

**INTER- AND INTRASPECIFIC VARIABILITY OF *Quercus cerris* L.
AND *Quercus frainetto* Ten. IN THE ŠUMADIJA REGION (SERBIA) BASED
ON LEAF GEOMETRIC MORPHOMETRICS**

Marija JOVANOVIĆ^{1*}, Jelena MILOVANOVIĆ¹, Marina NONIĆ²,
Mirjana ŠIJAČIĆ-NIKOLIĆ²

¹Singidunum University – Environment and Sustainable Development, Belgrade, Serbia

²University of Belgrade – Faculty of Forestry, Belgrade, Serbia

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In this study, we used leaf geometric morphometrics to quantify the differences among *Quercus cerris* L. and *Quercus frainetto* Ten. and their populations in the Šumadija region in Serbia. We sampled 2200 leaves from eight populations and on each leaf recorded 13 landmarks. To analyze how leaf morphology varies across species and populations, we used Procrustes ANOVA, a two-block partial least-squares analysis, a principal component analysis and a canonical variate analysis. The results showed a clear discrimination between species, followed by different levels of leaf shape variability in *Q. cerris* and *Q. frainetto*. The leaves of *Q. cerris* displayed higher levels of variability and higher differences among populations compared to *Q. frainetto*. The patterns of population grouping in the two species greatly differed, indicating that in these species different factors contribute to intraspecific variability. This study provides preliminary results of the leaf morphometric variability of oak species in the Šumadija region in Serbia. Future studies should include genetic and environmental analyses to understand the origins of phenotypic differences between species and populations, and to fully recognize which factors mostly determine relationships between taxa.

Key words: geometric morphometrics, Hungarian oak, leaf morphometrics, morphometric variability, Turkey oak

Corresponding author: Marija Jovanović, Singidunum University – Environment and Sustainable Development, Danijelova 32, Belgrade, Serbia. E-mail: marija.jovanovic.202@singimail.rs, telephone: +381 64 1154298

INTRODUCTION

Geometric morphometrics represents the approach that provides a mathematical description of biological structures according to geometric definitions of their size and shape (SAVRIAMA, 2018). It is often used in taxonomic studies for the identification of shape variation of morphological traits at the interspecific and intraspecific levels.

In plants, a great number of phenotypic traits can be used to differentiate taxa, such as leaf morphology, anatomical characteristics and other structural features. Among them, leaf morphology is one of the most important, particularly in tree species that are characterized by the low variation of floral structures and short flowering seasons. This is especially true for oaks (genus *Quercus*), where leaf traits are one of the most important for species discrimination (STACE, 1982), making it suitable for the application of geometric morphometrics methods. Additionally, at the intraspecific level, leaf morphological variation could be an indicator of the mutual influence of the genetic factors and local environmental conditions (e.g., geography and climate) (LI *et al.*, 2021). Previous studies confirmed that leaf morphology is determined by the simultaneous action of several genes (BAKER *et al.*, 2015; FU *et al.*, 2017), and shown that geographical isolation can initiate leaf morphological differentiation (MAYA-GARCÍA *et al.*, 2020). Some studies also suggested that leaf characteristics vary with climatic factors (GUERIN *et al.*, 2012; FU *et al.*, 2017), indicating they can express different phenotypes in response to environmental variation, i.e., exhibit phenotypic plasticity (RAMÍREZ-VALIENTE *et al.*, 2010; NONIĆ and ŠIJAIĆ-NIKOLIĆ, 2021). The variability of oak leaf shape has been analyzed by various authors using geometric morphometrics approach, to distinguish between species, their hybrids or populations of the same species (JANSEN *et al.*, 1993; PEÑALOZA-RAMIREZ *et al.*, 2010; VISCOSI and CARDINI, 2011; VISCOSI, 2015; LIU *et al.*, 2018; LI *et al.*, 2021; SU *et al.*, 2021; PROILETTI *et al.*, 2021). Although intensely investigated, due to its wide area of distribution, many populations of oak species have not yet been studied.

The diversity of oaks in Europe is not very high compared to America or Asia, although they are distributed throughout the continent (DENK *et al.*, 2017). However, for the largest part of Europe, genus *Quercus* species represent the most important vegetational and floristic phenomenon. In every oak species in Europe, dynamic processes of speciation are observable, suggested by the high intraspecific polymorphism and the high diversity of subspecies, varieties and forms. In Europe, the Balkan Peninsula is one of the most significant areas in the phylogenesis of the genus *Quercus* due to its refugial characteristics, specific climate and orographic factors, being the region where the postglacial evolution of all European oak species took place (JANKOVIĆ, 1973; LEROY *et al.*, 2017).

In order to contribute to the overall knowledge of leaf variability of oak species in Europe, the main aim of this study was to quantify the differences among the Turkey oak (*Quercus cerris* L.) and the Hungarian oak (*Quercus frainetto* Ten.) and their populations in the Šumadija region in Serbia using geometric morphometrics. These are the dominant species of the association *Quercetum frainetto-cerris* (RUDSKI, 1949), which has been widely distributed in the Šumadija region (VUJADINOVIĆ and GAJIĆ, 2005). This association occurs on placoric terrains (VUKIN and RAKONJAC, 2013) and represents climatogenic forest of this area. However, various kinds of human impact (e.g., grazing of the domestic animals, timber exploitation, agriculture) limited the actual distribution of these forests and in many cases changed their structure and

floristic composition (MEDWECKA-KORNAŠ *et al.*, 1986). This is particularly the case in the Šumadija region where oak forests have been greatly deforested and now occur as complexes of different sizes. Forest fragmentation can result in a decrease in genetic variation and an increase in genetic differentiation in tree species (YÜCEDAĞ *et al.*, 2021), making oak species to become more endangered, especially in deforested areas with fragmented populations such as the Šumadija region. Many populations have become so fragmented and small that they are vulnerable to genetic and demographic stochasticity and unable to recover on their own (SEGELBACHER *et al.*, 2021). Thus, the estimation of variability using morphological markers is one of the key steps for the development of the appropriate conservation programs (ŠIJACIĆ-NIKOLIĆ *et al.*, 2021).

This study aimed to determine differences in oak leaf shape at the inter- and intraspecific levels. At the interspecific level, we investigated the differences between *Q. cerris* and *Q. frainetto* in leaf shape to determine which species shows higher levels of variability. At the intraspecific level, we investigated the differences among populations of each species to determine whether these differences follow the same pattern in the same populations of the two species.

MATERIALS AND METHODS

Sampling

In the autumn of 2021, we sampled leaves from 220 randomly selected adult trees in eight *Q. cerris* and *Q. frainetto* populations throughout the Šumadija region in Serbia (Fig. 1, Table 1). Šumadija is the largest subregion of central Serbia. Its territory includes the areas between Sava and Danube rivers in the north, Velika Morava in the east, Zapadna Morava in the south and Dičina, Ljig and Kolubara in the west. In this paper, we analysed populations in the central part of the Šumadija region, in the areas around the cities Kragujevac and Aranđelovac (Fig. 1).



Figure 1. Population locations in the study area of the Šumadija region: 1) Topola, 2) Čumic, 3) Milatovac, 4) Resnik, 5) Komarice, 6) Erdeč, 7) Adžine livade, 8) Knić. The whole area of the Šumadija region is represented in gray shading, while the sampling area is contoured and magnified on the right.

Each population was at least 10 km apart and all sampled individuals were located at least 5 m from each other to minimize the risk of clone selection (LI *et al.*, 2021). We collected 10

fully developed leaves from each individual, at the height of 8 to 10 m around the crown of each tree (VISCOSI, 2015), mainly under shaded conditions. Leaves were pressed, herbarized and scanned by placing the abaxial surface facing upwards on an Epson Stylus DX4050 scanner (300 dpi resolution).

Table 1. Sample size (number of sampled individuals, *N*), altitude, latitude and longitude of sampled populations of *Q. cerris* and *Q. frainetto*.

Population	<i>Q. cerris</i> (N)	<i>Q. frainetto</i> (N)	Altitude	Latitude	Longitude
Topola	13	13	222 m	44°14'18" N	20°42'40" E
Čumić	15	15	288 m	44°7'30" N	20°49'12" E
Milatovac	21	5	150 m	44°5'15" N	20°58'49" E
Resnik	18	11	202 m	44°6'59" N	20°56'43" E
Komarice	13	13	347 m	44°2'3" N	21°0'42" E
Edreč	15	15	232 m	43°58'51" N	20°54'1" E
Adžine livade	18	7	373 m	43°55'2" N	20°52'4" E
Knić	18	10	272 m	43°55'44" N	20°41'7" E
Σ	131	89			

Landmark configuration

On each leaf of the entire dataset (2200 leaves), 13 landmarks were recorded on the left and right sides (Fig. 2), in accordance with the methodology suggested by VISCOSI (2015). Three landmarks (1-3) were unpaired and distributed along the midrib of the leaves, while the other landmarks (4-13) were paired and distributed symmetrically on both sides of the leaves. X and Y coordinates of the 13 landmarks for each leaf were recorded using tpsDig and tpsUtil software (ROHLF, 2015).

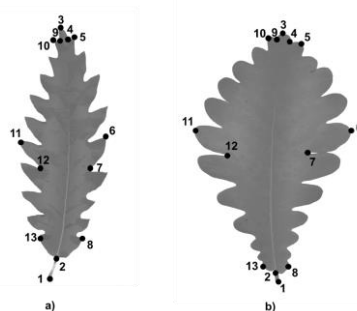


Figure 2. Configuration of *Q. cerris* (a) and *Q. frainetto* (b) leaves showing 13 landmarks: 1) beginning of the petiole, 2) junction of the blade and the petiole, 3) apex of the leaf blade, 4 and 9) base of the apical sinuses of the blade tip (right and left side), 5 and 10) tip of the lobe immediately above the apex of the leaf blade (right and left side), 6 and 11) tip of the lobe at the largest width of the blade (right and left side), 7 and 12) base of the sinus immediately beneath the lobe of the landmarks 6 and 11, 8 and 13) the first basal lobe of the blade (right and left side).

Statistical analyses

In order to separate the variation into a symmetric component (the variation in averages of original and mirrored configurations) and asymmetric component (the differences between the original and mirrored configurations), the matrix of the raw coordinates of the landmarks was imported and a generalized Procrustes analysis (GPA) was performed using MorphoJ software (KLINGENBERG, 2011). This analysis minimizes the sum of squared distances between corresponding landmarks to extract shape data by removing the information of size, location and orientation (SAVRIAMA, 2018). Procrustes ANOVA was performed to quantify the relative amounts of variation of symmetric and asymmetric components among individuals, populations and species (KLINGENBERG, 2003). The magnitude of the effects was measured as F ratios and the percentages of variance explained by each effect (LI *et al.*, 2021). Additionally, allometric patterns of covariation between leaf size and shape were assessed by the two-block partial least-squares analysis (2B-PLS) (ROHLF and CORTI, 2000). The principal component analysis (PCA) based on the covariance matrix was computed on the symmetric and the asymmetric components between the two species and the symmetric component among populations of each species. Subsequently, on intraspecific levels, canonical variate analysis (CVA) was used to generate Mahalanobis distances, which represented the baseline data for cluster analysis performed in Statistica 10 software (STATSOFT INC., 2011).

RESULTS

Procrustes ANOVA of leaf size and shape

The leaf size and shape subjected to Procrustes ANOVA showed that for both leaf size and shape most of the total variance was associated with between-individual effects. Between-species and between-population effects also showed significant differences, but explained a lower percentage of variation (Table 2).

Table 2. *Procrustes ANOVA of leaf size and shape of all samples.*

Response	Effect	% of Variation	SS	d.f.	F	p
Size	Species	23.28	18,183,739.37	1	194.40	<0.0001
	Population	27.32	21,344,157.44	15	16.85	<0.0001
	Individual	49.40	38,574,716.59	219	6.71	<0.0001
Shape	Species	22.99	0.94	11	0.09	<0.0001
	Population	28.19	1.15	165	108.01	<0.0001
	Individual	48.82	1.99	2409	17.80	<0.0001

SS – the sum of squares, d.f. – degrees of freedom, F – F values, p – p values.

Leaf morphological variation and allometric patterns

The two-block partial least square analysis of the symmetric component showed distinct separation of *Q. cerris* and *Q. frainetto* individuals (Fig. 3a). For the asymmetric component, separation was much less distinctive, with many overlapping individuals (Fig. 3b). However, a significant allometric pattern was observed in the symmetric component – when leaf size

increased, the length of the petiole declined and the greatest width of the leaf blade increased (Fig. 3a).

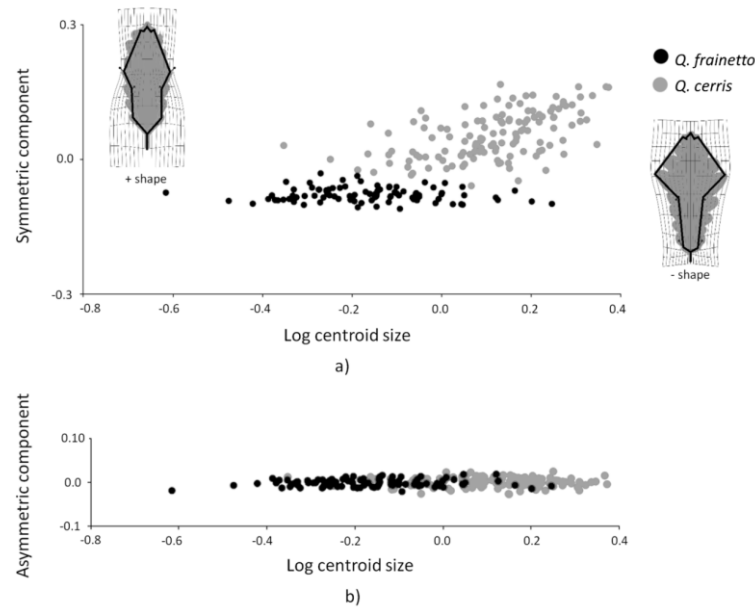


Figure 3. Scatterplots from the two-block partial least square analysis showing the relationship between log centroid size and a) symmetric component and b) asymmetric component of the leaf shape of *Q. cerris* and *Q. frainetto*. Deformation grids in Fig. 3a represent leaf shapes reflecting the negative extremes (- shape) and positive extremes (+ shape) of the partial least square axis for the symmetric component.

Leaf morphological discrimination between species

Principal component analysis computed on the symmetric component of the two species (*Q. cerris* and *Q. frainetto*) showed that the first principal component explained 62.75% of the total variation. The scatterplot of the PCA exhibited clear discrimination of the two groups along the first axis (Fig. 4a). The change in shape was observable within negative extremes of the first principal component, occupied mainly by *Q. cerris*, and positive extremes, occupied by *Q. frainetto*. The leaves of *Q. frainetto* exhibited shorter petiole, narrower leaf at the basal region, higher values of the greatest width and a flatter apex of the leaf blade, in contrast to *Q. cerris* leaves which showed longer petiole, wider leaf at the basal region, narrower greatest width and a more pointer apex of the leaf blade. Additionally, the greatest width of the leaf blade in *Q. frainetto* was located in the upper parts of the leaf compared to *Q. cerris*. On the asymmetric

component, the principal component analysis showed considerable overlap (Fig 4b), due to which further analyses among populations were represented for the symmetric component only.

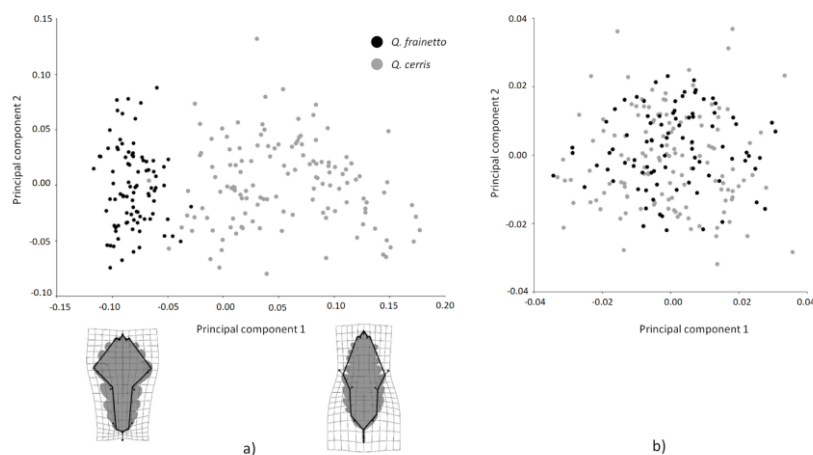


Figure 4. Ordination plots of the first principal component computed on the symmetric component (a) and the asymmetric component (b) obtained by the principal component analysis of *Q. cerris* and *Q. frainetto*. Deformation grids in Fig. 4a represent leaf shape variation along the first principal component for the symmetric component.

Leaf morphological discrimination among populations

For *Q. cerris*, principal component analysis computed on the symmetric component of the eight populations showed that the first principal component explained 47.76% of the total variation, while the second principal component explained 20.46%. Populations Milatovac and Resnik showed the tendency of grouping in the positive extreme of the first principal component by having longer petiole and the greatest width of the leaf blade located in the lower parts of the leaf, while populations Topola and Čumic grouped within negative extreme by having shorter petiole and the greatest width of the leaf blade located in the upper parts of the leaf. Within the second principal component, population Komarice showed a tendency of grouping in the positive extreme, by having shorter petiole and the greatest width of the leaf blade located in the lower parts of the leaf, while population Knić grouped within the negative extreme by having longer petiole and less pronounced lobes at the greatest width of the leaf blade (Fig. 5). Mahalanobis distances, obtained by the canonical variate analysis, confirmed the results of the principal component analysis, with three distinctive groups – 1) Adžine livade, Knić, Čumic, 2) Komarice, Milatovac, Resnik and 3) Erdeč, Topola (Fig. 6).

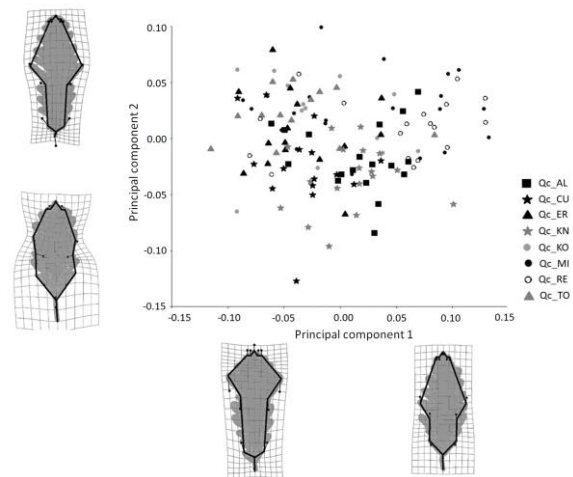


Figure 5. Ordination plots and leaf shape variation of the first principal component computed on the symmetric component obtained by the principal component analysis of *Q. cerris* populations. Abbreviations: Qc_AL – Adžine livade, Qc_CU – Čumic, Qc_ER – Erdeč, Qc_KN – Knić, Qc_KO – Komarice, Qc_MI – Milatovac, Qc_RE – Resnik, Qc_TO – Topola.

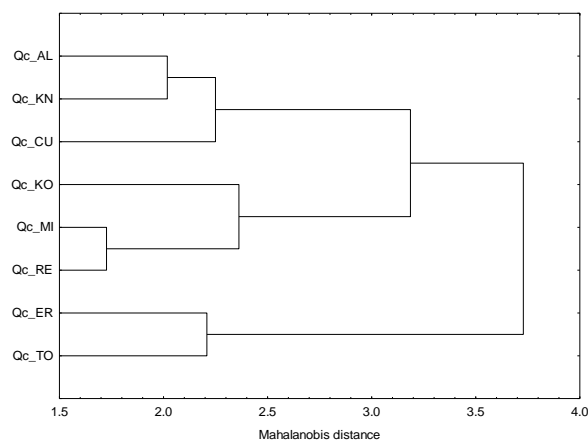


Figure 6. Cluster of the *Q. cerris* populations based on the Mahalanobis distances obtained by the canonical variate analysis. Abbreviations: Qc_AL – Adžine livade, Qc_CU – Čumic, Qc_ER – Erdeč, Qc_KN – Knić, Qc_KO – Komarice, Qc_MI – Milatovac, Qc_RE – Resnik, Qc_TO – Topola.

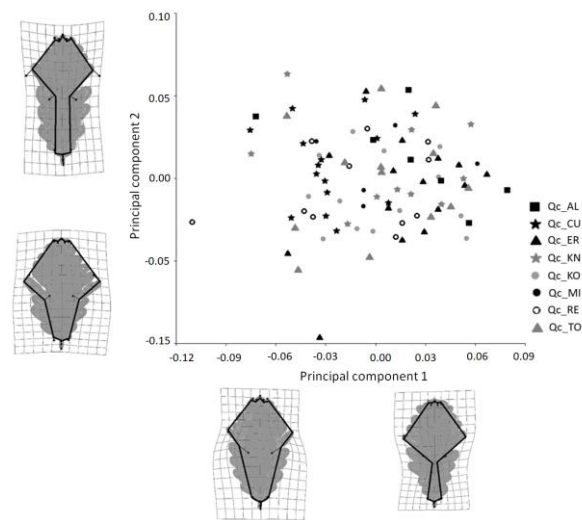


Figure 7. Ordination plots and leaf shape variation of the first principal component computed on the symmetric component obtained by the principal component analysis of *Q. frainetto* populations. Abbreviations: Qc_AL – Adžine livade, Qc_CU – Čumic, Qc_ER – Erdeč, Qc_KN – Knić, Qc_KO – Komarice, Qc_MI – Milatovac, Qc_RE – Resnik, Qc_TO – Topola.

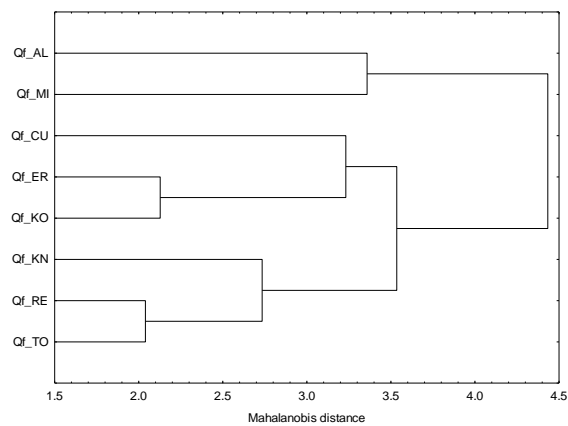


Figure 8. Cluster of the *Q. frainetto* populations based on the Mahalanobis distances obtained by the canonical variate analysis. Abbreviations: Qc_AL – Adžine livade, Qc_CU – Čumic, Qc_ER – Erdeč, Qc_KN – Knić, Qc_KO – Komarice, Qc_MI – Milatovac, Qc_RE – Resnik, Qc_TO – Topola.

For *Q. frainetto*, principal component analysis computed on the symmetric component of the eight populations showed that the first principal component explained 39.33% of the total variation, while the second principal component explained 21.54%. Compared to *Q. cerris*, populations of *Q. frainetto* showed lower levels of differentiation. However, some grouping patterns were observed – population Čumic showed a tendency of grouping within the negative extreme of the first principal component by having less pronounced lobes at the greatest width of the leaf blade (Fig. 7). Mahalanobis distances, obtained by the canonical variate analysis, showed grouping of the populations in three groups – 1) Adžine livade, Milatovac, 2) Čumic, Erdeč, Komarice, and 3) Knić, Resnik, Topola (Fig. 8).

DISCUSSION

This study provides the preliminary results of the leaf morphometric variability of oak species in the Šumadija region in Serbia. Our results showed different levels of leaf shape variability in *Q. cerris* and *Q. frainetto*. Species discrimination was observable only for the symmetric component of the leaf shape, along with the specific allometric pattern in the two species – as the leaf size increased, the length of the petiole decreased and the width of the leaf blade increased. This allometric pattern showed that *Q. frainetto* leaves were larger and widest closer to the apex, and with shorter petiole compared to *Q. cerris*. The observed differences are not surprising, as the detected pattern is one of the well-known ways to distinguish analyzed oak species (DE RIGO *et al.*, 2016; MAURI *et al.*, 2016). Thus, the high diagnostic power of the geometric morphometrics approach, previously suggested by VISCOSI *et al.* (2010) in other oak species, was confirmed by our results.

The leaves of *Q. cerris* displayed higher levels of variability and higher differences among populations compared to *Q. frainetto*. The patterns of population grouping in the two species greatly differed – in *Q. cerris*, patterns of differentiation followed geographical distribution in some populations (such as the grouping of Milatovac, Resnik and Komarice, or Adžine livade and Knić, which are geographically close to one another), whereas in *Q. frainetto* no geographical pattern was observed. Only one population of *Q. frainetto* (population Čumić) showed a tendency of grouping, while other populations greatly overlapped. This indicated that in the analyzed oak species, morphology can diverge in apparently random directions. ENESCU *et al.* (2010) reported a similar pattern of relatively low variability in *Q. robur* populations across Romania, while FORTINI *et al.* (2015) showed higher variability in *Q. pubescens* compared to *Q. frainetto* and *Q. petraea* in Italy, suggesting that the degree of leaf morphological variability can vary among species.

As the results obtained in our study are preliminary, concern only oak leaf morphometrics, and do not include environmental and genetic analyses, we emphasize that more research is needed on greater number of populations to complement the observed patterns of variability. Different geographic, genetic, and climatic factors can contribute to leaf morphological changes (MAYA-GARCÍA *et al.*, 2020). Accordingly, there are a few possible reasons for the observed differences in population differentiation and allometric patterns of the two analyzed oak species that need to be addressed in the future. Firstly, as morphometric patterns in oak leaves follow genetic differentiation (PEÑALOZA-RAMÍREZ *et al.*, 2010), it is possible that genetic factors greatly influenced the observed patterns of leaf shape variability.

The investigation of leaf morphology in terms of genetic differentiation provides information on between-population and within-population variability and can be the basis for the determination of species and lower taxa as well as intraspecific or interspecific hybrids (BATOS *et al.*, 2017). As this study is performed on a small-scale geographical range, low levels of variability in *Q. frainetto* may indicate a more pronounced gene flow between its populations compared to *Q. cerris*. Secondly, as geography and climate also influence leaf variation in oaks, the species responses to the same environmental conditions differ (LI *et al.*, 2021). For example, *Q. cerris* adapts well to different habitat conditions, it is relatively tolerant of drought (more than other oak species in the same area) and air pollution, it can grow on different types of soils, it requires good lighting, but can also grow in the shade in light forests (DE RIGO *et al.*, 2016). On the other hand, *Q. frainetto* has a narrower ecological amplitude compared to *Q. cerris*, it grows on acidic soils, tolerates high soil water saturation, it requires plenty of light and does not tolerate shading, but it is more drought-tolerant compared to *Q. cerris* (MAURI *et al.*, 2016). Consequently, different patterns in leaf shape variation can be the result of different ways of adaptation to the environmental conditions, dictated by the species biology. This indicates that the identification of mechanisms that determine adaptation ability is crucial for understanding how population morphological variation is shaped.

It is important to indicate the complexity of the systematic approach in the development of the conservation programs, especially when consequences of climate change and forest management are unpredictable (FORTINI *et al.*, 2015; SANDURSKA *et al.*, 2019; ŠIJAČIĆ-NIKOLIĆ *et al.*, 2021), and the use of morphological markers is one way to achieve this goal. The efficiency of *ex situ* management partly relies on the profound knowledge of a species' taxonomic status, and for many species of conservation concern, such information is either missing or incomplete (SEGELBACHER *et al.*, 2021). To fully recognize which factors mostly determine relationships between taxa and to understand the origins of phenotypic differences between species and populations, future studies should include molecular and environmental analyses of the oak populations in the Šumadija region. If that were the case, morphological and molecular markers, together with environmental indicators, would be useful tools for taxonomical identification and for establishing conservation strategies in this area.

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INTER- I INTRASPECIJSKA VARIJABILNOST VRSTA *Quercus cerris* I *Q. Frainnetto* U ŠUMADIJSKOM REGIONU (SRBIJA) NA OSNOVU GEOMETRIJSKE MORFOMETRIJE LISTA

Marija JOVANOVIĆ^{1*}, Jelena MILOVANOVIĆ¹, Marina NONIĆ²,
Mirjana ŠIJACIĆ-NIKOLIĆ²

¹Univerzitet Singidunum – Životna sredina i održivi razvoj, Beograd, Srbija

²Univerzitet u Beogradu – Šumarski fakultet, Beograd, Srbija

Izvod

U ovom istraživanju, geometrijska morfometrija lista korišćena je za kvantifikaciju razlika između vrsta *Quercus cerris* i *Quercus frainetto* i njihovih populacija u Šumadijskom regionu u Srbiji. Uzorkovano je ukupno 2200 listova iz osam populacija i na svakom listu zabeleženo je 13 specifičnih tačaka. Za analizu variranja morfologije lista korišćene su Prokrastova ANOVA, dvoblokovska analiza parcijalnih najmanjih kvadrata, analiza glavnih komponenti i kanonijska diskriminantna analiza. Rezultati su pokazali jasno odvajanje vrsta i različite nivoe varijabilnosti oblika lista kod *Q. cerris* i *Q. frainetto*. Listovi *Q. cerris* su pokazali više nivoe varijabilnosti i veće razlike između populacija u poređenju sa *Q. frainetto*. Obrasci grupisanja populacija kod dve vrste su se značajno razlikovali, ukazujući da kod ovih vrsta različiti faktori doprinose intraspecijskoj varijabilnosti. Ovo istraživanje prikazuje prliminarne rezultate morfometrijske varijabilnosti lista hrasta u Šumadijskom regionu u Srbiji. Naredne studije bi trebalo da uključe genetičke i sredinske analize koje bi doprinele boljem razumevanju porekla fenotipskih razlika između vrsta i populacija i faktora koji u najvećoj meri doprinose vezama između taksona.

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