

## Genetic and phenotypic relationships among *Pinus sylvestris* populations in the Pieniny National Park

Lech Urbaniak<sup>1</sup>, Paulina Lesiczka<sup>1</sup>, Ewa Gąsiorowska<sup>1</sup>, Ewa M. Pawlaczyk<sup>1,\*</sup>, Piotr Androsiuk<sup>2</sup>, Anetta Lewandowska-Wosik<sup>1</sup> and Grzegorz Vončina<sup>3</sup>

<sup>1</sup> Adam Mickiewicz University, Faculty of Biology, Institute of Experimental Biology, Department of Genetics, Umultowska 89, 61-614 Poznań, Poland

<sup>2</sup> University of Warmia and Mazury in Olsztyn, Faculty of Biology and Biotechnology, Department of Plant Physiology, Genetics and Biotechnology, M. Oczapowskiego 1A, 10-719 Olsztyn, Poland

<sup>3</sup> Pieniny National Park, Jagiellońska 107B, 34-450 Krościenko nad Dunajcem, Poland

\*Corresponding author: ewapaw@amu.edu.pl

Received: May 25, 2017; Revised: October 23, 2017; Accepted: October 23, 2017; Published online: November 27, 2017

**Abstract:** The phenotypic and genetic relationships among five *Pinus sylvestris* populations in the western part of the Carpathian Mountains were analyzed based on seven morphological needle traits and 67 loci using 10 RAPD primers. Three of the studied populations were on the western side (Macelowa Góra, Czubatka, Łazowa Skałka) and two were located in the central part (Sokolica, Zamkowa Góra) of the Pieniny National Park. The examined populations had short needles, which is characteristic for mountain habitats of the Scots pine. The populations from the Pieniny Mts. had fewer rows of stomata on both sides of the needles than others studied stands of Eurosiberian Scots pine. The number of stomata on both flat and convex sides of the needles was similar to the values observed for populations growing on the nearby Tatra Mountains. Moreover, when these two traits are considered, the populations from Sokolica and Łazowa Skałka share the highest degree of similarity. Nei's genetic distances and genetic identities between the populations were calculated on the basis of RAPD loci. The results show that the lowest Nei's distance exists between the populations from the western part of the Pieniny Mts. – Macelowa Góra and Czubatka, and its central part – Sokolica and Zamkowa Góra, with the highest values recorded between the populations of Zamkowa Góra and Czubatka, and Łazowa Skałka and Czubatka. A similar pattern of phenotypic and genetic relations that was observed between the populations in both regions of the Pieniny Mountains could be the result of planting-seed material sourced from the central Pieniny Mts. in the area of western Pieniny Mts.

**Key words:** Scots pine; Western Carpathians; RAPD; pine needle morphology; population differentiation

### INTRODUCTION

When examining the differentiation of a species such as *Pinus sylvestris*, it is necessary to take into consideration the impact of three basic factors: history, the geographic range of distribution and human influence [1-4]. The continuous geographical range of the Scots pine stretches over the European lowlands and central parts of Siberia up to isolated populations near the Okhotsk Sea [5]. Around this giant area there are numerous isolated stands, such as those in the Carpathians, which include the Pieniny Mountains.

Many elements of Scots pine morphology and anatomy are subject to environmental modification,

including needle traits. However, the adaptability of traits remains, to a smaller or larger extent, under genetic constraints [6, 7], and making conclusions on their basis is difficult [6,8].

Among all of the forests in the Pieniny National Park, which occupy ca. 70% of its area, one can distinguish xerothermic and rocky pinewoods located in the central and western Pieniny Mountains. The Scots pine in the Pieniny National Park is found in small populations that cover the peaks of numerous mountain ridges and rocks. A small number of individuals in a population can lead to genetic erosion caused by inbreeding [9-11]. The results of dendrochronological

research show that at least some populations, especially in the central Pieniny Mts., are natural [12, 13] – in many cases the trees were estimated to be a few hundred years old (slightly above 400), which proves their relict character [14].

In the western Pieniny Mts., apart from native pinewoods that dominate the slopes or isolated hills, there are numerous stands of *P. sylvestris* of different origin. These seem to be the remains of an afforestation process conducted on strongly eroded slopes [1,15-17]. The spread of Scots pines in this area may also have resulted from a spontaneous afforestation in abandoned fields [2].

During the last glacial period (Vistulian, ca.16000 years BP), the nearest refuges of *P. sylvestris* were situated in the Carpathians and Sudetes Mts. and along the northern sides of these ranges [4]. This is in concordance with Staszkievicz's [18-20] assumption that this species has existed in the mountain region in southern Poland since the last interglacial period. Moreover, research by Cheddadi [3] based on an extensive survey of DNA, pollen and macrofossil remains and on vegetation modeling has identified numerous *P. sylvestris* glacial refuges located between 40° N and 50° N. The sufficiently warm climate east of the Alps, on the Hungarian Plain, the Czech Republic, the Danube region, the Carpathians, Bulgaria, northern Ukraine and southwestern Russia has enabled *P. sylvestris* to survive. These regions seem to have played a major role during the entire recolonization process, which took place between 14000 and 8000 years BP [3,21,22]. Therefore, it can be assumed that Scots pine gene resources persisting in the populations found south of the Carpathians may have contributed to the differentiation of its populations observed in the Pieniny and Tatra Mountains [19,20,23].

The main objective of this study was to evaluate the pattern of phenotypic and genetic relationships among *P. sylvestris* across the Pieniny National Park using morphological needle traits and molecular markers. The questions posed here concern: (i) molecular differentiation of five groups of *P. sylvestris* found in the central and western parts of the Pieniny National Park, and (ii) evaluating to what extent the differentiation resulting from analysis of the morphological traits of needles correlates with the molecular

data. The description of morphological and genetical differentiation appears to be essential for conservation of their genetic resources and an understanding of the processes that have led to the observed differentiation.

## MATERIALS AND METHODS

### The characteristics of the investigated *P. sylvestris* populations

The Scots pine, *Pinus sylvestris*, can be found in the central and western Pieniny Mountains, occupying part of the Pieniny National Park in two different communities. The research material, i.e. the needles, was collected from five stands of *P. sylvestris* from two regions, the central and western parts of the Pieniny National Park (Table 1, Fig. 1). *P. sylvestris* needles used as the study material were collected during the first week of October 2007. They were collected from the top southern branches, which were strongly insolated.

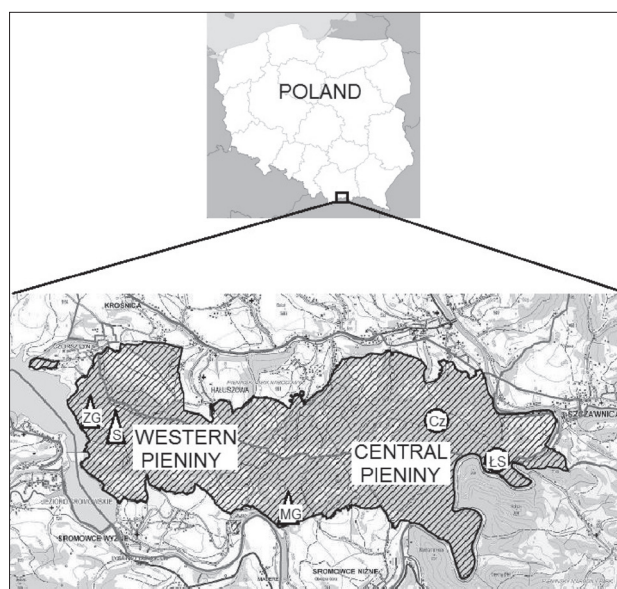
### Analyses of morphological traits

Seven morphological needle traits were analyzed: (1) the length of the needles (mm), (2) the number of stomata rows on the flat sides of the needles, (3) the number of stomata per 2 mm on the flat sides of the needles, (4) the number of stomata rows on the convex sides of the needles, (5) the number of stomata per 2 mm on the convex sides of the needles, (6) the number of serrations per 2 mm needle-length on the right-hand edges, (7) the number of serrations per 2 mm needle-length on the left-hand edges. Traits from 2 to 7 were analyzed in the middle part of the 2-year-old needles. Each tree was represented by 10 needles. The obtained data were used for statistical analyses: determination of the arithmetical mean, standard deviation and coefficient of variation (CV). Tukey's *t*-test was used to estimate whether the mean values of each trait differed significantly between the populations. Principal component analysis (PCA) was applied to present the scattering of five Scots pine populations from the Pieniny Mts. on the plane of first two principal components based on the morphometric characteristics of the needles. Additionally, the Mahalanobis distances were computed to describe the differences between populations based on 7 morphometric nee-

**Table 1.** Characteristics of environmental conditions for the analyzed populations.

Part of Pieniny National Park	Communities	Population	Geographic position	Number of trees	Elevation m a.s.l.	Growth conditions	Type of soil	Habitus of trees	Sign of human interference
Central Pieniny Mountains	<i>Pinus sylvestris</i> – <i>Calamagrostis varia</i> (rocky pinewoods)	S	E 20°26'24" N 49°25'04"	14	660 – 747	slopes facing south and west and open mountain tops	rendzic – lithic leptosols	umbrella-like crown, low trunk, twisted limbs	no
		ZG	E 20°25'19" N 49°25'12"	31	560 – 799	narrow rocky edge, facing south west and east	rendzic – lithic leptosols	umbrella-like crown, low trunk, twisted limbs	no
Western Pieniny Mountains	<i>Pinus sylvestris</i> – <i>Carex alba</i> (xerothermic pinewoods)	MG	E 20°22'53" N 49°24'21"	31	470 – 560	slopes facing south and west and open mountain ridges	rendzic – lithic leptosols, caloric cambisols + rendzic cambic leptosols	umbrella-like crown, low trunk, twisted limbs or slender trunk and typical crown	no
		Cz	E 20°19'48" N 49°25'22"	32	640 – 670	exposed mountain ridge on slopes facing south and west	rendzic – lithic leptosols	slender trunk and typical (narrow) crown	no
		ŁS	E 20°20'11" N 49°25'17"	22	650 – 670	slopes facing west and south and open mountain tops	rendzic – lithic leptosols	slender trunk and typical crown	minimal

S – Sokolica, ZG – Zamkowa Góra, MG – Macelowa Góra, ŁS – Łazowa Skalka, Cz – Czubatka



**Fig. 1.** *Pinus sylvestris* sampling localities in the Pieniny National Park. S – Sokolica, ZG – Zamkowa Góra, MG – Macelowa Góra, Cz – Czubatka, ŁS – Łazowa Skalka. Hatched area represents the Pieniny National Park.

dle characteristics. In this case, each population was treated as one point (a centroid). Next, the F statistics were calculated. Statistically significant F values point to significant differences between populations.

## Molecular analyses

The material for our investigation consisted of *P. sylvestris* needles that were stored at  $-20^{\circ}\text{C}$  after collection. The needles were cleaned by rinsing with distilled water. Analyses were conducted with the use of bulked samples. Each sample represented 10 trees randomly selected from each population.

A modified version of the cetyl trimethylammonium bromide (CTAB) method [24] was used for DNA isolation. DNA was isolated for each population from 1.5 g of frozen *P. sylvestris* needles that had been cleaned and sterilized with 70% ethanol. Needles ground in liquid nitrogen were thoroughly mixed with 2 mL of a preheated isolation buffer (2% CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl and 2%  $\beta$ -mercaptoethanol) and subsequently with 1 mL 20% CTAB, 1 mL 10% polyvinylpyrrolidone (PVP), and 330  $\mu\text{L}$  30% sarkosyl, and incubated at  $60^{\circ}\text{C}$  for 2 h. After three chloroform extractions, the DNA was precipitated with 96% ethanol, dried and dissolved in sterile, deionized  $\text{H}_2\text{O}$ .

PCR reactions were performed in 20  $\mu\text{L}$  containing 80 ng of template DNA, 0.3  $\mu\text{M}$  primer, 2 mM

MgCl<sub>2</sub>, 200 μM of each dATP, dGTP, dCTP and dTTP, 2 μL PCR buffer (400 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 1 M Tris-HCl pH 9.0), 4 μL of enhancer with betaine, and 1 unit of Tfl DNA polymerase (Epicentre Technology). The PCR reaction for random amplification of polymorphic DNA (RAPD) was processed at 94°C for 3 min, followed by 45 cycles at 94°C for 1 min, at 36°C for 1 min and at 72°C for 2.5 min, with a final extension step at 72°C for 5 min. Nineteen arbitrary primers were initially screened for their effective utilization in RAPD analysis. Of these 19 primers, 10 primers that gave polymorphic, clearly identifiable and repeatable bands were used further in PCRs. The nucleotide sequences of 10 RAPD primers which were used in the study are as follows: OPD-03: GTCGCCGTCA, OPD-05: TGAGCGCACA, OPD-06: ACCTGAACGG, OPD-13: GGGGTGACGA, OPA-12: TCGGCGATAG, OPA-15: TTCCGAACCC, OPB-16: TTTGCCCGGA, OPB-20: GGACCCTTAC, OPA-17: GACCGCTTGT, OPD-16: AGGGCGTAAG.

DNA fragments generated by amplification were separated in a 1.5% agarose gel in 1xTBE buffer at 100 V constant voltage, stained with 0.5 μg/mL ethidium bromide and visualized by UV light (312 nm). All the bands that could be reliably read were treated as single dominant loci and scored either as present (1) or absent (0) across the genotypes. On the basis of the obtained binary matrix of amplification products (bands), the Nei genetic similarity and distance (GD) were estimated [25,26]. A dendrogram was created by the unweighted pair group method with arithmetic mean (UPGMA) on the basis of genetic distance

values. The results were analyzed statistically with STATISTICA v. 12 software (StatSoft).

## RESULTS

### Analysis of needle morphology

The mean and Tukey's test values (Table 2) indicate that all analyzed morphological traits of needles influenced the pattern of interpopulation differentiation. The slightest effect is observed for traits 2 and 4, which describe the number of stomata rows on both sides of the needle. These traits are also characterized by high values of the coefficient of variation, which ranges from 16.7 to 21.2%. The rest of the morphological traits show the separateness of some population groups or individual characters of some *P. sylvestris* locations (trait 1: CV: 14.2-23.2%; trait 3: CV: 5.5-9.6%; trait 5: CV: 5.7-11.4%; trait 6: CV: 16.2-21.1%, and trait 7: CV: 16.2-21.1%). The *P. sylvestris* population from Łazowa Skalka is particularly distinguished by three morphological traits: the number of stomata per 2 mm on the flat sides of the needles (trait 3), the number of serrations per 2 mm needle-length on the right-hand edges (trait 6), and trait 7, the number of serrations per 2 mm needle-length on the left-hand edges (range of CV: 16.2-21.1%). The same needle traits allowed us to differentiate the Scots pines from Sokolica.

PCA is presented as a 2-D scattering plot of individuals from five studied *P. sylvestris* populations from the Pieniny Mountains based on the 7 morphometric

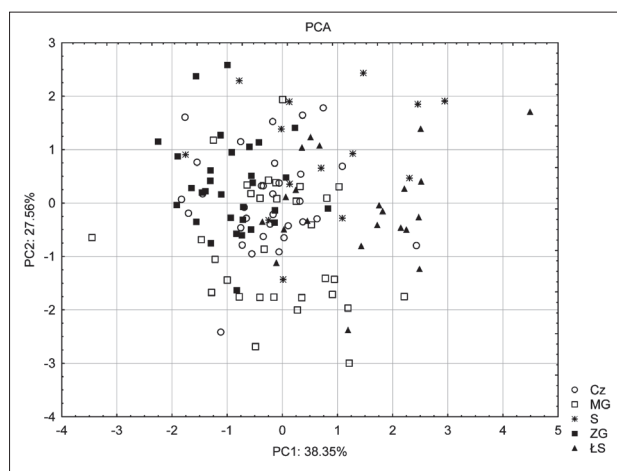
**Table 2.** Mean values of analyzed morphological traits with their standard deviations [SD], calculated for five Scots pine (*Pinus sylvestris* L.) populations.

Population	Morphological traits of needles						
	1** [mm]±SD	2 [n]±SD	3 [n]±SD	4 [n]±SD	5 [n]±SD	6 [n]±SD	7 [n]±SD
Cz	42.47±6.73 <sup>b</sup>	7.57±1.46 <sup>ab</sup>	20.42±1.76 <sup>a</sup>	7.69±1.28 <sup>ab</sup>	20.16±1.32 <sup>ab</sup>	8.41±1.78 <sup>b</sup>	8.43±1.78 <sup>ab</sup>
MG	38.31±8.90 <sup>a</sup>	7.79±1.52 <sup>b</sup>	20.37±1.65 <sup>a</sup>	7.93±1.40 <sup>b</sup>	20.52±1.90 <sup>b</sup>	8.59±1.72 <sup>b</sup>	8.66±1.70 <sup>b</sup>
S	43.94±6.83 <sup>bc</sup>	7.43±1.57 <sup>a</sup>	21.56±2.06 <sup>b</sup>	7.85±1.59 <sup>ab</sup>	21.37±2.44 <sup>c</sup>	7.78 <sup>a</sup> ±1.27 <sup>a</sup>	7.94±1.48 <sup>a</sup>
ZG	44.60±6.35 <sup>c</sup>	7.45±1.51 <sup>a</sup>	20.07±1.10 <sup>a</sup>	7.82±1.47 <sup>ab</sup>	19.91±1.14 <sup>a</sup>	8.46±1.31 <sup>b</sup>	8.40±1.36 <sup>ab</sup>
ŁS	40.55±6.68 <sup>a</sup>	7.61±1.30 <sup>ab</sup>	22.03±1.84 <sup>b</sup>	7.54±1.28 <sup>a</sup>	21.79±2.00 <sup>c</sup>	9.29±1.78 <sup>c</sup>	9.46±1.84 <sup>c</sup>

\*The same letters indicates a lack of statistically significant differences (Tukey's test, p=0.05) between the populations.

\*\*Symbols: 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 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 on the convex sides of the needles, 6 – number of serrations per 2 mm needle-length on the right-hand edges, 7 – number of serrations per 2 mm needle-length on the left-hand edges.

S – Sokolica, ZG – Zamkowa Góra, MG – Macelowa Góra, ŁS – Łazowa Skalka, Cz – Czubátka



**Fig. 2.** Result of principal components analysis of *Pinus sylvestris* populations from the Pieniny Mountains. The distances were calculated on the basis of seven morphological needle traits and presented on the plane of the first two principal components (PC1 and PC2).

needle characteristics (Fig. 2). This graph reveals the differentiation between the studied populations. The most different from other populations were those of Łazowa Skałka and Macelowa Góra, both from the western Pieniny Mts. whose individuals are predominantly located on the outskirts of this agglomeration. Individuals from the remaining three populations formed one cluster. The differentiation between the studied stands was confirmed by Mahalanobis distances (Table 3). F statistics showed that this differentiation was statistically significant. The most decisive influences on the pattern of interpopulational differentiation are possessed by traits 3 and 5 (that describe the number of stomata), and traits 6 and 7 (that are concerned with the number of serrations), which have a substantial effect on the first principal component (PC1), whereas the needle length (trait 1) impacts the second principal component (PC2).

### Molecular analysis

Ten RAPD primers [27] were chosen for the analysis of five *P. sylvestris* populations. In total, 67 loci were scored in the populations, and 12 of them (17.9%) were polymorphic. The highest number of loci were detected by primers OPA-17 and OPD-13 (10 and 9 loci, respectively). The lowest number of loci was detected by primers OPB-16 and OPB-20 (5 loci each)

**Table 3.** Mahalanobis distances between *Pinus sylvestris* L. populations from the Pieniny Mountains with F statistics calculated on the basis of seven morphological needle traits.

Cz						
MG	0.73					
	F=11.77**					
S	1.07	1.38				
	F=15.89**	F=25.95**				
ZG	0.45	1.06	1.27			
	F=4.56**	F=24.62**	F=21.97**			
ŁS	1.21	1.21	1.12	1.53		
	F=26.79**	F=26.53**	F=15.07**	F=42.32**		
	Cz	MG	S	ZG	ŁS	

\*\* – value significant at the level of  $p < 0.01$

S – Sokolica, ZG – Zamkowa Góra, MG – Macelowa Góra, ŁS – Łazowa Skałka, Cz – Czubatka.

**Table 4.** Factor changes among seven morphological traits of needles and the first two principal components.

Traits	PC1 55.96%	PC2 31.83%
1	2.61	22.99
2	0.71	3.82
3	26.66	1.39
4	1.85	2.83
5	27.44	2.07
6	22.30	0.01
7	21.34	0.04

and OPD-16, which amplified only 4 bands. The highest efficiency in detecting differences between analyzed populations was observed for primer OPD-03 that detected 6 loci, among which 50% were polymorphic. Primer OPD-13 showed very similar efficiency (44.4% polymorphic loci). Using primers OPA-17, OPB-16, OPB-20 and OPD-05, no differences were found among the analyzed populations (Table 5).

The genetic distance (GD) values estimated on the basis of RAPD markers ranged from 0.028 to 0.134. The lowest GD values were found between two pairs of populations: Czubatka (Cz) and Macelowa Góra (MG) and also for Sokolica (S) and Zamkowa Góra (ZG). The highest values of genetic distance were displayed for the populations of Zamkowa Góra and Czubatka (0.134), and also between Łazowa Skałka (ŁS) and Czubatka (0.118) (Table 6).

The dendrogram constructed on the basis of the GD based on 67 RAPD loci segregated the *P. sylvestris* populations from the Pieniny Mts. into two groups:

**Table 5.** Efficiency of RAPD markers in identifying genetic variability in *Pinus sylvestris* populations from the Pieniny Mts.

Primer	Number of identified loci	Polymorphic loci	
		N <sup>o</sup>	Percentage
OPA-12	6	1	16.7%
OPA-15	7	2	28.6%
OPA-17	10	0	0
OPB-16	5	0	0
OPB-20	5	0	0
OPD-03	6	3	50%
OPD-05	8	0	0
OPD-06	7	1	14.3%
OPD-13	9	4	44.4%
OPD-16	4	1	25%

**Table 6.** Nei's genetic identity (Nei and Li 1979) (above diagonal) and Nei's distance (Nei 1972) (below diagonal) based on 67 RAPD loci in five populations of *Pinus sylvestris*.

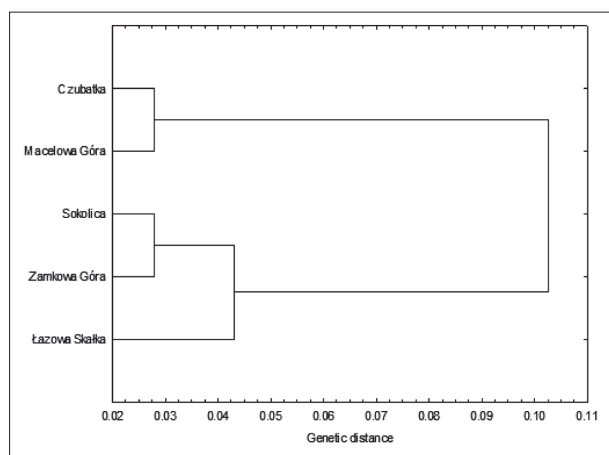
		Populations				
		Cz	MG	S	ZG	ŁS
Populations	Cz	***	0.972	0.903	0.875	0.889
	MG	0.028	***	0.931	0.903	0.917
	S	0.102	0.072	***	0.972	0.958
	ZG	0.134	0.102	0.028	***	0.958
	ŁS	0.118	0.087	0.043	0.043	***

S – Sokolica, ZG – Zamkowa Góra, MG – Macelowa Góra, ŁS – Łazowa Skalka, Cz – Czubatka

the first consists of populations from Czubatka and Macelowa Góra, and the second cluster gathered two closely related *P. sylvestris* populations from Sokolica and Zamkowa Góra, and the slightly different population from Łazowa Skalka (Fig. 3).

## DISCUSSION

The interest in *P. sylvestris* populations found in the Pieniny Mts. is due to their relict character. Therefore, they are treated as a unique element of the flora of the mountain range that is a part of the Western Carpathians. Populations in the central Pieniny Mts. in particular may contain elements of an ancestral gene pool. This is indicated by the research in cone trait variability of the populations located in the Pieniny Mts., the Tatra Mts. and comparable European stands, both in the mountains and lowlands [18]. Mejnartowicz and Bergmann [28] have argued on the basis of genetic analyses that a glacial refuge of *P. sylvestris* existed in the Pieniny Mts. The *polonica* type of

**Fig. 3.** UPGMA dendrograms based on Nei's genetic distances, calculated from RAPD data for five *Pinus sylvestris* populations from the Pieniny Mountains.

Scots pine differentiated in the area of the Pieniny and Tatra Mts. may have been one of the sources of species migration to the Polish lowlands and farther on to the northeast during the postglacial period [18-20]. Therefore, while considering *P. sylvestris* phenotypic and genetic variability in the Pieniny Mts., we also refer to the changeability of the examined traits on a scale representative for the species.

In comparison to the Polish lowland populations of *P. sylvestris* whose needle length is 48.2-68.3 mm [29], the pines found in the Pieniny Mts. have short needles. The shortest needles (38.31 mm) were observed in Macelowa Góra and the longest (44.60 mm) in Zamkowa Góra. In the Tatra mountain range located nearby, the *P. sylvestris* populations have even shorter needles: 33.01 (Wielkie Korycińska, 1120 m a.s.l.), 36.59 mm (Siwarowe Pański, 1300 m a.s.l.) [30]. The altitude difference between the stands in the two mountain ranges is ca. 500 m. This proves the rule pointing to the correlation between population altitude (above sea level) and needle length. It was apparently observed in native pinewoods in Scotland where the needle lengths at altitudes of 270 and 400 m a.s.l. were 32 and 40 mm, respectively [31]. Needle length is a trait that is to a significant extent modified by environmental impact [6,32,33]. However, in comparative experiments conducted in lowlands, the Pieniny populations retained short needles (40.34 mm) as compared to the lowland pine (57.29 mm) [34]. Short needles are traits that distinguish not only mountain populations. Pines characterized by this trait are also

found in peat bogs (e.g. in the north of Poland (Bory Tucholskie, the peat bog population Jelenia Wyspa has needles that are 32.8 mm long) [35]. Also, in the northern taiga behind the Urals, *P. sylvestris* has short needles, from 38 to 39 mm long [36].

Within the geographical range of *P. sylvestris* in Eurasia, populations are characterized by diverse numbers of stomata rows occurring on the convex and flat surfaces of the needles [7]. In Polish lowlands, the number ranges from 9.37 to 11.13 on the convex and from 10.05 to 11.72 on the flat sides of the needles [29]. Some northern populations in Russia have fewer stomata rows (Nowgorod: 8.41 and 9.75, respectively; Arkhangelsk: 8.82 and 9.66, respectively) [7]. Also, in the south, outside the continuous geographical range, a lower number of stomata rows was observed (Ukraine, Kharkov: 8.91 and 9.99 on the convex side; Turkey, Kars: 8.69 and 9.69 on the flat side of needle) [7]. A small number of stomata rows in needles was found in pines growing in lowland peat bogs in Poland, in the Bory Tucholskie and Puszcza Notecka forests (8.0 and 7.7 on the convex, and 8.3 and 9.0 on the flat side) [6,37]. The *P. sylvestris* populations growing in the Pieniny Mts. have the fewest stomata rows, from 7.43 to 7.79, and from 7.54 to 7.93 on the flat and convex sides of the needles, respectively. Also, a small number of stomata rows was observed in pines growing in the Tatra Mts. (from 7.48 to 8.54, and from 8.43 to 8.79 on the flat and convex sides of needles, respectively) [30]. The statistically significant value of the trait differentiates in Tukey's test the population in Macelowa Góra (western Pieniny Mts.) from Zamkowa Góra (central Pieniny Mts.) (trait 2), and Łazowa Skalka (western Pieniny Mts.) (trait 4) (Table 2).

When examining the differentiation of *P. sylvestris*, special attention was paid to the numbers of stomata per 2 mm on the flat and convex sides of the needles. These traits are subject to strict genetic control [6]. As a result, they are hardly susceptible to strong selective pressure and barely changeable within the population range [7,23,38-42]. The examined populations had from 20.07 to 22.03, and from 20.16 to 21.79 stomata per 2 mm on the flat and convex sides of the needles, respectively. Among them, the pines in Sokolica (central Pieniny Mts.) and Łazowa Skalka (western Pieniny Mts.) had the most, i.e. 21.56 and 22.03, and 21.37

and 21.79, respectively. For these characteristics, the populations in Sokolica (central Pieniny Mts.) and Łazowa Skalka (western Pieniny Mts.) were statistically significantly separated from other populations. Similar numbers of stomata per 2 mm in specified rows were found in populations representative of the Tatra Mts., Łysa Skalka and Wielkie Koryciska (22.43 and 21.52, 22.17 and 21.24, on flat and convex sides, respectively) [30]. A similar number of stomata was found in the populations from northeastern European and Siberian parts of the geographical range of the species, and the German foothills [7], whereas in the Polish lowlands, *P. sylvestris* had different numbers of stomata per 2 mm in specified rows on both sides of the needles [29]. The Masurian populations in Miłomłyn and Ruciane (northeastern Poland), and the ones in Bolewice in Wielkopolska (western Poland) were representative for the regions; they had similar numbers of stomata to the populations of the Pieniny Mts. (from 20.37 to 21.91 on the flat side, and from 20.05 to 21.29 on the convex side of the needle) [29]. Other populations that are representative for a significant part of Poland, Supraśl (northeastern Poland), Spała (central to southern Poland), Gubin (western Poland), Rychtal (southern Poland), Janów Lubelski (southeastern Poland), had more stomata per 2 mm needle: from 24.07 to 24.77 on the flat side, and from 23.35 to 24.26 on the convex side [29]. On the basis of the analysis by Tukey's test of both traits, the populations of Sokolica (central Pieniny Mts.) and Łazowa Skalka (western Pieniny Mts.) were grouped. These traits also make the greatest contribution to the shaping of the distribution of the population along the first principal component C1. As a result, both populations are positioned close to one another. Similarly, close relations were shown for Czubatka and Macelowa Góra from the western Pieniny Mts.

The usefulness of RAPD markers in describing diversity in the genus *Pinus* has been indicated earlier [27,43-45]. Based on the genetic distances obtained by RAPD markers, the western Pieniny populations of Czubatka and Łazowa Skalka are also part of two separate groups distinguished on the basis of phenotypic traits. The population of Czubatka is similar to that of Macelowa Góra. Due to the small GD values, the population of Łazowa Skalka is close to those of Sokolica and Zamkowa Góra. Despite the fact that RAPD primers

revealed a relatively low level of polymorphism (17.9% of all detected loci), the estimated values of GDs between particular populations of two groups were very similar to those published earlier for the same species [46,47]. Moreover, a similar GD (0.105) was observed between two Lithuanian pine populations located in ecologically different environments [27]. Naydenov et al. [21] also proved on the *P. sylvestris* population in Bulgaria that significant differentiation between populations and small genetic similarities are the result of population history and ecologic conditions, but not the geographical distance between them.

The presented coincident system of interpopulation relations in the area of the Pieniny Mts. with regard to the phenotypic and genetic trait variability can be partially explained by the history of these populations. It must be stressed that in the area of the western Pieniny Mts., tracing *P. sylvestris* history is hindered by the possible consequences of processes connected to mixing of the gene pools of populations with a different origin. Some are of clearly anthropogenic origin [1,15-17]. The available data show that *P. sylvestris* growing on state-owned land in the western Pieniny Mts. was not registered until 1937 [1]. Bodziarczyk and Pancer-Kotejowa [2] claimed that the source of seeds for the western Pieniny populations may have been pines growing in relict pine groves, as well as individual specimens growing on balks. This is the simplest explanation for the observed system of interpopulation connections. Nevertheless, the interpopulation differentiation in *P. sylvestris* in that relatively small area is significant. This is because the GD between distinct Pieniny populations exceeded the parameter values obtained for Polish populations [48,49]. Also, population differentiation based on the morphological traits of needles is evident, and is proved by the Mahalanobis distances. This is yet additional proof of the considerable, wide-ranging adaptability of *P. sylvestris* in changing environmental conditions.

**Acknowledgments:** This project was supported by the Polish National Science Centre under Grant No. NN305 019140

**Author contributions:** Study concept: LU; laboratory work: LU, PL, EG, EMP, PA, ALW; writing: LU, PL, EMP; plant material to the study: LU, GV.

**Conflict of interest disclosure:** The authors declare no conflict of interest.

## REFERENCES

1. Dziewolski J. Przemiany strukturalne lasów w Pieninach. *Chrońmy Przyrodę Ojczystą*. 1985;41(2):17-25.
2. Bodziarczyk J, Pancer-Koteja E. Naturalne i wtórne sośniny w Pienińskim Parku Narodowym (Natural and secondary pine woods in the Pieniny National Park). *Studia Naturae*. 2004;49:123-30.
3. Cheddadi R. Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. *Global Ecol Biogeogr*. 2006;15:271-82.
4. Latałowa M, Tobolski K, Nalepka D. *Pinus* L. subgenus *Pinus* (subgen. *Diploxylon* (Koehne) Pilger- Pine. In: Ralska-Jasiewiczowa M, Latałowa M, Wasylkowa K, Tobolski K, Madeyska E, Wright Jr HE, Turner Ch, editors. Late glacial and Holocene history of vegetation in Poland based on isopollen map). Kraków: Szafer Institute of Botany Polish Academy of Science; 2004. p. 165-79.
5. Critchfield WB, Little EL. Geographic distribution of the Pines of the World. Washington DC: U. S. Dept. Of Agriculture, Forest Serv; 1966. 116p.
6. Urbaniak L, Karliński L, Popielarz R. Variation of morphological needle characters of Scots pine (*Pinus sylvestris*L.) populations in different habitats. *Acta Soc Bot Pol*. 2003;72(1):37-44.
7. Urbaniak L. Zróżnicowanie geograficzne sosny zwyczajnej (*Pinus sylvestris* L.) z terenu Eurazji na podstawie cech morfologicznych i anatomicznych igieł. Poznań: Wyd. Naukowe UAM; 1998. 58p. (Seria Biologia)
8. Reusch TBH, Ehlers A, Hämmerli A, Worm B. Ecosystem recovery after climatic extremes enhanced by genotypic diversity. *P Natl Acad Sci USA*. 2005;102:2826-31.
9. Lande R. Risk of populations extinction from demographic and environmental stochasticity and reveal catastrophes. *Am Nat*. 1993;142:911-27.
10. Korol L, Gil SL, Climent MJ, Zehavi A, Schiller G. Canary Island pine (*Pinus canariensis* CHR. Im. ExDC) Gene flow among native populations. *For Genet*. 1999;6(4):277-82.
11. Frankham R. Genetics and extinction. *Biol Conserv*. 2005;126:131-40.
12. Kulczyński S. Die Pflanzenassoziationen der Pieninen. *Bull Acad Pol Sci Lett*. 1927;B:57-203. .
13. Pancer-Kotejowa E. Zbiorowiska leśne Pienińskiego Parku Narodowego - Forest Communities of Pieniny National Park (Western Carpathians). *Fragm Florist Geobot*. 1973;19(2):197-258.
14. Niedzielska B. Wiek sosen reliktowych na Sokolicy w Pienińskim Parku Narodowym - The age of relict pine trees on the Sokolica peak in the Pieniński National Park. *Sylwan*. 2001;145:57-62.
15. Dziewolski J. Lasy Pienińskiego Parku Narodowego. *Chrońmy Przyrodę Ojczystą*. 1974;30(6):22-29.
16. Dziewolski J. Naturalny rozwój drzewostanów Pienińskiego Parku Narodowego w czasie 51 lat (1936-1987). *Ochrona Przyrody*. 1991;49:112-28.
17. Dziewolski J. Zastosowanie inwentaryzacji lasu na przykładzie wybranych terenów Pienińskiego Parku Narodowego. *Chrońmy Przyrodę Ojczystą*. 1992;48(4):16-30.



18. Staszkiwicz J. Zmienność współczesnych i kopalnych szyszek sosny zwyczajnej (*Pinus sylvestris* L.). *Fragm Florist Geobot.* 1961;7(1):97-160.
19. Staszkiwicz J. Sosna w historii naszych lasów. Od trzeciorzędu do holocenu. In: Białobok S, Boratyński A, Bugała W, editors. *Biologia sosny zwyczajnej*. Poznań-Kórnik: Wyd. Sorus; 1993a. p. 11-7.
20. Staszkiwicz J. Zmienność morfologiczna szpilek, szyszek i nasion. In: Białobok S, Boratyński A, Bugała W, editors. *Biologia sosny zwyczajnej*. Poznań-Kórnik: Wyd. Sorus; 1993b. p. 33-43.
21. Najdenov KD, Tremblay FM, Alexandrov A, Fenton NJ. Structure of *Pinus sylvestris* L. populations in Bulgaria revealed by chloroplast microsatellites and terpenes analysis: provenance testes. *Biochem Syst Ecol.* 2005;33:1226-45.
22. Pyhäjärvi T, Salmela MJ. Colonization routes of *Pinus sylvestris* inferred from distribution of mitochondrial DNA variation. *Tree Genet Genomes.* 2008;4:247-54.
23. Urbaniak L, Chudzińska E, Faferek S. Differentiation of *Pinus sylvestris* L. populations of the Tatra Mts and the Tuchola forest expressed in the needle anatomical traits. *Dendrobiology.* 2008;60:35-43.
24. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 1980;8(19):4321-25.
25. Nei M. Genetic distance between populations. *Am Nat.* 1972;106:283-92.
26. Nei M, Li WH. Mathematical model for studying genetic variation in terms of restriction endonucleases. *P Natl Acad Sci USA.* 1979;76:5269-73.
27. Naugžemys D, Žvingila D, Aučina A, Rančelis V. Comparison of DNA polymorphism in seedlings of *Pinus sylvestris* L. from different populations by RAPD markers. *Biologija.* 2006;1:30-5.
28. Mejnartowicz L, Bergmann F. Genetic differentiation among Scots pine populations from the lowlands and the mountains in Poland. *Lect Notes Biomath.* 1985;253-65.
29. Bobowicz MA, Korczyk AF. Interpopulational variability of *Pinus sylvestris* L. in eight Polish localities expressed in morphological and anatomical traits of needles. *Acta Soc Bot Pol.* 1994;63:67-76.
30. Androsiuk P, Urbaniak L. Differentiation of Scots pine (*Pinus sylvestris* L.) populations in the Tatra Mountains based on needle morphological traits. *Biodiv Res Conserv.* 2006;3-4:227-31.
31. Taberlet P, Fumagalli L, Wust-Saucy AG, Cosson JF. Comparative phylogeography and postglacial colonization routes in Europe. *Mol Ecol.* 1998;7:453-64.
32. Ninemetes U, Ellsworth DS, Lukianow A, Tobias M. Site fertility and the morphological and photosynthetic acclimation of *Pinus sylvestris* needles to light. *Tree Physiol.* 2001;21:1231-44.
33. Lechner RAB, Rigal A. Indicators of drought effects in *Pinus sylvestris*: genetic analyses to corroborate the results of empirical methods. *iForest.* 2010;3:89-91.
34. Zajączkowski M. *Studia nad sosną zwyczajną w Tatrach i Pieninach*. Kraków: Prace Rolniczo-Leśne, PAU; 1949. 97 p.
35. Mamaev SA. *Formy wnutrividowej zmienności drewnianych rastenij*. Moskwa: Nauka; 1972.
36. Urbaniak L, Karliński L, Popielarz R. Variation of morphological needle characters of Scots pine (*Pinus sylvestris* L.) populations in different habitats. *Acta Soc Bot Pol.* 2003;72:37-44.
37. Woźniak T, Androsiuk P, Nowak D, Urbaniak L. The expression of morphological needle characters of Scots pine (*Pinus sylvestris* L.) populations growing in various habitats in Puszcza Notecka. In: Prus-Głowacki W, Pawlaczyk E, editors. *Variability and Evolution - New Perspectives*. Poznań: Wyd. Naukowe UAM; 2005. p. 449-62.
38. Urbaniak L, Ślusarz M, Karliński L. Description of the relict Scots pine (*Pinus sylvestris* L.) populations in the Tatra and Pieniny Mountains by needle characters. In: Third Balkan Scientific conference. Study, conservation and utilization of forest resources. 2000 Oct 2-6; Sofia, Bulgaria. Sofia: Academic Publishers; 2001. p.191-8.
39. Urbaniak L, Androsiuk P, Ślusarz M, Vončina G. Zróżnicowanie populacji sosny zwyczajnej (*Pinus sylvestris* L.) w Pieninach Właściwych na podstawie cech morfologicznych igieł. *Pieniny - Przyroda i Człowiek.* 2006a;9:71-78.
40. Urbaniak L, Gąsiorowska E, Klasa A. Próba wytypowania reliktowych populacji sosny zwyczajnej na terenie Ojcowskiego Parku Narodowego. *Prądnik: Prace i Materiały Muzeum im. Prof. W. Szafera;* 2006b.16:119-24.
41. Urbaniak L, Vončina G. Charakterystyka sosny zwyczajnej *Pinus sylvestris* L. z Sokolicy, Czertezika i Kazalnicy w cechach morfologicznych igieł (The characteristics of Scots pine (*Pinus sylvestris* L.) from Sokolica, Czertezik and Kazalnica peaks on the basis of morphological traits of needles). *Pieniny - Przyroda i Człowiek.* 2008;10:19-25.
42. Androsiuk P, Ciągło-Androsiuk S, Urbaniak L. Genetic diversity and differentiation of *Pinus sylvestris* L. from the IUFRO 1982 provenance trial revealed by AFLP analysis. *Arch Biol Sci.* 2015;67(4):1237-49.
43. Lu MZ, Szmidt AE, Wang XR. Inheritance of RAPD fragments in haploid and diploid tissues of *Pinus sylvestris* (L.). *Heredity.* 1995;74:582-9.
44. Nkongolo KK, Michael P, Gratton WS. Identification and characterization of RAPD markers referring genetic relationships among Pine species. *Genome.* 2002;45:51-8.
45. Androsiuk P, Urbaniak L. Genetic variability of *Pinus sylvestris* populations from IUFRO 1982 provenance trial. *Dendrobiology.* 2014;71:23-33.
46. Polok K, Pisarek W, Urbaniak L, Korzekwa K, Zieliński R. Genetic characteristic of *Pinus sylvestris* fo. *turfosa* Woerl from Gązwa Reserve, North-Eastern Poland. In: Prus-Głowacki W, Pawlaczyk E, editors. *Variability and Evolution - New Perspectives*. Poznań: Wyd. Naukowe UAM; 2005a. p. 225-39.
47. Polok K, Urbaniak L, Korzekwa K, Androsiuk P, Ciągło S, Kubiak K, Zieliński R. Genetic similarity of *Pinus sylvestris* populations on the base of DNA markers. In: Prus-Głowacki W, Pawlaczyk E, editors. *Variability and Evolution - New Perspectives*. Poznań: Wyd. Naukowe UAM; 2005b. p. 253-67.
48. Nowakowska JA. Zróżnicowanie genetyczne wybranych populacji sosny zwyczajnej (*Pinus sylvestris* L.) na podstawie analiz RAPD. *Sylwan.* 2003;11:26-37.
49. Nowakowska JA. Zmienność genetyczna polskich wybranych populacji sosny zwyczajnej (*Pinus sylvestris* L.) na podstawie analiz polimorfizmu DNA. *Sękocin Stary: Prace Instytutu Badawczego Leśnictwa;* 2007. 118 p.