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Geographic variation of *Pinus heldreichii* Christ from the Western Balkans based on cone and seed morphology

Abstract: *Pinus heldreichii* (Bosnian pine) is a Balkan-Apennine endemic and relict pine species that inhabits high mountains in the Mediterranean and sub-Mediterranean regions. Nineteen populations of *P. heldreichii* from the Western Balkans encompassing 187 individual trees were examined to evaluate morphological variation, a rarely studied aspect of the species. Univariate and multivariate statistics were applied in order to assess the variation of morphological traits of cones and seeds, evaluate the relationships among the sampled populations and verify geographic differentiation in the Dinaric Alps *versus* Scardo-Pindic mountains. The observations of *P. heldreichii* covering the populations from the western margins and the centre of the species distribution range indicated a morphological variation among populations and their geographic structure. In general, the southern populations (Scardo-Pindic group) had lower values for the most of morphological traits than the northern ones (Dinaric group). The observed geographic differences between these populations exhibit a north-western to south-eastern gradient, with a few inconsistencies. The southernmost sampled population, Tomorr in Albania, showed remarkable morphological divergence from the other studied populations and appeared to be a distinct morphological group. The pattern of morphological variation in Bosnian pine most likely resulted from multiple effects of long-term isolation and fragmentation in high mountain systems, adaptation to extreme environments and human disturbances.

Keywords: Bosnian pine, Dinaric Alps, Scardo-Pindic massif, morphometry, population differentiation

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Introduction

The Balkan peninsula represents one of the main European Pleistocene refugia and hotspots of species and genetic diversity (Petit et al., 2003; Hewitt, 2011). An exceptional level of plant diversity is a result of contrasting eco-climate conditions, edaphic diversity, palaeogeological history and migratory processes of different biogeographic plant groups, as well as a long-term human impact (Thompson, 2005; Hewitt, 2011; Nieto Feliner, 2014; Tomović et al., 2014). Furthermore, a recent study of diversity and

distribution of tree species in the Mediterranean Basin confirmed the highest rate of taxonomic diversity in the Balkan peninsula (Médail et al., 2019).

Bosnian pine (*Pinus heldreichii* Christ, syn. *P. leucodermis* Antoine) is a Balkan-Apennine endemic and relict species of the Tertiary flora (Vidaković, 1991) belonging to the group of mountain Mediterranean pine species (Gernandt et al., 2005). The tree exhibits the common pattern of amphi-Adriatic distribution (Frajman & Schönswetter, 2017). It occurs mostly in high mountains of the sub-mediterranean region and continental parts of the western and southern Balkan Peninsula with few isolated populations in the Apennine Peninsula. Its populations are patchily distributed in Bosnia and Herzegovina, Montenegro, North Macedonia, Kosovo, Albania, northern Greece, southwestern Bulgaria and southern Italy (Vidaković, 1991). It grows mostly on steep limestone and dolomite slopes and occurs on siliceous and ultrabasic bedrocks often forming the alpine tree line (Dinić & Janković, 2006; Panayotov et al., 2010; Caković et al., 2017). It is a slow-growing tree, adapted to extreme environmental conditions and thus often used for high-altitude reforestation. It is also used for ornamental and landscaping purposes due to lower susceptibility to pests compared to other pine species (Vendramin et al., 2008). In most of the countries in its range, the Bosnian pine is protected by national legislation; at the European level, its forests are included in Annex 1 habitat type of the EU habitat directive (code 95A0). *Pinus heldreichii* is globally assessed as a Species of Least Concern, but can be endangered by local risks such as fire, illegal logging and overgrazing of seedlings (Caković et al., 2017).

Many aspects of the species biology and ecology are still insufficiently known. A limited number of studies have aimed at classical morphology and phenology in a particular geographic region (Tucović & Stilinović, 1972, 1975, 1977; Popnikola, 1975, 1978; Ballian et al., 2005), molecular genetic variation (Morgante et al., 1991; Boscherini et al., 1994; Naydenov et al., 2005) and genome organization (Bogunić et al., 2006). The chemical composition and its variation are the most thoroughly studied aspects of this pine (Nikolić et al., 2007, 2015; Bojović et al., 2011).

Despite the fact that molecular investigations have been predominant over the last two decades, detailed biometric studies of morphological characters still prove critically informative in solving problems of geographic differentiation of pine species (Boulli et al., 2001; Boratyńska et al., 2005, 2015; Jasińska et al., 2014), hybridisation (Christensen & Dar, 1997), taxonomy (Boratyńska & Bobowicz, 2001) and plasticity (Gil et al., 2002). More recently, morpho-anatomic studies of needle variation of

P. heldreichii populations have indicated a geographic pattern between the Dinaric and Scardo-Pindic mountains (Nikolić et al., 2014, 2019).

Previous research on different pine species suggested that cone, seed and apophysis characters had adaptive significance and were related to geographic and ecological factors (Dawson et al., 1997; Gil et al., 2002; Leslie et al., 2017). In this study, we carried out a detailed morphometric analysis of cones and seeds of *P. heldreichii* populations from the Western Balkans. According to our best knowledge, this is the first detailed morphometric study dealing with cone and seed variation of this species at a larger geographical scale. The analyzed populations originate from the north-western margin of its distribution area and the central part of the species range as well. Morphological characters represent useful indicators given that morphological variation may be associated with various factors that are often difficult to evaluate. Our main objectives were (1) to assess the morphological variability of cone and seed traits, (2) to evaluate the relationships among the populations, and (3) to verify possible geographic differentiation of populations from the Dinaric and Scardo-Pindic mountains.

Material and methods

Plant material

A total of 187 individuals from 19 natural populations of *P. heldreichii* from Bosnia and Herzegovina, Montenegro, Kosovo and Albania were sampled (Fig. 1, Table 1). The distance between the sampled trees was at least 50 m. Mature closed cones were collected from the centre of the crown's southern exposure. Assignment of populations into the Dinaric and Scardo-Pindic groups was done following the classification of floristic regions proposed by Stevanović (1996).

Twelve morphological traits and four derived traits were analysed: length of cone (LC), diameter of cone (DC), length of winged seed (LWS), width of seed wing (WW), length of seed (LS), width of seed (WS), thickness of seed (TS), length of cone scale (LCS), width of cone scale (WCS), thickness of cone scale (TCS), length of apophysis (LA), width of apophysis (WA), ratio of length of cone/diameter of cone (LC/DC), ratio of length of seed/width of seed (LS/WS), ratio of length of cone scale/width of cone scale (LCS/WCS), ratio of length of apophysis/width of apophysis (LA/WA) (Fig. 2). The traits of 15 to 30 cones and 30 seeds per tree were measured with a digital caliper. The cone scales from the middle part of the cone were used for measurements.
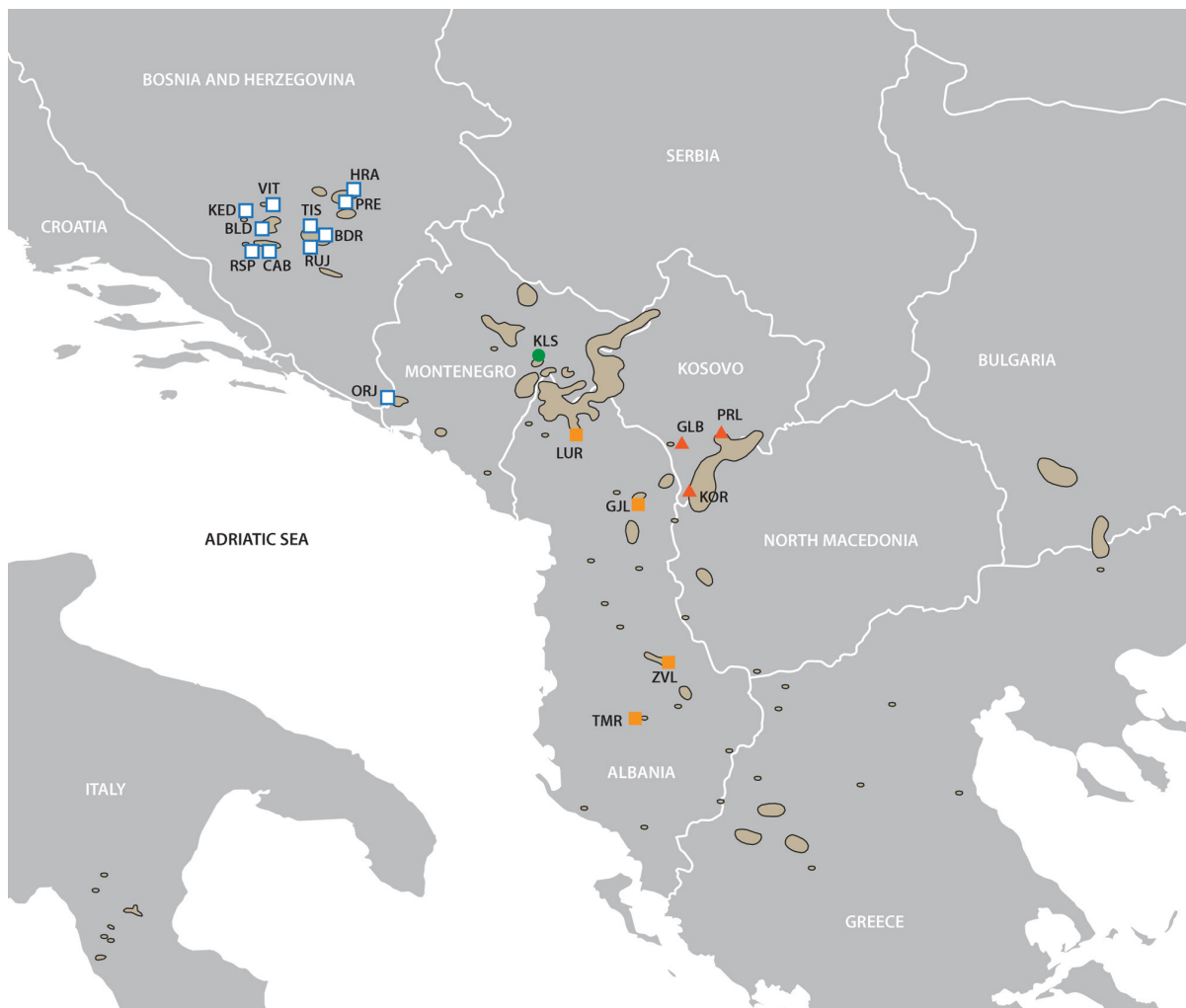
Fig. 1. Geographic range of *Pinus heldreichii* according to Caudullo et al. (2017) with sampled populations (acronyms and symbols correspond to those in Table 1 and Fig. 3)

Table 1. Geographic origin of sampled *Pinus heldreichii* populations

Country	Population	Population ID	N	Latitude (DD)	Longitude (DD)	Altitude (m)
Bosnia and Herzegovina*	Hranisava, Mt. Bjelašnica	HRA	11	43.734	18.118	1690
	Preslica, Mt. Bjelašnica	PRE	9	43.718	18.033	1290
	Boračka draga, Mt. Prenj	BDR	11	43.547	17.973	1450
	Tisovica, Mt. Prenj	TIS	10	43.592	17.873	1700
	Rujište, Mt. Prenj	RUJ	11	43.463	17.958	1115
	Vitlenica, Mt. Plasa	VIT	9	43.672	17.654	1532
	Krupna navala, Mt. Čabulja	CAB	10	43.492	17.688	1490
	Rosne poljane, Mt. Čabulja	RSP	10	43.529	17.518	1340
	Blidinje, Mt. Čvrsnica	BLD	10	43.626	17.534	1264
	Kedjara, Mt. Vran	KED	8	43.727	17.563	1640
	Obli svitavac, Mt. Orjen	ORJ	10	42.606	18.468	1410
Montenegro*	Kolašin, Mt. Bjelasica	KLS	10	42.688	19.619	1800
Kosovo**	Zhur, Koritnik, Mt. Korab	KOR	10	42.111	20.611	1515
	Golem bor, Kodza Balkan, Mt. Sharr	GLB	9	42.232	20.867	1590
	Prevelac, Ošljak, Mt. Sharr	PRL	10	42.201	20.896	1800
Albania**	Lure, Mt. Prokletije	LUR	10	42.307	19.851	1650
	Mt. Gjallica	GJL	10	42.034	20.494	1350
	Mt. Tomorr	TMR	10	40.664	20.148	1650
	Zavaline, Mt. Mali i Polisit	ZVL	10	41.128	20.317	1450

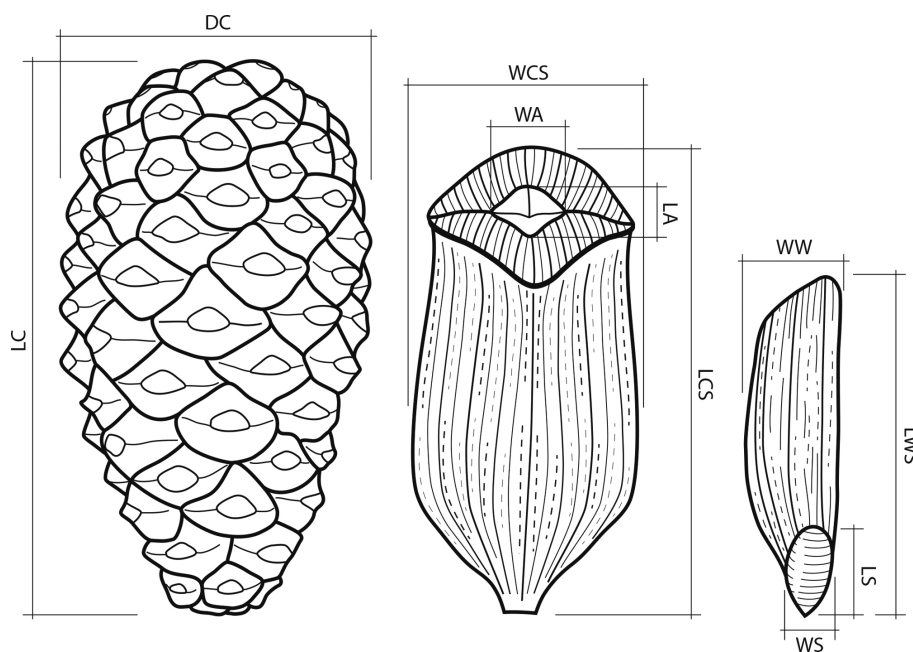*Dinaric mountains, **Scardo-Pindic mountains.

Fig. 2. Measured cone and seed traits of *Pinus heldreichii* used in the study (TS and TCS are not illustrated)

Data analysis

The cone and seed measurements were averaged for each individual to construct two data sets: population matrix and individual matrix. The population matrix was based on means of all morphological measurements at the population level and the individual matrix was based on means of the individual trees. To evaluate the morphological variation of the sampled populations, descriptive statistics (mean, standard deviation and coefficient of variation for each of the analysed traits at the population level), univariate statistics (one-way ANOVA followed by Tukey HSD test) and multivariate techniques, including Principal Component Analysis (PCA), Cluster Analysis (CA), and Canonical Discriminant Analysis (CDA), were applied. Significant differences among examined populations were revealed using one-way ANOVA followed by Tukey HSD test. Both PCA and CA were used to check affinities among populations based on the population matrix. PCA based on the individual matrix aimed at displaying a general pattern of variation and relationships among individuals, while CDA focused on determining the most distinguishing morphological traits and classifying individual trees into *a priori* specified groups (Dinaric *vs.* Scardo-Pindic).

All data were standardized before analyses due to different scales of trait scoring (measurements and ratios) (Quinn & Keough, 2009). Pearson correlation coefficients were calculated to determine highly correlated character pairs ($r > 0.85$) since these could distort the results of discriminant analysis (Legendre & Legendre, 1998). PCA of populations and individuals were performed on correlation matrices of all scored traits and the axes corresponding to principal components with Eigenvalues >1 were retained in the analysis. The cluster analysis by Ward's method was performed using Euclidean distances (Sokal & Rohlf, 1994; Boratyńska et al., 2015). CDA was based on the Mahalanobis distances of 10 variables included in the model (see Results). The histogram of frequency of individual discriminant function scores was used to display the distribution of examined individuals following the group assignment. Next, CDA was used to determine the proportion of individual trees to be assigned to each group by cross-validation procedure. The Mantel test (Mantel, 1967) was used to test whether morphological variation or differentiation of populations is affected by geographic isolation. The Mahalanobis' distances for morphological data and geographic distances between latitudes and altitudes were used for calculation. Data analyses were performed in PAST ver. 3.25 (Hammer et al., 2001) and SPSS ver. 20 for Windows (SPSS, Chicago, USA).

Results

Variation and correlations of individual morphological traits

Mean values, standard deviations and coefficients of variation of analysed morphological traits were calculated for each population (Table 2). In general, coefficients of variation had low (CVs $\leq 10\%$, 234 cases) to moderate values (CVs from 10 to 20%, 69 cases). The coefficient of variation (CV%) values did

Table 2. Descriptive statistics for analysed *Pinus heldreichii* populations (SD = standard deviation; CV = coefficient of variation)

Population	Trait (mm)	CL	CW	LWS	WW	LS	WS	TS	LCS	WCS	AL	AW	TCS	CL/CW	SL/SW	LCS/WCS	AL/AW
HRA	Mean±SD	74.8±7.7	34.1±3.4	22.3±2.4	7.3±0.6	7.5±0.7	3.9±0.4	2.7±0.1	11.9±0.7	8.4±0.5	4.7±0.4	2.7±0.1	3.1±0.4	2.2±0.1	1.9±0.1	1.4±0.1	1.8±0.1
(N = 11)	CV%	10.3	10.0	10.6	7.7	8.9	9.3	4.6	6.1	6.0	7.9	4.7	14.0	6.4	6.7	7.9	5.5
PRE	Mean±SD	60.3±4.8	28.9±1.5	23.2±2.1	6.4±0.6	7.1±0.6	4.4±0.4	2.7±0.2	10.8±0.8	8.6±0.7	4.8±0.3	2.7±0.2	3.3±0.3	2.1±0.1	1.6±0.1	1.3±0.1	1.8±0.1
(N = 9)	CV%	7.9	5.1	9.2	9.0	7.8	9.4	6.6	7.7	8.3	6.6	9.1	7.8	5.1	5.3	7.6	7.4
BDR	Mean±SD	71.9±6.6	35.0±2.5	22.5±1.5	7.2±0.4	7.1±0.5	4.1±0.4	2.8±0.2	11.3±1.1	8.7±0.5	5.2±0.4	2.9±0.3	3.2±0.4	2.1±0.2	1.7±0.1	1.3±0.1	1.8±0.1
(N = 11)	CV%	9.2	7.0	6.7	5.0	6.6	8.6	5.6	9.6	5.2	7.3	9.2	12.6	7.8	5.8	11.4	6.7
TIS	Mean±SD	56.5±6.0	28.7±1.9	20.5±2.0	6.8±0.3	6.6±0.3	3.8±0.2	2.5±0.1	10.7±0.4	8.0±0.2	4.4±0.4	2.6±0.1	3.0±0.3	2.0±0.1	1.7±0.1	1.3±0.1	1.7±0.1
(N = 10)	CV%	10.6	6.5	9.5	4.9	5.2	4.5	4.6	3.9	2.7	8.1	5.6	10.4	4.5	6.6	4.8	5.5
RUJ	Mean±SD	73.1±4.1	34.1±1.3	22.5±1.9	6.9±0.5	7.2±0.4	4.0±0.3	2.7±0.1	11.5±0.5	8.1±0.3	5.6±0.1	2.9±0.2	3.3±0.3	2.1±0.1	1.8±0.1	1.4±0.1	1.9±0.1
(N = 11)	CV%	5.6	3.9	8.2	6.6	6.2	6.9	4.7	4.4	4.0	1.8	5.6	8.4	4.2	4.2	7.2	4.4
VIT	Mean±SD	60.3±7.2	29.6±3.2	21.8±1.7	6.9±0.5	7.3±0.3	4.2±0.3	2.6±0.1	11.3±1.1	8.1±0.6	4.9±0.9	2.9±0.5	3.2±0.3	2.0±0.1	1.7±0.1	1.4±0.1	1.7±0.2
(N = 9)	CV%	11.9	11.0	7.9	7.0	4.8	7.2	5.6	9.9	7.0	17.8	17.4	8.4	4.9	6.6	7.5	12.5
CAB	Mean±SD	60.5±4.2	31.8±1.6	22.3±1.3	6.9±0.4	7.1±0.4	4.2±0.3	2.7±0.1	11.3±0.5	8.6±0.7	4.5±0.4	2.7±0.1	3.3±0.2	1.9±0.1	1.7±0.1	1.3±0.1	1.7±0.1
(N = 10)	CV%	6.9	5.1	5.8	6.4	5.8	6.3	5.4	4.6	8.7	8.2	4.9	6.9	5.4	6.6	7.5	4.9
RSP	Mean±SD	57.1±6.0	28.8±2.4	21.5±2.7	6.4±0.3	6.7±0.4	3.6±0.2	2.4±0.2	9.1±1.1	7.9±0.6	4.8±0.3	2.5±0.2	3.2±0.4	2.0±0.2	1.8±0.0	1.2±0.2	1.9±0.2
(N = 10)	CV%	10.5	8.4	12.6	4.9	5.7	5.6	8.1	11.9	7.7	7.2	9.5	12.0	9.3	2.3	14.2	9.9
BLD	Mean±SD	63.7±6.6	33.4±1.5	20.4±2.6	6.7±0.6	7.0±0.6	4.0±0.2	2.7±0.2	11.5±0.9	8.5±0.6	4.4±0.3	2.5±0.2	2.9±0.4	1.9±0.1	1.8±0.1	1.4±0.1	1.7±0.1
(N = 10)	CV%	10.3	4.4	12.7	8.6	7.8	6.3	6.8	7.5	7.4	7.2	7.6	12.4	7.6	5.9	9.5	7.3
KED	Mean±SD	64.3±8.2	30.6±2.8	22.9±1.5	7.2±0.3	7.2±0.4	4.3±0.1	2.7±0.2	11.5±0.9	8.2±0.2	4.7±0.2	2.6±0.2	3.2±0.2	2.1±0.1	1.7±0.1	1.4±0.1	1.8±0.1
(N = 8)	CV%	12.8	9.0	6.4	3.7	6.0	3.4	6.8	7.8	2.9	3.3	5.9	7.6	5.0	5.0	7.3	3.8
ORJ	Mean±SD	66.5±5.7	33.8±2.2	22.3±2.2	6.8±0.8	6.8±0.4	3.9±0.2	2.4±0.2	11.7±1.2	8.6±1.2	5.2±0.4	2.6±0.2	3.7±0.3	2.0±0.2	1.8±0.1	1.4±0.1	2.1±0.2
(N = 10)	CV%	8.6	6.4	9.9	11.7	6.3	6.3	7.3	10.5	14.1	7.6	9.5	8.1	7.8	5.4	9.8	11.3
KLS	Mean±SD	57.4±10.0	28.7±4.2	21.2±2.8	6.7±0.5	7.0±0.5	3.7±0.2	2.5±0.3	10.5±2.0	8.1±1.5	5.1±0.7	2.7±0.5	3.4±0.4	2.0±0.2	1.9±0.2	1.3±0.2	1.9±0.4
(N = 10)	CV%	17.4	14.7	13.4	7.7	7.8	4.4	11.1	18.7	18.0	13.3	17.3	12.2	11.4	10.5	15.1	21.5
KOR	Mean±SD	73.0±4.8	36.6±1.5	23.4±2.1	7.3±0.5	7.1±0.5	4.1±0.3	2.9±0.2	12.5±1.3	8.2±0.7	5.1±0.5	2.7±0.2	3.3±0.4	2.0±0.1	1.7±0.1	1.5±0.1	1.8±0.2
(N = 10)	CV%	6.5	4.0	9.1	6.9	6.8	6.7	5.8	10.2	8.0	10.4	6.3	10.9	5.8	4.1	8.7	8.4
GLB	Mean±SD	64.7±6.9	31.3±2.4	21.2±2.2	6.1±0.5	6.2±0.3	3.4±0.2	2.3±0.2	10.6±0.8	7.2±0.9	4.0±0.4	2.4±0.2	2.7±0.4	2.1±0.2	1.8±0.2	1.5±0.1	1.7±0.2
(N = 9)	CV%	10.7	7.7	10.3	7.8	4.6	6.2	6.7	8.0	13.1	9.9	8.1	13.8	11.0	9.5	10.1	10.4
PRL	Mean±SD	77.3±5.4	35.6±2.4	25.3±1.7	7.6±0.6	6.8±0.5	3.9±0.2	2.5±0.2	11.9±1.3	9.1±0.9	5.0±0.4	2.6±0.1	3.2±0.6	2.2±0.1	1.8±0.1	1.3±0.2	2.0±0.2
(N = 10)	CV%	7.0	6.7	6.8	8.1	7.2	6.0	8.5	10.7	9.7	8.6	4.0	20.0	6.2	4.7	12.7	9.6
LUR	Mean±SD	62.7±5.6	31.5±2.7	19.6±2.3	5.8±0.5	6.3±0.4	3.4±0.2	2.3±0.2	10.7±1.2	7.9±1.0	5.5±0.5	2.6±0.3	2.9±0.3	2.0±0.1	1.9±0.1	1.4±0.2	2.1±0.2
(N = 9)	CV%	8.9	8.4	11.5	9.0	6.1	6.5	8.0	11.3	13.3	8.5	13.0	8.8	4.2	5.9	15.3	10.4
GJL	Mean±SD	70.7±5.4	33.4±3.1	21.5±2.6	6.1±0.4	6.2±0.5	3.1±0.3	2.3±0.1	12.9±1.1	9.4±0.8	5.1±0.4	2.3±0.4	2.8±0.3	2.1±0.1	2.0±0.1	1.4±0.1	2.3±0.3
(N = 10)	CV%	7.6	9.3	11.9	6.7	8.8	8.5	5.5	8.4	8.8	8.0	18.7	11.4	6.6	5.7	6.8	15.2
TMR	Mean±SD	46.2±2.9	26.2±2.5	17.2±1.6	5.6±0.5	5.6±0.3	3.0±0.2	2.2±0.2	10.1±0.6	6.5±0.9	4.7±0.4	2.1±0.3	2.4±0.3	1.8±0.2	1.8±0.1	1.6±0.2	2.3±0.2
(N = 10)	CV%	6.3	9.6	9.4	8.2	5.2	6.9	7.8	5.5	13.1	7.7	13.0	13.4	12.8	5.3	12.2	7.9
ZVL	Mean±SD	61.8±4.1	31.3±2.1	21.7±1.4	6.3±0.5	6.1±0.3	3.2±0.2	2.4±0.3	11.0±0.6	8.2±0.7	5.3±0.6	2.4±0.4	2.4±0.2	2.0±0.1	1.9±0.1	1.4±0.1	2.2±0.4
(N = 10)	CV%	6.6	6.7	6.5	7.5	5.1	6.9	10.4	5.9	8.8	10.6	15.1	8.2	5.6	5.6	6.8	15.9

not exceed 20% except for trait LA/WA in population KLS (CV = 21.5%). The populations PRE, BDR, TIS, RUJ, CAB and KED had the lowest overall variation (the vast majority of traits with CVs ≤ 10%). The populations with the highest CVs were VIT, RSP, KLS, GLB, LUR and TMR, with at least one-third of the traits having CVs > 10%. The population KLS was the most variable, with most traits having CVs > 10%. The least variable traits in all populations were LS and WS, followed by DC, WW, TS, and LS/WS, while the most variable traits were LC, LWS, TCS, LCS/WCS and LA/WA.

Ninety-three out of 120 pairs of examined morphological traits were correlated at p ≤ 0.01 (Table 3). The correlation values did not exceed r ≥ 0.85 (Table 3). The highest correlation values were found between traits LC and DC (r = 0.83), LS and WS (r = 0.83) and WS and TS (r = 0.74). Positive correlations with high values were determined among all seed characters (Table 3). As expected, cone traits (LC and DC) were positively correlated with seed traits (LCS, LWS). The seed-related traits were also positively correlated with dimensions of apophysis (LA and WA). Contrarily, negative values were

Table 3. Pearson coefficients of correlation among morphological traits of sampled *Pinus heldreichii* populations

Character	LC	DC	LWS	WW	LS	WS	TS	LCS	WCS	LA	WA	TCS	LC/DC	LS/WS	LCS/WCS
DC	0.83														
LWS	0.61	0.47													
WW	0.48	0.48	0.54												
LS	0.39	0.31	0.52	0.63											
WS	**0.20**	0.19	**0.39**	**0.62**	0.77										
TS	0.27	0.35	0.35	0.56	0.64	0.74									
LCS	**0.45**	**0.51**	**0.26**	**0.41**	**0.26**	0.18	**0.35**								
WCS	0.41	0.30	0.52	0.38	0.35	0.29	0.32	0.53							
LA	**0.26**	**0.24**	0.15	0.17*	0.13	0.04	0.12	**0.32**	0.26						
WA	**0.32**	**0.22**	**0.32**	**0.39**	**0.52**	**0.50**	**0.40**	0.10	**0.30**	**0.42**					
TCS	0.28	0.29	0.48	0.39	0.48	0.47	0.29	0.15*	**0.35**	**0.24**	**0.47**				
LC/DC	**0.65**	0.12	**0.45**	0.21*	**0.27**	0.11	0.01	0.11	**0.32**	0.13	0.28	0.12			
LS/WS	0.16*	0.09	0.01	−0.21*	0.02	**−0.61**	**−0.37**	0.04	0.00	0.10	−0.13	−0.16*	0.15*		
LCS/WCS	0.02	0.20*	**−0.28**	−0.01	−0.15*	−0.15*	0.00	**0.46**	**−0.50**	0.07	**−0.21**	**−0.22**	**−0.25**	0.05	
LA/WA	−0.10	0.00	−0.18*	−0.25*	**−0.40**	**−0.45**	**−0.29**	0.18*	−0.04	**0.46**	**−0.59**	**−0.24**	−0.18*	**0.22**	**0.24**

* p ≤ 0.05 significance; bold print indicates p ≤ 0.01.

Table 4. Number of morphological traits significantly different (p ≤ 0.01) between pairs of *Pinus heldreichii* populations based on Tukey HSD test (547 significant differences)

Population	HRA	PRE	BDR	TIS	RUJ	VIT	CAB	RSP	BLD	KED	ORJ	KLS	KOR	GLB	PRL	LUR	GJL	TMR
PRE	4																	
BDR	1	2																
TIS	4	1	3															
RUJ	1	3	0	3														
VIT	3	0	2	0	2													
CAB	3	0	1	0	2	0												
RSP	4	1	5	0	6	3	2											
BLD	2	0	1	1	1	0	0	3										
KED	1	0	0	0	1	0	0	3	0									
ORJ	1	1	0	4	0	0	1	2	2	0								
KLS	2	2	3	0	2	1	2	0	1	2	1							
KOR	1	4	0	3	0	2	2	6	0	1	1	4						
GLB	3	3	7	0	5	2	2	1	3	4	2	3	7					
PRL	0	4	0	3	0	2	2	5	4	2	0	2	1	5				
LUR	6	3	4	3	4	4	5	0	4	4	3	1	4	2	3			
GJL	5	6	7	6	4	5	4	6	5	6	3	6	6	4	4	2		
TMR	12	12	13	6	12	10	12	9	8	10	8	10	12	5	10	2	6	
ZVL	6	5	6	3	5	4	5	3	4	5	2	3	8	2	4	0	0	4

Grey: 6–9 traits; orange: 10 or more traits.

observed between ratio LA/WA and seed characters (Table 3).

One-way ANOVA displayed significant differences between mean values of morphological traits for all populations at a statistically significant level with $p \leq 0.001$ (electronic supplementary materials, Table S1). As revealed by Tukey's post-hoc test, the total number of observed significant differences among populations is 547 (Table 4). The Albanian population TMR differed considerably, with the highest total number of significant differences (161) per morphological trait compared to the others (Table 4). Also, the Albanian population GJL followed a similar pattern and differed in most of the morphological traits (85 differences) compared to other populations (Table 4).

Population differentiation pattern

Principal Component Analysis based on the population matrix generated four significant principal components (three components are displayed). They accounted for 77.39% of the total variance (PC1 = 53.41%, PC2 = 17.54%, PC3 = 10.04%, PC4 = 6.4%) with moderate correlation to the majority of corresponding morphological traits (Table 5). The seed-related variables (LS, WW, LWS and WS) had the highest contribution to PC1; derived ratio traits (LA/WA and LS/WS) together with LC, DC and LCS mainly contributed to PC2; the ratio LCS/WCS had the highest contribution to PC3 (Table 5). Populations were separated mostly along the first and second axes (Fig. 3A). The PC1 separated the majority of the Dinaric from the Scardo-Pindic populations, and the PC2 showed separation between most of the Albanian and Kosovo populations from the rest. The

Dinaric populations were intermingled with KOR and PRL accessions that were initially assigned to the Scardo-Pindic group. The Tomorr population was the most differentiated in relation to all others (Fig. 3A).

Cluster analysis using Ward's method (Fig. 3B) generated two major clusters and showed a similar pattern to the PCA based on the population matrix. The first cluster contained two subgroups, one containing populations from Bosnia and Herzegovina and Kosovo (intermixed Dinaric and Scardo-Pindic) and the second one containing populations solely from the Dinaric mountains (Bosnia and Herzegovina and Montenegro) (Fig 3B). The second major cluster contained populations originating from Albania and Kosovo, which all corresponded to the Scardo-Pindic group (Fig. 3B).

The PCA based on the individual matrix displayed a similar pattern to the one based on the population matrix. Morphological variation was complex and was explained by five principal components with Eigenvalues > 1 (PC1 = 34.92%; PC2 = 16.9%; PC3 = 11.58%; PC4 = 8.06%; PC5 = 6.61%), which accounted for 76.78% of the total variance (three components are displayed). A pattern of separation among individuals originating from the Dinaric and Scardo-Pindic populations was evident (Fig. 3C). Most of the individuals from populations Koritnik and Prelevac were interspersed within the Dinaric cluster (Fig. 3A and C). On the other hand, the individuals from the Tomorr population were shown to be the most differentiated of all and constituted a unique group (Fig. 3C). The same variables similarly contributed to principal components as in the PCA based on the population matrix (Table 5).

The CDA model was significant (Wilks' λ = 0.434, $F_{10, 176}$ = 22.56, $p < 0.00001$) and included 10

Table 5. Principal components (PC) revealed by the Principal Component Analysis (PCA) for the *Pinus heldreichii* populations

|	Population matrix			Individual matrix		
	PC1	PC2	PC3	PC1	PC2	PC3
LC	0.265	0.332	0.117	0.294	0.325	−0.139
DC	0.238	0.325	0.208	0.253	0.325	0.135
LWS	0.310	0.103	−0.095	0.313	0.090	−0.217
WW	0.322	−0.014	0.083	0.330	−0.002	0.162
LS	0.323	−0.146	−0.037	0.338	−0.125	0.029
WS	0.302	−0.280	0.036	0.315	−0.323	0.219
TS	0.298	−0.134	0.191	0.293	−0.152	0.308
LCS	0.192	0.330	0.357	0.201	0.358	0.313
WCS	0.251	0.219	−0.225	0.263	0.120	−0.213
LA	0.068	0.328	−0.350	0.127	0.296	0.051
WA	0.296	−0.121	−0.130	0.279	−0.157	−0.100
TCS	0.277	−0.119	−0.242	0.261	−0.088	−0.058
LC/DC	0.218	0.244	−0.072	0.180	0.132	−0.434
LS/WS	−0.163	0.375	−0.126	−0.079	0.358	−0.306
LCS/WCS	−0.092	0.122	0.669	−0.080	0.248	0.535
LA/WA	−0.193	0.385	−0.209	−0.163	0.410	0.133
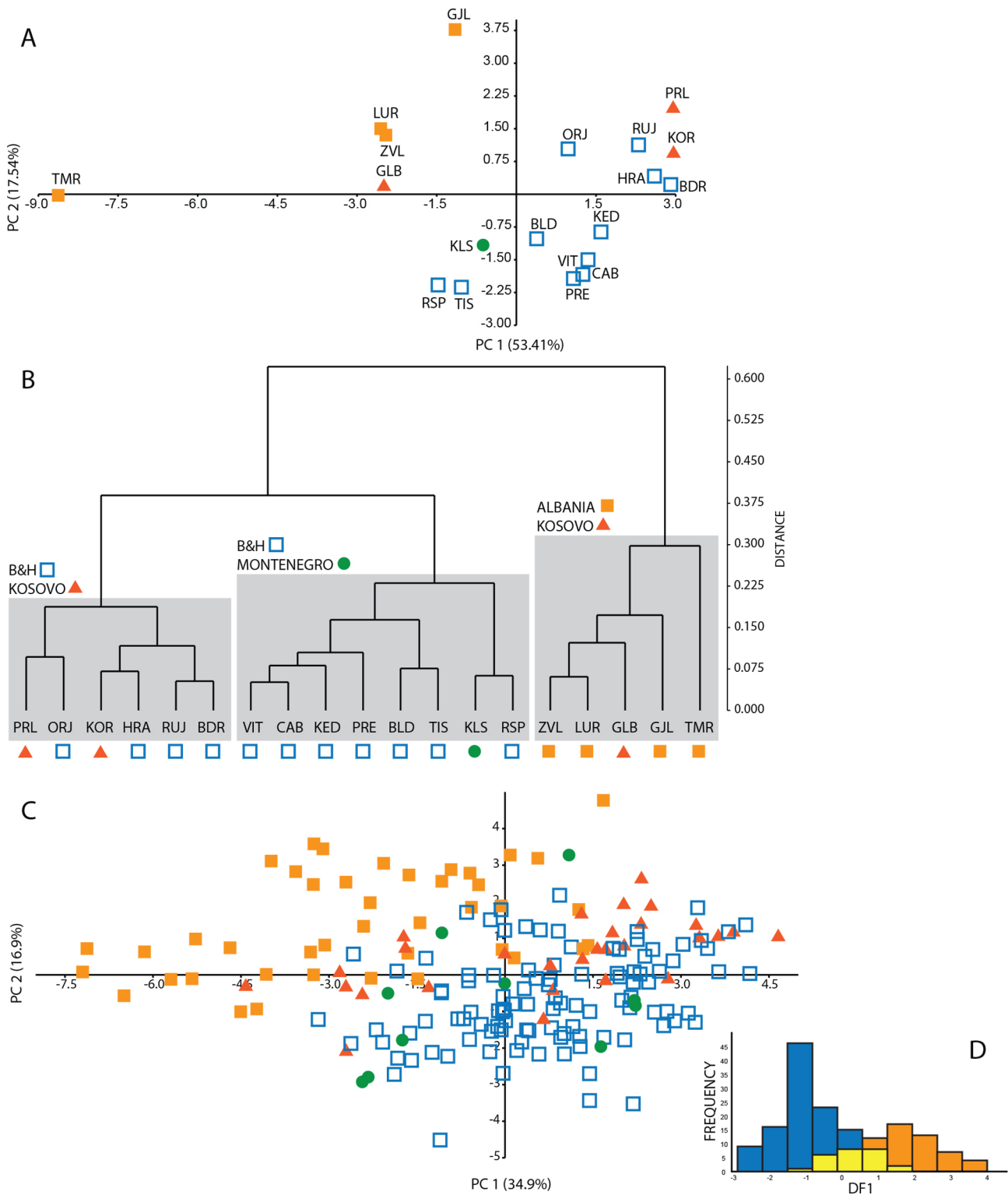
Fig. 3. Results of multivariate analyses for 19 analysed *Pinus heldreichii* populations: A – PCA ordination of morphological traits for populations; B – dendrogram showing morphological relationships among populations using Ward's method; C – PCA ordination of morphological traits for individuals; D – distribution graph of individual canonical scores for assigned groups (Dinaric vs. Scardo-Pindic). Acronyms, symbols and colours correspond to those in Fig. 1 except for D where blue represents Dinaric populations, yellow represents incorrectly assigned individuals and orange represents Scardo-Pindic populations

morphological variables. A canonical axis with low Eigenvalue (1.30) was extracted explaining a total variance among the two groups. The distribution of individuals along the first canonical axis with no clear gap was displayed on the distribution graph (Fig. 3D). The seed traits mainly revealed discrimination between the two groups (TCS and LS, partial Wilks' λ values: 0.88 and 0.92, p < 0.0000) followed by ratio AL/AW (partial Wilks' λ = 0.96, p < 0.0008). The results of classificatory discriminant analysis favoured weak discrimination between the two groups, resulting in 86.6% correct classification of all cases (N = 187), while 89% (N = 106) of the Dinaric specimens were correctly determined *versus* 82% (N = 56) of the Scardo-Pindic specimens. The highest number of incorrectly assigned individuals for the Scardo-Pindic group were determined for KOR (N = 7) and PRL (N = 3) populations, and the Dinaric populations HRA (N = 3) and RUJ (N = 3). The results of the Mantel test showed a very weak but significant relationship between the Mahalanobis and geographic distances (r = 0.16, p <0.05), but no significant relationship was determined between Euclidean and geographic distances (r = 0.17, p < 0.06).

Discussion

Variation of individual morphological traits

Cone and seed variation of *P. heldreichii* covering the western marginal populations and those from the central part of the species distribution range (Fig. 1) indicated a relatively low morphological plasticity mirrored in significant differences among populations and geographic structuring of populations. Correlations between examined quantitative traits of *P. heldreichii* populations showed that the seed traits were positively correlated with the length and diameter of cone and apophysis traits. As expected, the populations with smaller cones had smaller seeds. The pattern of variation of seed traits in this study, evidenced from both uni- and multivariate analyses, demonstrated that southern populations (Scardo-Pindic group) had lower values than the northern ones (Dinaric group) (Table 2), although some accessions from both groups (Rosne poljane – RSP, Obli svitavac – ORJ and Zhur – KOR) deviated from this pattern. This is in contrast with a common trend of seed size decreasing along the latitudinal gradient (Moles & Westoby, 2003). The same was documented for wind-dispersed *Pinus* spp., which is in line with the general hypothesis that pine species with smaller seeds might have a larger latitudinal range, having an advantage in colonizing northern latitudes after the retreat of glaciers (Grotkopp et al., 2004).

Different patterns of cone and seed variation were observed among Mediterranean pines (Boulli et al., 2001; Gil et al., 2002; Dangasuk & Panetsos, 2004; Wahid et al., 2006; Bogunić et al., 2007). Seed-related traits did not display any correlations with different eco-geographic variables, but strong associations between latitude/longitude were observed for cone and needle traits implying a clinal pattern of variation in *P. pinaster* Ait. (Wahid et al., 2006). Furthermore, no correlation was found between seed related traits (seed mass, height, length, width) and latitude/longitudes across the entire Mediterranean range of *P. nigra* Arnold (Bogunić et al., 2007). Contrarily, seed and cone variation in *P. halepensis* Mill. showed a strong clinal pattern of variation across the native range in Morocco (Boulli et al., 2001), where a strong positive correlation between seed related traits and latitude, longitude and altitude was found. A similar pattern was detected for *P. brutia* Ten. as well (Dangasuk & Panetsos, 2004).

The seed size and seed mass are linked with growing conditions and age. For this reason, they positively influence plant physiology by producing more vigorous seedlings (Dunlap & Barnett, 1983). The observed variation pattern for seed traits in our study might be explained by an interaction of several factors. Generally, the populations of the Scardo-Pindic mountains thrive in more xeric conditions and higher altitudes, mostly on acid siliceous soils. Contrarily, the populations of the Dinaric Alps predominantly inhabit sites with mesic conditions on basic calcareous soils with pronounced water deficiency. Thus, the adaptive significance of production of larger seeds in xeric conditions, with more nutrient and mineral content, will enhance chances for higher seedling establishment, growth and survival in early phases of their development (Bladé & Vallejo, 2008). Production of larger seeds with bigger wings means a better dispersal capacity of the populations (Gil et al., 2002). Certainly, this explanation refers to the observed trend because the seed variation also depends on local factors, as shown by the present study. Some authors considered a variation in seed functional morphology as a result of ecological and physiological specialization of the species (Boulli et al., 2001; Dangasuk & Panetsos, 2004; Wahid et al., 2006), while others considered it as phylogenetically constrained (Grotkopp et al., 2004; Leslie et al., 2017). All in all, seed-related traits (seed size, mass, number, dispersal mode and growth rate contribution) are recognized as key determinants affecting life-history strategies in pines and other conifers (Grotkopp et al., 2004).

A notable association between seed traits and apophysis traits (WA and TCS) is evidenced (Table 3). The apophyses (particularly their thickness) are considered to have adaptive significance related to the mechanism of cone opening and the resistance

of closed cones to high temperatures (Dawson et al., 1997). Fire has had a vital role in shaping the native range of *P. heldreichii* throughout the species history (Panayotov et al., 2010). Although fire has been a dominant factor influencing the distribution range dynamics of *P. heldreichii*, no evidence of cone serotiny (mirrored in apophysis traits) as an adaptation to fire was observed. Cone serotiny is a life-history strategy for adaptation of pines to intense fire (Dawson et al., 1997). In the absence of clear evidence, the patterns of association between apophyses and seed traits in *P. heldreichii* are presumably connected with a function of allometric growth of cones.

Geographic pattern of morphological variation

Multivariate analyses pointed out the general pattern of distinction of populations originating from the Dinaric Alps in relation to the Scardo-Pindic mountains (Fig. 3A–D). This pattern was previously demonstrated by Nikolić et al. (2015, 2019). In the first study, the authors had analysed the terpene composition and variation of the needle oils in *P. heldreichii* from the Scardo-Pindic populations Ošljak, Mt. Sharr (Kosovo) and Mt. Galičica (Northern Macedonia), and confirmed geographic differentiation from the Dinaric populations (Nikolić et al., 2015). Afterwards, using the material from the same populations, they conducted an analysis of morpho-anatomic variability of needles, which confirmed the geographic differentiation but with a lower resolution compared to terpene markers (Nikolić et al., 2019). Differentiation between the populations of *P. heldreichii* from the two mountain massifs at chemical and morpho-anatomic level was argued in the context of different geological substrates and climatic conditions (Nikolić et al., 2015, 2019). In our study, based on a larger sample and using reproductive morphological traits less prone to the influence of environmental conditions, similar geographic differentiation of populations from the Dinaric Alps and Scardo-Pindic mountains was observed. This pattern may actually be translated into geographic differentiation along the north-western to south-eastern gradient. The obtained differentiation was not sharp or discontinuous, and due to the presence of certain populations the observed pattern throughout the studied area could not be confirmed. Certain inconsistencies were present, namely, populations Zhur (KOR) and Prevelac (PRL) were clearly nested within the Dinaric group (Fig. 3A–C). The corresponding inconsistency in cone and seed variation implies that the current pattern of variation can be a result of intermixing separate lineages at this part of the range or may simply mirror the adaptation to local environmental

conditions. Absence of clear geographic structuring of populations is not rare in conifers with discontinuous distribution and complex life history (Gil et al., 2002; Brus et al., 2010; Sękiewicz et al., 2016).

Although multivariate analyses confirmed the weak but significant correlation between morphological variation and geographic distances of *P. heldreichii* populations, this pattern does not necessarily imply causality. Geographic isolation (to mountain ranges) between the Dinaric and Scardo-Pindic populations caused by orographic barriers and followed by adaptation to unfavourable climatic conditions (the cold and dry environment) could affect the complex pattern of variation within the sampled area of the species. The modern distribution and life history of Bosnian pine have been closely related to historical climatic conditions in the Peninsula. During the last glaciation, as did many other trees, the Bosnian pine presumably survived in mid- to high-altitude sites, areas with probable humid conditions (Willis, 1994), from where it started expanding its range. Severely fragmented *P. heldreichii* populations, restricted to high mountain ranges and summits across the Balkans, suggest complex distribution range dynamics throughout glacial and postglacial history. Long periods of isolation in different Pleistocene refugia throughout the Dinaric Alps, Scardo-Pindic massif, Balkan-Rhodope massif and southern Apennines, contrasting climatic conditions, different fire frequencies and anthropogenic influences drove dissimilar evolutionary processes within populations (Thompson, 2005; Panayotov et al., 2010; Hewitt, 2011). It is intriguing that Italian and Greek populations of *P. heldreichii* belonged to the same ancestral genetic lineage despite their separation for more than 15,000 years (Boscherini et al., 1994).

Bulgarian populations, which belong to the Balkan-Rhodope mountain system, showed a dissimilar structural pattern of population variation, which Naydenov et al. (2005) explained as a consequence of historical events as well as topography within high altitudes between watersheds during the glaciations, which prevented gene flow between the populations. Our results may go in favour of the 'refugia-within-refugia' hypothesis (Gómez & Lunt, 2007) which assumes the existence of multiple small refugia dispersed throughout the Balkan Peninsula. The presence of more than two refugia in the Balkans was identified in research on *Juniperus oxycedrus* L. subsp. *oxycedrus* (Brus et al., 2010), *Daphne blagayana* Freyer (Fišer Pečnikar et al., 2017), *Edraianthus tenuifolius* A.DC. (Glasnović et al., 2018) and *Salvia officinalis* L. (Jug-Dujaković et al., 2020). Presumably, the populations of Bosnian pine following the glaciation cycles were physically isolated and gene flow was restricted. Afterwards, during the postglacial periods, populations from multiple refugia most likely immigrated

upward and established themselves in the high mountain ridges and summits due to their better colonizing capacities in extreme environments relative to other forest tree species, which were more competitive in moderate and mesic habitats. In that context, the southernmost population Tomorr (TMR) showed remarkable morphological distinctiveness in relation to the Dinaric and to all other studied populations as well (Fig. 3A–C). Although we assumed the existence of two groups (northern and southern) in the study, population Tomorr (TMR) appeared as a distinct morphological group which most likely represents a specific southern *P. heldreichii* lineage.

Concluding remarks

Activities concerning selection of seed sources for forestry practice should consider the observed north-western to south-eastern gradient of variation in this region since the seed traits harbour genetic variation and adaptation of local populations (Ballian & Kajba, 2011). Special attention should be also paid to a high level of self-fertilization, which leads to inbreeding in small *P. heldreichii* populations (Morgante et al., 1991), and consequently a loss of genetic diversity. In that context, the 'dispersed sampling' model (Hoban & Schlarbaum, 2014) should be the most appropriate one, as it would ensure an adequate level of genetic diversity for seed collections in regeneration processes and reintroductions. The 'dispersed sampling' model was introduced to preserve genetic diversity in taxa having relatively small to moderate population size and poor geographic connections between the populations. Under this model, moderate sampling (from 25−30 individuals per population) from few but widely-spaced populations should ensure optimal performance (Hoban & Schlarbaum, 2014).

In absence of solid molecular and palaeobotanical evidence, morphometric analyses prove to be useful tools to understand the patterns of variation and relationships among the populations. Further studies based on more morphological characters and DNA markers should encompass the entire species range in order to advance understanding of the overall variation of this relict pine.

Acknowledgments:

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