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SPATIAL VARIATION OF MORPHOLOGICAL NEEDLE TRAITS OF SILVER FIR (*ABIES ALBA* MILL.) POPULATIONS IN THE BALKAN PENINSULA IN RELATION TO CLIMATIC FACTORS

PROSTORNA VARIJABILNOST MORFOLOŠKIH OBILJEŽJA IGLICA POPULACIJA JELE (*Abies alba* Mill.) NA BALKANSKOM POLUOTOKU U ODNOSU NA KLIMATSKE ČIMBENIKE

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SUMMARY

Interpopulation and intrapopulation variability of three morphological needle traits (length, width and thickness) was investigated in 16 natural silver fir populations in the Balkan Peninsula. The populations represent refugial areas of silver fir (*Abies alba* Mill.). This paper aims to provide a comprehensive analysis of the influence of climatic factors (mean annual temperature, number of days with temperatures < 0, > 5, < 18, > 18°C, Hargreaves climatic moisture deficit and De Martonne aridity index, on the pattern of morphological needle traits within each population. Populations showed variation in the analyzed morphological needle traits, which could not be clearly defined by any of the analyzed climatic factors. The De Martonne aridity index and Hargreaves climatic moisture deficit had the greatest impact on the trait values, whereas the mean annual precipitation had the lowest. Evolutionary ecology research of the silver fir needle morphology is a valuable contribution to the comprehention of the present genetic variability as a prerequisite for adaptation to the rapid climate change and conservation of the species area in the Balkan Peninsula region.

KEY WORDS: needle morphology, climatic factors, silver fir, Balkan Peninsula

INTRODUCTION
UVOD

Natural loss of forests is a consequence of stress caused by fluctuations in environmental factors due to rapid climate change as well asof the species sensitivity to temperature changes, water scarcity, anthropogenic factors (air pollution and human-driven deforestation), their genetic variability and adaptive capacity to adapt to such changes (Post-

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olache *et al.* 2013). The pattern of variability at the interpopulation and intrapopulation level has been modified by changing environmental conditions (Turunen *et al.* 1999; Klančnik*et al.* 2014). Studies confirm that abiotic factors (edaphic and climatic conditions) significantly affect the morphological and anatomical traits of pine needles (Schoettle and Rochelle 2000; Jankowski *et al.* 2017), intrapopulation differentiation of morphological traits of Scots pine needles (*Pinus silvestris*), and geographical differentiation of species (Niinemets *et al.* 2001; Urbaniak *et al.* 2003; Pensa *et al.* 2004).

Degradation of silver fir (*A. alba* Mill.) is the result of lower interpopulation and intrapopulation (genetic) variability, which leads to decrease in the adaptive capacity compared to other forest species (Musil and Hamernik 2007). The abilities of plant species in forest ecosystems toadapt to climate change, and their distribution along a large scale of heterogeneous environmental conditions are determined by the genetic resources of species (Falk and Hempelmann 2013; Naudiyal *et al.* 2021). Climate changes modify the growth of forest ecosystem species, so that decades-old trees present an archival imprint of environmental change (Bradshaw*et al.* 2000; Pandey 2021). Abiotic factors (altitude, air temperature, atmospheric pressure, photoperiod, precipitation, wind speed, mean annual temperatures), as well as nutrients, affect the physiological, anatomical and morphological traits of leaves or needles (Kašpar *et al.* 2017; Miljković *et al.* 2019; De La Torre *et al.* 2021). The morphology and anatomical structure of needles reflect their adaptation to changing environmental conditions (Xing *et al.* 2014). The most of needle traits are stable at the species level, but phenotypic variations in morpho-anatomical needle traits are the result of physiological adaptive evolution (Radu *et al.* 2014; Huang *et al.* 2016; Zhang *et al.* 2017; Wang *et al.* 2020). The characteristics of leaves / needles as the basic photosynthetic apparatus and the phenological phase of plants possess interspecific, interpopulation and intrapopulation differences due to changing environmental conditions (Turunen *et al.* 1999; Klančnik *et al.* 2014), as well as to age classes, and the position of leaves / needles in the canopy (Robakowski *et al.* 2004, Lukeš *et al.* 2013, Olascoaga *et al.* 2014). The morphological silver fir needle traits were used to determine the variability of natural populations in Northern Macedonia (Popnikola 1974), interindividual differentiation of trees in the western Serbia (Ratknić et al. 2013) and Tisovik Reserve (Pawlaczyk et al. 2005), and the influence of light on the seedling development (Robakowski et al. 2004; Dörken and Lepetit 2018).

The silver fir (*A. alba* Mill., Pinaceae) has the tallest tree in the genus *Abies* in Europe. In favorable environmental conditions it can live to the age of 500-600 years. Some mature trees can reach a height of 60-65 m and a diameter at breast height of 150-200 (380) cm. Natural habitats of silver fir are mountainous areas of Eastern, Western, Southern and Central Europe, where it grows mainly with beech (*Fagus silvatica* L.) at lower and middle altitudes and with spruce (*Picea abies* L. Karst) at higher altitudes (Liepelt *et. al.* 2009). The silver fir habitats spread from 52° N in the north (Poland) to 40° N in the south (northern border of Greece) and from 5° E in the west (western Alps) to 27° E in the east (Romania, Bulgaria). It occurs mainly at altitudes of 500 to 800 meters, and when going from north to south, the altitude increases. Due to the large distribution, this species is not yet endangered, although in the last 200 years silver fir forests have been significantly reduced in most European countries (Wolf 2003).

The aim of this research was to determine interpopulation and intrapopulation variability of the silver fir (*A. alba* Mill.) morphological needle traits and the contribution of certain climatic factors to its variability.

MATERIAL AND METHOD
MATERIJALI I METODE

Plant material and needle traits

Plant material for morphometric analysis was collected in 16 natural, geographically distant, silver fir populations in the Balkan Peninsula during August 2019. Populations were located at altitudes ranging from 720 to 1860 meters above sea level. The following geographical and climatic characteristics were used in the analyses: altitude, latitude, longitude, MAT (mean annual temperature (°C)), degree-days: < 0, > 5, < 18, > 18 (days), CMD (Hargreaves climatic moisture deficit (mm)) and I_{DM} (De Martonne aridity index) (Table 1). Hargreaves climatic moisture deficit is the climatic parameter calculated as the sum of the monthly (m) difference between a reference evaporation (Eref) and precipitation (P) (ClimateWNA, Wang et al. 2012). In the case Eref ≤ P (m), then CMD = 0, and if Eref (m) > P (m), then CMD = Eref (m) - P (m). Location aridity was estimated using the de Marton aridity index I_{DM} = MAP / (MAT + 10). Mean annual temperature and precipitation were estimated for the period from 1961 to 2018 according to the ClimateEU v4.63 software package, available at http://tiniurl.com/ClimateEU (Hamann *et al.* 2013).

Each population was represented by 20 trees (approximately 80-100 years old). Branches with needles were sampled from the northeast side of the canopy at a height of about 6 meters. Analyzes were performed on 320 trees. A sample of 10 two-year old needles was randomly taken from the each branch (3200 needles in total).

The needle lenght (NL) was measured with a vernier caliper with an accuracy of 0.01 mm. The needle width (NW) and needle thickness (NT) were measured on microscope slides using a light microscope (Carl Zeiss Jena, Laboval 2),

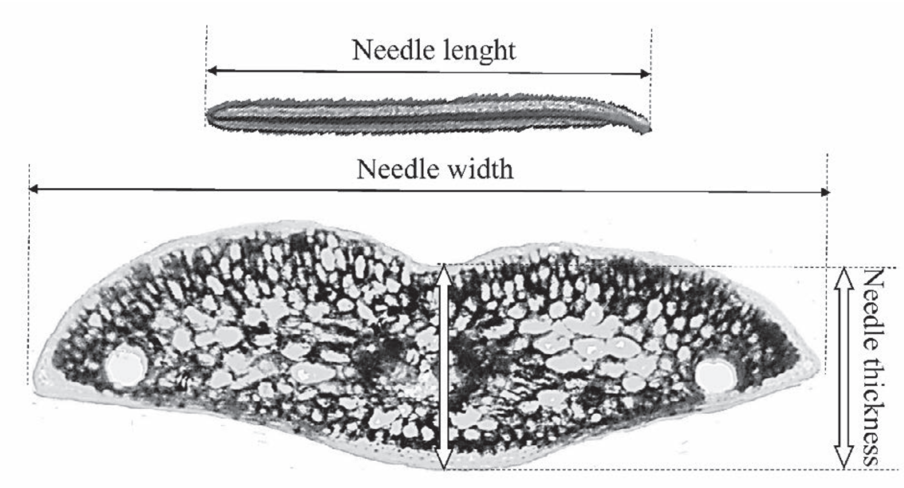

Figure 1. The *Abies alba Mill.* (L.) Karst. needle and transverse section (40 x magnification), with labeled morphological traits.
Slika 1. *Abies alba Mill.* (L.). igla i poprečni presjek (40 x povećanje), s označenim morfološkim osobinama.

a camera (microK) and a software package for calibration and measurement (TopView) (Figure 1). Microscope slides were obtained by cutting the middle of the needle with a scalpel (about 100 µ thick) by hand. Images were taken in JPG format with an image size resolution of 2592 × 1944 pixels.

Statistical analyses

The mean values of morphometric needles traits (lenght, width and thickness) were evaluated by PROC MEANS procedure in SAS software. 3D scatterplot was used for the presentation of the morphological trait mean values in each population. Statistically significancant differences in the sil-

Table 1. Geographic information: altitude, latitude, longitude and climatic parametars MAT (mean annual temperature (°C), degree-days: < 0, > 5, < 18 and > 18 (days), CMD (Hargreaves climatic moisture deficit (mm)), I_{DM} (De Martonne aridity index) in 16 silver fir (*Abies alba* Mill.) populations in the Balkan Peninsula.
Tablica 1. Geografski podaci: nadmorska visina, zemljopisna širina, dužina i klimatski parametri MAT (srednja godišnja temperatura (°C), stupanj-dani: < 0, > 5, < 18 i > 18 (dani), CMD (Hargreavesov deficit klimatske vlage (mm)), I_{DM} (De Martonneov indeks aridnosti) u 16 populacija jele (*Abies alba* Mill.) na Balkanskom poluotoku.

Populations Populacija	Elevation Nadmorska visina (m)	Latitude Zemljopisna **širina** WGS84	Longitude Zemljopisna dužina WGS84	MAT	Degree-days Stupanj-dani				CMD	I_{DM}
					<0	>5	<18	>18		
Pohorje (SLO)	720	46.6039	15.3614	6.6	445	1623	4212	40	0	75.4
Goč (SRB)	900	43.5603	20.7086	7.7	331	1801	3817	70	110	48.3
Lisina (BIH)	980	44.4038	17.0027	6.9	355	1610	4086	38	72	63.7
Pirin (BG)	1100	41.5689	23.5583	8.3	236	1903	3592	102	364	58.2
Tara (SRB)	1100	43.9094	19.4131	6.8	362	1561	4128	22	38	32.1
Stara planina (SRB)	1160	43.2494	22.8380	6.1	429	1449	4369	10	149	44.2
Javor (SRB)	1200	43.4147	20.0647	6.5	394	1501	4243	15	44	60.8
Kovač (MNE)	1250	43.5033	19.1667	6.3	380	1420	4317	1	46	66.8
Osogovo(BG)	1260	42.2144	22.4825	6.7	354	1529	4171	20	298	36.0
Zlatar (SRB)	1290	43.4267	19.7983	6	425	1392	4402	0	38	64.5
Dubočica (SRB)	1310	43.1628	19.8775	6.1	413	1399	4382	0	50	67.1
Kopaonik (SRB)	1360	43.2841	20.8522	5.5	463	1286	4606	0	46	60.8
Romanija (BIH)	1380	43.9297	18.4964	5.3	457	1237	4675	0	33	68.6
Golija (SRB)	1390	43.3480	20.4472	5.3	490	1266	4662	0	41	63.5
Hajla (MNE)	1520	42.7806	20.1886	5.1	465	1193	4735	0	51	73.0
Rila (BG)	1860	42.1517	23.5597	2.9	676	774	5522	0	125	58.7

ver fir needle traits were estimated by the analysis of variance (ANOVA, procedure PROC GLM in SAS software). Testing the significance of differences between populations (as fixed factor) and trees nested in population (as a random factor) was performed by F-test, using RANDOM options in PROC GLM procedure. The PROC CORR option in the SAS software was used to estimate Pearson's correlation coefficient between morphological needle traits and climatic factors. The Principal Component Analysis (PCA), a multivariate method, was applied for the analysis of population variability. In order to examine the patterns of population variability in relation to the morphological needle traits and climatic factors PCA based on the correlation matrix (Pearson's method) was performed. We used scaterplot graph to visually present results both on populations, morphological needle traits and climatic factors. The Microsoft Excel XLSTAT add-in software package Agglomerative Hierarchical Clustering (AHC) (Ward's method) was performed on standardized mean values of needle traits using Euclidean distance, which provided the optimal classification of the analyzed populations into homogeneous groups, so-called clusters. The cluster analysis was visually presented by a dendogram.

Statistical data analyses were performed using the appropriate procedures in the software package SAS 9.1.3. Graphic presentations of the obtained results were performed by XLSTAT in Microsoft Excel add-in software package.

RESULTS
REZULTATI

Mean values of the morphological needle traits varied between populations. The needle length varied from 2.08 to

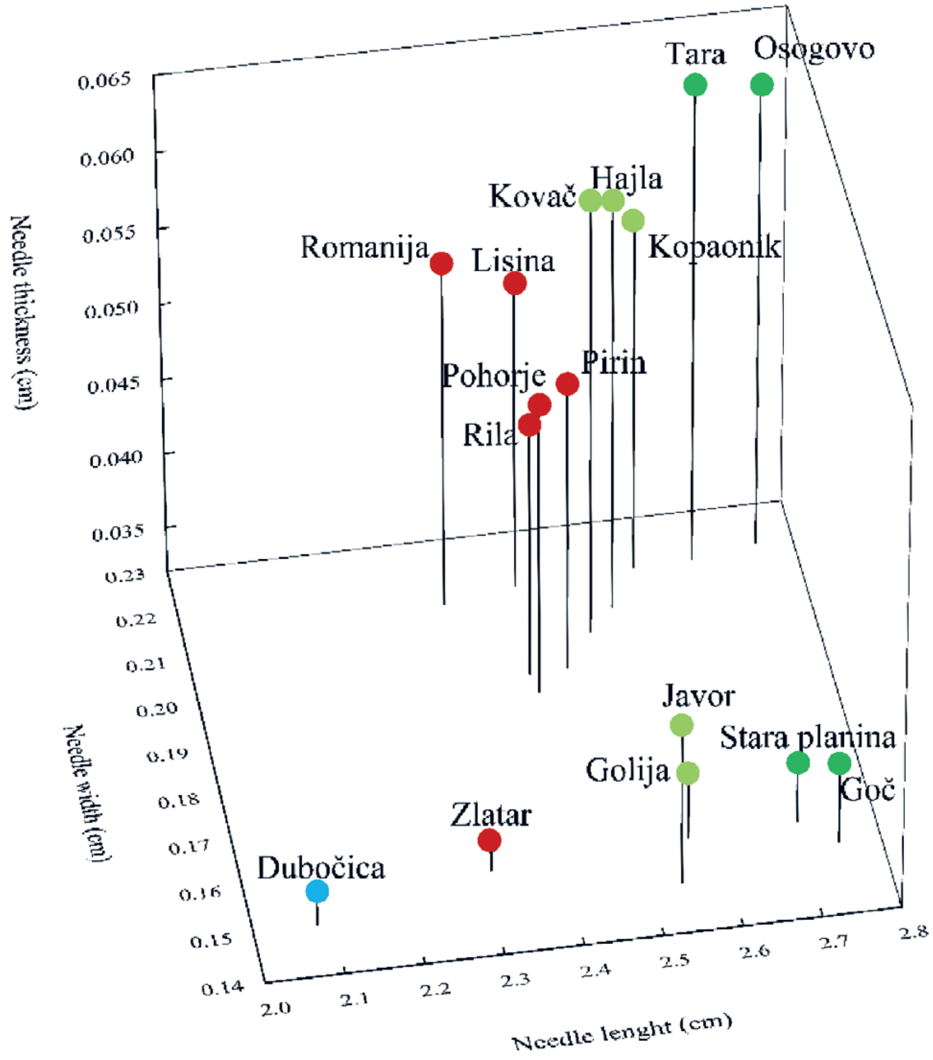

Figure 2. 3D scatter plot of the needle lenght, width and thickness of the silver fir (*Abies alba* Mill.) populations in the Balkan Peninsula. The colors correspond to the cluster analysis of populations differentiation (see below Figure 4.).

Slika 2. 3D dijagram raspršenosti dužine, širine i debljine iglica populacija jele (Abies alba Mill.) na Balkanskom poluotoku. Boje odgovaraju klaster analizi diferencijacije populacija (vidi sliku 4 dolje).

Table 2. Results of ANOVA analysis with fixed factor population (P), trees nested in population (Tree (P)) as a random factor for the silver fir (*Abies alba* Mill.) morphological needle traits.

Tablica 2. Rezultati ANOVA analize s populacijom kao fiksnim faktorom (P), stablima ugniježđenim u populaciji (Stablo (P)) kao slučajnim faktorom za morfološka obilježja iglica jele (*Abies alba* Mill.).

Source of variation Izvor varijacije		Needle lenght Dužina iglice		Needle width Širina iglice		Needle *thickness* Debljina iglice	
	df	MS	F	MS(x10^{-3})	F	MS(x10^{-3})	F
Population (P)	15	6.17	5.20****	162.44	60.61****	22.61	77.59****
Tree (P)	304	1.19	28.21****	2.68	35.57****	0.29	16.51****
Error	2880	0.04		0.08		0.02	

**** $P < 0.0001$.

2.75 cm, the needle width from 0.15 to 0.22 cm and the needle thickness from 0.034 to 0.064 cm. On the 3D scatter plot can be noticed that Dubočica population had the lowest needle mean values. The populations of Zlatar, Golija, Javor, Stara planina and Goč made a group with smaller NW and NT compared to the other populations (Figure 2).

The populations of Tara and Osogovo were singled out as the populations with the highest mean values of the needle traits. High negative correlation ($r = -0.83$, $p < 0.00001$) was obtained between these indices (CMD and I_{DM}). A negative statistically significant correlation was obtained for the ratio of altitude and evaporation ($r = -0.51$, $p < 0.0018$) indicating that the higher the altitude the less the evaporation. The correlation between CMD and elevation was weakly negative, but also significant ($r = -0.44$, $p < 0.0054$).

Interpopulation and intrapopulation variability was statistically significant and confirmed by the results of the applied ANOVA model. Namely, all three morphological needle traits showed highly significant differences between populations and between trees within populations (all $p < 0.0001$) (Table 2).

Based on the results of the applied PCA analysis, the obtained first principal component axis (PC1) described 56.28% of the total variability, and the second (PC2) 23.83% (Figure 3A). The variability of the needle samples could be clearly separated and the first two axes described 80.01% of the population variability of the silver fir morphological needle traits (Figure 3B). MAT and degree days (< 0, > 5, < 18 and > 18) contributed the most to the separation of populations based on the first PCA axis (make sharp angle < 90°) (Figure 3). According to the second PCA axis, the characteristics of NW and NT contributed the most to the separation of populations (the factor values of the second principal component were > 0.90). The lowest mean values of these

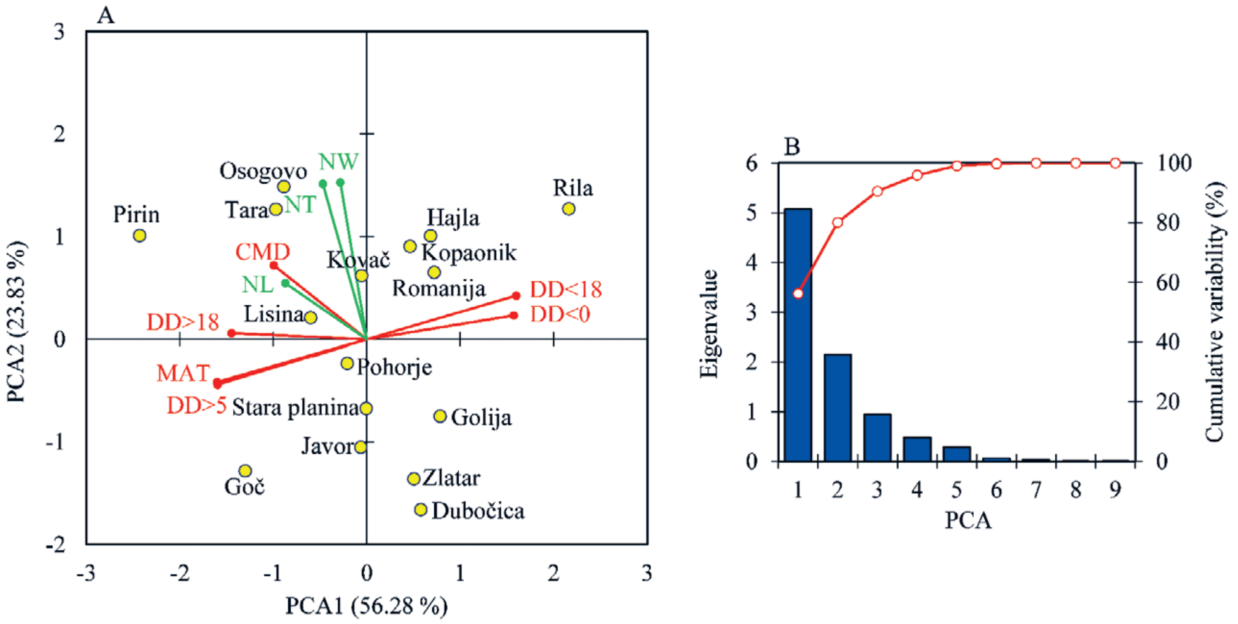

Figure 3. PCA triplot showing the first two principal component axes for the silver fir morphological needle traits, climatic factors and populations in the Balkan Peninsula (A); Eigenvalues of all nine principal components with percentage of cumulative variability (B).

Slika 3. PCA triplot koji prikazuje prve dvije glavne komponente osi za morfološka obilježja iglica jele, klimatski čimbenici i populacije na Balkanskom poluotoku (A); Vlastite vrijednosti svih devet glavnih komponenti s postotkom kumulativne varijabilnosti (B).

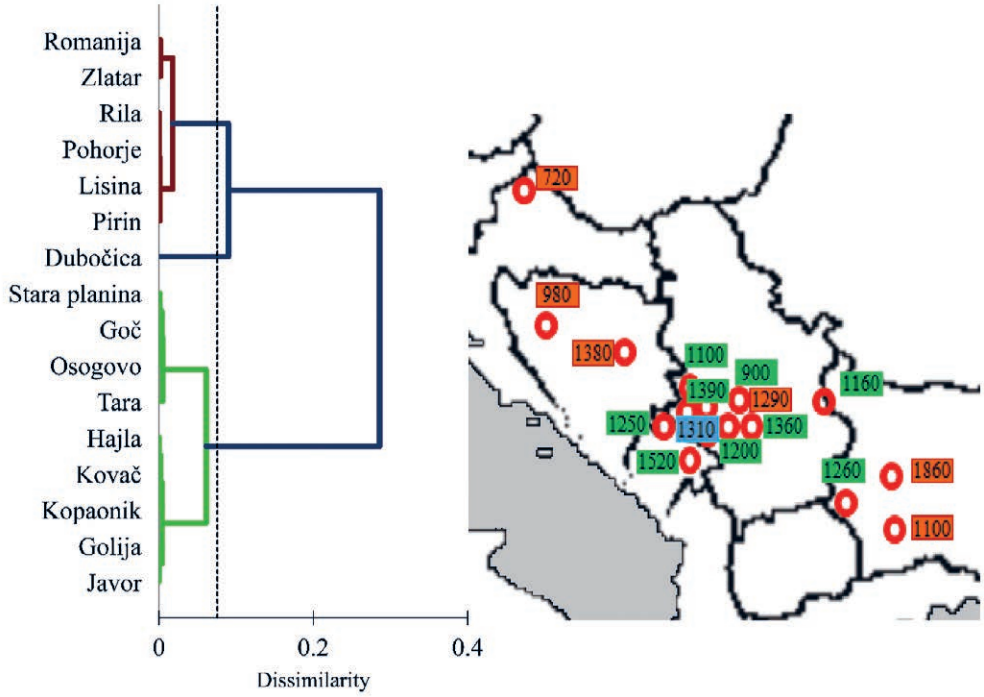

Figure 4. Dendrogram showing the grouping of 16 populations based on the silver fir (*Abies alba* Mill.) morphological needle traits with map of population distribution and altitude (m a.s.l).

Slika 4. Dendrogram koji prikazuje grupiranje 16 populacija na temelju morfoloških obilježja iglica srebrne jele (*Abies alba* Mill.) i mapu distribucije populacija sa nadmosrskom visinom (m a.s.l).

two needle traits contributed to the separation of the populations Zlatar and Dubočica. The highest mean values of the same needle traits contributed to the separation of the populations Tara and Osogovo, as well as the CMD values that were considerably higher than in the other populations (364 and 298; respectively). The NL vector was the shortest, which indicated that it was poorly described by the first two principal components and had less effect on population separation compared to the other two morphological needle traits (Figure 3).

Three clusters could be observed according to the agglomerative hierarchical clustering (AHC) analysis of the morphological needles traits of 16 silver fir populations (with a dendrogram cut at 0.076 Euclidean distances) (Figure 4). One cluster consisted of the two groups of populations: Stara planina, Goč, Osogovo and Tara (with NL > 2.67 cm, lower I_{DM} values < 50 and higher values of CMD), and Hajla, Kovač, Kopaonik, Golija and Javor (NL > 2.50 cm, the indices did not stand out in values compared to other populations). Dubočica population had the lowest mean values (for example NL = 2.08 cm, NW = 0.15 cm and NT = 0.03 cm) of the analyzed traits, although none of the climatic factors differed significantly relative to the other populations. Other populations formed the third cluster with two subclusters according to the NL values: Pohorje, Lisina, Pirin, Zlatar, Romanija and Rila populations had less values (NL < 2.50 cm and CMD values < 72, and I_{DM} values > 63) compared to the populations from the other cluster (Figure 4). Optimal classification of the analyzed populations into homogeneous groups - clusters was obtained on the basis of the large differences between (75.64%) and within (24.36%) homogeneous groups.

DISCUSSION
RASPRAVA

Tree populations continuously face new and rapidly changing selective pressures, such are more frequent extreme droughts that threaten the conservation of forest ecosystems (Lindner *et al.* 2010; Griesbauer *et al.* 2021). Since the global warming has caused water shortages and high temperatures in recent decades, the genetic diversity remains one of the most important factors that contribute to species adaptability (Thompson *et al.* 2009; Sánchez-Velásquez *et al.* 2021). Forest degradation leads to a reduction in population size that may lead to a reduction in genetic diversity in the next generation (Leimu *et al.* 2006). Reduction of forest areas, caused by drought, happens more often at the southern borders than in the center of the species distribution according to the biogeographic distribution scale (Hampe and Petit 2005; Jump *et al.* 2009). Numerous studies of interpopulation and intrapopulation (genetic) variability enable the understanding of differential responses of tree populations to drought stress (Allen *et al.* 2010).

Silver fir populations are a type of forest ecosystem that are autochotonous and originate from the glaciation period. The pattern of genetic differentiation fully corresponds to the morphological variation in the silver fir. Southern populations are much more variable (in some of the morphological traits like the number of stomates, needle thickness and width, etc.) compared to morphologically uniform northern populations (Paule *et al.* 2002).

The spatial variation of the silver fir morphological needle traits was observed in this study. Namely, the humidity index made the greatest impact on the obtained differences in the values of the morphological neeedle traits between populations. The values of the CMD water deficit index indicate the existence of drier environment in relation to other localities and depend on the degree of evaporation and precipitation on seasonal basis. Pirin and Osogovo populations had the lowest amount of precipitation compared to the others (about 600 mm/m^2), whereas Pohorje as a population with lowest altitude (720 m a.s.l) had the amount of precipitation about 1251 mm/m^2.

The morphological needle traits that are partly influenced by edaphic and climatic conditions were a useful tool in assessing the intrapopulation and interpopulation differentiation of Scots pine (Irvine *et al.* 1998, Niinemets *et al.* 2001, Pensa *et al.* 2004, Poljak *et al.* 2020, Ergül Bozkurt*et al.* 2021). They have also proven their importance in the characterization of the *Pinus silvestris* population and in the description of the geographical differentiation of this species (Urbaniak *et al.* 2003).

With increasing altitude environmental factors become more stressful, as a decrease of temperature and atmospheric pressure and an increase of precipitation and wind strength directly affect the growth and development of plants. The adaptive potential to environmental stress conditions is species-specific. The pattern of leaf morphology traits is determined by temperature and available water resources compared to spatial gradients (Zhu *et al.* 2022). Comparing the *Fagus sylvatica* morphological leaf traits and *Picea abies* morphological needle traits in a long period of time (two decades), it was found that the summer and autumn air temperatures in the previous year and reduced atmospheric pressure affected the Europeen beech leaf morphology, wherase spring air temperatures of the current year affected the Norway spruce needle morphology (Zhu *et al.* 2022). For example, the size of wild cherry leaves (as a broadleaves species) was influenced by the amount of precipitation in May and the De Martonne aridity index (Miljković *et al.* 2019). The number of days with temperatures below 0° C, the amount of precipitation and the altitude affected growth and phenology of *Pseudotsuga menziesii* (De La Torre *et al.* 2021). The interpopulation variability of *P. tabuliformis* morpho-anatomical needle traits also showed a positive correlation between the needle size and the amount of annual precipitation (Zhang *et al.* 2017), in contrast to *P. yunnanensis* needle size that showed a negative correlation with the amount of annual precipitation and temperature but a positive correlation with the latitude (Huang *et al.* 2016). In *Pinus roxburghii*, the needle length was shorter at higher altitudes (Tiwari *et al.* 2013). In our work, the the silver fir needle length was not correlated with altitude. Climate change increases aridity, and due to rising temperatures the rate of evapotranspiration increases, so that higher temperatures and less precipitation lead to climate stress caused by humidity. CMD as a quantification of stress caused by water deficit, which defines the dynamics of vegetation in ecosystems, is a parameter that is expected to increase in the future. In the Balkan Peninsula region, the projection is that these values will be three times higher than those recorded in the analyzed locations (Girvetz and Zganjar 2014). They will be close to the values recorded in the populations Pirin and Osogovo (CMD values 364 *vs.* 298; respectively), which have been confirmed to have influenced the silver fir morphological needle traits. The aridity index is in the semi-arid category (Nunes *et al.* 2016) in the areas where the populations Pirin and Osogovo are located (I_{DM} values 32.13 *vs.* 35.99; respectively). Tara population is the central area of the silver fir distribution and belongs to the category of sub-humid area ($I_{DM} = 58.15$). However, the microenvironmental conditions in the area indicate that the change in climatic factors occurred according to the projection of Girvetz and Zganjar (2014), *i.e.* to drier climate conditions.

CONCLUSIONS
ZAKLJUČCI

The analyzed populations varied in the mean values of the silver fir morphological needle traits. None of the silver fir morphological needle traits was statistically significantly correlated with altitude, wherase the climatic factors were significantly correlated . Namely, the number of days with temperature < 0°C and > 18°C showed positive correlation, and the number of days with temperature > 5°C and > of 18°C showed negative with altitude. The Hargreaves climatic moisture deficit was the highest in the Pirin and Osogovo populations, as a consequence of the lowest values of mean annual precipitation in relation to the rest, although the impact on the silver fir morphological needle traits was not clear (the needle lenght was shorter in Pirin population compared to Osogovo, 2.47 cm *vs.* 2.75 cm; respectively). Research of the larger southern area of the silver fir distribution gives a better picture on the variability of the morphological needle traits in relation to the climatic characteristics that are both spatially and temporally divergent. The obtained pattern of variability in local environmental conditions and adaptation of the species to more stressful environmental conditions will enable better design of breeding programs in order to adapt to projected climate change in the future. As a part of natural population monitoring, evolutionary ecology research of the silver fir needle morphology is the valuable contribution to the comprehension of present genetic variability as a prerequisite for the possibility of adaptation to rapid climate change and conservation of the species area in the Balkan Peninsula. Defining forest response to the spatial environmental changes through the acclimatization abilities of the evergreen conifer trees (for example morphological needles traits) is essential for predicting the survival of these ecosystems.

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ZAHVALE

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SAŽETAK

Interpopulacijska i intrapopulacijska varijabilnost triju morfoloških osobina iglica (dužina, širina i debljina) istraživana je u 16 prirodnih populacija jele na Balkanskom poluotoku. Populacije predstavljaju refugijalna područja jele (*Abies alba* Mill.). Ovaj rad ima za cilj pružiti opsežnu analizu utjecaja klimatskih čimbenika (srednja godišnja temperatura, broj dana s temperaturama < 0, > 5, < 18, > 18°C, Hargreavesov klimatski deficit vlage i De Martonneov indeks aridnosti) na obrazac morfoloških obilježja iglica unutar svake populacije. Populacije su pokazale varijacije u analiziranim morfološkim obilježjima iglica koje se nisu mogle jasno definirati niti jednim od analiziranih klimatskih čimbenika. Najveći utjecaj na vrijednosti obilježja imao je klimatski deficit vlage po Hargreavesu, a zatim najniže vrijednosti srednje godišnje količine oborina. Evolucijska ekološka istraživanja morfologije iglica jele vrijedan su doprinos razumijevanju sadašnje genetske varijabilnosti kao preduvjeta za prilagodbu na brze klimatske promjene i očuvanje vrste na području Balkanskog poluotoka.

KLJUČNE RIJEČI: morfologija iglica, klimatski čimbenici, jela, Balkanski poluotok.