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THE ESTIMATION OF GENETIC VARIATION WITHIN AND BETWEEN POLISH PROVENANCES OF NORWAY SPRUCE (*PICEA ABIES* (L.) KARST.) ON THE BASIS OF RAPD POLYMORPHISM

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ABSTRACT

In order to determine the genetic variation of Norway spruce (*Picea abies* (L.) Karst.) investigations on the polymorphism of the genomic DNA were conducted. The DNA was extracted from needles of trees belonging to 20 Polish provenances of Norway spruce. For the analysis of DNA the RAPD method with four 10- and 11-nucleotide primers was employed. In the pilot study the optimal concentration of magnesium ions used for the PCR reaction was determined. That allowed for obtaining a restricted right number of discrete and easily identifiable bands. In total, 45 products of amplification were obtained; they were further used for determining the variation of the analysed provenances. The usefulness of RAPD method for determining the inter- and intrapopulation genetic variation in Norway spruce was demonstrated. In this study the characteristic for each population monomorphic bands, allowing to identify each population, were obtained. The similarities of individuals within population (based on the Jaccard's coefficient) ranged from 0.39 (Miedzygorze (8)) to 0.58 (Rycerka (15)). The genetic distance among populations (expressed using the City-Block coefficient) ranged from 87 to 220. On the basis of a dendrogram constructed using the UPGMA method it was shown, that the analysed populations tend to form two group: one of them contained mainly provenances from the north-eastern Poland, while the second one contained mainly provenances from southern Poland.

Key words: *Picea abies*, genetic markers, RAPD, genetic diversity, identification

INTRODUCTION

Norway spruce (*Picea abies* (L.) Karst.) is the second - after the Scots pine (*Pinus sylvestris* L.) - important forest tree species in Poland. Spruce forests cover 5.9% of the total forest area in Poland, while its share in montane and submontane regions (Sudety, Beskidy) is over 30% of forest area. Norway spruce plays an important role in forestry in Poland, both because of its economic and ecological importance.
The genetic structure of Norway spruce in Poland is still far from being well-known. There are several valuable ecotypes of Norway spruce, e.g. the Istebna ecotype. Because of their supreme quality, these ecotypes have been studied for many years by researchers dealing with tree genetics and selection, both in Poland and abroad [13, 15, 29].

Norway spruce, among other Pinaceae species, belongs to the group of very highly polymorphic forest trees. In studies on genetic variation and on identifying individuals and provenances the most efficient and most accurate are the DNA markers (among them, also the RAPD markers). Results of the analysis employing these markers should in principle not to be biased by the influence of such causes as: the environmental factors, the phase of ontogenic development of analysed individuals, the kind of tissue used for the analysis, and the regulation of genes activity as well as modifications taking place after transcription and translation. The RAPD method is currently widely used for identifying species or clones of forest trees, among the others white spruce (*Picea glauca)* [23], the Engelmann spruce (*Picea engelmanni)* and their putative hybrids [23], Norway spruce (*Picea abies)* [19, 5*, 6*] and black spruce (*Picea mariana)* [31]. This paper aims at determining the inter- and intra-population genetic diversity in Norway spruce and at determining the possibility of identifying provenances of Norway spruce on the basis of the occurrence of characteristic RAPD markers.

MATERIAL AND METHODS

The material was collected in the experimental plantation of the Institute of Dendrology in Kórnik, belonging to the Polish Academy of Sciences. That plantation is a part of an international project called the "IUFRO 1972 NS Provenance Experiment". During that project 20 Polish provenances of Norway spruce have been planted in 28 locations in Europe and in 4 in Canada [14, 24]. The list of provenances and their geographic characteristics are given in Table 1 and in Figure 5.

Table 1. List of analysed provenances of *Picea abies* (L.) Karst. [24].

Name of provenance	Number according to IUFRO	Latitude	Longitude	Altitude (m)	Region
Zwierzyniec	1	52°48'	23°47'	160	NE
Zwierzyniec	2	52°42'	23°46'	180	NE
Wigry	3	54°03'	23°03'	170	NE
Przerwanki	4	54°10'	22°05'	180	NE
Borki	5	54°'06'	22°04'	180	NE
Nowe Ramuki	6	53°41'	20°34'	160	NE
Miedzygorze	8	50°13'	16°45'	580	S
Stronie Slaskie	9	50°14'	16°50'	820	S
Wisla	10	49°38'	18°58'	710	S
Istebna Bukowiec	11	49°34'	18°53'	630	S
Istebna Zapowiedz	12	49°32'	18°57'	600	S
Rycerka	13	49°31'	19°01'	620	S
Rycerka	14	49°29'	19°00'	700	S
Rycerka	15	49°'29'	19°00'	950	S
Orawa	16	49°34'	19°33'	1050	S
Witow (Tatra Mountains)	17	49°13'	19°48'	1420	S
Tarnawa	18	49°05'	22°52'	750	S
Zwierzyniec Lubelski	19	50°34'	22°58'	260	S
Blizyn	20	51°04'	20°41'	310	S
Kartuzy	21	54°23'	18°08'	200	O

NE - north-east region,
S - south region,
O - outside natural range

The number of trees for investigation was defined on the basis of the initial experiment which demonstrated that the increase of the tree number over 15 does not result in the increase of the number of bands in the electrophoretic pictures. What is more in such a case (increase of the tree number over 15] the bands previously detected occur with almost the same frequency (data not shown).

When the shoot growth was completed the current year shoots were collected from 15 random trees from each provenance. Needles were cut, frozen in liquid nitrogen, and stored at the temperature -70°C until the DNA extraction was completed.

Extraction of DNA

The genomic DNA was extracted using the CTAB method [4]. About 100 mg of frozen needles were ground in a mortar. The pulverised tissue was put into 1.5 ml sterile Eppendorf test-tubes; the tissue was than mixed with 1000 µl of a buffer containing: 2% CTAB (Hexadecyltrimethylammonium bromide) (Sigma), 1.4 M NaCl, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1% of polyvinylpyrrolidine (PVP), 0.2% mercaptoethanol. After stirring the test-tubes were placed in a water bath at the temperature of 60°C for two hours. Then the solution was centrifuged for 10 minutes at the speed of 10000 rpm. The aqueous phase was transferred to another test-tube, mixed with 500 µl of a mixture of chloroform and isoamyl alcohol in proportion 25:1 and centrifuged for ten minutes at 13000 rpm. The whole procedure was repeated twice. After collecting the aqueous phase 400 µl of cooled isopropanol were added in order to DNA precipitate. That process was conducted at the temperature of –20°C for more than 10 hours. After that time the test-tubes were centrifuged for 15 minutes at the speed of 13000 rpm. The obtained in such a way pellet of DNA was rinsed with 70% ethanol and dried in ambient temperature.

DNA amplification

The polymorphism of genomic DNA was determined using the RAPD (Random Amplified Polymorphic DNAs) method [37, 38]. For the amplification of DNA four 10- and 11-nucleotide primers were used; all of them have been previously used in analyses involving other species from the genus *Picea*. These primers were: MMUL 118 with the sequence of nucleotides CGTGGTAAACT [21], FCP 1 - GCTTACCACC, FCP 3 - CCATTCACCG [7], MOS 116 TACGATGACG [27]. The amplification was conducted in sterile 0.5 ml Eppendorf's test-tubes in 25 µl of reaction mixture containing: PCR buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl) (GibcoBRL), $MgCl_2$ (concentration of which was adjusted to the results obtained in the pilot study), 200 µM of each dATP, dCTP, dGTP, dTTP, 0.25 µM of primer, approximately 25 ng DNA, 0.75 U of polymerase DNA Taq (GibcoBRL). That mixture was covered with 30 µl of the mineral oil (Sigma). The process of amplification was conducted in a Thermocycler (Biometra). The template DNA was denatured at the temperature of 95°C for 5 minutes. The parameters of amplification were given in Table 2.

Table 2. Conditions of amplification in analysed provenances of Norway spruce

primer	reference	number of cycles	denaturation		annealing		elongation*	
			temp. (°C)	time (s)	temp. (°C)	time (s)	temp. (°C)	time (s)
MMUL 118	[21]	45	94	60	37	60	72	120
FCP 1, FCP 3	[28]	45	95 92	5 55	35	60	72	120
MOS 116	[27]	42	94	45	37	60	72	90

*** - while working with the primer MMUL 118, the time of elongation of the last 25 cycles was prolonged by 5 seconds per one cycle. After completing the last cycle for each primer test-tubes were kept at the temperature of 72°C for ten minutes to make sure, that the synthesis of DNA was completed. Then the test-tubes were cooled down to 4°C**

For each DNA sample the amplification was conducted twice in order to estimate the repeatability of the results and to eliminate possible mistakes. As a negative control, the mixture without DNA was used.

The amplification products were separated electrophoretically in a 2% agarose gel (Sigma) immersed in TAE buffer. 0.2 µg × ml^{-1} of ethidium bromide was added to the gel for bands visualization. For the electrophoresis - depending on the primer and on the analysed provenance - 2-6 µl of the reacting mixture with the loading buffer was used. The electrophoresis was conducted at the voltage of 60 V for 3-4 hours; then photographs were made in the UV light. The picture was than analysed using computer software.

For preparing the DNA size markers for the amplification products the Bluescript II SK(+) plasmid was used. The DNA of the plasmid was extracted from the bacteria E. coli XL-1 Blue, employing the alkaline lysis. 50 µg of the plasmid was digested overnight at the temperature of 37°C separately with each of the following enzymes: Bgl I and Hpa II. As a result of digestion the bands were formed: their sizes were: Bgl I - 1931, 1027, HPa II - 710, 489, 404, 346, 242, 190, 157, 147, 110, 67, 57, 34, 26. For the electrophoresis 6 µl of marker (150 ng) were put per each path.

In order to determine the optimum concentration of Mg^{++} for the primers used in analyses a series of pilot experiments was conducted. Three trees from different provenances (Blizyn, Witow, Wisla) were chosen, and the PCR reaction was conducted. As a negative control a sample without DNA was used. For separate primers three or more concentrations of Mg^{++} were used: MMUL 118 - 1.5 mM, 3.0 mM, 6.0 mM; FCP 1 - 1.5 mM, 3.0 mM, 6.0 mM, FCP 3 - 1.5 mM, 3.0 mM, 6.0 mM, 9.0 mM, 12 mM; MOS 116 - 1.5 mM, 3.0 mM, 6,0 mM. Then the electrophoresis was conducted and the photographs of gels in the UV light were taken. The best concentrations of magnesium ions for further analyses were selected on the basis of number, sharpness and intensity of bands obtained in introductory experiment.

Analysis of data

The length of obtained fragments of DNA making bands on the gel was determined using the computer program developed by the AAB (Advanced American Biotechnology). For each band one of two states was ascribed: 1 - the band present, 0 - the band absent. The frequency of occurrence of certain bands (A) in populations and the degree of polymorphism of bands (B) were calculated according to the formulas:

$$A = \frac{\text{number of trees with a certain band present}}{\text{number of analysed trees}}$$

$$B = \frac{\text{number of bands occuring with a frequency equal or greater than } 0.87}{\text{number of all bands for a given primer}}$$

The bands were divided into two groups in relation to their frequency: the bands with the frequency equal or greater than 0.87 and those of frequency less than 0.87. For bands of the first group the frequentness of the recessive homozygote (aa) in the particular provenances has been counted on the basis of the parameter A (Table 5). Both above mentioned measures were used as estimators of the genetic diversity of individuals within population. Next, for each population coefficient of similarity of pairs of trees on the basis of the Jaccard's coefficient [18, 22] were calculated. The genetic distances among populations were calculated employing the City Block coefficient. The cluster analysis was conducted using the UPGMA method, similarity among analysed populations were presented graphically in a form of a dendrogram.

All examined populations were described with basic statistical measures like: the arithmetic average of Jaccard's coefficient, variance, range and coefficient of variance. These data are presented in the table 6. The proportional relation between intra- and interpopulative variability was calculated on the basis of the previously determined statistical parameters.

RESULTS AND DISCUSSION

Optimisation of the Mg^{++} concentrations

To obtain clear and discrete bands on gels while using the RAPD method one needs to perform an initial optimisations of the circumstances of conducting the PCR reaction. This refers mainly to the choice of proper Mg^{++} concentrations, which differ among both primers and plant species [1, 4, 7, 10]. In order to obtain the proper concentrations for conducting the PCR reactions, trials were conducted for certain primers using the genomic DNA extracted from three specimens of spruce. Magnesium ions concentration, at which it were obtained streaks either without clear bands or with hardly visible bands, were considered sub-optimal. The obtained results suggest, that for the primers employed in the experiment it is quite easy to choose such a concentration of magnesium ions, at which bands are clear, easy to read and suitable for further analysis. The best results for the FCP I primer were obtained using a concentration of 1.5 mM, for MOS 116 - 6 mM, while for the MMUL 118 it was necessary to choose medium concentrations between 3 and 6 mM, and for FCP 3 between 6 and 9 mM (Table 3).

Table 3. Comparison of Mg^{++} concentrations for DNA amplification in Norway spruce (present study) and for other species of the genus *Picea*

primer	Optimum concentration of Mg^{++} in mM for		
	*Picea abies**	other species of the genus *Picea*	
MMUL 118	4.0	*Picea mariana* [21, 32]	1.5
FCP 1	1.5	*Picea glauca* [7]	1.9
FCP3	7.5	*Picea glauca* [7]	1.9
MOS 116	6.0	*Picea mariana, Picea glauca* [27]	1.5

*** - present study**

As shown by these experiments, for *Picea abies* the optimal magnesium ions concentration was close to that used by the other authors only for the FCP 1 primer, while for other primers it was several times higher (Table 3). It cannot be excluded, that this difference was due to employing in this study the polymerase produced by the GibcoBRL firm, while in the researches of the above mentioned authors the enzyme produced by the Promega Corp. or Perkin Elmer Cetus was used.

Numerous authors described the problem of optimisation of Mg^{++} ions in the reaction mixture. BOSQUET *et al.* [4] suggested that choosing the proper concentrations of Mg^{++} permits to minimise the errors caused by the polymerase Taq in the PCR reaction. Devos and Gale [10] determined the optimal magnesium ions concentration for ten primers in wheat (*Triticum aestivum*), BARCACCIA [1] in *Medicago sativa* , HOWARD *at al.* [20] in *Plasmodium falciparum,* SKOV [35] in *Picea abies*. The optimisation of magnesium ions concentration in *Picea glauca* using several primers FCP was carried out by CARLSON *et al.* [7]. They found, that while using too high magnesium ions concentration in a gel appeared a streak resulting from the production during the PCR reaction of the fragments of DNA with differences in length too small to permit their separation during electrophoresis. Too low concentration of Mg^{++} results in occurrence on the gel bands which are hardly visible. The choice of proper magnesium ions concentration is therefore of utmost importance for obtaining proper and easy to interpret electrophoretic pictures.

Identification of provenances

The RAPD method allowed for determining characteristic for each provenance bands occurring with a frequency of at least 0.87, which can be used for identification of trees belonging to that provenance (Table 4). For each provenance one can determine a group of characteristic bands, which permits - with a reasonably high probability - to tell them apart from the other populations of spruce. Using four primers did not allow to find for each provenance bands which were fully monomorphic (in 100 %). That refers especially to those populations, which yield a small amount of products of DNA amplification. This group is represented by both populations from Zwierzyniec and population from Witow. Other provenances - like Przerwanki, Miedzygorze, Stronie Slaskie, Wisla, Rycerka can be identified on the basis of bands which do occur in all trees belonging to that provenance. Considering the fact that numerous bands are formed relatively often (frequency >= 0.87), it is also possible to identify other provenances with relatively high probability It was also shown, that for certain primers no bands with high frequency were produced by some provenances. That refers to the populations from Rycerka, for which no such bands were found for the primer MOS 116. Similar result was found using the same primer for the population from Zwierzyniec; that population was characterised by a small number of DNA fragments being produced during the PCR reaction. Both populations did not yield any bands with high frequency for the FCP 1 primer (Table 4).

Table 4. Comparison of sizes of bands occurring with a frequency of at least 0.87 for analysed populations of Norway spruce

Provenance (number according to IUFRO)	Name of the primer			
	MMUL 118	FCP 1	FCP 3	MOS 116
	sizes of DNA fragments making the monomorphic band (number of nucleotides)			
Zwierzyniec (1)	887[c]		1406[c], 941[b]	
Zwierzyniec (2)	887[c]		941[b], 595[c]	
Wigry (3)	1051[b], 797[b]	1264[c], 814[a], 695[c], 475[c]	941[a]	1164[c], 802[c]
Przerwanki (4)	1874[c], 1363[c], 1051[b], 887[a]	1082[c], 814[b], 695[c], 475[c]	1406[a], 1208[b], 941[b], 728[b], 595[b]	802[b]
Borki (5)	887[b]	814[c]	1406[b], 941[b]	1164[a], 802[b]
Nowe Ramuki (6)	887[c]	358[b]	1208[a], 941[b]	802[c]
Miedzygorze (8)	887[b]	1082[c], 814[c], 475[c]	1406[b], 1208[a], 941[a], 728[b]	1509[c], 1164[a], 802[a]
Stronie Slaskie (9)	1051[b],	952[b], 814[b], 695[b], 475[b]	1406[c]	863[b], 802[c]
Wisla (10)	1051[b], 887[b]	1264[c], 814[a], 695[c], 475[c], 358[b]	1326[b], 1208[b], 941[b],	
Istebna Bukowiec (11)	887[b]	1264[b], 814[b], 358[c]	941[b]	1164[a], 802[c]
Istebna Zapowiedz (12)	1363[c], 887[c]		1406[b], 1326[c], 1208[b],	1164[a], 802[b]
Rycerka (13)	1555[c]	814[a], 475[b]	1208[a], 941[a]	1164[c]
Rycerka (14)	1051[c]	1082[c], 814[c], 695[b]	1326[a], 941[a]	
Rycerka (15)	887[b],	814[c]	1406[b], 1208[a], 941[c]	1164[c]
Orawa (16)	797[b],		1406[c], 1208[a], 941[a]	1509[b], 1164[a], 863[b]
Witow (Tatra Mountains) (17)			941[b], 665[c]	1164[b], 802[b]
Tarnawa (18)	887[a]		1406[c], 1208[c], 941[a], 595[b]	1164[b], 863[a], 802[a]
Zwierzyniec Lubelski (19)	1363[c], 887[b]	475[c]	1406[b], 1208[a], 941[a]	1509[c], 1164[b], 802[b]
Blizyn (20)	1051[c], 797[c]		1208[b]	1164[c], 640[c]
Kartuzy (21)		814[c]	1406[b], 1326[c], 1208[c], 941[b]	1164[b]

a - bands occurring in all analysed trees
b - bands occurring in 93% of analysed trees
c - bands occurring in 87% of analysed trees

Table 5. Frequences of recessive homozygote (aa) in the investigated provenances (for bands which occur with frequency of at least 0.87)

Provenances → / Primer (Size of DNA fragment) ↓	1	2	3	4	5	6	8	9	10	11	12	13	14	15	16	17	18	19	20	21
MMUL118 (797)	0.67	0.80	0.07	0.53	0.33	0.33	0.33	0.40	0.40	1.00	0.20	0.40	0.67	0.40	0.07	0.47	0.60	0.47	0.13	0.27
MMUL118 (887)	0.13	0.13	0.73	0.00	0.07	0.13	0.07	0.33	0.07	0.07	0.13	0.20	0.20	0.07	0.60	0.47	0.00	0.07	0.60	0.33
MMUL118 (1051)	0.33	0.33	0.07	0.07	0.60	0.33	0.27	0.07	0.07	0.53	0.33	0.27	0.13	0.20	0.40	0.40	0.53	0.33	0.13	0.47
MMUL118 (1363)	0.33	0.40	0.40	0.13	0.33	0.53	0.40	0.27	0.20	0.67	0.13	0.40	0.47	0.20	0.27	0.67	0.40	0.13	0.53	0.20
MMUL118 (1555)	0.73	0.40	0.33	0.33	0.53	0.53	0.87	0.47	0.33	0.53	0.53	0.13	0.60	0.73	0.60	0.53	0.67	0.47	0.60	0.60
MMUL118 (1874)	0.67	0.93	0.60	0.13	1.00	0.67	0.60	0.67	0.60	0.93	0.93	0.67	0.73	0.80	0.73	0.87	0.87	0.87	1.00	0.60
FCP1 (358)	0.53	0.80	0.20	0.33	0.60	0.07	0.60	0.40	0.07	0.13	0.80	0.40	0.33	0.20	0.27	1.00	0.20	0.67	0.47	0.33
FCP1 (475)	0.53	0.47	0.13	0.13	0.33	0.40	0.13	0.07	0.13	0.13	0.67	0.07	0.20	0.20	0.47	0.93	0.33	0.13	0.47	0.33
FCP1 (695)	0.40	0.33	0.13	0.13	0.27	0.53	0.20	0.07	0.13	0.27	0.47	0.27	0.07	0.20	0.27	0.60	0.53	0.20	0.40	0.40
FCP1 (814)	0.40	0.40	0.00	0.07	0.13	0.20	0.13	0.07	0.00	0.07	0.27	0.00	0.13	0.13	0.27	0.33	0.33	0.33	0.40	0.13
FCP1 (952)	0.93	0.33	0.93	0.33	0.87	0.87	0.40	0.07	1.00	0.93	0.40	0.80	0.27	0.20	0.33	1.00	0.93	0.27	0.20	0.73
FCP1 (1082)	0.60	0.73	0.27	0.13	0.27	0.47	0.13	0.33	0.40	0.67	0.47	0.27	0.13	0.33	1.00	0.60	0.33	0.27	0.40	0.47
FCP1 (1264)	0.80	0.47	0.13	0.93	0.40	0.47	0.60	0.40	0.13	0.27	0.60	0.40	0.20	0.33	0.33	0.40	0.53	0.60	0.80	0.40
FCP3 (595)	0.53	0.13	0.20	0.07	0.20	0.47	0.20	0.80	0.47	0.20	0.40	0.40	0.93	0.40	0.33	0.33	0.07	0.20	0.40	0.20
FCP3 (665)	0.47	0.20	0.27	1.00	0.47	0.87	0.33	0.53	0.20	0.20	0.73	0.40	0.60	0.40	0.47	0.13	0.27	0.87	0.33	0.27
FCP3 (728)	0.67	0.40	0.47	0.07	0.20	0.33	0.07	0.60	0.60	0.20	0.27	0.27	0.67	0.47	0.93	0.67	0.60	0.20	0.53	0.60
FCP3 (941)	0.07	0.07	0.00	0.07	0.07	0.07	0.00	0.20	0.07	0.13	0.80	0.00	0.00	0.13	0.00	0.13	0.00	0.00	0.33	0.07
FCP3 (1208)	0.53	0.53	0.20	0.07	0.20	0.00	0.00	0.80	0.07	0.33	0.07	0.00	0.47	0.00	0.00	0.33	0.13	0.00	0.07	0.13
FCP3 (1326)	0.33	0.47	0.27	0.27	0.27	1.00	1.00	0.27	0.07	0.27	0.13	0.33	0.00	0.27	0.73	0.20	0.60	0.20	0.67	0.13
FCP3 (1406)	0.13	0.47	0.40	0.00	0.07	0.20	0.07	0.13	0.20	0.67	0.07	0.33	0.47	0.07	0.13	0.40	0.13	0.07	0.20	0.07
MOS116 (640)	0.53	0.93	0.27	0.60	0.67	0.87	0.33	0.73	0.67	0.47	0.33	0.27	0.40	0.80	0.27	0.40	0.53	0.67	0.13	1.00
MOS116 (802)	0.20	0.33	0.13	0.07	0.07	0.13	0.00	0.13	0.33	0.13	0.07	0.53	0.47	0.47	0.20	0.07	0.00	0.07	0.20	0.27
MOS116 (863)	0.67	0.40	0.20	0.20	0.33	0.87	0.33	0.07	0.60	0.27	0.40	0.40	0.27	0.20	0.07	0.40	0.00	0.60	0.33	0.27
MOS116 (1164)	0.20	0.40	0.13	0.27	0.00	0.20	0.00	0.20	0.20	0.00	0.00	0.13	0.33	0.13	0.00	0.07	0.07	0.07	0.13	0.07
MOS116 (1509)	0.40	0.47	0.47	0.33	0.27	0.47	0.13	0.33	0.67	0.47	0.27	0.20	0.40	0.60	0.07	0.47	0.73	0.13	0.33	0.40

The investigations showed, that none of the obtained DNA fragments is monomorphic for all analysed spruce provenances (Table 4). The most frequent are two bands: 941 bases (for the FCP 3 primer) and 1164 bases (for the MOS 116 primer); for those bands the mean frequency in all analysed provenances is 0.89 and 0.87, respectively. Conducting similar research employing other primers would allow obtaining a higher number of RAPD markers for the Norway spruce; that would result in a more precise recognition of provenances.

The problem of identification of clones and individuals of forest trees, as well as using that in taxonomy and in forestry, was discussed by numerous authors; that referred especially to the species important from the economic point of view, like Norway spruce. For example, Yeh and Arnott [39] in their paper concerning *Picea sitchensis*, *Picea glauca* and their hybrids suggested, that the wrong identification of tree species causes problems in nurseries, especially in respect to choosing proper conditions for seedling productions. Therefore it is essential to identify tree species or their hybrids in order to collect seed from the right tree stands and to optimise the conditions of growing seedlings in nurseries. The morphological and phenological indicators, which have been used traditionally, are controlled by numerous genes and to a large extent they depend upon the environmental factors and upon the phase of ontogenetical development of a given specimen [26, 34, 36]. Moreover, the large and frequently continuous variability of plant traits makes it difficult to draw clear conclusions [9]. Using morphological traits as indicators it is almost hopeless to solve problems associated with identifying the especially valuable clones of forest trees, cases of out-crossing or avoiding doubling of genotypes in gene banks [8].

Determining intra- and interpopulation polymorphism in provenances of spruce

To evaluate the intrapopulation variability the Jaccard's coefficient was employed; for evaluating interpopulation variability the City Block distance method was used. While conducting the PCR reactions in analysed spruce populations 45 products of amplification were obtained in total; the number of DNA fragments differed depending upon the employed primer and upon analysed population. Typical electrophoretic pictures obtained in analyses for the MMUL 118 primer are presented in Figures 1, 2 and 3. The degree of polymorphism for separate primers and for the whole analysis was given in Table 7, whereas the statistical characteristic of intrapopulative variability is presented in Table 6.

Figure 1. The banding pattern obtained as a result of amplification of DNA using the MMUL 118 primer for 15 trees of the provenance Istebna Zapowiedz (12). K - control sample, M - marker, 1-15 - numbers of analysed trees

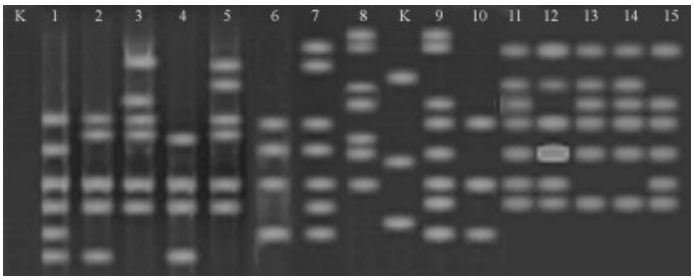


Figure 2. The banding pattern obtained as a result of amplification of DNA using the MMUL 118 primer for 15 trees of the provenance Zwierzyniec Lubelski (19). K - control sample, M - marker, 1-15 - numbers of analysed trees

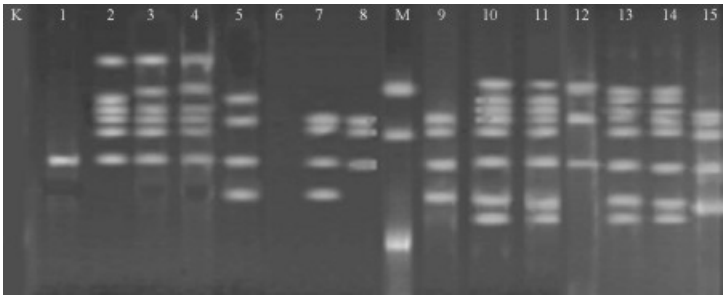
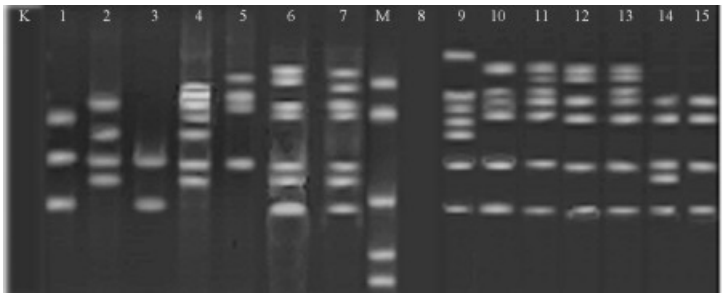


Figure 3. The banding pattern obtained as result of amplification of DNA using the MMUL 118 primer for 15 trees of the provenance Zwierzyniec (1). K - control sample, M - marker, 1-15 - numbers of analysed trees



The investigations showed that Polish provenances of Norway spruce differ substantially in respect to the degree of intra-population variability. That was supported by a wide range of the mean values of Jaccard's coefficient of similarity, varying from 0.39 for the provenance number 8 from Miedzygorze to 0.58 for the provenance number 15 from Rycerka (Table 6, 7). No significant relationship between the level of intrapopulation variability and the geographical location (as well as altitude) of certain populations was found for the whole sample; however, such relationships occurred locally. The results of investigation show that Jaccard’s coefficient of similarity is 0.50 for the mountain provenances (southern part of Poland) and 0.48 for the lowland provenances (north – east part of

Poland). It was shown, that both analysed populations from Istebna, occurring at similar altitudes (600 and 630 m above sea level) had similar level of intra-population similarity (0.55), while three populations from Rycerka, occurring at various altitudes (from 620 to 950 m above sea level) display the degree of similarity declining with increasing altitude. Also in the spruce populations from Miedzygorze in the Sudety Mountains (580 m above sea level) displays greater variability (lower value of the Jaccard's coefficient of similarity - 0.39) than the other population from the Sudety mountains, namely from Stronie Slaskie (820 m above sea level), where the Jaccard's coefficient amounted to 0.55. According to some authors, there is a positive relationship between the altitude and the level of intra-population variability [12, 24]. The ascertained in this study much lower level of intra-population variability in spruce from higher altitudes as compared with lower altitudes in Rycerka and in Sudety Mountains can be a result of much stronger selection due to more severe climatic conditions. This seems to support the results of Barzdajn [2], who found that populations of spruce from higher elevations in Rycerka and Sudety Mountains were characterised by a lower coefficient of variability of tree heights, lower variability in annual height increments and in tree diameters; that suggested that they were more homogeneous than the populations from lower elevations.

Table 6. Statistical characteristics of variance of investigated provenances of Norway spruce

Provenance (number according to IUFRO)	Region	Average Jaccard's Coefficient	Variance	Range	Coefficient of variance
Zwierzyniec (1)	NE	0.50	0.0511	0.93	0.45
Zwierzyniec (2)	NE	0.47	0.0270	0.81	0.35
Wigry (3)	NE	0.49	0.0452	0.92	0.43
Przerwanki (4)	NE	0.57	0.0412	0.81	0.36
Borki (5)	NE	0.45	0.0303	0.91	0.39
Nowe Ramuki (6)	NE	0.41	0.0495	0.92	0.54
Average for region NE		0.48			
Miedzygorze (8)	S	0.39	0.0130	0.68	0.29
Stronie Slaskie (9)	S	0.55	0.0416	0.85	0.37
Wisla (10)	S	0.53	0.0323	0.93	0.34
Istebna Bukowiec (11)	S	0.55	0.0367	0.91	0.35
Istebna Zapowiedz (12)	S	0.55	0.0619	0.96	0.45
Rycerka (13)	S	0.45	0.0304	0.79	0.39
Rycerka (14)	S	0.50	0.0641	0.96	0.50
Rycerka (15)	S	0.58	0.0411	0.92	0.35
Orawa (16)	S	0.47	0.0646	0.97	0.54
Witow (Tatra Mountains) (17)	S	0.47	0.0486	0.94	0.47
Tarnawa (18)	S	0.48	0.0338	0.88	0.38
Zwierzyniec Lubelski (19)	S	0.47	0.0369	0.89	0.40
Blizyn (20)	S	0.52	0.0437	0.95	0.40
Average for region S		0.50			
Kartuzy (21)	O	0.42	0.0259	0.75	0.39

NE – north-east region,
S – south region,
O – outside natural range

Table 7. The mean Jaccard's coefficient and the degree of polymorphism for Polish provenances of Norway spruce as determined using primers: MMUL 118, FCP 1, FCP 3, MOS 116

Provenance (number according to IUFRO)	Mean Jaccard's coefficient	degree of polymorphism for a primer				Entire study
		MMUL 118	FCP 1	FCP 3	MOS 116	
Zwierzyniec (1)	0.50	0.93	1.00	0.80	1.00	0.93
Zwierzyniec (2)	0.47	0.93	1.00	0.8	1.00	0.93
Wigry (3)	0.49	0.87	0.56	0.90	0.82	0.80
Przerwanki (4)	0.57	0.73	0.56	0.50	0.91	0.69
Borki (5)	0.45	0.93	0.89	0.80	0.82	0.87
Nowe Ramuki (6)	0.41	0.93	0.89	0.80	0.91	0.89
Miedzygorze (8)	0.39	0.93	0.67	0.60	0.73	0.76
Stronie Slaskie (9)	0.55	0.93	0.56	0.90	0.82	0.82
Wisla (10)	0.53	0.87	0.44	0.70	1.00	0.78
Istebna Bukowiec (11)	0.55	0.93	0.67	0.90	0.82	0.87
Istebna Zapowiedz (12)	0.55	0.87	1.00	0.80	0.82	0.84
Rycerka (13)	0.45	0.93	0.78	0.80	0.91	0.91
Rycerka (14)	0.50	0.93	0.67	0.80	1.00	0.87
Rycerka (15)	0.58	0.93	0.89	0.70	0.91	0.89
Orawa (16)	0.47	0.93	1.00	0.70	0.73	0.84
Witow (Tatra Mountains) (17)	0.47	1.00	1.00	0.80	0.82	0.91
Tarnawa (18)	0.48	0.93	1.00	0.60	0.73	0.84
Zwierzyniec Lubelski (19)	0.47	0.87	0.89	0.70	0.73	0.80
Blizyn (20)	0.52	0.87	1.00	0.90	0.82	0.89
Kartuzy (21)	0.42	1.00	0.89	0.60	0.91	0.89
Number of amplification products obtained		15	9	10	11	45

The genetic distances between the analysed populations were determined employing the City Block method. The obtained matrix of genetic distances between pairs of populations was than used to conduct analysis using the cluster analysis (UPGMA method); the results were presented graphically in a form of a dendrogram (Figure 4).

The obtained results allow to state, that the genetic distance between populations expressed by the means of the City Block coefficient ranged from 87 (for the pair Istebna Zapowiedz and Zwierzyniec Lubelski) to 220 (for the pair Witow and Przerwanki); that supported the hypothesis, that analysed populations were very diverse. On the basis of City Block coefficient and the resulting dendrogram it was found, that analysed populations of Norway spruce display a tendency to form two large clusters (Figure 4). One of those clusters includes populations from the north-eastern Poland, representing the Nordic-Baltic range of spruce distribution. The second cluster includes mainly populations from southern Poland, from the so called Harz-Sudety-Carpathians range. However, there are some exceptions; populations from Przerwanki and from Borki were included in the second group, though they belong to the Nordic-Baltic range of distribution. Populations from Istebna Bukowiec, Wisla, Witow and Tarnawa were included in the first cluster, though they belong to the southern range. The likely cause of those discrepancies in classification was the fact, that the RAPD analysis was restricted to four primers; all of them gave numerous bands, but that was probably not enough to classify those populations in a proper way. Another fact to be kept in mind is that the geographical distance could be not the main reason for their genetic diversity. This diversity could be also affected by climatic factors and by the former human activity. It could be suggested, that further researches, employing more primers, would allow to obtain additional RAPD markers, which would permit to classify and identify the populations in a more precise way.

Similarly, two clusters of populations were obtained by Scheepers [33]; one of those clusters was made of populations from the Nordic-Baltic region (provenances from Sweden and from White Russia), the second was made of the populations coming from France, Austria, Germany and Belgium.

Figure 4. The dendrogram for 20 Polish provenances of Norway spruce (*Picea abies* (L.) Karst.) obtained employing the UPGMA method

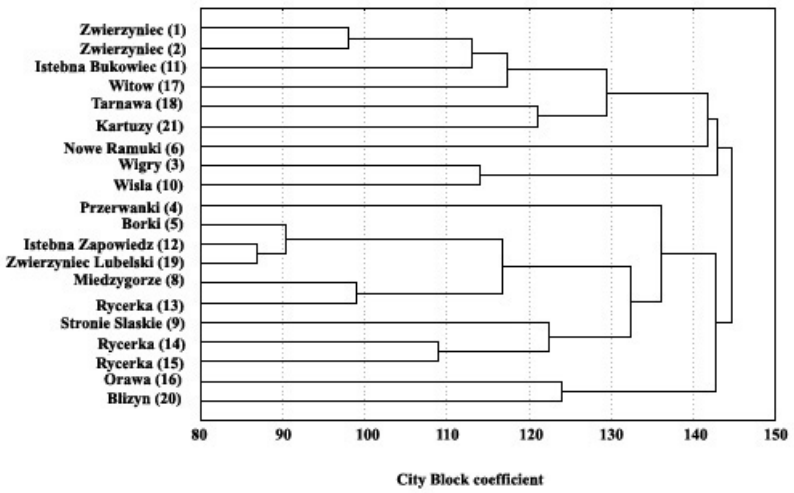
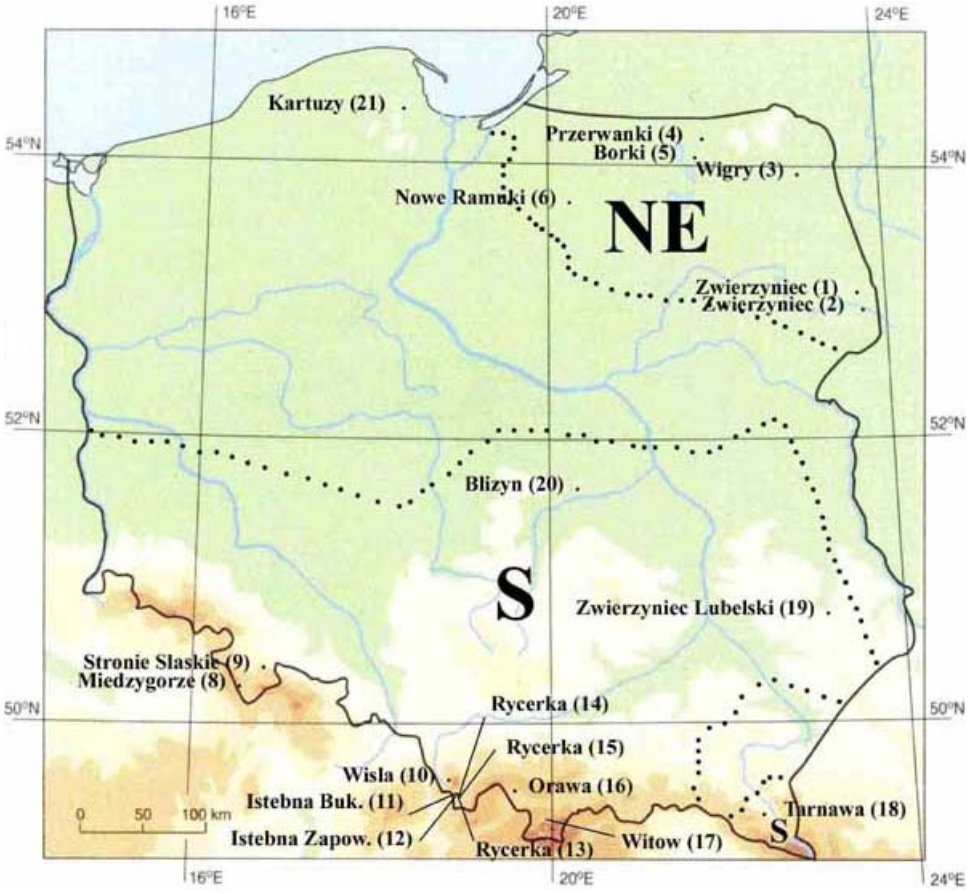


Figure 5. Localization of Norway spruce (*Picea abies* (Karst.) L.) provenances



The research showed, that in Polish populations of Norway spruce 93.63 % of total variability was the intra-population variation, while the inter-population variation accounts for 6.37 % of the total variability. That agrees with the results of studies conducted on Norway spruce using the isoenzyme method [11, 16, 17, 30, 25]. In those researches the intra-population variation accounted for 95% of total variability. In conifers - like in other

angiosperms - the level of genetic variation is usually high, and most of that is the intra-population variation. This is due to the fact, that they are generally wind-pollinated, what results in a high level of gene flow. What is also important, most of conifers of northern latitudes display continuous geographical distribution, which positively influences the gene flow [3].

CONCLUSIONS

1. The RAPD method was proved to be a useful tool to identify Polish provenances of Norway spruce (*Picea abies* (L.) Karst.) and for determining the level of their intra- and inter-population genetical variation.
2. In the research there were found numerous bands for certain polish provenances of spruce (Table 4) which can be useful for identification ecotypes of that species.

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