ARTICLES

Assessment of Genetic Diversity in Plus Tree Clones of *Pinus sylvestris* L. using RAPD Markers

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Random amplified polymorphic DNA (RAPD) method was used to assess genetic diversity and to identify genotype-specific markers in Scots pine plus tree clones. Analysis of 12 genotypes originally collected from different forest districts of Lithuania and now growing in a clonal archive at Šlienava showed polymorphic DNA bands with 25 oligonucleotide primers. A total of 225 RAPD bands were scored, of which 176 were polymorphic. These results confirm, that studied pine clones exhibit a high genetic variation. The estimated genetic distance on average was of 0.252, and varied from 0.175 to 0.338. Fourteen possible genotype-specific DNA bands were identified. We have revealed five primers, each of which used separately can identify all studied genotypes. Cluster analysis did not detect correlation between arrangement of genotypes in dendrograms constructed on the basis of the RAPD markers and geographic origin of studied Scots pine plus tree clones.

Key words: RAPD, genetic diversity, plus tree clones, *Pinus sylvestris*

Introduction

Pinus sylvestris is the most widely distributed coniferous species. In Lithuania Scots pine occupies up to 37.2% of the total forest area and is the most widespread tree species. Pine stands are a second most productive in Lithuania. Therefore, *P. sylvestris* plus tree selection and establishment of the clonal archives are a crucial step in genetic improvement of forests. To get a genetically superior forest it is desirable to use the genes of the best clones. The characterization of individual tree clones will be very important in the future with wider commercial applications of clonal forests (Tammisola et al. 1992; Lindgren 2001). Commercial management and breeding of plant species often leads to changes in the pool of genes and can cause the loss of genetic diversity (Harlan 1975; Thomas et al. 1999). Therefore, the evaluation of genetic diversity in the collection of tree clones is also important. The investigation of individual tree clones of several species was carried out by different authors through morphological, biochemical and molecular analyses (Danusevičius 1987, 2000; Gabrilavičius 1995; Van de Ven and McNicol 1995; Nilsson 1995; Scheepers et al. 1997; Kalil Filho et al. 1999; Takahashi 2000). The relative genetic diversity among individuals of Scots pine was determined using morphological markers (Ozolinčius 1998). It is essential to use markers, which usefully reflect the identity of the genetic material of the plant. Morphological features are inadequate for studying genetic diversity, because of their limited representation of the total genome and to the high degree of influence by environmental factors and development stage of the plant (Garcia et al. 1998). On the contrary, molecular markers based on DNA sequence polymorphism, are independent of environmental conditions, and show higher levels of polymorphism (Goto et al. 1998; Forrest et al. 2000). On the other hand the evaluation of genetic diversity of plus trees using molecular markers becomes much more important when a different response of individuals to the changes of environmental factors such as soil and environment acidity, ozone etc. is revealed (Ozolinčius, 1998).

The random amplified polymorphic DNA (RAPD) method is based on the amplification of random DNA sequences in low-stringency polymerase chain reac-

tion using arbitrary 10-base oligonucleotides as primers (Williams et al 1990). This method has been widely employed for its simplicity and capacity to detect genetic variation among very closely related genotypes in a number of species (Van de Ven, McNicol 1995; Garcia et al. 1998; Goto et al. 1998).

The aim of this study was to monitor the genetic diversity of *Pinus sylvestris* plus tree clones and identify genotype specific markers by the random amplified polymorphic DNA (RAPD) method.

Materials and methods

Plant material. Twelve plus tree clones of *P. sylvestris* were selected from the clonal archive in Šilienava (Lithuania). The origins and identity numbers of genotypes are given in Table 1.

Table 1. Plant material used in this study.

No.	Plus tree (No.)	Origin
1.	68	Alytus forest enterprise, Punia forest district
2.	79	Alytus forest enterprise, Punia forest district
3.	204	Kuršių Nerija NP, Juodkrantė forest district
4.	166	Švenčionėliai forest enterprise Laukagalis forest district
5.	77	Alytus forest enterprise, Punia forest district
6.	83	Alytus forest enterprise, Punia forest district
7.	875	Kretinga forest enterprise, Darbėnai forest district
8.	187	Dzūkija NP, Marcinkonys forest district
9.	855	Jurbarkas forest enterprise, Viešvilė forest district
10.	189	Dzūkija NP, Marcinkonys forest district
11.	164	Druskininkai forest enterprise, Latežeris forest district
12.	266	Jurbarkas forest enterprise, Viešvilė forest district

DNA extraction. DNA was isolated from fresh needles using Genomic DNA purification kit #K0512 (MBI Fermentas, Lithuania). The DNA concentration was estimated by spectrophotometer (BioPhotometer, Eppendorf) at 260 nm.

PCR analysis. Thirty decamer random primers (Table 2) were used in the PCR analysis. A standard 25-µl reaction contained 400 ng template DNA, 1.5 units of Taq DNA polymerase (MBI Fermentas, Lithuania), 0.4 µM primer, 0.2 mM of each dNTP, 3.0 mM $MgCl_2$, 2.5 µl 10× reaction buffer (MBI Fermentas, Lithuania). Samples were amplified on a DNA thermal cycler (Biometra). After an initial denaturation at 94°C, 45 cycles of 60s denaturation at 94°C, 60s annealing at 35°C, and 120s extension at 72°C were performed before a final extension of 5 min at 72°C and subsequent cooling to 4°C. Each reaction was performed at least twice. After amplification, the products were separated on

Table 2. Number of total and polymorphic DNA bands from RAPD primers used to assess genetic diversity in Scots pine plus tree clones.

RAPD primer	Sequence 5' - 3'	Total bands[1]	Polymorphic bands[2]	Size range of DNA fragments (bp)	Genotype-specific bands[3]	Number of patterns[4]
A3	GACAGACAGA	13	12	800 - 2500	1	12
A4	CGGGTACCA	Uninformative	-	-	-	-
A6	GACCCGTCCC	12	11	1000 - 3000	0	11
A7	GAAACGGGTG	11		1000 - 3000	1	12
B6	TGCTCTGCCC	13	9	400 - 2500	1	10
B7	GGTGACGCAG	11	7	700 - 2500	1	10
MP1	GAAACACCCC	7	7	500 - 1300	0	11
MP2	AGTCGTCCCC	6	5	650 -1350	1	8
MP3	CCATCCCCCA	8	5	500 - 1400	0	8
MP5	GTCATGCCTGGA	6	1	900 - 2500	0	2
MP6	TGAGCCTCAC	3	3	620 - 3000	1	5
MP7	TCGGCACGCA	5	3	550 - 2200	0	9
Roth 170-01	CATCCCGAAC	4	3	500 - 1400	1	4
Roth 170-02	CAGGGTCGAA	8	4	900 - 2000	1	7
Roth 170-03	ACGGTGCCTG	5	3	650 - 2500	0	6
Roth 170-04	CGCATTCCGC	8	6	500 - 3000	0	10
Roth 170-07	ATCTCCCGGG	Uninformative	-	-	-	-
Roth 170-08	CTGTACCCCC	9	7	850 - 4000	0	12
Roth 170-09	TGCAGCACCG	10	5	550 - 3000	2	8
Roth 170-10	CAGACACGGC	Uninformative	-	-	-	-
Roth 370-01	TCCCTGTGCC	Uninformative	-	-	-	-
Roth 370-02	GCTCTCCGTG	Uninformative	-	-	-	-
Roth 370-03	GAGACGTCCC	10	8	650 - 2400	1	11
Roth 370-04	GTATGCCGCG	11	11	650 - 3000	1	12
Roth 370-05	GCACCGAACG	11	7	500 - 3000	0	11
Roth 370-06	CCGGCGTATC	5	4	600 - 2500	1	7
Roth 370-07	AGCCTGACGC	10	8	600 - 3000	1	11
Roth 370-08	GCTCTGGCAG	14	12	650 - 2500	0	11
Roth 370-09	CGCACTCGTC	3	3	550 - 3000	0	6
Roth 370-10	CTGTCCGGTC	13	10	600 - 3000	0	12

1 – Total number of scorable bands detected;
2 – Total number of bands detected as polymorphic in at least one genotype;
3 – Number of possible genotype-specific bands, generated with corresponding primer.
4 – Number of amplification patterns revealed with corresponding primer.

1.6% agarose (Roth, Germany) gel by electrophoresis in Tris-borate-EDTA (TBE) buffer and stained with ethidium bromide. The gels were then photographed on an UV-transilluminator using BioDocAnalyse system (Biometra).

Data analysis. Faint bands were excluded from screening. For each sample, the presence and absence of DNA bands were recorded as 1 or 0, respectively. Bands with the same mobility were considered as identical DNA fragments. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis. Pairwise comparison of banding patterns was evaluated using Program TREECON for Windows (Van de Peer, De Wachter 1994). The genetic distance (GDxy) was calculated using the formula described by Nei and Li (1979):

$$GDxy = 1 - 2Nxy/(Nx + Ny),$$

where Nxy is the number of DNA bands shared in lines x and y; Nx is the number of bands in line x; Ny refers to the number of fragments in line y. Dendrograms were constructed by applying UPGMA (unweighed pair-group method of arithmetic averages) and WPGMA (weighed pair-group method of arithmetic averages) clustering methods and the Nei and Li's distance matrix.

Results

Of the 30 primers used to study DNA polymorphism, 5 gave no amplification at all, while 25 primers amplified polymorphic products (Table 2). Amplification of 12 Scots pine plus tree genotypes with these primers yielded a total of 225 scorable bands, of which 176 were polymorphic. A total of 9 bands on average were obtained per primer, and the amplification products ranged in size from 400 bp to 4000 bp. The highest number of bands (14) was obtained with primer 370-08, while primers MP6 and 370-09 revealed the lowest number (3).

Figure 1 shows a representative amplification pattern obtained using primers 370-03 and 370-08. Each of these primers generated 11 different DNA amplification patterns. Five primers (A3, A7, Roth 170-08, Roth 370-04 and Roth 370-10) revealed 12 different amplification patterns each (Table 2). Thus the RAPD polymorphism among the plus tree clones of *P. sylvestris* is high an we have been able to separate them by using each of these five primers.

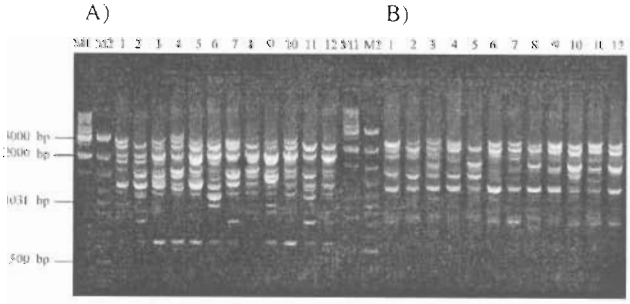

Figure 1. RAPD patterns of twelve Scots pine plus tree clones analyzed with primers 370 - 08 (A) and 370 - 03 (B). Lanes 1 to 12 correspond to the plus tree clones listed in Tab. 1. Size markers (M1 and M2) are given in base pairs (bp).

A close examination of the RAPD gel patterns revealed a number of amplification products that were genotype-specific and could also identify individual clones (Table 2). Possible genotype specific DNA bands were found for clones 68, 77, 79, 164, 187, 204, 855, and 875. Four genotype specific RAPD markers with four different primers (MP6, Roth 170-09, Roth 370-04, Roth 370-03) were identified in the investigation of clone 187. Two genotype-specific bands were identified for clones 168 (primers Roth 370-06 and Roth 370-07) and 875 (primers B6 and Roth 170-01).

RAPD based genetic distance (Nei, Li 1979) between pairs of clones on average had 0.252 and varied from 0.1752 (clone 77 vs 189) to 0.3384 (clone 83 vs 875) (Table 3).

Table 3. Pairwise genetic distance values between Scots pine plus tree clones. Calculations were based on RAPD data and performed using computing programme TREECON (Van der Peer, De Wachter 1994).

79	0.2253										
204	0.3053	0.2674									
166	0.2713	0.2491	0.2446								
77	0.2462	0.2620	0.2214	0.2319							
83	0.2813	0.2659	0.2869	0.2574	0.2701						
875	0.2698	0.2852	0.2427	0.2687	0.2741	0.3384					
187	0.2896	0.3111	0.2617	0.2291	0.2274	0.2601	0.2788				
855	0.2863	0.2786	0.2664	0.2431	0.1847	0.2438	0.2401	0.2448			
189	0.2969	0.2809	0.2246	0.2427	0.1752	0.2963	0.2256	0.2381	0.2156		
164	0.2741	0.2222	0.2473	0.2218	0.2447	0.2601	0.2699	0.2826	0.2518	0.2161	
266	0.2755	0.2391	0.2281	0.2029	0.2156	0.2473	0.2582	0.2128	0.2055	0.2258	0.2340
Clones	68	79	204	166	77	83	875	187	855	189	164

On the basis of genetic distance matrix the dendrograms were generated using cluster analysis methods UPGMA and the WPGMA (Fig. 2).

Discussion

When selecting the trees for cloning by vegetative propagation, it is important to ensure that those that are highly superior for the desired traits are chosen and also to ensure that the selected individuals are genetically diverse as possible. Although investigations on diversity of Scots pine plus tree clones in Lithuania have been reported (Baliuckas, Danusevičius 1994, Gabrilavičius 1995), nobody analysed these genotypes on DNA level. Therefore it was interesting to assess DNA polymorphism in pine clonal archive because molecular markers have the advantages as compared with morphological and biochemical (Powell et al. 1996; Goto et al. 1998) ones. Of the available molecular markers RAPD is one of the simplest to use, because hybridization steps are not required and only small amounts of DNA are needed to detect a large number of polymorphisms in short time (Williams et al. 1990, Welsh and McClelland 1990). In the present study RAPD markers were very effective in identifying genetic differences among pine plus tree clones. All used informative oligonucleotide primers identified

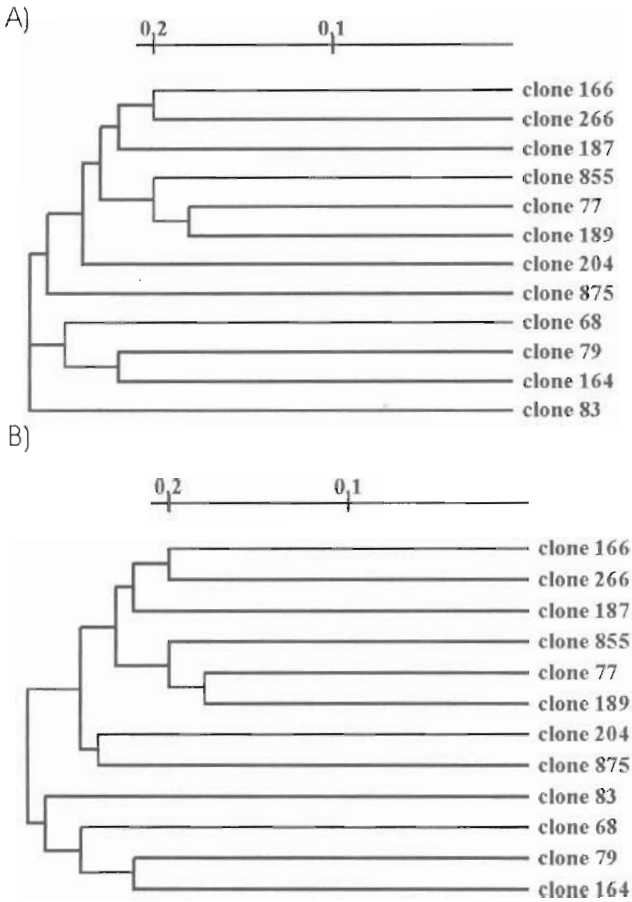

Figure 2. Dendrograms of the genetic relationships between twelve Scots pine plus tree clones based on genetic distance (Nei, Li 1979); (A) dendrogram revealed using UPGMA cluster analysis, (B) dendrogram generated by WPGMA clustering method. The numerical scale indicates genetic distance.

polymorphic DNA bands in the studied genotypes. Of the total amplification products scored in the RAPD analysis, 78.2% were polymorphic. These results show the effectiveness of used RAPD technology to reveal a high level of DNA polymorphism in Scots pine plus tree clones from a relatively small geographic area.

UPGMA and WPGMA dendrograms were constructed from the genetic distance values (Fig. 2). The dendrogram generated by the UPGMA clustering method did not show clear separate clusters. The dendrogram obtained using the WPGMA method separated all genotypes in two clusters. The pattern of grouping of genotypes in both dendrograms is very similar (Fig. 2), especially in the upper part (clones 166, 266, 187, 855, 77, 189, 204, 875). Small differences can be seen in arrangement of four genotypes (83, 68, 79, 164) in the lower part of both dendrograms. These genotypes in the WPGMA dendrogram form a separate cluster, which is not clearly expressed in the UPGMA dendrogram. Clones 83, 68, 79, 164 were obtained from plus trees in South Lithuania. Three of them (68, 79 and 83) originate in the same forest area (Punia, Alytus district). In the upper cluster of the WPGMA dendrogram we see clones of very different origin. Present results show lack of correlation between genetic and geographic distance. For example, the genetic distance between clones 83 and 68 from the same geographical area (Table 1) is 0.2813, while clones 166 and 266 according to the RAPD data were genetically more related to each other (GDxy = 0.2029), in spite of their geographically distinct origin. These results are consistent with the previously reported results on pine (Yeh et al. 1985; Rehfeldt 1988; Thomas et al. 1999), aspen (Tuskan et al. 1996) and beech (Gallois et al. 1998). Rehfeldt (1988) found that clinal variation of ecological traits in ladgepole pine was negligibly affected by geographic differences. Thomas et al. (1999) reported the insignificant effect of geographic location or stand type on genetic similarity of *P. cantora* var. latifolia evaluated using microsatellite and the RAPD markers. It was established in many studies that wind-pollinated conifers typically show very high levels of a within-population genetic variation and relatively less differentiation among populations (Thomas et al. 1999).

In the present study we have identified genotype-specific DNA bands and genotype-specific amplification patterns. Particularly informative were five primers (A3, A7, Roth 170-08, Roth 370-04, Roth 370-10), that were able to differentiate all studied genotypes. These primers showed genotype specific amplification patterns. Fourteen genotype-specific DNA fragments were also found. Unfortunately, we could identify such specific bands only for eight of the twelve investigated genotypes. Of course, we cannot confirm the uniques of a specific marker for given genotype, if the number of examined genotypes would be increased. This problem could be solved by detecting more genotype-specific markers for each particular genotype.

Thus by examining genomic DNA, we can more clearly distinguish varieties among selected plus tree clones and ensure that genetic diversity is maintained in the clones used for commercial production. Using specific RAPD profiles developed by RAPD technology, it is possible to identify individual plus tree clones.

Conclusions

The RAPD technique appears to be a valuable tool to assess genetic diversity in *P. sylvestris* plus tree clones from a small geographical region.

Genotype-specific DNA markers were found for eight studied plus tree clones. These markers could be useful to identify particular genotypes and may be important in Scot pine breeding, because identification of hybrids by morphological markers in many cases is a difficult task. The remaining four plus tree clones could be identified on the basis of genotype-specific amplification patterns.

Dendrograms did not indicate any clear pattern of division among investigated Scots pine plus tree clones based on putative geographic origin.

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ОЦЕНКА ГЕНЕТИЧЕСКОГО ПОЛИМОРФИЗМА В КЛОНАХ ПЛЮСОВЫХ ДЕРЕВЬЕВ *PINUS SYLVESTRIS* L. МЕТОДОМ RAPD

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Резюме

Исследован генетический полиморфизм 12 клонов *Pinus sylvestris* с помощью RAPD-анализа с 25 информативными праймерами. Для количественной оценки полиморфизма отобрано 225 ампликонов, из которых 176 были полиморфные. На основе этих данных расчитанное генетическое расстояние между исследованными генотипами составляло в среднем 0.252. Установлены специфические по отношению к генотипу паттерны амплификации геномной ДНК, а в восми случаях – генотип-специфические RAPD-маркеры, которые могут быть изполэованы для идентификации соответствующих клонов. Различными методами построены филогенетические дендрограмы не показали четкой зависимости между групированием исследуемых генотипов в кластеры и географическим происхождением клонов.

Ключевые слова: генетический полиморфизм, клоны плюсовых деревьев, *Pinus sylvestris*