



*Zoltán Attila Köbölkuti\**, *Endre György Tóth\**, *Márta Ladányi*,  
*Mária Höhn*

## Morphological and anatomical differentiation in peripheral *Pinus sylvestris* L. populations from the Carpathian region

Received: 9 September 2016; Accepted: 28 December 2016

**Abstract:** Carpathian Scots pine populations having peripheral distribution within the species' range are often sustained in specific types of habitats, such as peat bogs and rocky surfaces or lime consisting sandy substrates. Due to their long time adaptive processes, which involve genotypes that can be preadapted to a given selection pressure, historically isolated populations are subjects of particular interest in studies of *in situ* adaptation. In this study we focus on detecting the level of phenotypic differentiation based on cone morphology and needle anatomy in marginal populations of *Pinus sylvestris* L. in the Pannonian Basin and the Carpathian Mountains. Six cone morphological and eight needle anatomical characters were measured and four cone morphological and four needle anatomical ratios were calculated. Our results in concordance with paleobotanical data indicate a common origin of the populations from the Northern Carpathians and the Pannonian Basin. High levels of variation were observed in cone morphology. Discriminant function analysis based on the eight cone characteristics revealed clearly discernible groups of populations and indicated significant differentiation among populations growing in peat bogs and on rocky substrates. Significant differences among populations from different habitats were also revealed by comparing needle anatomical variables. The phenotypic differentiation by habitat type based on the measured characters might be evaluated as a sign of local adaptation with detectable phenotypic patterns.

**Keywords:** adaptation, cone morphology, geographic differentiation, needle anatomy

**Address:** Z. A. Köbölkuti, E. Gy. Tóth, M. Ladányi, M. Höhn, Szent István University, Faculty of Horticulture, 1118, Ménesi út 44., Budapest, Hungary, e-mail: zoltanattila.kobolkuti@gmail.com

\*The authors contributed equally to this article and the research on which it was based.

### Introduction

Important shifts in the geographic distribution of forest tree species caused by recent climate change make the study of edge populations a topic

of particular importance for biologists. The importance of the persistence of some species in peripheral 'small refugia' is being increasingly recognized (Noss, 2001; Petit et al., 2003). While the term was originally introduced to refer to locations where

species survived the last glacial period (Bennett & Provan, 2008), now it is often also used to refer to areas that should be conserved to minimize the impacts of changes in the global ecosystem (Rull, 2009). The properties of tree populations in these peripheral refugia have evolved through the interaction of migration and selection (Davis & Shaw, 2001). Although trees are usually well adapted to local environments (González-Martínez et al., 2006) with high phenotypic plasticity, the composition and structure of peripheral populations is influenced or determined by a combination of historical events, geological changes in the growing sites, and in situ adaptation to ecological factors (Losos, 1996). The adaptedness and the adaptability of edge populations to varying environmental conditions are dependent on the diversity accumulated in the gene stock and long potential exposure to divergent selection pressures (Gregorius, 1989). Although each peripheral population possesses a specific migration history, natural selection over extended periods of time leaves traces on gene based morphological and anatomical traits. As the most widely distributed member of the family Pinaceae (Critchfield & Little, 1966; Turna, 2003) in Central and Eastern Europe (including the Carpathians), Scots pine is present only in isolated, peripheral localities. Also, it has been postulated that in Europe Scots pine survived the last glaciations in five main refugia, namely the Iberian Peninsula, the north Apennine Peninsula, south of the Carpathians, the Sudetes, and the Balkans (Prus-Głowacki et al., 2012). Nowadays, these refugial populations represent gene pools of the species and are affected by isolation, mutations and selection due to ecological conditions and genetic drift. Moreover, these small populations persist in extreme habitats types with elevated groundwater tables (such as peat bogs) or grow on sunny, rocky substrates (Urbaniak, 1998). They continue to survive under diverse environmental conditions within a scattered geographic area. Natural selection forces individuals within these specific habitat types to adapt to the local environmental conditions (Lenormand, 2002). Accordingly, it is presumed that Carpathian populations of *P. sylvestris* exhibit considerable variation in morphological characteristics (Boratyński, 1991; Turna, 2003).

Morphological and anatomical traits are important indicators of responses to varying environmental conditions (Urbaniak et al., 2003). As phenological marks, needles are directly exposed to the environment's physical factors, such as altitude, air temperature, atmospheric pressure, precipitation, and wind velocity (Friend & Woodward, 1990; Körner, 2007; Tiwari et al., 2013). They are also responsible for photosynthesis, carbon assimilation, and exchange of gas and water, and they may vary in

overall dimensions, as well as in details of key anatomical characteristics important in processes of adaptation (Donnelly et al., 2016). Cone formation is also known to be variable, depending greatly upon climatic factors (Ovington, 1957). Our hypothesis is that natural populations in the refugial areas, in addition to possessing imprints of historical events, are also distinctive due to adaptations to different habitat extremes on the periphery. This hypothesis is supported by studies performed on populations from Central and Northern Europe, the Balkans, Iberia, and Anatolia (Staszkiwicz, 1961; Tobolski & Hanover, 1971; Mejnartowicz, 1979; Prus-Głowacki & Stephan, 1994; Alía et al., 2001; Prus-Głowacki et al., 2003; Turna, 2003; Labra et al., 2006; Bilgen & Kaya, 2007; Pyhäjärvi et al., 2007; Semiz et al., 2007; Dzialuk et al., 2009; Jasińska et al., 2014). Based on these works, it can be concluded that populations in refugial areas display not only 'geographic marks', but also genetic differences and differentiation at the phenotypic level. In this study, we focus on detecting the level of phenotypic differentiation of Scots pine in peripheral populations from the Carpathians and the Pannonian Basin based on morphological and anatomical data in cones and needles. As our finding demonstrates, morphological and anatomical trait variation within these populations shows a specific level of individual homeostasis as a consequence of the phenotypic stability. Our findings may yield information concerning the genetic background of populations and provide indication about the origin and formation of the current gene pool, which has been shaped by the consequences of local adaptation to extreme ecological sites.

## Materials and methods

### Study sites

16 natural populations of *Pinus sylvestris* L. from the Carpathians and the Pannonian Basin were sampled between 2011 and 2015 (Table 1, Fig. 1). All of these marginal populations within the natural range of the species occupy specific habitat types, such as raised bogs, dry rocky substrates, or mixed forests on specific substrates with low nutrient content. These mixed forests, which have developed on different substrates, are characterized by specific competition features due to the vegetation mixture of conifers and broad-leaved deciduous trees, in which Scots pine seems to be less competitive (Fig. 1).

### Sampling design

Trees were chosen using randomized sampling design at each stand. 25 to 40 fully ripened, brown,

Table 1. List of studied *Pinus sylvestris* populations from the Central and Eastern European peripheral distribution of the species

No.	Code	Country	Residential area	Latitude (N)	Longitude (E)	Altitude (m)	Size (km <sup>2</sup> )	Habitat
1	HKO	Hungary	Kőszeg	47.22	16.33	630	0.04	Rocky substrate
2	HFE	Hungary	Fenyőfő	47.35	17.77	252	4.49	Mixed forest
3	HVE	Hungary	Pethőhenye	46.87	16.92	306	0.04	Mixed forest
4	HZA	Hungary	Szalafő	46.87	16.30	231	0.08	Mixed forest
5	HOR	Hungary	Csörötnek	46.93	16.35	296	0.10	Mixed forest
6	SKV	Slovakia	Kvacany	49.18	19.54	799	0.48	Rocky substrate
7	STU	Slovakia	Svarin	49.02	19.91	1107	0.70	Rocky substrate
8	SLI	Slovakia	Liptovský Hrádok	49.04	19.74	729	0.02	Rocky substrate
9	RFE	Romania	Fântâna Brazilor	46.50	25.26	953	0.32	Peat bog
10	RPO	Romania	Poiana Stampei	47.30	25.12	878	1.43	Peat bog
11	RMO	Romania	Băile Tusnad	46.13	25.91	1052	0.58	Peat bog
12	RPA	Romania	Voineasa	45.38	23.91	753	3.42	Rocky substrate
13	RBI	Romania	Roșia	46.84	22.37	393	0.13	Rocky substrate
14	RBE	Romania	Poșaga de sus	46.49	23.36	524	0.84	Rocky substrate
15	RML	Romania	Ponor	46.33	23.34	925	0.10	Peat bog
16	RMH	Romania	Călățele	46.73	23.02	913	0.58	Peat bog

two-year-old cones from each population and 10–20 two-year-old brachyblasts with healthy needles were collected from each tree, from four trees per population. Following the collection, the cones were kept at room temperature. After three weeks, all of the seeds were extracted and stored in paper bags. Following seed extraction, the cones were desiccated at 30–40°C and then stored in airtight plastic bags. Needles were taken from 30–40-year-old adult trees at 2–3 m above ground level from a well-illuminated part of the crown. Leaf collections were stored in sealed plastic bags at –20°C in a freezer. Before measurements were taken, a cross section was made from the middle of each needle. Cross sectioning was carried out with an Ernst Leitz GMBH Wetzlar tissue microtome. Sections were stained with Toluidine blue, washed with 10% hydrochloric acid, and placed on glass slides in glycerinated water.

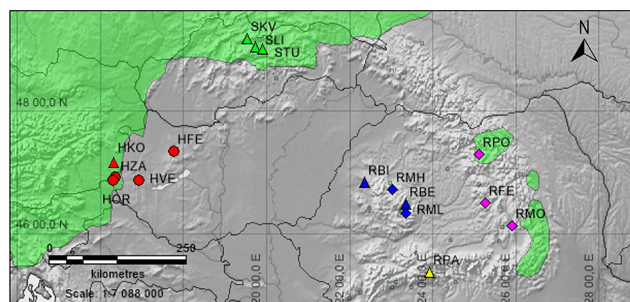


Fig. 1. Sampled populations of *Pinus sylvestris*. The colors indicate the geographical affiliation (red: Pannonian Basin (PB), green: Northern Carpathians (NC), blue: Central-Island Mountains/ Apuseni Mt. (CIM), pink: Eastern Carpathians (EC) and yellow: Southern Carpathians (SC)). The shapes of the markers (rectangles: peat bogs, triangles: dry rocky substrates, circles: mixed forests) refer to the type of the habitat. The Scots pine distribution area is highlighted in green, based on a Euforgen map

## Characters and their measurements

In reference to all twenty two studied traits, six cone morphological and eight needle anatomical characters were measured and four cone morphological and four needle anatomical ratios were calculated. Six morphological characters were measured on each cone: cone width (CW), cone length (CL), number of scales (NBS), width (AW), length (AL), and thickness of apophysis (AT). Four ratios were calculated: cone length/width (CL/CW), cone length/number of scales (CL/NBS), apophysis length/apophysis thickness (AL/AT), and apophysis length/width (AL/AW). These parameters were measured with an electronic caliper with 0.1 mm accuracy.

Needle anatomical data were obtained from 256 needles: four needles per tree on four trees per population (a total of 16 needles per population). The anatomical parameters (8 in total) measured for each needle were: number of resin ducts (NRD), number of layers of the armed palisade parenchyma on the concave side (NBRCC), convex side (NBRCV) and between central cylinder and needle edge (NBRCW). Additional characteristics (measured in  $\mu\text{m}$ ) were assessed, including needle height (NH) and width (NW), the height (CCH) and width (CCW) of the central cylinder, and four ratios: central cylinder width/height (CCW/CCH), needle width/height (NW/NH), central cylinder height/needle height (CCH/NH), and central cylinder width/needle width (CCW/NW). The number of resin ducts and parenchyma cells were examined under an Olympus XC21 microscope, with the ocular and objective magnification of 10 $\times$  and 40 $\times$ , respectively.

The height and width of the needle and central cylinder were measured in micrometers ( $\mu\text{m}$ ) using a Zeiss Axio Imager A2 microscope with 5 $\times$ /0.13

magnification, and they were photographed using a HRc AxioCam with AxioVision Microscopy software (Carl Zeiss, Germany).

## Data analysis

Statistical analysis was carried out on 4448 sampling data to investigate the cone traits and needle anatomical parameters. Methods were chosen according to the existing literature (Staszkiwicz, 1961; Marcysiak, 2006; Turna & Güney, 2009; Jasińska et al., 2014). Multivariate ANOVA (MANOVA), discriminant analysis, and the Mantel test were performed. Maximal-minimal values, arithmetic means, and standard deviations were calculated and analyzed for all populations and groups of populations. The one-way multivariate analysis of variance (MANOVA) test with geographical position or habitat type as a factor was used, followed by variable-wise between-subjects effects analysis, to evaluate the significance of differences among populations for particular characteristics. The normality of the residuals was accepted on the basis of their skewness and kurtosis (Tabachnick & Fidell, 2013). Since the assumption of homogeneity of variances was moderately violated by Levene's test ( $p < 0.05$ ), we separated the significantly different groups by using Games-Howell's post hoc test. We applied discriminant function analysis to predict a categorical dependent variable and determine whether a set of variables was effective in predicting category membership.

We considered previous molecular studies and macrofossil and pollen data analyses, which arrive at the conclusion that there is evidence of refugial locations in East-Central Europe, e.g. the Hungarian plain (Naydenov et al., 2005; Cheddadi et al., 2006; Bernhardsson et al., 2016), as well as studies and data

published by Magyari et al. (2014), who contended that the species persists in Eastern Carpathian refugia. Therefore, we decided to apply discriminant function analysis separately for both morphological and anatomical datasets to detect samples grouping after a previous sorting of populations according to geographical distribution (the Pannonian Basin, the Northern Carpathians, Central Island Mts., the Eastern Carpathians, and the Southern Carpathians). We also sorted the populations under examination according to the type of their habitat (peat bogs, rocky substrates, and mixed forests) to detect any grouping by traits, which are (or are not) suited to the specific environment. The analysis was performed according to the stepwise method by computing the group sizes within groups. Morphological variation was analyzed with IBM SPSS 20.0 (IBM Corp.) and Microsoft Excel. A Mantel test (Mantel, 1967) was performed to test the relationship between the geographical and morphological multi-character differences among the populations. Euclidean distances and geographical distances among populations were used for the evaluation using GenAlEx 6.5 (Peakall & Smouse, 2006) software.

## Results

### Differentiation by geographical distribution

Our average values with standard deviations of the analyzed cone characteristics by geographical distribution are summarized in Table 2. One-Way MANOVA test on morphological traits yielded significant differences ( $F(20;820) = 8.25$ ;  $p < 0.001$ ) with significant between-subjects effects ( $F(4;251) > 6.1$ ;

Table 2. Average values with standard deviations of the analyzed cone characteristics by geographical distribution

Code	Characteristics	Pannonian Basin (PB)	Northern Carpathians (NC)	Central Island Mts. (CIM)	Eastern Carpathians (EC)	Southern Carpathians (SC)
		(HFE, HVE, HZA, HOR)	(SKV, STU, SLI)	(RBI, RBE, RML, RMH)	(RFE, RPO, RMO)	(RPA)
CW	Cone width (cm)	4.1 ± 0.7	4.0 ± 0.5	4.8 ± 0.7	4.0 ± 0.9	4.4 ± 0.8
CL	Cone length (cm)	3.9 ± 0.7	3.4 ± 0.6	4.3 ± 0.8	3.8 ± 1.0	3.8 ± 0.8
NBS	Number of scales	72.3 ± 11.7	59.0 ± 8.4	81.5 ± 11.8	67.6 ± 11.2	63.8 ± 9.4
AW	Width of apophysis (mm)	6.7 ± 1.3	6.4 ± 1.0	6.3 ± 0.9	7.2 ± 1.0	6.4 ± 1.2
AL	Length of apophysis (mm)	6.9 ± 1.0	6.8 ± 0.8	6.8 ± 1.0	6.7 ± 1.6	7.2 ± 0.6
AT	Thickness of apophysis (mm)	2.6 ± 0.7	2.5 ± 0.6	3.3 ± 0.9	3.3 ± 1.1	2.7 ± 0.8
CL/CW	Cone length/cone width	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.2	0.9 ± 0.1	0.9 ± 0.1
CL/NBS	Cone length/number of scales	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
AL/AW	Apophysis length/apophysis width	1.1 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	0.9 ± 0.2	1.2 ± 0.2
AL/AT	Apophysis length/apophysis thickness	2.9 ± 1.0	2.7 ± 0.5	2.2 ± 0.9	2.2 ± 0.8	2.9 ± 0.9



$p < 0.001$ ). We have found that in the case of five variables (AW, AT, CW, and AL/AW, AL/AT), populations from the Northern Carpathians and the Pannonian Basin form one group, while cones from the Eastern Carpathians are separated by four (AW, AT and AL/AT and AL/AW ratios) variables (Fig. 2). According to the discriminant function analysis, on the basis of the first variable (Function 1), which was responsible for 60.6% of the variation, the centroids of the

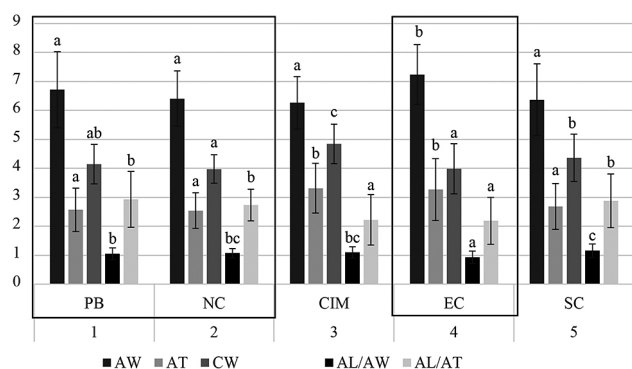


Fig. 2. Statistically significant morphometric variables of Scots pine among the geographical regions under study, detected by Games-Howell's post hoc test ( $p < 0.05$ ). The PB abbreviation stand for Pannonian Basin, NC: Northern Carpathians, CIM: Central-Island Mountains (Apuseni), EC: Eastern Carpathians and SC: Southern Carpathians. For morpho-anatomical abbreviations see Table 2. The unit of the measurement is mm (AW, AT, CW), except AL in the case of /AW and AL/AT

populations were split into two distinct groups: one comprises the Central Island Mts. (3), the Northern Carpathians (2), and the Southern Carpathians (5), and a second, separate group is formed by populations from the Pannonian Basin (1) and the Eastern Carpathians (4) (Fig. 3A). NBS and CW were discriminating variables of Function 1. The second variable (Function 2), which was responsible for 20.7% of the total variation, differentiated the populations from the Pannonian Basin (1) from populations in the Eastern Carpathians (4), while the Northern (2) and Southern Carpathians (5) still remained in one group. The second variable (Function 2) differentiated the populations by CL/CW, AW and AL/AW.

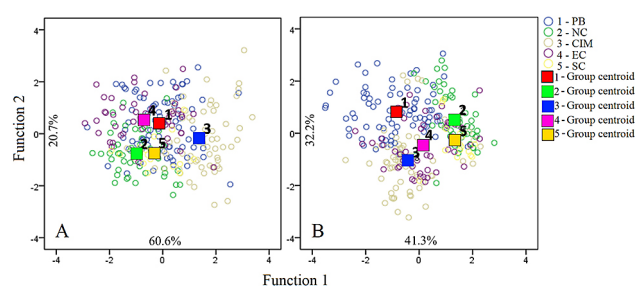


Fig. 3 A–B. Differentiation of *Pinus sylvestris* populations by geographical distribution based on cone morphological characters (A) and needle anatomical variables (B), detected by Games-Howell's post hoc test ( $p < 0.05$ ). PB: Pannonian Basin, NC: Northern Carpathians, CIM: Central-Island Mountains (Apuseni), EC: Eastern Carpathians and SC: Southern Carpathians

Table 3. Average values with standard deviations of the analyzed needle characteristics by geographical distribution

Code	Characteristics	Pannonian Basin (PB)	Northern Carpathians (NC)	Central Island Mts. (CIM)	Eastern Carpathians (EC)	Southern Carpathians (SC)
		(HFE, HVE, HZA, HOR)	(SKV, STU, SLI)	(RBI, RBE, RML, RMH)	(RFE, RPO, RMO)	(RPA)
NRD	Number of resin ducts	13.0 ± 3.1	12.1 ± 2.9	9.0 ± 3.2	10.7 ± 1.4	9.6 ± 2.0
NBRCC	Number of mesophyll layers on the concave (adaxial) side	2.4 ± 0.5	3.0 ± 0.2	2.6 ± 0.4	2.5 ± 0.5	2.9 ± 0.3
NBRCV	Number of mesophyll layers on the convex (abaxial) side	3.0 ± 0.6	3.0 ± 0.1	2.8 ± 0.5	3.1 ± 0.7	3.0 ± 0.0
NBRCW	Number of mesophyll layers between central cylinder and needle edge	5.0 ± 0.8	5.1 ± 0.4	4.7 ± 0.6	5.9 ± 0.9	5.0 ± 0.0
NH	Needle height (μm)	703.7 ± 97.7	620.0 ± 86.6	606.2 ± 95.8	613.1 ± 86.6	585.4 ± 59.4
NW	Needle width (μm)	1379.8 ± 220.1	1206.3 ± 169.8	1149.4 ± 193.2	1238.1 ± 184.2	1158.2 ± 110.3
CCH	Central cylinder height (μm)	313.5 ± 48.2	269.7 ± 34.8	282.2 ± 43.6	294.9 ± 37.1	274.6 ± 26.8
CCW	Central cylinder width (μm)	911.1 ± 166.2	724.4 ± 124.1	688.9 ± 143.3	764.1 ± 141.1	676.1 ± 95.2
CCW/CCH	Central cylinder width/central cylinder height	2.9 ± 0.4	2.7 ± 0.3	2.5 ± 0.5	2.6 ± 0.5	2.5 ± 0.4
NW/NH	Needle width/needle height	2.0 ± 0.2	2.0 ± 0.2	1.9 ± 0.3	2.0 ± 0.2	2.0 ± 0.1
CCH/NH	Central cylinder height/needle height	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
CCW/NW	Central cylinder width/needle width	0.7 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.6 ± 0.0

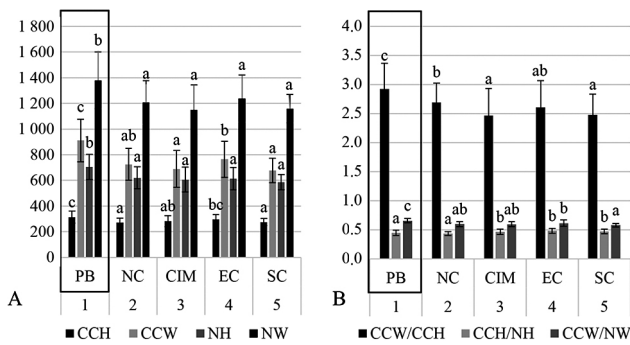


Fig. 4 A–B. Statistically significant anatomical variables of Scots pine among the geographical regions under study, detected by Games-Howell's post hoc test ( $p < 0.05$ ). PB: Pannonian Basin, NC: Northern Carpathians, CIM: Central-Island Mountains (Apuseni), EC: Eastern Carpathians and SC: Southern Carpathians. Unit of the measurement is  $\mu\text{m}$  (CCH, CCW, NH, NW), except CCW/CCH, CCH/NH and CCW/NW

Furthermore, by considering Function 3 (17%) we could discriminate the Pannonian Basin (1) from the Eastern Carpathians (4) on the basis of AL and AT variables.

We calculated the average values of the analyzed needle characteristics with standard deviations by geographical distribution (Table 3). Performing the MANOVA test with anatomical traits, we found significant differences based on four variables: CCH, CCW, NH, NW and three ratios: CCW/CCH, CCH/NH and CCW/NW ( $F(16;758)=10.35$ ;  $p < 0.001$ ;  $F(126;659)=12.62$ ;  $p < 0.001$ ;) with significant between-subjects effects ( $F(4;251) > 14.90$ ;  $p < 0.001$ ;  $F(4;251) > 9.94$ ;  $p < 0.001$ ). Populations from the Pannonian Basin were significantly separated from the Northern Carpathians by four variables: CCH, CCW, NH, and NW and three ratios: CCW/CCH, CCH/NH, and CCW/NW (Fig. 4A,B). By carrying out discriminant function analysis by the first variable (Function 1), which was responsible for 41.3% of the variation, we showed that the populations form two distinctive groups (Fig. 3B): the Pannonian Basin (1), the Central Island Mts. (3), the Eastern Carpathian (4) vs. Northern (4) and Southern Carpathians (5). NBRCC and CCH were discriminating variables. The second function (Function 2), which was responsible for 32.2% of the total variation, made evident the separation of the Pannonian Basin (1) from the Central Island Mts. (3) and Eastern Carpathians (4). Discriminating variables were: NRD, CCW, NW, and NH. Additionally, Function 3 (24%) indicated a difference between the populations in the Central Island Mts. (3) and the populations in the Eastern Carpathians (4) according to NBRCW and NBRCV.

The Mantel correlation test was not significant ( $R^2=0.017$ ,  $p < 0.05$ ) (Fig. 5).

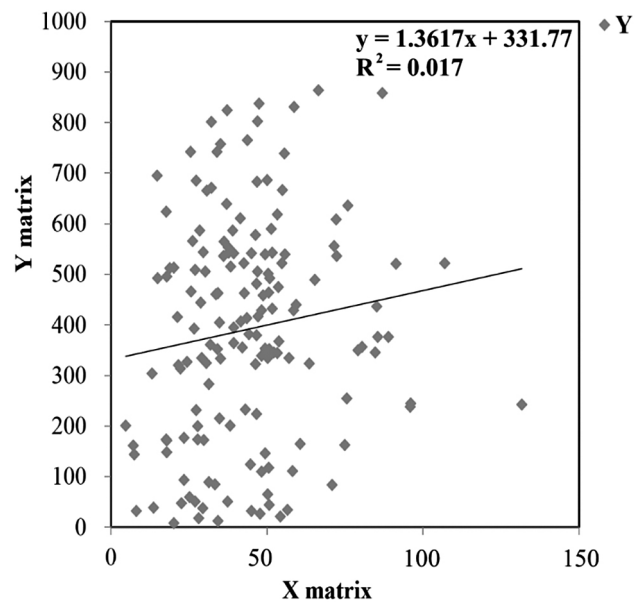


Fig. 5. The relationship between pairwise Euclidean distance and geographic distances ( $r_{xy}=0.130$ ,  $p=0.130$ ,  $R^2=0.017$ ) for the 16 *Pinus sylvestris* populations

## Differentiation by habitat type

First, we calculated the average values of the analyzed needle and cone characteristics with standard deviations by habitat type (Tables 4, 5). We performed a statistical analysis with three previously formed groups according to habitat type. By carrying out a one-way MANOVA test using the morphological cone dataset (AL, AT, AW, CL/CW, NBS), we detected significant differences ( $F(10;498)=13.69$ ;  $p < 0.01$ ) by revealing significant between-subjects effects ( $F(2;253) > 3.46$ ;  $p < 0.05$ ). Populations from peat bogs differed significantly from those of rocky substrates and mixed forests in the case of two variables: the length and thickness of apophysis (AL, AT). Samples originating from dry rocky substrates were significantly separated according to three variables: AW, NBS, and CL/CW (Fig. 6a). By performing a one-way MANOVA test with ratios (CCW/CCH, CCH/NH, CCW/NW) and with NRD, we detected significant differences again ( $F(8;500)=12.64$ ;  $p < 0.001$ ) with significant between-subjects effects ( $F(2;253) > 8.00$ ;  $p < 0.001$ ). We found significantly less resin ducts (NRD) among populations from peat bogs and on rocky substrates. In the case of three proportions (CCW/CCH, CCH/NH and CCW/NW), populations with mixed forest origin differed significantly by high values at CCW/NW and CCW/CCH and lower values at CCH/NH ratios (Fig. 6B). Therefore, these anatomical traits seem to be very useful in describing differentiation among habitats. Discrimination analysis based on the measured morphological datasets, with the first variable (Function 1) responsible for 69.6% of the variation and the second

Table 4. Average values with standard deviations of the analyzed needle characteristics by habitat type (peat bogs, rocky substrates, mixed forests)

Code	Characteristics	Peat bogs	Rocky substrate	Mixed forests
		(RMO, RFE, RPO, RML, RMH)	(HKO, RBE, RBI, RPA, SLI, SKV, STU)	(HFE, HZA, HVE, HOR)
NRD	Number of resin ducts	10.4 ± 2.2	10.3 ± 3.2	13.6 ± 3.1
NBRCC	Number of mesophyll layers on the concave (adaxial) side	2.4 ± 0.5	2.7 ± 0.5	2.3 ± 0.5
NBRCV	Number of mesophyll layers on the convex (abaxial) side	3.0 ± 0.6	2.9 ± 0.3	2.9 ± 0.7
NBRCW	Number of mesophyll layers between central cylinder and needle edge	5.5 ± 0.9	5.0 ± 0.6	4.8 ± 0.8
NH	Needle height (μm)	630.1 ± 93.0	592.4 ± 75.8	733.0 ± 85.5
NW	Needle width (μm)	1220.3 ± 196.6	1154.4 ± 155.6	1451.4 ± 183.0
CCH	Central cylinder height (μm)	293.8 ± 39.5	272.4 ± 34.5	322.2 ± 49.1
CCW	Central cylinder width (μm)	745.9 ± 151.6	698.3 ± 113.1	958.7 ± 148.5
CCW/CCH	Central cylinder width/central cylinder height	2.5 ± 0.4	2.6 ± 0.4	3.0 ± 0.4
NW/NH	Needle width/needle height	1.9 ± 0.2	2.0 ± 0.2	2.2 ± 0.1
CCH/NH	Central cylinder height/needle height	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
CCW/NW	Central cylinder width/needle width	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.0

Table 5. Average values with standard deviations of the analyzed cone characteristics by habitat type (peat bogs, rocky substrates, mixed forests)

Code	Characteristics	Peat bogs	Rocky substrates	Mixed forests
		(RMO, RFE, RPO, RML, RMH)	(HKO, RBE, RBI, RPA, SLI, SKV, STU)	(HFE, HZA, HVE, HOR)
CW	Cone width (cm)	4.3 ± 0.9	4.3 ± 0.7	4.1 ± 0.7
CL	Cone length (cm)	4.1 ± 1.0	3.7 ± 0.7	4.0 ± 0.8
NBS	Number of scales	74.9 ± 13.8	66.7 ± 13.0	72.4 ± 11.4
AW	Width of apophysis (mm)	6.9 ± 1.1	6.3 ± 0.9	6.9 ± 1.4
AL	Length of apophysis (mm)	6.6 ± 1.3	6.9 ± 0.9	7.0 ± 1.0
AT	Thickness of apophysis (mm)	3.5 ± 1.0	2.7 ± 0.6	2.5 ± 0.8
CL/CW	Cone length/cone width	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.1
CL/NBS	Cone length/number of scales	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
AL/AW	Apophysis length/apophysis width	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2
AL/AT	Apophysis length/apophysis thickness	2.0 ± 0.7	2.7 ± 0.8	3.1 ± 1.0

variable (Function 2) responsible for 30.4% of the variation, revealed a slight pattern of populations by the separation into two groups: mixed forests (3) with rocky substrates (2) vs. peat bogs (1). The highest level of differences between the populations was defined by the following variables; for function 1: AT, AL/AT, AL/AW, NBS, AW, and for function 2, which separated mixed forests (3) from rocky substrates (2): CL/CW (Fig. 7A).

Discriminant function analysis with anatomical needle variables after a previous sorting of populations by habitat type showed that the first variable (Function 1) was responsible for 59.2% of the variation and the second variable (Function 2) was responsible for 40.8% of the variation. Three groups were identified: mixed forests (3), rocky substrates (2), and peat bogs (Fig. 7B). Differences among populations were defined by three variables represented by Function 1: central cylinder width (CCW), number

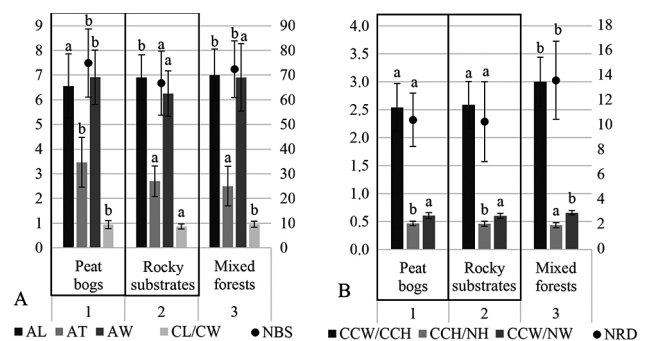


Fig. 6 A–B. Statistically significant morphometric and anatomical variables of Scots pine individuals within the habitat types under study (peat bogs, rocky substrates and mixed forests), detected by Games-Howell's post hoc test ( $p < 0.05$ ). See abbreviation from Table 4,5. Units of the measurement are mm (AL, AT, AW) and piece (NBS, NRD), except CL/CW, CCW/CCH, CCH/NH and CCW/NW

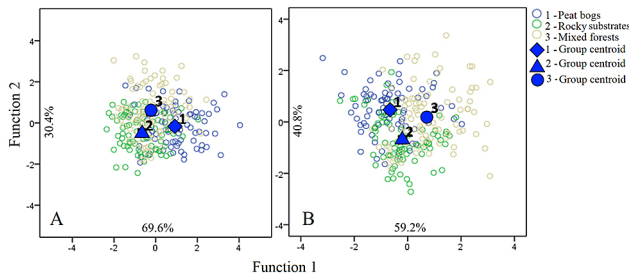


Fig. 7 A–B. Differentiation of *Pinus sylvestris* L. populations by habitat type detected on the basis of morphological cone characteristics (A) and needle anatomic variables (B), found using by Games-Howell's post hoc test ( $p < 0.05$ ): 1 – peat bogs (RMO, RFE, RPO, RML, RMH), 2 – rocky substrates (HKO, RBE, RBI, RPA, SLI, SKV, STU), 3 – mixed forests (HFE, HZA, HVE, HOR)

of resin ducts (NRD), and needle width (NW). Populations from mixed forests were discriminated by Function 2 with five variables: central cylinder height (CCH), needle height (NH), number of parenchyma layers between central cylinder and needle edge, and on the concave and convex sides (NBRCW, NBRCV, NBRCC) (Fig. 7B).

## Discussion

Peripheral populations of Scots pine involved in our study are considered to be of natural origin and have persisted in refugial territories with specific local ecological conditions. Plants are systems integrated in their habitats, therefore the study of traits that influence their survival and reproduction in these habitats can reveal diversification in contrasting environments (Reich et al., 2003). However, populations or genotypes can be preadapted to a given selection factor or environmental condition. When they colonize new habitats or geographic areas, their survival can depend on their functional traits, which are or are not suited to the environment (Reich et al., 2003). Thus, current species distributions may reflect ecological pre-sorting processes, in addition to *in situ* adaptive evolution (Losos, 1996), indicating their origin and their historical colonization routes.

The level of differences in peripheral populations of Scots pine found in our study can be treated as congruent with the earlier described variation of the species. Differentiation of populations from the Iberian Peninsula was described using needle (Pardos et al., 1990; Boratynska & Hincă, 2003; Jasińska et al., 2010; Jasińska et al., 2014) and cone characteristics (Staszkiwicz, 1993; Marcysiak, 2006). Urbaniak et al. (2003) also found differentiation among populations, detected on the basis of morphological character expression, influenced by both the edaphic conditions and the distinct genetic structure. Morphological and anatomical differences among

populations are also listed as distinguishing characteristics among populations in the work by Bobowicz (1984) and Bobowicz & Korczyk (2000). Oleksyn et al. (1998) suggested that new models in predicting species' adaptation to the changing environmental conditions needs study on intraspecific differentiation level. Cone characteristics are traits of the highest discriminating power in inter-population comparisons among regions in the works by Bobowicz and Korczyk (2000) and Jasińska et al. (2014). In work of Androsiuk et al. (2011), the principal variables which proved to be indicative to discriminate populations were the needle characteristics. Several studies which describe differentiation among populations of other species from the genus *Pinus* can also be found in papers by Marcysiak (2004), Bączkiewicz et al. (2005) and Sobierajska et al. (2010).

## Geographical differentiation

Our analyses showed in the first instance a grouping of populations by their geographical position. Populations from the Northern Carpathians and the Pannonian Basin formed a well-distinguished group on the basis of five cone (Fig. 1) and two needle characteristics (Fig. 3). These findings are generally congruent with previous molecular studies (Naydenov et al., 2005; Cheddadi et al., 2006; Bernhardsson et al., 2016) and macrofossil and pollen data analyses (Stieber, 1967; Rudner et al., 1995; Haesaerts et al., 1996; Damblon, 1997; Richardson & Rundel, 1998; Rudner & Sümegei, 2001; Willis & Van Andel, 2004; Jankovská & Pokorný, 2008). According to them, one main recolonization route in Europe presumably originated from around the Eastern Alps and the surroundings of the Danube plain, and there is evidence of refugial locations in the Eastern Alps and East-Central Europe, e.g. the Hungarian plain (Naydenov et al., 2005; Cheddadi et al., 2006; Bernhardsson et al., 2016). The data in the literature are also consistent with our findings based on needle anatomical characteristics, according to which populations from the Pannonian Basin are differentiated significantly according to six anatomical variables. On the other hand, on the basis of four morphometric variables in the MANOVA test and discriminant function analysis, the Eastern Carpathian region proved to be distant from the rest of the populations. The study by Magyari et al. (2014), based on pollen analysis, predicted *Pinus* persistence in the Eastern Carpathians between 22,870 and 19,150 cal yr BP. The application of the Mantel test to correlate the morpho-anatomical and geographic distances and seek for spatial patterns, yielded no significant correlation. According to this finding, we assume that Scots pine from the Carpathians represents only a small geographic range of the species' large distribution. Fossil evidences,



paleoclimatic modeling, and genetic research suggest that conifers and some broadleaf trees were continuously present throughout LGM in refugial territories around the Pannonian Basin (Willis & Van Andel, 2004; Ronikier, 2011; Mitka et al., 2014).

## Differentiation by habitat type

Under extreme conditions, peripheral populations with specific structures are exposed to dramatic environmental changes which will impose novel selection pressures and may therefore cause adaptive responses (Bone & Farres, 2001). Morphological traits of conifer species are known to vary adaptively with geographic, climatic, and edaphic variables (Ji et al., 2011). In several works in the existing literature, significant differences between isolated populations were observed, mostly with regard to their morphological features (Szweykowski & Urbaniak, 1982). On the other hand, abiotic factors, such as temperature, light, soil type, available nutrients, and other derived factors (Pawlaczyk et al., 2010) lead to the development of local phenotypes, i.e. populations with distinguishing characteristics. These populations are present in a given area within the range of the species (Remlein et al., 2015). The samples of Scots pine, which we examined, were mainly from specific habitat types, such as raised bogs, dry rocky substrates and sandy substrates, and they were from different altitudinal gradients which varied from 252 m (HFE) to 1107 m (STU).

Discriminant function analysis based on the eight cone characteristics revealed clearly discernible groups of populations. Nevertheless, cone size and weight can be influenced by tree age, general health of the trees, and the macro- and micro- habitat of the parent trees (Dangasuk & Panetsos, 2004). The high levels of variation observed in cone morphology might be explained by the long-term adaptation of populations to diverse and changing environmental conditions, and they also can be due to the lack of competition with other pines (Gil et al., 2002). However, the results of a one-way MANOVA test with cone datasets revealed significant differentiation among populations growing in peat bogs and on rocky substrates in the case of the following traits: length, width, and thickness of apophysis (AL, AW, AT), number of scales (NBS), and the cone length/cone width (CL/CW) ratio. If we take into consideration the fact that cones from several geographically different peat bog locations were not significantly larger than those from other habitat types, it can be concluded that in peat bogs cone structure, not cone size represents a difference that can be evaluated as a sign of local adaptation. Lack of geographic correspondence among populations with similar phenotypes was also observed in *P. canariensis* (Gil et al.,

2002), *P. radiata* (Forde, 1964), and *P. tecunumanii* (Eguiluz Piedra, 1984). Beaulieu and Simon (1995) showed that no general geographical grouping could be detected in the observed variation in *P. strobus* collected from ten natural populations in the Canadian province of Quebec. Furthermore, cones from individuals on dry rocky substrates were separated as significantly smaller and, on the basis of apophysis width (AW), number of scales (NBS), and cone length/width ratio (CL/CW), well-proportioned but less dense in structure.

Significant differences in needle anatomy were found among populations with different habitat types. A one-way MANOVA test applied to needle anatomical data shows significantly less resin ducts (NRD) among populations from peat bogs and rocky substrates or significantly more NRD among populations with mixed forest provenience. Though not well understood, pine resin may play a role in water regulation (Farrell et al., 1991; Bell, 2010). All our samples from mixed forests were obtained from areas in the Pannonian Basin with the lowest altitudinal gradient. Accordingly, this could be regarded as a sign of altitudinal adaptation, or it might be due to common geographical origin. However, similar findings were described in *Pinus brutia* by Dangasuk and Panetsos (2004), who reported the number of resin canals as a useful trait for identifying altitudinal and longitudinal adaptation variations within and among populations.

The calculated CCH/NH and CCW/NW proportions actually estimate the dimensional relationship of central cylinder with the photosynthetically active mesophyll. Lower values indicate a thicker mesophyll and, consequently, increased photosynthetic activity. We have found significantly higher values of needle width (NW) and needle height (NH) and significantly lower values of CCH/NH proportion among populations from mixed forests. Considering that our mixed forest samples were collected from areas in the Pannonian Basin with the lowest altitudinal gradient, our NW data is in agreement with the findings of Wahid et al. (2006), who found that needle width correlated negatively with altitude in maritime pine (*P. pinaster*). The higher NH value and lower CCH/NH proportion indicate a larger leaf area, thicker mesophyll and, therefore, increased photosynthetic activity. Plants growing under strong light have well developed palisade parenchyma, thicker leaves, relatively larger leaf area, higher biomass, increased photosynthesis, and lower contents of chlorophyll, carotenoid, and nitrogen (Je et al., 2006; Yang et al., 2007; Huang et al., 2008; Volkova et al., 2010). Structural characteristics of conifer needles are often strongly related to gradients in long-term light availability within canopies and across stands (Richardson et al., 2000; Richardson et al., 2001; Niinemets et al., 2002;

Lhotáková et al., 2007; Niinemets et al., 2007). The lower NBRCC and NBRWC values and higher CCH/NH proportion in peat bog populations and populations growing on rocky substrates can be explained as a response to increasing environmental stress, which is accompanied by a decline in photosynthetic and growth rate, since higher leaf age compensates for low photosynthesis (Schoettle et al., 1994; Reich et al., 1995). According to some authors, Scots pine needles from the sites with limited phosphorus availability (such as peat bogs or rocky substrates) are narrower and thinner than needles from the nutrient-rich site (Niinemets et al., 2001). Our findings are congruent with those data, since the width and height of needles in populations from peat bogs and on rocky substrates were significantly lower. Scots pine, however, typically occurs on different types of well-drained mineral soils, representing a broad range of variation in pH, nutrient availability, and vegetation (Persson, 1980). Consequently, not only the geographical range, but also the ecological tolerance of the species is very wide. Variation in leaf traits is an important characteristic of ecological processes that are driving forces for biogeochemical cycles in ecosystems (Reich et al., 1992). Studies of needle traits allow us to gain important insights into these processes and predict ecosystem responses to changes in the environment.

## Conclusions

A common group membership of the Northern and Southern Carpathian populations, obtained as a result of discriminant analysis (based on six needle anatomical characteristics) and concurring with previous molecular data, can also be evidence of a common geographical origin. The phenotypic differentiation by habitat type based on five cone and eight needle characteristics might be evaluated as a sign of local adaptation with detectable phenotypic patterns. Finally, one can conclude that both geographical and habitat type differentiation support the hypothesis that the structure of the Carpathian peripheral Scots pine populations will be shaped by a combination of their colonization history and the influence of *in situ* ecological factors.

## Acknowledgments

The authors are grateful to Tamás Pócs (Hungary) and Tibor Baranec (Slovakia) for advice and for help during sample collection. Furthermore, we would like to express our thanks to Anett Zahorecz and Krisztina Tóth for their support with the morphological and anatomical measurements. This work was supported by the National Research, Development

and Innovation Office, Hungary with a grant provided by the Hungarian Scientific Research Fund [OTKA K101600].

## References

- Alía R, Moro-Serrano J & Notivol E (2001) Genetic variability of Scots pine (*Pinus sylvestris*) provenances in Spain: growth traits and survival. *Silva Fennica* 35: 27–38.
- Androsiuk P, Kaczmarek Z & Urbaniak L (2011) The morphological traits of needles as markers of geographical differentiation in European *Pinus sylvestris* populations. *Dendrobiology* 65: 3–16.
- Bączkiewicz A, Buczkowska K & Wachowiak W (2005) Anatomical and morphological variability of needles of *Pinus mugo* Turra on different substrata in the Tatra Mountains. *Biological Letters* 42: 21–32.
- Beaulieu J & Simon J-P (1995) Variation in cone morphology and seed characters in *Pinus strobus* in Quebec. *Canadian Journal of Botany* 73: 262–271.
- Bell G (2010) Fluctuating selection: the perpetual renewal of adaptation in variable environments. *Philosophical Transactions of the Royal Society of London B: Biological Sciences* 365: 87–97.
- Bennett K & Provan J (2008) What do we mean by ‘refugia’? *Quaternary Science Reviews* 27–28: 2449–2455.
- Bernhardsson C, Floran V, Ganea S & García-Gil M (2016) Present genetic structure is congruent with the common origin of distant Scots pine populations in its Romanian distribution. *Forest Ecology and Management* 361: 131–143.
- Bilgen B & Kaya N (2007) Allozyme variations in six natural populations of Scots pine (*Pinus sylvestris*) in Turkey. *Biologia* 62: 697–703.
- Bobowicz M (1984) Variability of needles in Polish populations in Scotch pine (*Pinus sylvestris*). *Bulletin de la Société des Amis des Sciences et des Lettres de Poznań, D* 24: 97–104.
- Bobowicz M & Korczyk A (2000) The variability of the oldest trees of Scots pine (*Pinus sylvestris* L.) from the Białowieża Primeval Forest. II. Variability of needle morphology traits in old-growth Scots pine trees from the Białowieża Primeval Forest as compared to the variability in eight Polish populations. *Biological Bulletin of Poznań* 37: 5–15.
- Bone E & Farres A (2001) Trends and rates of microevolution in plants: Microevolution rate, pattern, process. Springer, pp. 165–182.
- Boratynska K & Hincă M (2003) Morphological characteristic of *Pinus sylvestris* L. in the southernmost, isolated locality in the Sierra de Baza (S Spain) as expressed in the needle characters. *Dendrobiology* 50: 3–9.

- Boratyński A (1991) Range of natural distribution: Genetics of Scots pine (ed. by M Giertych & C Mátyás) Akadémiai Kiadó, Budapest, pp. 19–30.
- Cheddadi R, Vendramin GG, Litt T, François L, Kagayama M, Lorentz S, Laurent JM, De Beaulieu JL, Sadori L & Jost A (2006) Imprints of glacial refugia in the modern genetic diversity of *Pinus sylvestris*. *Global Ecology and Biogeography* 15: 271–282.
- Critchfield WB & Little EL (1966) Geographic distribution of the pines of the world. US Department of Agriculture, Forest Service, pp. 11–32.
- Damblon F (1997) Palaeobotanical study of representative upper palaeolithic sites in the central European plain: a contribution to the SC-004 Project. *Préhistoire européenne* 11: 245–254.
- Dangasuk OG & Panetsos KP (2004) Altitudinal and longitudinal variations in *Pinus brutia* (Ten.) of Crete Island, Greece: some needle, cone and seed traits under natural habitats. *New Forests* 27: 269–284.
- Davis MB & Shaw RG (2001) Range shifts and adaptive responses to Quaternary climate change. *Science* 292: 673–679.
- Donnelly K, Cavers S, Cottrell JE & Ennos RA (2016) Genetic variation for needle traits in Scots pine (*Pinus sylvestris* L.). *Tree Genetics & Genomes* 12: 40.
- Dzialuk A, Muchewicz E, Boratyński A, Montserrat JM, Boratyńska K & Burczyk J (2009) Genetic variation of *Pinus uncinata* (Pinaceae) in the Pyrenees determined with cpSSR markers. *Plant Systematics and Evolution* 277: 197–205.
- Eguiluz Piedra T (1984) Geographic variation in needles, cones and seeds of *Pinus tecunumanii* in Guatemala. *Silvae Genetica* 33: 72–79.
- Farrell BD, Dussourd DE & Mitter C (1991) Escalation of plant defense: do latex and resin canals spur plant diversification? *American Naturalist*: 881–900.
- Forde MB (1964) Variation in natural populations of *Pinus radiata* in California: Part 3. Cone characters. *New Zealand Journal of Botany* 2: 459–485.
- Friend AD & Woodward FI (1990) Evolutionary and ecophysiological responses of mountain plants to the growing season environment. *Advances in Ecological Research* 20: 59–124.
- Gil L, Climent J, Nanos N, Mutke S, Ortiz I & Schiller G (2002) Cone morphology variation in *Pinus canariensis* Sm. *Plant Systematics and Evolution* 235: 35–51.
- González-Martínez SC, Krutovsky KV & Neale DB (2006) Forest-tree population genomics and adaptive evolution. *New Phytologist* 170: 227–238.
- Gregorius H-R (1989) The importance of genetic multiplicity for tolerance of atmospheric pollution: Genetic effects of air pollutants in forest tree populations. Springer Berlin Heidelberg, pp. 163–172.
- Haesaerts P, Damblon F, Bachner M & Trnka G (1996) Revised stratigraphy and chronology of the Willendorf II sequence, Lower Austria. *Archaeologia Austriaca* 80: 25–42.
- Huang X, Yin C, Duan B & Li C (2008) Interactions between drought and shade on growth and physiological traits in two *Populus cathayana* populations. *Canadian Journal of Forest Research* 38: 1877–1887.
- Jankovská V & Pokorný P (2008) Forest vegetation of the last full-glacial period in the Western Carpathians (Slovakia and Czech Republic). *Preslia* 80: 307–324.
- Jasińska AK, Boratyńska K, Dering M, Sobierajska KI, Ok T, Romo A & Boratyński A (2014) Distance between south-European and south-west Asiatic refugial areas involved morphological differentiation: *Pinus sylvestris* case study. *Plant Systematics and Evolution* 300: 1487–1502.
- Jasińska AK, Wachowiak W, Muchewicz E, Boratyńska K, Montserrat JM & Boratyński A (2010) Cryptic hybrids between *Pinus uncinata* and *P. sylvestris*. *Botanical Journal of the Linnean Society* 163: 473–485.
- Je S-M, Son S-G, Woo S-Y, Byun K-O & Kim C-S (2006) Photosynthesis and chlorophyll contents of *Chloranthus glaber* under different shading treatments. *Korean Journal of Agricultural and Forest Meteorology* 8: 54–60.
- Ji M, Zhang Q, Deng J, Zhang X & Wang Z (2011) Intra-versus inter-population variation of cone and seed morphological traits of *Pinus tabulaeformis* Carr. in northern China: impact of climate-related conditions. *Polish Journal of Ecology* 59: 717–727.
- Körner C (2007) The use of ‘altitude’ in ecological research. *Trends in Ecology & Evolution* 22: 569–574.
- Labra M, Grassi F, Sgorbati S & Ferrari C (2006) Distribution of genetic variability in southern populations of Scots pine (*Pinus sylvestris* L.) from the Alps to the Apennines. *Flora-Morphology, Distribution, Functional Ecology of Plants* 201: 468–476.
- Lenormand T (2002) Gene flow and the limits to natural selection. *Trends in Ecology & Evolution* 17: 183–189.
- Lhotáková Z, Albrechtová J, Malenovský Z, Rock BN, Polák T & Cudlín P (2007) Does the azimuth orientation of Norway spruce (*Picea abies* L./Karst.) branches within sunlit crown part influence the heterogeneity of biochemical, structural and spectral characteristics of needles? *Environmental and Experimental Botany* 59: 283–292.
- Losos JB (1996) Phylogenetic perspectives on community ecology. *Ecology* 77: 1344–1354.



- Magyari EK, Veres D, Wennrich V, Wagner B, Braun M, Jakab G, Karátson D, Pál Z, Ferenczy G, St-Onge G, Rethemeyer J, Francois JP, von Reumont F & Schäbitz F (2014) Vegetation and environmental responses to climate forcing during the Last Glacial Maximum and deglaciation in the East Carpathians: attenuated response to maximum cooling and increased biomass burning. *Quaternary Science Reviews* 106: 278–298.
- Mantel N (1967) The detection of disease clustering and a generalized regression approach. *Cancer Research* 27: 209–220.
- Marcysiak K (2004) Interpopulational variability of *Pinus uncinata* Ramond ex DC. in Lam. & DC. (Pinaceae) cone characters. *Dendrobiology* 51: 43–51.
- Marcysiak K (2006) Scots pine (Pinaceae) from the Crimea compared to the species variation in Europe on the basis of cone traits. *Phytologia Balcanica* 12: 203–208.
- Mejnartowicz L (1979) Genetic variation in some isoenzyme loci in Scots pine (*Pinus sylvestris* L.) populations. *Arboretum Kórnickie* 24: 91–104.
- Mitka J, Bąba W & Szczepanek K (2014) Putative forest glacial refugia in the Western and Eastern Carpathians. *Modern Phytomorphology* 5: 85–92.
- Naydenov KD, Tremblay FM, Alexandrov A & Fenton NJ (2005) Structure of *Pinus sylvestris* L. populations in Bulgaria revealed by chloroplast microsatellites and terpenes analysis: provenance tests. *Biochemical Systematics and Ecology* 33: 1226–1245.
- Niinemetts Ü, Ellsworth DS, Lukjanova A & Tobias M (2001) Site fertility and the morphological and photosynthetic acclimation of *Pinus sylvestris* needles to light. *Tree Physiology* 21: 1231–1244.
- Niinemetts Ü, Ellsworth DS, Lukjanova A & Tobias M (2002) Dependence of needle architecture and chemical composition on canopy light availability in three North American *Pinus* species with contrasting needle length. *Tree Physiology* 22: 747–761.
- Niinemetts Ü, Lukjanova A, Turnbull MH & Sparrow AD (2007) Plasticity in mesophyll volume fraction modulates light-acclimation in needle photosynthesis in two pines. *Tree Physiology* 27: 1137–1151.
- Noss RF (2001) Beyond Kyoto: forest management in a time of rapid climate change. *Conservation Biology* 15: 578–590.
- Oleksyn J, Tjoelker MG & Reich PB (1998) Adaptation to changing environment in Scots pine populations across a latitudinal gradient. *Filva Fennica* 32: 129–140.
- Ovington J (1957) Dry-matter production by *Pinus sylvestris* L. *Annals of Botany* 21: 287–314.
- Pardos JA, Lange W & Weißmann G (1990) Morphological and chemical aspects of *Pinus sylvestris* L. from Spain. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood* 44: 143–146.
- Pawlaczyk EM, Bobowicz MA & Korczyk AF (2010) Zmienność trzech naturalnych populacji *Pinus sylvestris* L. z różnych siedlisk Puszczy Białowieskiej oszacowana cechami igieł. *Leśne Prace Badawcze* 71: 83–92.
- Peakall R & Smouse PE (2006) GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.
- Persson H (1980) Fine-root dynamics in a Scots pine stand with and without near-optimum nutrient and water regimes. *Acta Phytogeographica Suecica* 68: 101–110.
- Petit RJ, Aguinagalde I, de Beaulieu J-L, Bittkau C, Brewer S, Cheddadi R, Ennos R, Fineschi S, Grivet D, Lascoux M, Mohanty A, Müller-Starck G, Demesure-Musch B, Palme A, Martin JP, Rendell S & Vendramin GG (2003) Glacial refugia: hotspots but not melting pots of genetic diversity. *Science* 300: 1563–1565.
- Prus-Głowacki W & Stephan BR (1994) Genetic variation of *Pinus sylvestris* from Spain in relation to other European populations. *Silvae Genetica* 43: 7–14.
- Prus-Głowacki W, Stephan BR, Bujas E, Alia R & Marciniak A (2003) Genetic differentiation of autochthonous populations of *Pinus sylvestris* (Pinaceae) from the Iberian peninsula. *Plant Systematics and Evolution* 239: 55–66.
- Prus-Głowacki W, Urbaniak L, Bujas E & Curtu AL (2012) Genetic variation of isolated and peripheral populations of *Pinus sylvestris* (L.) from glacial refugia. *Flora-Morphology, Distribution, Functional Ecology of Plants* 207: 150–158.
- Pyhäjärvi T, García-Gil MR, Knürr T, Mikkonen M, Wachowiak W & Savolainen O (2007) Demographic history has influenced nucleotide diversity in European *Pinus sylvestris* populations. *Genetics* 177: 1713–1724.
- Reich PB, Walters MB & Ellsworth DS (1992) Leaf life-span in relation to leaf, plant, and stand characteristics among diverse ecosystems. *Ecological Monographs* 62: 365–392.
- Reich PB, Wright IJ, Cavender-Bares J, Craine JM, Oleksyn J, Westoby M & Walters MB (2003) The evolution of plant functional variation: traits, spectra, and strategies. *International Journal of Plant Sciences* 164: S143–S164.
- Reich PB, Koike T, Gower ST & Schoettle AW (1995) Causes and consequences of variation in conifer leaf life-span: *Ecophysiology of Conifers* (ed. by



- WK Smith & TM Hinckley) Academic Press, San Diego, CA, pp. 225–254.
- Remlein A, Jelonek T, Tomczak A, Jakubowski M & Grzywinski W (2015) Morphological architecture of different ecotypes of Scots pine (*Pinus sylvestris* L.) in Poland. *Annals of Warsaw University of Life Sciences-SGGW. Forestry and Wood Technology* 92.
- Richardson AD, Berlyn GP, Ashton PMS, Thadani R & Cameron IR (2000) Foliar plasticity of hybrid spruce in relation to crown position and stand age. *Canadian Journal of Botany* 78: 305–317.
- Richardson AD, Berlyn GP & Gregoire TG (2001) Spectral reflectance of *Picea rubens* (Pinaceae) and *Abies balsamea* (Pinaceae) needles along an elevational gradient, Mt. Moosilauke, New Hampshire, USA. *American Journal of Botany* 88: 667–676.
- Richardson DM & Rundel PW (1998) Ecology and biogeography of Pinus: an introduction: Ecology and biogeography of Pinus (ed. by DM Richardson) Cambridge University Press, pp. 3–46.
- Ronikier M (2011) Biogeography of high-mountain plants in the Carpathians: An emerging phylogeographical perspective. *Taxon* 60: 373–389.
- Rudner E, Sümegei P, Tóth I, Beszedá I & Hertelendi E (1995) The vegetation of the upper-Weichselian in the central and southern part of the Great Hungarian Plain. 7th European Ecological Congress 1: 108.
- Rudner ZE & Sümegei P (2001) Recurring Taiga forest-steppe habitats in the Carpathian Basin in the Upper Weichselian. *Quaternary International* 76: 177–189.
- Rull V (2009) Microrefugia. *Journal of Biogeography* 36: 481–484.
- Schoettle AW, Fahey TJ & Shoettle AW (1994) Foliage and fine root longevity of pines. *Ecological Bulletins* 43: 136–153.
- Semiz G, Heijari J, Isik K & Holopainen JK (2007) Variation in needle terpenoids among *Pinus sylvestris* L. (Pinaceae) provenances from Turkey. *Biochemical Systematics and Ecology* 35: 652–661.
- Sobierajska K, Boratyńska K & Marcysiak K (2010) Variation of cone characters in *Pinus mugo* (Pinaceae) populations in the Giant Mountains (Karkonosze, Sudetes). *Dendrobiology* 63: 33–41.
- Staszkiewicz J (1961) Biometric studies on the Cones of *Pinus Sylvestris* L., growing in Hungary. *Magyar Tudományok Akadémia*.
- Staszkiewicz J (1993) Zmienność morfologiczna szpilek, szyszek i nasion. *Biologia sosny zwyczajnej*. PAN Instytut Dendrologii, Poznań-Kórnik.
- Stieber J (1967) A magyarországi felsőpleisztocén vegetációtörténete az. anthrakotómiai eredmények (1957-IG) Tükrében. *Földtani Közlemények* 97: 308–317.
- Szweykowski J & Urbaniak L (1982) An interesting chemical polymorphism in *Pinus sylvestris* L. *Acta Societatis Botanicorum Poloniae* 51: 441–452.
- Tabachnick BG & Fidell LS (2013) Using multivariate statistics, 6th ed. Allyn and Bacon, Boston.
- Tiwari SP, Kumar P, Yadav D & Chauhan DK (2013) Comparative morphological, epidermal, and anatomical studies of *Pinus roxburghii* needles at different altitudes in the North-West Indian Himalayas. *Turkish Journal of Botany* 37: 65–73.
- Tobolski JJ & Hanover JW (1971) Genetic variation in the monoterpenes of Scotch pine. *Forest Science* 17: 293–299.
- Turna I (2003) Variation of some morphological and electrophoretic characters of 11 populations of Scots pine in Turkey. *Israel Journal of Plant Sciences* 51: 223–230.
- Turna I & Güney D (2009) Altitudinal variation of some morphological characters of Scots pine (*Pinus sylvestris* L.) in Turkey. *African Journal of Biotechnology* 8: 202–208.
- Urbaniak L (1998) Morphometric differentiation of *Corex ligericá* Gay in Poland. *Acta Societatis Botanicorum Poloniae* 67: 263–268.
- Urbaniak L, Karlinski L & Popielarz R (2003) Variation of morphological needle characters of Scots pine [*Pinus sylvestris* L.] populations in different habitats. *Acta Societatis Botanicorum Poloniae* 72: 37–44.
- Volkova L, Bennett LT, Merchant A & Tausz M (2010) Shade does not ameliorate drought effects on the tree fern species *Dicksonia antarctica* and *Cyathea australis*. *Trees* 24: 351–362.
- Wahid N, González-Martínez SC, El Hadrami I & Boulli A (2006) Variation of morphological traits in natural populations of maritime pine (*Pinus pinaster* Ait.) in Morocco. *Annals of Forest Science* 63: 83–92.
- Willis KJ & Van Andel TH (2004) Trees or no trees? The environments of central and eastern Europe during the Last Glaciation. *Quaternary Science Reviews* 23: 2369–2387.
- Yang Y, Liu Q, Han C, Qiao Y, Yao X & Yin H (2007) Influence of water stress and low irradiance on morphological and physiological characteristics of *Picea asperata* seedlings. *Photosynthetica* 45: 613–619.