grams constructed, could point towards a conservative approach regarding the origin of plant material for artificial regeneration. In light of the above discussion, and provided that the results presented are coupled with provenance studies, the use of plant material originating from Thasos island should be limited to this locale. Nevertheless, the opposite practice could be beneficial in a program of breeding through intraspecific hybridization.


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# Analysis of Random Amplified Polymorphic DNA Markers in Three Conifer Species


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### Abstract

From 40 arbitrary primers tested with one DNA from each species, two, three and four were chosen to generate RAPD profiles in Norway spruce, silver fir and Scots pine respectively. Sample sites were Northern Vosges, Hunsrück mountains and, between them, the forest around the University of Saarbrücken. The similarity indices of intra- as well as between-sampling sites averaged for all primers, were distinctly lower (0.48) in all spruce and fir stands than in Scots pines' (0.79). Almost all variability resided within samples. Mean genetic distances among sampling sites were generally small and seemed slightly elevated in Scots pine only. Some applications of RAPD data are discussed.



*Key words: Abies alba* MILL., *Picea abies* L. KARST., *Pinus sylvestris* L., RAPDs, polymorphism.

*FDC:* 165.5; 165.3; 174.7; *Abies alba;* 174.7 *Picea abies;* 174.7 *Pinus sylvestris.*


### Introduction

Allozyme studies have shown that conifers are one of the genetically most variable groups of forest tree species, probably because they are long-lived, have large geographic distributions, exhibit wind pollination linked to high fecundities, shed winged seeds and are common in later successional stages (MITTON, 1983). Some species, however, such as red pine, Torrey pine and western red cedar harbour almost no genetic variation for soluble proteins. This may possibly be due to their restricted and isolated populations, where genetic drift is known to erode allozyme variability more quickly in smaller populations. Though conifers, in large and continuous stands, shed more than 90% outcrossed seed, small stands and isolated trees are increasingly forced to selfing. However, inbred progeny suffer severe inbreeding depression and it is thought that selection against deleterious alleles found in homozygotes arising from consanguineous matings results in an overall excess of heterozygotes in older stands. On account of these counteracting evolutionary strategies and contradictory find-

[1]) Correspondence

ings every prediction concerning the genetic variability of a given population seems questionable. A rapid procedure for comparative screening of given stands of forest trees is therefore desirable.

The random amplified polymorphic DNA (RAPD) assay (WILLIAMS et al., 1990) has become an increasingly popular tool in genetic studies. Since its simplicity and rapidity permit identification of a large number of polymorphic DNA markers distributed throughout the genome it should have become the method of choice to analyse coniferous populations, too. Instead, after a few early studies on forest trees which revealed the MENDELian inheritance of amplified sequences but emphasized that dominant RAPD markers of diploid tissue would be unsuitable for populational surveys, they have been exclusively used in genomic mapping (JERMSTAD et al., 1994; BUCCI and MENOZZI, 1995; ISABEL et al., 1995; PLOMION et al., 1995; DEVEY et al., 1996).

The objective of the study described here was to fill this gap by revealing the extent of genetic variability of RAPDs in and between stands of silver fir (*Abies alba* MILL.), Norway spruce (*Picea abies* L. KARST.) and Scots pine (*Pinus sylvestris* L.). The statistical methods used were those developed for multilocus fingerprint evaluation.

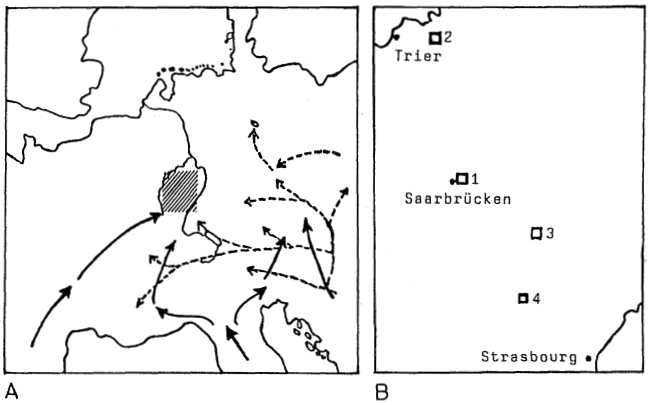


*Fig. 1.* – Location of sampling sites. A) shaded area between the rivers Rhine and Moselle in relation to the putative migratory routes of silver fir (solid arrows) and Norway spruce (dotted arrows) from the glacial refugia. B) enlarged area showing the sampling sites Scheidt (1), Osburger Hochwald (2), Hasselfurter Weiher (3) and Neuwiller (4).

## Material and Methods

*Plant material and DNA extraction*

Young needles of naturally regenerated trees not older than 20 years were collected and stored at –80 °C. Sampling sites were (*Fig. 1*)

Neuwiller-lès-Saverne (Northern Vosges, France),

Hasselfurter Weiher (near Bitche, France),

Scheidt (around the University campus, Saarbrücken, Germany),

Osburger Hochwald (Hunsrück, Germany).

For DNA extraction from needles ground in liquid nitrogen, three procedures were used. DNA-Isolator™ (Genosys, Cambridge) resulted in the lowest yield for all species. The PVP method of GUILLEMAUT and MARÉCHAL-DROUARD (1992) and the CTAB procedure of MURRAY and THOMPSON (1980) both worked satisfactorily. The DNA, which was not further purified in CsCl, was diluted to a working concentration of 100 ng/µl.

*RAPD protocol*

Using 0.2 ml tubes, amplifications were performed in volumes of 25 µl consisting of 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCL_2$, 0.1% Triton X-100, 0.2 mM dNTPs, 1 µM primer, 0.5 unit of Prime Zyme DNA polymerase (Biometra, Göttingen, Germany) and 100 ng genomic DNA. The DNA thermal cycler (Personal cycler from Biometra with heated lid) was programmed for 1.5 min at 94 °C followed by 45 cycles of 30s at 94 °C (denaturation), 1 min at 42 °C (annealing), 2 min at 72 °C (extension), and a final 10-min extension at 72 °C. Amplification products were separated by electrophoresis on 1.4% agarose gels using 1 x Tris-borate-EDTA buffer and detected by staining with 1.5 x $10^{-6}$M bisbenzimide H 33258 (Serva, Heidelberg, Germany). The Kilobase DNA-marker (Pharmacia Biotech, Uppsala, Sweden) and the DNA molecular weight marker VI (Boehringer, Mannheim, Germany) were used for sizing PCR products. The arbitrary decamer primers initially screened with one DNA of each of the three species consisted of 10 (kit 2–50) purchased from Genosys (Cambridge,

*Table 1.* – Attributes of primer kits, each composed of ten (in parenteses), and selected single primers showing reproducible RAPD patterns in *Abies alba*, *Pinus sylvestris* and *Picea abies*.

| Primer (or total kit) | Nucleotide sequence 5`to 3` | % GC content | (Average) number of fragments A. alba | P. sylv. | Pic. abies |
|---|---|---|---|---|---|
| (2-50) | | 50 | (5.06) | (2.3) | (4.7) |
| 2-50-07 | ACGATTCCTG | 50 | 7 | | |
| (160) | | 60 | (5.3) | (4.6) | (5.5) |
| (170) | | 70 | (5.5) | (7.3) | (7.1) |
| 170-03 | ACGGTGCCTG | 70 | | 6 | |
| 170-04 | CGCATTCCGC | 70 | | 8 | 10 |
| 170-05 | GAGATCGGCG | 70 | 9 | | |
| 170-06 | GGACTCCACG | 70 | | | 9 |
| (180) | | 80 | (5.0) | (6.8) | (7.0) |
| 180-02 | CGCCCAAGCC | 80 | | 9 | |
| 180-06 | GCACGGAGGG | 80 | 9 | | |
| 180-10 | CGCCCTGGTC | 80 | | 10 | |

England) and of 30 (kits 160, 170 and 180) purchased from Roth (Karlsruhe, Germany). Only primers yielding reproducible and unambiguous fragments (3 primers for silver fir, 2 for Norway spruce and 4 for Scots pine DNA) were chosen for within and between stand comparisons (*Table 1*).

*Analysis of RAPD data*

Only RAPD profiles of the same gel were compared pairwise within and between stands. Amplified fragments were scored as the presence or absence of a fragment. Non-reproducible RAPD bands, generally not found with the primers used, are typically very faint and have often a molecular weight >2000 bp or <200 bp. The level of polymorphism was quantified by using NEI's estimator of similarity, based on the probability that an amplified fragment from one tree will also be found in another according to the formula $S_{xy} = 2n_{xy}/(n_x + n_y)$ where $n_{xy}$ is the number of fragments shared by individuals x and y and $n_x$ and $n_y$ are the number of fragments scored for each individual (NEI and LI, 1979; LYNCH, 1991). Within sample similarity ($S_i$ or $S_j$) is calculated as the average of $S_{xy}$ throughout all possible comparisons between individuals within a sample (i or j), and between sample similarity ($S_{ij}$) is the average of all paired individuals from samples i and j. $S_{ij}$ was converted to a measure of genetic distance ($D_{ij}$) using the equation (LYNCH, 1991): $D_{ij} = -\ln [S_{ij}/\sqrt{(S_i S_j)}]$. $D_{ij}$ values were used to construct dendrograms using the unweighted pair-group method of analysis (UPGMA). In our tables $S_i$, $S_j$ and $S_{ij}$ values are uniformly recorded under $P_b$ (band-sharing probability). The mean population frequency q of an amplified sequence was provisionally derived from $1-\sqrt{1-P_b}$ (JEFFREYS and MORTON, 1987) and the mean probability h for a band to be present in the heterozygous state was calculated from 2(1-q)/(2-q) (GEORGES et al., 1988).

*Table 2.* – RAPD values of n silver firs from Scheidt (1), Osburger Hochwald (2) and Neuwiller (4) generated with 3 primers. The averaged statistical parameters of intra- and between-sample comparisons are the similarity indices ($P_b$) and the genetic distances ($D_{ij}$).

| Primer | Stands | n | $P_b$ | $D_{ij}$ |
|---|---|---|---|---|
| 2-50-07 | 1 | 2 | 0.46 | |
| | 2 | 4 | 0.47 | |
| | 4 | 6 | 0.51 | |
| | 1/4 | 8 | 0.46 | 0.05 |
| | 2/4 | 10 | 0.56 | 0.13 |
| | 1/2 | 6 | 0.52 | 0.11 |
| 170-05 | 1 | 2 | 0.63 | |
| | 2 | 5 | 0.45 | |
| | 4 | 6 | 0.53 | |
| | 1/4 | 8 | 0.52 | 0.10 |
| | 2/4 | 11 | 0.47 | 0.04 |
| | 1/2 | 7 | 0.52 | 0.02 |
| 180-06 | 1 | 2 | 0.50 | |
| | 2 | 5 | 0.45 | |
| | 4 | 6 | 0.47 | |
| | 1/4 | 8 | 0.53 | 0.09 |
| | 2/4 | 11 | 0.50 | 0.08 |
| | 1/2 | 7 | 0.45 | 0.05 |

## Results

*Abies alba MILL.*

All 40 primers tested with one silver fir DNA resulted in different banding profiles. The two primers 2-50-08 and 160-07 amplified only one sequence, but on an average, all primer kits regardless of their % GC content, yielded the same number of bands (*Table 1*).

RAPD patterns of 11 silver firs from Osburger Hochwald (01 to 011) and of 2 trees from Scheidt (S1, S2) exhibit a considerable polymorphism (*Fig. 2*). With primer 170-05 average intrasample band-sharing rates ($S_i$ or $P_b$) of 0.4 (±0.15) and 0.63 (±0), respectively, result. Further primers (2-50-07, 180-06), though they amplified totally different sets of sequences (profiles not shown), yielded similar band-sharing rates (0.38 ± 0.15 and 0.38 ± 0.15) in the same trees from Osburger Hochwald. Eight silver firs from Neuwiller exhibited average band-sharing rates of 0.57 (± 0.18) and 0.38 (± 0.16) and 0.52 (± 0.22)

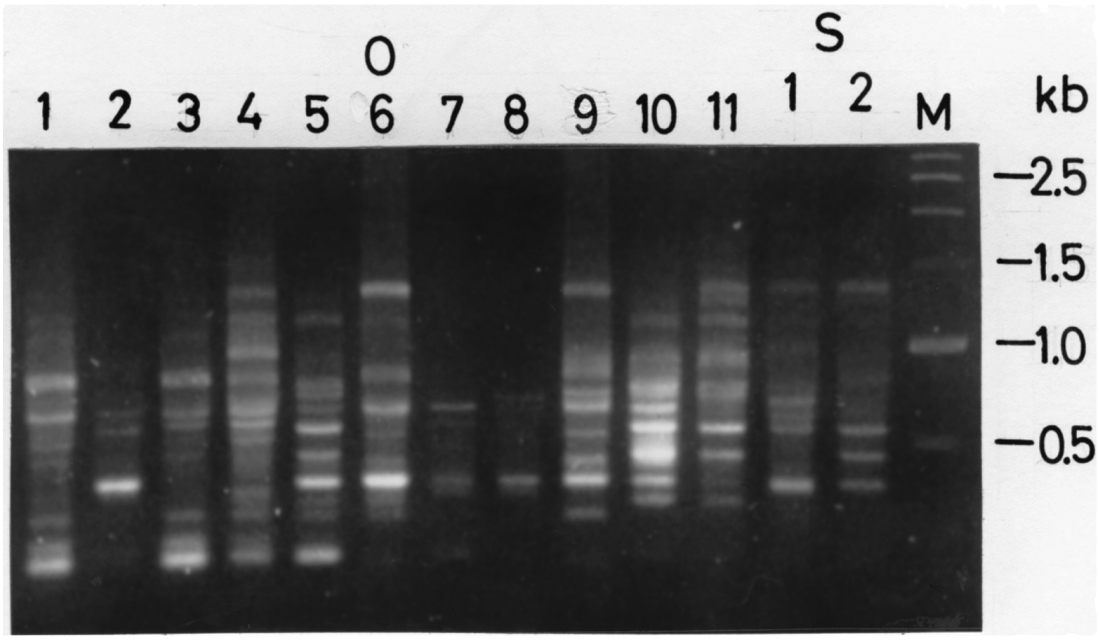


*Fig. 2.* – RAPD patterns generated in 11 silver firs from Osburger Hochwald (O) and 2 from Scheidt (S) by primer 170-05. M the KiloBase DNA Marker lane.

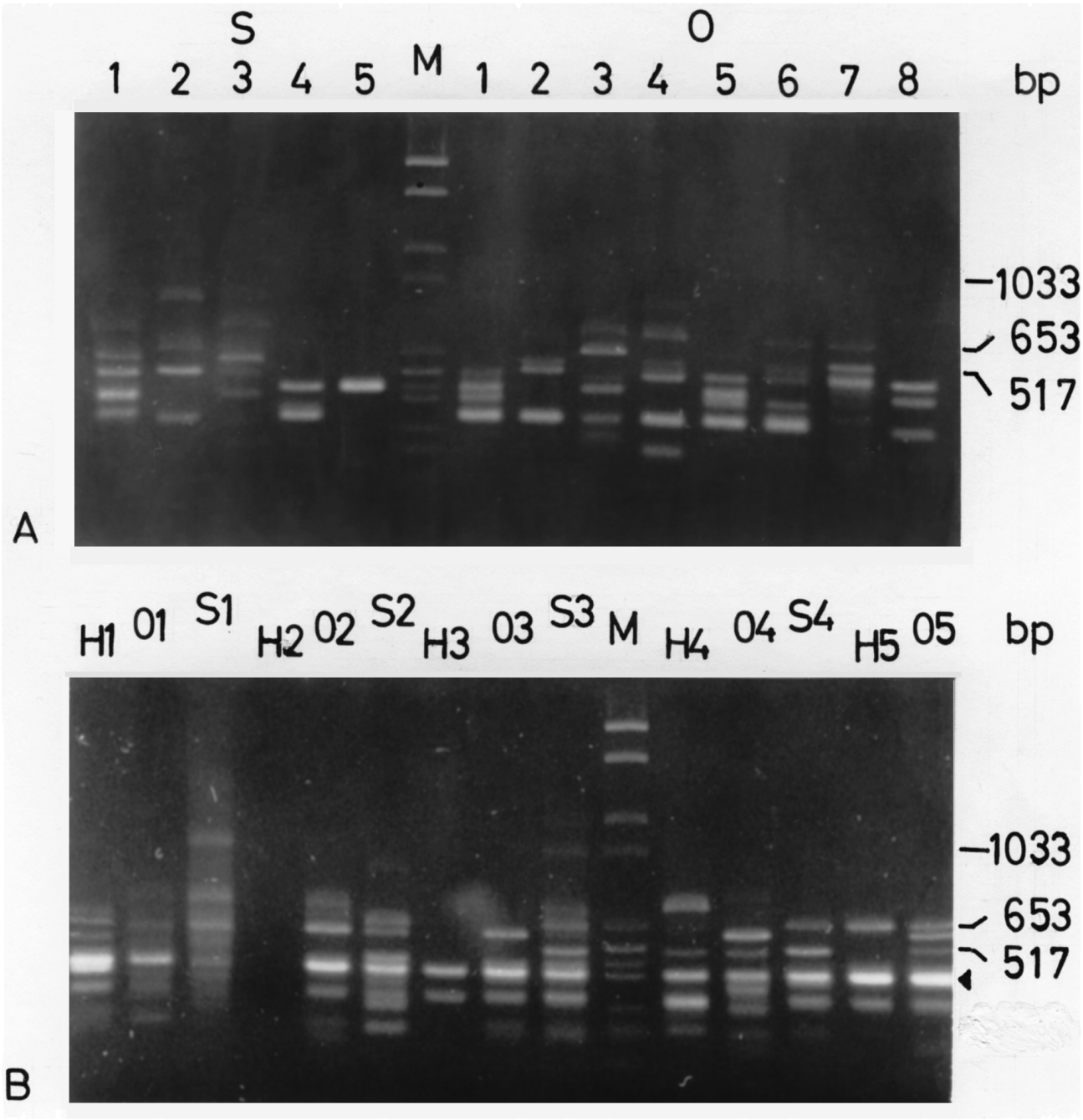


*Fig. 3.* – RAPD profiles of Norway spruce DNAs from Scheidt (S), Osburger Hochwald (O) and Hasselfurter Weiher (H). Some fragment sizes of the molecular weight marker VI (lane M) are given in basepairs (bp). A) patterns of DNA fragments amplified with primer 170-04. B) bands amplified from trees of 3 stands by primer 170-06. The arrowhead points to a monomorphic band. DNA of H2 did not amplify.

with the primers 2-50-07, 170-05 and 180-06. In order to compare intrasample and between-sample similarity, amplification profiles of the same agarose gel had to be evaluated. This was done with a necessarily restricted number of silver firs from Scheidt, Osburger Hochwald and Neuwiller by using 3 primers (*Table 2*). Betweensample similarity ($P_b$) indices were not found to be significantly lower than intrasample similarities. Therefore, most variability is contained in each sample and scarcely any between-sample differentiation can be expected. The tentatively evaluated frequencies for an amplified sequence (allele) are rather low and lie between 0.26 and 0.34 (with the exception of 0.39 for 2 silver firs from Scheidt, planted in an area otherwise free of this species). Consequently, the preliminary average probabilities h for amplified bands to be heterozygous are high (0.79 to 0.85). Since true heterozygosities, however, cannot be derived from dominantly manifesting RAPD bands, our data must be compared at a later date with those obtained from haploid megagametophytes of conifer seeds.

*Picea abies (L.) KARST.*

To start with, one Norway spruce DNA was used to screen all 40 primers (*Table 1*, last column). Primers with a 70% and 80% GC content generally amplified more sequences than those with a 50% and 60% GC content. Two primers (170-04 and 170-06) were then chosen to determine intrasample similarities. Five trees from Scheidt, 8 from Osburger Hochwald and 9 from Hasselfurter Weiher were used. Primer 170-04

resulted in very different profiles exhibiting no monomorphic bands (*Fig. 3A*). Band-sharing rates of both samples slightly exceed 0.40, whereas a value of 0.50 ± 0.12 was obtained with trees from Hasselfurter Weiher.

*Table 3.* – RAPD values of n trees of Norway spruce from Scheidt (1), Osburger Hochwald (2) and Hasselfurter Weiher (3) generated with 2 primers.

| Primer | Stands | n | $P_b$ | $D_{ij}$ |
|---|---|---|---|---|
| 170-04 | 1 | 4 | 0.44 | |
| | 2 | 5 | 0.42 | |
| | 3 | 5 | 0.62 | |
| | 1/3 | 9 | 0.52 | 0.015 |
| | 2/3 | 10 | 0.52 | 0.07 |
| | 1/2 | 9 | 0.46 | 0.02 |
| 170-06 | 1 | 4 | 0.37 | |
| | 2 | 5 | 0.46 | |
| | 3 | 5 | 0.48 | |
| | 1/3 | 9 | 0.51 | 0.19 |
| | 2/3 | 10 | 0.48 | 0.02 |
| | 1/2 | 9 | 0.44 | 0.06 |

To compare intrasample similarities with between-sample band-sharing rates, RAPD profiles of all 3 stands generated by both primers and run on the same gel were evaluated two and two in all possible combinations. The amplification patterns with primer 170-06, exhibiting one monomorphic band of 394 bp, are shown in *figure 3B*. As far as between-sample similarities are concerned, the data for both primers (*Table 3*) do not reveal a general trend. Obviously, each Norway spruce stand harbours such a lot of genetic variability that $P_b$ values of between-stands are not lower than intrastand similarities, but rather exceed them. As in silver fir, the preliminary mean allele frequency q is 0.28 and the mean value of 0.82 for h is similarly high.

*Pinus sylvestris L.*

One Scots pine DNA was used to screen all 40 primers. As with Norway spruce DNA, primers with 70% and 80% GC content generally amplified a few sequences more (*Table 1*). Four primers were chosen for intra- and between-sample comparisons. Scots pines from Hasselfurter Weiher, Osburger Hochwald and Scheidt were analysed. Primer 170-04, for example, revealed a marked similarity within and between stands (*Fig. 4*) and generated two monomorphic fragments of 900 bp and 745 bp. That this is not a characteristic peculier to the primer 170-04, has been shown by the data of additional primers tested (*Table 4*). The intrastand band-sharing rate $P_b$ averaged from all primer tests and stands is 0.81. Since stands of different populations are concerned, this may be attributed to either the same or totally different loci amplified by the primers. Only between-sample similarity values can help to decide between these alternatives. Since the between-sample band-sharing rate, again averaged from all sample pairs and primers, is 0.78, we conclude that the considerable similarity in Scots pine stands is caused by identical alleles, even though 3 comparisons (1/3 and 2/3 with primer 180-10 and 1/2 with primer 170-04, *Table 4*) exhibit somewhat reduced similarities.

*Table 4.* – RAPD values of n Scots pines from the sampling sites given in *table 3.*

| Primer | Stands | n | $P_b$ | $D_{ij}$ |
|---|---|---|---|---|
| 180-02 | 1 | 5 | 0.92 | |
| | 2 | 4 | 0.83 | |
| | 3 | 5 | 0.74 | |
| | 1/3 | 10 | 0.82 | 0.006 |
| | 2/3 | 9 | 0.81 | 0.03 |
| | 1/2 | 9 | 0.81 | 0,08 |
| 180-10 | 1 | 5 | 0.84 | |
| | 2 | 4 | 0.88 | |
| | 3 | 4 | 0.75 | |
| | 1/3 | 9 | 0.70 | 0.06 |
| | 2/3 | 8 | 0.73 | 0.02 |
| | 1/2 | 9 | 0.85 | 0.10 |
| 170-03 | 1 | 5 | 0.81 | |
| | 2 | 4 | 0.78 | |
| | 3 | 4 | 0.77 | |
| | 1/3 | 9 | 0.79 | 0.15 |
| | 2/3 | 8 | 0.82 | 0.21 |
| | 1/2 | 9 | 0.80 | 0.045 |
| 170-04 | 1 | 5 | 0.87 | |
| | 2 | 4 | 0.69 | |
| | 3 | 4 | 0.89 | |
| | 1/3 | 9 | 0.74 | 0.17 |
| | 2/3 | 8 | 0.79 | 0.12 |
| | 1/2 | 9 | 0.68 | 0.33 |

## Discussion

*General applicabilities of RAPD data*

About 29.6% (7.37 Mio ha) of the total area of the Western Federal Republic are covered with forest. The three main coniferous species *Picea abies* (L.) KARST., *Pinus sylvestris* L. and *Abies alba* MILL., analysed by RAPD fingerprints in this study, comprise 61.3% of the total forest area (MUHS, 1989). Due to their economic importance it is of interest to document and compare the levels of genetic variation in the three species, even though this has been done here only in a restricted area in South-West Germany and the neighbouring French area.

Our results may be useful for the following 4 reasons:

First, does the extent of genetic variation revealed by molecular RAPD markers agree with that formerly obtained from allozyme polymorphisms? With regard to the latter, conifers are known to be one of the most genetically variable groups of species and it is generally accepted that most of the genetic variation is found within a single population (MITTON, 1983; MÜLLER-STARCK, 1991). Beyond all doubt, this is true for silver fir and Norway spruce stands whose amplification profiles, generated with three and two arbitrary primers respectively, result in intra- and between-sample similarity values of approximately 0.5, preliminarily in average allele frequencies of about 0.30 and in mean heterozygosities of more than 0.80. Observed heterozygosities of alIozyme loci as reviewed by MÜLLER-STARCK (1991) for silver fir and Norway spruce are 0.49 and 0.12. The more abundant polymorphisms of DNA markers (RAPDs and microsatellite loci) presumably originate in being likely to fall within noncoding DNA (PLOMION et al., 1995; LIN and FURNIER, 1993; SMITH and DEVEY, 1994). Whereas our RAPD data on silver fir and Norway spruce conform with allozyme studies, it is surprising that our results from Scots pine do not. Similarity values within stands of approx. 0.80 are very high, correspondingly the presumed average allele frequencies are elevated and the mean heterozygosities are reduced to 0.58. At a first glance, it appears that different alleles may be fixed in particular Scots pine stands.

*Fig. 4.* – Patterns of amplification of Scots pines from Hasselfurter Weiher (H), Osburger Hochwald (O) and Scheidt (S) by primer 170-04 showing 2 monomorphic bands (arrowheads). Lane H4 without amplification. Molecular weight marker VI (lane M) fragment sizes are given in basepairs (bp).

However, inspection of banding profiles generated from different samples with 4 arbitrary primers as well as between-sample band-sharing rates (*Table 4*) signify that all Scots pine stands predominantly harbour the same alleles. This is contradictory to all allozyme studies (MÜLLER-STARCK, 1991) which indicate an intrapopulation variation for Scots pine substantially greater than that reported e.g. for Norway spruce. At present we have no explanation for the exceptional position of the Scots pine. We cannot totally exclude that if additional primers were used for Scots pine greater diversity would result. In other cases where very low intrapopulation variability has been found, such as in *Pinus merkusii* (SZMIDT et al., 1996), disjunct populations reveal great differentiation. Genetic drift, known to erode genetic variation quickly in smaller populations seems to be as improbable for the continuous distribution of Scots pine as any speculation about a bottleneck in population size after the last glaciation (except restricted seed use for afforestation).

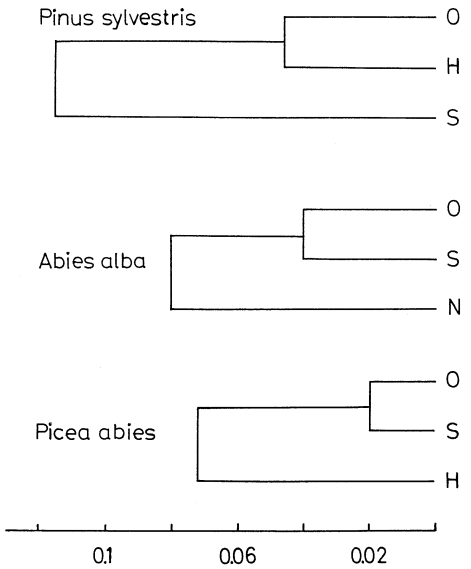

*Fig. 5.* – UPGMA dendrograms based on Lynch's genetic distances between the stands from Osburger Hochwald (O), Hasselfurter Weiher (H), Scheidt (S) and Neuwiller (N). $D_{ij}$ values of *tables 2* to *4* averaged for all primers were used.

Pertaining to our distance values among silver fir, Norway spruce and Scots pine stands the UPGMA dendrograms (*Fig. 5*) reveal minor differentiation in the first two species which harbour the highest variabilities, but a somewhat more pronounced differentiation among Scots pine stands. In spite of (or perhaps due to) their high similarity indices, a very small fraction of the genome with different fixed alleles may be sufficient to raise the genetic distance.

Second, are RAPD profiles related with quantitative traits? Surely not directly since amplified sequences are predominantly noncoding. However, they reflect either genetic uniformity or variability and accordingly the degree of heterozygosity, which has been found to be positively correlated to viability in beech (ZIEHE and MÜLLER-STARCK, 1991), to growth in birch (WANG, 1996) and to height, circumference and female fertility in maritime pine (DUREL et al., 1996). Conifers generally suffer severe inbreeding depression (MITTON, 1983) and high heterozygosity is expected to result in somatic, reproductive and adaptive (= greater homeostasis and better competition) heterosis. The abundant variability found in all our silver fir and Norway spruce stands seems to meet all prerequisites whereas the genetically more uniform Scots pines should be enriched with distantly related material all the more than they may have been purified from deleterious mutant alleles by selection.

Third, do RAPD data supply information on the migration routes of silver fir and Norway spruce from their glacial refugia? As outlined in *figure 1A,* Norway spruce and silver fir are assumed to have different postglacial recolonization pathways originating from 2 and 3 refugia, respectively

(SCHMIDT-VOGT, 1978; MORGANTE and VENDRAMIN, 1991; BERGMANN, 1991; KONNERT and BERGMANN, 1995). Since our sampling sites exhibit equally high variabilities in both species, we would speculate a) that silver firs of Northern Vosges as well as of Hunsrück mountains originated from both Central Italy and the Pyrenees (or possibly Southern France) and b) that Norway spruce, too, arrived there completely intermixed (SCHMIDT-VOGT, 1978). On account of the high intra- and between-sample variability and low genetic distances among the stands we conclude for the present that both species had at least a dual postglacial origin.

Fourth, can RAPD profiles be connected to forest decline? In our study the forest area damaged by air pollution and acid rain is: *Picea abies* 54.1%, *Pinus sylvestris* 54% and *Abies alba* 82.9%. These species are registered with the highest priority for conservation (under 1: extremely urgent) (MUHS, 1989). That high genetic variability at allozyme loci correlates with resistence against environmental stress has been documented for Norway spruce (BERGMANN and SCHOLZ, 1985) and beech (MÜLLER-STARCK, 1989). We presume that this is the result of adaptive heterosis which may also operate in silver fir and Norway spruce stands analysed in this study, but to a lesser extent if at all in Scots pine. Another strategy (in preparation) is the comprehensive primer screening with DNA of healthy and damaged trees in order to uncover a RAPD band which is linked to a gene for either stress resistence or sensitivity. Such a marker or its STS (sequence tagged site) would permit to select future healthy trees as early as in their seedling stage.


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