SHORT NOTE

DNA AMPLIFICATION FROM SINGLE POLLEN GRAINS OF BEECH (*FAGUS SYLVATICA* L.)

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ABSTRACT

Gene flow via pollen is one of the forces which contribute to the evolutionary potential of forest tree populations by influencing their genetic structure and variation. The lack of sufficiently variable gene markers in haploid tissue from angiospermous forest trees gives cause to develop a method for analyzing the DNA from single pollen grains. Using the PCR-technology the DNA from single pollen grains of beech (*Fagus sylvatica* L.) could be amplified. The pattern generated from a 10-base random oligonucleotide of haploid and diploid tissue was compared. The described method will offer a new approach for paternity-analysis in angiosperms.

Key words: *Fagus sylvatica* L., PCR, pollen grain

INTRODUCTION

The evolutionary potential of forest tree populations depends on their genetic structure and variation which will be influenced by the gene flow via pollen and seed dispersal. In former times the amount of gene flow among forest tree populations was thought to be too low to have evolutionary significance because pollen and seed dispersal is limited. But some recent studies (see references in ADAMS 1992) showed that gene flow could thoroughly be an important evolutionary force. Hence, the extent of gene movement contributes to the levels of genetic diversity within populations, to the effective population size and to the degree to which populations become subdivided as a result of selection or drift.

In the case of gymnosperms, the measurement of gene flow can easily be done by assaying, *e.g.* with isozyme analysis, the megagametophyte tissue of seeds to determine the female contribution and at the same time the genotype of the successful male gamete can be inferred. Therefore, gene flow by pollen could be analyzed by using the paternity-exclusion procedures (STARKE & (MÜLLER-STARCK) 1977, ADAMS & BIRKES 1991).

For angiosperm populations, the paternity-exclusion analysis generally underestimates the gene flow because the foreign and local gametes within a population cannot be sufficiently distinguished (DEVLIN & ELLSTRAND 1990). Therefore, the development of molecular markers and techniques which leads to more efficient paternity-exclusion and allows a rapid screening of progeny will be a requisite for analyzing gene flow in angiosperm populations. The present study describes a method for the DNA extraction and amplification from a single pollen grain of beech (*Fagus sylvatica* L.) as a first approach facilitating the measurement of population genetics parameters. As beech is one of the carrier tree species of various forest ecosystems, this method offers the opportunity to study the extent of factors such as differential pollen fertility, distance dependent male mating success, floral phenology and stand structure which affect the pattern of outcrossing in natural stands. So the investigations of STARKE & MÜLLER-STARCK (1992) showed that the patterns of outcrossing and by this means the composition of the pollen cloud in beech stands were affected by the density and distribution pattern of the trees. Unfortunately, the lack of sufficiently variable gene markers in haploid material has not allowed an explanation of these effects.

Furthermore, these additional genetic markers could be used to determine the taxonomic identity of *Fagus* species which originated from interspecific hybridization in introgression zones. In the context of gene conservation it will be important to define the limits and direction of the gene flow between species and to characterize the diversity within the genus *Fagus* as pointed out by PAULE (1995) and GÖMÖRY *et al.* (1993).

MATERIAL AND METHODS

Plant material

The pollen was harvested from closed but fully differentiated male flowers of single trees of beech (*Fagus sylvatica*) in the laboratory under laminar flow in order to avoid bacterial contact therefore an antibacterial pre treatment was not necessary. From harvest to the preparation of single pollen grains the pollen was stored at –20 °C. The diploid bud tissue was harvested from the same trees.

Separation and germination of single pollen grains

Single pollen grains were separated according to the procedure described by KRABEL *et al.* (1996) and transferred to 3 5l germination medium (16 mmol·l^{-1} H_3BO_3, 7 mmol·l^{-1} $CaCl_2$, 584 mmol·l^{-1} sucrose) which were placed in 0.5 ml reaction tubes. For germination the samples were stored at 21 °C for 20 hours. The effectiveness of this step was microscopical controlled. The samples were centrifuged at 10 000 rpm for 1 min, treated with ultrasonic for 3 min in order to break the pollen tubes, and directly used for PCR amplification.

DNA extraction

The DNA from buds were isolated according the procedure described by ZIEGENHAGEN *et al.* (1993).

The DNA from several pollen grains of the same tree was isolated by adding 1ml extraction buffer according to METTLER (1987) (1% lauryl sarcosine, 50 mmol·l^{-1} NaCl, 20 mmol·l^{-1} EDTA, 50 mmol·l^{-1} Tris /HCl pH 8.0) to the germinated pollen on solid media followed by an incubation step at room temperature for 30 min. After an ultrasonic treatment for 3 min the samples were extracted with one volume phenol /chloroform then the DNA was precipitated by adding to volumes of ethanol at –20 °C. The DNA was resuspended in TE (10 mmol·l^{-1} Tris/HCl pH 8.0, 1 mmol·l^{-1} EDTA).

DNA amplification

For DNA amplification the conditions recommended by WILLIAMS *et al.* (1990) were modified as follows:

The reaction mixtures had a total volume of 50 ml containing 30 pmole of a single 10-mer oligonucleotide (Operon Techn. Inc. Alameda, CA, kit Y), all four dNTP4s (each at 200mM), 50mM KCl, 10mM Tris/HCl (pH 8,3), 2.5 mM $MgCl_2$, and 1 unit Taq polymerase (Boehringer, Mannheim, Germany). The reaction mixtures were overlayered with parafin oil (Merck, Darmstadt, Germany) before being placed in a Biometra thermal cycler with the following profile (*i*) 95 °C for 3 min for 1 cycle; (*ii*) 94 °C for 1 min, 36 °C for 1 min and 72 °C for 3 min for 44 cycles; and (*iii*) 72 °C for 10 min for the last cycle.

In the case of DNA amplification from buds and several pollen grains with the same oligonucleotide 12 ng DNA served as template DNA. Control reactions were done with all components on the one hand without DNA and on the other hand without primer to exclude amplification of exogenous DNA

The PCR amplification products were precipitated by addition of 0.1 volume 3 M NaAc and 2.5 volume of 96% EtOH, washed in 70 % EtOH, dried, and redissolved in 10 5l TE buffer (10 mmol·l^{-1}, pH 8.0, 1 mmol·l^{-1} EDTA, pH 8.0) to concentrate the amplification products for agarose gel electrophoresis. DNA fragments were separated by length on 1.8 % agarose gels with 1 X TAE (40 mmol·l^{-1} Tris acetate, pH 7.8, 1 mmol·l^{-1} EDTA) as running buffer.

DNA fragments were stained with ethidium bromide and gels were photographed with a MP4-Polaroid camera under UV light (302 nm). The plasmid pBR 328 digested with *BglI* and *HinfI* (Boehringer DNA molecular weight marker VI) served as a standard for fragment size determination.

RESULTS AND DISCUSSION

The intention of this study was to develop a method which allows the detection of molecular markers for the analysis of haploid angiosperm tissue especially of single pollen grains of beech.

For analyzing gene flow by paternity analysis, which also includes a rapid screening of progeny, the method may offer the opportunity to exclude a higher rate of potential pollen donors by these gene markers. In the sense of gene conservation it might be a new tool for the analysis of gene flow between different species of the genus *Fagus* – *e.g. F. sylvatica* and *F. orientalis* – in introgressive hybrid zones.

At first, single pollen grains were separated and germinated in liquid germination medium. Subsequently, the amplification reaction was done by the polymerase chain reaction in the same reaction tube. Therefore, no further manipulations were necessary which could inhibit this procedure. Figure 1 shows the amplification products generated from the 10-mer primer Y 10 (Operon Techn.) of the DNA from haploid (pollen) and diploid (buds) tissue of two different beech trees. The band with the length of about 350 bp marked by an arrow discriminates the haploid as well as the diploid tissue of tree 1 (lane 2–5) from that of tree 2 (lane 6–11). The comparison between the banding

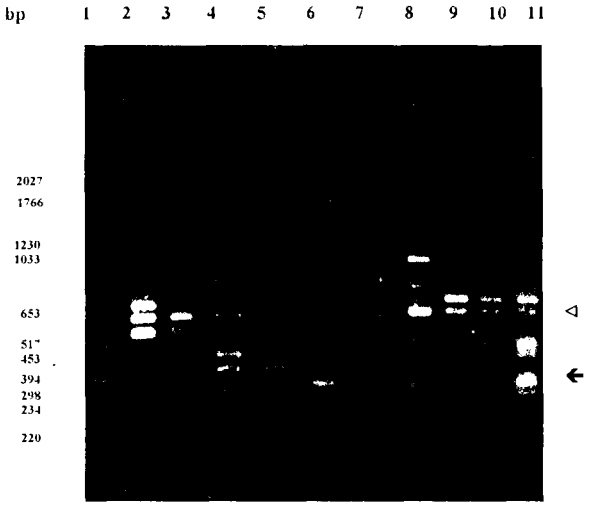

Figure 1 Amplification products generated with the primer Y 10 (Operon Tech.) of haploid and diploid tissue from two beech trees. Lane 1: molecular weight marker (Boehringer marker VI), lane 2-4: three different single pollen grains of tree 1, lane 5: buds of tree 1, lane 6: buds of tree 2, lane 7-11: five different single pollen grains of tree 2

patterns of single pollen grains (lane 2–4: tree 1 and lane 7–11: tree 2) and the diploid tissue of buds (lane 5: tree 1 and lane 6: tree 2) rom the same tree shows differences especially for the larger amplification products. This might could be explained by recombination events in the diploid tissue.

Larger amplification products of the DNA from haploid tissue were obtained by using 12 ng of template DNA from several pollen grains (KRABEL *et al.* 1996).

The band of about 650 bp length which is marked with an open triangle and which is present in all haploid tissue so far tested but absent in the diploid tissue (lanes 5, 6) could represent a pollen specific sequence.

For parentage analysis it might be better to reduce the complex banding pattern which is generated by RAPD-markers to only a few bands, thus, the comparison between different single pollen grains from the same pollen cloud will also be facilitated as shown in Fig. 1 five different amplification patterns generated from the same primer were different from five pollen grains of the same tree (lane 7–11). This could be achieved by PCR amplification of specific alleles (PASA) because in this case coding sequences of specific genes were used as primers (SARKAR *et al.* 1990).

However, the genotype of any one pollen could not be confirmed by a second PCR analysis, therefore for further characterization, *e.g.* by sequencing or by restriction fragment length analysis the amplification product must be amplified by cloning.

Further studies to determine the selfing rate in beech have just been started. Therefore, pollen grains were sampled from single isolated individuals of beech. The data from isozyme analysis of these individuals and their progeny indicated that even in this case the proportion of selfed beech nuts is less than 15% (KRABEL, unpublished data). Thus, the most likely reason for this low proportion is gene flow from moderately distant stands or the accumulation of pollen dispersed from distant individuals within the same habitat. The method described in this investigation might be a first requisite in order to examine the composition of the pollen cloud to determine the sources of pollen making up the self-fertilization and the outcrossing component.

ACKNOWLEDGMENT

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REFERENCES

ADAMS, W.T. 1992: Gene dispersal within forest tree populations. *In:* Adams, W. T., Strauss, S. H., Copes, D. L. and Griffin, A.R. (eds) Population Genetics of Forest Trees. pp.211 – 240. Kluwer Academic Publishers, Dordrecht.

ADAMS, W. T. & BIRKES, D. S. 1991: Estimating mating patterns in forest tree populations. *In:* Fineschi, S., Malvolti, M.E., Cannata, F. and Hattemer, H.H. (eds) Biochemical Markers in the Population Genetics of Forest Trees. pp. 157 –172. SPB Academic Publishing, The Hague.

DEVLIN, B. & ELLSTRAND, N. C. 1990: The development of a refined method for estimating gene flow from angiosperm paternity analysis. *Evolution* **44**:248–259.

GÖMÖRY, D., VYŠNÝ, J. & PAULE, L. 1995: Genetic differentiation of populations in the transition zone between *Fagus sylvatica* L. and *Fagus orientalis* Lipsky. *In:* Madsen, S.F. (ed) Genetics and Silviculture of Beech. *Forskningsserien* **11**:238–244

KRABEL, D., VORNAM, B. & HERZOG, S. 1996: PCR-based random amplification of genomic DNA from single pollen grains of trees. *Silvae Genetica*, submitted.

MÜLLER-(STARCK), G. 1977: Cross-fertilization in a conifer stand inferred from enzyme gene-markers in seeds. *Silvae Genet.* **26**:223–226.

PAULE, L. 1995: Gene conservation in European beech (*Fagus sylvatica* L.). *Forest Genetics* **2**:161–170.

SARKAR, G., CASSADY, J., BOTTEMA, C. D. K . & SOMMER, S.S. 1990: Characterization of polymerase chain reaction amplification of specific alleles. *Anal.Biochem.* **186:** 64–68.

STARKE, R. & MÜLLER- STARCK, G. 1992: Genetische Untersuchungen über die Reproduktion in zwei Beständen der Buche (*Fagus sylvatica* L.). *In:* Biochemische Untersuchungen zur Genetik von Waldbaumpopulationen. pp. 57–67. Schriftenreihe der Landesanstalt für Forstwirt-

schaft Nordrhein-Westfalen 1992.

WILLIAMS, J.G. K., KUBELIK, A. R., LIVAK, K. J., RAFALSKI, J. A. & TINGEY, S.V. 1990: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acid Res* **18**:6531–6535.

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

ERRATA

In the paper of **Luomajoki, A.: Adaptation of microsporogenesis of exotic conifers in Finland *Forest Genetics* 3(3):153–160, 1997**, the following errors appeared:

In tables 2 a 3 instead of "Punkharju" there should be "Punkaharju". This error appeared 27 times in both tables.

Since *Pinus mugo* is an exotic tree species in Finland there should be in the caption of Figure 2 full dot instead of empty one.

Thus the correct Figure caption should be:

Figure 2 Period unit heat sum average and corresponding average date at 50% completion of anthesis for the stands of each species shown: 1 – *Alnus incana* (○), 2 – *Corylus* (○), 3 – *A. glutinosa* (○), 4 – *Thuja* (•), 5 – *Populus* (○), 6 – *Larix gmelini* (•), 7 – *L. sibirica* (•), 8 – *L. decidua* (•), 9 – *Taxus* (○), 10 – *Betula pendula* (○), 11 – *B. pubescens* (○), 12 – *Abies balsamea* (•), 13 – *A. veitchii* (•), 14 – *Picea glauca* (•), 15 – *P. abies* (○), 16 – *A. sibirica* (•), 17 – *P. omorika* (•), 18 – *Quercus* (○), 19 – *P. mariana* (•), 20 – *Pinus banksiana* (•), 21 – *Juniperus* (○), 22 – *P. sylvestris* (○), 23 – *P. contorta* (•), 24 – *P. mugo* (•), 25 – *P. cembra* (•), 26 – *P. peuce* (•)

In the paper of **Toda, Y.: Karyomorphological studies of the *Taxodiacae*. *Forest Genetics* 3(3):141–147, 1996**, the pages in the header should be corrected as follows:

FOREST GENETICS 3(3):141–146, 1996.

In the paper of **Vornam B.: DNA amplification from single pollen grains of beech (*Fagus sylvatica* L.). *Forest Genetics* 3(4):213–216 , 1996** the following errors appeared:

p. 213, left column, line 7 from bottom	instead of "(STARKE & (MÜLLER-STARCK)" should be "MÜLLER-(STARCK)"
p. 214, left column, line 14 from top	instead of "3 5l" should be "3 µl"
p. 214, left column, line 13 from bottom	instead of "to" should be "two"
p. 214, left column, line 7 from bottom	instead of "50 ml" should be "50 µl"
p. 214, left column, line 4 from bottom	instead of "dNTP4s"should be "dNTP's
p. 214, right column, line 15 from top	instead of "10 5l" should be "10 µl"
p. 215, left column, line 3 from top	instead of "rom" should be "from"

We apologize for this inconvenience to the authors of the above mentioned papers and readers of Forest Genetics.