It also suggests that triploidy and mixploidy should be investigated by cytogenetic studies in elite phenotypes, especially when microsatellite profiling raises such a possibility. Microsatellite fingerprinting is also shown to be a potential tool for cytogenetics.

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MENDELian Inheritance and Tissue Expression of RAPD-markers in *Picea abies* (L.) KARST.

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Abstract

Due to a need to identify DNA-markers well suited for genome mapping in Norway spruce, an investigation was carried out with RAPD markers. The plant material – controlled crosses – made it possible to study MENDELian inheritance of the applied RAPD markers in the following tissues: Needles and buds (2n) as well as megagametophytes (n) of parent individuals, germinating embryos, young seedlings as well as 15 year old progenies of the progeny generation (2n). Prior to the MENDELian study, the RAPD assay from DNA extraction through PCR to agarose gel electrophoresis was optimized and an extensive primer screening revealed the available level of polymorphism. The RAPD markers behaved as a rule as reproducible dominant markers and was expressed in all tested tissues, haploid as well as diploid. Occasional occuring RAPD fragments in the haploid megagametophytes, not present in either of the parents, can in mapping projects where megagametophytes are the mapping population be discarded by including a reference sample of the diploid female parent.

Key words: Picea abies, RAPD, tissue expression, MENDELian segregation, repeatability, primer-screening.

FDC: 165.3; 165.41; 174.7 *Picea abies.*

Introduction

Dealing with identification of DNA-markers suitable for genome mapping in *Picea abies* (L.) KARST., a research programme has been initiated in Denmark. This programme serves as a part of a larger project concerning localizing of quantitative trait loci (QTL) expressed later in the development of coniferous trees.

Initial point to clarify is the type of methodology to select, which again causes a wide range of questions to be answered before deciding which markers to apply.

A retrospective view reveals that a lot of attempts have been made to examine the large sized and relative anonymous nuclear genomes of conifers.

Several fragments of genome maps have been constructed in an early period based on isozyme markers (GURIES et al., 1978; CONKLE, 1981; KING and DANCIK, 1983; POULSEN et al., 1985; O'MALLEY et al., 1986; MUONA et al., 1987). Although isozymes are a useful tool for forest genetics and tree improvement research, the small number of mapped loci provides only a limited view of the organization of the conifer genome (NEALE and WILLIAMS, 1991).

A major event in the history of marker methodology is the introduction of the DNA marker, restriction fragment length polymorphism – RFLP – (BOTSTEIN et al., 1980) which has been used to construct linkage maps for identifying quantitative trait loci – QTL – in a number of crop species (PATERSON et al., 1988; LANDER and BOTSTEIN, 1989; KJÆR, 1994) and a few tree species (DEVEY et al., 1991; NEALE and WILLIAMS, 1991; BRADSHAW and FOSTER, 1992; GROOVER et al., 1994). RFLP is a powerful technique considering detection of polymorphism but seems not quite suitable for our purpose due to several facts: i) The large size of the conifer genomes and a great deal of repetitive DNA sequences make standard RFLP analysis difficult (NEALE and WILLIAMS, 1991; WEINING and LANGRIDGE, 1991; WILKIE et al., 1993; NEALE et al., 1994). ii) The method is time consuming and involves technically complex laboratory procedures. iii) The DNA quantity for RFLP exceeds the DNA amount available in the megagametophytes and early – stage seedlings of *Picea abies.*

Recent advances of DNA technology resulted in the development of the polymerase chain reaction – PCR (SAIKI et al., 1985) and later, based on this the random amplified polymorphic DNA (RAPD) methodology entered the research field of genome mapping in 1990 (WELSH and MCCLELLAND, 1990; WILLIAMS et al., 1990). The RAPD technique provides a new way of detecting polymorphism at the DNA level. The assay is based on DNA amplification using only single primers of arbitrary nucleotide sequence. There is no prior requirement for template DNA sequence information and only nanogramme quantities of template DNA is necessary.

Despite a lot of criticism concerning repeatability and dominance (RIEDY et al., 1992; ELLSWORTH et al., 1993) and MENDELian inheritance (HEUN and HELENTJARIS, 1993; LU et al., 1997), the RAPD assay has developed into a widespread and generally accepted powerful tool for genome mapping in the plant kingdom (GRATTAPAGLIA et al., 1992; TINGEY and TUFO, 1993; DAVIS et al., 1995).

Pinaceae constitute an evident opportunity to test the inheritance of the dominant RAPD markers. The megagametophytes are haploid and are derived from the same single mothercell after meiosis which also produces the corresponding egg cell. In this way it is possible to analyse a DNA fragment expressed in diploid tissue for homo- or heterozygosity and to use the segregating loci in megagametophytes as a mapping population. Recently three publications on tissue expression and MENDELian segregation have been published, namely CARLSON et al. (1991) in *Picea glauca* and *Pseudotsuga menziesii,* BUCCI and MENOZZI (1993) in *Picea abies* and LU et al. (1995) in *Pinus sylvestris.*

A number of partial genome mapping attempts has been published in *Pinaceae* based on the segregation of RAPD-markers in the haploid megagametophytes of individual mother trees: *Pinus taeda* (GRATTAPAGLIA et al., 1991), *Picea glauca* (TULSIERAM et al., 1992), *Pinus elliottii* (NELSON et al., 1993), *Pinus palustris* (NELSON et al., 1994), *Picea abies* (BINELLI and BUCCI, 1994), *Pinus brutea* (KAYA and NEALE, 1995), *Pinus sylvestris* (YAZDANI et al., 1995), *Pinus pinaster* (PLOMION et al., 1995a and b).

This report describes the Danish version of optimization of the RAPD assay on four different tissue types (needles, leaf primordia, seedlings and megagametophytes) in *Picea abies.* Based on the obtained confidence in the development of the technique, successive investigations of level of polymorphism, repeatability of the method and MENDELian inheritance are reported.

Materials and Methods

Plant material

In this preliminary investigation, 4 available tissue types (needles, leaf primordia dissected from buds, seedlings and the corresponding megagametophytes) have been tested from two *Picea abies* L. KARST. pedigrees based on controlled crosses *(Fig. 1).*

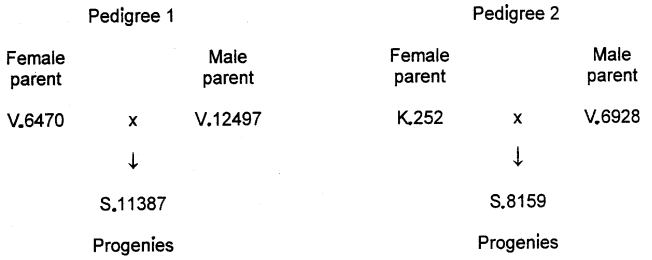


Figure 1. – The two applied pedigrees initiated by controlled crosses.

The female clones used, K 252 and V 6470, are non-related members of different sub populations in the Danish Norway spruce breeding programme (WELLENDORF et al., 1994). Male clones are unrelated with their partners and mutually unrelated as well.

Experiments performed

(1) Optimization procedures involved all 4 tissue types from both applied female clones. These were tested according to a number of variables concerning DNA isolation and the PCR-RAPD procedure *(Table 1).*

(2) Primer screening was carried out through a subset of 5 megagametophytes (n) from clone K 252 using 118 different 10-base primers to demonstrate the level of polymorphism obtained by the optimized procedure.

(3) Repeatability. To test the reliability of the whole process from DNA isolation through DNA-amplification, subsequent gel electrophoresis and observations on specified RAPD fragments, the following experiment was set up. DNA samples from one leafprimordia (2n) and from 5 haploid megagametophytes (no: 58,59,60,61,62) originating from clone V 6470, were analysed against 28 different 10-base primers. The repeatability was investigated by replication of the basic procedures 4 months later. χ^2 analyses of frequencies of identical/non-identical observations as well as frequencies of missing PCR at the first or second observation were carried out.

(4) MENDELian inheritance and tissue expression. Two controlled crossings, pedigree 1 and 2 shown in *figure 1* were utilized to determine the segregation pattern of the dominant RAPD markers in the parents, their megagametophytes as well as in the offspring and simultaneously demonstrate if agreement existed between the different tissue types.

Table 1. – Summary of experience with optimization of 10 components of the RAPD assay in the 4 investigated tissues in *Picea abies*. The underscored alternative was the best suited.

COMPONENTS	TESTED ALTERNATIVES	REMARKS
1) DNA isolation procedures	Sharp et al. 1988 (S-buffer method)	No DNA precipitation
	Qiagen method	No DNA precipitation
	Doyle and Doyle 1990	DNA isolated, but insufficient amount in megagametophytes
	Neale and Williams 1991	Excellent on needle tissue
	Carlson et al. 1991	Works well in all tested tissue types
	Tulsieram et al. 1992	DNA isolated, but PCR not reproducible using megagametophytes
2) Template DNA concentration	0.5 ng, 1.0 ng, 2.0ng, 10ng and 20ng	Below 2 ng PCR not reproducible, higher than 10 ng no reaction.
3) $MgCl_2$ concentration	1.5 mM, 2.0 mM, 2.5mM and 3.0 mM	Lesser background and brighter bands with 2.0 mM
4) Decamer primer concentration	8 ng , 16 ng and 32 ng / reaction	8 ng → ampl. products larger, 32 ng → ampl. products shorter
5) Amplitaq polymerase conc.	0.3 units and 0.5 units / reaction	0.5 units recommended by purchaser, and gives the best results
6) Brand of DNA polymerase	Supertaq versus Amplitaq	Supertaq resulted in too many amplification products to secure correct scoring
7) Individual thermocyclers	Two Perkin Elmer 480s tested	No differences observed
8) Wells in the heating block	Different positions in the heating block	No differences observed
9) Mineral oil in the heating block	+ /–	Only marginal differences observed
10) Type of reaction tubes	Thin walled tubes versus ordinary tubes	Thin walled tubes require another optimized cycler program

DNA isolation

Among several DNA isolation procedures tested (see *Table 1*), the CTAB procedure recommended by BOUSQUET et al. (1990) and modified by CARLSON et al. (1991) with some minor adjustments, proved to be the most efficient to obtain genomic DNA from needles, leaf primordia, seedlings and megagametophytes. The criterion for judging the applied DNA extraction was consistent amplification by PCR.

3 to 5 needles per vial, 2 to 7 leaf primordia (depending on developmental stage) per vial, a single megagametophyte per vial or a whorl of primary leaves from the seedlings per vial are sufficient quantity of material to isolate DNA for a large number of RAPD reactions.

The tissues were transferred aseptically into 1.5 ml sterile microcentrifuge tubes, with 150 µl CTAB isolation buffer (2% (w/v) CTAB (cetyltrimethylammonium bromide, Sigma), 1.4 M NaCl, 20 mM EDTA, 1% (w/v) PEG 8000 (polyethylene glycol, Sigma) and 100 mM TRIS-HCl (pH 9.5)). The tubes were frozen in liquid nitrogen for 30 sec., followed by grinding of the tissue with a fitted glass rod. The glass rod was rinsed with 700 µl CTAB buffer, in the microcentrifuge tube to be mixed with the homogenate and then incubated at 65 °C for 30 min. Afterwards the homogenate was extracted with 500 µl chloroform: isoamyl alcohol (24:1) and then placed at a shaking table (slow vibrations) for 30 min followed by a centrifugation in a microcentrifuge at 13,000 rpm for 5 min at 4 °C. DNA was precipitated from the aqueous phase by mixing with 500 µl isopropanol and pelleting by centrifugation at 4 °C for 5 min. The DNA pellet was rinsed in 70% ethanol, vacuum dried, and resuspended in 100 µl 1 x TE buffer (10 mM Tris, 0.25 mM EDTA, pH 8.0).

Concerning DNA isolation from needle tissue: The needles were washed in alcohol and grounded to powder in liquid nitrogen using sterile mortar and pestle. The powder was then transferred to separate microcentrifuge tubes and the above description was followed until the point of extraction, which was done twice or more on needle tissue.

Finally the DNA concentrations were measured using a fluorometric assay (CESARONE et al., 1979), and the samples were diluted to equal concentrations.

RAPD analysis

The optimal RAPD reaction conditions were established after testing a range of different factors (see *Table 1*) resulting in the following procedure.

Amplification reactions were performed according to WILLIAMS et al. (1990) with minor modifications. The reaction volumes of 20 µl contained 2 ng to 5 ng template DNA, 0.5 unit Amplitaq DNA polymerase (Perkin Elmer, Cetus), 2 µl 10 x PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl), 200 µM each dATP, dCTP, dGTP and dTTP (Pharmacia), 2.0 mM MgCl2 and 16 ng of a 10-base random primer (Operon technologies, Alameda CA). 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 (denaturation), 1 min at 36 °C (primer annealing) and 2 min at 72 °C + extension time 1 sec/cycle from the 6. cycle (primer extension). 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 ll and analyzed by electrophoresis in 1.4% agarose TBE gels with ethidium bromide (SAMBROOK et al., 1989). Bands were visualized by UV light and photographed with Polaroid film for instant interpretation.

Results

Optimization of the RAPD assay for Picea abies

According to *table 1* the procedures of CARLSON et al. (1991) were conclusive, at least in our laboratory, for DNA isolation because of its effectiveness in all tissue types tested. A striking demand to the method to be suitable for genome mapping in conifers based on haploid megagametophyte populations, is a sufficient amount of isolated DNA from individual megagametophytes to run a large number of RAPD reactions. This point was fulfilled with yields averaging 4 µg/megagametophyte. The method proposed by NEALE and WILLIAMS (1991) was excellent when used on needle tissue. It could be recommended if the demand is large quantities of DNA and the marker type co-dominant, for instance if the aim is RFLP linkage maps based on known pedigrees.

Within the frame given by WILLIAMS et al. (1990), different components in the RAPD assay were adjusted one by one, keeping the remaining components constant (see *Table 1*).

Template DNA concentrations were varied between 0.5 ng and 20 ng per reaction. The most critical tissue type – the megagametophytes – reacted with unreproducible amplification products in concentrations below 2 ng. Utilizing DNA concentrations higher than 10 ng, no amplification was observed. DNA from needles, leafprimordia and seedlings showed the same tendency although less pronounced. The optimal concentrations of template DNA were 2 ng to 5 ng/reaction.

In tests of $MgCl_2$ concentrations in the range of 1.5 mM to 3.0 mM, no crucial dissimilarities were found. All amplification products were reproduced in each concentration used, and no new products appeared. $MgCl_2$ concentration at 2.0 mM was chosen according to the brightness of the fragments.

Three different concentrations of decamer primer, 8.0 ng, 16 ng and 32 ng per reaction were tested. A general tendency appeared. Using high decamer concentrations the amplified fragment lengths were displaced in a direction of shorter products and low decamer concentrations amplified larger fragments. This is a key point in narrow defined jobs where specified fragments are sought. It might be useful in joint mapping projects where one may be forced to reproduce specific RAPD fragments run at other laboratories. To a certain degree variable success was obtained in the assay depending on the primer used, indicating that a few primers needed separate optimization.

Concerning Amplitaq DNA polymerase, the most expensive ingredient in the RAPD-assay, we unsuccessfully tried a reduction of the recommended concentration given by the purchaser. When moving from 0.5 to 0.3 units per reaction, several amplification products disappeared and by using that option we needed to run far more primers to reach the same level of polymorphism.

Attempts were made to change Amplitaq DNA polymerase with SuperTaq (HT BioTechnology). Amplification succeeded to a degree where interpretation was complicated by too many bands. Careful examination made it possible to re-create the fragments observed in Amplitaq DNA polymerase runs.

Two Perkin Elmer Thermal 480 Cyclers were utilized interchangeable and no differences were observed. Wells in the heating block of the thermocyclers were tested for uniformity regularly. No dissimilarities were found.

Primer screening

To identify the level of polymorphism achieved by the above mentioned optimization, a subset of DNA from 5 megagametophytes (n) belonging to clone K 252 was screened against 118 different decamer primers. Segregation of markers in haploid tissue is expected to follow a 1 : 1 MENDELian ratio of presence/absence of marker band and therefore the use of 5 megagametophytes will give reasonable confidence (94% of the time) in detecting heterozygotic loci in the maternal parent tree.

Each primer produced a unique banding pattern. Of 118 primers tested, 88 were found to be usable in the prospect of genome mapping. The 88 segregating primers gave rise to 223 potential polymorphic loci, which is an average of 2.5 polymorphism/primer. 8 primers were monomorphic and in 9 primers no reactions were found. The remaining group of 13 primers expressed a weak reaction which indicated that a further optimization of these primers might be necessary. Typically a suitable primer gave rise to 5 to 14 bands ranging 300 to 2600 base pairs.

Repeatability

We needed to demonstrate that under the same reaction conditions we could reproduce identical amplification products.

In order to separate tests of repeatability of the double-observations and the total response pattern, two sets of contingency tables were lined up: a) Test of repeatability, + : identical observations, – : non-identical observations, see *table 2* and *3*, and b) Test of total response pattern including missing observations ~ 0 : *table 4* and *5*. In each of the contingency tables, the standard G^2-test (SOKAL and ROHLF, 1981) was applied to test independence of factors (tissue and RAPD bands respectively) and the response counts. The χ^2 for each table has d.f. = (r-1)(c-1), where r is the number of rows and c is the number of columns in the contingency tables.

Table 2. – Observations on repeatability on tissue.

Tissue	Numbers of		
	+	–	total
Buds (2N)	80	2	82
MG58 (N)	88	4	92
MG59 (N)	74	3	77
MG60 (N)	92	2	94
MG61 (N)	71	5	76
MG62 (N)	86	4	91
Total	491	20	509

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

Table 3. – Observations on repeatability on RAPD bands.

RAPD bands	Numbers of		
	+	–	total
1	5	0	5
2	5	0	5
3	4	1	5
–	–	–	–
–	–	–	–
95	5	0	5
Total	491	20	509

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

Table 4. – Observations on total response pattern on tissue.

Numbers of			
+	–	0	total
80	2	13	95
88	4	3	95
74	3	18	95
92	2	1	95
71	5	19	95
86	4	5	95
491	20	59	570

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

Table 5. – Observations on total response pattern on RAPD bands.

Numbers of			
+	–	0	total
5	0	1	6
5	0	1	6
4	1	1	6
–	–	–	–
–	–	–	–
5	0	1	6
491	20	59	570

+ : identical observations in two successive analyses;
– : different observations in two successive analyses;
0 : missing observations due to fall-out of PCR.

The results of the 4 G^2-tests are shown in *table 6*. The mosaic plot in *figure 2* shows the proportion of + , – , and 0 observations corresponding to the total response pattern for tissues. Concerning conclusions on repeatability, i.e. the frequency of identical observations of defined RAPD bands, the overall frequency is 491 / 509 = 96.5%. The rare non-identical observations seem to be randomly distributed to tissues and primers. Concerning conclusions of the total response pattern, i.e. including the distribution of missing reactions during the first or second run, highly significant effects of the tested tissues appeared. However, according to *figure 2* the dependence seems not associated with the distinction between diploid or haploid tissue.

MENDELian inheritance and tissue expression

The genetic and experimental design used made it possible in one investigation to expose if discrepancies from MENDELian segregation and deviations between tissue types occurred.

An example of the original observations on two RAPD-fragment in pedigree 1 is presented in *figure 3* and the corresponding interpretation is presented in *table 7*.

Each RAPD fragment analysis included tissue from the female parent represented by replicated runs of DNA from needles (2N) and leafprimordia from dormant buds (2N) besides DNA from 7 megagametophytes (N) from her seeds. The male parent was subject to a similar sampling. Seedlings (2N) descending from a controlled cross between the involved parent trees were tested in order to follow the inheritance of specific RAPD fragments.

Although the sample size is too small for critical tests of distortions, the observed segregations are in accordance with expectations of dominant markers.

As is demonstrated here, crossings between the heterogeneous members of the wild, outcrossing base-populations results in all type of situations, two of which are realized here, i.e. a heterozygote x homozygote recessive (a test-cross situation for locus A) and a heterozygote x heterozygote (a F_2 situation for locus B).

Concerning tissue expression, agreement seems to occur:

V 6470 has the + phenotype in the two loci in both diploid tissues, needles and leafprimordia. The segregation in the female haploid megagametophytes indicates, that the female is heterozygotic in both loci.

V 12497 shows the phenotype of a 'silent' allele for locus A in both diploid tissues indicating that the male is homozygotic recessive. For locus B, the male shows the + phenotype in both diploid tissues. Looking at the male megagametophyte segregation the homozygotic recessive status of locus A is confirmed by lack of + phenotypes. For the other locus B, the male shows up to be heterozygotic as + and – phenotypes segregate.

In progeny S 11387 (V 6470 x V 12497) sampling in the diploid tissue of early-stage seedlings shows the expected segregations mentioned above and shown in *figure 3* and *table 7*.

A summary of 25 such tests of MENDELian inheritance is presented in *table 8*. For situations where segregation is expected in the progeny, G^2-tests are performed to check if the proportions are in accordance with the expected, i.e. 1:1 for the backcross to the homozygote recessive (test-cross situations) and 3:1 for the F_2-situations.

Backcross to the homozygotic dominant parent and two dominant homozygotes crossed with each other ("hom dom selfed") are not expected to generate segregation in the progeny if complete dominance is in operation.

A closer interpretation of the material in *table 7* and *8* confirms the general experience of RAPD fragments as fully dominant markers closely following MENDELian inheritance.

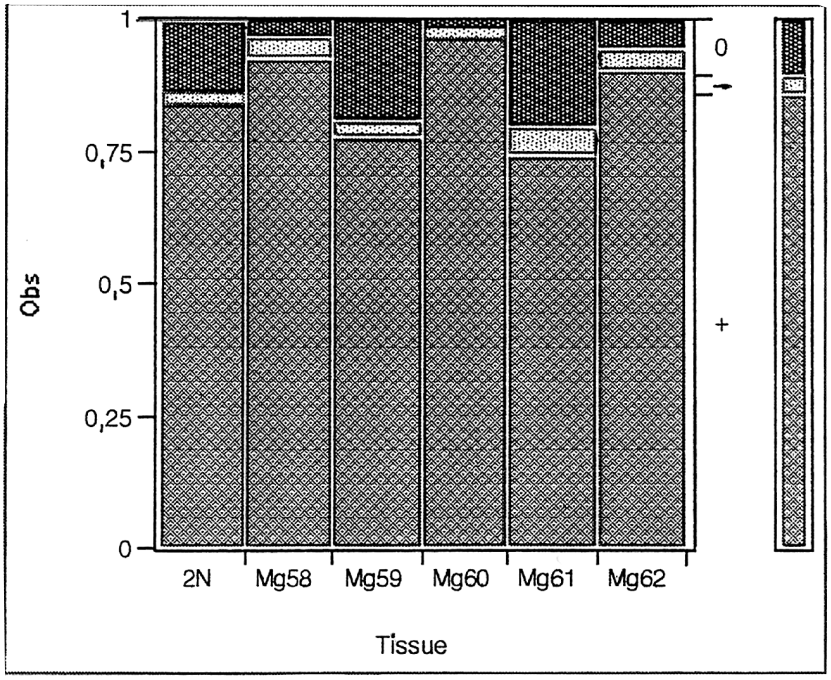
These markers are expressed in diploid tissues: needles, leafprimordia from dormant buds and early stage seedlings as well as in haploid tissue, the megagametophytes.

Conclusions and Discussion

Concerning the RAPD assay, establishment of standard conditions in *Picea abies* proved to be critical due to the sensi-

Table 6. – 4 tests of independence between i) tissue and response counts and ii) RAPD bands and response counts.

Respons Factors	Test of repeatability 2 response classes: + / –			Test of total response pattern 3 response classes: + / – / 0		
	X^2	d.f.	p	X^2	d.f.	p
i) Tissue	2.80 NS	5	.730	42.04***	10	.000
ii) RAPD bands	78.08 NS	94	.882	170.95NS	188	.809



0: Missing PCR at first or second run; –: non-identical observations; +: identical observations at first and second run.

Figure 2. – Mosaic plot of repeatability investigation.

tivity of the system to experimental variables. Consequently, amplification of random genomic sequences in a reproducible way is only possible with rigorously optimized reaction conditions as demonstrated in the present investigation. The importance of this general experience is shared by numerous authors.

With the developed RAPD assay a reproducibility of 96.5% in the present investigation matches remarkable well with the relative few other reported reproducibility figures (WEEDEN et al., 1992; HEMMAT et al., 1994; and SKROCH and NIENHUIS, 1995).

Concerning identification of components of the RAPD assay which are the most critical, a certain discrepancy appears between reports.

In the present investigation in *Picea abies* on different tissues, DNA extraction – influencing DNA purity and amount – as well as DNA concentration showed up to be key points. Other investigators agree on the importance of DNA purity (WEEDEN et al., 1992), whereas the same authors for template DNA find that the concentration could be varied 10-fold without seriously affecting the RAPD-pattern.

In *Picea abies* the importance of fluorometric or spectrophotometric analyses to standardize the DNA concentrations across all individuals is stressed.

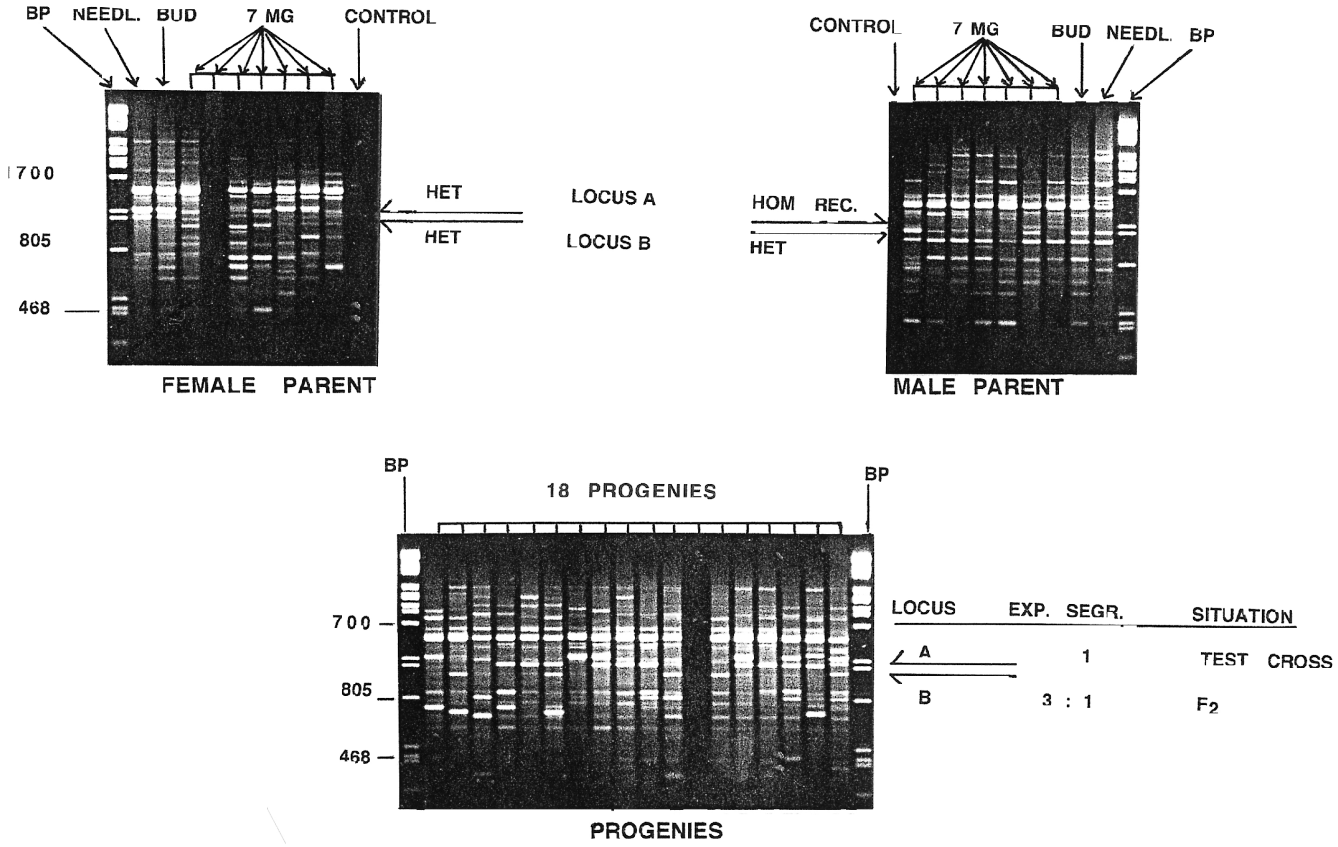
In principle each oligonucleotide primer requires optimization with respect to the amplification conditions, concentration of primer relative to the template, and magnesium concentra-

Table 7. – MENDELian inheritance and tissue expression. Example of two RAPD loci.

		Female parent - V 6470														Male parent – V 12497									
Loc	Zyg	2n tissue		n tissue (MG no)							n tissue (MG no)							2n tissue		Zyg
		needl	bud	1	2	3	4	5	6	7	1	2	3	4	5	6	7	needl	bud	
A	ht	+	+	+	0	–	+	–	+	+	–	–	–	–	–	–	–	–	–	hm r
B	ht	+	+	+	0	+	+	–	+	+	+	+	+	+	–	+	–	+	+	ht

18 progenies - S 11387 - seedlings																				
	2n tissue - (individual no)																		Segregation +/–	
Loc	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Situation	Obs	Exp
A	+	–	+	–	+	+	–	–	+	+	+	0	+	–	–	+	–	+	test cross	10 : 7	1 : 1
B	–	–	–	+	+	+	–	+	+	+	+	0	–	+	+	+	+	–	F_2	11 : 6	3 : 1

MG: Megagametophyte;
Loc: locus; Zyg: Zygosity; ht: heterozygote; hm r: homozygote recessive (–); +: band present; –: band absent; 0: missing observation (PCR-reaction drop-out).



BP: Base pair ladder, λ digested with Pst 1; MG: megagametophytes – haploid (n); HOM-REC: Homozygotic recessive; HET: Heterozygotic; TEST-CROSS: Back-cross to a homozygotic recessive.

Figure 3. – MENDELian inheritance and tissue expression. Example of two RAPD loci.

tion. However, in practice the great majority of primers requires nearly identical experimental conditions and these should therefore be followed strictly by the default.

With the RAPD method of fragment amplification an often occurring artefact on agarose gels is the resolution of some minor bands that are not repeatable. These unstable bands have been suggested to result from the formation of artificial heteroduplexes between multiple amplified fragments (AYLIFFE et al., 1994) or from non specific amplification, that is, amplification when primer/template homology is not perfect. These artefacts were minimized by optimizing PCR components.

Occasional, more bands were observed in megagametophyte DNA than expected from the parents DNA phenotype observed in their diploid tissue. Further investigations are needed to determine the cause of these aberrant patterns.

In the stage of demonstrating the MENDELian inheritance and tissue expression through a generation turn-over, the developed RADP assay seems to be reliable for subsequent genetic analyses. For a specified sample of RAPD fragments the inheritance and tissue expression have been checked and were found to be in accordance with expectations for a dominant marker. This infers monitoring the markers through the generation turn-over from the parents diplophase over the female parents haplophase to the diplophase of the progenies.

On this background, it is recommended to apply a diploid parent reference sample when analyzing segregation and recombinations in megagametophyte populations. In this way one avoid scoring loci that do not occur in diploid tissue.

The cited conclusions are in general agreement with other investigations of the formal genetics of RAPD fragments in *Picea glauca* and *Pseudotsuga menziesii* (CARLSON et al., 1991), in *Picea abies* (BUCCI and MENOZZI, 1993) and in *Pinus sylvestris* (LU et al., 1995).

A number of investigators have used RAPDs in megagametophytes from *Pinaceae* individuals as mapping populations (GRATTAPAGLIA et al., 1991; TULSIERAM et al., 1992; NELSON et al., 1993; BINELLI and BUCCI, 1994) and others. Implicit in these investigations are 1 : 1 tests of segregation of presence or absence of RAPD fragments in the mapping population. These observations are therefore supplying us with further information of the formal MENDELian genetics of specified RAPD fragments in *Pinaceae.*

The general conclusion then is, that amplification products in the form of RAPD fragments defining polymorphisms exhibit MENDELian inheritance and is expressed in diverse tissues.

The developed assay for RAPD markers thus fulfils the required demand for a type of DNA marker well suited for dense genome mapping in *Picea abies* and other *Pinaceae.* The expression in diverse tissues is important for use in mapping of quantitative trait loci (QTL), as we in such projects need to determine the genotype in successive developmental stages. For application in subsequent marker aided selection (MAS) it is furthermore convincing that RAPD markers are expressed in available tissues associated with somatic embryogenesis (ISABEL et al., 1993).

Table 8. – Summary table of performed tests of MENDELian inheritance of 22 RAPD fragments in two pedigrees.

RAPD fragment	Female parent			Male parent			Progeny population				
							Situation	Segregation		G^2 test	
Primer - locus	hom dom	het	hom rec	hom dom	het	hom rec		Observed	Expected	χ^2	d.f.
	K 252			V 6928			S 8159				
C 07 -a		x			x		F_2	12:5	3:1	0.1702	1
C 07 -b		x				x	BC to hom rec	8:9	1:1	0.0294	1
C 07 -c		x			x		F_2	12:5	1:1	0.1702	1
C 07 -d	x			x			self hom dom	17:0	1:0		
C 09 -a		x			x		F_2	15:3	3:1	0.7280	1
C 09 -b		x			x		F_2	13:5	3:1	0.0724	1
C 09 -c		x			x		F_2	13:5	3:1	0.0724	1
C 15 -a		x				x	BC to hom rec	9:9	1:1	0.0000	1
C 15 -b		x			x		F_2	13:5	3:1	0.0724	1
C 15- c		x			x		F_2	11:7	3:1	1.6802	1
D 20 -a	x					x	F_1	18:0	1:0		
D 20- b		x		x			BC to hom dom	18:0	1:0		
D 20- c		x				x	BC to hom rec	10:8	1:1	0.1113	1
D 20-d		x			x		F_2	12:6	3:1	0.6254	1
	V 6470			V 12497			S 11387				
A 02 -a	x			x			self hom dom	18:0	1:0		
A 02 -b		x				x	BC to hom rec	10:8	1:1	0.1113	1
A 02 -c		x			x		F_2	14:4	3:1	0.0760	1
A 02 -d			x		x		BC to hom rec	9:9	1:1	0.0000	1
B 12 -a		x		x			BC to hom dom	17:0	1:0		
B 12 -b		x				x	BC to hom rec	10:7	1:1	0.2661	1
B 12 -c			x		x		BC to hom rec	9:8	1:1	0.0294	1
B 12 -d	x			x			self hom dom	17:0	1:0		
C 15 -a		x			x		F_2	13:4	3:1	0.0199	1
C 15 -b		x				x	BC to hom rec	8:9	1:1	0.0294	1
C 15 -c		x			x		F_2	12:5	3:1	0.1702	1
Grand total	4	19	2	5	12	7				4.4342 NS	19

hom dom: homozygote dominant; het: heterozygote; hom rec: homozygote recessive; BC: backcross; 5% fractile for χ^2-tests with 1 d.f. is 3.84, i.e. none of the individual χ^2-tests contradicts MENDELean inheritance. The total χ^2 for all the independent tests is far from any upper limit fractile, i.e. the overall pattern is in accordance with MENDELean expectations.

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Timing of DNA Extraction from Megagametophytes for PCR during Initial Steps of Seedling Development in *Picea abies* (L.) KARST.

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Abstract

Within the framework of QTL investigations and subsequent marker aided selection in *Pinaceae,* a need to grow seedlings with saved megagametophytes for DNA extraction has evolved. The present investigation reports a timing experiment in which the optimum stage of seedling development is identified. DNA amount proved to be relative independent of seedling development, but successful PCR was, probable due to the occurrence of fat and carbohydrates, only possible at relative late stages of seedling development when the role of the megagametophyte as a nutrient source has decreased. Removing the megagametophyte at this stage is not a problem for the germinating seedlings, which then are relying on their own root uptake and photosynthesis.