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DIVERSITY AND STRUCTURE OF CROATIAN CONTINENTAL AND ALPINE-DINARIC POPULATIONS OF GREY ALDER (*Alnus incana* /L./ Moench subsp. *incana*): ISOLATION BY DISTANCE AND ENVIRONMENT EXPLAINS PHENOTYPIC DIVERGENCE

RAZNOLIKOST I STRUKTURIRANOST HRVATSKIH KONTINENTALNIH I ALPSKO-DINARSKIH POPULACIJA BIJELE JOHE (*Alnus incana* /L./ Moench subsp. *incana*): GEOGRAFSKA I OKOLIŠNA IZOLACIJA KAO UZROK FENOTIPSKE DIVERGENCIJE

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Summary

We studied the morphological variation and its correlation to the environment in a boreal tree species, *Alnus incana* (L.) Moench subsp. *incana*, across the Croatian continental and Alpine-Dinaric biogeographical regions. A total of seven grey alder populations from environmentally divergent habitats were included in the study. We combined descriptive and multivariate statistical methods by using morphological leaf traits and environmental features to examine the diversity and structure of grey alder populations. High phenotypic variation, and two distinct morphotypes were found: small-leaf in the Alpine-Dinaric region, and large-leaf in the continental region. We identified a biogeographical structuring of populations with a high level of among-tree variation within the populations. Multivariate phenotypic and environmental analysis confirmed the existence of small- and large-leaf ecotypes. Additionally, a lack of significant among-population variation within biogeographical regions was observed. Furthermore, we found that phenotypic divergence of the studied grey alder populations can be explained as a result of a significant level of isolation both by distance and by environment.

KEY WORDS: phenotypic variation, leaf morphology, adaptation, ecological divergence, clinal variation, altitudinal gradient

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INTRODUCTION
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Alnus incana (L.) Moench subsp. *incana* (hereafter *Alnus incana*), family *Betulaceae*, is a diploid (2n = 2x = 28), monoecious and wind-pollinated, relatively short-lived, small to medium-sized deciduous tree (Tallantire 1974; Mandák *et al.* 2016; Vít *et al.* 2017). It is a light-demanding, hygromesophilous species which tolerates low winter temperatures. The grey alder can be found on stream banks, lake shores, damp meadows and in wet mountain environments at elevations of up to 1800 m (Schütt and Lang 2014; Houston Durrant *et al.* 2016). Along with all other alder species, it is unique within the *Betulaceae* family since it can fix nitrogen in symbiotic root nodules with the actinomycetes bacteria from the genus *Frankia* (Benson *et al.* 2004; Vanden Heuvel 2011). In addition, it is an important pioneer species with the ability of fast regeneration and colonization of disturbed or damaged areas. Similar to the Norway spruce (*Picea abies* /L./ H.Karst), the range of the grey alder is divided into a northern (subsp. kolaensis /N.I.Orlova/Á.Löve et D.Löve) and a southern area, which meet in the Polish lowlands (Douda *et al.* 2014). The distribution of subsp. *incana* in the southern part of the range is linked with a patchy mountain occurrence (in the Alps, the northern Apennines, the Hercynian mountains, the Carpathians, the Bulgarian mountains, the Dinaric Alps, the Caucasus, and mountains in Turkey) and continues eastward across European Russia to western Siberia (Mandák *et al.* 2016). Grey alder is represented by two subspecies in North America: subsp. *rugosa* (Du Roi) R.T.Clausen and subsp. *tenuifolia* (Nutt.) Breitung.

In Croatia, *A. incana* inhabits north-western and north-eastern parts of the country at the southern border of its natural range of the Central European mountains (Vukelić and Rauš 1998; Vukelić 2012; Trinajstić 2008; Poljak *et al.* 2014). This part of the grey alder's natural distribution is completely separated from its southern natural range in the Balkan Peninsula. Here, the grey alder appears in two biogeographical regions with contrasting climates: the continental region, along the course of the Drava river; and in the mountainous Alpine-Dinaric region in Gorski kotar, along the course of the Kupa river and its tributaries. This biogeographical divergence caused by strong climatic gradients and complex topography resulted in a clear floristic differentiation of grey alder stands in Croatia.

In the continental region of Croatia, the grey alder occurs mainly in riparian and floodplain forests along the main watercourse of the river Drava, where it forms smaller and isolated populations. These populations are included within the association *Equiseto hyemali-Alnetum incanae* Moor 1958 (Trinajstić 1964; Franjić *et al.* 1999). This association is characterized by the presence of hygrophilous species, such as: *Fraxinus angustifolia* Vahl subsp. *oxycarpa* (Willd.) Franco et Rocha Afonso, *Ulmus laevis* Pall., *U. minor* Mill., *Prunus padus* L., *Equisetum hyemale* L., and others. The former forest areas that were studied by Trinajstić (1964) are today no longer covered by forest. Riparian and floodplain forests close to urban centres are being dried out, and their habitats are transformed into residential areas and municipal infrastructure so that grey alder forests along the course of the Drava river are nowadays in strong regression (Vukelić 2012; Poljak *et al.* 2014).

Stands from the north-western Dinarides are defined within the association *Lamio orvalae-Alnetum incanae* Dakskobler 2010, where a geographic variant with *Helleborus dumetorum* is described (Vukelić *et al.* 2012, 2017). In general, the main characteristic of these stands is floristic heterogeneity and high richness of species, mainly as a consequence of the biogeographical position and floristic development of the western Dinarides (Bennett *et al.* 1991; Petit *et al.* 2003; Magri *et al.* 2006; Liepelt *et al.* 2009; Temunović *et al.* 2013) – one of the floristically richest forest areas in Europe. The following species are particularly prominent: *Fraxinus excelsior* L., *Ulmus glabra* Huds., *Fagus sylvatica* L., *Acer pseudoplatanus* L., *Lamium orvala* L., *Scopolia carniolica* Jacq., *Helleborus dumetorum* Waldst. et Kit., *Lunaria rediviva* L., *Knautia drymeia* Heuff. subsp. *drymeia*, and others. The occurrence and growth of grey alder forests in this region is conditioned by occasional flooding of the river Kupa and its tributaries over a length of about thirty kilometres. The river partially flows through a canyon and partially extends along to horizontally elongated terraces, on which forests were cut down in the past. In the last 50 years, land cultivation has been gradually abandoned, giving place to natural succession of vegetation.

To date, several genetic studies have been carried out on the grey alder. Microsatellite primers specific for the *A. glutinosa* (L.) Gaertn and *A. incana*, developed by Lepais and Bacles (2011), were used to evaluate the population genetic structure and diversity of 65 populations, and to reconstruct the historical pattern of postglacial biogeographical range expansion of the boreal tree species *A. incana* in Europe (Mandák *et al.* 2016). Furthermore, genetic diversity and clonal structure of 24 populations covering the Central European part of the species range from the Scandinavian Peninsula to the Balkans were analysed utilizing four nuclear microsatellite markers (Dering *et al.* 2016). Additionally, several studies have addressed the morphological variability of grey alder populations (Krauze-Michalska and Boratyńska 2013; Poljak *et al.* 2014). Krauze-Michalska and Boratyńska (2013) revealed a high level of morphological variation of leaves and significant differences between the Scandinavian and all other European populations of *A. incana*. By contrast, in our previous research of grey alder populations in Croatia (Poljak *et al.* 2014), along the upper course of the Drava river, the absence of inter-population variability was observed. However, no research of the grey alder's morphologic variation on a larger geographical scale in Croatia has been performed thus far.

In this study, we combined descriptive and multivariate statistical methods using the morphological leaf traits and environmental features to examine the diversity of grey alder populations distributed across the Croatian continental and Alpine-Dinaric biogeographical regions. Our main objectives were: 1) to reveal the levels of population variation and divergence within and among natural populations of *A. incana*; and 2) to test correlations between geographic, environmental and morphological variation.

MATERIAL AND METHODS
MATERIJAL I METODE

Plant material and leaf characters studied – *Biljni materijal i istraživane značajke lista*

The material for morphometric analysis was collected in July 2016 in seven natural populations of *A. incana* in Croatia (Figure 1). The study encompassed four populations from the Alpine-Dinaric region (P01 - Gerovo, P02 – Turke, P03 – Čedanj; P04 – Lukovdol), and three populations from the continental region (P05 – Varaždin, P06 – Slatina; P07 – Donji Miholjac). Each of the studied populations was represented by 20 trees and each tree by 20 healthy and undamaged leaves of short shoots from the external, sunlit part of the crown. The minimum distance between individual trees was at least 50 m, to avoid the sampling of close relatives. Vouchers for the populations studied were deposited in the herbarium at the Faculty of Forestry of the University of Zagreb (DEND).

The leaves were scanned and measured using the WinFolia program (WinFolia TM2001). A total of nine characteristics were measured (Figure 2). The accuracy of measurements was 0.1 mm, and the following morphological characteristics

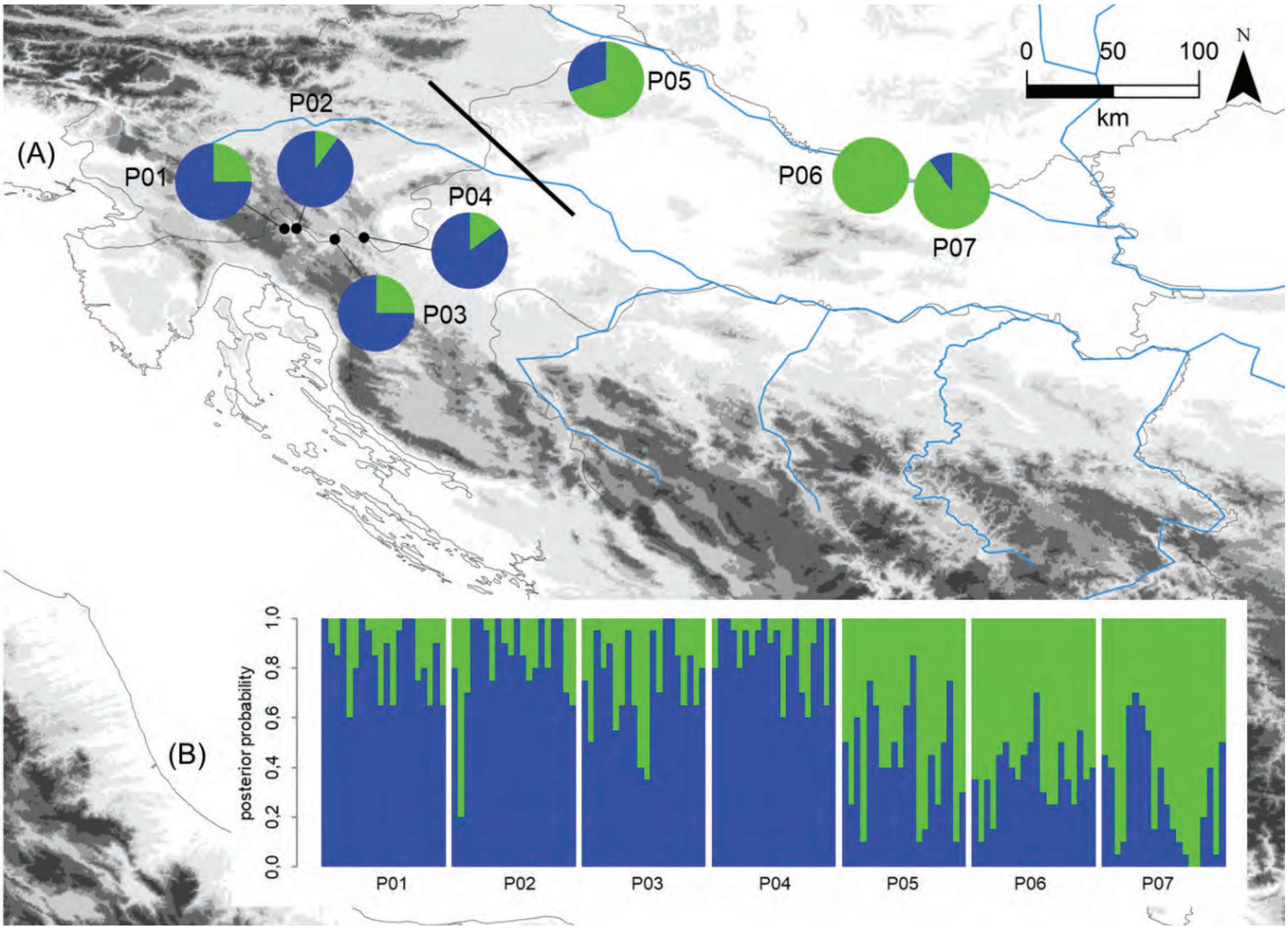

Figure 1. Locations of the seven sampled *Alnus incana* populations. (A) Geographical distribution of two groups of populations detected from K-means clustering (the proportions of the ancestry of each population in each of the defined clusters are colour-coded: cluster A–blue, cluster B–green); and (B) barplot with posterior probabilities of classification of each individual into each group from the results of the classification discriminant analysis. The main barrier as obtained by Barrier 2.2 is also shown. Acronyms of populations: P01 – Gerovo, P02 – Turke, P03 – Čedanj, P04 – Lukovdol, P05 – Varaždin, P06 – Slatina, P07 – Donji Miholjac.

Slika 1. Lokacije uzorkovanja sedam populacija bijele johe. (A) Geografski raspored dvije skupine populacija na temelju K-means klasterske metode (udjeli porijekla svake populacije u svakom od definiranih klastera određeni su bojom: klaster A – plava, klaster B – zelena); (B) Barplot s vjerojatnostima klasifikacije svakog stabala u svaku od istraživanih skupina temeljen na rezultatima klasifikacijske diskriminantne analize. Na slici je prikazana barijera protoka gena između sedam istraživanih populacija bijele johe utvrđena Monmonierovim algoritmom maksimalne razlike na temelju Euklidske udaljenosti. Akronimi populacija: P01 – Gerovo, P02 – Turke, P03 – Čedanj, P04 – Lukovdol, P05 – Varaždin, P06 – Slatina, P07 – Donji Miholjac.

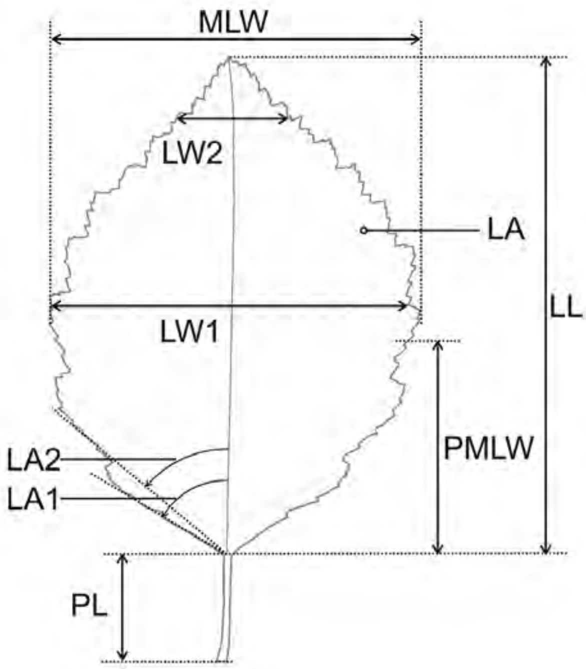

Figure 2. Measured leaf traits: leaf blade area (LA); leaf blade length (LL); maximum leaf blade width (MLW); leaf blade length, measured from the leaf base to the point of maximum leaf width (PMLW); leaf blade width at 50% of leaf blade length (LW1); leaf blade width at 90% of leaf blade length (LW2); angle closed by the main leaf vein and the line defined by the leaf blade base and a point on the leaf margin, at 10% of leaf blade length (LA1); angle closed by the main leaf vein and the line defined by leaf blade base and a point on the leaf margin, at 25% of leaf blade length (LA2); petiole length (PL).

Slika 2. Mjerene značajke lista: površina plojke (LA); dužina plojke (LL); maksimalna širina plojke (MLW); dužina plojke, mjerena od osnove plojke do mjesta najveće širine plojke (PMLW); širina plojke na polovici dužine plojke (LW1); širina plojke na 90 % dužine plojke (LW2); kut koji zatvaraju glavna lisna žila i pravac definiran osnovom plojke i točkom na rubu lista, koja se nalazi na 10 % dužine plojke (LA1); kut koji zatvaraju glavna lisna žila i pravac definiran osnovom plojke i točkom na rubu lista, koja se nalazi na 25 % dužine plojke (LA2); dužina peteljke (PL).

were measured on each leaf: leaf blade area (LA); leaf blade length (LL); maximum leaf blade width (MLW); leaf blade length, measured from the leaf base to the point of maximum leaf width (PMLW); leaf blade width at 50% of leaf blade length (LW1); leaf blade width at 90% of leaf blade length (LW2); angle closed by the main leaf vein and the line defined by the leaf blade base and a point on the leaf margin, at 10% of leaf blade length (LA1); angle closed by the main leaf vein and the line defined by leaf blade base and a point on the leaf margin, at 25% of leaf blade length (LA2); and petiole length (PL). Finally, a total of 2800 leaves, dried in a herbarium, were measured, and 25 200 simple data values were obtained.

Statistical analyses – *Statističke analize*

The measured morphological characteristics were described by standard descriptive statistical parameters: arithmetic mean, standard deviation, coefficient of variation (CV%), and the percentiles 0% (minimum), 5%, 25% (lower quartile), 50% (median), 75% (upper quartile), 95% and 100% (maximum). To assess the possibility of conducting multivariate statistical analyses and parametric tests, the symmetry, unimodality and homoscedasticity of data were verified (Sokal and Rohlf 2012). Assumptions of normality were checked using the Shapiro–Wilk test, and the assumption of homogeneity of variance using Levene's test. A hierarchical analysis of variance (ANOVA) was performed to examine the partition of phenotypic variation between the continental and the Alpine-Dinaric regions, among populations within the regions, and within populations.

The relationship between average values of morphological leaf characteristics and geographical longitude, latitude, and altitude (e.g. Krauze-Michalska and Boratyńska 2013) and the influence of climatic variables on leaf characteristics were tested using Spearman's coefficient (Sokal and Rohlf 2012). Climate data were obtained from the WorldClim database with a spatial resolution close to a square km (Hijmans *et al.* 2005). To evaluate the correlation between multicharacter differences among populations, a Mantel test (Mantel 1967) was performed on the matrices of Euclidean distances. First, the correlations among all 19 WorldClim bioclimatic variables and topographic variables for all presence points were calculated to exclude the highly correlated ones, whilst keeping the variables useful in predicting the distribution limits of trees, such as climatic averages and extremes (Zimmermann *et al.* 2009; Temunović *et al.* 2012). We computed and tested the correlations between: (1) the matrix of the geographical distances between pairs of populations and the matrix of morphological differences among populations – "isolation by distance" (Wright 1943); and (2) the matrix of environmental distances and the matrix of morphological differences among populations – "isolation by environmental distance" (Mendez *et al.* 2010). The significance level was assessed after 10,000 permutations, and the Mantel test was performed with the R package "Vegan" (Oksanen *et al.* 2017).

Multivariate statistical methods were used to identify the population differentiation (McGarigal *et al.* 2000). Pearson's correlation coefficient was used to identify interactions between leaf traits and to detect potential redundant variables, i.e. highly correlated variables were excluded from the analyses. The conducted cluster analysis resulted in a hierarchical tree, where the unweighted pair-group method with arithmetic mean (UPGMA) was used to join the clusters, and the Euclidean distance to define the distance between the studied objects. The K-means method was applied to detect phenotypic structure and define the number of K-groups that best explained the morphological variation of populations (e.g Douaihy *et al.* 2012; Boratyński *et al.* 2013; Sobierajska *et al.* 2016). In addition, the biogeographical structure of the studied populations was further analysed using the Monmonier's maximum difference algorithm, implemented in Barrier 2.2 software (Manni *et al.* 2004). A canonical discriminant analysis was performed to calculate the discriminatory power of characters among biogeographical regions. The proportion of correctly classified individuals into biogeographical regions was determined using classificatory discriminant analyses (Oksanen *et al.* 2017). To examine the level of environmental and phenotypic divergence between the studied biogeographical regions, principal component analysis (PCA) was used. The input data in multivariate statistical methods were previously standardized, i.e. standardization of characters to zero mean and unit standard deviation was performed prior to each multivariate analysis.

The above statistical analyses were conducted using the STATISTICA 8.0 statistical program (StatSoft, Inc. 2001), and "MorphoTools" R scripts in R v.3.2.2 (R Core Team, 2016) following the manual of Koutecký (2015).

RESULTS
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The results of the conducted descriptive statistical analysis are shown by biogeographical region (Figure 3). The highest mean values for all analysed characteristics were observed in the populations from the continental region. Coefficients of variation among populations for particular morphological traits were generally similar (data not shown). However,

Table 1. Hierarchical analysis of variance.
Tablica 1. Hijerarhijska analiza varijance.

Character Značajka	Variance component Sastavnice varijance	df	F	Percent of variation Postotak varijabilnosti	P-value P-vrijednost
LA	Region	1	35.19	29.85	0.0019
	Population (Region)	5	3.11	2.02	0.0109
	Tree (Region*Population)	133	7.45	16.62	0.0000
	Error			51.52	
LL	Region	1	35.79	21.92	0.0019
	Population (Region)	5	1.98	1.07	0.0863
	Tree (Region*Population)	133	7.48	18.84	0.0000
	Error			58.17	
MLW	Region	1	30.97	30.51	0.0026
	Population (Region)	5	3.56	2.48	0.0047
	Tree (Region*Population)	133	7.83	17.09	0.0000
	Error			49.92	
PMLW	Region	1	17.371	9.22	0.0088
	Population (Region)	5	1.725	0.81	0.1332
	Tree (Region*Population)	133	6.273	18.78	0.0000
	Error			71.19	
LW1	Region	1	33.30	29.75	0.0022
	Population (Region)	5	3.18	2.14	0.0096
	Tree (Region*Population)	133	7.82	17.35	0.0000
	Error			50.77	
LW2	Region	1	18.25	10.47	0.0079
	Population (Region)	5	1.41	0.60	0.2241
	Tree (Region*Population)	133	9.46	26.48	0.0000
	Error			62.45	
LA1	Region	1	14.92	8.57	0.0118
	Population (Region)	5	1.07	0.16	0.3786
	Tree (Region*Population)	133	14.54	36.94	0.0000
	Error			54.33	
LA2	Region	1	8.21	4.70	0.0352
	Population (Region)	5	1.12	0.24	0.3523
	Tree (Region*Population)	133	13.92	37.38	0.0000
	Error			57.69	
PL	Region	1	7.16	17.06	0.0457
	Population (Region)	5	9.82	10.14	0.0000
	Tree (Region*Population)	133	8.77	20.36	0.0000
	Error			52.44	

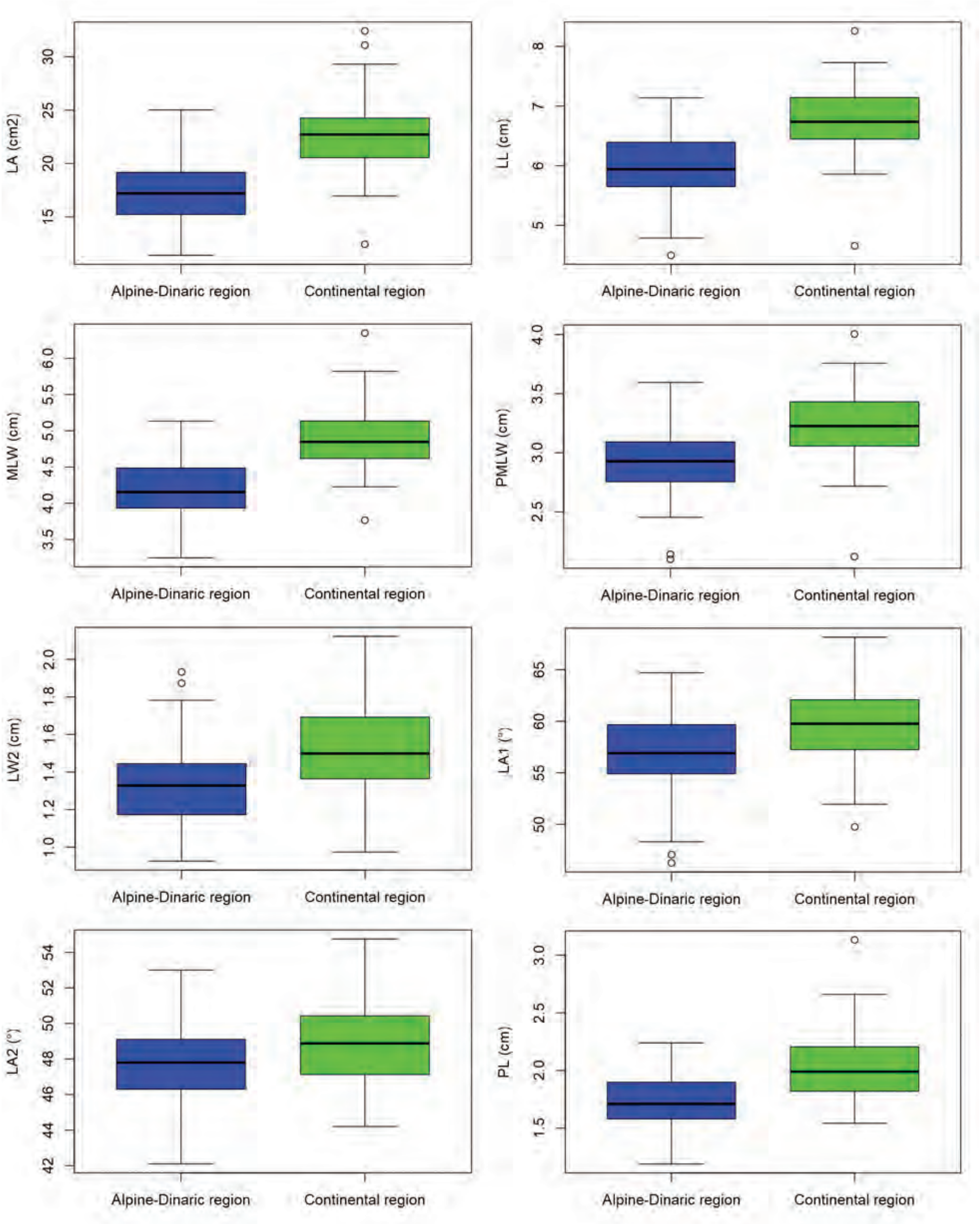

Figure 3. Morphological differentiation between continental and Alpine-Dinaric biogeographical regions. The box-and-whisker plot represents the observed measures for each region, with the centre bar indicating the median value. All the measured variables are presented except LW1, due to the occurrence of redundancy with the MLW.

Slika 3. Morfološka diferencijacija između kontinentalne i alpsko-dinarske biogeografske regije. Box-and-whisker dijagramima prikazani su deskriptivni statistički pokazatelji za mjerene značajke lista po regijama, na kojima središnja linija označava medijanu. Prikazane su sve varijable, osim LW1 zbog pojave redundantnosti sa značajkom MLW.

in the populations from the Alpine-Dinaric region, somewhat higher coefficients of variations were observed. The most variable characteristics with the CV above 25% were leaf blade surface (LA = 32.65%) and leaf blade width at 90% of leaf blade length (LW2 = 30.30%). In addition, a high degree of variability was also noted for petiole length

Table 2. Correlations between geographic, environmental, and morphological variables (level of significance).
Tablica 2. Korelacije između geografskih, okolišnih i morfoloških značajki (nivo signifikantnosti).

Variable Značajka	LA	LL	MLW	PMLW	LW1	LW2	LA1	LA2	PL
Latitude	0.54 (0.22)	0.64 (0.12)	0.61 (0.15)	0.64 (0.12)	0.61 (0.15)	0.64 (0.12)	0.61 (0.15)	0.57 (0.18)	0.75 (0.05)
Longitude	0.96 (0.00)	0.79 (0.04)	0.89 (0.01)	0.71 (0.07)	0.89 (0.01)	0.71 (0.07)	0.96 (0.00)	0.93 (0.00)	0.75 (0.05)
bio1	0.93 (0.00)	0.75 (0.05)	0.86 (0.01)	0.75 (0.05)	0.86 (0.01)	0.79 (0.04)	0.89 (0.01)	0.86 (0.01)	0.71 (0.07)
bio3	–0.89 (0.01)	–0.71 (0.07)	–0.79 (0.04)	–0.79 (0.04)	–0.79 (0.04)	–0.86 (0.01)	–0.86 (0.01)	–0.82 (0.02)	–0.64 (0.12)
bio5	0.93 (0.00)	0.75 (0.05)	0.86 (0.01)	0.75 (0.05)	0.86 (0.01)	0.79 (0.04)	0.89 (0.01)	0.86 (0.01)	0.71 (0.07)
bio6	0.60 (0.15)	0.51 (0.24)	0.60 (0.15)	0.47 (0.28)	0.60 (0.15)	0.36 (0.42)	0.33 (0.47)	0.44 (0.33)	0.40 (0.37)
bio12	–0.96 (0.00)	–0.79 (0.04)	–0.89 (0.01)	–0.71 (0.07)	–0.89 (0.01)	–0.71 (0.07)	–0.96 (0.00)	–0.93 (0.00)	–0.75 (0.05)
bio15	0.79 (0.04)	0.89 (0.01)	0.86 (0.01)	0.82 (0.02)	0.86 (0.01)	0.79 (0.04)	0.75 (0.05)	0.86 (0.01)	0.86 (0.01)
bio18	–0.94 (0.00)	–0.99 (0.00)	–0.99 (0.00)	–0.90 (0.01)	–0.99 (0.00)	–0.77 (0.04)	–0.81 (0.03)	–0.90 (0.01)	–0.95 (0.00)
bio19	–0.96 (0.00)	–0.79 (0.04)	–0.89 (0.01)	–0.71 (0.07)	–0.89 (0.01)	–0.71 (0.07)	–0.96 (0.00)	–0.93 (0.00)	–0.75 (0.05)
Altitude	–0.99 (0.00)	–0.89 (0.01)	–0.96 (0.00)	–0.82 (0.02)	–0.96 (0.00)	–0.79 (0.04)	–0.93 (0.00)	–0.96 (0.00)	–0.82 (0.02)
d-water	–0.89 (0.01)	–0.67 (0.10)	–0.76 (0.04)	–0.71 (0.07)	–0.76 (0.04)	–0.76 (0.04)	–0.87 (0.01)	–0.82 (0.02)	–0.60 (0.15)

with the total CV of 24.27%. As opposed to that, the lowest value of the coefficient of variation was found for the angle closed by the main leaf vein and the line defined by the leaf blade base and a point on the leaf margin, at 10% (LA1 = 10.46%) and 25% (LA2 = 7.82%) of leaf blade length.

The results of the hierarchical analysis of variance (ANOVA) for each characteristic are shown in Table 1. The studied regions differ significantly for all studied variables. Likewise, statistically significant differences among trees within populations were observed. The AMOVA analysis showed that most of the morphological diversity was attributable to the differences between regions, confirming the geographical structuring of populations. However, a highly significant percentage of variation was explained by the differences among individuals within populations for the variables: leaf blade length, measured from the leaf base to the point of maximum leaf width (PMLW); leaf blade width at 90% (LW2); petiole length (PL); and leaf angles LA1 and LA2. Among populations within regions, there were significant differences in four of the nine analysed traits (LA, MLW, LW1, PL), where only a small proportion of the total variation was explained.

As expected, strong correlations between leaf morphological traits were observed. Almost all measured leaf traits correlated with each other at a statistically significant level. The highest correlations, considering all populations, were found between leaf blade area (LA); leaf blade length (LL); maximum leaf blade width (MLW); and leaf blade width at 50% of leaf blade length (LW1). We also observed strong and significant correlations between the angles closed by the main leaf vein and the lines defined by leaf blade base and points on the leaf margin, at 10 % (LA1) and 25% (LA2) of leaf blade length. Non-significant correlations were found between petiole length (PL) and the above-mentioned leaf traits (LA1 and LA2).

Using Spearman's correlation coefficient (Table 2), a highly positive correlation was found between geographical longitude and the six measured variables (LA, LL, MLW, LW1, LA1, LA2). On the other hand, all measured variables were highly negatively correlated with the altitude and total precipitation of the warmest quarter of the year. Likewise, almost all leaf traits were significantly negatively correlated with the distance-to-water, annual mean precipitation, precipitation of the coldest quarter and isothermality. By contrast, almost all leaf traits were positively correlated with the annual mean temperature, maximum temperature of the warmest month and precipitation seasonality.

The Mantel test identified significant correlations between the morphological, geographical, and environmental distance matrices. Correlations were higher between morphological and geographical distance matrices ($r = 0.91$, $p = 0.0018$), and slightly smaller but still very strong between the morphological and environmental distance matrices ($r = 0.80$, $p = 0.0029$).

The structure of the seven grey alder populations was inferred by the K-means clustering method. The most probable division was detected at $K = 2$, and the estimated population structure is shown in Figure 1. If the proportion of a certain population was equal to or higher than 0.75, it was assumed that the population belonged to one cluster, and if it was lower than 0.75, it was assumed that the population had a mixed origin. The populations from the Alpine-Dinaric region grouped together into cluster A, and the populations from continental Croatia grouped together into cluster B. Only one population, P05 (Varaždin), was of mixed origin, with the dominant proportion from cluster B. The results obtained with the K-means clustering method were congruent with the hierarchical tree (Figure 4C), where the unweighted pair-group method with arithmetic mean (UPGMA) was used to join the clusters.

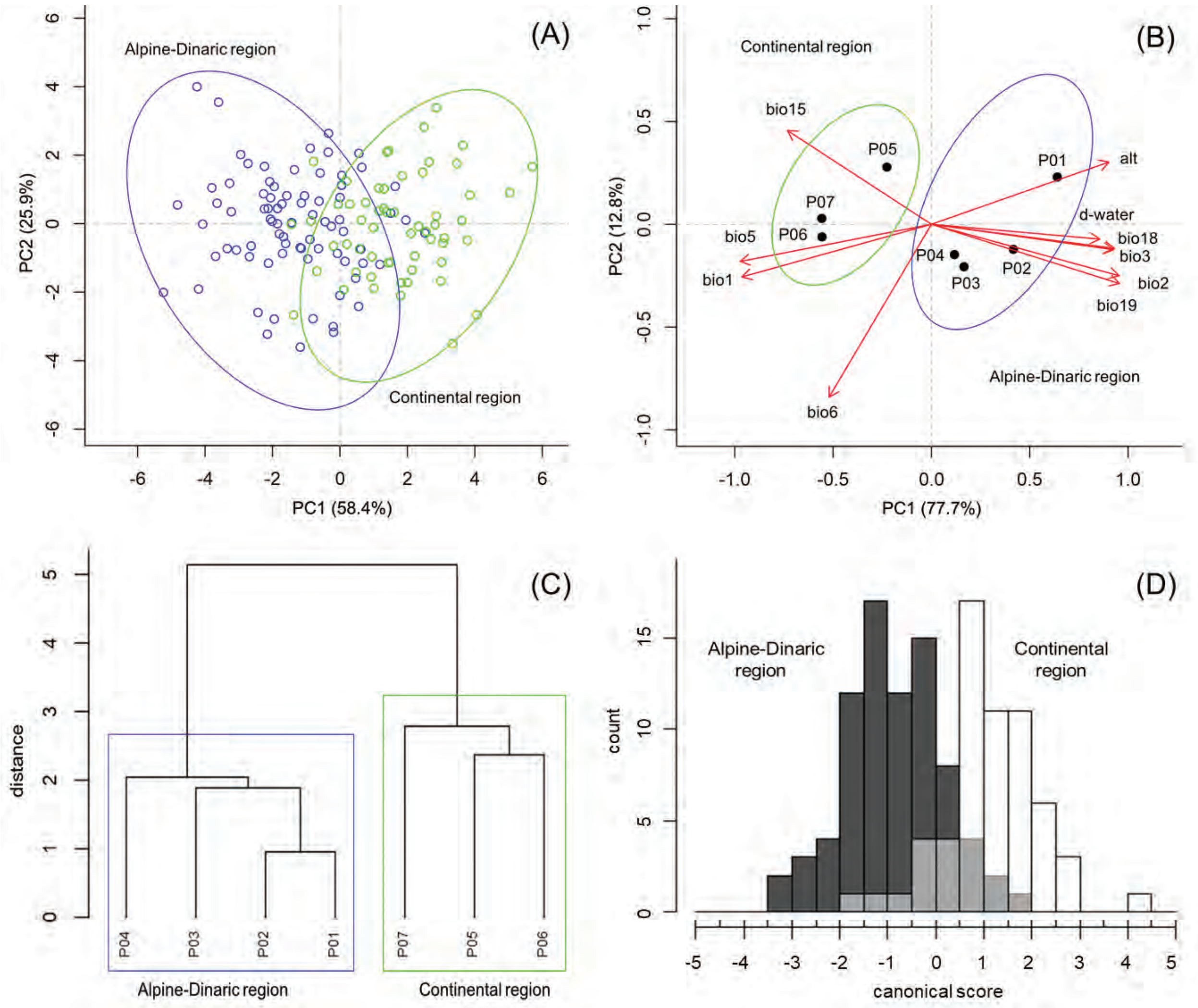

Figure 4. (A) Principal components analysis (PCA) ordination diagram of 140 *A. incana* individuals using morphometric leaf traits; (B) principal components analysis (PCA) ordination diagram based on environmental variables; (C) dendrogram constructed by the UPGMA method of cluster analysis on the closest Euclidean distances between analysed populations; (D) canonical discriminant analysis.

Slika 4. (A) PCA dijagram dobiven analizom glavnih sastavnica na temelju morfoloških značajki listova za 140 stabala bijele johe; (B) PCA dijagram dobiven analizom glavnih sastavnica na temelju okolišnih čimbenika za sedam lokaliteta bijele johe; (C) horizontalno hijerarhijsko stablo, pri čemu je za udruživanje klastera korištena UPGMA metoda, a za definiranje udaljenosti između istraživanih objekata Euklidova udaljenost; (D) kanonička diskriminantna analiza.

Table 3. Pearson correlation coefficients between ten environmental variables and scores for the first three principal components.

Tablica 3. Pearsonov koeficijent korelacije između deset sastavnica okolišnih značajki i vrijednosti prve tri glavne sastavnice.

Environmental variables Okolišne varijable		Principal component Glavne sastavnice		
		PC1	PC2	PC3
bio1	Annual Mean Temperature	0.9637	–0.2561	–0.0415
bio3	Isothermality (BIO2/BIO7) (*100)	–0.9314	–0.1172	0.3345
bio5	Max Temperature of Warmest Month	0.9723	–0.1810	–0.0919
bio6	Min Temperature of Coldest Month	0.5202	–0.8411	0.1459
bio12	Annual Precipitation	–0.9540	–0.2504	–0.0437
bio15	Precipitation Seasonality (Coefficient of Variation)	0.7317	0.4560	0.4562
bio18	Precipitation of Warmest Quarter	–0.9261	–0.1236	–0.0801
bio19	Precipitation of Coldest Quarter	–0.9560	–0.2883	–0.0271
Alt	Altitude	–0.9026	0.3027	–0.2448
d-water	Distance to Water	–0.8564	–0.0722	0.3873
Eigenvalue		7.7775	1.2855	0.5705
% of variance		77.77	12.85	5.70

The results of the discriminant analysis suggest that the differentiation between the studied biogeographical regions is significant. Although with some overlap, the analysis revealed a clear separation between the Alpine-Dinaric and continental populations (Figure 4D). The proportion of correctly classified individuals into two studied biogeographical regions "Alpine-Dinaric" and "continental" is shown in Figure 1B. The overall classification success was 87.1% of correctly classified individuals. Within the Alpine-Dinaric group, 88.7% of the individuals were included in the correct population group. A lower rate of correctly classified individuals was observed in the continental region (85.0%).

Principal component analysis (PCA) has clearly supported a separation of the continental and Alpine-Dinaric populations along the altitudinal, temperature and precipitation gradients (Figure 4B, Table 3). The results show that continental populations are characterised by low elevations and warmer habitats with higher temperatures and lower precipitation. By contrast, high-elevation populations from the Alpine-Dinaric region are distributed within cooler habitats with lower winter temperatures and higher precipitation. In addition, PCA analysis of all individuals has supported the divergence of two morphologically distinct groups (Figure 4A).

DISCUSSION
RASPRAVA

The results clearly demonstrate a high phenotypic diversity of grey alder populations in Croatia. In general, populations from the continental region had larger and wider leaves than populations from high altitudes in the mountainous Alpine-Dinaric region. In addition, large-leaf populations showed slightly lower variation possibly due to an overall reduction in the number of individuals, and fragmentation and isolation of populations due to human impact (Vukelić 2012; Poljak *et al.* 2014). On the other hand, environmental heterogeneity over very short distances within the Alpine-Dinaric region could result in higher phenotypic intra-population variability. A significant variability of morphological characteristics of the grey alder leaves was also reported by Krauze-Michalska and Boratyńska (2013), and Poljak *et al.* (2014). Nevertheless, in our previous study of grey alder populations, along the upper course of the river Drava, somewhat higher within-population variability was recorded. Furthermore, our results indicated that a natural hybridization has occurred between the common and grey alder in those populations, but at relatively low rates. However, these events may influence the diversity and structure of populations (Barton 2001; Poljak *et al.* 2017), and increase within-population morphological variability. In the current study, the above-mentioned influence was strongly reduced by examining the plant material for the analysis, i.e. hybrid individuals were subsequently excluded from the study.

The results of the hierarchical analysis of variance (ANOVA) were in line with the expectations of high morphological variation within populations and low differentiation between populations, as observed in alders (Krauze-Michalska and Boratyńska 2013; Poljak *et al.* 2014) and other wind-pollinated floodplain tree species (Jarni *et al.* 2011; Zebec *et al.* 2010, 2014). The relatively high level of among-tree variation within the populations is probably a result of both phenotypic adaptation to specific micro-environmental conditions experienced by each tree, and genetic differentiation among individual trees (Brus *et al.* 2011; Poljak *et al.* 2015). However, the AMOVA analysis showed that a great proportion of the total variation was attributable to the differences between regions, confirming the geographical structuring of populations. Moreover, most of the population pairs from both biogeographical regions had non-significant pairwise values (data not shown). The lack of significant among-population variation within biogeographical regions could be explained with the population longitudinal distribution along the rivers and floodplains with no barriers to gene flow, where free pollen and seed dispersion between the populations occurs (Temunović *et al.* 2012). More specifically, this can confirm the findings of our previous study of grey alder populations in Croatia (Poljak *et al.* 2014), along the upper course of the Drava river, where absence of inter-population variability was also observed.

The results of the hierarchical analysis of variance have been confirmed by multivariate statistical methods, suggesting the existence of a clear divergence between the populations from two biogeographical regions. Such differences in variability were also found in other woody species that occur in different biogeographical regions with contrasting climates (Škvorc *et al.* 2005; Temunović *et al.* 2012; Poljak *et al.* 2015; Zebec *et al.* 2016).

We found that the divergence among populations tended to follow an altitudinal cline along which populations from lower altitudes had larger and wider leaves than populations from high altitudes. In general, it is well known that leaf morphological traits, such as leaf length and width, are negatively correlated with altitude (Körner *et al.* 1986, 1989; Hovenden and Vander Schoor 2003; Bresson *et al.* 2011; Paridari *et al.* 2013). However, many environmental features accompany altitudinal changes to which plant populations adapt: those physically tied to meters above sea level, such as atmospheric pressure, temperature and clear-sky turbidity; and those that are not generally altitude specific, such as moisture, hours of sunshine, wind, season length, geology and even human land use (Körner 2007). In fact, our results showed that the leaf measured characteristics that varied in the grey alder populations were significantly related to climate features. Populations of the grey alder from high altitudes in the mountainous Alpine-Dinaric region occupy habitats with lower temperatures accompanied by high irradiance and larger temperature oscillations. On the other hand, low altitude populations from the continental region are exposed to higher temperatures, lower radiation and higher precipitation variability. In addition, growing season tends to increase toward warmer low-elevation sites. Furthermore, common-garden experiments have shown that populations from high elevations have lower growth rates than populations from high altitudes (Vitasse *et al.* 2009, 2013; Montesinos-Navarro *et al.* 2011). Similar trends were observed in our study (authors' personal observations). This pattern was likely due to differential selection pressure among the biogeographical regions, i.e. populations from low altitudes tend to be adapted to the warmer climate under which selection has favoured a high allocation to growth and competitive ability, whereas populations from high altitudes display lower growth rates and greater cold-tolerance (Körner 2003).

Surprisingly, low- vs high-elevation populations significantly increased leaf area at decreasing annual precipitation and precipitation of the warmest and coldest quarter. Similar results were also obtained by Meier and Leuschner (2008) for *Fagus sylvatica* stands in central Germany. Authors concluded that stand leaf area of the beech along this precipitation gradient is not a simple function of water availability, but is controlled by several abiotic factors including spring temperature and possibly also nitrogen supply, which both tend to increase toward drier sites, thus overlaying any negative effect of water shortage on leaf development. Since the grey alder is a hygro-mesophilous species which mostly occupied riversides, we assume that differences in precipitation among biogeographical regions did not influence population divergence. Similar conclusions were reported by Krauze-Michalska and Boratyńska (2013). Nevertheless, our results revealed that leaves of grey alder populations decrease in almost all measured leaf traits with increasing distance-to-water. This variation trend can be explained by the specific differences in the geomorphology of the terrain among biogeographical regions. The majority of grey alder populations in the Alpine-Dinaric region inhabit a drier riparian part, on elongated terraces and wet slopes along the course of the river Kupa and its tributaries, which are generally 2 to 6 m above the average water level. By contrast, continental populations of the grey alder, along the river Drava, grow along an active watercourse with intensive shifting of pebbly, and sometimes sandy sediments, on which aerated and humus soils are formed. The presence of different species in these stands also reflects different biogeographical characteristics (Trinajstić 1964; Franjić *et al.* 1999; Vukelić *et al.* 2012, 2017). In general, tree species have high levels of morphological variability (Poljak *et al.* 2012; McKown *et al.* 2014; Zebec *et al.* 2015), and phenotypic differences among populations are often a result of the environmental distances between populations. Nevertheless, our results suggest that phenotypic divergence of the studied grey alder populations is the result of a significant level of isolation both by distance and by the environment. The isolation by distance assumes that phenotypic differentiation between populations increases with increasing geographic distance because of limited pollen and seed dispersal (Wright 1943, 1946; Orsini *et al.* 2013). On the other hand, gene flow among ecologically divergent habitats is reduced because of reduced success of the establishment of immigrants from different environments, as a result of local genetic adaptation (Nosil and Crespi 2004; Noisl *et al.* 2005, 2008, 2009; Orsini *et al.* 2013; DeWoody *et al.* 2015). However, we cannot exclude the possibility that adaptive differentiation and historical migration processes acted in combination to produce the observed pattern of morphological variation in the studied populations (Temunović *et al.* 2012; DeWoody *et al.* 2015). Although the main refugium during the last glacial period was located in Central Europe (Huntley and Birks 1983; Douda *et al.* 2014; Mandák *et al.* 2016), *A. incana* probably survived in situ in river canyons and protected valleys along the north-western Dinaric Alps, while north-eastern populations from the continental biogeographical region could represent newly colonised populations from the refugium in Central Europe.

CONCLUSIONS
ZAKLJUČCI

The study revealed the existence of two morphologically and, to a large extent, geographically and environmentally distinct and well-defined groups of grey alder populations in Croatia. These patterns confirm that multiple evolutionary processes influence the morphological diversity and structure of the populations (DeWoody *et al.* 2015). In general, two distinct morphotypes were found: small-leaf in the mountainous Alpine-Dinaric region, and large-leaf in the continental region. These ecologically divergent habitats may have led to the general subdivision into two distinct ecotypes. Adaptation to local environmental conditions in the continental region probably resulted in a higher growth rate and large-leaf morphotypes which increase the species' competitive ability and long-term success, whereas in the Alpine-Dinaric region natural selection has probably favoured greater cold-tolerance (Körner 2003; Vitasse *et al.* 2009). We also believe that our results could be valuable baseline data for the development of more efficient management plans for this boreal tree species.

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Sažetak

Bijela, odnosno siva joha, *Alnus incana* (L.) Moench subsp. *incana*, listopadna je, jednodomna i anemofilna drvenasta vrsta iz porodice *Betulaceae*. Prirodno je rasprostranjena u sjevernoj, srednjoj i mjestimično južnoj Europi, zapadnom Sibiru i Turskoj, od nizinskoga pojasa do preko 1800 m nadmorske visine u alpskom području. Bijela joha je mezofilna do higrofilna vrsta koja dobro podnosi oštru planinsku i kontinentalnu klimu, a raste na karbonatnim, ali i silikatnim, pjeskovitim, šljunčanim, slabo humusnim tlima. Najčešća je u planinskim dolinama, uz potoke i rijeke gdje na vlažnim staništima s mnogim higrofilnim vrstama tvori guste, fragmentarne sastojine, često obnovljene iz panja. To su povremeno plavljeni lokaliteti uz vodotoke ili okolne padine kroz koje se postrano procjeđuje voda prema glavnom vodotoku.

Prisutnost bijele johe u Hrvatskoj vezana je za dva oštro ograničena područja: sjeveroistočno, u nizinskom dijelu Hrvatske (110 do 190 m nadmorske visine) uz tok rijeke Drave; i sjeverozapadno, u alpsko-dinarskom području uz rijeku Kupu i njene pritoke (220 do 550 m nadmorske visine).

U sjeveroistočnom području rasprostiru se sastojine definirane kao *Equiseto hyemali-Alnetum incanae* Moor 1958 (Trinajstić 1964; Franjić *et al.* 1999), u kojima se u odnosu na sastojine bijele johe u gorskome području kao razlikovne vrste izdvajaju: *Ulmus laevis* Pall., *U. minor* Mill., *Prunus padus* L., *Equisetum hyemale* L. i druge. Ove sastojine danas su rjeđe nego nekada, manje se koriste, a zbog nekadašnjeg načina sječe vrlo su često panjače. Nekadašnje šumske površine koje je Trinajstić istraživao (1964) danas više nisu pod šumom. Poplavne i vlažne šume u blizini urbanih središta isušuju se, a na njihovim su staništima izgrađena naselja i komunalna infrastruktura tako da su danas šume sive johe uz tok rijeke Drave u velikoj regresiji.

U alpsko-dinarskom području istraživane sastojine svrstane su u šumsku zajednicu *Lamio orvalae-Alnetum incanae* Dakskobler 2010, a opisane su kao geografska varijanta *Helleborus dumetorum* (Vukelić *et al.* 2012, 2017). Ona se ističe mnogim vrstama iz dinarske vegetacijske zone, poput *Fraxinus excelsior* L., *Ulmus glabra* Huds., *Fagus sylvatica* L., *Acer pseudoplatanus* L., *Lamium orvala* L., *Lunaria rediviva* L., *Scopolia carniolica* Jacq., *Helleborus dumetorum* Waldst. et Kit., *Knautia drymeia* Heuff. subsp. *drymeia* i druge. Siva joha ovdje raste u kanjonu Kupe i na obalnim terasama koje su uglavnom antropogenizirane, a nekoć su bile korištene u poljoprivrednoj proizvodnji.

Glavni ciljevi rada bili su utvrditi morfološku raznolikost i strukturiranost populacija bijele johe u Hrvatskoj te istražiti na koji način ekološki i geografski čimbenici utječu na varijabilnost populacija.

Materijal za morfometrijsku analizu sakupljen je iz sedam prirodnih populacija – tri iz kontinentalne i četiri iz alpsko-dinarske regije (slika 1). Svaka populacija predstavljena je s 20 stabala, a svako stablo s 20 zdravih i neoštećenih listova s kratkih fertilnih izbojaka iz vanjskog, osvijetljenog dijela krošnje. Listovi su sakupljeni početkom srpnja 2016. godine, odnosno sredinom vegetacijskog razdoblja, kada su s obzirom na dimenzije i oblik u potpunosti razvijeni. Listovi su skenirani i izmjereni pomoću programa WinFolia. Ukupno je izmjereno devet lisnih značajki (slika 2).

Deskriptivnim statističkim metodama utvrđena je visoka varijabilnost listova bijele johe, kao i jasan trend divergencije između kontinentalnih i alpsko-dinarskih populacija (slika 3). Najvarijabilnijim su se pokazale značajke: površina plojke, širina lista mjerena na 90 % dužine plojke i dužina peteljke. Za razliku od toga, najmanji stupanj varijabilnosti zabilježen je za varijable koje opisuju osnovu lisne plojke. Iako je trend variranja pojedinih značajki po populacijama bio sličan, u populacijama alpsko-dinarske regije zabilježena je nešto viša unutar-populacijska raznolikost. Kontinentalne populacije su imale veće vrijednosti svih mjerenih značajki lista u odnosu na populacije iz alpsko-dinarske regije.

Analizom varijance, utvrđene su statistički značajne razlike između istraživanih regija te između stabla unutar populacija, dok su se populacije unutar regija razlikovale samo za četiri istraživane značajke (tablica 1). Unutarpopulacijska varijabilnost veća je od međupopulacijske varijabilnosti, što je odlika mnogih vrsta drveća. Multivarijatne analize potvrđuju postojanje jasne razlike između kontinentalnih i alpsko-dinarskih populacija bijele johe (slika 1, slika 4, tablica 3), što upućuje na mogućnost da istraživane populacije predstavljaju dva različita ekotipa.

Morfološka udaljenost između populacija bila je korelirana s geografskom i ekološkom udaljenošću, što potvrđuje uzorak „isolation by distance“ i „isolation by environmental distance“. Drugim riječima, utvrđeno je da geografska udaljenost i ekološka divergencija imaju važan utjecaj na oblikovanje morfološke strukture populacija te da je ona posljedica njihove moguće lokalne prilagodbe na okolišne uvjete i njihove geografske udaljenosti (tablica 2).

Dobivene spoznaje o varijabilnosti populacija mogu doprinijeti razvoju učinkovitijih planova očuvanja i gospodarenja sivom johom u Hrvatskoj. Kako bi se potvrdili dobiveni zaključci o varijabilnosti populacija sive johe dobiveni morfometrijskim metodama, istraživanja je svakako potrebno proširiti i na molekularno-biološke metode.

KLJUČNE RIJEČI: fenotipska varijabilnost, morfologija lista, adaptabilnost, ekološka divergencija, klinalna varijabilnost, visinski gradijent