Plastid DNA Polymorphism of Megagametophytes and Pollen in Two *Abies* Species

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Dedicated to WOLFGANG LANGNER on the occasion of his 90th birthday

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Abstract

Single pollen grains of *Abies kawakamii* and megagametophytes of *Abies alba* and *A. kawakamii* were investigated for a *psb*C chloroplast gene region. This region was amplified and digested with endonuclease *Hae* III. Two different restriction fragment patterns occurred corresponding to the 2 variants detected in previous investigations on needles and embryos of *Abies.* The occurrence of plastid DNA in megagametophytes as well as in pollen of *Abies* is discussed with regard to the mechanisms of inheritance of plastid DNA in conifers. Prospects are given for PCR-based analysis of plastid DNA in single pollen grains and megagametophytes as a promising tool in population genetics.

Key words: Abies alba, Abies kawakamii, chloroplast DNA, PCR, RFLP, single pollen grains, primary endosperm.

FDC: 165.3; 168; 174.7 *Abies alba;* 174.4 *Abies kawakamii.*

Introduction

Many forest tree species are bisexual and thus individuals have capacity of both pollen and seed dispersal. Different approaches are reported for tracing pollen and seed migration and their relevance for genetic structures of populations. By experiment, migration rates of seeds and reproductively effective pollen were estimated using genetic markers. Already in the early 50s, migration of reproductively effective pollen of a few *Picea abies* trees was traced by means of an 'aurea mutant type' (LANGNER, 1953). Later on, dispersal of reproductively effective pollen was estimated by means of isozyme gene markers applied on seed or seedling progeny (review by ADAMS, 1992). By theoretical approach, ENNOS (1994) estimated relative rates of both pollen and seed migration among populations. The methods applied did not allow tracing of single pollen grains, as this would require genotyping them. Isozyme gene markers are limited in this respect and there is need for new techniques.

Novel PCR-based approaches allow analyses of extremely small amounts of template DNA, e.g., from a single cell (ZHANG *et al.,* 1992). Thus, they are the method of choice for obtaining information on DNA of a single pollen grain. In trees, just recently, RAPD analysis of genomic DNA from single pollen grains was successful for the angiosperm tree species *Fagus sylvatica* (VORNAM, 1996). As most of the polymorphic fragments in random amplified DNA banding patterns were shown to be of dominant gene action (WILLIAMS *et al.,* 1990; LU *et al.,* 1995), nuclear origin holds true for these fragments. Due to the predominantly paternal inheritance of chloroplast DNA in conifer species (review by WAGNER, 1992), conifer pollen offers the opportunity to be investigated for polymorphic plastid[4)] DNA. This approach excludes any contamination of PCR products by DNA fragments amplified from heterotrophic organisms which may colonize pollen grain surface.

Thus for our investigations of *Abies*, chloroplast DNA was chosen for PCR-amplification from single pollen grains. The investigations are based on a former detected chloroplast DNA polymorphism in needles and embryos of *Abies alba* (ZIEGENHAGEN *et al.,* 1995). Two distinct variants ('cytotype I' and 'cytotype II') occurred when a particular *psb*C gene region was PCR-amplified and digested by the restriction endonuclease *Hae* III. These 2 cytotypes were found to be paternally inherited and to be both present in numerous investigated populations of *A. alba.* So far, either cytotype I or cytotype II, were found also in some other *Abies* species.

In the following study, this particular PCR-RFLP analysis is applied on plastid DNA from single pollen grains of *Abies kawakamii.* In addition, it is tested on megagametophytes of *Abies alba* and *A. kawakamii.* If maternal plastid DNA can be detected in megagametophytes of *Abies* seeds such as just recently found in 2 *Pinus* species (WANG *et al.,* 1996) a tool will be provided for directly assessing migration of both seeds and pollen using chloroplast DNA polymorphisms as genetic markers. Moreover, potential of the tool for determination of an individual's contribution to the zygote and pollen population will be discussed.

Material and Methods

Abies alba: Megagametophytes, embryos and needles

For studying plastid DNA of megagametophytes, 20 seeds were available from a self-fertilized individual of *A. alba.* The corresponding embryos of the same seeds as well as needles of the seed tree were investigated for comparison.

Abies kawakamii: Pollen, megagametophytes and embryos

For studying plastid DNA of single pollen grains, pollen from the Asian *A. kawakamii* was available, harvested from different individuals in Taiwan in spring 1996. Harvesting was done by climbing the trees. The mature pollen was collected by putting plastic bags on selected male cones and shaking them. From this species, seeds were also at hand. Five embryos and the corresponding megagametophytes were investigated for comparison.

Preparation of megagametophytes and embryos

Seeds were cut through using a scalpel. Megagametophytes and the corresponding embryos were carefully separated using

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[4)] In this paper plastid DNA and chloroplast DNA (= cpDNA) are synonymously used.

the scalpel tip and directly transferred to eppendorf tubes for DNA extraction. By their yellowish color, embryos of *Abies* species can easily be distinguished from the white primary endosperm (= megagametophyte).

Pollen treatment

During the period of investigation, the pollen was stored in a desiccator at 4 °C. Samples were taken and germinated for 72 h on glass microscope slides according to VENNE *et al.* (1989). Under a microscope or binocular, single germinated pollen grains were easily selected and retrieved from the slide using an eye-lash (VORNAM, 1996). A total of 20 germinated pollen grains were directly transmitted to PCR reaction medium, one single pollen grain per PCR tube and subjected to amplification. Transmission of more than one single pollen grain is excluded as the eye-lash and later on the PCR medium were confirmed to be in contact with exactly one single pollen grain. Control was carried out under a binocular.

DNA extraction from needles, megagametophytes and embryos

Total DNA of needles, megagametophytes and embryos was isolated according to the mini-preparation by ZIEGENHAGEN *et al.* (1993).

PCR amplification

The particular *psb*C gene region was amplified by a pair of primers which represent sequences from 5' and 3' region of *psb*C gene (ps II 44kd): primer 1 (sense): 5´ -AGC ACT AAA GCT TGG ACA GG- 3´ (ZIEGENHAGEN *et al.,* 1995); primer 2 (anti-sense): 5´ -GGT CGT GAC CAA GAA ACC AC- 3´ (DEMESURE et al., 1995).

PCR was run in a pre-heated DNA thermal cycler THC1 (Perkin-Elmer & Co GmbH, Überlingen, Germany) with the following cycles and temperatures (DEMESURE et al., 1995): 94 °C for 4 min., followed by 35/40 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. Cycle counts were 35 for megagametophytes and embryos, and 40 for single pollen grains. Amplification was performed in 25 µl reaction mixture. Reaction mixture according to DEMESURE et al. (1995) was modified as follows: Either one single pollen grain or 20 ng DNA extracted from needles, megagametophytes or embryos, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8, 2 mM $MgCl_2$, 0.001% W_1 (Gibco BRL, Life Technologies GmbH, Eggenstein), 10 mM β-mercaptoethanol, 4.4 µg/ml bovine serum albumin, 200 mM of each four dNTP, 1 unit of Taq polymerase (Gibco BRL, Life Technologies GmbH, Eggenstein, Germany) and 0.6 µM of each primer.

Digestion

For restriction analysis, PCR products (16 µl) were digested by 10 units of restriction endonuclease *Hae* III. Digestion was performed overnight according to instructions of Boehringer Mannheim, Germany. Total digestion volume was loaded onto the gel.

Visualization of DNA fragments

Undigested PCR products as well as restriction fragments were separated by electrophoresis in 1.0% (w/v) agarose gel, run in Tris-borate buffer (pH 8.0) at 13 V/cm for 3 hours. DNA fragments were visualized by UV fluorescence after staining with ethidium bromide (0.25 µg/ml staining solution).

Results

Megagametophytes, embryos and needles of Abies alba

Amplification of the *psb*C gene region from megagametophytes of the *A. alba* individual resulted in a DNA fragment of about 1,310 bp for all investigated seeds. *Figure 1A* (lane 1) shows an example for the amplification product of one megagametophyte.

A DNA fragment of the same length is observed in agarose gel after application of the same procedure to the embryos of the same seeds (results not shown) and to needles of the seed tree (*Fig. 1B*, lane 1).

Digestion with endonuclease *Hae* III resulted in restriction fragment patterns which are the same for all 3 tissues, needles, megagametophytes and embryos (*Figs. 1A* and *1B*). They are

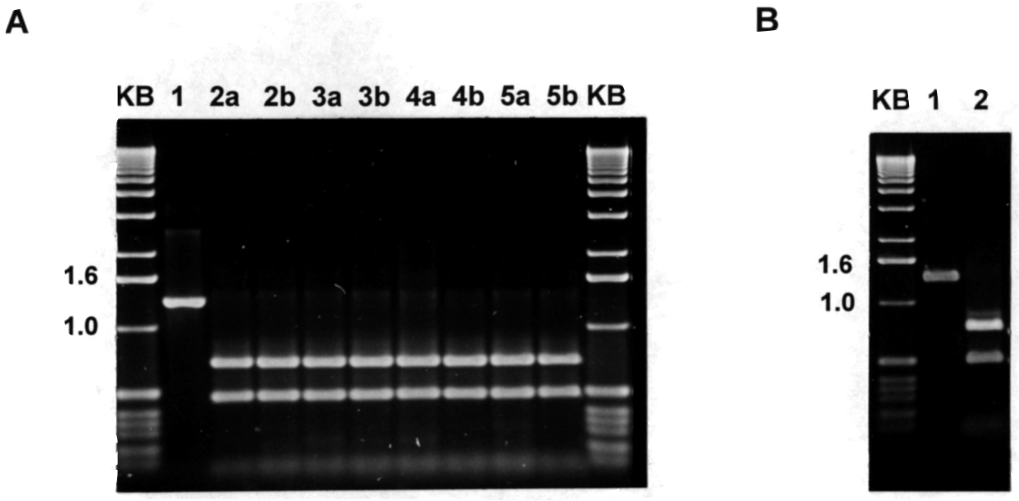

Figure 1. – PCR amplified *psb*C gene region and *Hae* III digested PCR product of megagametophytes, embryos and needles of an *Abies alba* individual. *A.* Megagametophytes and embryos. Lane 1: undigested PCR amplified *psb*C gene region of a megagametophyte, lanes 2 to 5: *Hae* III restriction patterns for 4 megagametophytes and the corresponding embryos of the same seeds, a = embryo, b = megagametophyte. *B.* Needles of the seed tree. Lane 1: undigested PCR amplified *psb*C gene region, lane 2: *Hae* III restriction pattern.
All *Hae* III restriction patterns represent cytotype I.
KB = size standard (1 KB ladder, Gibco BRL, Eggenstein, Germany).

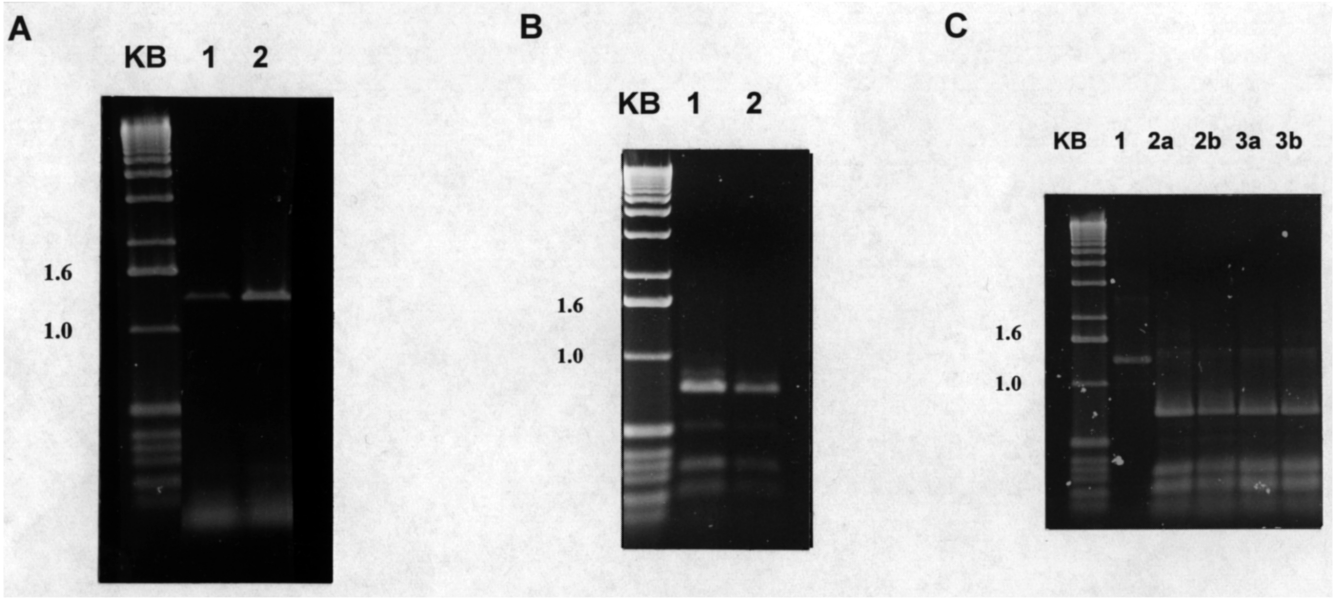

Figure 2. – PCR amplified *psb*C gene region and *Hae* III digested PCR product of single pollen grains, megagametophytes and embryos of *Abies kawakamii*. *A.* Single pollen grains. Lanes 1 and 2: undigested PCR amplified *psb*C gene region of 2 single pollen grains. *B.* Single pollen grains. Lanes 1 and 2: *Hae* III restriction patterns of 2 single pollen grains. *C.* Megagametophytes and embryos. Lane 1: undigested PCR amplified *psb*C gene region of a megagametophyte, lanes 2 and 3: *Hae* III restriction patterns of 2 megagametophytes and the corresponding embryos of the same seeds, a = embryo, b = megagametophyte.
All *Hae* III restriction patterns represent cytotype II.
KB = size standard (1 KB ladder, Gibco BRL, Eggenstein, Germany).

identical to the former detected *Abies* chloroplast DNA variant 'cytotype I' (ZIEGENHAGEN et al., 1995). Cytotype I is characterized by 2 fragments of about 700 bp and 490 bp in length.

Pollen, megagametophytes and embryos of Abies kawakamii

After amplification procedure, PCR products of single pollen grains could either be checked on the gel or be directly submitted to digestion. 16 out of 20 PCR products were checked for the presence of the expected amplification product. The remaining 4 were subjected to restriction analysis. The amplification products of 14 single pollen grains were distinctly visible in agarose gel, each exhibiting the expected size of 1,310 bp. PCR products of 2 single pollen grains failed to be visualized in agarose gel. *Figure 2A* gives the PCR products for 2 single pollen grains. A PCR product of the same length was observed also for megagametophytes and the corresponding embryos of this *Abies* species. In *Figure 2C* (lane 1) the undigested PCR product of one megagametophyte is shown, results from embryos are not shown.

After digestion of the relevant PCR products, restriction fragment patterns occur which are identical for all 4 single pollen grains and the 5 megagametophytes and embryos of *A. kawakamii*. *Figures 2B* and *2C* give the restriction patterns for 2 single pollen grains and for 2 embryos with the corresponding 2 megagametophytes. *Figure 2B* clearly reveals that restriction fragments originating from one single pollen grain are still detectable in agarose gel.

The observed restriction fragment pattern corresponds to the former identified *Abies* chloroplast DNA variant 'cytotype II' (ZIEGENHAGEN *et al.,* 1995). As compared to cytotype I, cytotype II is characterized by one additional band which is derived by restriction within the 'cytotype I' 490 bp fragment, splitting it into 2 fragments of 320 bp and 170 bp. As far as can be judged from agarose gel, cytotype II has 3 visible fragments of about 700 bp, 320 bp and 170 bp. The restriction patterns of all 3 tissues, pollen, megagametophytes and embryos reveal a faint band at 490 bp position (see cytotype I) indicating either incomplete digestion or heteroplasmic conditions. Incomplete digestion is a common phenomenon and can be observed also for the 1,310 bp fragment (see all figures).

Discussion

Megagametophytes

Cytological results do not give clear evidence for the presence of plastids in megagametophyte tissue of conifers (SINGH, 1978). BOSCHERINI *et al.* (1994) analyzed megagametophyte tissue of *Pinus leucodermis* using cpDNA markers. By PCR-based analysis using primers for coding and non-coding *trn*T – *trn*F plastid DNA region, they did not obtain amplification products. In contrast, our results give clear genetic evidence for the presence of maternal plastids in the megagametophytes of 2 *Abies* species. This is in accordance with the results of WANG *et al.* (1996) who, upon completing this manuscript, published on successful amplification of 2 particular chloroplast DNA segments of the same above mentioned *trn*T – *trn*F plastid DNA region from megagametophytes of *Pinus nigra* and *Pinus sylvestris.*

Question arises how these findings fit together with the predominantly paternal inheritance of plastid DNA in many conifer species (review by WAGNER, 1992). For species of the genera, *Pinus* and *Abies,* paternal inheritance of cpDNA was confirmed by the investigations of NEALE and SEDEROFF (1989), WAGNER *et al.* (1989) and ZIEGENHAGEN *et al.* (1995). Paternal inheritance of plastid DNA necessarily implies the elimination of maternal plastids taking place before embryo development at the latest. From the presence of maternal plastid DNA in the megagametophytes it can be assumed that elimination takes place during proembryo development. This is supported by cytological investigations during fertilization and proembryo development in Douglas fir (OWENS and MORRIS, 1990 and

1991). Thereafter, maternal plastids were still present in the egg cell where they were observed in large inclusions. Normally these inclusions were found not to be incorporated into the embryonic cytoplasm.

Pollen

Our investigations confirm chloroplast DNA to be present in mature pollen from *Abies kawakamii.* Upon completing this manuscript SUYAMA *et al.* (1996) and WANG *et al.* (1996) published on the presence of cpDNA in pollen of other *Abies* species and of 2 *Pinus* species. Transmission of plastids via pollen is a prerequisite for mode of paternal inheritance of this organelle in conifers. This is in accordance with cytological results (OWENS and MORRIS, 1990 and 1991).

While WANG *et al.* (1996) carried out their investigations on a bulked pollen sample our analysis was successful on single pollen grains. A tool is provided which is highly effective (18 amplifications successful out of 20) for amplification of a large cpDNA fragment (1,310 bp). The latter enables various secondary amplifications and sequence analyses as well as rising the probability for detecting polymorphisms by restriction analyses. As demonstrated by restriction analysis, even restriction fragments originating from one single pollen grain can easily be scored from agarose gels.

The tool was developed to investigate within species variation. SUYAMA et al. (1996) published on successful amplification of a cpDNA segment from single pollen grains for differentiation between *Abies* species. Our PCR-based approach towards plastid DNA in megagametophytes and single pollen grains offers a tool for investigating specific aspects of the mating system. So far it operates on two intraspecific cpDNA variants. Prerequisite for its application on pollen and seed migration within and among populations is sufficient polymorphism. First results on polymorphic chloroplast microsatellite loci in *Abies alba* reveal high variation of more than 30 haplotypes (VENDRAMIN and ZIEGENHAGEN, unpublished data). Thus, chloroplast microsatellites will serve as markers for directly assessing pollen and seed migration of bisexual conifer individuals. Hence, they might allow the determination of an individual's contribution to the pollen and zygote population. Applying them on embryos of the seeds will allow the determination of reproductively effective pollen of other individuals and moreover enable estimation of paternal gene flow in space and time. Applying these markers on megagametophytes will support estimations of maternal gene flow via seeds.

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