

Variability of Scots pine (*Pinus sylvestris* L.) called Tabórz pine (Forest District Miłomłyn) expressed in analysis of morphology of needle traits and polymorphism of microsatellite DNA

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Abstract. Scots pine (*Pinus sylvestris* L.) is one of the most widespread forest trees in the world, ranging from southern Mediterranean mountains to eastern Siberia. 30 trees of Scots pine from the Miłomłyn Forest District were analyzed in terms of 7 nuclear and 8 chloroplast microsatellite DNA loci as well as 7 morphological needle traits. Nuclear microsatellites (Simple Sequence Repeats) have proved to be useful in studying phylogeographic and gene flow patterns in conifers and are being used to infer the demographic history of tree species. A population's genetic diversity, in fact, represents an important criterion which could be applied in planning future forest management and breeding. Additionally, many elements of Scots pine morphology, including needle traits, are subject to environmental modification. However, the adaptability of those traits remains, to some extent, under genetic control. The trees we investigated here are called Tabórz pine located in the northeast of Poland. This population is described as one of the best in Poland and even in Europe regarding bio mass production, ductility and wood quality. The aim of the study was to determine variation and genetic structure within this population. An analysis of phenotypic differentiation showed that the traits which differed the most between individuals were needle length, the number of serrations per 2 mm of needle length along the right edge and the number of stomatal rows on the convex side of the needles. In Tabórz pine, we detected 30 different genotypes with 87 alleles as well as 28 haplotypes with 30 alleles. We also revealed a slight excess of homozygotes, but the population is still in Hardy-Weinberg equilibrium. The Scots pine population from Tabórz furthermore showed a higher level of genetic diversity compared to stands from other Polish and European regions. This diversity may be the main factor impacting on the population's wood quality and its breeding value.

Keywords: Scots pine from Tabórz, needle morphology, nuclear (nrSSR) and chloroplast (cpSSR) microsatellite DNA, genetic variation

1. Introduction

Scots pine (*Pinus sylvestris* L.) is the main forest species in Poland, where it accounts for about 71.5% of the species' composition of forests (Czerwiński 1993). Its enormous geographic range extends east-west for a distance of about 14,000 km, from eastern Russia to western Spain, and north-south from Norway to the Sierra Nevada Mountains in Spain for a distance of about 2,700 km, giving it the largest range of any *Pinus* species in the world. The great variety of habitats in which it occurs has resulted in numerous types, ecotypes, strains, forms and even varieties within the species (Boratyński 1993).

The currently observed genetic inter-population variability of Scots pine is due to its extensive Euro-Siberian range, its distinct geographic populations from various glacial refuges, the micro-evolutionary processes occurring during the migration of its pollen and seeds after the last glaciation, and dominant cross pollination (Critchfield, Little 1966; Rudin, Lindgren 1977; Shen et al. 1981; Sinclair et al.; 1998, Soranzo et al. 2000; Labra et al. 2006; Naydenov et al. 2007; Pyhäjärvi et al. 2008; Cheddadi et al. 2006). Populations of Scots pine in Europe also exhibit characteristic climatic variability associated with, among other things, the initiation of spring growth or the characteristic variations of needles observed in north-south transects (Gier-

Received: 12.02.2016 r., reviewed: 19.04.2016 r., accepted: 19.01.2017 r.

tych 1993, Urbaniak 1998). In addition, the large variability of the pine is influenced by its generational longevity, which can reach up to 500 years (Pravdin 1964). In Poland, the oldest dated tree of this species, 400 years old, is at Sokolica in the Pieniny Mountains (Niedzielska 2001). In addition, the current picture of the variability of Poland's Scots pine was significantly influenced by the import of foreign seeds, often of undetermined origin. The genetic image of the pine's intra specific structure has also been shaped by micro-evolutionary processes (natural selection, genetic drift, mutation, migration, etc.) occurring in the populations themselves. Evidence for this is the characteristic, distinctly featured adaptive Polish populations: western Polish (from Bolewice), Tabórz (from Tabórz), Supraśl (from Supraśl), Janów (from Janów Lubelski) and Rychtal (from Rychtal). There are also distinct mountain populations from the Tatra and Pieniny regions (Przybylski 1970; Staszkiwicz 1970; Giertych 1980).

The population of the Tabórz pine from north-eastern Poland deserves special attention (Miłomłyn Forest District). The Tabórz pine is considered to be a population of exceptional economic value in Poland and was well evaluated in provenance studies. The breeding value of this population, determined on the basis of quantitative and qualitative traits, results from its straight, tall and cylindrical trunk, delicate branching and immunity to fungal infections. It is characterised by highly energetic height increments, volume production and plasticity, as well as high quality wood. These features place it among the best provenances in Europe (Fabijanowski 1961; Giertych 1980; Cierniewski, Giertych 1982; Giertych 1986; Matras 1989; Giertych, Oleksyn 1992; Korczyk 2002). Tabórz pine owes its high breeding value to the optimal conditions of its habitat, which is deep silt loam (moraine), lying on loose sand, with significant (up to 5.1%) humus content. In addition to the fertility of its habitat, the development of the best specimens of this species also results from the stimulus received from competing trees and bushes in its surroundings (especially from oaks) (Giertych 1980; Giertych 1986).

In order to protect the Tabórz pine, the Tabórz Pine Reserve was established in 1957 in the vicinity of Tabórz (Łukta Township). Ancient pine trees of the so-called Tabórz ecotype have been protected in an area of about 95 hectares. The reserve harbours pine trees that are 250–270 years old, reaching up to 40 m high. The old pine growing in the Tabórz Pine Reserve continue to fruit. Their cones are half the size of the cones of fully developed pines, but they produce first class quality seeds (Boratyński 1993; http://podroze.gazeta.pl/podroze/1,114158,8682845,Polska_Sosna_Taborska.html).

This study aims to describe the variability and genetic structure of the economically valuable Tabórz pine growing in the reserve as well as outside of it in Miłomłyn Forest District. This pine was examined both in terms of phenotypic

characteristics (needle characteristics) and the microsatellite polymorphism of nuclear and chloroplast DNA.

2. Materials and methods

Needle analysis

The intra-population variability of Scots pine from Miłomłyn Forest District was analysed. The study used a total of 30 trees; 17 from the Tabórz Pine Reserve, the remaining 13 from the surrounding forests: Perskie, Ruś, Zakątek, Tabórz and Sarni Dół (Figure 1, Table 1). Ten two-year-old needles from the southern exposure collected from ten different boughs were analysed. The material was stored in 70% ethyl alcohol until the time of the analysis. A total of 300 needles were analysed. The studied morphological traits of the needles were: 1 – needle length, 2 – number of stomata rows on the flat side of the needle, 3 – number of stomata per 2 mm length on the flat side of the needle, 4 – number of stomata rows on the convex side of the needle, 5 – number of stomata per 2 mm length on the convex side of the needle, 6 – number of serrations per 2 mm length on the left-hand edge of the needle, 7 – number of serrations per 2 mm needle length on the right-hand edge of the needle.

Molecular research

Genomic DNA extraction

Genomic DNA was isolated from the needles. Frozen and cut needles (100 mg of tissue) were homogenised in liquid nitrogen. The DNA was isolated by the Doyle and Doyle method (Doyle, Doyle 1990) using the CTAB buffer. The

Table 1. Location of studied Scots pine trees in Miłomłyn Forest District

Tree number	Forest Department	Compartiment
1, 3	Leśnictwo Perskie – LP	7c
2	Leśnictwo Perskie – LP	28
4, 5	Leśnictwo Ruś – LR	91d
6–8	Leśnictwo Zakątek – LZ	146 g, i
9, 10	Leśnictwo Tabórz – LT	104 c
11, 12	Leśnictwo Sarni Dół – LSD	120 d
13	Leśnictwo Tabórz – LT	90 d
14–30	Rezerwat Sosny Taborskiej (RST) – Leśnictwo Tabórz	94 c

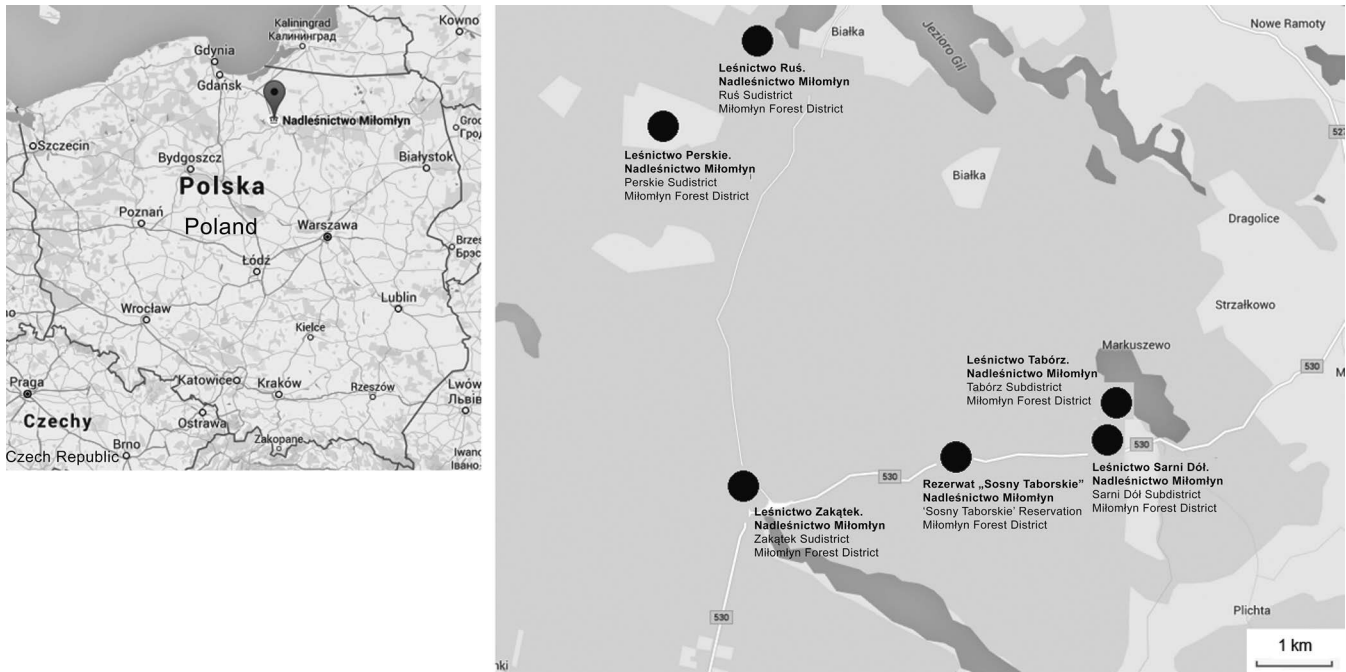


Figure 1. Location of studied Scots pine subpopulations in Miłomłyn Forest District

DNA was dissolved in a $0.1 \times$ TE buffer (10 mM pH 8.1 Tris-HCl; 1 mM pH 8.0 EDTA) for further use. The quality and amount of extracted DNA was checked with a Nano-drop™ ND-1000 (Thermo Scientific) spectrophotometer, and then diluted to a final concentration of 20 ng/ μ l.

DNA amplification

We studied seven nuclear microsatellite DNA loci (nrSSR): PtTX3107, PtTX3116, PtTX4001, PtTX4011, SPAC11.4, SPAC12.5, SPAG7.14 (Soranzo et al. 1998; Elsie et al. 2000; Auckland et al. 2002; Zhou et al. 2002) and eight chloroplast microsatellite DNA loci (cpSSR): PCP1289, PCP30277, PCP41131, PCP45071, PCP71987, Pt45002, Pt30204, Pt71936 (Vendramin et al. 1996; Provan et al. 1999). Primers sequences and repetitive motifs are provided in the aforementioned papers. PCR reactions were performed in a 2720 Thermal Cycler (Applied Biosystems®) thermocycler. Each amplification reaction for nrSSR was performed in a volume of 10 μ l. The reaction mixture contained 1 \times reaction buffer (Novazym), 0.2 mM dNTP, 0.25 μ M primers in both directions (F and R), 0.5 unit of HiFi Taq DNA (Novazym) polymerase, 60 ng of genomic DNA and deionised water. For the nuclear markers, the following PCR profile was used: 3 min 95°C, then 40 cycles – 15 s at 95°C, 1 min. At 58°C (for PtTX markers) or 60°C (for SPAC-markers), 30 s at 72°C and final elongation for 10 min at 72°C. Each of the nuclear microsatellite DNA markers was amplified in a single PCR reaction.

For the chloroplast loci, two multiplex PCRs were designed. Multiplex I included loci: Pt30204, Pt71936, PCP1289 and PCP41131. Multiplex II included loci: PCP30277, Pt71987, PCP45071 and Pt45002. The amplification reaction for cpDNA was performed in a volume of 6 μ l. The reaction mixture contained 1 \times Type-it® Microsatellite PCR Kit, 1x Q-Solution, 0.2 μ M primers in both directions (F and R) and deionised water. Chloroplast DNA was amplified at 95°C for 5 min, then 30 cycles – first 30 s at 95°C, 1 min and 30 s at 50°C and 30 s at 72°C. The final elongation lasted for 30 min at 60°C.

One of the primers of each locus, both nuclear and chloroplast (F – forward) was fluorescent labelled (dyes: 6FAM – blue, VIC – green, NED – yellow and PET – red) to avoid errors in reading different loci of similar allele lengths. Amplification products were capillary electrophoresed together on a 3130xl Genetic Analyzer (Applied Biosystems®) using the size standard. GeneScan™600LIZ™. The results were read using the Peak Scanner ver. 1.0. program (Applied Biosystems®).

Statistical methods

Based on data obtained from the measurement of needle traits, statistical analysis was performed using Statistica PL software from StatSoft. The calculations of the characteristics of the applied traits included: arithmetic mean (for each individual and the whole population), standard deviation, minimum and maximum, as well as coefficients of variation. We used agglomerative clustering with the UPGMA

method on the basis of Mahalanobis distances. Based on the *F* statistics in the analysis of variance (ANOVA), the most distinguishing traits of specific individuals were identified. Pearson correlation coefficients were also calculated between the investigated needle traits.

For each nuclear locus, we calculated: the number of alleles per locus, the effective number of alleles per locus, observed and expected heterozygosity, fixation index, the heterozygote deficiency test according to the Hardy-Weinberg equilibrium, and Nei's genetic distance (Nei 1972) using the PopGene32 programs (Yeh et al. 2000) and GenAlEx (Peakall, Smouse 2006). We also used a correction of the results of the presence of null alleles with the Inest 2.0 program (Chybicki, Burczyk 2009).

For each chloroplast locus, we calculated the number of alleles per locus, the effective number of alleles per locus, the coefficient of genetic variation based on the frequency of alleles and haplotypes, and Nei's genetic distance using the PopGene32 and GenAlEx programs. For both nuclear and chloroplast loci, we constructed dendrograms using the UPGMA method based on Nei's genetic distances with the MEGA program (Tamura et al. 2013).

3. Results and discussion

The results presented in this paper should be considered at two levels: the genetic variability, expressed by the polymorphism of nuclear DNA (nrDNA) and chloroplast DNA (cpDNA), and the variability of the morphological traits of the needles. The nuclear genome in gymnospermous plants is inherited from both parents, while the chloroplast genome is inherited from the paternal parent through pollen. It is significant that, due to the proportionally higher effective size of the nuclear genome relative to the chloroplast genome, the latter is more likely to lose genetic variability, especially in the case of population fluctuations (Pamilo, Nei 1988). As a consequence, the nuclear and organelle markers can differ in the way they present the demographic history of a population, the effects of the so-called 'bottleneck', as well as genetic drift.

Analysis of the morphological traits of the needles

Seven needle traits, illustrating the plasticity range of the studied population, were analysed. Based on the characteristics of the traits (Table 2), we determined that the variability of the traits was between 9 and 20% for the Miłomłyn population. The least variable traits were 3 and 5, whereas the most variable were 4 and 2. Needle length (trait 1) oscillated between 48 and 101 mm and the average was 73.1 mm. The number of stomata rows on both sides of a needle (traits 2 and 4) ranged between 1 and 18 and averaged lower on the flat side

(8.8) than on the convex side (9.6). These differences were tested with Student's *t*-test and were statistically significant ($p = 0.000006$). The number of stomata per 2 mm of length on either side of the needle (traits 3 and 5) oscillated between 8 and 29 and was slightly higher on the flat side of the needle (21.0) than on the convex side (20.8). These differences were not statistically significant ($p = 0.478$). The number of serrations on both sides of the needle (traits 6 and 7) oscillated between 4 and 11 and was slightly higher on the right-hand edge of the needle (7.6) than on the left (7.3). However, this difference was statistically significant ($p = 0.000195$).

In addition, we calculated the traits descriptive statistics of six subpopulations of Miłomłyn pine (Table 3). The longest needles (trait 1) were found in the pines of the Sarni Dół Forest Unit (LSD), and the shortest ones from the Perskie (LP) and Rus (LR) Forest Units. The highest numbers of stomata rows on the flat side of the needle (trait 2) were found in the pines of the Tabórz Forest Unit (LT) and the smallest number in the pines from Perskie (LP) and Sarni Dół (LSD). In terms of the convex side of the needle (trait 4), the pine trees of Zakątek (LZ) and Tabórz (LZ) Forest Units had the highest number of stomata rows, whereas Sarni Dół (LSD) had the lowest number. The highest number of stomata per 2 mm on the flat side of a needle (trait 3) was recorded in the pine trees from the Tabórz Forest Unit (LT), while the lowest number was from the Ruś (LR) Forest Unit. Perskie Forest Unit (LP) had the highest number of stomata on the convex side of a needle (trait 5) and Ruś Forest Unit (LR) had the lowest number.

Table 2. Descriptive statistics of 7 studied needles traits of Scots pine from Miłomłyn

Trait	Mean	Min	Max	V%	Asymmetry	Kurtosis
1	73.1	48	104	13.35	0.34	-0.04
2	8.8	6	14	17.83	0.52	0.33
3	21.0	8	27	9.46	-0.66	5.29
4	9.6	1	18	19.50	0.15	1.86
5	20.8	10	29	9.95	-0.65	4.49
6	7.3	4	10	16.33	0.23	-0.23
7	7.6	4	11	15.33	0.26	0.22

min – minimum value, max – maximum value, V% – coefficient of variation

Traits: 1 – length of the needles (mm), 2 – number of stomata rows on the flat sides of the needles, 3 – number of stomata per 2 mm length on the flat sides of the needles, 4 – number of stomata rows on the convex sides of the needles, 5 – number of stomata per 2 mm length on the convex sides of the needles, 6 – number of serrations per 2 mm needle length on the left-hand edges, 7 – number of serrations per 2 mm needle length on the right-hand edges.

Table 3. Descriptive statistics of 7 studied needles traits in subpopulations.

Trait	Subpopulation	Mean	Min	Max	V%	Trait	Subpopulation	Mean	Min	Max	V%
1	LP	67.8	56	78	7.63	5	LP	22.0	18	25	7.16
	LR	67.8	57	80	9.37		LR	19.5	17	24	8.56
	LZ	76.9	52	91	11.08		LZ	21.1	17	24	8.22
	LT	73.0	60	82	7.73		LT	21.5	19	27	9.68
	LSD	77.9	68	90	9.91		LSD	20.5	17	23	6.44
	RST	73.5	48	104	14.98		RST	20.6	10	29	10.62
2	LP	8.6	6	13	20.95	6	LP	7.5	6	10	12.50
	LR	8.9	6	10	12.84		LR	6.5	5	9	19.01
	LZ	8.8	6	13	22.15		LZ	6.4	4	9	15.13
	LT	9.2	7	12	13.55		LT	7.7	6	10	11.39
	LSD	8.6	6	11	13.40		LSD	6.6	5	8	10.31
	RST	8.8	6	14	18.14		RST	7.5	5	10	16.18
3	LP	21.2	18	25	8.25	7	LP	7.6	6	10	12.27
	LR	20.4	18	24	8.91		LR	7.1	5	9	18.22
	LZ	21.6	19	25	7.43		LZ	7.3	5	8	10.88
	LT	21.8	19	25	7.17		LT	7.9	7	10	11.90
	LSD	20.6	17	27	11.86		LSD	7.2	6	9	11.58
	RST	20.8	8	26	9.89		RST	8.4	4	67	75.61
4	LP	9.9	7	14	23.48						
	LR	9.4	7	12	13.10						
	LZ	10.4	6	14	20.96						
	LT	10.4	7	18	19.00						
	LSD	9.0	6	12	17.66						
	RST	9.4	1	13	18.53						

Symbols of the subpopulations the same as in tab. 1, list of traits – see tab. 2.

The highest number of serrations per 2 mm of the left-hand edge of a needle (trait 6) was found for Tabórz Forest Unit (LT) pines and the lowest number for the pines of the Zakątek Forest Unit (LZ). In turn, the most serrations per 2 mm on the right-hand edge of a needle (trait 7) were identified in the pines from the Tabórz Pine Reserve (RST), and the lowest number in the pines from the Ruś (LR) Forest Unit.

Studies from 1994 (Bobowicz, Korczyk 1994) showed that the Polish lowland populations of Scots pines have needle lengths from 48.2 mm for the population in Miłomłyn to 68.3 mm for the Bolewice population. By analyzing other populations, even shorter needles, 30.3 mm long, were found in the pine trees of the Tuchola Pine Forests (Urbanik et al. 2003).

Needle analysis performed for the IUFRO 1982 provenance experiments in Kórnik, where the provenance pines from Germany, Russia, Sweden, Latvia, Belgium, Slovakia, France, Hungary, Bosnia, Montenegro, Turkey and Poland were growing in similar habitat conditions, a pine from Miłomłyn had one of the longest needles (83.4 mm), a high number of serrations per 2 mm on the left-hand (7.6) and right-hand (7.6) edges of the needle, an average number of stomata rows on the convex and flat sides of the needle (13.5 and 12.8 respectively) and a mean number of stomata on the convex and flat sides of a needle per unit length (23.3 and 23.1 respectively) among the examined provenances (Androsiuk et al. 2011). On the other hand, a study on the progeny lines of pine trees from 8 Polish provenances (Miłomłyn, Ruciane, Supraśl, Janów

Lubelski, Gubin, Rychtal, Bolewice and Spała) tended on a plot in Sękocin showed that the Miłomłyn pines had shorter needles (71.59 mm), a lower number of stomata rows on the convex and flat sides of the needle (9.05 and 11.45 respectively) and a higher number of stomata on the convex and flat sides of the needle per unit length (23.86 and 22.68, respectively) compared to the other tested provenances (Bobowicz et al. 1995). Analysis of the same provenances growing on plots in Miłomłyn showed that compared to the trees grown in Sękocin, Miłomłyn provenance pines were characterised by longer needles (79.78 mm), more rows of stomata on the convex and flat sides of the needle (respectively 10.52 and 12.75) and a lower number of stomata on the convex and flat sides of the needle per unit length (20.12 and 20.11 respectively) (Breninek 1995). Other provenances exhibit a similar situation (i.e., the above-mentioned different values of traits of Miłomłyn provenance trees grown on plots in Miłomłyn and Sękocin) (Breninek 1995). The research conducted by Bobowicz and Korczyka (1994) for the same populations, but not growing in similar cultivation conditions, showed that the Miłomłyna population was characterised by the shortest needles (48.18 mm), an average number of stomata on the convex and flat sides of the needle (10.55 and 9.94 respectively) and a lower number of stomata on the convex and flat sides of the needle per unit of length (21.07 and 21.33 respectively) compared to the remaining 7 populations. It should be noted that the needle length is a phenotypic trait of multigene inheritance (Szyp-Borowska et al., 2011; Nowicka et al., 2013) and is strongly influenced by the environment. For the Miłomłyn population, the heritability of this trait calculated for the crop cultivated in Miłomłyn was 0, whereas for the crop cultivated at Sękocin, it was 0.31 (Bobowicz et al. 2007). In addition, this trait is an important indicator of pollution and the changes in habitat and climatic conditions occurring in a given area (Chudzińska et al., 2014). It is therefore important to study the traits of needles cyclically, as they are the assimilation apparatus of the plant and thus determine the growth and reproductive potential of the trees. Therefore, knowing the scope of this trait allows us to assess the adaptability of the species.

The current research on the Tabórz pine growing in its natural habitat near Miłomłyn shows that it has longer needles (73.1 mm) than was found in previous studies in the same location (Bobowicz, Korczyk 1994). This indicates that climatic conditions (short and mild winters, greater amounts of precipitation) strongly influence the development of the assimilation apparatus, and therefore, determine the good productivity of the Tabórz pine.

The smallest variation was found in the traits describing the number of stomata on both sides of the needle – the average per 2 mm of needle length on the flat side was 21, while on the convex side, it was 20.8. Compared to other Polish lowland populations, Tabórz pines have significantly fewer stomata per

2 mm of the defined rows on the flat and convex sides of their needles. The Mazury population of Ruciane has 28.2 and 28.1, while the Tuchola population has 24.4 to 25.4 on the flat side of the needle and 24.5 to 26.2 on the convex side. A similar, lower number is found in the eastern and northern European part of the pine's range, for example, in Russia (Tobolsk) – 21.8 and 21.5 and Finland (Inari) – 22.9 and 22.8. Pines from the German foothills are also found to have a small number of stomata per 2 mm of needle length – Elmstein, Germany (21.9 and 21.5) (Urbaniak 1998; Urbaniak et al. 2003, Urbaniak et al. 2006). The heritability coefficients calculated for these traits for Miłomłyn's provenance in trees grown in Sękocin are 0.642 for the number of stomata on the convex side of the needle and 0.777 for the number of stomata on the flat side of the needle. Significantly less inheritability characterised these traits for the pines cultivated in Miłomłyn (0 and 0.176 respectively) (Bobowicz et al. 2007). Stomata are located in fewer rows in these pines (8.8 and 9.6) than in some of the lowland populations. In Janów Lubelski in eastern Poland, 10.26 and 11.46 rows of stomata on the flat and convex sides of the needle were observed, while in western Poland at Bolewice – 10.51 and 11.33 rows were observed (Urbaniak 1998). The heritability coefficients calculated for the number of rows of stomata indicate the opposite situation for the Miłomłyn provenance. They are lower for the trees in the Sękocin crop than the individuals grown in Miłomłyn, and are for the number of stomata rows on the convex side of the needle and 0.095 for the number of stomata rows on the flat side of the needle. On the other hand, the coefficients are 0.156 and 0.111 respectively for the trees growing in Miłomłyn (Bobowicz et al. 2007).

The F distribution was calculated in order to confirm the existence of differences between the needle trait averages of specific pine trees. The results of the test are presented in Table 4, which shows that the 30 trees tested are varied in terms of the range of variability of all 7 traits. The traits with the highest variance distinguishing the trees in the Miłomłyn population were: needle length (trait 1), the number of serrations per 2 mm on the left-hand edge of the needle (trait 7) and the number of stomata rows on the convex side of the needle (trait 4). The smallest variance of the studied individuals was found for the number of stomata per 2 mm of length on the flat (trait 3) and convex (trait 5) sides of the needle. In addition, Wilks' λ (Table 4) was calculated to determine the level of internal population differentiation. The closer this value is to zero, the greater the population's variation, whereas the closer the value is to 1, the smaller the population's variation. The calculated Wilks' λ is 0.0428, which indicates a substantial, significant variation in the analysed traits among the 30 subjects tested.

The graphically detected variation within the studied pine population is presented on a dendrogram constructed with the use of the UPGMA method on the basis of Mahalanobis distances (Figure 2a). The figure shows the trees divided

into two groups: the first with trees number 17, 15, 19, 12, 18, 22, 20, 21, 9, 23 and 8, and the second group with the remaining trees. The second group is divided into two sub-

Table 4. The result of one-way analysis of variance (ANOVA) for 7 studied needles traits of Scots pine from Miłomłyn. *** – significant value at level $\alpha=0,001$.

Trait	F statistics
1	14.42***
2	6.25***
3	2.82***
4	6.80***
5	4.00***
6	6.62***
7	6.83***
Total	λ Wilks
	0.0428***

List of traits – see tab. 2.

groups: the first one consists of trees number 14, 13, 6, 3, 10, 30, 16, 5 and 25, and the second with trees number 29, 27, 28, 4, 7, 26, 24, 2 and 1. Tree no. 11 exhibits an individual character. The resulting image of variation does not reflect the division of trees into subpopulations.

The Pearson correlation coefficients calculated between the analysed traits show that the highest direct proportional statistically significant ($p < 0.05$) correlations occur consecutively between 6 and 7, 2 and 4, 3 and 5, 1 and 2, 3 and 4 (Table 5).

Analysis of the molecular markers

Nuclear genome

Populations of Scots pine in northeastern Poland have developed valuable ecotypes. This has been proven in comparative research, such as the provenance experiments. Scientists search for the reasons for uniqueness of this region's population in the history of species and the dynamic environment, where the changeable climate and competition from other species have exerted a certain selective pressure. Thus, one would expect that the Scots pine from this region should be characterised by high genetic variability on the

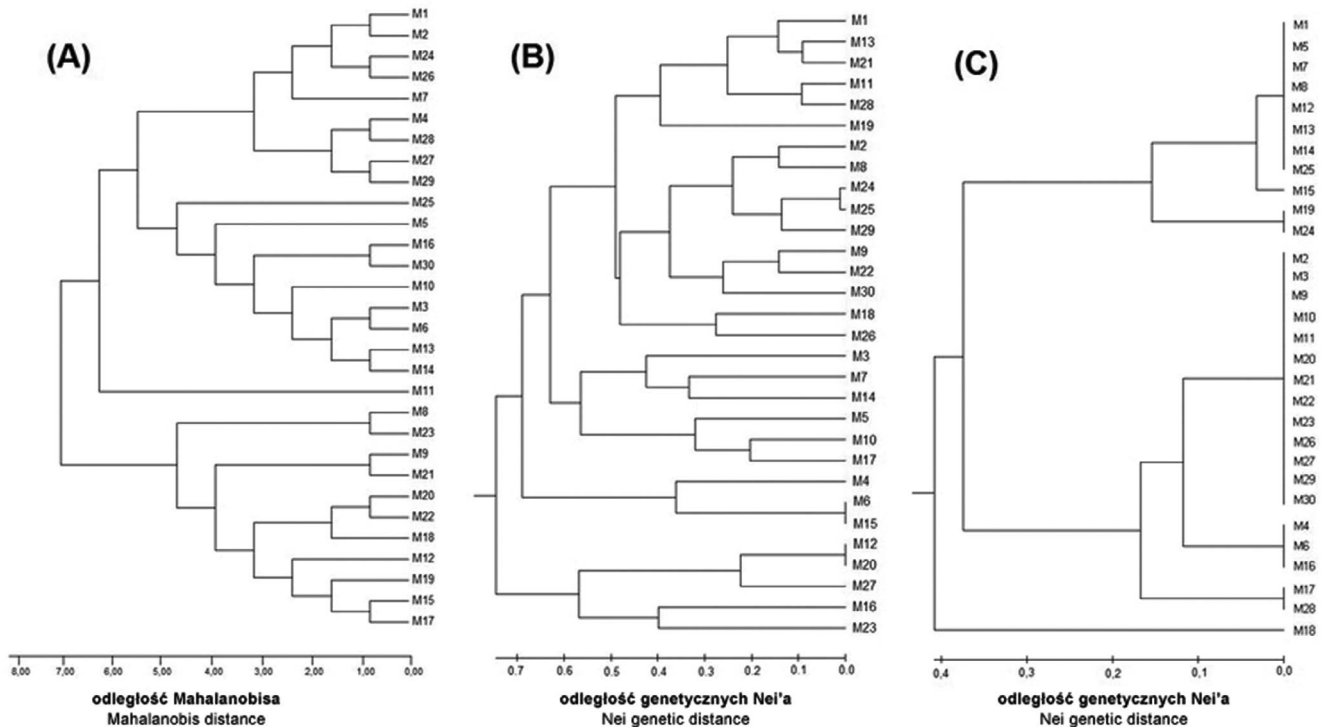


Figure 2. Dendrogram of 30 Scots pine trees from Miłomłyn constructed used the UPGMA method, (A) on the basis of Mahalanobis distances for 7 morphological needles traits, (B) on the basis of Nei genetic distances for nuclear DNA loci, (C) on the basis of Nei genetic distances for chloroplast DNA loci

Table 5. Pearson correlation coefficients between 7 morphological needles traits of Scots pine from Miłomłyn.

1							
2	0.49**						
3	0.16	0.18					
4	0.32	0.76**	0.46*				
5	-0.04	0.32	0.51**	0.36			
6	0.05	-0.01	0.11	-0.13	0.14		
7	0.19	-0.02	0.20	-0.10	0.16	0.88**	
	1	2	3	4	5	6	7

* – significant value at level $\alpha=0,05$; ** – significant value at level $\alpha=0,01$.

List of traits – see tab. 2.

one hand, but also that these populations should conform to the Hardy-Weinberg equilibrium. In other words, they should have the most favourable genetic homeostasis in the given conditions. It seems that the Tabórz pine population of the Miłomłyn Forest District meets these expectations. The presented results indicate that it has a significant level of genetic variability.

Of the 30 individuals tested, 30 different genotypes were detected, so none of them repeated. 7 to 20 alleles were found at the locus (Table 6). The highest number was detected for locus SPAG7.14 (20) and the lowest for PtTX3107 and PtTX4011 (7). An average of 12.4 alleles in a locus was detected for the population. The highest effective number of alleles was detected for locus SPAG7.14 (15.79) and lowest for PtTX4011 (2.86), with the average for the population at 7.78. The most frequent alleles for specific loci are: allele 161 for locus PtTX3107 (33% frequency), allele 157 for PtTX3116 (37%), allele 214 for PtTX4001 (38%), allele 257 for PtTX4011 (53%) allele 217 for SPAG7.14 (10%), allele 134 for SPAC11.4 (15%) and allele 139 for SPAC12.5 (18%). Nowakowska (2007) analysed 42 Polish populations of Scots pine, including the Miłomłyn population. She studied four nuclear loci, including two of the same ones from the present study (SPAG7.14 and SPAC12.5) and found that alleles 199 and 221 most frequently occurred for locus SPAG7.14, while alleles 155, 161 and 139 were most frequent for SPAC12.5. In general, she detected 14 alleles for locus SPAG7.14 and 11 alleles for locus SPAC12.5, and thus fewer than what was found in the present study. As in our analyses, the number of alleles in a locus for loci SPAG7.14 and SPAC12.5, respectively 20

and 19, was found for the pine stand in Oława (Nowakowska et al., 2014), whereas fewer alleles (6 to 15) were found for the Italian population from the southern end of the Scots pine range (Scalfi et al. 2009).

For all the studied loci, the population of Miłomłyn was in Hardy-Weinberg equilibrium. However, a heterozygous deficit was noted, highest for locus PtTX4011 ($F_{IS} = 0.282$) and lowest for PtTX4001 ($F_{IS} = 0.004$), with the fixation index at an average of 0.106. Correction of the F_{IS} fixation index for the presence of null alleles (Chybicki, Burczyk 2009) did not show significant deviations from the initial results (mean F_{IS} after correction 0.101). The heterozygote deficiency may be a result of several factors, among which special attention should be paid to kin correlation or the presence of substructures in the population. The highest observed heterozygosity was found for locus SPAC11.4 (0.87), the lowest for locus PtTX4011 (0.47), and the mean for all the loci was 0.73. The expected heterozygosity was higher than the observed and the mean for the population was 0.82. Nowakowska (2007) detected a higher expected heterozygosity (0.924) for SPAG7.14 than the observed (0.800) and the reverse for locus SPAC12.5 (0.910 and 1.000 respectively). On average, for all 4 analysed loci, she detected a small excess of heterozygotes (expected heterozygosity was 0.803 and observed 0.844). However, the study population was in Hardy-Weinberg equilibrium. This population belongs to the Baltic syntaxonomical region, for which Nowakowska (2007) detected the greatest diversity compared to the other five studied Polish regions. Other studies on pine pointed to a higher observed and expected heterozygosity (0.84 and 0.85 respectively), which was recorded for adult pine specimens from the Oława Forest District, and a lower fixation index (0.011) (Nowakowska et al. 2014). On the other hand, the Italian populations located at the extremity of the range exhibited a similar mean heterozygosity (0.81), but it ranged from 0.74 to 0.91 for specific populations. These populations were characterised by a higher average fixation index (0.16), which varied from 0.125 to 0.212 for individual populations (Scalfi et al. 2009).

Based on Nei's genetic distances, a dendrogram was constructed using the UPGMA method (Figure 2b). The figure shows that the trees form four groups. The first consists of trees numbered 23, 16, 27, 20 and 12; the second of 15, 6 and 4; and the third of 17, 10, 5, 14, 7 and 3. The fourth largest group is divided into two subgroups: Nos. 26, 18, 30, 22, 9, 29, 25, 24, 8 and 2, and a second smaller one with 19, 28, 11, 21, 13 and 1. It should be noted that the trees of the Tabórz Pine Reserve are mainly found in the first and fourth group, while the pine trees from the reserve's adjacent areas are in the second and third groups on the dendrogram. However, each of the distinguished groups contains trees from separate subpopulations. This is most likely the consequen-

Table 6. Statistic parameters of genetic diversity. Symbols: N – sample size, N_A – number of alleles per locus, N_E – number of effective alleles per locus, H_O – observed heterozygosity, H_E – expected heterozygosity, F_{IS} – fixation index, HWE – deviation from Hardy-Weinberg equilibrium AZ – mean number of null alleles, h – diversity index

Locus	nrSSR								Locus	cpSSR		
	N	N_A	N_E	H_O	H_E	F_{IS}	HWE	AZ		N_A	N_E	h
PtTX3107	30	7	4.749	0.700	0.789	0.113	ns	0.00	Pt71987	2	1.385	0.278
PtTX3116	30	8	3.797	0.667	0.737	0.095	ns	0.00	PCP45071	6	3.169	0.684
PtTX4001	30	10	4.348	0.767	0.770	0.004	ns	0.00	Pt45002	4	1.619	0.382
PtTX4011	30	7	2.857	0.467	0.650	0.282	ns	0.00	Pt30204	5	3.383	0.704
SPAG7.14	30	20	15.789	0.833	0.937	0.110	ns	0.00	Pt71936	3	1.718	0.418
SPAC11.4	30	16	11.392	0.867	0.912	0.050	ns	0.00	PCP1289	4	2.356	0.576
SPAC12.5	30	19	11.465	0.833	0.913	0.087	ns	0.00	PCP41131	2	1.220	0.180
									PCP30277	4	3.689	0.729
Mean	30.000	12.429	7.771	0.733	0.815	0.106	ns	0.00	Mean	3.75	2.317	0.494
SE	0.000	2.170	1.901	0.052	0.041	0.033			SE	0.49	0.345	0.074

ns – not significant value, SE – standard error of the mean

Table 7. Statistic parameters of genetic and morphological (Wilks λ) diversity in particularly subpopulations. Symbols the same like in tab. 6. *** – significant value at level $\alpha=0,001$

Subpopulation	N	nrSSR					cpSSR			Needles λ Wilksa
		N_A	N_E	H_O	H_E	F	N_A	N_E	h	
LP	3.000	4.143	3.696	0.810	0.698	-0.150	1.875	1.700	0.583	0.098***
LR	2.000	3.286	3.124	0.857	0.625	-0.371	1.750	1.750	0.750	0.150***
LZ	3.000	4.000	3.504	0.619	0.659	0.027	1.750	1.600	0.500	0.048***
LT	3.000	4.143	3.592	0.857	0.683	-0.262	1.750	1.650	0.458	0.386
LSD	2.000	3.286	3.048	0.786	0.661	-0.181	1.500	1.500	0.500	0.253**
RST	17.000	10.000	7.324	0.697	0.799	0.133	3.375	2.311	0.525	0.058***

ce of the common genetic pool of Scots pine concentrated in this region.

Chloroplast genome

28 haplotypes were detected in the chloroplast genome, with haplotypes 130/170/189/160/164/124/157/154 occurring in three individuals (nos. 21, 23 and 26) growing in the Tabórz Pine Reserve in the Tabórz Forest District. 2 to 6 alleles were found in the locus (Table 3). The highest number was detected for the locus PCP45071, and the lowest for Pt71987 and PCP41131. On average, we detected 3.8 alleles in a locus for the population, which is comparable to the

results obtained (3.6 alleles) for twelve populations of Scots pine from Bulgaria (Naydenov et al. 2005). The highest effective number of alleles was detected for locus PCP30277 (3.69) and the lowest for PCP41131 (1.22), the mean for the population was 2.32. In the Italian populations at the edge of the Scots pine range, 4 to 5 alleles were detected on the 12 to 19 individuals analysed. However, only two loci were studied, Pt30204 and Pt71936, and a total of 7 to 10 different haplotypes were detected, an average of 8.25 (Scalfi et al. 2009). Miłomłyn individuals had the highest values of the genetic diversity coefficient for locus PCP30277 (0.73), the lowest for locus PCP41131 (0.18), and the mean for all loci

was 0.49. The coefficient of genetic variation for the whole population, calculated on the basis of haplotype frequencies, was 0.96 and was higher than the mean of this coefficient for the Italian population (0.92, ranging from 0.88 to 0.95 for individual populations) (Scalfi et al. 2009) and for the relict populations of Caledonian Scots pine (0.91) (Provan et al. 1998). The high result of this parameter for the Caledonian Scots pine is significantly affected by the number of unique alleles in the population, at an average of 19 (Provan et al. 1998). The studies conducted by Naydenov et al. (2005) observed a similar number of alleles in the locus as it is shown in the presented results. Six length variants were found for locus Pt45002, ranging from 167 to 172, differing from each other by one nucleotide; eight different lengths of fragments were found for locus Pt30204, ranging from 137 to 144. In our study, the analysed loci were in the range of 187–190 and 157–162 for loci Pt45002 and Pt30204 respectively. A comparison of the lengths of amplified DNA to Bulgarian populations from the southern end of the species' range indicates that the Tabórz pine population may represent trees with a different evolutionary history.

Based on Nei's genetic distances, a dendrogram was constructed using the UPGMA method (Figure 2c). The figure shows that the trees form two groups with subgroups. The first group is divided into two subgroups, the smaller one includes trees number 16, 6 and 4, and the larger group has trees number 30, 29, 27, 26, 23, 22, 21, 20, 11, 10, 9, 3 and 2. These two subgroups are joined by trees 17 and 28. The second group is also divided into two subgroups: the first has two individuals, 19 and 24, and the other consists of trees number 25, 14, 13, 12, 8, 7, 5 and 1. Tree number 15 joins this group. Tree number 18 differs the most among the remaining individuals, confirming the greatest Nei distance seen in the figure.

Studies on intra-population variation based on DNA sequence polymorphism indicate significant variation within specific populations. A much smaller range of variation is found between populations. One of the promoters of genetic change in a population with a continuous range, which occurs in the Polish Lowlands, is the very structure of settlement of this area. This is an area with a clearly continuous range of Scots pine. This facilitates the development of local adaptations significantly (Holt, Gomulkiewicz 1997; Lenormand 2002). Populations from this geographic region are particularly genetically diverse compared to pine populations from other regions of the country (Nowakowska 2007, Nowakowska et al., 2014) and Europe (Scalfi et al. 2009). These are areas where the remaining forest areas have been impacted to a relatively limited degree by human activity, and the age of the surveyed trees, from 240 to 280 years, would seem to guarantee their nativeness.

Variability between subpopulations and a comparison of variability detected by various markers

The variation among the pine subpopulations of Miłomłyn Forest District was also determined (Table 7). The greatest variability in pine needle traits (the lowest Wilks' λ value) was found for the pine from the Zakątek Forest Unit (LZ), and the smallest in the Tabórz Forest Unit (LT). With the use of chloroplast loci, the highest variability (h) was found for pines from the Ruś Forest Unit and the lowest (as with the morphological marker) for the Tabórz Forest (LT). With the use of nuclear loci, the highest variability (Ho) was detected for pine from the Ruś (LR) and Tabórz (LT) Forest Units and the lowest from the Zakątek Forest Unit (LZ). The Pearson correlation coefficient was also calculated between these three variability parameters for each subpopulation. We found a weak positive correlation between the h index for chloroplast loci and Wilks' λ for phenotypic traits ($r = 0.31$; $p = 0.550$) and a medial negative correlation between observed heterozygosity (Ho) and Wilks' λ ($r = -0.67$; $p = 0.141$). In comparing the coefficients of variation for molecular markers, a weak positive correlation was found between the h index for chloroplast loci and observed heterozygosity (Ho) for nuclear loci ($r = 0.39$; $p = 0.447$).

Genetic and biological distances between the individual subpopulations were also calculated (Table 8). Needle trait differences were greatest between the pines of Perskie (LP) and Sarni Dół (LSD) Forest Units, as well as between Tabórz (LT) and Sarni Dół (LSD) Forest Units. Needle trait variability was lowest between Perskie (LP) and Tabórz (LT) Forest Units, as well as between the Tabórz Forest Unit (LT) and Tabórz Pine Reserve (RST). For chloroplast loci, the pine forests of Perskie (LP) and Zakątek (LZ) Forest Units exhibited the greatest difference; and, as with the phenotypic traits, Perskie (LP) and Sarni Dół (LSD) Forest Units, while the least difference was noted between Ruś (LR) and Tabórz (LT) Forest Units, and also between the Tabórz Forest Unit (LT) and Tabórz Pine Reserve (RST), as with the needle traits. For nuclear loci, the greatest differences were between Perskie (LP) and Ruś (LR), as well as Zakątek (LZ) and Sarni Dół (LSD) Forest Units, while the least variation was found between Zakątek (LZ) and Tabórz Pine Reserve (RST), Perskie (LP) and Zakątek (LZ), Perskie (LP) and Tabórz Pine Reserve (RST), as well as Tabórz Forest Unit (LT) and Tabórz Pine Reserve (RST). Generally, based on all three studied markers, we can assume that the pines from Tabórz Forest Unit and Tabórz Pine Reserve are the most similar, very likely due to the largest gene flow between these subpopulations, which grow closest to each other geographically.

However, it must be noted that 5 subpopulations had a population of 2 to 3 individuals, which does not provide a reliable

Table 8. Calculated biological and genetic distances between subpopulations.**a) Mahalanobis distances for morphological traits of needles**

LP	LR	LZ	LT	LSD	RST	
0.000					LP	
1.660	0.000				LR	
1.499	1.529	0.000			LZ	
0.765	1.694	1.344	0.000		LT	
1.805	1.392	1.046	1.699	0.000	LSD	
1.073	1.280	1.336	0.885	1.101	0.000	RST

b) Nei genetic distances for chloroplast markers

LP	LR	LZ	LT	LSD	RST	
0.000					LP	
0.254	0.000				LR	
0.394	0.342	0.000			LZ	
0.237	0.122	0.178	0.000		LT	
0.390	0.254	0.229	0.178	0.000	LSD	
0.157	0.140	0.242	0.127	0.173	0.000	RST

c) Nei genetic distances for nuclear markers

LP	LR	LZ	LT	LSD	RST	
0.000					LP	
0.990	0.000				LR	
0.367	0.838	0.000			LZ	
0.444	0.728	0.467	0.000		LT	
0.688	0.552	1.001	0.544	0.000	LSD	
0.369	0.690	0.336	0.374	0.663	0.000	RST

Symbols of the subpopulations the same as in tab. 1.

ble population sample. For this reason, the results of this study pertaining to the whole study population of 30 trees from the Miłomłyn Forest District seem to reflect its genetic structure.

4. Conclusions

We identified significant variability of the morphological characteristics of pine needles from the Miłomłyn Forest District: the largest variation was for the length of the needles (73.1 mm), and the smallest for the number of stomata

per 2 mm of length in the rows (an average of 21 per 2 mm on the flat side of the needles and 20.8 on the convex side).

We revealed significant genetic diversity of the studied population. Each tree had a separate genotype described from a set of 7 nuclear genome loci. For all loci studied, the population was in Hardy-Weinberg equilibrium. Based on an analysis of the polymorphism of 8 loci of the chloroplast genome, 28 haplotypes were detected and the coefficient of genetic diversity was 0.96.

Analysis of the Pearson correlation coefficient between the three variability parameters examined for individual subpopulations indicates that there is a weak positive correlation between the coefficient of genetic variation (h) for chloroplast loci and the level of intra-population variation (Wilks' λ indicator) for phenotype traits ($r = 0.31$; $p = 0.550$), whereas there is a medial negative correlation between the observed heterozygosity (H_o) and Wilks' λ ($r = -0.67$; $p = 0.141$). Comparing the coefficients of variation for molecular markers, we confirmed a weak positive correlation between the h index for chloroplast loci and the observed heterozygosity (H_o) for nuclear loci ($r = 0.39$; $p = 0.447$).

Conflict of interest

The authors declare no potential conflicts of interest.

Acknowledgements and source of funding

The study was financed by funds from the National Science Centre as research project N N305 019140.

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Authors' contribution

P.L. – conception of the study, genetic analysis, development of the results, manuscript writing; E.M.P. – conception of the study, genetic analysis, development of the results, manuscript writing; B.Ł. – development of the results, manuscript writing; L.U. – conception of the study, inventory and collection of biological material, development of the results, manuscript writing.