

## INTRA- AND INTERPROVENANCE VARIATIONS IN LEAF MORPHOMETRIC TRAITS IN EUROPEAN BEECH (*FAGUS SYLVATICA* L.)

Srdan Stojnić<sup>1,\*</sup>, Saša Orlović<sup>1</sup>, Danijela Miljković<sup>2</sup> and Georg von Wuehlisch<sup>3</sup>

<sup>1</sup> University of Novi Sad, Institute of Lowland Forestry and Environment, Novi Sad, Serbia

<sup>2</sup> University of Belgrade, Institute for Biological Research "Siniša Stanković", Department of Evolutionary Biology, Belgrade, Serbia

<sup>3</sup> Thünen-Institute for Forest Genetics, Grosshansdorf, Germany

\*Corresponding author: stojnics@uns.ac.rs

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**Abstract:** European beech (*Fagus sylvatica* L.) is one of the most important tree species in Europe. Due to substantial genetic diversity and phenotypic plasticity, beech has successfully adapted to different environments within its natural range. Provenance tests provide a good basis for studying within- and between-provenance genetic variation, due to homogeneous within-trial environmental conditions. The aim of the present study was to determine the within- and between-provenance genetic variability of certain leaf morphological traits among eleven beech provenances, grown in a common garden experiment. Univariate analysis of variance (ANOVA) was used to test for differences among the studied beech provenances. Principal component analysis (PCA) allowed a complex assessment of the relationships among the provenances and an estimation of multivariate relations among the analyzed characters. The results of the study revealed the existence of substantial variability among provenances ( $p < 0.001$ ). Likewise, high genetic variability was observed at the intra-provenance level ( $p < 0.001$ ). The first three principal components (PC1-PC3) explained approximately 81% of the total variance among the European beech provenances tested. The highest contribution on PC1 corresponded to variables related to leaf size: leaf area (-0.882) and leaf width (-0.876). Based on the position of provenances on a PCA scatter plot, it could be assumed that European beech is characterized by a more ecotypic pattern of genetic variation rather than by clinal variation. Also, the presence of considerable genetic diversity within provenances will be important.

**Key words:** European beech; provenance trial; leaf morphological traits; within- and between-provenance variation

### INTRODUCTION

Leaves are very important organs in plant production and adaptation to environmental conditions [1-3]. The analysis of leaf morphometric parameters could present a valuable tool in studying the genetics, taxonomy, biogeography and evolution of tree species [4]. Numerous studies demonstrated that plants are able to adjust to habitat conditions by complex alterations in leaf morphological structure. For example, Bussotti et al. [5] found that among beech stands in Italy, leaf area was the smallest and sclerophylly was the greatest in the southernmost stands and under drought conditions. Similarly, the increased sclerophylly in beech associated with water stress was demonstrated by other studies [6-8].

Studies of leaf morphological traits found an important place in plant taxonomy as well. A recent study [9] showed that leaf morphometry clearly sepa-

rated holm oak provenances into 'ilex' and 'rotundifolia' ecotypes. Also, morpho-anatomical analysis of 44 *Edraianthus graminifolius* populations confirmed that there are several morphologically distinct groups of populations in the central Balkans, supporting the taxonomic concept that recognizes many single taxa within the *E. graminifolius* complex [10]. Moreover, although it is widely accepted that in Europe only European beech (*Fagus sylvatica* L.) and Eastern beech (*Fagus orientalis* Lipsky) occur, several authors described a separate species, Balkan beech (*Fagus moesiaca* /Domin, Maly/Czeczott), which is identified on the basis of morphological traits, mainly of the leaf and fruit (cupule), which are found to be intermediate between those of *F. sylvatica* and *F. orientalis* [11-14].

Variation between clones, genotypes and populations, based on leaf morphology, has been studied

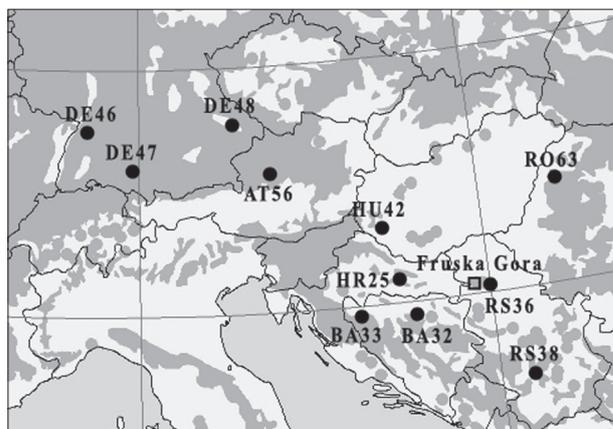
intensively within tree species [15-18]. Several studies have provided excellent evidence of tree growth improvement options by selection and breeding for certain leaf morphological, anatomical and physiological characters [19-21]. Ballian et al. [22] suggested that the variability of leaf morphological traits, observed at intra- and interpopulation levels, could provide a basis for the improvement and preservation of forest genetic diversity. Genetic diversity plays a crucial role in the adaptation processes of tree populations to environmental changes. Natural selection can take place only when enough genetic diversity is present. Diversity at species and ecosystem levels is an important prerequisite for tree adaptation to altered environmental conditions [23,24]. Furthermore, the presence of genetic diversity within and between tree populations might ensure a faster response to rapid climatic changes, allowing forest trees to survive, adapt and evolve in new environments [25].

European beech is ecologically and economically one of the most important tree species in Europe. Due to its high genetic diversity and phenotypic plasticity, the species extends across ecologically and climatically variable regions [26]. Provenance trials may provide a good basis to evaluate the genetic diversity of various provenances [27]. Since the environmental variability in provenance trials is minimized, differences in more uniform environments are likely to be the result of genetic differentiation [28]. The objective of the present study was to determine the level of genetic diversity and to assess the within- and between-provenance variation of leaf morphological traits in European beech cultivated in a common garden experiment. Additionally, a multivariate statistical technique was applied to estimate multivariate relations among leaf characteristics and to examine patterns of variation between the beech provenances tested.

## MATERIALS AND METHODS

### Experimental site

The European beech provenance trial is situated on Mt. Fruška Gora, in the northern part of Serbia. It was established in spring 2007 by planting 3-year-old seedlings. The geographical coordinates of the plot are: N 45°10'9.86", E 19°47'53.45". The trial was arranged in



**Fig. 1.** Geographical distribution of the studied beech provenances. The symbols indicate provenances (●) and the trial site, Mt. Fruška Gora (□). The dark shaded area represents the natural distribution range of beech [62].

a randomized complete block design at an altitude of

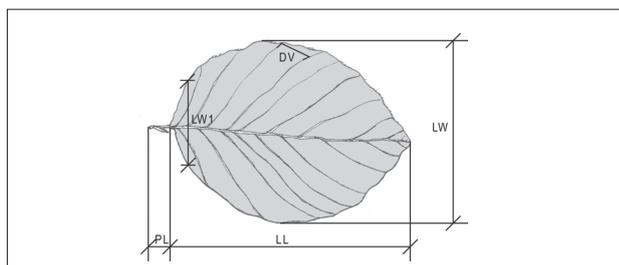
**Table 1.** European beech provenances studied.

Code	Provenance	Country	Geographical coordinates		Altitude (m)
			Lat. (N)	Lon. (E)	
HR25	Vrani Kamen	Croatia	45°37'	17°19'	600
BA32	Crni Vrh, Tešanj	Bosnia	44°33'	17°59'	500
BA33	Grmeč	Bosnia	44°46'	16°16'	650
RS36	Fruška Gora	Serbia	45°10'	19°50'	370
RS38	Kopaonik	Serbia	43°10'	20°50'	820
HU42	Valkonya	Hungary	46°30'	16°45'	300
DE46	Pfalzgrafeweiler	Germany	48°46'	08°35'	700
DE47	Schelklingen	Germany	47°59'	09°59'	650
DE48	Höllerbach	Germany	49°01'	13°14'	755
AT56	Scharnstein, Mitterndorf	Austria	47°54'	13°58'	480
RO63	Alesd	Romania	47°11'	22°15'	490

approximately 370 m a.s.l. with a northwestern exposition. The climate on the mountain is temperate continental. Mean air temperature is 11.1°C, and annual precipitation is 624 mm. Mean temperature during the vegetation period (April-September) is 17.8°C. The total precipitation, in the same period, amounts to 369 mm (45% of the total annual precipitation). The climate records are from the weather station Rimski Šančevi (N 45°20', E 19°51'; 84 m a.s.l.), 30 km from the trial stand. The soil is acid brown with a pH of 5.4 [29].

### Leaf morphology measurements

The study involved eleven beech provenances in their juvenile developmental stage, originating from Austria,



**Fig. 2.** Examination of leaf morphological traits. LL – lamina length; LW – lamina width; PL – petiole length; LW1 – leaf width at 1 cm above the leaf base; DV – distance between 3<sup>rd</sup> and 4<sup>th</sup> vein - left.

**Table 2.** Leaf morphological traits examined.

Abbreviation	Leaf morphological trait	Unit
LA	Leaf area	cm <sup>2</sup>
LMA	Leaf dry mass per unit area	mg/cm <sup>2</sup>
LL	Lamina length	mm
LW	Lamina width	mm
PL	Petiole length	mm
LW1	Leaf width at 1 cm above the leaf base	mm
DV	Distance between 3 <sup>rd</sup> and 4 <sup>th</sup> vein - left	mm
NVL	Number of veins on the left side of the leaf	
NVR	Number of veins on the right side of the leaf	
LI	Leaf index	
PI	Petiole index	

Bosnia and Herzegovina, Croatia, Germany, Hungary, Romania and Serbia (Fig. 1; Table 1). The leaves were sampled in mid-August 2011. Within each provenance 20 leaves from 10 saplings were collected. Sampling was performed on the third or fourth distal sun-leaf, fully expanded and hardened, from the spring flush, southwest-oriented and situated in the upper third part of the crown [30]. Only insect- and disease-free leaves were collected [31]. After sampling, the leaves were herbarized. The leaf area (LA) was measured with the ADC Bioscientific Ltd. AM300 Portable Leaf Area Meter on the leaves stripped of the petiole. Later, these leaves were dried at 70°C for 72 h and leaf mass was determined. For each leaf, eight morphological traits were examined directly (LA, LL, LW, PL, LW1, DV, NVL and NVR) (Fig. 2; Table 2). From the directly measured traits, three more variables were calculated. Leaf dry mass per unit area (LMA) was calculated as the ratio between leaf dry mass and leaf area (mg/cm<sup>2</sup>) [1]. Leaf index (LI) and petiole index (PI) were calculated according to following equations: a)  $LI = LL/LW \times 100$ , and b)  $PI = PL/LL \times 100$  [32].

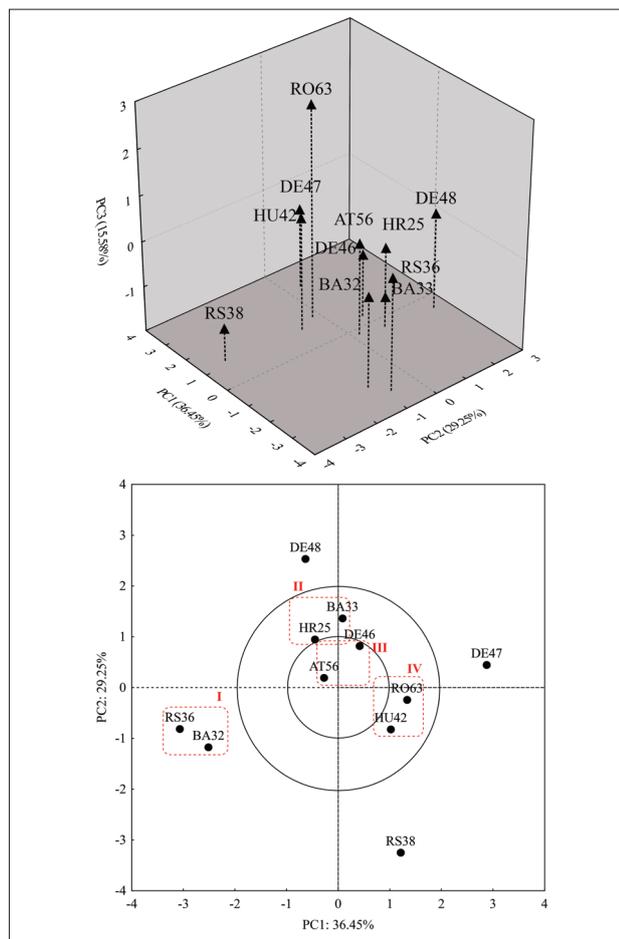
## Data analysis

All statistical analyses were performed with Statistica 10 data analysis software (StatSoft Inc., www.statsoft.com). The following parameters of descriptive statistics were evaluated for beech morphological traits: mean, minimum value, maximum value, range, standard deviation (SD) and coefficient of variation (CV%). To estimate the within- and between-provenance variation, analysis of variance (ANOVA) was performed. Significant changes were determined at  $p < 0.01$  throughout. Before statistical analyses, all data were tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Bartlett test) [33]. For the analysis of factors in the total variation, a model with two phenotypic variance sources was utilized: provenance (genetic variation between the provenances) and genotype (nested in a provenance, representing genetic differences within a provenance). The provenances were set as fixed factors (being clearly defined), while a genotype was regarded to be a random factor (since the selection of genotypes within the provenance was randomized) [34].

Principal component analysis (PCA) was performed in order to: a) examine patterns of variation between beech provenances [35], b) study the relationship among the provenances [36], and c) estimate multivariate relations among analyzed leaf morphological traits, and to study correlations among variables [37]. Provenance means were used to create a correlation matrix from which standardized principal component (PC) scores were extracted. To determine which of the PCs accounted for the greatest amount of variation, the eigenvalues of the 3 PCs were compared for each trait [36]. Traits LMA, LI and PI were not used in PCA since they are calculated from directly measured variables, and, thus, highly correlated with them.

## RESULTS AND DISCUSSION

The provenance average values of particular morphological traits found in our study did not differ notably from values reported by other authors [6,2,32]. Minimum, maximum and mean values were calculated for the analyzed leaf traits, as well as coefficient of variation and standard deviation as main indicators of variability (Table 3). The studied leaf traits showed the



**Fig. 3.** Distribution of eleven European beech provenances in three-dimensional (upper figure) and two-dimensional (lower figure) scatter diagrams showing the relationship between provenances and the PCs.

highest variation coefficient for LA and LW1 (17.80% and 17.14%, respectively). Contrarily, the lowest variation coefficient was observed for NVR and NVL (7.77% and 8.11%, respectively).

The extent to which tree populations are capable of adapting to new environments will depend on the level of genetic variation within and between tree populations [38]. The ANOVA analysis indicated that variation between provenances as well as variation between genotypes within provenances were highly significant ( $p < 0.001$ ) for all analyzed traits (Table 4). Since the level and distribution of genetic variation among beech provenances are usually accompanied by ecological and geographical variation across a species' natural distribution [39,40], the observed differences between provenances are likely the result of the differ-

ent provenances' genetic architecture developed as a result of local adaptation to diverse selective pressures existing across the beech distribution range. Similar results were reported by Bayramzadeh et al. [4], who studied the variation in certain leaf morphological traits in natural populations of *Fagus orientalis* in the Caspian forests of northern Iran. Similarly, variation between provenances, based on leaf morphological traits has also been found within other tree species [41,42,9]. With regard to within-provenance genetic variability, the high between-tree variation within the provenances may be the results of the complex effect of the microsite conditions experienced by each tree and genetic differences between individuals [31,43]. Several studies have already demonstrated a high intrapopulation variation of isozyme-gene markers in European beech populations [44-47]. A high intrapopulation variability of certain functional traits has been documented in beech as well [38,48,43].

The presence of significant differences among beech provenances in terms of leaf morphological traits corresponds to the findings reported by several authors [49-51]. However, these studies did not provide insight into the structure of the between-provenance variability. Therefore, PCA was performed to determine the relationships among provenances and to examine the patterns of genetic variation between them.

The three principal components (PC1-PC3) explained approximately 81% of the total variance among the European beech provenances tested, and the first two PCs accounted for approximately 65.7% of the multi-trait variation (Table 5). However, although total variance was explained by eight PCs, we used only an eigenvalue greater than one as a measure for the significance of a PC [37]. The rest of the components (PC4-PC8) varied to a lesser extent (18.7% of total variance). Thus, only correlations between the original variables and the first three PCs are shown in Table 5 and further analyzed. PC1 accounted for 36.5% of the total variance. The highest contribution on PC1 corresponded to variables related to the leaf size: LA (-0.882) and LW (-0.876). A relatively high score on PC1 was also determined for the LL (-0.625), as well. Similarly, Hatziskakis et al. [32] reported that the high morphological diversity of Greek beech populations is mainly explained by leaf size, leaf shape and

**Table 3.** Descriptive statistical analysis of studied leaf morphometric traits. The trait acronyms are defined in Materials and Methods.

Trait	Mean	Minimum value	Maximum value	Range	SD	CV (%)
LA	22.8	20.7	24.7	4.0	4.05	17.80
LMA	6.6	5.0	8.0	3.0	0.76	11.59
LL	65.9	62.9	68.1	5.2	7.22	10.91
LW	48.0	45.7	50.3	4.6	5.15	10.73
PL	7.9	6.5	8.9	2.4	1.33	16.92
LW1	32.7	31.4	35.0	3.6	5.61	17.14
DV	8.5	7.9	8.8	0.9	1.26	14.70
NVL	8.1	7.8	8.4	0.6	0.65	8.11
NVR	8.0	7.8	8.3	0.5	0.62	7.77
LI	139.5	131.2	151.4	20.2	15.63	11.05
PI	12.1	10.4	13.5	3.1	1.82	14.98

petiole length. Paridari et al. [52] found that lamina length and lamina width were the most strongly correlated with PC1 in *Carpinus betulus* populations located along an altitudinal gradient. PC2 explained 29.3% of the total variance. Variables responsible for the differentiation along the PC2 are the following morphological characters: LW1 (0.870), NVL (-0.683) and NVR (-0.662). The third PC accounted for 15.6% of the total variance. Characters contributing to differentiation along the PC3 are: PL (-0.805) and DV (0.609) (Table 5).

**Table 5.** Eigenvalues, proportion of total variability and correlations between the investigated variables and the first three principal components (PCs)

Trait	PC 1	PC 2	PC 3
LA	<b>-0.882</b>	0.316	0.177
LL	-0.625	0.384	-0.132
LW	<b>-0.876</b>	-0.010	0.199
PL	-0.176	0.467	<b>-0.805</b>
LW1	-0.150	<b>0.870</b>	-0.215
DV	-0.309	0.463	<b>0.609</b>
NVL	-0.622	<b>-0.683</b>	-0.268
NVR	-0.667	<b>-0.662</b>	-0.143
Eigenvalue	2.92	2.34	1.25
% Var.	36.45	29.25	15.58
Cum. %	36.45	65.71	81.29

**Table 4.** F-statistics for 11 leaf morphometric traits in European beech provenances, from a nested-crossed analysis of variance. The trait acronyms are defined in Materials and Methods.

Variance component		df	LA	LMA	LL	LW	PL	LW1	DV	NVL	NVR	LI	PI
Provenance	F	10	15.70	11.22	14.45	6.42	23.12	7.51	4.70	32.40	29.80	4.70	20.51
$p (<)$			0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Genotype (provenance)	F	99	10.58	18.10	8.09	6.74	12.13	10.94	13.48	8.70	8.50	6.42	8.01
$p (<)$			0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

The scatter plot of the first three PCs and the two PCs (Fig. 3) shows geometrical distances among the provenances in the plot that reflect a genetic similarity in terms of the studied leaf morphometric traits [35]. Because the first three PCs explained the majority of total variation (81%) among the provenances, the approximation of the real multivariate diversity of the provenances on the 3-PC axis is considered to be acceptable for the most important discriminating (contributing) traits [53,54,35]. Nevertheless, because the three-dimensional scatter plot does not provide a clear projection of the provenances in the plot, the distribution of provenances on the PC1 and PC2 was presented on a two-dimensional plane. Starting from the negative to the positive values of PC1, beech provenances demonstrated a general decrease in leaf area, leaf width and length. Starting from the negative towards the positive values of PC2, the provenances generally decreased in number of veins on both sides of the leaf blade and increased leaf width at 1 cm above the leaf base. Based on the geometrical distances among the provenances in the scatter plot, four groups of related provenances could be separated. Group I consists of two provenances, with a high negative value of PC1 and low negative of PC2 (RS36 and BA32). Group II includes provenances with both slightly negative and positive PC1 values and intermediate positive PC2 values (HR25 and BA33). Similar to the previous group, Group III also included two provenances with slightly negative and positive PC1 values, but low positive PC2 values (AT56 and DE46). Two provenances with low to intermediate positive PC1 values and low negative PC2 values were assigned to Group IV (HU42 and RO63). The rest of the provenances may be considered unique (DE46, DE47 and RS38) (Fig. 3).

The genetic background and possible adaptation of beech populations to different environmental conditions have resulted in a complex morphological pattern, especially in areas in which beech populations have been spreading from different glacial refugia [32].

The absence of association between the provenances from the same geographic regions revealed an ecotypic variation pattern related to seed origin that affected leaf morphometric traits in beech provenances. This is in agreement with previously reported findings, which demonstrated that beech is characterized by an ecotypic rather than clinal variation pattern [55,56,51]. According to Sułkowska [40], this differentiation of European beech is likely the result of many different factors, not only environmental and genetic but also anthropogenic ones. Comps et al. [57] proposed that the higher genetic diversity observed in beech populations toward the southern limit of species distribution (e.g. the Balkan Peninsula) is the result of different causes – e.g., more heterogeneous ecological conditions, older stands and the location of beech refugia were numerous. For example, during postglacial periods, in the Balkan Peninsula area genetically different beech populations spread from several glacial refugia, but never occupied larger areas [58,59]. Likewise, due to the common silvicultural management practice of natural regeneration in beech stands, the natural composition of pure and mixed beech forests has been preserved in many parts of the range (e.g. Balkan Peninsula) [60]. In contrast, Demesure et al. [44] believe that beech populations in the northern range are genetically more uniform, indicating a bottleneck at the time of postglacial recolonization. For example, Sułkowska [40] reported high genetic diversity of beech in Poland, similar to other neighboring European populations, with a slight decrease in the average number of alleles per locus and level of differentiation towards the north of the natural range limit. Lower genetic diversity is also the consequence of the conversion of beech forest to coniferous stands. In the past, many beech forests were converted into agricultural land, and later into coniferous forests (e.g. Scots pine and Norway spruce stands), particularly in the western and north-eastern distribution range (Czech Republic, Germany and Poland) [60,61]. Isajev et al. [24] consider that this practice in some cases resulted in a reduction in the genetic diversity to the level of risk of the species (e.g. in disjunctive and marginal populations). Nevertheless, our study showed that German provenances were assigned to different groups, being genetically distant from each other. The obtained results further support the findings of Magri et al. [59] who argued that beech in central and northern Europe originates

from different refugia that were located in southern France, the western Alps, eastern Alps-Slovenia-Istria and southern Moravia-southern Bohemia.

## CONCLUSIONS

The study was conducted on young beech plants in the juvenile stage of development. The results showed the presence of significant within- and between-provenance genetic variation with regard to all the parameters measured. Notable morphological differences between the investigated provenances are probably related to the specificity of their habitats and different selective pressures. PCA revealed an absence of association between the provenances from the same geographic regions (e.g. Germany and the Balkan Peninsula), revealing a more ecotypic pattern of genetic variation. The existence of substantial genetic variability both within and between provenances might be of crucial relevance in adaptation processes of beech to climate change.

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**Conflict of interest disclosure:** We certify that there is no actual or potential conflict of interest in relation to this article, including any financial, personal, or other relationships with people or organizations.

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