Empirical assessment of allozyme and RAPD variation in *Pinus sylvestris* (L.) using haploid tissue analysis

ALFRED E. SZMIDT*, XIAO-RU WANG & MENG-ZHU LU

Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences, S-901 83, Umeå, Sweden

We analysed 20 allozyme and 22 putative random amplified polymorphic DNA (RAPD) loci in two populations of *Pinus sylvestris* (L.) from northern Sweden. Genotypes for individual allozyme and RAPD loci were inferred from segregation patterns in haploid macrogametophytes. Therefore, it was possible to distinguish between homo- and heterozygotes carrying a RAPD fragment and to estimate directly the frequencies of RAPD fragments. The percentage of polymorphic loci and the expected and observed heterozygosity were lower for allozymes than for RAPDs. Average fixation indices for both types of markers were negative indicating a heterozygote excess over panmictic expectations. The apportionment of genetic variation within and among the investigated populations was similar for allozymes and RAPDs and showed that most of the variation resided within populations. RAPD genotypes inferred from haploid material were subsequently converted to diploid phenotypes and used to estimate indirectly the frequencies of RAPD fragments. Gene diversity measurements derived from indirectly estimated RAPD frequencies were distinctly lower than those based on directly estimated RAPD frequencies. This result was caused by the absence of the null homozygote at many loci which appeared as monomorphic in the diploid data set. Population differentiation coefficients based on the indirectly estimated RAPD frequencies were not concordant with those based on directly estimated RAPD frequencies. Our present results indicate that when complete genotype information can be obtained, RAPD analysis provides genetic information similar to that revealed by analysis of allozyme variation. On the other hand, our results are concordant with theoretical results suggesting that analysis of RAPD variation in diploid material can produce unreliable estimates of population-genetic parameters.

Keywords: allozymes, gene diversity, *Pinus sylvestris*, population differentiation, RAPDs.

Introduction

The random amplified polymorphic DNA (RAPD) technique (Williams *et al.*, 1990; Welsh & McClelland, 1990) is an increasingly popular tool in genetic studies. Unfortunately, population analysis with RAPD data is hampered by the generally dominant character of RAPD variation which precludes zygosity inference and direct estimation of allele frequencies. As noted by Lynch & Milligan (1994), this enhances the sampling variance associated with individual loci as well as inducing bias in estimation of population-genetic parameters.

When only diploid material is available for analy-

sis, frequencies of RAPD fragments are estimated by indirect methods employing the observed frequency of individuals lacking the fragment, i.e. the frequency of null homozygotes (Clark & Lanigan, 1993; Liu & Furnier, 1993; Lynch & Milligan, 1994). In this approach it is assumed that the population under study is in a Hardy–Weinberg equilibrium (HWE). When data for codominant markers are available, the estimated RAPD allele frequency can be corrected for potential deviations from HWE (Chong *et al.*, 1994). Studies employing allozymes suggest that wind-pollinated conifers, including *Pinus sylvestris* (L.), generally show only modest departures from HWE for this type of marker (Szmidt & Muona, 1985; Plessas & Strauss, 1986). This suggests that the same may be true for

*Correspondence.

well as indirect RAPD frequencies, were used for estimation of the following gene diversity parameters: percentage of polymorphic loci (P), unbiased expected heterozygosity (H_E), observed heterozygosity (H_O) and unbiased genetic distance (D) (Nei, 1987). Observed heterozygosities were corrected for macrogametophyte sampling (Morris & Spieth, 1978). Fixation indices (F) were computed as described by Curie-Cohen (1982). χ^2-tests were used to examine conformance of F estimates to Hardy–Weinberg expectations. H_O and F could be calculated only for allozymes and the direct RAPDs. Calculations were made using release 1.7 of the BIOSYS-1 program (Swofford & Selander, 1981). Coefficients of gene differentiation (G_{ST}) (Nei, 1987) were calculated using allozyme and putative RAPD loci that were polymorphic in at least one population. A locus was considered polymorphic when the frequency of the most common allele did not exceed 0.95. To limit the sampling bias resulting from the dominant character of RAPDs, Lynch & Milligan (1994) have recommended the restriction of RAPD analyses to loci for which the null homozygote frequency is more than $3/N$, where N is the number of individuals sampled in a population. With the sample size of 20 used in the present study this restriction applied to null homozygotes whose incidence was above 0.15. Therefore, genetic parameters based on indirect and direct RAPDs were also calculated for only those loci that satisfied this restriction.

Results

Allozymes vs. direct RAPDs

The two primers used in the present study revealed 22 reproducible RAPD loci. The size of fragments amplified by these primers ranged from 0.23–1.4 kb. An example of the RAPD profiles generated by the primers used in this study is presented in Fig. 1. Of the 20 allozyme and 22 putative RAPD loci examined, 16 and 21 loci, respectively, were polymorphic in at least one population. Fourteen allozyme loci were polymorphic in each of the two analysed populations. Twenty putative RAPD loci were polymorphic in the Svartberget population and 19 loci were polymorphic in the Korpilombolo population (Table 1). The mean variability measures (P, H_E and H_O) for allozymes were lower than similar measures for RAPDs (Table 2).

Average fixation indices for allozyme and RAPD markers were negative indicating heterozygote excess over panmictic expectations (Table 3). This excess was more pronounced for RAPDs than for allozymes. Three RAPD loci (OPA9–340, OPA9–400 and OPA10–1300) showed a statistically significant ($P<0.05$) excess of heterozygotes in the Svartberget population. In the Korpilombolo population, this excess was significant at two loci (OPA9–450 and OPA10–1250). There was much variation among allozyme loci with respect to the F estimates. On the other hand, F estimates for RAPDs were more uniform among loci (Table 3).

The genetic distance separating the Svartberget and Korpilombolo populations was low for allo-
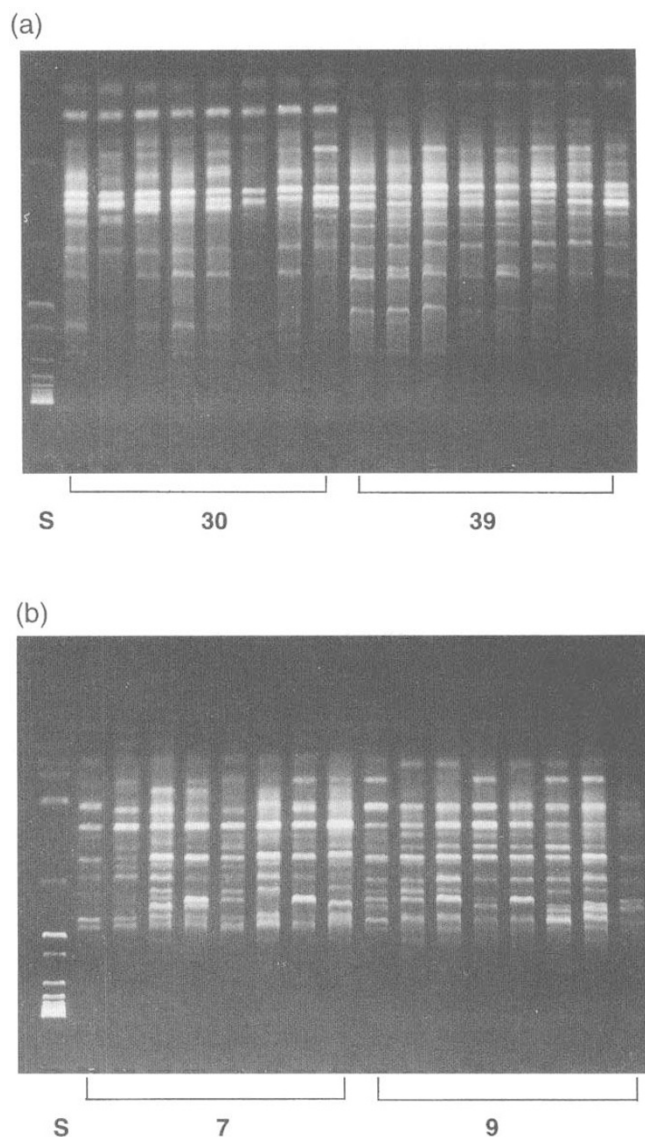
(a)

(b)

Fig. 1 RAPD profiles in haploid macrogametophytes from individual trees of *Pinus sylvestris* generated by (a) the OPA9 and (b) OPA10 primers. The leftmost lane contains a 1 kb DNA ladder. Trees are indicated by accession numbers.

Table 1 Frequencies of RAPD fragments in *Pinus sylvestris* estimated from haploid macrogametophytes (H) and diploid profiles (D)

Population locus		Svartberget			Korpilombolo		
		n	H	D	*n*	H	D
OPA9–230	*A*	20	0.975	1.000	18	1.000	1.000
	a		0.025	0.000		0.000	0.000
OPA9–320	*A*	20	0.375	0.401	18	0.194	0.215
	a		0.625	0.599		0.806	0.785
OPA9–340	*A*	20	0.325	0.401	18	0.389	0.463
	a		0.675	0.599		0.611	0.537
OPA9–400	*A*	20	0.575	0.746	18	0.694	0.647
	a		0.425	0.254		0.306	0.353
OPA9–450	*A*	20	0.750	1.000	20	0.675	1.000
	a		0.250	0.000		0.325	0.000
OPA9–650	*A*	20	0.825	1.000	20	0.950	1.000
	a		0.175	0.000		0.050	0.000
OPA9–700	*A*	20	0.650	0.665	20	0.700	1.000
	a		0.350	0.335		0.300	0.000
OPA9–750	*A*	20	0.900	1.000	20	0.925	1.000
	a		0.100	0.000		0.075	0.000
OPA9–800	*A*	20	0.725	0.746	20	0.925	1.000
	a		0.275	0.254		0.075	0.000
OPA9–1300	*A*	20	0.800	1.000	20	0.900	1.000
	a		0.200	0.000		0.100	0.000
OPA9–1380	*A*	20	0.525	0.598	20	0.525	0.490
	a		0.475	0.402		0.475	0.510
OPA10–410	*A*	18	0.611	0.733	19	0.553	0.656
	a		0.389	0.267		0.447	0.344
OPA10–480	*A*	18	0.306	0.370	19	0.500	0.430
	a		0.694	0.630		0.500	0.570
OPA10–520	*A*	20	0.800	1.000	19	0.816	1.000
	a		0.200	0.000		0.184	0.000
OPA10–600	*A*	20	0.925	1.000	19	0.895	1.000
	a		0.075	0.000		0.105	0.000
OPA10–750	*A*	20	0.825	1.000	20	0.875	1.000
	a		0.175	0.000		0.125	0.000
OPA10–800	*A*	20	0.850	1.000	20	0.974	1.000
	a		0.150	0.000		0.026	0.000
OPA10–850	*A*	20	0.600	0.665	20	0.575	0.598
	a		0.400	0.335		0.425	0.402
OPA10–1200	*A*	20	0.775	0.746	19	0.974	1.000
	a		0.225	0.254		0.026	0.000
OPA10–1250	*A*	20	0.350	0.401	20	0.425	0.541
	a		0.650	0.599		0.575	0.459
OPA10–1300	*A*	20	0.675	1.000	19	0.737	0.740
	a		0.325	0.000		0.263	0.260
OPA10–1400	*A*	20	0.975	1.000	20	0.950	1.000
	a		0.025	0.000		0.050	0.000

n, plant sample size for each locus; *A*, fragment present; *a*, fragment absent.

zymes and RAPDs ($D = 0.006$ and 0.003, respectively). The G_{ST} coefficients for allozyme and RAPD markers were similar and showed that most of the variation resided within populations (Table 4).

Direct RAPDs vs. indirect RAPDs

The null homozygote was absent at 10 RAPD loci identified in this study (data not shown). Consequently, only 12 of the 22 putative RAPD loci

Table 2 Mean measures of genetic diversity at 16 allozyme and 22 putative RAPD loci for the investigated populations of *Pinus sylvestris*

Population marker	$n \pm SE$	P	$H_O \pm SE$	$H_E \pm SE$
Svartberget				
Allozymes	19.8 ± 0.2	70.0	0.269 ± 0.054	0.262 ± 0.054
RAPD*	19.8 ± 0.1	90.9	0.444 ± 0.043	0.356 ± 0.030
RAPD†	19.8 ± 0.1	50.0	—§	0.223 ± 0.049
RAPD‡	19.7 ± 0.3	100.0	—	0.482 ± 0.007
Korpilombolo				
Allozymes	19.7 ± 0.2	70.0	0.296 ± 0.058	0.276 ± 0.053
RAPD*	19.3 ± 0.2	86.4	0.343 ± 0.050	0.298 ± 0.039
RAPD†	19.3 ± 0.2	40.9	—	0.192 ± 0.051
RAPD‡	19.2 ± 0.4	100.0	—	0.480 ± 0.025

n, mean sample size per locus; P, percentage of polymorphic loci; H_O, observed heterozygosity; H_E, unbiased expected heterozygosity.
*Based on direct RAPD frequencies, 0.95 criterion.
†Based on indirect RAPD frequencies, 0.95 criterion.
‡Based on indirect RAPD frequencies, for loci that satisfied the 0.15 restriction.
§Cannot be calculated from frequency data alone.

Table 3 Fixation indices for allozyme and putative RAPD loci in the investigated populations of *Pinus sylvestris*

Locus	Population Svartberget	Korpilombolo	Locus	Population Svartberget	Korpilombolo
Lap-1	—†	—	OPA9–230	—	—
Lap-2	—	−0.053	OPA9–320	−0.173	−0.241
Got-1	—	—	OPA9–340	−0.481*	−0.403
Got-2	−0.414	−0.303	OPA9–400	−0.535*	0.084
GDh	0.026	0.200	OPA9–450	−0.333	−0.481*
FEst	−0.129	−0.042	OPA9–650	−0.212	−0.053
Aco	−0.053	—	OPA9–700	−0.099	−0.429
Pgm-1	−0.081	−0.081	OPA9–750	−0.111	−0.081
Pgm-2	—	—	OPA9–800	−0.129	−0.081
Pgi-1	—	—	OPA9–1300	−0.250	−0.111
Pgi-2	0.134	−0.053	OPA9–1380	−0.303	0.098
Pgd-1	0.134	−0.103	OPA10–410	−0.403	−0.384
Pgd-2	−0.212	−0.190	OPA10–480	−0.440	0.263
APh	−0.176	−0.290	OPA10–520	−0.250	−0.226
SDh-1	−0.187	−0.178	OPA10–600	−0.081	−0.118
SDh-2	−0.081	—	OPA10–750	−0.212	−0.143
MDh-1	—	−0.053	OPA10–800	−0.176	—
MDh-2	−0.330	−0.538*	OPA10–850	−0.250	−0.125
ADh-1	−0.173	−0.099	OPA10–1200	−0.004	—
Mnr	0.626*	0.000	OPA10–1250	−0.319	−0.535*
			OPA10–1300	−0.481*	−0.086
			OPA10–1400	—	−0.053
Mean:	−0.065	−0.127	Mean:	−0.262	−0.163

†Not computed when the frequency of the most common homozygote exceeds 0.98.
*χ^2-test, $P < 0.05$.

Table 4 Comparison of G_{ST} coefficients for *Pinus sylvestris* based on allozymes, direct and indirect frequencies of RAPD fragments

Allozyme locus		RAPD locus	Direct	Indirect
Lap-2	0.004	OPA9–230	0.013	—*
GDh	0.005	OPA9–320	0.040	0.041
FEst	0.011	OPA9–340	0.004	0.004
Aco	0.026	OPA9–400	0.015	0.012
Pgi-2	0.024	OPA9–450	0.007	—
Pgd-1	0.135	OPA9–650	0.039	—
Pgd-2	0.020	OPA9–700	0.003	0.201
APh	0.009	OPA9–750	0.002	—
SDh-1	0.025	OPA9–800	0.069	0.145
SDh-2	0.013	OPA9–1300	0.020	—
MDh-1	0.004	OPA9–1380	0.000	0.012
MDh-2	0.006	OPA10–410	0.003	0.007
ADh-1	0.001	OPA10–480	0.039	0.004
Mnr	0.019	OPA10–520	0.000	—
		OPA10–600	0.003	—
		OPA10–750	0.005	—
		OPA10–800	0.048	—
		OPA10–850	0.001	0.005
		OPA10–1200	0.090	0.145
		OPA10–1250	0.006	0.020
		OPA10–1300	0.005	0.149
		OPA10–1400	0.004	—
Mean	0.022		0.019	
Mean†	—		0.023	0.062

*Locus monomorphic.
†Based on 12 polymorphic loci for both direct and indirect RAPDs.

remained polymorphic (0.95 criterion) following conversion of the RAPD genotypes inferred from haploid macrogametophytes to diploid phenotypes (Table 1). The P and H_E estimates derived from indirect RAPD frequencies at these loci were lower than those based on the direct RAPDs (Table 2). In the diploid data set, the frequency of null homozygotes was above 0.15 for only six loci (data not shown). The measures of intrapopulation diversity based on the indirect RAPD frequencies for these six loci were much higher than similar measures based on the direct RAPD frequencies (Table 2).

The estimates of population differentiation based on the indirect frequencies for polymorphic RAPD loci were different from those based on the direct RAPD frequencies. The genetic distance between the Svartberget and Korpilombolo populations ($D = 0.019$) was several times greater than distance coefficients based on the direct RAPD frequencies. The average G_{ST} for indirect RAPD frequencies was much higher than the average G_{ST} for the direct RAPD frequencies and allozymes (Table 4). The high D and G_{ST} obtained for the indirect RAPD frequencies were mainly caused by four loci: OPA9–700, OPA9–800, OPA10–1200 and OPA10–1300. Two of these loci (OPA9–800 and OPA10–1200) showed true differences between the Svartberget and Korpilombolo populations (Table 4). However, the other two loci had a much lower G_{ST} for the direct than for the indirect RAPDs (Table 4). This discrepancy was caused by the absence of the null homozygote at the OPA10–1300 locus in the Svartberget population, and at the OPA9–700, OPA9–800 and OPA10–1200 loci in the Korpilombolo population (data not shown). Consequently, in the diploid data set the Svartberget population becomes fixed for the presence of the OPA10–1300 fragment and the Korpilombolo population becomes fixed for the presence of the fragments at OPA9–700, OPA9–800 and OPA10–1200

loci. When analysis of population differentiation was based on six loci that satisfied the 0.15 restriction, the D and G_{ST} coefficients were low (0.001 and 0.013, respectively).

Based on the multilocus allozyme genotypes it was possible to discriminate between all individuals from the Svartberget and Korpilombolo populations. The same was true for the RAPD multilocus genotypes inferred from analysis of macrogametophytes. Based on the diploid RAPD phenotypes it was still possible to discriminate between 95 per cent of the investigated individuals (data not shown).

Discussion

Allozymes vs. direct RAPDs

Earlier studies have suggested that RAPD analysis usually reveals a high number of polymorphic loci (Carlson *et al.*, 1991; Bucci & Menozzi, 1993; Nelson *et al.*, 1994; Lu *et al.*, 1995). Our present results are concordant with these suggestions. Although only two primers were employed in the present analysis, as many as 22 putative RAPD loci could be identified.

The present analysis of RAPD variation in haploid macrogametophytes provides the first direct estimates of population-genetic parameters based on this new type of marker. In plants, RAPDs generally show similar or higher levels of polymorphism than other types of markers (Liu & Furnier, 1993; Chong *et al.*, 1994; Wu *et al.*, 1995). Our present measurements of genetic variability based on the direct RAPD frequencies are concordant with these results. All but one putative RAPD loci identified in the present study were polymorphic (0.95 criterion) and produced higher measures of gene diversity than allozymes. This result can be explained by more random and efficient genome sampling by RAPDs than by allozymes which are restricted to genes coding for proteins. The RAPD distance and G_{ST} coefficients for the investigated populations were low and comparable to similar measures for *P. sylvestris* populations from Scandinavia based on allozymes (Szmidt & Muona, 1985; Szmidt & Wang, 1993; this study). Low levels of population differentiation for both types of markers found in this study can be explained by unrestricted gene flow among populations and the relatively recent history of *P. sylvestris* in Scandinavia.

It has been suggested that because of the generally neutral character of allozyme and RAPD markers potential deviations from HWE should affect both types of markers in a similar way (Chong

et al., 1994). Results of the present study provide the first empirical evidence for this suggestion. Fixation indices for both allozyme and RAPD loci were negative, indicating an excess of heterozygotes over panmictic expectations. In conifers, inbred individuals commonly express inbreeding depression and are removed from a population during the early stages of life (Eriksson *et al.*, 1973). The material analysed in this study came from two mature stands of *P. sylvestris*. Selection against the more homozygous, relatively inbred individuals was the likely cause of the negative fixation indices observed in this and other studies (e.g Szmidt & Muona, 1985; Plessas & Strauss, 1986; Kärkkäinen & Savolainen, 1993). It thus appears that when complete genotype information is available, analysis of RAPD variation yields population genetic parameters that are comparable to those based on allozymes. Nearly all individuals analysed in this study could be distinguished by RAPD profiles generated with only two primers. This confirms the usefulness of RAPD markers for fingerprinting purposes suggested in many previous studies (e.g. Koller *et al.*, 1993; Keil & Griffin, 1994).

Direct vs. indirect RAPDs

Theoretical studies suggest that the usefulness of RAPDs is limited when only diploid material is available (Clark & Lanigan, 1993; Lynch & Milligan, 1994). Our present results provide the first empirical information on this subject. Dominance is widely observed for RAPD fragments in *P. sylvestris* and other forest conifers (Carlson *et al.*, 1991; Bucci & Menozzi, 1993; Lu *et al.*, 1995). Because of this property of RAPDs, gene frequency estimates for such loci derived from diploid material are less accurate than those obtained with codominant markers. When only diploid material is available and the null homozygote is not detected, a locus is scored as fixed for the fragment presence. As demonstrated by Lynch & Milligan (1994) the bias in the estimation of the null-allele frequency because of small sample size can be substantial when the null homozygote is rare. Our results revealed that in the diploid data set, 10 of the 21 polymorphic RAPD loci appeared as fixed for the fragment presence because of the absence of null homozygotes. Consequently, intrapopulational diversity parameters derived from the indirect RAPD frequency were much lower than similar parameters derived from complete genotype information. Statistical methods to analyse population differentiation using RAPD data include the assumption that the genotype frequencies are in

HWE (Lynch & Milligan, 1994). Under this assumption, 2 to 10 times more individuals should be sampled per RAPD locus than per codominant allozyme locus to reduce the fraction of loci that will yield biased parameter estimates in diploid material (Lynch & Milligan, 1994). Most of the putative RAPD loci identified in the present study had negative *F*-values indicating that the frequency of homozygotes was substantially lower than expected under HWE. This result implies that RAPD analysis in diploid material would probably require a much larger sample size than that suggested by Lynch & Milligan (1994). Recently, Chong *et al.* (1994) have proposed a new method for the estimation of null-allele frequencies for putative RAPD loci corrected for deviations from HWE. In this method, frequencies of null-alleles are estimated using the F_{IS} value (Wright, 1965) previously estimated with codominant markers, e.g. allozymes for the same population. The generally higher deviations from HWE for the RAPD loci than for allozyme loci found in our study indicate that such correction may be insufficient. The necessity of carrying out additional analyses employing codominant markers further limits the feasibility of this approach.

Lynch & Milligan (1994) have suggested that in diploid material unbiased estimates of population-genetic parameters can be achieved with RAPDs provided the analysis is restricted to markers that are not too common. In our diploid data set, this restriction was satisfied for only a few highly polymorphic loci and caused substantial overestimates of gene diversity measures. Moreover, the loci that are rejected may differ from population to population (Lynch & Milligan, 1994). In the present study, such differences were observed for several RAPD loci and produced biased estimates of population differentiation.

It has been suggested that RAPDs may be particularly useful in genetic conservation programmes, where they could allow distinct gene pools to be recognized from small population samples (Wu *et al.*, 1995). Results of the present study clearly warn against such application of RAPD markers when only diploid material is available for analysis. Both earlier theoretical and the present empirical results demonstrate that RAPD analysis can produce seriously biased estimates of genetic parameters when only diploid material is available for analysis and the sample size is small. In conifers, this bias can be compounded by deviations from HWE, especially those involving heterozygote excess.

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