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

Meng-Zhu Lu, Xiao-Ru Wang & Alfred E. Szmidt*

Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S–901 83 Umeå, Sweden
* corresponding author

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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

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.

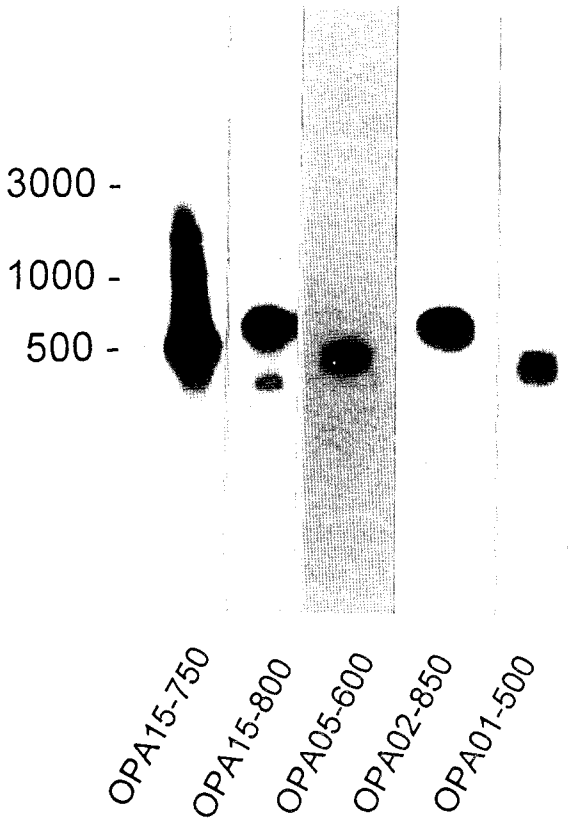

Figure 2. Hybridization pattern of the purified RAPD fragments (probe type II) to their re-amplification profiles (C-blots).

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