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Genetic variability of *Pinus sylvestris* populations from IUFRO 1982 provenance trial

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Abstract: Provenance trials were designed to analyse the quantitative responses of tree species to environmental variables found in different experiment location. However, we have still limited knowledge how natural and artificial selection affects genetic variation of the species populations gather in such experimental sites. We have used bulked DNA-based RAPD and ISJ analysis to investigate genetic diversity and differentiation of Scots pine populations from two Polish locations of IUFRO 1982 provenance trial placed in Kórnik and in Supraśl. Applied categories of DNA markers differed in terms of revealing genetic diversity of the species. Ten RAPD primers applied in the study yielded a total of 75 bands, of which 21 (28%) and 15 (20%) were polymorphic in Kórnik and in Supraśl, respectively. Six ISJ primers revealed 42 bands of which 4 (9.52%) and 14 (33.3%) were polymorphic in Kórnik and in Supraśl, respectively. The genetic diversity and differentiation was low, as expressed by H_e=0.071 and H_e=0.085, and by genetic distance values which ranged from 0.0 to 0.240 (on average 0.081) and from 0.017 to 0.188 (on average 0.094) for Kórnik and Supraśl, respectively. Location of provenance trial appeared to have a significant influence on revealed level of genetic polymorphism and pattern of interpopulation differentiation. However, genetic structure found for analysed Scots pine provenances from IUFRO 1982 in Kórnik was also confirmed for Supraśl experimental site. In the light of available data we also discussed the influence of historical migration routes and gene flow on observed genetic variation of the species.

Additional key words: interpopulation variability, molecular markers, selection, bulking

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Introduction

The Scots pine (*Pinus sylvestris* L.) is one of the most important forest-forming species of Europe and Asia. It is characterized by extremely vast distribution area, in fact the biggest distribution area of all *Pinus* species, which stretches from beyond the Arctic Circle in Scandinavia to southern Spain and from western Scotland to the Okhotsk Sea in eastern Siberia (Meusel et al. 1965). Within its range *Pinus sylvestris* grows at elevations from sea level up to 2500m, with the elevation generally increasing from north to south (Boratyński 1993). High level of phenotypic plasticity and genetic variation, together with wide ecological tolerance contributed to the success of the species. Scots pine populations are found both in the

areas characterized by high level of groundwater, like peat bogs or fresh water swamp forests, as well as in dry, lichen Scots pine forest (Cladonio-Pinetum). It is also tolerant for poor soils and extreme temperatures: hot summers in Spain and freezing winters in Siberia (Pravdin 1964).

Climatic conditions of the last glacial period restricted the range of Scots pine to patchy, discontinuous and climatically constrained areas of glacial refugia localized in the Balkans, the Alps and the Iberian Peninsula and in the Russian Plains (Huntley and Birks 1983, Bennett et al. 1991, Soranzo et al. 2000, Naydenov et al. 2007). Its early postglacial expansion is likely to have taken place both from remaining local populations as well as by the northward expansion of southern refugial populations following the retreat of the ice sheets. Also anthropogenic factors such as deforestation or industrial pollution have exerted a strong influence on the present configuration of the genetic structure and on the genetic differentiation of the Scots pine (Oleksyn et al. 1994).

High genetic diversity makes *Pinus sylvestris* ideal material for breeding and conservation studies. One of the most convenient sources of material for examining Scots pine variability is provenance trial. In the present study we investigated genetic diversity and differentiation of the Scots pine populations from IUFRO 1982 provenance trial. This experiment gather 20 populations of the species originated from sampling sites situated along transects running across Europe from north to the south (20° of western longitude) and from the west to the east (52° of northern latitude) (Oleksyn 1988). Previous analyses of that material, provide valuable information concerning growth, plasticity and productivity, survival, susceptibility for biotic and abiotic factors (e.g. Stephan and Liesbah 1996; Barzdajn 2000, 2008; Oleksyn et al. 2001) as well as morphological variation and physiological properties of investigated provenances (e.g. Oleksyn 1988, Oleksyn et al. 1992a, 1992b, Reich et al. 1994, Oleksyn et al. 1999, Oleksyn et al. 2000, Oleksyn et al. 2003, Androsiuk et al. 2011a).

Recently, use of DNA technology has been shown to be effective in the characterization of forest trees (Newton et al. 1999). Molecular marker techniques based on the PCR approach have become of common use to define the genetic structure of natural plant populations, including Scots pine, on the basis of genetic information contained in nuclear, chloroplast and mitochondrial DNA. However, despite many advantages that approach is associated with significant laborious and financial effort. One of the possibilities to overcome these constraints is to analyse one or several bulked samples per population, rather than individual plants. That approach can reduce considerably the financial effort, however bulking of DNA samples also results in the potential non-detection of rare alleles and the loss of information concerning the amount of heterozygosity within samples (Reif et al., 2005). Nevertheless, there are many examples of application of molecular markers in detection of DNA polymorphism in bulk samples of many plant species (e.g. Goto et al., 2001; Herrmann et al., 2005; Reif et al., 2005).

Among various molecular tools, Randomly Amplified Polymorphic DNA (RAPD) markers (Williams et al., 1990) have been widely used to study patterns of genetic variability of many species, including conifers (e.g. Szmidt et al. 1996; Newton et al. 2002; Xia et al. 2008), due to their advantages over other molecular methods such as less complex and labour-intensive procedures and more arbitrary sampling of the genome. Semi-specific ISJ (Intron-Exon Splice Junction) markers are based on the sequences which are commonly found in plants and which are indispensable for post-transcription DNA processing (Weining and Langridge 1991). ISJ primers are partly complementary to the sequences on the exon-intron boundary. These markers had been successfully applied in studies of genera *Polygonatum* (Szczecińska et. al 2006), *Chamaedaphne* (Szczecińska et al. 2009) and also *Pinus* (Polok et al. 2005). Application of these two categories of DNA markers enables estimating of genetic variability of investigated populations not only in random sequences (RAPD markers) but also within functional regions of a genome (ISJ markers), what might be of a great importance during surveying an adaptative variability.

Unfortunately, molecular data concerning genetic diversity and differentiation of Scots pine from IUFRO 1982 provenance trial, related to geographic and racial aspects or tracing the changes in genetic characteristic of populations due to natural and artificial selection remain still largely incomplete. Therefore, the objectives of the study were (*i*) to investigate the genetic variation and geographic differentiation of *Pinus sylvestris* populations from IUFRO 1982 provenance trial in Kórnik and in Supraśl by the means of RAPD and ISJ markers, (*ii*) to analyse how the natural and artificial selection shape the genetic variation of the Scots pine populations gather in two different experimental site locations, (*iii*) to study how the RAPD and ISJ based grouping correlates with previously reported morphological and molecular diversity of the species from IUFRO 1982 provenance trial and (*iv*) to discuss the implication of the results on Scots pine genetic diversity studies.

Material and Methods

Plant Material

Material for analyses was collected from two Polish IUFRO 1982 provenance trial locations in Kórnik and

Table 1. The origin of Scots pine (*Pinus sylvestris* L.) provenances used in the study. Provenances are ordered and grouped by latitude of origin

Region	Provenance	Country	Latitude	Longitude	Altitude
North (>55°N)	1. Roshtshinsaya Datsha	Russia	60° 15'	29° 54'	80
	15. Sumpberget	Sweden	60° 11'	15° 52'	185
	2. Kondezhskoe	Russia	59° 58'	33° 30'	70
	3. Serebyanskoe	Russia	58° 50'	29° 07'	80
	4. Silene	Latvia	55° 45'	26° 40'	165
Central (55–48°N)	5. Miłomłyn	Poland	53° 34'	20° 00'	110
	6. Supraśl	Poland	53° 12'	23° 22'	160
	10. Neuhaus	Germany	53° 02'	13° 54'	40
	11. Betzhorn	Germany	52° 30'	10° 30'	65
	9. Bolewice	Poland	52° 24'	16° 03'	90
	7. Spała	Poland	51° 37'	20° 12'	160
	8. Rychtal	Poland	51° 08'	17° 55'	190
	13. Ardennes	Belgium	50° 46'	04° 26'	110
	12. Lampertheim	Germany	50° 00'	10° 00'	95–100
	14. Haguenau	France	48° 49'	07° 46'	130–180
	16. Zahorie	Slovakia	48° 46'	17° 03'	160
South (<48°N)	17. Pornóapáti	Hungary	47° 20'	16° 28'	–
	19. Prusačka Rijeka	Bosnia	44° 06'	17° 21'	800–970
	18. Maočnica	Montenegro	43° 10'	19° 30'	1200
	20. Catacik	Turkey	40° 00'	31° 30'	1380–1420

in Supraśl. The IUFRO 1982 provenance trial comprised of 20 Scots pine populations from indigenous stands representing almost whole European range of that species (Table 1). Cones collected from selected trees representing particular stand were polled together into one lot and extracted jointly. These pools of cleaned seeds representing given stand were used for establishing each location of the provenance trial (Oleksyn 1988).

The material for our investigation consisted of Scots pine needles, which were stored at –20°C after being collected and cleaned. Needles from 10 trees per population were sampled. Only Turkish provenance Catacik (20) from IUFRO 1982 in Kórnik consisted of six individual trees, the last which survived on this plot. Moreover, to ensure that the samples are representative of the population of origin, needles were collected from original trees at the age of 24 years.

Experimental sites

IUFRO 1982 provenance trial in Kórnik is a permanent plot in the Institute of Dendrology in Kórnik experimental forest located in central Poland (52°15'N and 17°04'E). In the following analysis IUFRO 1982 in Kórnik was represented only by 19 Scots pine populations because Bolewice provenance (9) did not survived and was not represented by any tree when needles were collected. The climate of the region is transitional between maritime and continental. The average annual precipitation is 526 mm and average temperature 7.7°C, with a mean growing season length of 220 days. Soils at this site are clayey, light sands (Oleksyn et al. 1999).

IUFRO 1982 provenance trial in Supraśl is an experimental area localized in south-eastern part of Knyszyńska Forest (53°11'N and 23°18'E), supervised by Department of Silviculture of Poznań University of Life Sciences. The humid climate of the region is characterized by the clear influence of continental climate. The average annual precipitation is 617 mm and average temperature is 7.0°C. The average length of grooving season for this part of Poland is about 202 days (Kożuchowski and Degrimendžić 2005). Soils at this site are dark and lixiviated with light clayey sands. Upper layers of soil are intensively dusty and lower are gravelled (Rzeźnik 1991).

DNA isolation

CTAB procedure (Murray and Thompson 1980) with modifications (Polok 2007) and further adjustment for *Pinus sylvestris* was used for the DNA extraction. DNA was extracted from frozen Scots pine needles previously cleaned and sterilized with 70% ethanol and white spirit (Shell). Briefly, the liquid nitrogen-ground needles were thoroughly mixed with 2

ml of a preheated CTAB isolation buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2% β-mercaptoethanol), 1 ml 20% CTAB, 1 ml 10% PVP, and 330 μl 30% sarcosyl and subsequently, incubated at 60°C for 2 hours. After three chloroform extractions, the DNA was precipitated and dissolved in sterile, deionised H_2O. The quality of DNA was verified on 1% agarose while purity of DNA samples was assessed spectrophotometrically. The bulked samples were generated with equal amount (150 mg) of the frozen needle tissue of 10 trees selected randomly from each population.

Molecular analyses

The PCR reactions were performed in a volume of 20 μl containing 1 μl PCR buffer (400 mM $(NH_4)_2SO_4$ and 1 M tris-HCl pH 9.0); 2 mM $MgCl_2$; 2 μl Enhancer with betaine (Epicentre Technology); 200 μM of dATP, dGTP, dCTP, dTTP; 0.3 μM primer; 1 U of *Tfl* DNA polymerase (Epicentre Technology); and 80 ng template DNA. The PCR reaction for RAPD was processed at 94°C for 3 min. followed by 45 cycles at 94°C for 1 min., 36°C for 1 min., and 72°C for 2.5 min., with a final extension step of 72°C for 5 min. A total of 19 arbitrary 10-mer primers were screened, for their effective utilization in RAPD analysis. Of these 19 primers, 10 primers (Table 2) which gave polymorphic, clearly identifiable and repeatable bands were used further in PCRs. The PCR reaction for ISJ was processed at 94°C for 3 min. followed by 45 cycles at 94°C for 1 min., 48°C for 1 min., and 72°C for 2.5 min., with a final extension step of 72°C for 5 min. A total of 12 ISJ primers were screened, for their effective utilization in analysis. Of these 12 primers, 6 primers (Table 2) which gave polymorphic, clearly identifiable and repeatable bands were used further in PCRs. PCR products were separated on 1.5% agarose gels in 1 × TBE buffer at 100V constant power, stained with 0.5 g/ml ethidium bromide and visualised by illumination with UV light (312 nm).

Table 2. Sequences of RAPD and ISJ primers used in the study

Primer	Sequence	Number of amplified bands
OPA-12	5'-TCGGCGATAG-3'	7
OPA-15	5'-TTCCGAACCC-3'	6
OPA-17	5'-GACCGCTTGT-3'	7
OPA-20	5'-GTTGCGATCC-3'	8
OPB-16	5'-TTTGCCCGGA-3'	8
OPB-20	5'-GGACCCTTAC-3'	8
OPD–03	5'-GTCGCCGTCA-3'	7
OPD–06	5'-ACCTGAACGG-3'	10
OPD-13	5'-GGGGTGACGA-3'	10
OPD-16	5'-AGGGCGTAAG-3'	4
ISJ-2	5'-ACTTACCTGAGGCGCCAC-3	7
ISJ-4	5'-GTCGGCGGACAGGTAAGT-3'	7
ISJ-6	5'-ACTTACCTGAGCCAGCGA-3'	8
ISJ-7	5'-TGCAGGACAGGACCCT-3'	4
ISJ-8	5'-GACCGCTTGCAGGTAAGT-3'	9
ISJ-11	5'-TGCAGGTCAAACGTCG-3	7
Total		117

Data analysis

All bands that could be reliably read were treated as single dominant loci and scored either present (1) or absent (0) across genotypes. Genetic similarity (I_N) and pairwise genetic distance (D_N) among all analysed populations were estimated based on the number of shared amplification products (Nei, 1972), method implemented in POPGENE 1.32 software (Yeh et al. 2000). The matrix of genetic distances was used to generate dendrogram showing the clustering of analysed Scots pine provenances using UPGMA (unweighted pair-group method with arithmetical averages), algorithm implemented in STATISTICA 7.1 software. Basic genetic parameters were also calculated, using the POPGENE 1.32. They include mean number of alleles per locus (N_a), effective number of alleles (N_e) and percentage of polymorphic loci (P). Genetic diversity was estimated using Shannon's diversity (H_o) and Neis gene diversity statistic (H_e). The results were analyzed statistically with the use of STATISTICA 7.0 software. Following the results of previous studies (e.g. Oleksyn 1999; Androsiuk 2011a) analysed Scots pine populations were structured into three groups representing Northern, Central and Southern range of the species in Europe. For mentioned above groups of populations parameters describing genetic diversity and differentiation were estimated.

The data was also tested for presence of population structure by AMOVA using Arlequin 3.5 software (Excoffier 2005). For this purpose the analysed Scots pine provenances were also structured according to their origin, to the three groups of populations (North, Central and South). For that analysis, joined RAPD and ISJ data was treated as haplotypic comprising of a combination of alleles at one or several loci (Excoffier 2005). The significance of the fixation indices were tested using a non-parametric permutation approach according to Excoffier et al. 1992.

Results

Efficiency of RAPD and ISJ primers

Our analysis of *Pinus sylvestris* populations from IUFRO 1982 provenance trial, using 16 primers representing two DNA markers categories, enabled 117 amplification products (bands) to be distinguished (Table 2). The highest number, 75 bands, were revealed by 10 RAPD primers, on average 7.5 per primer. The six ISJ primers showed similar efficiency, revealing a total of 42 bands (on average 7 loci per primer). The highest number of bands (10) was amplified for primers OPD-06 and OPD-13, while the smallest number of bands (4 loci) was scored for primer OPD-16 and ISJ-7.

Considering Scots pine populations from IUFRO 1982 provenance trial in Kórnik, out of all identified loci 22.22% were polymorphic. The number and proportion of polymorphic loci detected by RAPD and ISJ primers amount to 22 (29.33%) and 4 (9.52%), respectively. In case of Scots pine from IUFRO 1982 experimental site in Supraśl the ISJ markers turned out to be the most polymorphic among the populations examined – 14 (33.3%) polymorphic band were found, whereas RAPD primers reveal 15 (20%) bands which shows differences between populations; summarizing out of all identified loci 24.79% were polymorphic. No population specific bands were found.

Genetic diversity and differentiation

In case of both provenance trial locations AMOVA (Table 3) revealed presence of population structure when populations were clustered into three groups (North, Central and South) according to the latitude of their origin. On the basis of AMOVA analysis, the differences among populations were significant, but greater variance was recorded among populations within groups which accounted for 79.98% of the total variance in case of IUFRO 1982 in Kórnik and 83.76% for IUFRO 1982 in Supraśl, while among groups variance accounted for 20.02% and 16.24% for provenance trial in Kórnik and in Supraśl, respectively.

The values of effective number of alleles, gene diversity and Shannon's diversity index (Table 4) showed that in case of both provenance trial locations Northern group of Scots pine populations is characterized by the lowest values of mentioned above parameters. The highest genetic variation was found in Southern group of Scots pine populations from both IUFRO 1982 locations. Nevertheless, only in case of IUFRO 1982 in Kórnik differences observed between mentioned above genetic parameters were significant.

Table 3. Analysis of molecular variation (AMOVA).

IUFRO 1982 location	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-valuea
Kórnik	Among populations	2	18.432	0.94193	20.02	0.05
	Within populations	16	60.200	3.76250	79.98	0.05
Supraśl	Among populations	2	20.086	0.90431	16.24	0.01
	Within populations	17	79.264	4.66257	83.76	0.01

[a]Significance tests after 1023 permutations.

Table 4. Genetic variation parameters summarized for *Pinus sylvestris* populations represented in IUFRO 1982 in Kórnik and in Supraśl, structured into three groups (North, Central and South). N_a=mean number of alleles, N_e=effective number of alleles, P=percentage of polymorphic loci, H_e=gene diversity, H_o=Shannon's diversity index, D=mean genetic distance (Nei 1972).

IUFRO 1982 location	Group	N_a	N_e	P	H_e	H_o	D
Kórnik	North	1.017	1.012[a]	1.71	0.007[a]	0.010[a]	0.009
	Central	1.222	1.094[b]	22.22	0.064[b]	0.103[b]	0.077
	South	1.197	1.162[c]	19.66	0.088[b]	0.125[b]	0.125
	Total	1.222	1.108	22.22	0.071	0.110	0.081
Supraśl	North	1.145	1.095[a]	14.53	0.056[a]	0.083[a]	0.073
	Central	1.222	1.112[a]	22.22	0.069[a]	0.106[a]	0.080
	South	1.179	1.145[a]	17.95	0.079[a]	0.113[a]	0.112
	Total	1.248	1.140	24.79	0.085	0.129	0.094

Statistically significant values at P = 0.05 for the North, Central and South group are indicated by different letters.

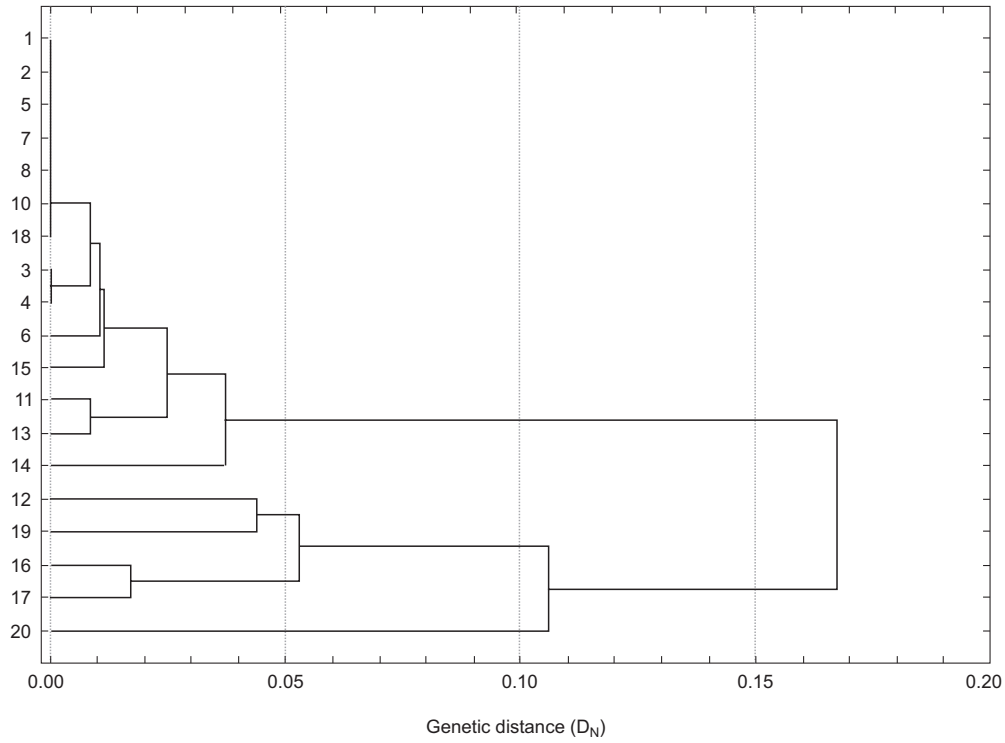

Fig. 1. UPGMA grouping of P. sylvestris populations from IUFRO 1982 in Kórnik based on Nei (1972) genetic distance

In order to estimate the genetic differentiation between Scots pine populations pairwise genetic distance values were calculated. Values of that parameter calculated on the basis of joined RAPD and ISJ data ranged from 0.00 to 0.240 (on average 0.081) and from 0.017 to 0.188 (on average 0.094) for IUFRO 1982 in Kórnik and in Supraśl, respectively (data not shown). On the base of genetic distance values Scots pine populations were subjected to grouping based on UPGMA.

Considering the IUFRO 1982 in Kórnik, 19 populations of Scots pine were grouped into three clusters at the D_N=0.10 (Fig. 1). Cluster I is the largest and consist of consists of 14 Scots pine provenances: Ro-

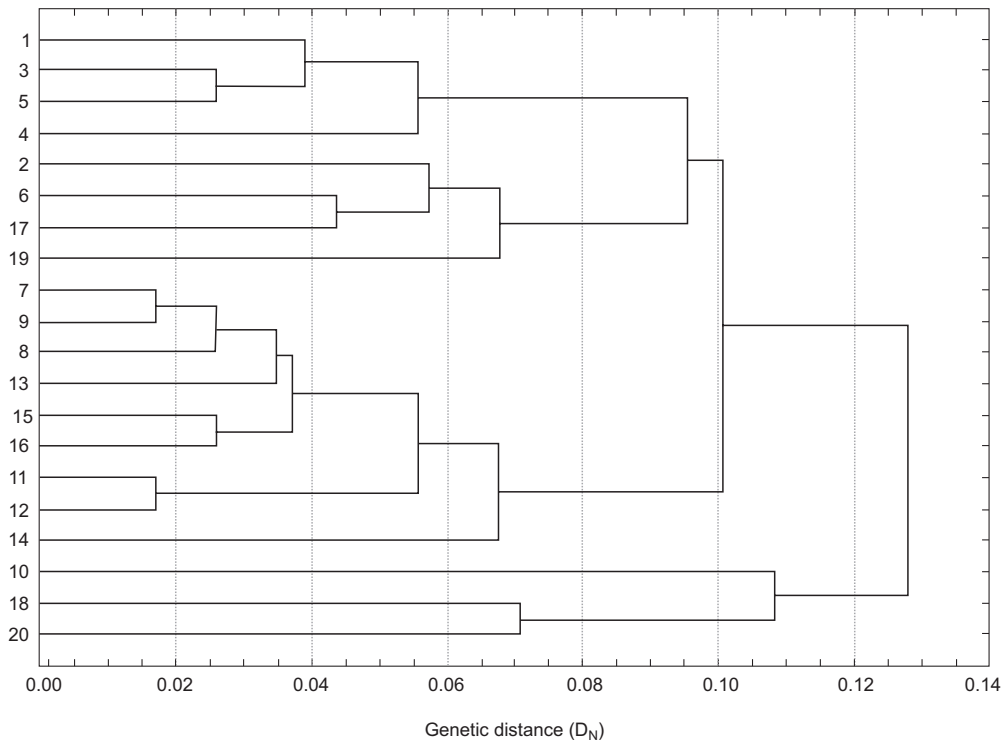

Fig. 2. UPGMA grouping of P. sylvestris populations from IUFRO 1982 in Supraśl based on Nei (1972) genetic distance

shtshinsaya Datsha (1), Kondezhskoe (2), Serebyanskoe (3), Silene (4), Miłomłyn (5), Supraśl (6), Spała (7), Rychtal (8), Neuhaus (10), Betzhorn (11), Ardennes (13), Haguenau (14), Sumpberget (15) and Maočnica (18). Cluster II is formed by four populations (Lampertheim (12), Zahorie (16), Pornóapáti (17) and Prusačka Rijeka (19)). Cluster II is composed of only one population Catacik (20).

Taking into account IUFRO 1982 provenance trial in Supraśl molecular markers markers discriminate populations into four main groups at the D_N=0.10 (Fig. 2). Among the four clusters, cluster II was the largest with nine populations (Spała (7), Rychtal (8), Bolewice (9), Betzhorn (11), Lampertheim (12), Ardennes (13), Haguenau (14), Sumpberget (15) and Zahorie (16)), followed by cluster I consisting of eight populations (Roshtshinsaya Datsha (1), Kondezhskoe (2), Serebyanskoe (3), Silene (4), Miłomłyn (5), Supraśl (6), Pornóapáti (17) and Prusačka Rijeka (19)) and cluster IV formed by two populations Maočnica (18) and Catacik (20). The remaining cluster III was composed of only one population Neuhaus (10).

Discussion

Values of the genetic distance (D_N) obtained for the analyzed populations of the Scots pine, determined on the basis of RAPD and ISJ markers, are in agreement with those reported previously for IUFRO 1982 in Kórnik by the means of B-SAP markers (which ranged from 0.0 to 0.252, and on average reached value of 0.069) (Androsiuk et al. 2011b).

UPGMA grouping based on RAPD and ISJ markers revealed, for IUFRO 1982 in Kórnik, presence of group of closely related populations originated mainly from north-eastern and central Europe (Fig. 1 and 2). Similarity of Scots pine provenances from northern and north-eastern part of Europe was also previously reported by i.e. Prus-Głowacki et al. (1993), Prus-Głowacki and Stephan (1994), Stephan and Liesbach (1996) on the base of the results of isoenzymatic analyses and also by Urbaniak (1997), who used morphological traits of seeds to describe interpopulational differentiation of the species. Similar geographic pattern of genetic differentiation of Scots pine was also reported in our previous paper (Androsiuk et al. 2011b), where north European populations of the species form separate, genetically very homogenous cluster. Individual character of northern and north-eastern Scots pine populations was pointed also by some physiological features like higher N and P resorption efficiency from senescing foliage (Oleksyn et al. 2000, 2003), earlier bud set (Garcia-Gil et al. 2003), lower average maximum of photosynthetic rate (Luoma 1997) and slower above ground growth (Oleksyn et al. 1999). These populations also share a great deal of similarity of first-year growth response to simulated 50° and 60° N photoperiod and are subsumed to the same photoperiod genotype (Giertych and Oleksyn 1992). That geographical pattern of interpopulational differentiation of the Scots pine is in agreement with the phenotype-based taxonomic division of the species (Pravdin 1964; Giertych and Mátyás 1991).

Also a heterogeneous group of low differentiated Scots pine populations from central European locations can be observed in the results of cluster analysis based on RAPD and ISJ markers both for IUFRO 1982 in Kórnik and in Supraśl. This geographic pattern of differentiation of the species is concordant with observations made by Oleksyn et al. (1999) who analyzed biomass production of root system of Scots pine from IUFRO 1982 in Kórnik and with results of analyses of intraspecific differentiation of *P. sylvestris* from IUFRO 1982 provenance trials in Kórnik and in Supraśl based on morphological traits of the needles (Androsiuk et al. 2011a). A low level of genetic differentiation of Scots pine populations from central European plains was also observed by e.g. Giertych (1979), Stephan and Liesbach (1996), Urbaniak (1998) and Sinclair et al. (1999). There are several reasons which may explain such an occurrence: intensive gene flow between geographically close populations, lack of sufficient physiographic barriers which may limit that process or intense, long-lasting silviculture.

Our results showed that the populations which undeniably deserve attention are those from the southern part of Europe: Pornóapáti from Hungary (17), Maočnica from Montenegro (18) and Prusačka Rijeka from Bosnia (19). Although they are not geographically distant from each other, taking into account the area of whole Europe, they belong to distinct genetic groups (Fig. 1 and 2). This is in agreement with results of our previous molecular analyses with the use of B-SAP markers and observations based on analyses of morphological traits of the needles (Androsiuk et al. 2011a, b) which also pointed at high differentiation of those populations.

Radical climatic changes which took place during the last glaciation limited the distribution area of *Pinus sylvestris* to spacially discontinuous and isolated areas of glacial refugia (Bennet et al. 1991, Birks and Line 1993, Huntley and Birks 1983). Long-lasting spacial isolation of the Scots pine refugia resulted in constriction of gene flow or, in extreme cases, it could make a gene exchange between them impossible. The above mentioned populations, two of which occupy mountain massifs of the Balkan Peninsula – Maočnica (18) and Prusačka Rijeka (19), are so genetically distant that an assumption can be made that the gene migration between them was very limited or even did not take place. As a result, changes of the

genetic structure and formation of new genetic variability could be observed in these populations. After the continental glacier retreat these refugia could become a new starting point for the Scots pine settlement of the continent, introducing new, distinctive genetic features in this way.

Cheddadi et al. (2006) hold a similar view, claiming that during the last glacial period *Pinus sylvestris* was pushed to the south of Europe and survived there in the form of refugia placed between 40 and 50° of northern latitude, from where it came back to the north after the continental glacier retreat. On the basis of paleobotanic and molecular data, the authors proved the existence of many fronts of the Scots pine migration to the north of Europe: from the Iberian Peninsula, Apennine Peninsula as well as areas of the Hungarian Lowlands, the Danube Valley and the Balkans. What is more, they claim that *Pinus sylvestris* refugia from the eastern parts of the Alps massif, the Hungarian lowlands and the Danube Valley played a crucial role in recolonization, which took place around 14000 and 8000 years ago.

Distant character of the south European populations from Montenegro (18) and Turkey (20) was also noticed by Wójnicka-Półtorak (1997) based on genotype frequencies at 11 isoenzymatic loci. Oleksyn (1988) confirmed the distinctivness of populations from Montenegro (18), Bosnia (19) and Turkey (20). The author noticed their considerably poorer growth, in comparison with populations found not only in Kórnik, but also in the rest of areas where the provenance trial IUFRO 1982 took place. Similarly, Stephan and Liesbah (1996) emphasise high mortality found in these populations in the IUFRO 1982 in Bensheim, Germany. In case of the Turkish population (20), its distinct character, shown previously (Oleksyn 1988, Giertych and Oleksyn 1992, Oleksyn et al. 1999) was also confirmed by our research, especially for IUFRO 1982 in Kórnik (Fig. 1). High mortality of the Scots pine from the Turkish provenance, as well as its lowest biomass growth of all the populations from the experiment area IUFRO 1982 in Kórnik proves its narrow adaptability to native climatic and habitat conditions (Oleksyn et al. 1999).

Comparison of molecular data obtained by the means of RAPD and ISJ markers for both IUFRO 1982 provenance trials allow us to estimate the influence of experimental site location on genetic variation and genetic relationships among European Scots pine populations. Our previous observations based on morphological traits of the needles showed that Scots pine populations react differently to dissimilar environmental conditions stated for IUFRO 1982 provenance trials in Kórnik and in Supraśl (Androsiuk et al. 2011a). Nevertheless, Scots pine populations from both provenance trials locations maintained common geographic pattern of differentiation, in which north European and south European provenances manifest individual character, different from provenances from Central Europe. In current research analogous pattern of interpopulational differentiation can be observed. However, comparison of results of cluster analyses showed some differences in process of grouping of particular populations, which may be explained by the differences in selection pressure found in both IUFRO 1982 locations. IUFRO 1982 provenance trial localized in Supraśl is characterized by noticeable influence of harsh continental climate which is manifested by lower annual average temperature and shorter vegetation period in comparison to IUFRO 1982 in Kórnik. Therefore selection pressure of more severe climate in case of Supraśl is generally due to frost damages which are most frequently noticed for provenances of southern origin, especially when they are moved from mild climate of their origin to the north (Oleksyn 1993). Selection, which acts on the particular phenotypes, is also shaping the genetic structure of populations. Therefore, severe climate found in Supraśl, accompanied by more intensive natural selection of individuals, might emphasize the differences between analysed Scots pine provenances: genotypes which survived in Kórnik, (those which were probably responsible for shearing similar genetic pattern between provenances and, as a consequence, responsible also for presence of groups of indistinguishable populations) were eliminated in Supraśl. As a result all provenances from IUFRO 1982 in Supraśl are distinguishable from each other and form separate branches of the dendrogram. It need to be emphasized, that average mortality estimated for IUFRO 1982 in Kórnik is 35% (Oleksyn et al. 1999), whereas for IUFRO 1982 in Supraśl 50% (Barzdajn 2008).

Apart from natural selection also silvicultural operations, such as thinning, may had an influence on observed differences in pattern of interpopulational differentiation of Scots pine provenances from IUFRO 1982. Selective thinning, performed in order to improve growing conditions for selected trees, was carried out in both locations of provenance trial, and as a result the number of individuals was substantially reduced, even by 46% in case of IUFRO 1982 in Kórnik (Oleksyn et al. 2000).

However, even more intensive selection is observed during the first, initial stages of young tree development (seedling stage) when they are particularly vulnerable to infection by pathogenic fungi. According to Myczko (2005), as a result of the infection about 32% of Scots pines seedlings die during germination, whereas 69% of successfully germinated seedlings die because of the same reason before the end of first year of their life. Furthermore, about 50% of seedlings which avoid infection and survive undergo selection which aim is to select these of the best

quality which become a planting material used for establishing Scots pine plantations, seed orchards or provenance trials. In summary, only about 10.5% of the Scots pine seeds used for propagation may be represented by mature trees in the field (Myczko 2005). This fact is of significant meaning for interpretation of results obtained for both IUFRO 1982 provenance trial locations. It has to be emphasized, that in case of both experimental sites trees representing particular provenance originate from the same pool of seeds collected from the same natural stand so they represent the same genetic pool. However, later on they were germinated and grown independently in different provenance trial location and undergone different selection pressure characteristic for particular experimental site.

Differences observed in our results are thus consequences of diversified natural and artificial selection observed in both locations of IUFRO 1982 provenance trial. However, this modifying influence was not strong enough to erase the presence of common geographic pattern of genetic differentiation of analysed Scots pine populations, as it was also shown previously for the same material by the means of morphological traits of the needles (Androsiuk et al. 2011a). Furthermore, the parameters describing the genetic variability showed very similar overall level of variation of Scots pine from both experimental areas. However, to draw any wider conclusions about changes in genetic structure of Scots pine populations from different locations of IUFRO 1982 provenance trial further, more detailed studies are required. That knowledge may be essential for proper understanding and correct interpretation of results of other analyses performed on provenance trials.

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