Short Note: Development of Mitochondrial Markers for Population Genetics of Norway Spruce [*Picea abies* (L.) Karst.]

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Abstract

Norway spruce is an important commercial tree species in northern and central Europe. Pure mitochondrial DNA isolated from tissue culture materials grown in the dark were used to construct a partial mitochondrial library. 100 clones were randomly selected and 19 markers were isolated. Three of these markers proved to be polymorphic and two showed maternal inheritance in controlled crosses. These markers will be useful for population genetic studies in *P. abies*.

Key words: mtDNA, marker development, maternal inheritance, population genetics, length polymorphism, null alleles, *Picea abies*.

Norway spruce is one of the commercially and ecologically most important coniferous species in Europe. Therefore, knowledge about the existing genetic variation and population differentiation is helpful for various aspects of management of its genetic resources. Plant mitochondrial DNA (mtDNA) is generally maternally inherited (NEALE et al., 1989; NEALE and SEDEROFF, 1989; GRIVET et al., 1999), and thus indicative of seed dispersal, while chloroplast DNA (cpDNA) is generally maternally inherited in angiosperms, but paternally (or biparentally) in gymnosperms and thus indicative of gene flow by both pollen and seeds (VENDRAMIN et al., 1996; LATTA et al., 1998). Among gymnosperms, mitochondria are inherited predominantly from the female parent in the *Pinaceae* and probably in the *Taxaceae*, but in four other coniferous families mitochondrial inheritance appears to be predominantly paternal (NEALE et al., 1989; MOGENSEN, 1996). This differential mode of inheritance makes organellar genomes an ideal tool for studies of gene flow and population structures (MITTON et al., 2000; GUGERLI et al., 2001). From the geographic distribution of variation in the organellar DNA it is possible to draw conclusions about past gene flow through migration. In contrast to bi-parentally transmitted markers, maternally inherited alleles are expected to exhibit higher levels of population differentiation at equilibrium, due to the absence of meiotic recombination and to the fact that haploid genomes are more sensitive to the effects of random drift (PETIT et al., 1993; ENNOS, 1994).

The mitochondrial (mt) genome of plants is not conserved in size and structure between species (WOLFE et al., 1987; PALMER, 1992; PALMER et al., 2000). Therefore, markers developed for a particular species usually cannot be transferred to other taxa.

Up to now, some polymorphic regions have already been identified in the mitochondrial genome of the Norway spruce (SPERISEN et al., 2001; GUGERLI et al., 2001; JEANDROZ et al., 2002; BASTIEN et al., 2003; JARAMILLO-CORREA et al., 2003). Nevertheless, since not all these markers might be polymorphic in some geographic regions of this widespread species, it is reasonable to search for additional markers which will be useful not only for population genetics and forest management, but also for detecting instances of mtDNA recombination and heteroplasmy.

In this study, we developed new primers for the mitochondrial genome of *P. abies*. Pure mtDNA of Norway spruce was isolated from cell cultures according to MACKENZIE (1994) with minor modification in speed and time of centrifugation. Out of 600 g of tissue (dark grown embryogenic callus cultures), 1–2 µg of mtDNA could be isolated. The purity of mtDNA was tested by PCR using published primer pairs specific for cpDNA, nuclear DNA and mtDNA. The DNA was considered as pure if amplification was successful exclusively with the mtDNA specific primers (data not shown). Due to the limited amount of mtDNA available, the Random Amplified Polymorphic DNA (RAPD) technique was used to generate clonable fragments for the construction of partial mt genome libraries. Each RAPD amplification reaction contained 1x buffer (Dynazyme), 2 mM $MgCl_2$, 10 pmol primer (OPERON: OPD-02, OPE-20, OPH-20, OPJ-20), 0.75 unit Dynazyme II DNA polymerase, 20–30 ng DNA template and PCR water up to 25 µl. PCR-cycling conditions consisted of an initial denaturation step of 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 36 °C for 1 min, 72 °C for 2 min, and a final extension step at 72 °C for 8 min. PCR products obtained with different RAPD primers were cloned using the TOPO TA cloning Kit for sequencing (Invitrogen). Purified plasmid DNA of the clones was sequenced (BigDye sequencing Kit, PE Applied Biosystem) on a capillary sequencer (ABI 3100 Genetic Analyser) and analysed using the software Sequencher™ 4.0 and DNAsis 2.5. Out of 7200 positive identified clones a total of 100 were randomly selected and sequenced. Primers were designed (Oligo Primer Analysis Software; version 4.1) for 19 mtDNA-bearing clones. The clones were selected

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on the basis of homology to known mt sequences or the length of the insert (> 1000 bp). Out of 19 potential loci, 14 were effectively amplified, but 11 of them proved to be monomorphic even with PCR-RFLP analysis. In total, three of the investigated loci (*Table 1*) were polymorphic: mt15-D02, mt23-D02, and mt1H01. Maternal inheritance was tested in a set of 11 controlled crosses (5–8 offsprings of each family) using different combinations of five male and six female parents (obtained from SkogForsk/Sweden and the Federal Research Centre for Forestry and Forest Products BFH Hamburg-Grosshansdorf/Germany).

For the analysis of polymorphism at the three loci, PCR amplifications were conducted in a total volume of 25 µl according to the HotStarTaq polymerase (QIAGEN) supplier's instructions. PCR-cycling consisted of an initial denaturation step of 95°C for 15 min, followed by 35 cycles of 1 min at 95 °C, 1 min at annealing temperature (see *Table 1*), 2 min at 72 °C, and a final extension step of 10 min at 72 °C.

Maternal inheritance was proved for markers mt15-D02 and mt23-D02 in controlled crosses. For marker mt15-D02 two alleles corresponding to PCR fragments of 1107 bp (mitotype *a*) and 1249 bp (mitotype *b*) were detected. All female parents yielded mitotype *a*, while all male parents yielded mitotype *b*. Analysis of all 70 F_1 individuals from the 11 families possessed the maternal mitotype *a*, thus confirming maternal inheritance (*Figure 1a* and *Table 1*). For marker mt23-D02 the primers yielded fragments of 1117 bp (mitotype *a*) or 1122 bp (mitotype *b*), and in several cases no amplification product (mitotype *c*) (*Figure 1b* and *Table 1*). For the analysis of marker mt23-D02, a high-resolution method such as PCR-RFLP was necessary to visualize the 5 bp size difference between mitotypes *a* and *b* (*Figure 2*). The PCR products were digested with restriction enzyme *Hinf* I (5 U; 3h at 37 °C) and separated by 8 % non-denaturing polyacrylamide gels [Rotiphorese® Gel 30 (37,5:1)] at 14 °C for 3 h using Dual Vertical Slab System DSG-250 (C.B.S. Scientific), followed by silver staining for band detection (DUMOLIN et al., 1995; DEMESURE et al., 1996). Of the 40 F_1 individuals from the various crosses all possessed the maternal mitotype (data not shown). The third marker (mt1H01) showed a monomorphic 720 bp fragment in all eleven parents (mitotype *a*). In a population study (MAGHULY et al., 2007), however, some individuals were found for which no amplification product could be obtained. The lack of a PCR fragment was interpreted as a null allele (mitotype *b*) that may be caused by putative mutations in the primer binding sites *(Table 1)*.

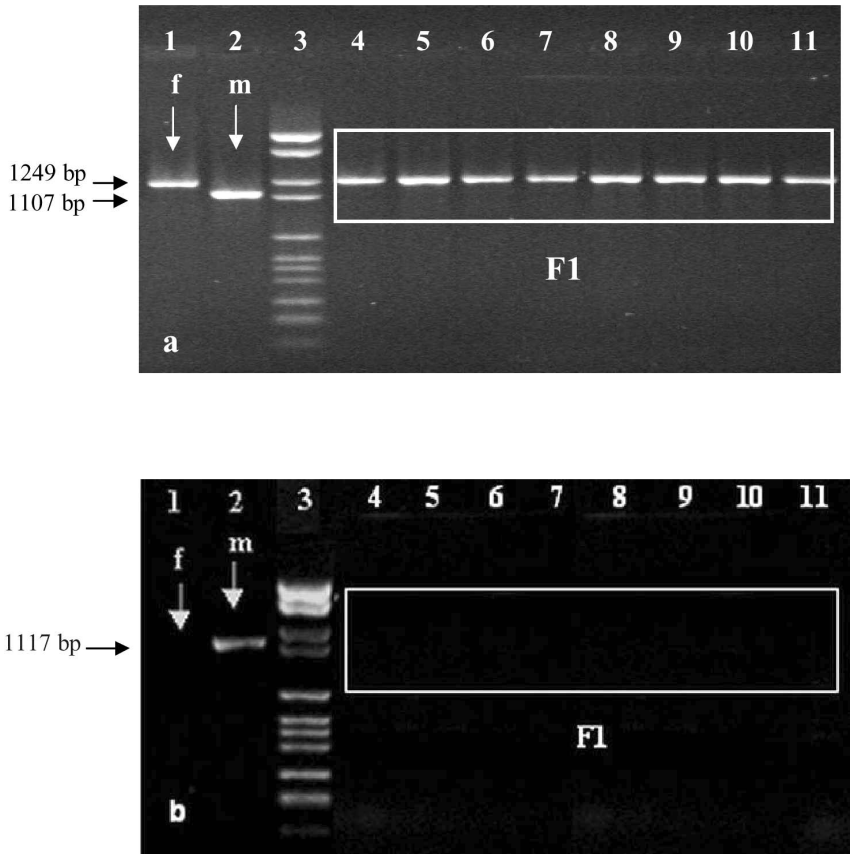

Figure 1. – Amplification products obtained with the three markers separated on agarose gels (1.2%): a) mt15-02, b) mt23-DO2. Parents and offspring of controlled crosses are shown. Lane 1: female parent, lane 2: male parent, lane 3: length marker VI, lanes 4-11: F_1 offspring. In mt15-02 the F_1 progeny shows the same fragment as the female parent. In mt23-DO2 the female parent and the F_1 offspring show the null allele whereas the male parent carries the 1117 bp allele.

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Table 1. – PCR primers for amplification of three mitochondrial markers.

Marker name	primer sequence 5'-3'	Mitotypes and fragment size	GenBank Acc. no.	Annealing temperature
mt15-D02	TATCTGACTTGCCTTATC	***a***: 1107	AY897577	54 °C
	ATCCGAATACATACACC	***b***: 1249		
mt23-D02	CACCCTTGGGTAGACTGG	***a***: 1117	AY897578	54 °C
	GGTTCACGCAGTGCTTCT	***b***: 1122		
		c: n.a.		
mt1H01	AAGATGGATCGCCCTTACGC	***a***: 702	AY897576	52 °C
	GAGGAGGAGGCTTCGTCGTC	***b***: n.a.		

n.a. = no amplification.

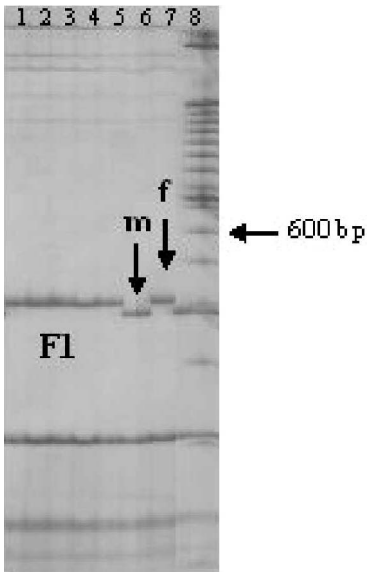

Figure 2. – PCR-RFLP for mitochondrial markers mt23-DO2. PCR fragments were digested with restriction Enzyme *Hinf* I. Fragments were separated on a high resolution on acrylamide gel. Lane 1-5: F_1, lane 6: male, lane 7: female, lane 8: 100 bp ladder (Boehringer).

The DNA sequence analysis of the two mitotypes *a* and *b* (*Table 1*) detected in the marker mt15-D02 revealed the presence of a 142 bp insertion/deletion (indel). On the other hand, the difference between two of the three mitotypes (*a* and *b*) of the locus mt23-D02 was due to a 5 bp indel. The third mitotype (*c*) from this locus was a null allele (*Table 1*).

BLASTN searches revealed that marker mt1H01 is highly similar (91%) to a 729 bp section of the mt *nad1* gene of the Evening primrose *Oenothera berteriana* [Score = 908 bits (458), E-value = 0.0]. For the other two markers similarity was found only to very short sections of known mt sequences, which is quite meaningless with respect to homology.

All three mtDNA makers were employed successfully in the analysis of 37 populations (185 individuals) originating from various geographic regions of Austria (Maghuly et al., 2006, 2007) and thus provide a valuable tool for further population studies of *P. abies*. They will be also useful for forest management programs and for reconstructing the phylogeographic history and post-glacial migration routes of *P. abies*.

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