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Use of RAPD patterns for clone verification and in studying provenance relationships in Norway spruce (*Picea abies*)

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Abstract We have used the RAPD technique to analyse samples of *Picea abies* obtained from an improvement forestry station. Two types of plant material were harvested, the first being clones and the second provenances from various regions. We first checked the clonal identity of elite tree cuttings and clones; some differences in the RAPD patterns resulting from misplanting or mis-labelling of cuttings were found. We also established a reference library of RAPD fingerprints for 96 clones, which will serve as a reference source in cases of litigation concerning clone identity. The RAPD technique was also used to study the genetic relationship between nine European provenances of Norway spruce. A dendogram was obtained by individual pairwise comparison of 42 RAPD bands, which separated the nine provenances into two major groups, one containing the Nordic provenances (Sweden and Bielorussia) and another the Alpine provenances (France, Austria, Germany and Belgium). The Belgian provenance, which is not indigenous, is most closely related to the German provenance. We conclude that the RAPD technique is a useful tool for forestry stations in managing propagation operations.

Key words RAPD · Clones · Natural populations · *Picea abies*

Introduction

Because of its high productivity and resistance to cold climates, Norway spruce is one of the most common trees in Europe, especially in the North. This fast-growing species produces wood used in paper-mills and saw-yards. At first, improvement programs were based solely on individually selected provenances, but clonal forestries are now slowly starting to be used. When properly performed, the vegetative propagation method is a powerful means of capturing the genetic superiority of selected individuals. However, labelling and sampling errors are unfortunately very common during these operations and are difficult to detect by visual inspection of plants; therefore tools are needed to check that the cutting operations are properly performed.

Many genetic properties are still unknown in the majority of tree species and it is therefore important that the gene pool be preserved and the level of intra-population diversity and interpopulation differentiation evaluated. Among forest trees, conifers are known for their high genetic variability. The evaluation and conservation of this genetic diversity is of crucial importance for the perpetuation of forests, both in the context of environmental constraints illustrated by the recent forest decay and in the prospect of the announced climate modifications. In Belgium, *Picea abies* is not native, being introduced only in the last century, but constitutes 80% of conifers planted (Rondeux et al. 1986). Information on the origin of the Belgian populations could be useful in future improvement programs. Most studies have been based on morphological traits or isozymes (Tigerstedt 1973; Lundkvist and Rudin 1977; Borghetti et al. 1988; Langercrantz and Ryman 1990). Recently, however, the use of molecular markers, such as random amplified polymorphism DNA (Welsh and McClelland 1990; Williams et al. 1990), has been applied to the detection of plant genetic polymorphism.

The objective of the present research was to use random amplified polymorphic DNA (RAPD) markers:

(1) to check the clonal identity of hedge clones,
(2) to establish a fingerprinting reference library for the *P. abies* multiclonal variety, and

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(3) to estimate the genetic relatedness between nine European populations of *P. abies* (L.) Karst, using the DNA bulking procedure, and to provide more detailed information on the origin of the Belgian *P. abies* populations.

Materials and methods

Plant material

All plant material (*P. abies*) was supplied by the Station de Recherches Forestières du Ministère de la Région Wallonne at Gembloux, Belgium (Nanson 1974; Piroton 1992).

Clones

The original stockplant of a clone is called the "ortet", whereas the plants derived from a cutting from the ortet are referred to as "ramets". Samples of clones were obtained from three different sites in Belgium:

(1) Fenffe, clones of elite trees; needles from ten ramets of six different elite trees (A9-A23-A58-A60-A61-A106) were analysed to check their clonal identity.
(2) Vielsalm, (a) needles from nine ramets of 22 selected clones were collected to check the clonal identity; (b) ten to fifteen 6-year-old ramets from 96 different clones (sibling of elite trees) of the multiclonal variety were collected at a nursery.
(3) Groenendaal, the Vielsalm ramets were derived from ortets at this site; ortets from the 22 clones selected in Vielsalm were also collected.

Needles were frozen in liquid nitrogen before storage at $-70\,^{\circ}C$.

Provenances

Nine European provenances of *P. abies* (Karst) were studied (Table 1). Needles from 20 individual trees per provenance were collected in the Tahanfagne plantation (Belgium). These provenances were planted 30 years ago from seed harvested in the different regions of origin. All provenances are indigenous, except those from Belgium, which were imported.

DNA extraction

DNA was extracted from needles or buds, using the Doyle and Doyle (1990) protocol (Focus BRL). Needles were ground in liquid nitrogen, using a pestle and a mortar. Buds were ground directly in an Eppendorf tube in 50 µl of CTAB buffer, using a small pestle. The amount of purified DNA was measured by fluorimetry.

For phylogenetic analysis DNA was extracted from individual trees and pooled together in the same proportion for each provenance after purification and quantification. Equal amounts of genomic DNA from 20 individual trees were mixed for the bulked RAPD analyses.

Table 1 List of the provenances used in the RAPD analysis

Code	Name	Country	Longitude	Latitude
06	Görtschitztal	Austria	14.30	46.52
16	Schwarzwald	Germany	8.17	48.17
20	Sandvik	Sweden	13.33	57.18
22	Tidaholm	Sweden	13.97	58.17
29	Wolozin	Bielorussia	26.50	54.08
31	Mignovillard	France	6.20	46.75
32	St Laurent	France	6.03	46.57
33	Gerardmer	France	6.90	48.18
B(5820)	Bullange	Belgium	6.33	50.42

RAPD procedure

RAPD reactions were performed in a volume of 25 µl containing 100 ng of template DNA, 10 pmoles of primer DNA (0.4 µM), 100 µM of each dNTP, 1 U *Taq* polymerase (Pharmacia) and 2.5 µl of *Taq* polymerase 10× buffer Pharmacia (500 mM KCl, 15 mM MgCl2, 100 mM Tris-HCl pH 9.0). The sample was covered with 25 µl of mineral oil. For DNA amplification, the reaction was performed in a Perkin-Elmer Cetus DNA 480 Thermal Cycler, using a PCR profile consisting of one cycle of 3 min at 93°C, 1 min at 37°C, 2 min at 72°C and then 35 cycles of 1 min at 93°C, 1 min at 37°C, 2 min at 72°C. The last cycle was followed by 10 min at 72°C. After amplification, the products were separed by 1.3% agarose-gel electrophoresis and detected using ethidium bromide. A total of 140 primers (Operon Technologies, Alameda, California; kit A to H) was used.

Data analysis

All polymorphic bands were scored as 1 for present and 0 for absent. Estimations of similarity were based on the number of shared bands amplified (Nei 1987). The phylogenetic tree was constructed by the unweighted pair-group method with arithmetic average (UPGMA) and the Neighbor-joining method using PHYLIP (Phylogenetic Inference Package).

Results

Clone analysis

Elite trees

Elite trees were selected from provenances and planted in a seed orchard; the seeds were then used to produce the multiclonal variety (600 clones are selected and propagated by cuttings for this purpose). It is therefore important to be certain of the identity of the original elite tree, since it was to be used as a parent in open or controlled pollination to produce the multiclonal variety. We analysed ten ramets from six different elite trees, using three different primers. No differences in RAPD profile were detected in five of the six clones. Figure 1 shows an example of one of these clones (A106), the ramets of which all exhibit the same RAPD profile. However, the sixth clone (A61) showed two kinds of RAPD profiles with all three primers, with ramets A61 (0-6-7-8–9) presenting a different profile to ramets A61 (1-2-3-4–5) (Fig. 1), indicating that two different genotypes had been mixed during the cutting or labelling operations.

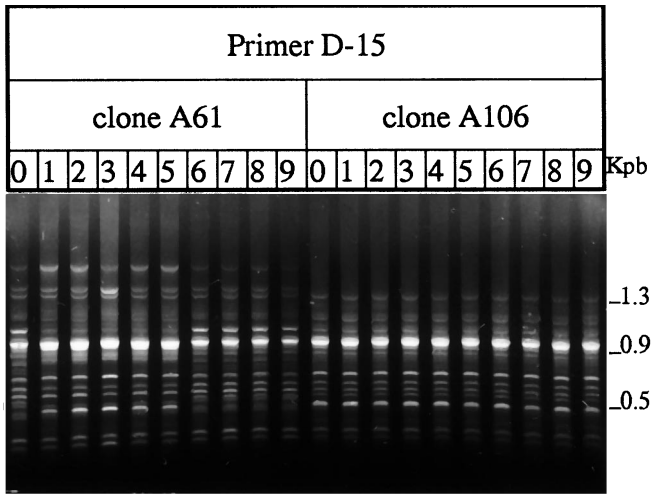

Fig. 1 RAPD profiles of *P. abies* ramets of two elite tree clones, A61 and A106, using primer D-15. Clone A61 is not homogenous, with ramets 0-6-7-8-9 showing a different profile to ramets 1-2-3-4-5. Clone A106 is homogenous, with all the ramets showing the same RAPD profile

Multiclonal variety

To check clonal identity, we compared the RAPD profiles of nine ramets and the ortet of 22 clones, again using three different primers. Each reaction was performed at least twice to check the reproducibility of the RAPD method. No differences in RAPD profiles were detected for the 18 clones.

Figure 2a shows the results obtained for one of these clones, clone 1068 (primer OPG-05). The other clones showed differences in their RAPD profiles. With both clones 1120 and 1095, ramet 1 showed a different profile from the original ortet and therefore does not belong to this clone (Fig. 2a). A special case was found when clones 1093 and 1094 were analysed. During sampling, we noted that each of these clones consisted of a mixture of trees with early buds and trees with late buds. Since the early/late character of the bud is clone-specific, it seemed that two clones had been mixed and misplanted. However, on RAPD analysis of seven ramets from each clone, three different RAPD profiles

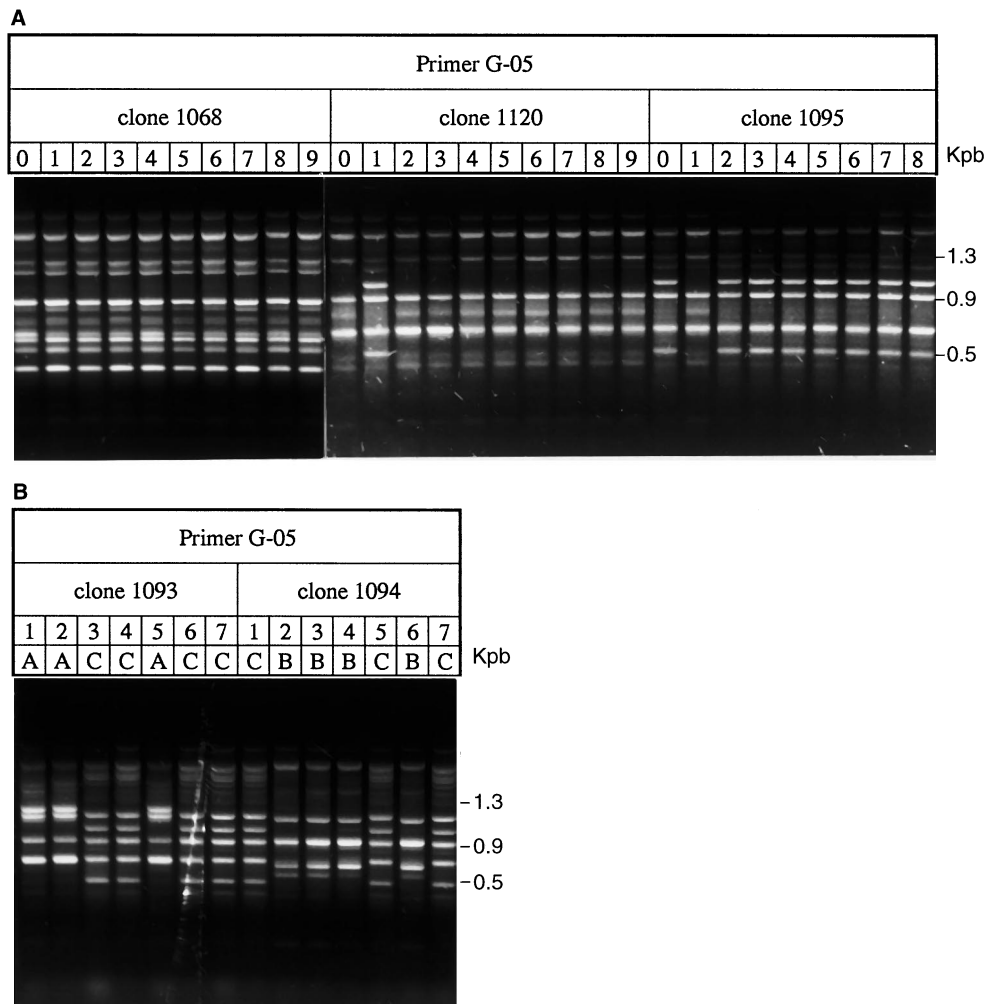

Fig. 2A, B RAPD profiles of ramets of *P. abies* clones using primer G-05. **A** 0 is the ortet and numbers 1 to 9 are ramets of the clone. With clone 1068, the ortet and all ramets show the same RAPD profile. With clones 1120 and 1095, ramet 1 shows a different RAPD profile from the ortet and the other ramets. **B** RAPD profile of two clones (1093 and 1094) using primer G-05. *A*, *B*, *C* represent the three types of profiles seen. Profiles *A* and *B* are associated with clones 1093 and 1094, respectively, whereas profile *C* is not derived from either or these

(A, B and C) were seen (Fig. 2b), with the profile type corresponding to the early/late character of the bud: A and B corresponding to trees with late buds and C to trees with early buds. Thus the original clones 1093 and 1094 are trees with late buds (1093 1-2-5 and 1094 2-3-4-6) mixed with ramets from a contaminant early bud clone (1093 3-4-6-7 and 1094 1-5-7).

To establish a fingerprint reference library and to check the identity of propagated clones, samples of 96 clones of the multiclonal variety were collected; this library will serve as a reference source of RAPD profiles for each clone to allow them to be identified unambiguously. To test the validity of the library, 18 clones from the Forestry Reseach Station were tested blind with the aim of identifying them by comparing their RAPD profiles with those in the library. Four primers were used to establish the data base. Two (primers OPG-05 and OPG-12) gave a RAPD profile which was highly specific for each clone. Approximately 20 bands of various intensities were amplified with the primers. Figure 3

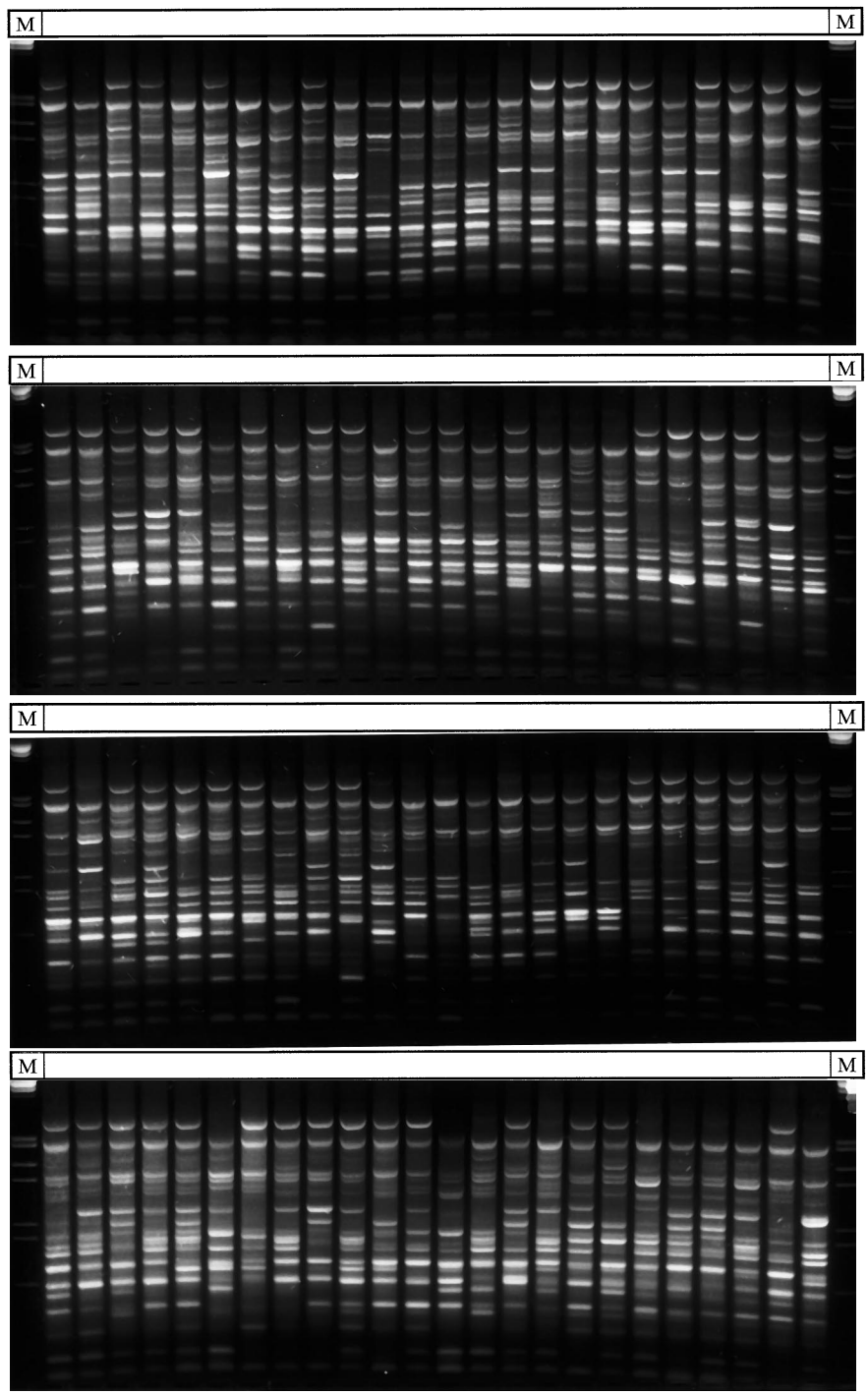

Fig. 3 Fingerprint library. RAPD profiles of 96 clones from the multiclonal variety, using primer G-05. Each lane represents a single individual of the multiclonal variety. *M* is the lambda molecular-size marker digested by *Eco*RI and *Hin*dIII

shows the RAPD profiles of the 96 clones amplified using the G-05 primer. The DNA from the 18 test clones were amplified with the same primers and the RAPD profiles compared with those in the data base. Of the 18 clones, four appeared not to belong to the multiclonal variety, since their RAPD profile was not present in the library. All the remaining clones were found in the library and were identified unambiguously. Using this method, it was possible to distinguish clones even as closely related as half-sib.

Provenance

To estimate the relationship between heterogeneous populations (nine European *P. abies* provenances), the bulking DNA method of analysis was used on DNA from 20 individuals per provenance. The reproducibility of the RAPD reaction was tested by repeating the process at least twice for each primer and the RAPD patterns were found to be highly reproducible. Of the 160 primers tested, 156 produced a total of 998 amplified bands, giving an average frequency of 6.4 RAPD bands per primer. Of these 998 bands, 78 (7%) were polymorphic. Figure 4 shows a representative example of the electrophoretic pattern obtained after RAPD amplification of the nine European provenances of *P. abies*, using primers D-17 and D-19.

The analysis used to generate the dendogram was the UPGMA method, employing the Nei index (Nei 1987). The data were, in addition, subjected to another method of calculation of the genetic distance matrix; no significant diffrences were seen in the dendograms obtained. The relationship between the nine provenances estimated by UPGMA cluster analysis is shown in Fig. 5. Two groups can be seen, one of which contains the Nordic provenances from Sweden (20 and 22) and Bielorussia (29) and the other the provenances from France (31, 32, 33), Germany (16), Austria (06) and Belgium (5820). The two groups are well separated and this result is consistent with the theory of probable migratory routes in the post-glacial area, which presents a common origin for the provenances from Scandinavia and Russia to constitute the actual Baltico-Nordic domain of naturally distributed Norway spruce.

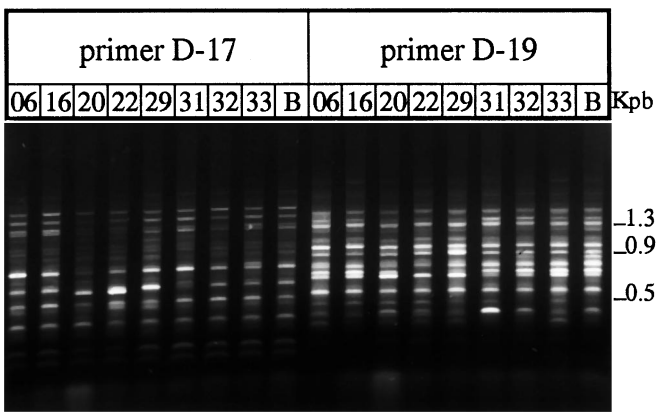

Fig. 4 RAPD analysis on provenances. DNA from 20 individuals tree from the same provenance were bulked together and amplified, using primers D-17 and D-19. The *numbers* are the codes for the provenances (see Table 1)

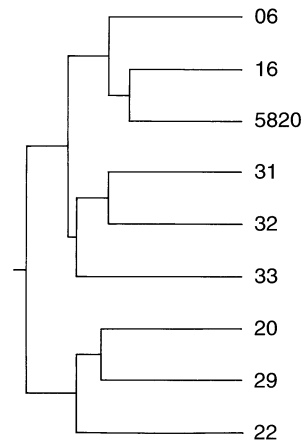

Fig. 5 The dendogram generated by clustering using UPGMA analysis computed from a pairwise comparison of RAPDs from nine *P. abies* provenances. The *numbers* are the codes for the provenances (see Table 1)

Discussion

A high reproducibility of RAPD patterns is a prime requirement for the use of the RAPD method in verifying clonal identity. Care must be taken during all the operations involved in the RAPD reaction. We found that one of the most important parameters in terms of reproducibility is the amount of DNA used in the reaction, as the RAPD profile is dependent on this (data not shown). Very precise quantification of DNA after purification is therefore a crucial step.

This study shows that the RAPD method can be used to check the clonal identity of *P. abies* plants propagated from cuttings. Of the six clones of elite trees, one showed a mixture of two different patterns and therefore seems to be a mixture of two different clones. It is absolutely critical to be certain of the identity of the starting material. Of the 22 clones of the multiclonal variety analysed, four presented polymorphism in their RAPD profiles. Mis-planting or mislabelling of clones appears to be a common occurrence during forestry operations (Harju and Muoana 1989; Wheeler and Jech 1992; Keil and Griffin 1994). The intraclonal differences detected by RAPDs do not seem to arise from somaclonal variation. Studies on the embryogenic mass of *P. abies* have been unable to detect somaclonal variation by RAPDs, even after 5 years of regeneration of the mass (Fourré 1995).

Similar results have been reported by other authors (Heinze 1993; Isabel et al. 1993).

Our results demonstrate that, using the protocol described, it is possible to establish a "fingerprint" data bank of RAPD patterns of 100 different clones of *P. abies*, and using a single primer to distinguish all 100. Such data banks are needed both as reference libraries and to protect the forestry reseach station's patent rights for clonal varieties.

The bulking procedure has been successfully used to estimate the relatedness between alfalfa populations (Yu and Pauls 1993). In the present study, we used the method to compare heterogeneous natural populations of *P. abies*, as this would be expected to lead to a more representative population of bands for a natural provenance than the use of DNA from a single plant.

Norway spruce is found naturally in three zones in Europe; the Alpine domain (from the French Alps to the Jura and ex-Yugoslavia), the Carpatho-Hercynian domain (Germany, Romania, ex-Czechoslovakia and Ukraine), and the Baltico-Nordic domain (from Scandinavia to Russia, including Poland and the Baltic States). In other regions, Norway spruce has been introduced, e.g. North America, Canada, parts of France and Belgium. Our results from the phylogenetic tree for the different provenances show the same distribution according to geographical origin, with the Belgian provenance (B5872) being most closely related to the German provenance (16). This result is consistent with what is know about the origin of the Belgian provenance, which was probably planted by the Prussian forestry commission in 1894.

An interesting application of this RAPD survey will be to establish provenance-specific primers. We are currently investigating this approach using the SCAR (sequence characterized amplified regions) technique (Paran and Michelmore 1993) to select specific bands.

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