosges Mountains with only cytotype II, require further investigations with enlarged sample sizes. From our preliminary results there is no hint on when and where the mutation occurred. Also, the role of the mutation in the silver fir biogeography is not known. However, there is evidence for a geographical differentiation suggesting that cytotype I is predominant in the east and south-east and cytotype II predominant in the west and south-west of the natural range. At least in the Pyrenees, as the uppermost south-west locality of silver fir, an overwhelming occurrence of cytotype II could be verified by adequate sample sizes. This might indicate a situation of reproductive isolation, thus confirming the results of KONNERT & BERGMANN (1995). On the basis of isozyme investigations they postulated that the Pyrenees formed a glacial refugium of *A. alba* and even that the Pyrenean populations were isolated and did not contribute to post-glacial recolonisation of the species. With regard to cpDNA, enlarged sample sizes (populations and individuals) will be needed for frequency studies of our variants. This might strengthen the basis for discussion of a geographical cline as was reported for an *Abies mariesii* cpDNA polymorphism in Japan (TSUMURA *et al.* 1994). Also, this will contribute to other results on geographical patterns of physiological and genetic variation in the species. LARSEN & MEKIĆ (1991) found a distinct geographical differentiation for the variation of gas exchange and corresponding morphological traits, the behaviour of the provenances from Calabria totally different from that of central and eastern Europe provenances. Similar results were obtained for parameters of genetic multiplicity and diversity in isozyme studies of BERGMANN *et al.* (1990).

The suitability of cpDNA polymorphism for geographical differentiation has been reported for the European angiosperms *Quercus robur* and *Qu. petraea* (KREMER *et al.* 1991; FERRIS *et al.* 1993). FERRIS *et al.* (1993) identified a strong geographical polarisation of two distinct chloroplast types as a western and an eastern type.

We are interested in widening our investigations to other species of the genus *Abies*. The four species involved in our interspecific crosses showed the same *Hae* III restriction patterns as described for cytotypes I and II in *A. alba*. Most striking were the findings in another genus of the family *Pinaceae*. We looked at *Hae* III and *Apa* I restriction sites within the sequence between the *trnS* and *psbC* genes of *P. thunbergii*. Very similar to cytotype II of *Abies*, we identified *Hae* III restriction fragments (**719 bp, 522 bp, 195 bp, 81 bp**, 30 bp, 8 bp) and analogous restriction fragments of *Apa* I cytotype II. Two restriction sites are shared by *Hae* III and *Apa* I, both of which are situated in the *psbC* gene of *P. thunbergii*. This encourages us to look more closely at the *psbC* gene of conifers with regard to phylogenetic and introgressive aspects.

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REFERENCES

ALI, I. F., NEALE, D. B., & MARSHALL, K .A. 1991: Chloroplast DNA restriction fragment length polymorphism in *Sequoia sempervirens* D. Don Endl., *Pseudotsuga menziesii* (Mirb.) Franco, *Calocedrus decurrens* (Torr.), and *Pinus taeda* L. *Theor. Appl. Genet.* **81**:83–89.

BERGMANN, F., GREGORIUS, H.–R. & LARSEN, J .B. 1990: Levels of genetic variation in European silver fir (*Abies alba*) – Are they related to the species' decline? *Genetics* **82**:1–10.

BOSCHERINI, G., MORGANTE, M., ROSSI, P. & VENDRAMIN, G. G. 1994: Allozyme and chloroplast DNA variation in Italian and Greek populations of *Pinus leucodermis*. *Heredity* **74**:284–290.

CLEGG, M. T., LEARN, G. H. & GOLENBERG, E. M. 1991: Molecular evolution of chloroplast DNA, pp. 135–149. In: Evolution at the Molecular Level (Selander, R.K. *et al.* eds). Sinauer Assoc., Sunderland, Mass.

DEMESURE, B., SODZI, N. & PETIT, R.J . 1995: A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. *Mol. Ecol.* **4**: 129–131.

DONG, J. & WAGNER, D. B. 1994: Paternally inherited chloroplast polymorphism in *Pinus*: estimation of diversity and population subdivision, and tests of disequilibrium with a maternally inherited mitochondrial polymorphism. *Genetics* **136**:1187–1194.

FERRIS, C., OLIVER, R. P., DAVY, A. J. & HEWITT, G. M. 1993: Native oak chloroplasts reveal an ancient divide across Europe. *Mol. Biol.* **2**:337–344.

GOVINDARAJU, D. R. & WAGNER, D. B. 1988: Chloroplast DNA variation within individual trees of *Pinus banksiana – Pinus contorta* sympatric region. *Can. J. For. Res.* **18**:1347–1350.

GOVINDARAJU, D. R., DANCIK, B. P . & WAGNER, D. B. 1989: Novel chloroplast DNA polymorphism in a sympatric region of two pines. *J. Evol. Biology* **2**:49–59.

HONG, Y.-P., HIPKINS, V. D. & STRAUSS, S. H. 1993: Chloroplast DNA diversity among trees, populations and species in the California Closed-Cone Pines (*Pinus radiata, Pinus muricata* and *Pinus attenuata*). *Genetics* **135**: 1187–1196.

KONNERT, M. & BERGMANN, F. 1995: The geographical distribution of silver fir (*Abies alba, Pinaceae*) in relation to its migration history. *Pl. Syst. Evol.* **196**: 19–30.

KORMUŤÁK, A., SZMIDT, A. E. & WANG, X.-R. 1993: Restriction fragment length polymorphism of chloroplast DNAs in some species of fir (*Abies* sp.). *Biologia Plantarum* **35**:113–119.

KREMER, A., PETIT, R., ZANETTO, A., FOUGÈRE, V., DUCOUSSO, A., WAGNER, D. & CHAUVIN, C. 1991: Nuclear and organelle gene diversity in *Quercus robur* and *Quercus petraea. In:* Genetic Variation of Forest Tree Populations in Europe (Ziehe, M. & Müller-Starck, G. eds). Sauerländer's-Verlag, Frankfurt am Main, 141–166.

KREMER, A. & PETIT, R.J. 1993: Gene diversity in natural populations of oak species. *Ann. Sci. For.* **50** (Suppl. 1):186–202.

LARSEN, J.B. & MEKIĆ, F. 1991: The geographic variation in European silver fir (*Abies alba* Mill.). *Silvae Genetica* **40**: 188–198.

NEALE, D. B., WHEELER, N. C. & ALLARD, R. W. 1986: Paternal inheritance of chloroplast DNA in Douglas-fir. *Can. J. For. Res.* **16**:1152–1154.

NEALE, D. B., MARSHALL, K. A. & SEDEROFF, R. R. 1989: Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. *Proc. Natl. Acad. Sci. USA* **77**:212–216.

NEALE, D. B., MARSHALL, K. A. & HARRY, D .E. 1991: Inheritance of chloroplast and mitochondrial DNA in incense cedar (*Calocedrus decurrens*). *Can. J. For. Res.* **21**:217–220.

NEALE, D. B. & SEDEROFF, R. R. 1989: Paternal inheritance of chloroplast DNA and maternal inheritance of mitochondrial DNA in loblolly pine. *Theor. Appl. Genet.* **77**: 212–216.

PALMER, J. D. 1987: Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. *Am. Nat.* **130**:6–29.

SACHS, L. 1984: Angewandte Statistik: Anwendung statistischer Methoden. Springer Verlag, Berlin, Heidelberg, New York, 6th edition, 846 p.

SZMIDT, A. E. 1991: Phylogenetic and applied studies on chloroplast genome in forest conifers. *In*: Biochemical markers in the population genetics of forest trees. (S. Fineschi *et al.* eds). SPB Academic Publishing bv, The Hague, The Netherlands, 185–196.

SZMIDT, A. E., ALDÉN, T. & HÄLLGREN, J.-E. 1987: Paternal inheritance of chloroplast DNA in *Larix. Plant Mol. Biol.* **9**:59–64.

STINE, M., SEARS, B. B. & KEATHLEY, D. E. 1989: Inheritance of plastids in interspecific hybrids of blue spruce and white spruce. *Theor. Appl. Genet.* **76**:841–845.

STRAUSS, S. H., BOUSQUET, J., HIPKINS, V. D. & HONG, Y.-P. 1992: Biochemical and molecular genetic markers in biosystematic studies of forest trees. *New Forests* **6**:125–158.

TABERLET, P., GIELLY, L., PAUTOU, G. & BOUVET, J. 1991: Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Mol. Biol.* **17**:1105–1109.

TSUMURA, Y., TAGUCHI, H., SUYAMA, Y. & OHBA, K. 1994: Geographical cline of chloroplast DNA variation in *Abies mariesii. Theor. Appl. Genet.* **89**:922–926.

VICARIO, F., VENDRAMIN, G. G., ROSSI, P., LÌO, P. & GIANNINI, R. 1995: Allozyme, chloroplast DNA and RAPD markers for determining genetic relationships between *Abies alba* and the relic population of *Abies nebrodensis. Theor. Appl. Genet.* **90**(7–8):1012–1018.

WAGNER, D. B. 1992: Nuclear, chloroplast, and mitochondrial DNA polymorphisms as biochemical markers in population genetic analyses. *New Forests* **6**:373–390.

WAGNER, D. B., FURNIER, G. R., SAGHAI-MAROOF, M. A., WILLIAMS, S. M., DANCIK, B. P. & ALLARD, R. W. 1987: Chloroplast DNA polymorphisms in lodgepole pines and jack pines and their hybrids. *Proc. Natl. Acad. Sci. USA* **84**:2097–2100.

WAGNER, D. B., GOVINDARAJU, D. R., YEATMAN, C. W. & PITEL, J.A. 1989: Paternal chloroplast DNA inheritance in a diallel cross of jack pine (*Pinus banksiana* Lamb.). *J. Hered.* **80**:483–485.

WAKASUGI, T., TSUDZUKI, J., ITO, S., SHIBATA, M. & SUGIURA, M. 1994: A physical map and clone bank of the black pine (*Pinus thunbergii*) chloroplast genome. *Plant Mol. Biol. Reptr.* **12**:227–241.

ZIEGENHAGEN, B., GUILLEMAUT, P. & SCHOLZ, F. 1993: A procedure for mini-preparations of genomic DNA from needles of silver fir (*Abies alba* Mill.). *Plant Mol. Biol. Reptr.* **11**:117–121.