MOLECULAR PROPERTIES OF RAPDS IN *PINUS SYLVESTRIS* (L.) AND THEIR IMPLICATIONS FOR GENETIC ANALYSIS

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ABSTRACT

To characterize the molecular properties of RAPD variation in *Pinus sylvestris* (L.), we isolated 10 polymorphic and 10 monomorphic RAPD fragments and used them as probes in Southern analysis of the RAPD profiles and genomic DNA digests of *P. sylvestris* individuals from different populations. The results showed that: (*i*) individual RAPD bands often contain more than one non-homologous DNA sequence, though one sequence usually predominates; (*ii*) Southern hybridization revealed that the presence/absence polymorphism of RAPD bands is due to the lack of amplification of the corresponding sequence rather than to the absence of the target sequence in the individuals lacking the band; (*iii*) despite of a large genome in conifers, most of the analysed RAPD fragments did not appear to originate from repetitive sequences. Of the 20 RAPD fragments tested, only one fragment appeared to be of repetitive origin. The implications of the observed molecular properties of RAPD variation for genetic analysis are discussed.

Key words: *Pinus sylvestris*, RAPD, sequence homology, repetitive sequence, presence/absence polymorphism

INTRODUCTION

In the recent years, random amplified polymorphic DNAs (RAPDs) have become popular markers in genetic studies in conifers and other plants (*e.g.*, BINELLI & BUCCI 1994; CHONG *et al.* 1994; MOREAU *et al.* 1994; NELSON *et al.* 1993; WEISING *et al.* 1995). However, despite the wide use of the technique, the molecular basis underlying RAPD variation and its significance for the validity of genetic inferences based on this type of markers are still poorly understood (HEUN & HELENTJARIS 1993; ROTHUIZEN & VAN WOLFEREN 1994). Several studies have shown that RAPDs are commonly amplified from nuclear genome (LU *et al.* 1996 and references therein). However, in a recent study, nearly half of the RAPDs have been amplified from mitochondrial genome (AAGAARD *et al.* 1995). The same authors also reported that RAPDs showed higher differentiation (G_{ST} = 0.73) among populations than allozymes, assuming Hardy-Weinberg equilibrium. However, as demonstrated in other studies, such assumption may not be true for RAPD markers (ISABEL *et al.* 1995; SZMIDT *et al.* 1996). Furthermore, some studies have suggested that RAPDs are amplified from repeated sequences (DEVOS & GALE 1992; KAZAN *et al.* 1993; N'GORAN *et al.* 1994; WILLIAMS *et al.* 1990; PLOMION *et al.* 1995). On the other hand, results reported by BODENES *et al.* (1997) showed that majority of the RAPDs originated from single or low copy sequences. This problem gains special importance in conifers, which are believed to possess large genomes (RAKE *et al.* 1980) where more repetitive sequences are expected (LAPITAN 1992). Most of the evidence for the repetitive origin of the RAPD fragments is based on Southern analysis of the RAPD bands directly isolated from agarose gels or their total re-amplification products (WEISING *et al.* 1995). It is likely, that such RAPD bands contain additional DNA fragments of similar size which cannot be resolved by electrophoresis (QUIROS *et al.* 1995). When used as probes, such composite fragments can give multiple hybridization signals in the Southern analysis of RAPD profiles and genomic DNA digests, similar to those expected for repetitive sequences. Therefore, for this purpose, it would be preferable to use at least partially purified RAPD fragments containing fewer unrelated sequences.

In a previous study we demonstrated that in *Pinus sylvestris* (L.), most of the RAPD fragments show presence/absence polymorphism and segregate in the Mendelian fashion (LU *et al.* 1995). However, deviations from the Mendelian expectation were found for a few RAPD markers (LU *et al.* 1995). The molecular basis of the presence/absence character of the RAPD

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variation is not clear. Therefore, to better evaluate the usefulness of RAPDs for genetic analysis it is necessary to obtain more precise information about the molecular properties of this type of markers. To provide such information, we employed Southern analysis of the randomly amplified DNA products and genomic DNA of *P. sylvestris*. We addressed the following questions: (1) do individual RAPD bands include more than one DNA sequence?; (2) do RAPD products come from repetitive sequences?; and (3) what is the molecular basis of the presence/absence character of the RAPD variation in *P. sylvestris*?

MATERIALS AND METHODS

Plant material, DNA isolation and digestion

Buds and needles were collected from ten unrelated individuals of *P. sylvestris* in northern Sweden (LU *et al.* 1995). In addition, bulked samples of 30 seedlings of *P. sylvestris* from one Turkish population (SZMIDT & WANG 1993) and five young individuals of *Zea mays* and ten individuals of *Arabidopsis thaliana* were included in the Southern analysis.

Genomic DNA was isolated using the CTAB method according to DOYLE & DOYLE (1990), and suspended in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). About 10 μg of genomic DNAs were digested separately with four enzymes: *Hind* III, *Kpn* I, *Bcl* I and *Dra* I. The digestion products were separated in 0.8% agarose gels in 1 x TAE and vacuum-transferred to Hybond™-N nylon membrane (Amersham) according to the manufacturer's instruction (Pharmacia). These genomic DNA blots are hereafter referred to as A-blots.

RAPD amplification and probe isolation

PCR amplifications were performed using nine random primers (OPA01 through OPA05, OPA07, OPA14, OPA15 and OPA18, Operon Technologies) as described by LU *et al.* (1995). The amplification products generated by each primer were resolved in 1.5% agarose gels in 0.5 x TBE and in 2% low melting point agarose (LMP) gels (SEAPLAQUE™, FMC) in 1 x TAE. The RAPD products separated in 1.5% agarose were blotted to nylon membrane (hereafter referred to as B-blots). The products separated in 2% LMP gel were used for the isolation of individual RAPD bands. Twenty RAPD bands from *P. sylvestris* were selected in the present study (Table 1). Twelve of these bands have been identified in our previous analysis of the

Table 1. RAPD fragments used as probes in the Southern analysis of genomic DNA digests (A blots), RAPD profiles (B blots) and profiles of the re-amplified RAPD bands (C blots).

Fragment	Host tree	Probe type	Polymorphism	A-blots	B, C-blots	Copy number
OPA01–1000	AC1019	II	P	–	+	L
OPA01–600	BD1178	II	P	–	+	L
OPA01–500	AC1014	II	P	+	+	L
OPA02–850	BD1178	II	P	+	+	H
OPA03–2500	AC1005	I	M	+	–	L
OPA04–1500	AC1005	I	M	+	–	L
OPA04–750	AC1005	I	M	+	–	L
OPA05–1500	AC4210	II	P	+	+	L
OPA05–1200	AC1016	II	M	+	+	L
OPA05–600	AC1016	II	P	+	+	L
OPA07–1400	AC1005	I	P	+	–	L
OPA14–2100	AC1019	I	M	+	–	L
OPA15–1600	AC1019	I	P	+	–	L
OPA15–1500	AC1019	I	M	+	–	L
OPA15–800	AC1019	II	P	+	+	L
OPA15–750	AC1019	II	P	+	+	L
OPA18–850	BD1032	II	M	+	+	L
OPA18–800	BD1032	II	M	+	+	L
OPA18–650	Y3020	I	M	+	–	L
OPA18–450	Y3020	I	M	+	–	L

Notes: **I**: probes derived from isolated RAPD bands; **II**: probes derived from the re-amplified products of isolated RAPD fragments; +: tested; – : not tested; P: fragment showing presence/absence variation among the investigated individuals; M: monomorphic fragment; L: low; H: high.

controlled progeny of *P. sylvestris* (LU *et al.* 1995). Of the 20 RAPD bands analysed, nine bands were isolated by cutting a piece of LMP agarose gel containing the band. These nine bands were used directly as probes (type I, Table 1) in hybridisations to the genomic DNA digests (A-blots).

The remaining 11 fragments (Table 1) were isolated for PCR re-amplification by picking up a small section of the LMP agarose gel containing the band of interest with a yellow tip and then placing it in 50 µl TE buffer. This mixture was subsequently melted at 65°C for 5 min and mixed. Two µl of the mixture was used for re-amplification with the corresponding primer under the same PCR conditions as in the first amplification. The re-amplification products were separated in 1.5% agarose as well as in 2% LMP agarose gels. The products separated in 1.5% agarose gels were blotted to nylon membrane (hereafter referred to as C-blots). On the LMP gels, the fragments with the expected sizes were re-isolated by cutting the slice of gel (type II probes, Table 1). These probes were subsequently hybridised to B, C and A-blots containing the RAPD profiles, the profiles of the re-amplified individual RAPD bands and genomic DNA digests respectively (Table 1).

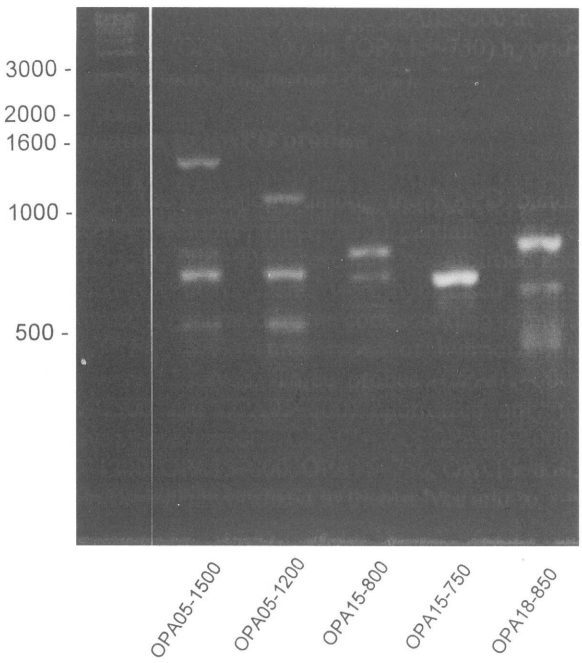

Figure 1. Re-amplification of the selected RAPD bands with corresponding random primer.

Probe labelling and DNA hybridisation

The probes were labelled with α-^{32}P-dCTP using the Oligolabelling kit (Pharmacia) according to the manufacturer's instruction. The DNA hybridizations were performed in rotating tubes at 65°C as described by SAMBROOK *et al.* (1989). Ten to 15 min exposure to the X-ray film (Amersham) was sufficient for B- and C-blots, but two days were usually needed for A-blots. After exposure, filters were washed for 15 min in 0.1 x SSC, 0.1% SDS solution at 100°C to remove the radioactivity and reused for subsequent hybridizations. Eighteen probe/enzyme combinations were tested on the genomic digests A-blots. Three probes failed to give signal on A-blots. Therefore, they were further tested in slot-blot analysis. For this purpose, about 10 µg DNA for each individual was blotted on Hybond™-N membrane with the Minifold II ™ Micro-sample Filtration Manifold (Schleicher & Schuell, Germany). The probe labelling and hybridization conditions were the same as described above.

RESULTS

Re-amplification of RAPD bands

To determine whether individual RAPD bands isolated from agarose gels contain more than one sequence, 11 RAPD bands (type II in Table 1) were selected for re-amplification. Only one band OPA15–750 gave a single product of the expected size (though with an additional smaller but very weak band, Fig. 1). Re-amplification of the remaining 10 bands produced the fragment of the expected size as well as several addi tional fragments (*e.g.*, OPA18–850, OPA15–800, OPA05–1200 and OPA05–1500, Fig. 1).

To determine the homology of these additional fragments detected after the re-amplification of individual RAPD bands, the 11 re-isolated and thus partially purified RAPD fragments (type II probes, Table 1) were hybridized to the re-amplification profiles of individual RAPD bands (C-blots). Of the 11 probes tested, nine probes (OPA01–1000, OPA01–600, OPA01–500, OPA02–850, OPA05–1500, OPA05 –1200, OPA05–600, OPA18–850, and OPA18–800) hybridized only to themselves (*e. g.* OPA05–600 in Fig. 2). Two probes (OPA15–800 and OPA15–750) hybridized to two or more fragments (Fig. 2).

Hybridisation to RAPD profiles

To analyse the homology among the RAPD bands amplified by a random primer, the 11 partially purified probes (type II, Table 1) representing re-amplified and re-isolated RAPD fragments were hybridised to the RAPD profiles generated by the corresponding primers (B-blots) (Fig. 3A). Three types of hybridization pattern were observed. Three probes (OPA01–600, OPA05–1500 and OPA05–600) hybridized only to themselves. Seven probes (OPA01–500, OPA01–1000, OPA05–1200, OPA15–800, OPA15–750, OPA18–850, and OPA18–800) hybridized to themselves and to 1 –

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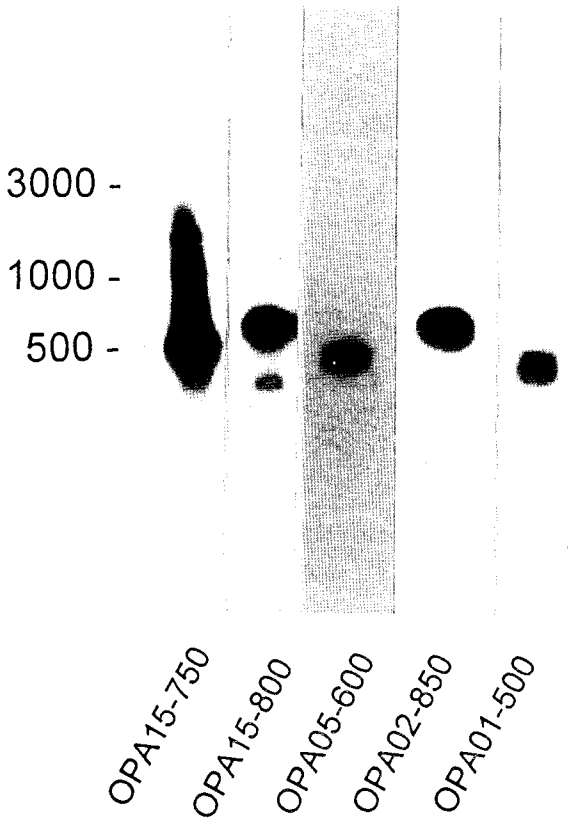

Figure 2. Hybridization pattern of the purified RAPD fragments (probe type II) to their re-amplification profiles (C-blots).

4 additional, usually very weak fragments. For example, the probe OPA01–500 hybridized to three different fragments on the OPA01 RAPD profile, of which one fragment had the same size as the probe, and the other two fragments were very weak (Fig. 3B). Among the 11 probes tested only one probe, OPA02–850, gave a smeared hybridization pattern (Fig. 3B).

Presence/absence polymorphism

To determine the molecular basis of the presence /absence character of the RAPD polymorphism, eight polymorphic and three monomorphic partially purified RAPD fragments (type II probes, Table 1) were hybridized to the RAPD profiles from ten individuals of *P. sylvestris* analysed in our previous study (LU *et al.* 1995). Of the eight polymorphic probes, six probes gave hybridization signals only in individuals that showed the presence of the band on the RAPD profiles. For example, the OPA05–600 band was present in tree AC1016 but was absent in tree AC4210 (Fig. 3A). The OPA05–600 probe hybridized to a corresponding 600bp fragment in AC1016, but did not give any signal in AC4210 (Fig. 3B). On the other hand, one probe (OPA01–500) gave a strong signal in two trees (AC-1019, AC1014) possessing this fragment, and a weaker signal in the two trees (AC1005, BD1178) lacking it (Fig. 3A and 3B). The probe OPA02–850 gave a smeared hybridization pattern on the RAPD profiles of tree BD1178 and AC1014, but the signal at the 850bp position was stronger in tree BD1178 (Fig. 3B) that showed the presence of this band on its RAPD profile (Fig. 3A). The three probes representing monomorphic RAPD bands hybridized to the corresponding fragments on the RAPD profile in all analysed trees (results not shown).

Hybridisation to the genomic DNA digests

Eighteen RAPD fragments (Table 1) were used as probes in the Southern analysis of the genomic DNA digests (A-blots). Fourteen probes hybridized to single or low copy sequences, *i.e.*, fewer than three bands were observed (Fig. 4). Three probes (OPA05–1200, OPA18–850 and OPA18–800) did not give any signals. However, further slot-blot analysis showed the presence of these three sequences in the genomic DNA (results not shown). Visual inspection of the intensity of these signals suggested that they also corresponded to low-copy sequences. Only one probe (OPA02–850) hybridised to several fragments (Fig. 5), suggesting the repetitive character of this fragment. The hybridization patterns generated by each of the 15 probe/enzyme combinations were different. However, no variation was observed among individual trees for any of the probe/enzyme combinations tested. Individuals from the Turkish population of *P. sylvestris* gave the same hybridization patterns as trees originating from Sweden (Fig. 4). Total DNA digests of *Z. mays* and *A. thaliana* gave no hybridization signals to *P. sylvestris* probes (Fig. 4 and Fig. 5).

DISCUSSION

Individual RAPD bands contain more than one DNA sequence

We are aware that the best way to clarify the presence of additional sequences in individual RAPD band is to clone and sequence the DNA fragments in the band. Multiple transformants from a single RAPD band would need to be sequenced in order to answer if more sequences are present in the band, especially if the additional sequences are many but in minority. Large scale sequencing analysis is not in the scope of the present experiment. Instead, we used re-amplification of individual RAPD bands to investigate the presence

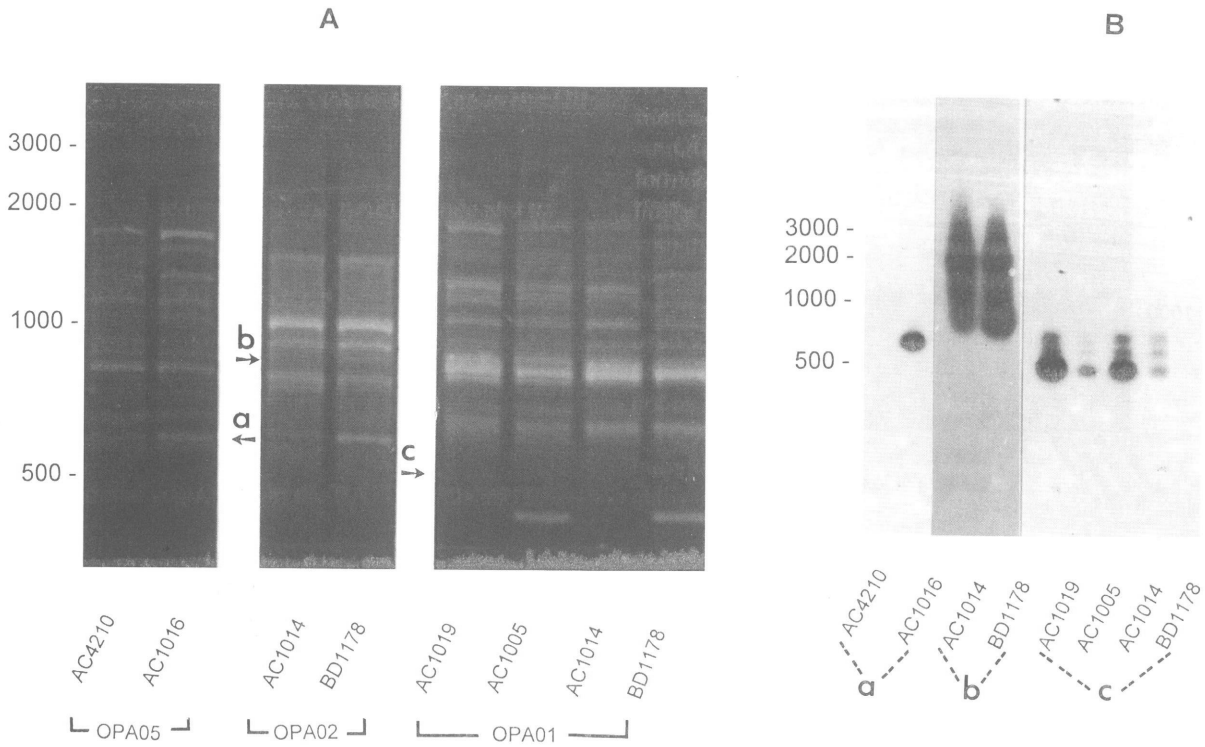

Figure 3. Southern analysis of the presence/absence polymorphism observed on RAPD profiles. **A:** RAPD profiles. Arrows indicate the polymorphic fragments analysed. **B:** hybridisation patterns of the selected fragments to corresponding RAPD profiles in A.

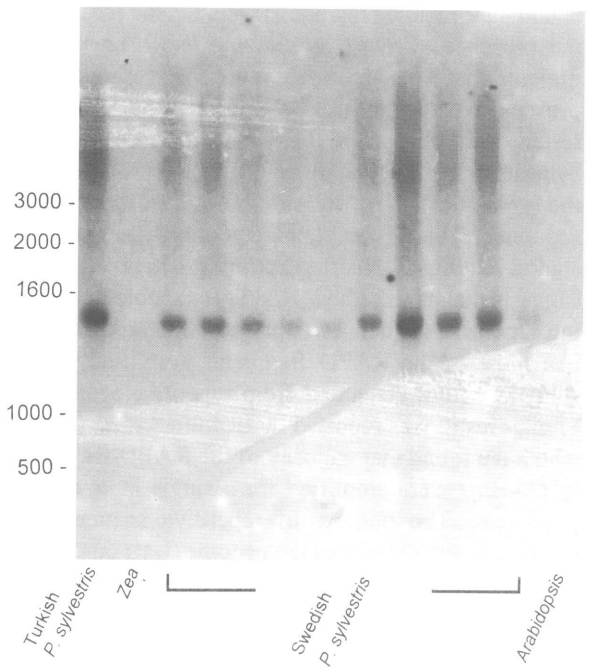

Figure 4. Hybridization of the OPA18–450 probe to *Hind* III genomic digests (A-blots).

of additional sequences in the RAPD bands and to purify the fragments of interest for further Southern analysis of RAPD profiles and total DNA digests.

Re-amplification of individual RAPD bands often produced several additional fragments and further Southern analysis revealed that most of these fragments were not homologous. Several factors can contribute to the production of additional fragments in the re-amplification of a RAPD band. The additional re-amplification products can represent contamination with fragments of the sheared genomic DNA that was used in the RAPD reaction. Alternatively, these additional fragments may result from contamination with other RAPD fragments during fragment isolation. Finally, such additional fragments may also represent single stranded DNA fragments which failed to re-anneal or formed secondary structures that changed their mobility in the gels. All these sequences, if isolated together with the target RAPD band, could serve as templates in the second amplification and produce visible additional fragments. Such a total re-amplification product, when used as a probe, would give more signals on the genomic DNA digests. Thus, this kind of product should not be used for detection of repetitive sequences.

QUIROS *et al.* (1995) found that hybridisation of individual RAPD bands to RAPD profiles detected many fragments in *Brassica nigra*. The authors sug-

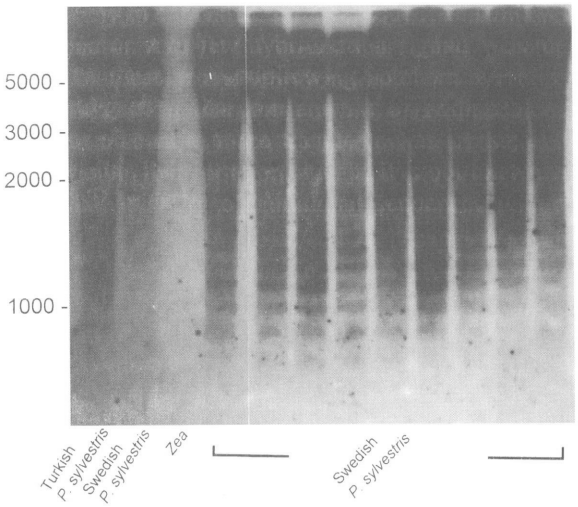

Figure 5. Hybridization of the OPA02–850 probe to *Kpn* I genomic digests (A-blots).

gested that this result was due to the occurrence of multiple co-migrating sequences within a single RAPD fragment used as a probe. In the present study, hybridization of re-isolated RAPD bands to RAPD profiles also often gave more than one signal. However, these additional signals were generally few and weak. In most earlier experiments, the RAPD bands used as probes were directly isolated from the first PCR products (KAZAN *et al.* 1993; N'GORAN *et al.* 1994; QUIROS *et al.* 1995; REITER *et al.* 1992; WILDE *et al.* 1992). In contrast, the probes used in our hybridizations to RAPD profiles were partially purified through re-amplification and re-isolation and, therefore, were likely to contain fewer sequences that could give rise to additional signals. This suggestion is concordant with results reported by BODENES *et al.* (1997) who used cloned RAPD fragments as probes and also found only a few signals following hybridization to genomic DNA digests. Such probes did not contain additional comigrating RAPD fragments that could generate multiple signals in the hybridization.

It has been suggested that the presence of additional sequences in a single RAPD band may lead to distortion of segregation proportions (QUIROS *et al.* 1995). In the present study, nine probes used in hybridisations to the genomic digests represented RAPD bands that were directly isolated from the first PCR products which were likely to contain additional sequences. However, only one or very few hybridisation signals were detected by these probes following Southern hybridization, suggesting that the amount of additional sequences present in these fragments was too small to yield visible hybridisation signals. It is not likely that the small quantity of additional sequences will be detectable on the ethidium bromide stained agarose gel in individuals lacking the band which could produce distortions in the expected segregation proportions. In fact, many inheritance studies in conifers have demonstrated the Mendelian segregation of most of the RAPD fragments (BINELLI & BUCCI 1994; BUCCI & MENOZZI 1993; CARLSON *et al.* 1991; LU *et al.* 1995).

Are RAPD bands generated from repetitive DNA sequences?

It has been suggested that individual RAPD bands are sometimes generated from homologous repetitive sequences (DEVOS & GALE 1992; N'GORAN *et al.* 1994; WILLIAMS *et al.* 1990). Two approaches (Southern analysis of RAPD profiles and genomic DNA digests) were employed in this study to establish whether the RAPD bands in *P. sylvestris* were generated from such sequences. If RAPD bands amplified by a single primer are derived from repetitive sequences they will be likely to show homology to each other and produce multiple signals on the autoradiogram when one of the bands was used as a probe (WEISING *et al.* 1995). Similarly, the probe (RAPD fragment) represents single or low copy sequence if only one or a few signals appear after hybridization to blots of genomic DNA digests (WEISING *et al.* 1995 and the references therein), unless the repetitive sequences are identical copies both in length and in their flanking regions as well. On the other hand, the presence of many hybridizing bands or a smear indicate that mid- or highly repetitive sequences are present (WEISING *et al.* 1995). Of the total 20 probes tested, only one probe (OPA02-850) gave smeared hybridization patterns both on the RAPD profile and on the genomic DNA digests indicating a repetitive sequence. This probe could potentially be useful in studies requiring DNA fingerprinting. This finding also suggests that as a method, RAPD is capable of detecting repetitive sequences. Our present results suggest that most of the RAPD fragments amplified in this study were not generated from repetitive sequences. Similar result was reported by BODENES *et al.* (1997), who have found that majority of the RAPD fragments in *Quercus* were amplified from single or low copy sequences. The number of repetitive sequences is correlated with the size of the genome (LAPITAN 1992). Therefore, taking into account the large genome size of conifers (RAKE *et al.* 1980) the paucity of the repetitive sequences observed in this study is surprising. More empirical data on the genome structure and organization in conifers are needed to explain these present results.

Molecular basis of the presence/absence character of RAPD variation

A characteristic feature of the RAPD variation in *P. sylvestris* and other diploid organisms is its dominant (presence/absence) character (*e.g.*, BINELLI & BUCCI 1994; LU *et al.* 1995; ROY *et al.* 1992; SZMIDT *et al.* 1996). Little is known, however, about the molecular basis of this phenomenon. To our knowledge, this particular aspect of RAPD variation has not been studied previously. Our present results suggest that in most cases the presence/absence polymorphism commonly observed among individual RAPD bands reflects true absence of the amplification product in individuals lacking a band rather than the difference in the amplification efficiency. Of the eight polymorphic RAPD fragments analysed, six fragments were truly absent in trees lacking the band in their RAPD profiles. Only one RAPD fragment (excluding the OPA02–850, which was found to represent a repetitive sequence) was amplified in all analysed trees but to a different extent. Such situation was also observed in some other studies (*e.g.*, HEUN & HELENTJARIS 1993).

The absence of an amplification product can arise through several entirely different mechanisms such as the abortion and/or variation of primer binding sites, the absence of target sequence in some individuals, and length mutation between the binding sites (WILLIAMS *et al.* 1990). All the *P. sylvestris* individuals analysed in this study (including those lacking a RAPD band) gave hybridization signals on the genomic DNA blots to all the RAPD fragments used as probes. This result suggests the presence of the probe sequences in the genomic DNA of all individual trees analysed. Therefore, the absence of bands in RAPD profiles of some trees is not due to the absence of a target sequence in the genome but due to the lack of its amplification. Length mutations between the primer binding sites are likely to produce restriction fragment length polymorphisims (RFLPs) detectable by the Southern analysis of the genomic DNA digests. The observed lack of RFLPs among analysed *P. sylvestris* individuals with all the probe/enzyme combinations suggests that this mechanism is not the prime cause for the presence /absence character of the RAPD variation. It is more likely that the RAPD polymorphism in *P. sylvestris* is due to the absence or alteration of one or both binding sites which is less likely to result in RFLPs detectable by Southern analysis of genomic DNA with a limited number of restriction enzymes. Cloning and sequencing of the amplified regions would provide additional information on this subject.

The lack of hybridisation signals in *Z. mays* and *A. thaliana* indicates that none of the *P. sylvestris* sequences amplified in this study was present in these two species. Similar results were found even among much more closely related species of *Brassica* (QUIROS *et al.* 1995).

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