Theor Appl Genet (2003) 106:1353–1367
DOI 10.1007/s00122-002-1174-z

J. P. Jaramillo-Correa · J. Bousquet · J. Beaulieu ·
N. Isabel · M. Perron · M. Bouillé

Cross-species amplification of mitochondrial DNA sequence-tagged-site markers in conifers: the nature of polymorphism and variation within and among species in Picea

Received: 9 April 2002 / Accepted: 20 August 2002 / Published online: 13 February 2003
© Springer-Verlag 2003

Abstract Primers previously developed to amplify specific non-coding regions of the mitochondrial genome in Angiosperms, and new primers for additional non-coding mtDNA regions, were tested for their ability to direct DNA amplification in 12 conifer taxa and to detect sequence-tagged-site (STS) polymorphisms within and among eight species in *Picea*. Out of 12 primer pairs, nine were successful at amplifying mtDNA in most of the taxa surveyed. In conifers, indels and substitutions were observed for several loci, allowing them to distinguish between families, genera and, in some cases, between species within genera. In *Picea*, interspecific polymorphism was detected for four loci, while intraspecific variation was observed for three of the mtDNA regions studied. One of these (SSU *rRNA* V1 region) exhibited indel polymorphisms, and the two others (*nad1* intron b/c and *nad5* intron1) revealed restriction differences after digestion with *Sau*3AI (PCR-RFLP). A fourth locus, the *nad4*L-*orf25* intergenic region, showed a multibanding pattern for most of the spruce species, suggesting a possible gene duplication. Maternal inheritance, expected for mtDNA in conifers, was observed for all polymorphic markers except the intergenic region *nad4*L-*orf25*. Pooling of the variation observed with the remaining three markers resulted in two to six different mtDNA haplotypes within the different species of *Picea*. Evidence for intra-genomic recombination was observed in at least two taxa. Thus, these mitotypes are likely to be more informative than single-locus haplotypes. They should be particularly useful for the study of biogeography and the dynamics of hybrid zones.

Keywords Conifer genes · Mitochondrial haplotypes · Mitochondrial molecular markers · Plant mitochondrial genome · *Picea*

Communicated by D.B. Neale

J. P. Jaramillo-Correa · J. Bousquet (✉) · J. Beaulieu · N. Isabel · M. Bouillé
Chaire de recherche du Canada en génomique forestière et environnementale and Centre de recherche en biologie forestière,
Pavillon Charles-Eugène-Marchand,
Université Laval, Sainte-Foy Québec, Canada, G1K 7P4
e-mail: bousquet@rsvs.ulaval.ca
Fax: +1-418-656-7493

J. Beaulieu · N. Isabel
Natural Resources Canada,
Canadian Forest Service – Laurentian Forestry Centre,
1055 du P.E.P.S., P.O. Box 3800, Sainte-Foy Québec,
Canada, G1V 4C7

M. Perron
Direction de la recherche forestière,
Ministère des ressources naturelles du Québec,
2700, rue Einstein, Sainte-Foy, Québec, Canada, G1P 3W8

Introduction

Mitochondrial DNA (mtDNA) has proved to be a useful source of haploid DNA markers for the study of population genetics and biogeography. They have been used to decipher population structure, migration routes, mating systems and reproductive behavior (e.g. Lunt et al. 1998; Palmer et al. 2000). This is particularly true for animal taxa where mtDNA is maternally inherited with a relatively rapid rate of sequence evolution. However, the use of mtDNA markers for population genetics studies in plants, especially in angiosperms, has progressed at a slow pace, as compared to chloroplast DNA (cpDNA) markers. The development of mtDNA markers in plants has been hampered by the complex structure and changes in the gene order and organization of the mitochondrial genome, and also because of the frequent exchange of DNA material between the mitochondria and the nucleus (Palmer et al. 2000). These features of the plant mitochondrial genome reduce the opportunities for wide cross-species transferability of primers designed to amplify more variable non-coding regions. With plant mtDNA, the likelihood of detecting intraspecific polymorphisms in coding and non-coding regions also appears to be reduced, especially because of the typically slow rate of sequence evolution of plant mtDNA as compared to nuclear and cpDNA (Laroche et al. 1997).

Table 1 Target regions, annealing temperatures and expected size of PCR products for primer pairs used to amplify mtDNA regions for the conifer multi-genus panel

Genomic region	Annealing temperature (°C)	PCR product size (bp)	Primer source
coxI (intron 1)	58	500–800	Lu et al. 1998
matR (intron 1)	58	300–500	Present study[c]
mp6[a]	62	~2000	Present study[c]
nad1 (intron b/c)	58	~3000	Demesure et al. 1995
nad3-rps12 (i.r.)[b]	58	320–350	Soranzo et al. 1999
nad3 (intron 1)	58	120	Soranzo et al. 1999
*nad4*L-*orf25* (i.r.)[b]	53	300–500	Kubo et al. 2000
nad5 (intron 1)	62	600–900	Present study[c]
nad7 (intron 3)	50	~1000	Bonen et al. 1994
rps14-cob (i.r)[b]	60	~2000	Demesure et al. 1995
SSU *rRNA* (V1 region)	64	350–800	Duff and Nickrent 1999
SSU *rRNA* (V7 region)	58	300–500	Duff and Nickrent 1999

[a] Mitochondrial plasmid-like DNA repeat region from *P. abies*
[b] i.r.: Intergenic region
[c] Primers developed herein (see Materials and methods):
*matR*a Forward: 5'-CGACAGAAGCACGAAATTCC-3'
*matR*b Reverse: 5'-ACCCGACGATAACTAGCTTC-3'
*mp6*a Forward: 5'-CGCTTCACTCTAACCCTTCC-3'
*mp6*b Reverse: 5'-CCTTCACTCTTACAAACGCC-3'
*nad5*a Forward: 5'-AGTCCAATAGGGACAGCAC-3'
*nad5*b Reverse: 5'-ACCCGACGATAACTAGCTTC-3'

In angiosperms, both cpDNA and mtDNA are maternally inherited and dispersed through seeds. Any geographic structure detected through variation in these genomes will reflect the contribution of seed movement to the total gene flow for a given species (Campbell 1991). Hence, cpDNA markers have been used extensively in angiosperms for the fine-scale study of population structure (McCauley 1997; Petit et al. 1997; Schaal et al. 1998). However, in most conifers, cpDNA is paternally inherited (Birky 1995). Thus the patterns of gene flow deciphered by cpDNA markers reflect the contribution of pollen. Accordingly, maternally inherited mtDNA remains the sole source of markers to track the effects of seed dispersion on population structure in conifers.

During the past few years, the most common approach to find mtDNA polymorphisms in conifers has been the use of specific mitochondrial probes on previously digested DNA, allowing for the detection of restriction fragment length polymorphisms (RFLPs) (e.g. Strauss et al. 1993; Tsumura and Suyama 1998; Sinclair et al. 1999). The development of primers specific to certain regions (e.g. Demesure et al. 1995) has allowed the study of different regions of the plant mitochondrial genome more simply by polymerase chain reaction (PCR). However, most of the regions studied to date have failed to show enough intraspecific variation for useful population genetics studies (Soranzo et al. 1999). Perhaps the most remarkable exception to this trend is the *nad1* intron b/c, where two polymorphic tandem repeats have been reported in *Picea abies* (Sperisen et al. 2001). The polymorphism in this intron has been widely used in population studies of other conifers such as in *Pinus* spp. (Senjo et al. 1999; Mitton et al. 2000; Soranzo et al. 2000; Latta and Mitton 2001) and *Abies* spp. (Isoda et al. 2000). However, it is rather likely that one polymorphism is insufficient to represent the variation of a genome, which is reputed to be around 1,000 kb long in conifers (Kumar et al. 1995). Indeed, the presence of several multi-locus haplotypes or mitotypes due to recombination of mtDNA has been shown for several conifer species, especially in the genus *Pinus* (Strauss et al. 1993; Wu et al. 1998).

Using a hierarchical, simple and accessible approach, the aim of this study was to test a group of primers specific to diverse regions of the plant mitochondrial genome to assess cross-species amplification in conifers, and to survey polymorphisms among eight different species in the genus *Picea*. Intraspecific polymorphism was further checked for different spruce species and maternal inheritance was verified. As well, the nature of the observed polymorphisms has been documented by DNA sequencing.

Materials and methods

Cross-species amplification

Primer pairs previously shown to amplify specific regions of the mitochondrial genome in different angiosperm taxa (Bonen et al. 1994; Demesure et al. 1995; Lu et al. 1998; Duff and Nickrent 1999; Soranzo et al. 1999; Kubo et al. 2000; See Table 1) were screened in a conifer multi-genus panel consisting of two trees for each of the following taxa: *Picea mariana* (Pinaceae), *Tsuga canadensis* (Pinaceae), *Abies balsamea* (Pinaceae), *Pinus strobus* (Pinaceae), *Pinus banksiana* (Pinaceae), *Larix laricina* (Pinaceae), *Pseudotsuga menziesii* (Pinaceae), *Thuja occidentalis* (Cupressaceae), *Taxus canadensis* (Taxaceae), *Araucaria heterophylla* (Araucariaceae), *Decussocarpus rospigliosii* (Podocarpaceae) and *Podocarpus oleifolius* (Podocarpaceae). Three more primer pairs were developed based on published sequences of the genes *R-maturase* from *Pinus* sp. (Qiu et al. 1999), *nad5* from *Picea smithiana* (Wang et al. 2000) and the mitochondrial plasmid-like DNA repeat region (*mp6*) from *Picea abies* (Chandelier et al. 1999). These primer pairs were tested using the same species panel as above.

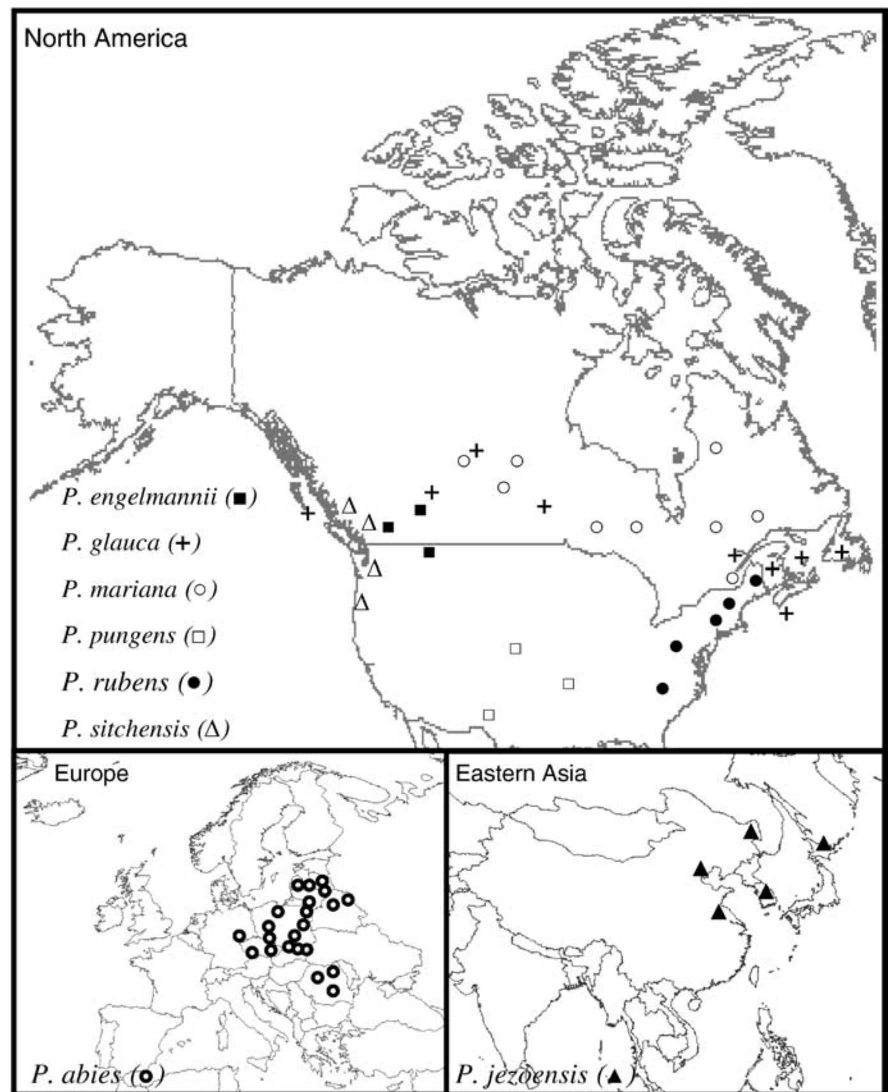

Fig. 1 Geographical locations of the origins of trees included in the various screening panels for eight species in the genus *Picea*

DNA was extracted from leaves or needles using a DNeasy Plant Mini Kit (Qiagen) and amplified in a DNA thermal cycler (PTC-225, MJ Research) using 0.1 μM of each primer, 0.1 mM of each dNTP, 1 × reaction buffer, 1.5 mM of $MgCl_2$, and 0.125 units of Platinum *Taq* DNA polymerase (Invitrogen). PCR conditions were as follows: denaturation during 2 min at 94°C, 35 cycles consisting of 30 s at 94°C, 30 s at the appropriate annealing temperature (see Table 1) and 1 min at 72°C, and a final elongation at 72°C for 10 min. Products were examined by gel electrophoresis (2% agarose in TAE), and those exhibiting one single DNA fragment for both trees of each species were tentatively considered as positives and conserved for further analysis.

Detection of polymorphism among and within species in Picea

Primer pairs retained at the previous step were further screened on range-wide panels of individuals for eight different species in the genus *Picea* (*P. abies*, *P. mariana*, *P. rubens*, *P. glauca*, *P. engelmannii*, *P. sitchensis*, *P. pungens* and *P. jezoensis*) consisting from three to 22 trees distributed across the species' ranges (Fig. 1). Products were first examined by gel electrophoresis (2% agarose in TAE), and those exhibiting one single DNA fragment were conserved for further analysis. In order to detect small indel polymorphism, they were electrophoresed through 8% non-denaturing polyacrylamide gels (in TBE). PCR products, apparently monomorphic, were further digested with different restriction enzymes (*Cfo*I, *Hae*III, *Rsa*I, *Sau*3AI and *Taq*I) and separated in polyacrylamide gels under the same conditions as above to detect possible restriction fragment length polymorphisms (PCR-RFLP markers). Undigested PCR products of markers that failed to show polymorphism with both previous approaches were denaturated for 5 min at 94°C and electrophoresed in 6% polyacrylamide gels at 10 mA and 10° C for 18 h to detect possible single-strand conformation polymorphisms (PCR-SSCP markers) following procedures reported elsewhere (Fournier et al. 2002).

The mitochondrial nature of the polymorphisms observed was tested with black spruce F_1 progeny trees from independent controlled crosses. These crosses consisted of 12 receptor trees from various populations, each one being pollinated with a mix of pollen from the other 11 parent trees (polymix). As mtDNA is maternally inherited in conifers, it was expected that all progeny trees should exhibit the maternal haploid fragment.

To determine the structural nature of the polymorphisms at the DNA sequence level, the various alleles detected for the new polymorphic loci were sequenced on an automated DNA sequencer (ABI 377). Direct sequencing of the two DNA strands was conducted with the dideoxynucleotide chain-termination procedure using the appropriate amplification primers and a Sequenase GC-

Table 2 Amplification trials for 12 specific mtDNA primer pairs on a panel of 12 conifer taxa. Each primer pair was scored in two individuals per taxon for success (+), failure (–) or the presence of multiple DNA fragments (m.f.) from DNA amplification. Presence (P) or absence (NP) of polymorphism among taxa is indicated in the last column

Genomic region	Pinaceae							Cupressaceae	Taxaceae	Araucariaceae	Podocarpaceae		Poly-morphism
	Picea mariana	*Tsuga canadensis*	*Abies balsamea*	*Pinus strobus*	*Pinus banksiana*	*Larix laricina*	*Pseudotsuga menziesii*	*Thuja occidentalis*	*Taxus canadensis*	*Araucaria heterophylla*	*Decussocarpus rospigliosii*	*Podocarpus oleifolius*	
coxI (intron 1)[a]	+	+	+	+	+	+	+	+	+	+	+	+	NP
matR (intron 1)[a, b]	+	+	+	+	+	+	+	–	+	–	–	–	P
mp6[b]	m.f.	–	–	m.f.	m.f.	–	–	–	–	–	–	–	–
nad1 (intron b/c)[a]	+	+	+	+	+	+	+	+	+	+	+	+	P
nad3-rps12 (i.r.)[a, c]	+	+	+	+	+	+	+	+	+	+	+	+	NP
nad3 (intron 1)[a]	+	+	+	+	+	+	+	+	+	+	+	+	NP
nad4L-orf25 (i.r.)[a, c]	+	–	+	+	+	–	+	+	+	–	+	+	P
nad5 (intron 1)[a, b]	+	+	+	+	+	+	+	+	–	+	+	+	P
nad7 (intron 3)[a]	+	+	+	+	+	+	+	+	+	+	+	+	NP
rps14-cob (i.r)[c]	m.f.	–	–	m.f.	m.f.	–	+	–	–	m.f.	–	–	–
SSU rRNA (V1 region)[a]	+	+	+	+	+	+	+	+	+	+	+	+	P
SSU rRNA (V7 region)	m.f.	m.f.	+	+	+	+	+	–	+	–	–	–	P

[a] Primer pairs retained from the screening of panels from eight species in the genus *Picea*
[b] Newly designed primers also resulting in positive amplifications for Angiosperms (see Results)
[c] i.r.: Intergenic region

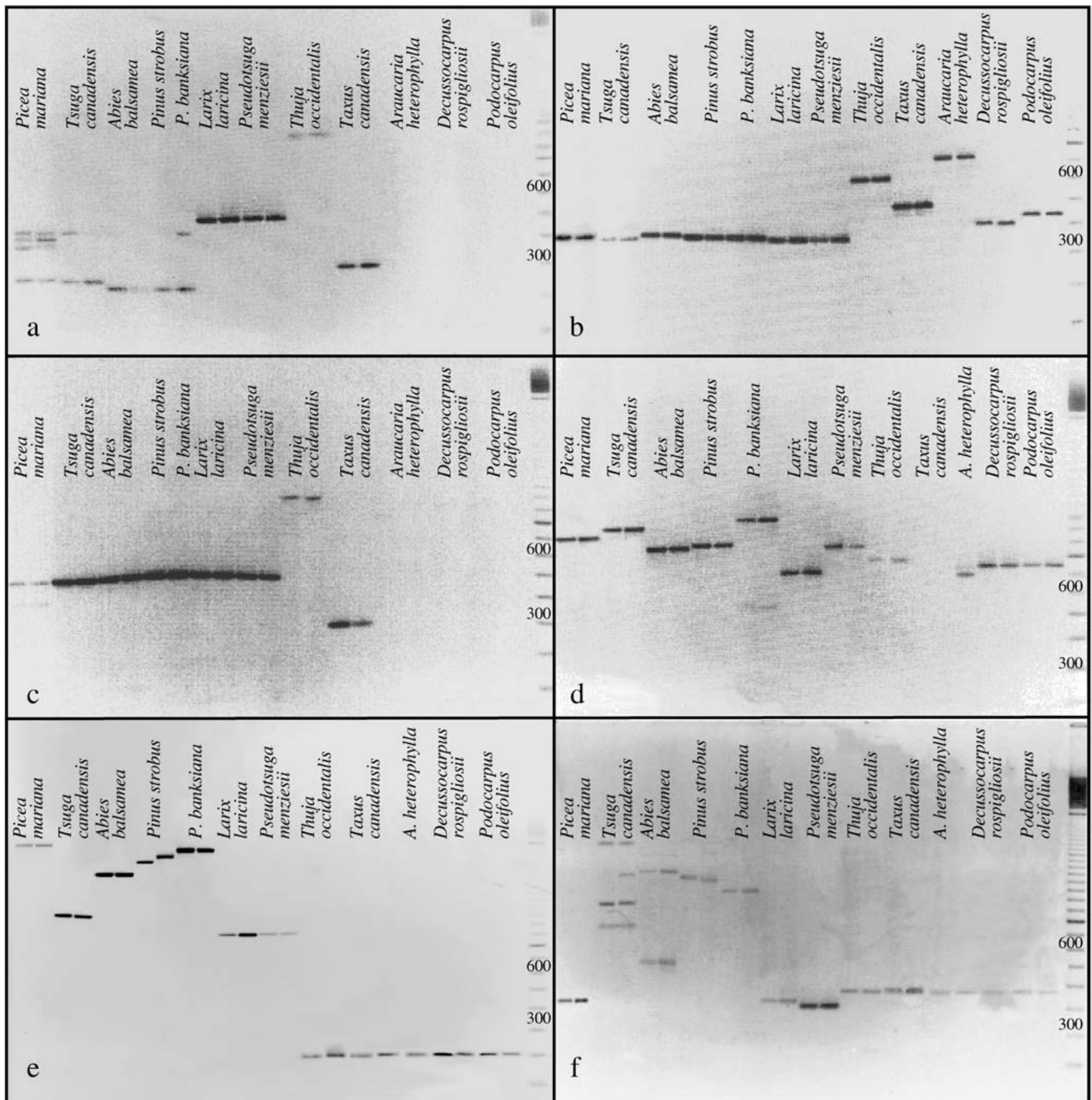

Fig. 2a–f Results of amplification trials for a panel of 12 conifer taxa for **a** SSU *rRNA* V7 region, **b** SSU *rRNA* V1 region, **c** *matR* intron 1, **d** *nad5* intron 1, **e** *nad1* intron b/c and **f** the *nad4*L-*orf25* intergenic region. Negative images of ethidium bromide-stained agarose gels are shown. The 100-bp ladder (Pharmacia) is located at the right

rich kit (Applied Biosystems). The identity of the sequences was verified by comparing them with the non-redundant nucleotide sequence database at the National Center of Biotechnology Information using BLASTN (Altschul et al. 1990).

Results

Amplification trials in conifers

The 12 primer pairs were first tested on a conifer multi-genus panel and those primer pairs not resulting in the amplification of a unique DNA fragment were discarded. Three primer pairs failed such a test and resulted in no amplification product or in the amplification of multiple

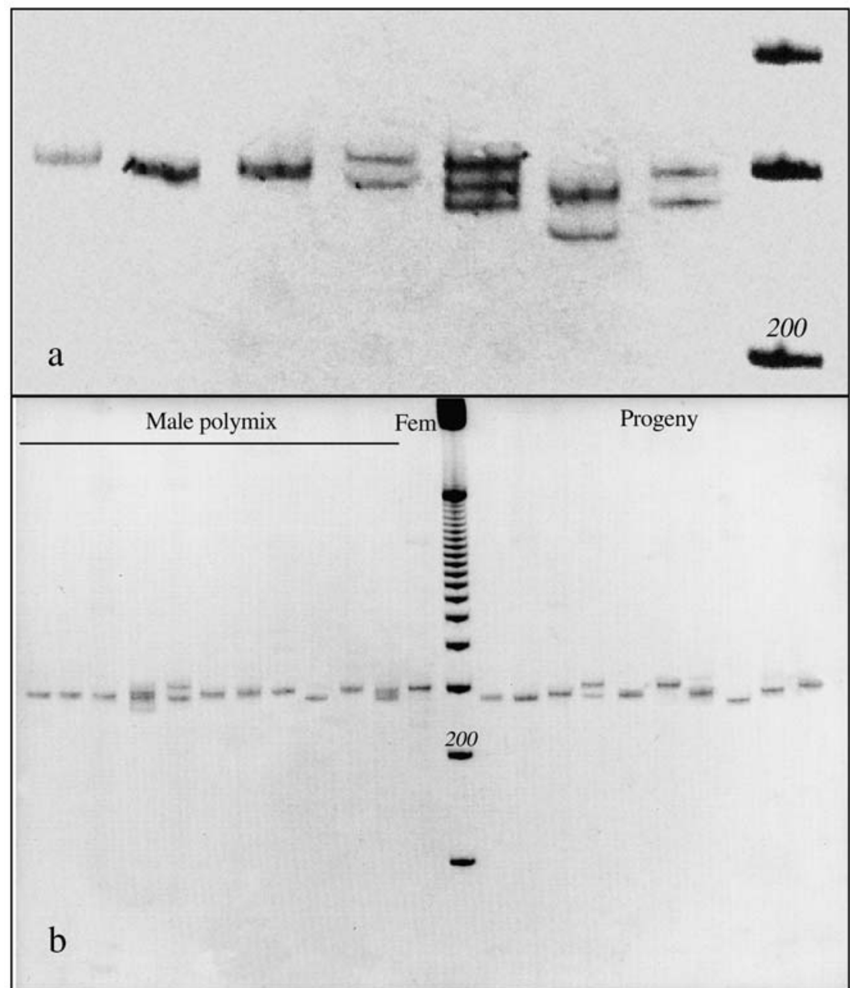

Fig. 3 **a** Polymorphisms observed in the *nad4*L-*orf25* intergenic region in *P. mariana*. **b** Inheritance pattern of the *nad4*L-*orf25* intergenic region from a controlled polycross in *P. mariana* (see Materials and methods). Negative images of ethidium bromide-stained polyacrylamide gels are shown. The 100-bp ladder (Pharmacia) is located at the right (**a**) and in the middle (**b**)

fragments, those for the *mp6* repeat region, the *rps14-cob* intergenic region and the V7 region of the SSU *rRNA* gene (Table 2). It should be noted that for the three new pairs of primers designed in this study (*matR* intron 1, *mp6* and *nad5* intron 1, see Table 1), DNA amplification was successful for a monocot (*Zea mays*) and a dicot (*Betula nigra*), and a single DNA fragment was obtained for the first intron of *matR* and for the first intron of *nad5*, while *mp6* primers produced multiple DNA fragments (data not shown). Thus, two of the three newly designed primer pairs appear gene-specific and universal. They should be useful for most seed plants.

The absence of amplification of the mitochondrial plasmid-like DNA repeat region (*mp6*) from *P. abies*, in almost all the conifer taxa tested, suggests that this repeat region might be exclusive to a subset of *P. abies*. Indeed, attempts to amplify this mitochondrial region in the *P. abies* panel produced a single DNA fragment only in individuals from Poland and no fragment in the others (data not shown). This marker was excluded from the analysis with the various panels of *Picea*.

The intergenic region located between the genes *rps14* and *cob* could not be amplified, or resulted in multiple fragments for all species except *P. menziesii* where a single fragment of approximately 1,000 bp was observed. Varying PCR conditions ($MgCl_2$, template DNA and dNTP concentrations, and annealing time and temperature) did not affect the results observed when more amplification attempts were made. Thus, this marker was also excluded from the analysis with the various panels of *Picea*.

DNA amplification of the V7 region of the SSU *rRNA* gene revealed multiple fragments in *P. mariana* and no fragment in *T. occidentalis*, *A. heterophylla* and the two taxa of the Podocarpaceae (Fig. 2a). As it was not possible to obtain a single fragment in *P. mariana*, even after modifying the annealing temperature, this marker was also excluded from the analysis with the various panels of *Picea*.

Among the remaining nine primer pairs, four did not reveal any fragment length polymorphism on agarose gel. For the remaining five primer pairs, fragment length polymorphism was detected among conifers. DNA sequencing revealed that the observed polymorphisms were due to indels ranging from 20 to 600 bp, such as those observed in the introns of the genes *matR*, *nad1* and *nad5*, in the V1 region of SSU *rRNA* and in the *nad4*L-*orf25* intergenic region (Genbank accessions AY159838 to

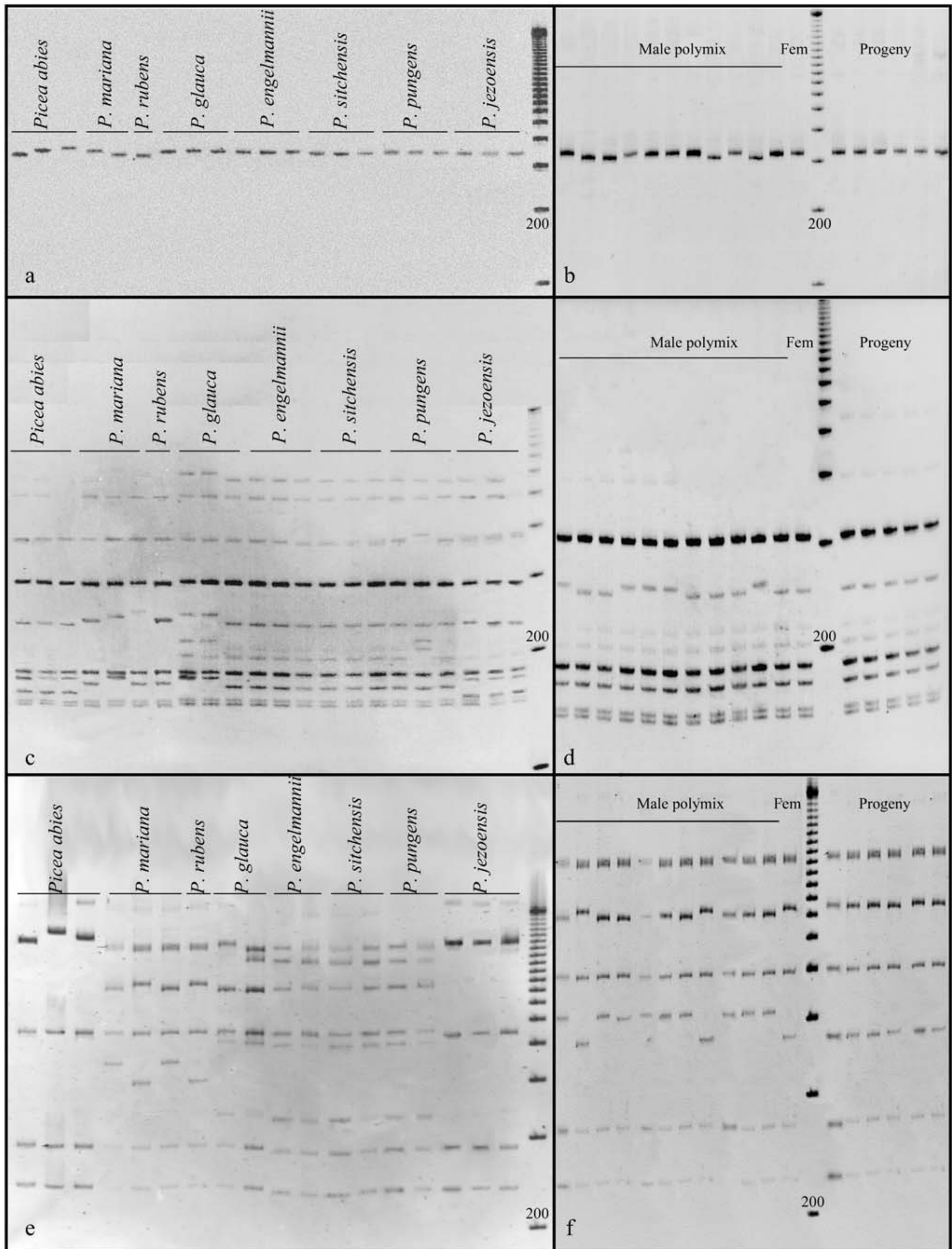

Fig. 4 a, c, e Polymorphic markers observed among trees for eight species in the genus *Picea*, and **b, d, f** patterns of inheritance from controlled polycrosses in *P. mariana* (see Materials and methods). **a, b** SSU *rRNA* V1 region. **c, d** *nad5* intron 1. **e, f** *nad1* intron b/c. Negative images of ethidium bromide-stained polyacrylamide gels are shown. The 100-bp ladder (Pharmacia) is located at the right (**a, c, e**) and in the middle (**b, d, f**)

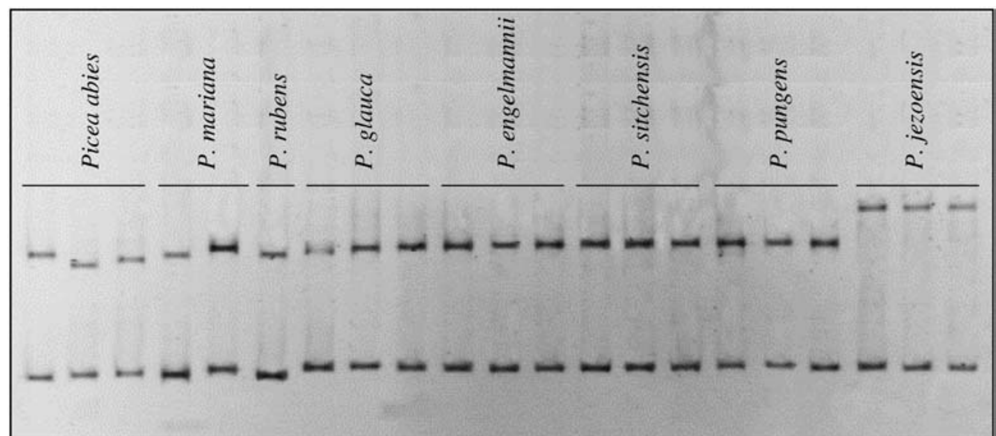

Fig. 5 Single-strand conformation polymorphisms (SSCP) observed among trees for eight species in the genus *Picea* in the SSU *rRNA* V1 region. Negative images of ethidium bromide-stained polyacrylamide gels are shown

AY159878). In general, most of these primers were useful to amplify all the conifers tested, revealing indel polymorphisms between families, genera and, in some cases, between and within congeneric species.

The indels observed in the V1 region of the SSU *rRNA* gene distinguished all taxonomic families surveyed. Two indels were observed within some families for this region, one occurring between *A. balsamea* and the rest of the Pinaceae, and the second one between the two genera of Podocarpaceae (Fig. 2b). For the first intron of *matR*, no polymorphisms were observed within the Pinaceae. However, it was possible to distinguish the Pinaceae from the Cupressaceae and the Taxaceae due to the presence of multiple indels in this region (Fig. 2c). No amplification was observed for the Araucariaceae and the Podocarpaceae (Fig. 2c). A large number of indels was observed in the first intron of *nad5* and in the intron b/c of *nad1*. For the first intron of *nad5*, the high level of polymorphism made it possible to distinguish all taxa tested except the two genera of Podocarpaceae (Fig. 2d). For the intron b/c of *nad1*, indels ranging between 200 and more than 2,000 bp allowed distinguishing between all taxa except *T. occidentalis, Taxus canadensis, A. heterophylla, D. rospigliosii* and *P. oleifolius* where a single 190-bp fragment was observed (Fig. 2e). Some indels were also observed in the *nad4*L-*orf25* intergenic region, allowing us to distinguish between most of the taxa (Fig. 2f). This region was difficult to amplify in some taxa and specific adjustments in PCR conditions (annealing temperature and time) were necessary. The annealing temperature was set to 50°C for *T. occidentalis, Taxus canadensis, A. heterophylla, D. rospigliosii* and *P. oleifolius*, while the concentration of $MgCl_2$ was reduced to 0.5 mM for *Tsuga canadensis, A. balsamea, P. strobus, P. banksiana* and *Taxus canadensis*.

Polymorphism among and within species in *Picea*

Nine of the 12 primer pairs used in the conifer multi-genus panel were retained for the screening of eight species in *Picea*, based on the criterion that each exhibited a single DNA fragment on agarose gel (Table 3). However, one of these markers (the *nad4*L-*orf25* intergenic region) was further discarded after exhibiting multiple fragments on polyacrylamide gel (Fig. 3a) and departing from the expected maternal inheritance (Fig. 3b).

On polyacrylamide gel, only one of the eight remaining primer pairs (the V1 region of the SSU *rRNA* gene) revealed indel polymorphisms within *P. abies* and within *P. mariana* (Fig. 4a). Some of the alleles observed at this locus had the same length between various spruce species (see Table 3), but they could be unambiguously distinguished by SSCP analysis (Fig. 5). Differences among PCR products of *P. abies, P. mariana, P. glauca* and *P. jezoensis* were further confirmed by sequencing, revealing indels located at different sites in each species (Fig. 6a, other sequences in Genbank, accessions AY196179 to AY196195). The variant B_3 was observed in *P. glauca, P. engelmannii, P. sitchensis*, and *P. pungens*, and the variant B_2 was shared between *P. mariana* and *P. rubens* (Table 3).

Two more mtDNA regions (*nad1* intron b/c and *nad5* intron 1) revealed restriction fragment length polymorphisms within and among spruce species on polyacrylamide gels when digested with the enzyme *Sau*3AI (Fig. 4c and e). Polymorphisms at these two loci clearly differentiated *P. abies* and *P. jezoensis* from all other taxa. Within-species polymorphisms were observed for the intron 1 of *nad5* in *P. mariana, P. glauca* and *P. pungens* (Fig. 4c). For intron b/c of *nad1*, more than one variant was observed in *P. abies, P. mariana, P. glauca* and *P. pungens* (Fig. 4e). Allele homology among species was likely for each of these two regions because some digestion patterns were shared (Fig. 4c and e). These homologies were later confirmed at the sequence level. *P. mariana* and *P. rubens*, as well as *P. engelmannii, P. sitchensis* and *P. pungens* shared alleles for each of these two introns (Table 3). All detected variants were caused by a few indels and substitutions that in some cases suppressed or added *Sau*3AI restriction sites (Fig. 6b). In the first intron of *nad5*, one of the indels was caused by the insertion of a 5-bp repeated motif. A small poly-T

Table 3 Alleles detected at nine mtDNA loci analysed with three different methods in range-wide panels of eight species in *Picea*, and the number of mtDNA haplotypes observed after pooling the genotypes at three acceptable polymorphic loci (*nad1* intron b/c, *nad5* intron 1 and SSU *rRNA* V1 region)

Genomic region	Method[b]	*P. abies* *n*=22	*P. mariana* *n*=15	*P. rubens* *n*=5	*P. glauca* *n*=20	*P. engelmannii* *n*=3	*P. sitchensis* *n*=4	*P. pungens* *n*=3	*P. jezoensis* *n*=5
	Indel	A	A	A	A	A	A	A	A
coxI (intron 1)	PCR-RFLP	A	A	A	A	A	A	A	A
	SSCP	A	A	A	A	A	A	A	A
	Indel	A	A	A	A	A	A	A	A
matR (intron 1)	PCR-RFLP	A	A	A	A	A	A	A	A
	SSCP	A	A	A	A	A	A	A	A
	Indel	A	A	A	A	A	A	A	A
nad1 (intron b/c)	PCR-RFLP	B[d], C, D	E, F[d], G	E	H[d], I, J	J	J	J	K
	SSCP	–	–	–	–	–	–	–	–
	Indel	A	A	A	A	A	A	A	A
nad3-rps12 (i.r.)[a]	PCR-RFLP	A	A	A	A	A	A	A	A
	SSCP	A	A	A	A	A	A	A	A
	Indel	A	A	A	A	A	A	A	A
nad3 (intron 1)	PCR-RFLP	A	A	A	A	A	A	A	A
	SSCP	A	A	A	A	A	A	A	A
	Indel	m.f.[e]	m.f.	m.f.	m.f.	m.f.	m.f.	m.f.	m.f.
*nad4*L-*orf25* (i.r.)[a]	PCR-RFLP	–	–	–	–	–	–	–	–
	SSCP	–	–	–	–	–	–	–	–
	Indel	A	A	A	A	A	A	A	A
nad5 (intron 1)	PCR-RFLP	I	B, C[d], D	B	E[d], F	F	F	F[d], G	H
	SSCP	–	–	–	–	–	–	–	–
	Indel	A	A	A	A	A	A	A	A
nad7 (intron 3)	PCR-RFLP	A	A	A	A	A	A	A	A
	SSCP	A	A	A	A	A	A	A	A
	Indel	A, B[d], C	A, B[d]	A[d], B	B	B	B	B	B
SSU *rRNA* (V1 region)	PCR-RFLP	–	–	–	–	–	–	–	–
	SSCP[c]	A_1, B_1[d], C	A_2, B_2[d]	A_2[d], B_2	B_3	B_3	B_3	B_3	B_4
Total number of mtDNA haplotypes		6[f]	5[f]	2	3	1	1	2	1

[a] i.r.: Intergenic region
[b] Indel: Insertion-deletion polymorphism, RFLP: restriction fragment length polymorphism, SSCP: single strand conformation polymorphism
[c] Numbers in subscript indicate different alleles only detectable by SSCP
[d] Most common allele
[e] m.f.: multiple DNA fragments observed when analysed by polyacryamide gel
[f] including recombinant mtDNA haplotypes

Fig. 6a, b Alignment of partial DNA sequences of **a** SSU *rRNA* V1 region and **b** *nad5* intron 1 from *Picea* sp. The corresponding alleles are indicated in parentheses next to the species names (see Table 3). Indels are shadowed in *gray*, substitutions are *underlined*, and polymorphic *Sau*3AI restriction sites responsible for the various alleles at *nad5* intron 1 are shown in *bold*

a.

Species	Allele	55 – 104
P. abies	(A_1)	GGGGGCAG.A AGG.CAAGAA .GGAATGGCC CCCTCCTA.. ....GC
P. mariana	(A_2)	GGGGGCAG.A AGGGCAAGA. ..GAATGGCC CCCTCCTA.. ....GC
P. abies	(B_1)	GGGGGCAGCA AGGGCAAGTA GGGAATGGCC CCCTCCTA.. ....GC
P. mariana	(B_2)	GGGGGCAG.A AGGGCAAGA. ..GAATGGCC CCCTCCTA.. ....GC
P. glauca	(B_3)	GGGGGCAG.A AGGGCAAGAA GGGAATGGCC CCCTCCTA.. ....GC
P. engelmannii	(B_3)	GGGGGCAG.A AGGGCAAGAA GGGAATGGCC CCCTCCTA.. ....GC
P. sitchensis	(B_3)	GGGGGCAG.A AGGGCAAGAA GGGAATGGCC CCCTCCTA.. ....GC
P. pungens	(B_3)	GGGGGCAG.A AGGGCAAGAA GGGAATGGCC CCCTCCTA.. ....GC
P. jezoensis	(B_4)	GGGGGCAG.A AGGGCAAGA. GGGAATGGCC CCCTCCTATC CTATCCTAGC
P. abies	(C)	GGGGGCAG.A AGGGCAAGA. .GGAATGGCC CCCTCCTA.. ....GC

Species	Allele	– 154
P. abies	(A_1)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGC.AAATGA
P. mariana	(A_2)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGCCAAATGA
P. abies	(B_1)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGC.......
P. mariana	(B_2)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCG... .....AATGA
P. glauca	(B_3)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGCC......
P. engelmannii	(B_3)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGCC......
P. sitchensis	(B_3)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGCC......
P. pungens	(B_3)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGCC......
P. jezoensis	(B_4)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCG... ....
P. abies	(C)	GCGTGCTCAC TCACTCAAAT GATATGCAAG TGGTGCGCCG GGC.......

Species	Allele	– 204
P. abies	(A_1)	TA.......G GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. mariana	(A_2)	TA.......G GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. abies	(B_1)	.......GTG GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. mariana	(B_2)	TATGCGCGTG GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. glauca	(B_3)	TA.....GTG GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. engelmannii	(B_3)	TA.......G GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. sitchensis	(B_3)	TA.......G GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT
P. pungens	(B_3)	TA.......G GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACACGT
P. jezoensis	(B_4)	.......GTG GGGCACAGCA AGCAAAGAGG CGAACGGGTG CGTAACGCGT
P. abies	(C)	.......GTG GGGCACAGCA AGCAAAGTGG CGAACGGGTG CGTAACGCGT

Species	Allele	– 254
P. abies	(A_1)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. mariana	(A_2)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. abies	(B_1)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. mariana	(B_2)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. glauca	(B_3)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. engelmannii	(B_3)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. sitchensis	(B_3)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. pungens	(B_3)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. jezoensis	(B_4)	GG.AATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA
P. abies	(C)	GGGAATCTGC CGAACAGTTC ACTCCAAATC CTGAATAATT CATAAAGCAA

Species	Allele	– 304
P. abies	(A_1)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. mariana	(A_2)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. abies	(B_1)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT ATTATCACAG GTAGTTGGTT
P. mariana	(B_2)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. glauca	(B_3)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. engelmannii	(B_3)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. sitchensis	(B_3)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. pungens	(B_3)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT
P. jezoensis	(B_4)	GCTAATTCGC GCTGTTCGAT GAGCCCGCGT AGTATGT..G GTAGTTGGTT
P. abies	(C)	GCTAATTTGC GCTGTTTGAT GAGCCCGCGT AGTATCA..G GTAGTTGGTT

Fig. 6 (continued)

b.

```
                                231                                                 280
P. abies          (I)   ATGCCG.TAC ACTT.ACTTG .......... ....AGTGGC AAAGGAAAGC
P. mariana        (B)   ATGCCG.TAC ACTT.ACTTG .....CTTGA CTTGAGTGGC AAAGGAAAGC
P. mariana        (C)   ATGCCG.TAC ACTT.ACTTG .....CTTGA CTTGAGTGGC AAAGGAAAGC
P. glauca         (E)   ATGCCG.TAC ACTT.ACTTG ACTTGCTTGA CTTGAGTGGC AAAGGAAAGC
P. sitchensis     (F)   ATGCCG.TAC ACTT.ACTTG .........A CTTGAGTGGC AAAGGAAAGC
P. pungens        (G)   ATGCCGGTAC ACTT.ACTTG .....CTTGA CTTGAGTGGC AAAGGAAAGC
P. jezoensis      (H)   ATGCCG.TAC ACTTTACTTG .....CTTGA CTTGAGTGGC AAAGGAAAGC
                                                                                    330
P. abies          (I)   GATAACGAAT GGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
P. mariana        (B)   GATAACGAAT GGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
P. mariana        (C)   GATAACGAAT GGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
P. glauca         (E)   GATAACGAAT GGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
P. sitchensis     (F)   GATAACGAAT GGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
P. pungens        (G)   GATAACGAAT GGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
P. jezoensis      (H)   GATTACGAAT TGCGCCTTTC TTTACTGATT CACTTTGGTA GGTTATAACA
                                                                                    380
P. abies          (I)   CGCGCGAGGG GAAGGTCCTG AAAG.TAAAC CCAGTAAGGT CCTGTCCTGA
P. mariana        (B)   CGCGCGAGGG GAAGGTCCTG AAAGGTAAAC CCAGTAAGGT CCTGTCCTGA
P. mariana        (C)   CGCGCGAGGG GAAGGTCCTG AAAGGTAAAC CCAGTAAGGT CCTGTCCTGA
P. glauca         (E)   CGCGCGAGGG GAAGGTCCTG AAAG.TAAAC CCAGTAAGGT CCTGTCCTGA
P. sitchensis     (F)   CGCGCGAGGG GAAGGTCCTG AAAGGTAAAC CCAGTAAGGT CCTGTCCTGA
P. pungens        (G)   CGCGCGAGGG GAAGGTCCTG AAAGGTAAAC CCAGTAAGGT CCTGTCCTGA
P. jezoensis      (H)   CGCGCGAGGG GAAGGTCCTG AAAGGTAAAC CCAGTAAGGT GCTGTCCTGA
                                                                                    430
P. abies          (I)   TCAAC..... ......CCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTC
P. mariana        (B)   TCAAC..... ......CCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTA
P. mariana        (C)   TCAACTCCTG ATCAACCCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTA
P. glauca         (E)   TCAAC..... ......CCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTA
P. sitchensis     (F)   TCAAC..... ......CCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTC
P. pungens        (G)   TCAAC..... ......CCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTA
P. jezoensis      (H)   TCAAC..... ......CCTT CTAACTCTTT GAGGGGAAGA AATTCTAGTA
                                                                 470   751        760
P. abies          (I)   AAAACCCTAT AGAAGGGGAA GGGGGG.ATC ..TGTTCGGA // TAG.CCCTCG
P. mariana        (B)   AAAACCCTAT AGAAGGGGAA GGGGGG.ATC C.TGTTCGGA // TAGCCCCTGG
P. mariana        (C)   AAAACCCTAT AGAAGGGGAA GGGGGG.ATC C.TGTTCGGA // TAGGCCCTCG
P. glauca         (E)   AAAACCCTAT AGAAGGGGAA GGGGGG.ATC C.TGTTCGGA // TAG.CCCTCG
P. sitchensis     (F)   AAAACCCTAT AGAAGGGGAA GGGGGG.ATC C.TGTTCGGA // TAG.CCCTCG
P. pungens        (G)   AAAACCCTAT AGAAGGGGAA GGGGGG.ATC CTTGTTCGGA // TAG.CCCTCG
P. jezoensis      (H)   AAAACCCTAT AGAAGGGGAA GGGGGGTATC CTTGTTCGGA // TAGCCCCTCG
                                                                                    810
P. abies          (I)   AATTTATGTG TCA.TTCATC CATT.CCTAC TTTTTTT.AC GCCAATGTCG
P. mariana        (B)   ATTTAATGTG TCAATTCAAC CATT.CCTAC TTTTTTT.AC GCCAATGTCG
P. mariana        (C)   GATTTATGTG TCA.TTCATC CATTTCCTAC TTTTTTT.AC CCCAATTTCG
P. glauca         (E)   AATTTATGTG TCA.TTCATC CATT.CCTAC TTTTTTT.AC GCCAATGTCG
P. sitchensis     (F)   AATTTATGTG TCA.TTCATC CATT.CCTAC TTTTTT..AC GCCAATGTCG
P. pungens        (G)   AATTTATGTG TCA.TTCATC CATT.CCTAC TTTTTTTTAC GCCAATGTCG
P. jezoensis      (H)   AATATATGTG TCAATTCATC CATTT.CTAC TTTTTTT.AC GCCAATGTCG
```

pattern located at the end of the intron exhibited polymorphism among species (Fig. 6b).

None of the polymorphisms observed in the introns of the genes *nad5* and *nad1* were detectable with the SSCP method, probably because of the long size of the PCR fragments (3 kb for the intron b/c of *nad1* and 1 kb for the intron 1 of *nad5*). No other polymorphisms were detected in these regions when more restriction enzymes were used (*Cfo*I, *Hae*III, *Rsa*I and *Taq*I). No other conformation polymorphisms were observed in the remaining monomorphic PCR products when analysed by SSCP.

Hence, suitable polymorphisms excluding multiple fragment patterns were detected for three loci; that is, one caused by an indel detectable by polyacrylamide gel electrophoresis for the V1 region of the SSU *rRNA* gene, and two others detectable by PCR-RFLP with polyacrylamide gel electrophoresis for the second intron of *nad1* and the first intron of *nad5*. It should be noted that fragment length polymorphism was previously observed at these three loci among the 12 conifers (see Table 2). In contrast, the four genes that failed to show fragment length polymorphism on agarose gel among conifers (the

first intron of *coxI*, the intergenic region *nad3-rps12*, the first intron of *nad3* and the third intron of *nad7*) (Table 2), showed no fragment length polymorphism among or within spruce species (Table 3).

All three suitable polymorphic markers within *Picea* exhibited the expected maternal inheritance after validation with F_1 progeny trees of *P. mariana* obtained from controlled polycrosses (Fig. 4b, d, e). The combination of genotypes at these three polymorphic loci revealed six different mitotypes within *P. abies*, five within *P. mariana*, three within *P. glauca* and two in each of *P. rubens* and *P. pungens* (Table 3). For two of these species (*P. abies* and *P. mariana*), recombinant mitotypes were observed between the SSU *rRNA* V1 region region on one hand, and the *nad1* intron b/c and the *nad5* intron 1 regions on the other hand. Sequencing of the three mtDNA markers showed that single-locus haplotypes found in different mitotype configurations were homologous, thus confirming true recombination.

Discussion

The plant mitochondrial genome is much larger than its animal counterpart. It is also structurally complex and highly variable, due to numerous rearrangements and changes in gene order, and to the frequent exchange of DNA material between the nucleus and the organelles during its evolutionary history (Palmer et al. 2000). These structural and evolutionary parameters represent formidable constraints for developing universal mitochondrial primers in plants. However, the slow rate of evolution of plant mitochondrial exons at the sequence level (Laroche et al. 1997), and the association of mitochondrial genes in clusters which are often co-transcribed in many taxa (e.g. Perrota et al. 1996; Kubo et al. 2000), should facilitate the development of more or less universal primers for conserved regions bordering more variable non-coding regions such as introns or intergenic spacers. Indeed, new pairs of primers were designed for three mtDNA non-coding regions in this study (see Table 1), which resulted in positive amplifications for both Gymnosperms and Angiosperms.

A large proportion of the primers tested produced the expected PCR product in more than half of the genera analysed, revealing a high amount of polymorphism among conifers. In some cases, these tests also suggested the loss or the duplication of specific regions in some taxa. Once the genomic regions with multiple fragment patterns were excluded, indels were observed in about half of the mtDNA regions surveyed. This trend was expected, considering that most of these regions are non-coding. Most importantly, and as a general rule of thumb, those genomic regions showing suitable fragment length polymorphism among conifers were more likely to show suitable variation among and within taxa in the genus *Picea*, such as for the intron b/c of *nad1*, the first intron of *nad5* and the V1 region of the gene coding for SSU *rRNA*. In contrast, those regions apparently well conserved among conifers, such as the first intron of *coxI*, the intergenic region *nad3-rps12*, the first intron of *nad3* and the third intron of *nad7*, failed to show any fragment length polymorphism among and within taxa in the genus *Picea*. Thus, in spite of the non-coding nature of most of the mtDNA regions sampled in this study, there seems to be region specific evolutionary constraints well conserved across the various levels of taxonomic organization within conifers. This trend confirms a similar pattern previously observed for introns in Angiosperms (Laroche et al. 1997).

The universal primers developed previously to amplify the intergenic region between the mitochondrial genes *rps14* and *cob* have produced PCR products in a wide variety of angiosperm taxa (Demesure et al. 1995). However, in our study, they failed to direct amplification in all the conifers surveyed except *P. menziesii*. These results suggest a mutation at the annealing site for one or both primers, an insertion long enough to limit the PCR success, or even the loss or splicing of the whole gene cluster. In addition, the first intron of the gene *matR* could not be amplified in the two Podocarpaceae taxa analysed herein, *D. rospigliosii* and *P. oleifolius*. This observation suggests the loss of a mtDNA region in the Podocarpaceae, confirming a similar report for *Podocarpus lawrencii* (Qiu et al. 1999). Such variable features of the plant mitochondrial genome have recently proved to be useful in establishing plant phylogenies at the local and broader scales (e.g. Qiu et al. 1999; Gugerli et al. 2001a).

Multiple DNA fragments were observed when PCR products of the *nad4*L-*orf25* intergenic region were analysed in *P. mariana* (Fig. 3). It has been shown previously that this intergenic region is conserved in most angiosperms except in the Poaceae (Kubo et al. 2000). Our results suggest that this region is present in *Picea*, and that it has probably been duplicated, generating a great amount of polymorphism (up to six alleles in *P. mariana* alone, see Fig. 3a), presumably by homologous recombination. In some conifers, including white spruce and black spruce, the presence of two copies of the gene *orf25* has also been suggested following results of hybridization tests (DeVerno et al. 1993). The presence of several copies of other mitochondrial genes has also been reported in pines (Wu et al. 1998). If the *nad4*L-*orf25* intergenic region is located within a repeated DNA element which can undergo homologous recombination with other repeated elements, genomic instability could be promoted and multiple copies of the same region could be produced. Such homologous recombination events involving the genes *orf25* and the *atp6* have been reported for different species of *Triticum* (Mohr et al. 1993). On the other hand, heteroplasmy caused by paternal leakage does not appear as likely to explain the observed pattern, because individuals of *P. mariana* exhibiting more than one DNA fragment for the *nad4*L-*orf25* intergenic region did not harbor multiple DNA fragment patterns at the three other mtDNA loci found polymorphic in this species.

Other than the *nad4*L-*orf25* intergenic region, three more mtDNA regions revealed polymorphisms among and within species in *Picea*, and these polymorphisms followed expected maternal inheritance. Intraspecific polymorphisms in the intron b/c of *nad1* had been previously reported for many conifers including *P. abies* (Sperisen et al. 2001), several species in the genus *Pinus* (Senjo et al. 1999; Mitton et al. 2000; Soranzo et al. 2000; Latta and Mitton 2001) and in the genus *Abies* (Isoda et al. 2000). Intraspecific polymorphisms in the first intron of the gene *nad5* had also been revealed recently in *Pinus albicaulis* (Richardson et al. 2002) but not in the genus *Picea*. As for the V1 region of the SSU *rRNA* gene, a loop structure, the current study appears to be the first report of an intraspecific mtDNA polymorphism at this locus for a plant taxon.

Within the genus *Picea*, variation at the sequence level was found to be low. The detection of polymorphism with three different methods (PCR, PCR-RFLP and PCR-SSCP) was possible for only three of the 12 mtDNA regions surveyed, if one rejects the uncertain nature of the multifragment polymorphism detected for the *nad4*L-*orf25* intergenic region. In Angiosperms, the high amount of variation in the structure and order of mitochondrial genes contrasts with the low diversity observed at the sequence level, in either coding or non-coding regions (Laroche et al. 1997; Laroche and Bousquet 1999; Palmer et al. 2000). The present study suggests that variation in the mtDNA of *Picea* follows similar trends, implying a slow rate of evolution at the sequence level. Indeed, most of the polymorphisms that were observed among and within taxa in *Picea* were caused by a few substitutions and indels (Fig. 6). Some of the indels detected were produced by small direct tandem repeats. In the first intron of *nad5*, a 5-bp pattern was repeated between two and five times in the species studied, while a poly-T motif was found at the end of the same intron. Such tandem repeats and small mononucleotide sequences have been observed frequently in non-coding regions of the nuclear and mitochondrial genomes of conifers (Perry and Bousquet 1998; Soranzo et al. 1999; Sperisen et al. 2001).

Pooling the genotypes of linked genes or genomic regions (e.g. mitochondrial genes) in haplotypes allows studying the whole region as a single polymorphic locus. In recent years, mtDNA haplotypes ("mitotypes") have been one of the most powerful tools for the study of biogeography in conifers, permitting the identification of migration routes and glacial refugia, among others (e.g. Sinclair et al. 1999; Mitton et al. 2000; Soranzo et al. 2000; Gugerli et al. 2001b). In this study, the consideration of three different polymorphic loci revealed between two and six mitotypes within five out of eight species of *Picea* (see Table 3). If we assume that all three loci are linked and that there are no rearrangements producing new associations between the alleles of different loci, only two or three mitotypes per species would have been observed. On the other hand, if all polymorphic markers were allowed to recombine freely as non-linked genes, between six and 18 different mitotypes would have been expected. A few more mitotypes than expected under a strict non-recombination hypothesis were observed for *P. abies* and for *P. mariana*. Instead of three mitotypes, six and five mitotypes were observed for *P. abies* and for *P. mariana*, respectively. In angiosperms, recombination due to structural rearrangements and heteroplasmy has been recognized as a potent source of new mitochondrial types (Dumolin-Lapègue et al. 1998; Saville et al. 1998; Palmer et al. 2000). In conifers, most of the polymorphisms previously observed in the mitochondrial genome appear to result from structural rearrangements (Strauss et al. 1993; Tsumura and Suyama 1998; Wu et al. 1998). Recombination in plant mtDNA would be promoted by the presence of repeated DNA elements which would interact among them and generate plasmid multimers and new arrangements of mitochondrial genes (Palmer 1985; Lonsdale et al. 1988). In heteroplasmic cells, this process could theoretically occur after the interaction of homologous repetitive sequences present in the various types of mtDNA (Lonsdale et al. 1988). Mitochondrial heteroplasmy has not been frequently reported in plants, but evidence for this has been noticed in maize (Fauron et al. 1995). It has also been suspected in *P. abies* (Sperisen et al. 2001).

To date, most of the conifer studies using PCR-based mtDNA markers relied almost exclusively on a unique locus, the *nad1* intron b/c (Senjo et al. 1999; Isoda et al. 2000; Mitton et al. 2000; Soranzo et al. 2000; Latta et al. 2001). Several polymorphisms have been found within this 3-kb intron (Sperisen et al. 2001) and, when considered simultaneously, these polymorphisms have produced different mitotypes. However, it is unlikely that the polymorphims located in a single region could represent appropriately the whole mitochondrial genome, especially if homologous recombination is taking place such as observed in the present study. The simultaneous consideration of several polymorphic markers dispersed over the mitochondrial genome should allow for a broader and more precise estimation of the intraspecific haplotype diversity, providing a powerful tool for studying the microevolution of conifer mtDNA, and leading to a more accurate view of population structures and biogeographical processes.

Several of the polymorphisms detected among spruce species involved groups of potentially hybridizing taxa, such as the progenitor *P. mariana* and the derivative *P. rubens* in eastern North America (Perron and Bousquet 1997; Perron et al. 2000), *P. glauca*, *P. sitchensis* and *P. engelmannii* in western Canada (Taylor 1959; Fowler 1987; Sutton et al. 1994), or *P. engelmannii* and *P. pungens* in western United States (Mitton and Andalora 1981). Although the number of individuals sampled in this study was at a minimum for several species, useful observations could be drawn. In the case of *P. rubens*, the unique polymorphism detected at the V1 region of the SSU rRNA gene was also detected in *P. mariana*, while polymorphisms that were detected for two additional loci in *P. mariana* (the intron b/c of *nad1* and the intron 1 of *nad5*, see Table 3) were not detected in *P. rubens*, even

after expanding the sampling by a factor of ten (data not shown). This observation reinforces the concept of a progenitor-derivative relationship previously reported from nuclear ESTP loci between *P. mariana* and *P. rubens* (Perron et al. 2000).

In *P. glauca*, polymorphisms were detected for two mtDNA loci (the intron b/c of *nad1* and the intron 1 of *nad5*, see Table 3). For each of these loci, rare alleles were detected in individuals from western Canada that matched the common alleles of potentially hybridizing species such as *P. engelmannii* and *P. sitchensis*. Such a trend could be indicative of introgressive leakage, and additional studies and sampling are needed to ascertain this observation. Together with nuclear or cpDNA markers, variation at these mtDNA loci should help to assess the extent of introgressive hybridization as well as the structures of the present zones of contact between these species.

Acknowledgements We thank S. Plante and N.O. Tremblay (C.R.B.F., Univ. Laval) for valuable help in the laboratory, S. Boisclair and S. Senneville (C.R.B.F., Univ. Laval) for help with the collection of samples, and two anonymous reviewers for constructive comments and suggestions to improve the manuscript. This research was supported by grants from Fonds Québécois de la Recherche sur la Nature et les Technologies (F.Q.R.N.T.), the National Sciences and Engineering Research Council of Canada (N.S.E.R.C.), and the Canadian Forest Service (C.F.S.) to J. Bousquet, J. Beaulieu and N. Isabel, and an international fellowship from the Québec Ministry of Education to J.P. J.-C.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Birky CW Jr (1995) Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. Proc Natl Acad Sci USA 92:331–338

Bonen L, Williams K, Bird S, Wood C (1994) The NADH dehydrogenase subunit 7 gene interrupted by four group II introns in the wheat mitochondrial genome. Mol Gen Genet 244:81–89

Campbell DR (1991) Comparing pollen dispersal and gene flow in a natural population. Evolution 455:1965–1968

Chandelier A, du Jardin P, Avril C, Pâques M (1999) Identification of mitochondrial plasmid-like DNAs in *Picea abies* (L.) Karst. Plant Cell Rep 18:841–847

Demesure B, Sodzi N, Petit RJ (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

DeVerno LL, Charest PJ, Bonen L (1993) Inheritance of mitochondrial DNA in the conifer *Larix*. Theor Appl Genet 86:383–388

Duff RJ, Nickrent DL (1999) Phylogenetic relationships of land plants using mitochondrial small-subunit rDNA sequences. Am J Bot 86:372–386

Dumolin-Lapègue S, Pemonge M-H, Petit RJ (1998) Association between chloroplast and mitochondrial lineages in oaks. Mol Biol Evol 15:1321–1331

Fauron CMR, Moore B, Casper M (1995) Maize as a model of higher plant mitochondrial genome plasticity. Plant Sci 112:11–32

Fournier D, Perry DJ, Beaulieu J, Bousquet J, Isabel N (2002) Optimizing expressed sequence tag polymorphisms by single strand conformation polymorphism in spruces. For Genet 9:11–17

Fowler DP (1987) The hybrid white × Sitka spruce: species crossability. Can J For Res 17:413–417

Gugerli F, Sperisen C, Büchler U, Brunner I, Brodbeck S, Palmer JD, Qiu Y-L (2001a) The evolutionary split of Pinaceae from other conifers: evidence from an intron loss and a multigene phylogeny. Mol Phylogenet Evol 21:167–175

Gugerli F, Sperisen C, Büchler U, Magni F, Geburek T, Jeandroz S, Senn J (2001b) Haplotype variation in a mitochondrial tandem repeat of Norway spruce (*Picea abies*) populations suggests a serious founder effect during postglacial re-colonization of the western Alps. Mol Ecol 10:1255–1263

Isoda K, Shiraishi S, Watanabe S, Kitamura K (2000) Molecular evidence of natural hybridization between *Abies veitchii* and *A. homolepis* (Pinaceae) revealed by chloroplast, mitochondrial and nuclear DNA markers. Mol Ecol 9:1965–1974

Kubo T, Yamamoto MP, Mikami T (2000) The *nad4*L-*orf25* gene cluster is conserved and expressed in sugar beet mitochondria. Theor Appl Genet 100:214–220

Kumar R, Lelu M-A, Small I (1995) Purification on mitochondria and mitochondrial nucleic acids from embryogenic suspension cultures of a gymnosperm, *Larix* × *leptoeuropaea*. Plant Cell Rep 14:534–538

Laroche J, Bousquet J (1999) The evolution of angiosperm mitochondrial *rps3* group II introns in perennial and annual taxa: substitutions, insertions-deletions and homology to *nad5* intron 1. Mol Biol Evol 16:441–452

Laroche J, Li P, Maggia L, Bousquet J (1997) Molecular evolution of angiosperm mitochondrial introns and exons. Proc Natl Acad Sci USA 94:5722–5727

Latta RG, Mitton JB (2001) Historical separation and present gene flow through a zone of secondary contact in ponderosa pine. Evolution 53:769–776

Lonsdale DM, Brears T, Hodge TP, Melville SE, Rottmann WH (1988) The plant mitochondrial genome: homologous recombination as a mechanism for generating heterogeneity. Phil Trans R Soc Lond B 319:149–163

Lu M-Z, Szmidt AE, Wang XR (1998) RNA editing in gymnosperms and its impact on the evolution of the mitochondrial *coxI* gene. Plant Mol Biol 37:225–234

Lunt DH, Whipple LE, Hyman BC (1998) Mitochondrial DNA variable number of tandem repeats (VNTRs): utility and problems in molecular ecology. Mol Ecol 7:1441–1455

McCauley DE (1997) The relative contributions of seed and pollen movement to the local genetic structure of *Silene alba*. J Hered 88:257–263

Mitton JB, Andalora R (1981) Genetic and morphological relationships between blue spruce, *Picea pungens*, and Engelmann spruce, *Picea engelmannii*, in the Colorado Front Range. Can J Bot 59:2088–2094

Mitton JB, Kreiser BR, Latta RG (2000) Glacial refugia of limber pine (*Pinus flexilis* James) inferred from the population structure of mitochondrial DNA. Mol Ecol 9:91–97

Mohr S, Schulte-Kappert E, Odenbach W, Oettler G, Kück U (1993) Mitochondrial DNA of cytoplasmic male-sterile *Triticum timopheevi*: rearrangement of upstream sequences of the *atp6* and *orf25* genes. Theor Appl Genet 86:259–268

Palmer JD (1985) Evolution of chloroplast and mitochondrial DNA in plants and algae. In: MacIntyre RJ (ed) Molecular evolutionary genetics. Plenum Press, New York, pp 131–240

Palmer JD, Adams KL, Cho Y, Parkinson L, Qiu Y-L, Song K (2000) Dynamic evolution of plant mitochondrial genomes: mobile genes and introns and highly variable mutation rates. Proc Natl Acad Sci USA 97:6960–6966

Perron M, Bousquet J (1997) Natural hybridization between black spruce and red spruce. Mol Ecol 6:725–734

Perron M, Perry DJ, Andalo C, Bousquet, J (2000) Evidence from sequence-tagged-site markers of a recent progenitor-derivative species pair in conifers. Proc Natl Acad Sci USA 97:11,331–11,336

Perrotta G, Regina TMR, Ceci LR, Quagliariello C (1996) Conservation of the organization of the mitochondrial *nad3* and *rps12* genes in evolutionarily distant angiosperms. Mol Gen Genet 251:326–337

Perry DJ, Bousquet J (1998) Sequence-tagged site (STS) markers of arbitrary genes: development, characterization and analysis of linkage in black spruce. Genetics 149:1089–1098

Petit RJ, Pineau E, Demesure B, Bacilieri R, Ducousso A, Kremer A (1997) Chloroplast DNA footprints of postglacial recolonization by oaks. Proc Natl Acad Sci USA 94:9996–10,001

Qiu Y-L, Lee J, Bernasconi-Quadroni F, Soltis DE, Soltis PS, Zanis M, Zimmer EA, Chen Z, Savolainen V, Chase MW (1999) The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. Nature 402:404–407

Richardson BA, Bunsfeld SJ, Klopfenstein NB (2002) DNA from bird-dispersed seed and wind-disseminated pollen provides insights into postglacial colonization and genetic structure of whitebark pine (*Pinus albicaulis*). Mol Ecol 11:215–228

Saville BJ, Kohli Y, Anderson JB (1998) MtDNA recombination in a natural populations. Proc Natl Acad Sci USA 95:1331–1335

Schaal BA, Hayworth DA, Olsen KM, Rauscher JT, Smith WA (1998) Phylogeographic studies in plants: problems and prospects. Mol Ecol 7:465–474

Senjo M, Kimura K, Watano Y, Ueda K, Shimizu T (1999) Extensive mitochondrial introgression from *Pinus pumila* to *P. parviflora* var. *pentaphylla* (Pinaceae). J Plant Res 112:97–105

Sinclair WT, Morman JD, Ennos RA (1999) The postglacial history of Scots pine (*Pinus sylvestris* L.) in western Europe: evidence from mitochondrial DNA variation. Mol Ecol 8:83–88

Soranzo N, Provan J, Powell W (1999) An example of microsatellite length variation in the mitochondrial genome of conifers. Genome 42:158–161

Soranzo N, Alia R, Provan J, Powell W (2000) Patterns of variation at a mitochondrial sequence-tagged-site locus provides new insight into the postglacial history of European *Pinus sylvestris* populations. Mol Ecol 9:1205–1211

Sperisen C, Büchler U, Gugerli F, Mátyás G, Geburek T, Vendramin GG (2001) Tandem repeats in plant mitochondrial genomes: application of the analysis of population differentiation in the conifer Norway spruce. Mol Ecol 10:257–263

Strauss SH, Hong Y-P, Hipkins VD (1993) High levels of population differentiation for mitochondrial DNA haplotypes in *Pinus radiata, muricata* and *attenuata*. Theor Appl Genet 86:605–611

Sutton BCS, Pritchard SC, Gawley JR, Newton CH, Kiss GK (1994) Analysis of Sitka spruce-interior spruce introgression in British Columbia using cytoplasmic and nuclear DNA probes. Can J For Res 24:278–285

Taylor TMC (1959) The taxonomic relationship between *Picea glauca* (Moench) Voss and *P. engelmannii* Parry. Madrono 15:111–115

Tsumura Y, Suyama Y (1998) Differentiation of mitochondrial DNA polymorphisms in populations of five Japanese *Abies* species. Evolution 52:1031–1042

Wang X-Q, Tank DC, Sang T (2000) Phylogeny and divergence times in Pinaceae: evidence from three genomes. Mol Biol Evol 17:773–781

Wu J, Krutovskii KV, Strauss SH (1998) Abundant mitochondrial genome diversity, population differentiation and convergent evolution in pines. Genetics 150:1605–1614