Are RAPD-markers Reproducible Between Different Laboratories?

A Case Study of *Picea abies* (L.) KARST.

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Abstract

The widespread application of RAPD-markers in diverse organisms has highlighted a need to demonstrate the repeatability of these markers across different laboratories, using deviating protocols, as well as across different genetic material within our organism, *Picea abies* (L.) KARST..

The present investigation has i) compared the segregation of RAPD-markers in the haploid megagametophytes of the same tree analyzed at two different laboratories and ii) compared the zygosity of two geographical diverse individuals.

Under the assumption that the same band positions represent identical RAPD fragments, a repeatability of 93% across laboratory environments in a sample of 43 markers distributed to 18 decamer Operon primers was obtained. Further, of 40 observable heterozygotic loci in tree no. 1, 75% were scored as heterozygotic in tree no. 2.

Key words: Picea abies (L.) KARST., RAPD-markers, repeatability between laboratories.

FDC: 165.3/4; 174.7 *Picea abies.*

Introduction

On the background of the developed procedures for using RAPD-markers in Norway spruce, which have included check of MENDELian inheritance and tissue expression (SKOV, 1998a), timing of DNA extraction from megagametophytes during initial steps of seedling development (SKOV, 1998b) and using the recombination of markers in the haploid megagametophytes for mapping (SKOV and WELLENDORF, 1998), the present investigation is aiming to demonstrate to which degree RAPD-markers are reproducible between different laboratories and compare the zygosity of two geographical diverse individuals.

Seen in the light that every laboratory has developed its own optimization procedure to obtain reliable reproducible results, it will be interesting to compare results from another laboratory with results from the Danish optimization procedure on material common to both places. This might give a much needed expression of stability of the RAPD assay across different laboratory environments.

The present investigation tries to uncover problems concerning repeatability of RAPD-markers in an Italian initiated dataset based on laboratory work carried out at USDA Southern Forest Experiment Station, Gulfport, Mississippi and a dataset of Danish origin. Both datasets concerned scoring of RAPD-markers segregating in haploid megagametophytes of an Italian tree. Megagametophytes of this tree served as mapping population for the partial linkage map reported by BINELLI and BUCCI (1994). Further, we compare the zygosity of the Italian tree with the zygosity of a Danish cloned tree (V6470) on specific RAPD loci observable in both trees.

A reasonable resemblance of such comparisons is a prerequisite for accumulating joined maps based on two or more partial maps developed at different laboratories on different genetic material.

Material and Methods

Plant material

Seeds from the Danish plantation-grown elite clone V6470 of West continental origin, which basically served as the first mapping unit in the Danish genome mapping program (SKOV and WELLENDORF, 1998), were combined with seeds from one Italian wild stage tree from the autochtone North Italian stand Campolino. Megagametophytes from this particular tree have earlier served as mapping population in the published partial genome map of *Picea abies* (BINELLI and BUCCI, 1994).

The seeds were germinated according to the 'KNUDSEN' method described by (SKOV, 1998b), and megagametophytes were stored at −80°C in separate microcentrifuge tubes until DNA extraction.

DNA extraction, – amplification, and gel electrophoresis

From each of the two parent trees, total genomic DNA was extracted from 20 individual megagametophytes. Sampling was carried out at a late stage during seed germination, just before spontaneous release of megagametophytes as described by SKOV (1998b). For V6470 supplementary sampling and DNA extraction were carried out on diploid leafprimordia to use as reference tissue of that particular parent.

Amplification of the DNA was performed according to WILLIAMS et al. (1990) with the adjustment given by SKOV (1998a and b) for optimization in Norway spruce. The following extension concerned application of two types of DNA polymerases in order to cover a broader range of length variation in the amplified PCR products.

Two types of reaction mixes were thus prepared according to the DNA polymerase used. Reaction volumes of 20 µl contained as follows:

1) AmpliTaq DNA polymerase, AS (Perkin Elmer, Cetus) 0.5 unit per reaction, 2 µl 10 x PCR Buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.0 mM $MgCl_2$, 200 mM of each dATP, dCTP, dGTP and dTTP (Pharmacia), 16 ng of a ten base random primer (Operon Technologies, Alameda, CA) and 2 ng to 5 ng template DNA, or –

2) AmpliTaq DNA polymerase, Stoffel Fragment (Perkin Elmer, Cetus) 1.5 to 3.0 units per reaction, 2 µl 10 x Stoffel Buffer (100 mM KCl, 100 mM Tris-HCl, pH 8.3), 2.5 mM $MgCl_2$ and the remaining group of components as mentioned above.

The reactions were prepared on ice and transferred to a 93°C thermocycler (Perkin Elmer, Cetus, 480) programmed for an initial denaturation stage at 93°C in 5 min and subsequent 45 cycles consisting of 1 min at 93°C, 1 min at 36°C and 2 min at 72°C. The fastest attainable transitions between each temperature were employed. After cycling the reactions were soaked at 4°C until recovery.

A volume of 16 µl of amplification product was added to 4 µl loading buffer II and analyzed by electrophoresis in 1.4%

Table 1. – Summary of experience with repeatability of 18 decamer primers from Operon Technologies, constituting 43 loci, investigated in megagametophytes (N) of *P. abies* (L.) KARST. See text for further information.

PRIMER:	INVESTIGATED LOCI (bp) (Binelli & Bucci ,1994)	REPRODUCED IN ITALIAN MEGAGAMETOPHYTES	REPRODUCED IN DANISH MEGAGAMETOPHYTES	AMPLIFICATING DNA-POLYMERASE
OA06	1680	x (1680)	Homozygotic recessive	2 (3.0 U)
OA06	1000	x (1020)	Segregating	2 (3.0 U)
OA06	0790	x (0790)	Segregating	2 (3.0 U)
OA07	1100	x (1090)	Segregating	1 (0.5 U)
OA08	2410	x (2410)	Segregating (indistinct)	1 (0.5 U)
OA08	1285	x (1250)	Segregating	1 / 2 (0.5U / 2.5 U)
OA08	0383	x (0370)	Homozygotic recessive	2 (2.5 U)
OA08	0340	x (0340)	Homozygotic recessive	2 (2.5U)
OA08	0302	–	–	1 / 2 (0.5 U / 1.5 U and 2.5 U)
OA11	0595	x (0590)	Segregating	2 (2.5 U)
OA11	0400	x (0440)	Segregating	2 (2.5 U)
OA11	0337	x (0310)	Segregating	2 (2.5 U)
OA12	0790	x (0760)	Segregating	2 (3.0 U)
OA12	0582	x (0560)	Segregating	2 (3.0 U)
OA12	0451	x (0450)	Segregating	2 (3.0 U)
OB02	1750	x (1750)	Segregating	1 (0.5 U)
OB02	1150	x (1150)	Segregating	1 (0.5 U)
OB05	1150	x (1150)	Segregating (indistinct)	1 (0.5 U)
OB05	1020	x (1020)	Segregating	1 (0.5 U)
OB08	0632	x (0630)	Segregating	1 (0.5 U)
OB13	1986	–	–	1 / 2 (0.5 U / 1.5 U and 3.0 U)
OB13	1900	x (1900)	Homozygotic dominant	1 (0.5 U)
OB13	1093	x (1093)	Homozygotic dominant	2 (3.0 U)
OB13	1050	x (1050)	Segregating	2 (3.0 U)
OC10	0830	x (0830)	Homozygotic recessive	1 (0.5 U)
OC10	0805	x (0805)	Segregating	2 (1.5 U)
OC10	0514	x (0514)	Segregating	2 (1.5 U)
OC10	0339	x (0339) Homozygotic dominant	Segregating	2 (1.5 U)
OD14	1159	x (1159)	Segregating	1 (0.5 U)
OD14	0300	x (0300)	Homozygotic recessive	1 (0.5 U)
OE12	1300	x (1300) Homozygotic dominant	Homozygotic dominant	1 (0.5U)
OE17	1720	x (1800)	Segregating	1 (0.5 U)
OE17	1550	x (1550)	Segregating	1 / 2 (0.5 U / 3.0 U)
OF14	1000	x (1030) Homozygotic dominant	Segregating	1 (0.5 U)
OF14	0850	x (0805)	Segregating	1 / 2 (0.5 U / 3.0 U)
OF14	0750	x (0740)	Homozygotic recessive	2 (3.0 U)
OF16	2000	x (2000) indistinct	Segregating	1 (0.5 U)
OF16	1950	–	–	1 (0.5 U)
OF16	1920	x (1900)	Segregating	1 (0.5 U)
OG10	0470	x (0450)	Homozygotic recessive	2 (3.0 U)
OJ01	0550	x (0560)	Segregating	2 (3.0 U)
OJ06	1250	x (1275)	Segregating	2 (3.0 U)
OJ06	0820	x (0850)	Segregating	2 (3.0 U)

Concerning column five. 1 = AmpliTaq DNA polymerase, AS and 2 = AmpliTaq DNA polymerase, Stoffel Fragment. Numbers in brackets = Units enzyme used per reaction.

agarose TBE gels with ethidiumbromide (SAMBROOK et al., 1989). Bands were visualized by UV light and photographed with Polaroid film for instant interpretation.

With the intention to obtain a complete picture of the investigated loci special optimization was performed for the individual primer. Focus was put on primer concentration and use of different DNA polymerases as well as the concentration of the these.

Each set-up for PCR consisted of at least one diploid reference and 10 megagametophyte from V 6470 (the Danish clone) run side by side with 10 megagametophytes from the Italian mapped tree.

Choice of primers

The primers investigated are listed in *table 1.* They constitute a random sample of eighteen amongst the applied Operon decamer primers in the Italian mapping work (BINELLI and BUCCI, 1994). These primers cover a sample of 43 segregating mapped loci in the Italian tree.

Choice of DNA polymerase

AmpliTaq DNA polymerase, AS, and AmpliTaq DNA Polymerase, Stoffel fragment were used interchangeable in separate runs of the same primer. The last mentioned has been reported to facilitate the creation of smaller PCR products with enhanced reproducibility (Perkin Elmer, personal communication, 1994).

Results

Results are summarized in *table 1.* In the first column are listed a test sample of eighteen original investigated primers in Gulfport of the Italian tree. These primers constitute the foundation of the present investigation. In the Gulfport run the primers were scored according to the second column resulting in 43 segregating loci named by basepairs of the segregating fragment with reference to a Pst 1 digested λ marker. Column three documents to which degree it had been possible to reproduce the segregating loci established in Gulfport. The estimates of basepairs are regarded as an approximation, with the Danish scoring mentioned in brackets. A '–' sign indicates, that the respective loci not could be reproduced. Column four demonstrates the success in refinding the defined loci in another Norway spruce tree of diverse geographic origin, the Danish clone V6470. The status of the PCR fragments is here noted as segregating, homozygotic dominant, homozygotic recessive or '–' : missing observation. The base pair sizes are identical with the estimates of column three as these two samples were run side by side on the same gel. In the fifth column are mentioned the amplificating polymerase used for PCR. To avoid confusion in the polymerase nomenclature, AmpliTaq DNA polymerase, AS and AmpliTaq DNA polymerase, Stoffel Fragment are referred to as respectively 1 and #2 in *table 1,* and the number of units used are mentioned in brackets for the individual locus.

Thus the presentation of results in *table 1* is divided into two parts, i) comparing the same tree analyzed at the two laboratories (column 2 and 3) and ii) comparing the zygosity of the two parent trees analyzed at our laboratory (column 3 and 4).

Comparison of the Italian tree analyzed at the two laboratories

The following three points proved to be decisive when comparing the two laboratories:

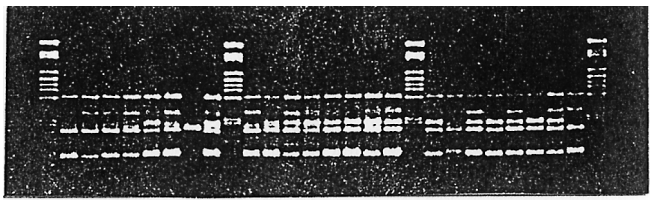
a) Resemblance between the general amplification pattern for individual primers;

b) The range of observable fragment sizes must correspond to each other;

c) Evidence of identity of specified RAPD fragments.

Ad a) – The general amplification pattern

For AmpliTaq DNA polymerase, AS, each primer utilized in this comparative study, demonstrates clearly a specific amplification pattern which is common for the original Gulfport investigation and the present investigation – see *figure 1* and *2* for examples. In these two figures are shown the specific amplification pattern for the two primers OA07 and OE12 respectively. Primer A07 is a good example because of the homozygotic dominant status given at basepair position 1800 and 620. Compared to the original Gulfport run there is a striking resemblance. Such situations are general for the tested primers. It is especially rewarding, as the applied DNA polymerases are of different brand in the Gulfport and in the Danish investigation – Taq DNA polymerase (Boehringer, Mannheim) and AmpliTaq DNA polymerase (Perkin Elmer, Cetus) – respectively.



a) RAPD analysis of Italian megagametophytes originally obtained in Gulfport using primer OA07.

Ad b) – Specific optimization for each primer

As earlier reported (SKOV, 1998a) a change in the primer concentration changes the size of the amplified RAPD fragments. When using high primer concentration shorter fragments are achieved and vice versa. This is a valuable aspect in closely defined jobs as the present, in which specific locus positions are seeked.

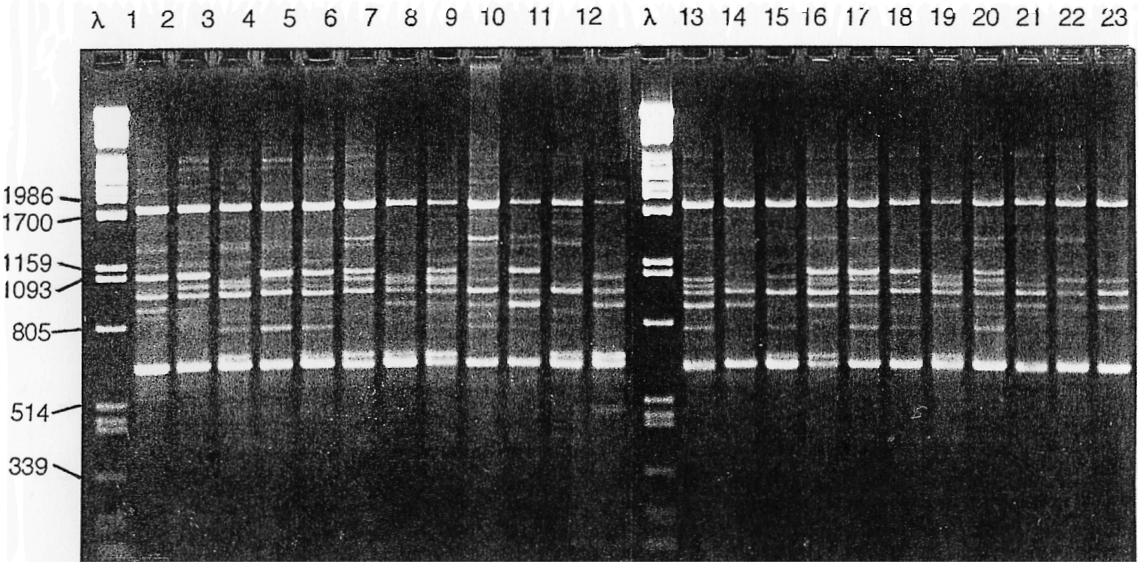
However, in practice this method was unable to change the range of observable fragment sizes much, so the shorter fragment sizes were still difficult to score.

Another approach is to use two different DNA polymerases in two different runs, which complement each other. In this way it was possible to expand the range of scoreable loci per primer. As documented it was found convenient to use AmpliTaq DNA polymerase, AS and AmpliTaq DNA polymerase, Stoffel Fragment alternatively or in combination – see *table 1* column 5 for the applied combination for individual loci.

Figure 3 shows an example of how the two DNA polymerases in combination expand the range of scoreable RAPD fragment sizes for a particular primer, OA11. The actual ranges vary between primers.

Ad c) Identity of loci

The most decisive point is to verify the identity of RAPD loci run in Gulfport and in the present investigation. As mentioned, a sample of at least 10 megagametophytes from the same Italian tree as was mapped in Gulfport, has been re-run in the Danish environment. By lining-up column 2 and 3 in *table 1,* the base pair positions of 43 RAPD loci are comparable. The majority of loci are segregating in both laboratories. For primer OC10 we can observe a complete matching of readings of the band positions. However, the situation is due to the fact, that in this particular case, the three RAPD fragments have the exact same base pair position as the applied base pair ladder of the Pst 1 digested λ marker. For most of the other RAPD fragments approximation due to interpolation of the base pair positions is necessary. This is reflected in the deviations of the listed base pair positions. Conditions for best separation of



b) RAPD analysis of Italian and Danish megagametophytes obtained in Denmark using primer OA07.

Figure 1. – The characteristic amplification pattern of primer OA07. Panel a) shows the original amplifications achieved in Gulfport of 24 individual megagametophytes of the Italian tree and run due to their optimization protocol (BINELLI and BUCCI, 1994). Panel b) demonstrates the Danish version according to our optimization (SKOV, 1998a, b) of both Danish (lane 1 to 12) and Italian (lane 13 to 23) megagametophytes. Base pair ladder: Pst 1 digested Lambda marker. The megagametophytes from the two trees of geographic diverse origin both seem to be homozygotic dominant at position 1800 bp and 620 bp. A closer examination demonstrates the seeked polymorphic locus at bp 1100 (1090) in both materials.

RAPD fragments and thereby most precise interpolations of base pair sizes are completely run electrophoresis; this has apparently not in all cases been obtained in Gulfport. Nevertheless, the identity of the scored RAPD fragments is supported by their position in the overall amplification pattern mentioned above.

Conclusion about identity of specified RAPD fragments

A key point to manifest in this investigation is to which degree it is possible for the Danish laboratory to refine loci originally scored in the Italian megagamethophytes run in Gulfport.

Table 2 shows a summary of the correspondence of base-pair positions between interpretations in the two laboratories.

Table 3 shows the correspondence of zygosity of the common parent tree.

The overall conclusion then is, that out of 43 heterozygotic loci used for mapping in Gulfport, successful reproduction was obtained in the Danish laboratory for 40 of these, corresponding to a repeatability of 93%. The discrepancy was due to either missing observations or no correspondence in zygosity of the parent trees judged on segregation among 10 megagametophytes. There was no association between discrepancy of zygosity and correspondence of base-pair positions – from *table 1* it can be extracted, that the band positions show exact correspondence in two of the three cases where zygosity is judged different.

Table 2. – Correspondence of base-pair positions. Summary of correspondence of interpretation between the two laboratories, Gulfport and Denmark for 43 specified RAPD fragments.

Correspondence of base-pair positions	Number of cases
Exact	20
Approximate	20
Missing obs	3
Total	43

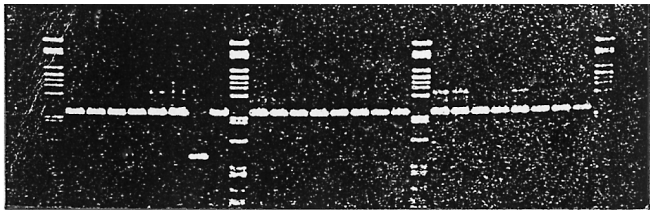
Table 3. – Correspondence of zygosity of common parent tree. Summary of correspondence of interpretation between the two laboratories, Gulfport and Denmark for 43 specified RAPD fragments.

Correspondence of zygosity	Number of cases
Both heterozygotes	40
No correspondence	3
Total	43

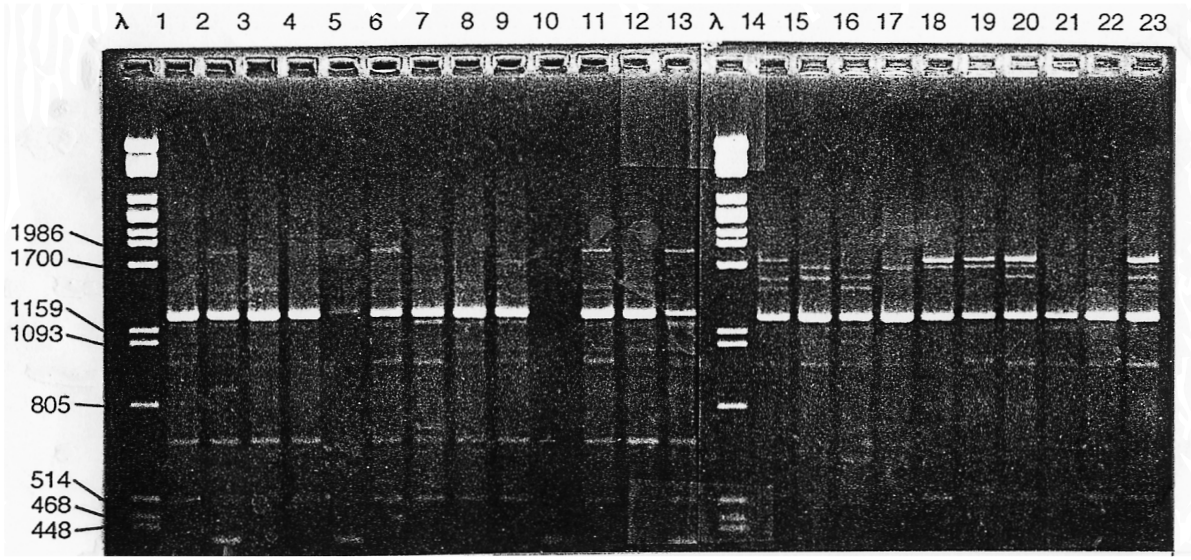
Comparison of zygosity of the two trees analyzed at the Danish laboratory

Table 4 shows an extract from the source, *table 1,* of the correspondence in zygosity between the Italian Campolino tree and the Danish clone V6470 where both materials are analyzed side by side on the same gels. This means the identity of RAPD fragments in this case is determined with near certainty across trees.

As can be seen, in 30 out of the 43 investigated cases the two trees have common loci in which they are both heterozygotes.



a) RAPD analysis of Italian megagametophytes originally obtained in Gulfport using primer OE12.



b) RAPD analysis of Danish and Italian megagametophytes obtained in Denmark using primer OE12.

Figure 2. – The characteristic amplification pattern of primer OE12. Panel a) shows the original amplifications achieved in Gulfport of 24 individual megagametophytes of the Italian tree and run due to their optimization protocol (BINELLI and BUCCI, 1994). Panel b) demonstrates the Danish version according to our optimization (SKOV, 1998a, b) of both Danish and Italian megagametophytes. Base pair ladder: Pst 1 digested Lambda marker.
Lane 1 to 12 megagametophytes from V6470 (Danish origin), lane 13 diploid reference tissue from V6470, and lane 14 to 23 megagametophytes from the Italien tree.
Interpretation documents a homozygotic locus at bp 1300.
A segregating locus exists in the Italian material at bp 1800.

Table 4. – Correspondence of zygosity of the two parent trees. The Italian Campolino tree and the Danish clone V 6470 were both analyzed at the Danish laboratory.

Correspondence of zygosity	Number of cases
Both heterozygotes	30
Italian het Danish hom dom	3
Italian het Danish hom rec	7
Missing obs	3
Total	43

hom: homozygote; het: heterozygote; dom: dominant; rec: recessive

This proportion is close to 70%. If the missing observations are excluded, the proportion rises to 75%.

Discussion

The repeatability of RAPD markers between laboratories is enveloped in several levels of confidence.

Normally in linkage map construction using RAPD markers, the primers preliminary are selected through a screening procedure. According to the high number of different primers available and the elevated level of polymorphism of these markers in proportion to for instance isozymes there exists no need for optimizing single questionable primers.

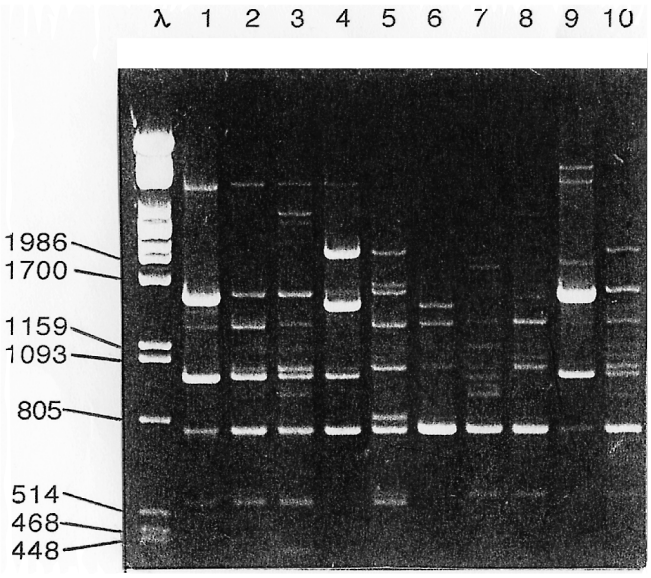
However, the opposite situation arises in comparative studies of repeatability between different laboratories. The defined segregating loci are given beforehand which consequently means that special optimization sometimes must be developed for loci of individual primers.

In the present investigation a reasonable agreement was found between the results from the two different laboratories, keeping in mind that the procedures used in each environment were quite dissimilar. Specific examples of deviations of methods are megagametophyte dissection, DNA extraction, DNA amplificating polymerase, reaction mix constitution, thermocycler type and thermocycler program. For details consult BINELLI and BUCCI (1994) versus SKOV (1998a and b).

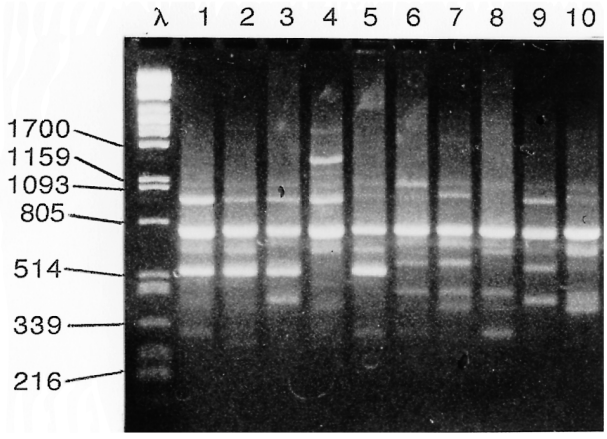
The approximate nature of deciding upon the identity of specific RAPD fragments run in the two laboratories must be underlined. The segregating loci are investigated and scored with reference to a base pair ladder.

What is required to check the ultimate identity of a sample of RAPD markers run at different laboratories is other methods to document identity of DNA fragments, i.e. hybridization or sequencing.

The relative high proportion – 75% – of loci heterozygotic in the Italian tree which also showed up to be heterozygotic in the Danish clone of West Continental origin seems promising for future join-mapping of these and other individuals of *Picea abies.*



a) RAPD analyses of 10 megagametophytes from V6470 of Danish origin using primer OA11. Amplified with AmpliTaq DNA polymerase, AS.



b) RAPD analyses of the same megagametophytes as mentioned above using primer OA11, but utilizing Stoffel Fragment DNA polymerase as amplificating enzyme.

Figure 3. – Demonstration of how two complementary DNA polymerase enzymes expand the range of loci to be scored in a single primer. In both runs, panel a) and b), lanes 1 to 10 constitute the same 10 megagametophytes of Danish origin. An overlapping zone at the homozygotic dominant locus at base pair 780 is observed. AmpliTaq DNA polymerase enables a scoring in the approximate range of bp 1700 to bp 514 whereas Stoffel Fragment DNA polymerase amplificates in the approximate range of bp 780 to bp 310 in this particular primer.

Acknowledgements

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