# LEAF STOMATAL TRAITS VARIATION WITHIN AND AMONG FOURTEEN EUROPEAN BEECH (*Fagus sylvatica* L.) PROVENANCES

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In the present study, variations of leaf stomatal traits for fourteen European beech provenances (*Fagus sylvatica* L.), originating from six countries (Bosnia and Herzegovina, Croatia, Germany, Hungary, Romania and Serbia), were evaluated at the inter- and intra- provenances levels. Ten stomatal traits (stomatal density (SD), guard cell length ($L_A$) and width ($W_B$), stomatal aperture length ($L_a$) and width ($W_b$), pore surface of guard cell ($SPS_{LAWB}$), pore surface of a widely opened pore ($SPS_{LaWb}$), coefficient (SSC), potential conductance index (PCI) and relative stomatal pore surface (RSPS)) were examined using methods of univariate (ANOVA and Tukey's HSD test) and multivariate statistical analyses (principal component analysis (PCA), discriminant analysis and cluster analysis).
The results obtained provide significant insight into the genetic diversity and differentiation of European beech provenances and confirmed high genetic variability within and between examined European beech provenances, according to all examined parameters. Differences at the intra- provenance level had a bigger impact on most of the

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examined stomatal traits (with 70-80% of total variation), while differences at the inter-provenances level accounted for only 20-30% to the total expected variance. The results of PCA and canonical analysis suggest ecotypic pattern of genetic variability related to seed origin, which influenced leaf stomatal traits in *F. sylvatica* provenances. Forward stepwise discriminative analysis showed that $W_B$, SD, PCI, $SPS_{LaWb}$ and $W_b$ traits accounted for the same level of discrimination, as all the examined stomatal parameters together.

However, the analysis detected considerably small correct allocation of the model that included all examined traits (only 38.85%). In order to discriminate examined beech provenances more precisely, additional phenotypical traits (e.g. morphological, physiological, etc.) should be included in the model.

*Keywords*: *Fagus sylvatica* L.; provenance trial; leaf stomatal traits; within- and between-provenance variation; ecotypic genetic variability

## INTRODUCTION

Stomata are morphological structures in the epidermis of leaves, which are essential for two of the most important of plant processes: photosynthesis and transpiration (REZAEI NEJAD *et al.*, 2006; XU and ZHOU, 2008; SARWAR *et al.*, 2013). These passages on a plant surface regulate the exchange of gasses, especially water vapor and $CO_2$ (ZHANG *et al.*, 2012), between internal plant tissue and the atmosphere. With this procedure, plants maintain optimal photosynthesis (DASZKOWSKA-GOLEC and SZAREJKO, 2013) and reduce water loss by adjusting the level of transpiration. Due to their role in the regulation of transpiration, stomata also have an important role in leaf cooling (HETHERINGTON and WOODWARD, 2003), long distance signaling (JIA and ZHANG, 2008) and the creation of a barrier for the ozone and other pollutants (WEI *et al.*, 2017).

Moreover, understanding the adaptation mechanism of leaf anatomical variation can help in understanding the adaptation strategies of species, as well as forecasting their reaction to future climate conditions (TIAN *et al.*, 2016). Generally, plants in arid regions have smaller and densely arranged stomata, to minimize water loss and avoid dehydration (KUSVURAN *et al.*, 2009). The benefit of smaller stomata is a quick reaction to a sudden change in habitat conditions which results in a faster reduction of conductance (DRAKE *et al.*, 2013; ELLIOTT-KINGSTON *et al.*, 2016). In those terms, species with more effective stomatal control are expected to have a higher chance of survival in the upcoming climate change (HAWORTH *et al.*, 2011). Another strategy of plants pressured by unfavorable conditions is facilitated through stomata behavior, which presents a short turn-reaction when experiencing drought stress (LUAN, 2002; MARTIN-STPAUL *et al.*, 2017), salinity (MUNNS and TESTER, 2008; SOLMAZ *et al.*, 2011), a high $CO_2$ concentration (YOUNG *et al.*, 2006; ENGINEER *et al.*, 2014) or a vapor pressure deficit (BALOTA *et al.*, 2012; MCADAM *et al.*, 2016). For instance, when in water deficit, plants can react by closing their stomata when water supply is limited (ZWIENIECKI *et al.*, 2016).

European beech (*Fagus sylvatica* L.) is a main forest tree species with high economic value and ecological importance in Central European forests (BALANDIER *et al.*, 2007; RAJSNEROVÁ *et al.*, 2015). According to projected climate scenarios with more intense and frequent drought, *F. sylvatica* is thought to be a severely threatened species (STOJANOVIĆ *et al.*, 2013; PFLUG *et al.*, 2018). Intraspecific genetic diversity is of fundamental importance for the adaptation and evolution of forest ecosystems under changing climate conditions (FRANKHAM *et al.*, 2002; SCHUELER *et al.*, 2012). Species with higher intraspecific genetic variations are

presumed to have a greater chance of survival and adaptation to changing biotic and abiotic stresses (JUMP et al., 2009; KIRK and FREELAND, 2011; TOOKER and FRANK, 2012). High levels of genetic variation within populations can be the result of outcrossing matting systems, diversifying selection, continuous distribution, a long life span and a large population (HAMRICK *et al*., 1992; PETIT and HAMPE, 2006). Considering that the same species might be endangered by a loss of genetic diversity, its determination could help the formation of preservation strategies, breeding and improvement programs (AMOS and BALMFORD, 2001; KESIĆ *et al*., 2018).

In order to determine within- and between- population genetic variations, provenance trials are of key importance, since the environmental variation in these trials is minimized and the majority of differences can be explained by genetic differentiation (MEIER and LEUSCHNER, 2008). However, provenance trials are usually affected by factors of variable soil conditions and microclimate, therefore spatial patterns of these trials must be taken into consideration when geographical trends are shown as a result of adaptation (GÖMÖRY *et al*., 2011). In addition to adaptation determination, these trials could be used to find populations with the most desireable charactersitics (e.g. good growth and adaptational properties, etc.) that could be used for future reforestation (GÖMÖRY, 2010).

The present study focus on evaluation of intra- and inter- provenance variation of leaf stomatal traits of examined European beech provenances using methods of univariate (ANOVA, Tukey's HSD test), and multivariate (PCA, discriminant analysis and cluster) statistical analyses. Although a certain study (KUČEROVÁ *et al*., 2018) of the variability of European beech provenances included the measurement of stomatal traits, to the best of our knowledge, this study is the first to demonstrate an analysis of variation based on the above-mentioned statistical methods. Furthermore, the present study has shown the importance of an assessment of examined stomatal traits in the discrimination of provenances. This finding could be of great importance for further research, due to possibility of providing the number of traits which is sufficient for discrimination between provenances and reducing the time spent for investigation. Moreover, we have attempted to give insight into the structure of variability between the stomatal traits of European beech provenances, which could contribute to the further understanding of the genetic variation of stomata.

## MATERIAL AND METHODS

European beech provenance trial in Serbia was established in 2007 on Fruška Gora Mountain (N 45°10', E 19°47'), as the part of a pan-European network of beech provenances trials initiated in the framework of the *COST Action E52 - Evaluation of the Genetic Resources of Beech for Sustainable Forestry*. The climate of Fruška Gora Mountain is temperate continental. Mean annual temperature is approximately 11.1°C, while the annual sum of precipitation amounts to about 624 mm. The experimental plot is located at an altitude of 370 m a.s.l. Detailed description of the trial site environmental conditions was given by STOJNIĆ *et al*. (2012).

The provenance trial was founded with 2- and 3-year-old seedlings produced in the nursery of the Thünen-Institute for Forest Genetics (Grosshansdorf, Germany), and arranged in a randomized complete block design, with three replications (i.e. 50 seedlings per each block, planted with 1 x 2 m spacing). The present study involved 14 provenances originating from six countries (Bosnia and Herzegovina, Croatia, Germany, Hungary, Romania and Serbia) (Table 1.; Figure 1.).

*Table 1. General data about provenances involved in study*

| Abbrev. | Provenance | Country | Latitude (dd.mm) | Longitude (dd.mm) | Altitude (m) | Mean air temperature (°C) | Sum of precipitation (mm) |
|---------|-----------|---------|---------|---------|---------|---------|---------|
| HR24 | Sjeverini Dilj Čaglinski | Croatia | 45°17' | 18°01' | 350 | 10.8 | 779 |
| HR25 | Vrani Kamen | Croatia | 45°37' | 17°19' | 600 | 8.5 | 972 |
| BA30 | Tajan, Žepče | Bosnia | 44°23' | 18°03' | 700 | 10.3 | 804 |
| RS36 | Fruška Gora | Serbia | 45°10' | 19°55' | 370 | 11.1 | 624 |
| RS38 | Kopaonik | Serbia | 43°10' | 20°50' | 820 | 3.6 | 985 |
| HU42 | Valkonya | Hungary | 46°30' | 16°45' | 300 | 9.5 | 800 |
| DE47 | Schelklingen | Germany | 48°00' | 10°00' | 650 | 6.0 | 840 |
| DE48 | Höllerbach | Germany | 49°01' | 13°14' | 755 | 5.0 | 1200 |
| BA59 | Vranica-Bistrica | Bosnia | 43°33′ | 17°49′ | 750 | 8.8 | 826 |
| BA60 | Crni Vrh | Bosnia | 44°33′ | 17°59′ | 500 | 9.6 | 1069 |
| BA61 | Grmeč, Bosanska Krupa | Bosnia | 44°46' | 16°16' | 650 | 10.3 | 1304 |
| RO64 | Alba-Iulia | Romania | 46°10' | 23°05' | 860 | 8.0 | 850 |
| RS66 | Avala | Serbia | 44°23′ | 20°45′ | 475 | 11.9 | 668 |
| RS67 | Boranja | Serbia | 44°01' | 19°45' | 410 | 668 | 10.9 |
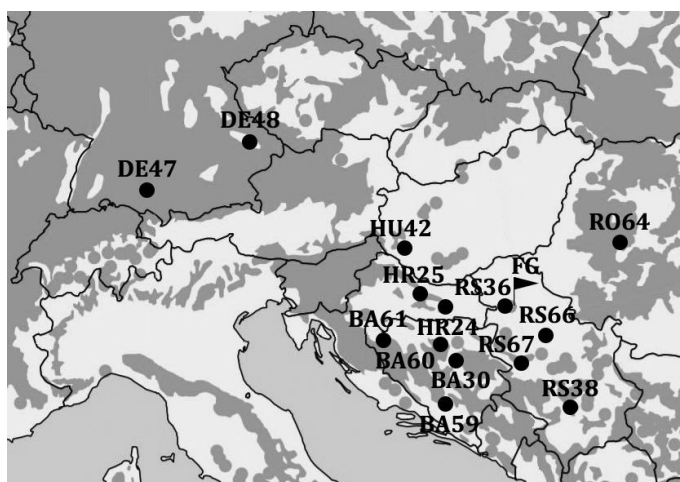


Figure 1. Distribution of European beech provenances involved in the study. The abbreviation indicates provenances (●) as shown in Table 1. Trial site (FG) is marked with the flag icon. The dark shaded area represents natural distribution range of beech (after: von Wuehlisch 2008)

Leaf imprints were collected from the upper third of the crown of fully expanded leaves (ORLOVIĆ *et al*., 2003) at the end of July 2011, in the timescale between 9:00 AM and 11:00 AM, on a sunny and windless day. Per each studied provenance, imprints were made on the abaxial leaf surface, between the 3$^{rd}$ and 4$^{th}$ leaf vain with one replication on 10 randomly chosen seven and eight-year-old seedlings.

Stomatal density per mm$^2$ (SD), stomata guard cell length (L$_A$ [μm$^2$]) and width (W$_B$ [μm$^2$]), and stomatal aperture length (L$_a$ [μm$^2$]) and width (W$_b$ [μm$^2$]) were determined from leaf prints following the protocol described by STOJNIĆ et al. (2015). Measurements of stomata imprints were performed using the *Olimpus Vanox* light microscope. SD was determined at five randomly chosen fields of view using *tpsDIG 2* freeware software (http://life.bio.sunysb.edu/ee/rohlf/software.html). The size of stomata guard cells and stomata aperture were measured with *QuickPhoto Camera 3.2.* software, on five stomata per five randomly chosen fields of view. Beside mentioned stomatal characters, following parameters were calculated, as well:

-stomatal pore surface of stomata guard cell (SPS$_{LAWB}$=(L$_A$*W$_B$*π)/4 [μm$^2$]) (BALASOORIYA *et al*., 2009);

-stomatal pore surface of a widely opened stomatal pore (SPS$_{LaWb}$=(L$_a$*W$_b$*π)/4[μm$^2$]) (BALASOORIYA *et al*., 2009);

-stomatal shape coefficient (SSC=100*W$_b$/L$_a$) (BALASOORIYA *et al*., 2009);

-potential conductance index (PCI=L$_A$$^2$*SD*10$^{-4}$) (HOLLAND and RICHARDSON, 2009);

-relative stomatal pore surface (RSPS=(SPS$_{LaWb}$*SD)*100 [%]) (KARDEL *et al*., 2010).

The variability of the examined group of provenances was described by methods of univariate (ANOVA, Tukey's HSD (honestly significant difference) test), and multivariate analysis (principal component analysis, discriminant analysis and cluster analysis), performed by STATISTICA for Windows version 13 (TIBCO Software Inc, 2017).

## RESULTS AND DISCUSSION

In the present study, the differences among provenances, were found to be statistically significant for all ten measured stomatal parameters (Table 2). This result suggests a high genetic diversity of European beech, which has been already evidenced with respect to certain anatomical (STOJNIĆ *et al*., 2016a; HAJEK *et al*., 2016), morphological (IVANKOVIĆ *et al*., 2011; MEKIĆ *et al*., 2010), phenological (CHMURA and ROZKOWSKI, 2002; BALLIAN *et al*., 2015) and physiological (STOJNIĆ *et al*., 2010; KUČEROVÁ *et al*., 2018) traits. The difference between provenances, applies to different functional traits, are assumed to be the result of the various genetic architecture of provenances, which has evolved as a result of local adaptation, in response to diverse selective pressure in areas of beech distribution (JUMP *et al*., 2006; STILL *et al*., 2005; STOJNIĆ *et al*., 2016b). This is characteristic of species that occupy large geographic ranges (ECKERT *et al*., 2008). According to PLUESS *et al*. (2016), species existing under different environmental conditions may contribute to substantial genetic diversity, reducing the risk of extinction under changed environmental conditions. Increased genetic diversity contributes to a species faster response to climate changes and is of key importance for survival and adaptation to changed environmental conditions (ALBERTO *et al*., 2013; FAO, 2014; POTTER *et al*., 2017). FETZNER and CRANDALL (2001) noted that species with low genetic variation are under a high risk of extinction as they might not be able to evolve a solution even in instances of smaller changes in site conditions. Large quantity of genetic variation is necessary for natural selection

to alter the frequency of specific alleles in response to forthcoming environmental conditions (VORNAM *et al*., 2004).

A high degree of inter-population variation, concerning different stomatal traits, has also been confirmed for other tree species originating from regions with a temperate-continental climate. For instance, BATOS *et al*. (2010) found significant inter-population variation for SD, PCI, SSC and $L_A$ between five natural populations of *Quercus robur* L. in Serbia. Likewise, BRUSCHI *et al*. (2003) noted statistically important variations between five natural Italian populations of *Quercus petrea* (Matt.) Liebl., in SD, LS and WS, as well as for the stomatal area. Furthermore, ČORTAN *et al*. (2017) reported significant genetic differentiations among four native populations of *Populus nigra* L. originating from Serbia for $L_a$ and SSC.

*Table 2. Results of the analysis of variance for measured parameters of examined beech provenances*

| Examined traits | $SS_B$[1] | $MS_B$ | $SS_W$ | $MS_W$ | $F_B$ value | $CV_B(\%)$ | $CV_W(\%)$ |
|---|---|---|---|---|---|---|---|
| SD | 107198.5 | 152906.34 | 8246.04 | 1223.25 | 6.74 *** | 9.12 | 11.99 |
| $L_A$ | 33.3 | 68.94 | 2.56 | 0.55 | 4.64 *** | 2.04 | 3.38 |
| $W_B$ | 58.1 | 42.63 | 4.47 | 0.34 | 13.11 *** | 3.46 | 3.13 |
| $L_a$ | 26.4 | 74.55 | 2.03 | 0.60 | 3.41 *** | 2.87 | 5.82 |
| $W_b$ | 5.0 | 9.73 | 0.39 | 0.08 | 4.96 *** | 2.77 | 4.39 |
| $SPS_{LAWB}$ | 38199.5 | 36891.16 | 2938.43 | 295.13 | 9.96 *** | 5.06 | 5.33 |
| $SPS_{LaWb}$ | 1862.2 | 4035.14 | 143.24 | 32.28 | 4.44 *** | 5.04 | 8.56 |
| SSC | 277.5 | 949.09 | 21.35 | 7.59 | 2.81 ** | 2.45 | 5.73 |
| PCI | 180.8 | 390.42 | 13.91 | 3.12 | 4.45 *** | 7.40 | 12.55 |
| RSPS | 3.3 | 8.37 | 0.25 | 0.07 | 3.76 *** | 7.08 | 13.43 |

Labels concerning significance of F-test: \*\*p < 0.01; \*\*\*p < 0.001;

$SS_B$ – sum of square between provenances, $MS_B$ – mean square between provenances, $SS_W$ – sum of square within provenances and of residual, $MS_W$ – mean square within provenances and of residual, $F_B$ value – F value for variation between provenances, $CV_B$ – coefficient of variation between provenances (%), $CV_W$ – coefficient within provenances (%)

Stomata may vary in density and size among species (HULTINE and MARSHALL, 2001; CASSON and GRAY, 2008; BAYRAMZADEH, 2011; WANG *et al*., 2015.), provenances (*STOJNIĆ* et al*., 2015)*, varieties (SOLANGI *et al*., 2010; BOSO *et al*., 2016), clones (ORLOVIĆ *et al*., 2003; ORLOVIĆ *et al*., 2004; MILJKOVIĆ *et al*., 2013) and cultivars (SAGARAM *et al*., 2007; AVCI and AYGÜN, 2014). Moreover, they depend on the phase of leaf development (KOUWENBERG *et al*., 2004; VATÉN and BERGANN, 2012), the position of the leaf (BRUSCHI *et al*., 2003; BATOS *et al*., 2010), a part of the leaf (*SMITH* et al*., 1989)*, and environmental conditions (HETHERINGTON and WOODWARD, 2003; *CASSON and GRAY, 2008*). The present study has confirmed the variation of stomatal traits between, as well as within provenances. SD, PCI and RSPS were estimated to be the most heterogenous traits with values of 9.12%, 7.40% and 7.08% coefficient of variation between provenances, respectively. On the contrary, $L_A$ (2.00%) and SSC (2.34%) varied to a lesser extent between provenances. In the same way, RSPS (13.34%), PCI (12.47%) and SD (11.73%) demonstrated the highest values of coefficient of variation within provenances.

Meanwhile, $W_B$, $L_A$ and $W_b$ were found to be the most homogenous, with a variation coefficient less than 5%.

In addition, in order to acquire a complete picture of the significance of differences between provenances, in respect of the examined stomatal traits, Tukey's **HSD post-hoc test** was performed. The results of the test for all examined traits have been presented in Table 3. The highest number of homogenous groups was determined for $SPS_{LAWB}$ (six homogenous groups) and $W_B$ (five homogenous groups). In contrast, provenances related to SSC of stoma formed only two homogenous groups, suggesting that this trait had the smallest influence on the differentiation between the examined provenances.

*Table 3. HSD-test for measured parameters of examined European beech provenances. Differences between values followed by the same letter are not statistically significant (p ≤ 0.05)*

| Provenance | SD | $L_A$ | $W_B$ | $L_a$ | $W_b$ | $SPS_{LAWB}$ | $SPS_{LaWb}$ | SSC | PCI | RSPS |
|---|---|---|---|---|---|---|---|---|---|---|
| HR24 | 276.13 [cd] | 22.93 [a] | 19.53 [a] | 14.10 [a] | 6.57 [a] | 351.56 [a] | 72.80 [a] | 46.66 [ab] | 14.52 [ab] | 2.01 [ab] |
| HR25 | 285.37 [bcd] | 22.23 [abc] | 18.64 [bcd] | 13.77 [ab] | 6.65 [a] | 325.57 [bcd] | 71.92 [a] | 48.36 [ab] | 14.08 [ab] | 2.04 [ab] |
| HU42 | 261.43 [cd] | 22.09 [abcd] | 19.04 [ab] | 13.63 [abc] | 6.45 [ab] | 330.33 [abcd] | 69.07 [ab] | 47.43 [ab] | 12.76 [bc] | 1.81 [bc] |
| DE47 | 351.40 [a] | 21.02 [d] | 17.95 [de] | 12.63 [bc] | 6.30 [abc] | 296.26 [ef] | 62.57 [bc] | 49.96 [a] | 15.53 [a] | 2.20 [a] |
| DE48 | 311.93 [abc] | 21.49 [bcd] | 18.46 [bcd] | 12.77 [bc] | 6.24 [abc] | 311.67 [cdef] | 62.74 [bc] | 48.91 [ab] | 14.42 [ab] | 1.96 [abc] |
| RO64 | 287.27 [bcd] | 22.11 [abcd] | 19.15 [ab] | 12.94 [bc] | 6.51 [ab] | 332.66 [abcd] | 66.10 [abc] | 50.37 [a] | 14.06 [ab] | 1.90 [abc] |
| RS36 | 282.43 [bcd] | 22.24 [abc] | 18.83 [abc] | 13.54 [abc] | 6.46 [ab] | 329.25 [abcd] | 68.90 [ab] | 47.81 [ab] | 13.92 [ab] | 1.94 [abc] |
| RS38 | 305.93 [abc] | 21.32 [cd] | 17.21 [e] | 12.59 [c] | 5.89 [c] | 288.18 [f] | 58.17 [c] | 47.04 [ab] | 13.91 [ab] | 1.78 [bc] |
| RS66 | 275.77 [cd] | 21.87 [abcd] | 17.86 [de] | 13.17 [abc] | 6.26 [abc] | 307.00 [def] | 64.90 [abc] | 47.70 [ab] | 13.17 [abc] | 1.78 [bc] |
| RS67 | 272.60 [cd] | 22.54 [ab] | 18.01 [cde] | 13.66 [abc] | 6.13 [bc] | 319.04 [bcde] | 65.86 [abc] | 44.92 [b] | 13.87 [abc] | 1.79 [bc] |
| BA30 | 238.63 [d] | 21.67 [bcd] | 18.67 [abcd] | 13.46 [abc] | 6.38 [ab] | 317.68 [bcde] | 67.48 [ab] | 47.51 [ab] | 11.25 [c] | 1.62 [c] |
| BA59 | 292.70 [bc] | 22.12 [abcd] | 19.26 [ab] | 13.11 [abc] | 6.43 [ab] | 334.65 [abc] | 66.24 [abc] | 49.16 [a] | 14.34 [ab] | 1.94 [abc] |
| BA60 | 333.26 [ab] | 21.89 [abcd] | 19.14 [ab] | 13.18 [abc] | 6.27 [abc] | 329.48 [abcd] | 65.07 [abc] | 47.76 [ab] | 15.88 [a] | 2.14 [ab] |
| BA61 | 307.57 [abc] | 22.46 [ab] | 19.28 [ab] | 13.09 [abc] | 6.51 [ab] | 339.98 [ab] | 66.91 [ab] | 49.79 [a] | 15.51 [a] | 2.06 [ab] |

The intra- and inter-populational variation in stomatal traits of European beech provenances have not been completely understood thus there is a need for proof, which could provide a clear perception of the structure of variability between provenances. Numerous studies into the gene markers of European beech have confirmed that most of the variation is harbored within population or stand, and that the diversity between population or stands rarely exceeds 5% (KONNERT, 1995; KRZAKOWA and MATRAS, 2005; KEMPF and KONNERT, 2016; SZASZ-LEN and CONNERT, 2018). For example, SANDER *et al*. (2000) detected that 99% of the diversity is within the stands and only 1% is between them. In terms of phenotypic traits, BRESSON *et al*. (2011) examined the leaf morphological and physiological traits and found that, in a common garden experiment, genetic differentiation between *F. sylvatica* populations accounted for only 0–28% of total phenotypic variation. Our results are in concordance with these results, as differences within provenances had a bigger impact on most of the examined traits (70-80% of total variation), while differences between provenances contributed by only 20-30% to the total expected variance (Figure 2). In case of variability within population, the greatest contribution of

this source of variation was observed for SSC (84.6%) and $L_a$ (80.5%). On the other hand, the highest impact of variation between provenances was found for $W_B$ (55.0%). High contribution of within-population variance could be explained by the fact that in wind-pollinated forest tree species, such as the European beech (VORNAM *et al.*, 2004; CHYBICKI *et al.*, 2009; COMPS *et al.*, 2001; PIOTTI *et al.*, 2012), effective pollen dispersal facilitates extensive gene flow, which results in high genetic diversity between individuals of the same population, low diversity among the population (HAMRICK *et al.*, 1992; BURCZYK and CHYBICKI, 2004; SHANJANI *et al.*, 2010; KRAMER, 2012; POLJAK *et al.*, 2014), and low spatial genetic structure (JUMP *et al.*, 2012).
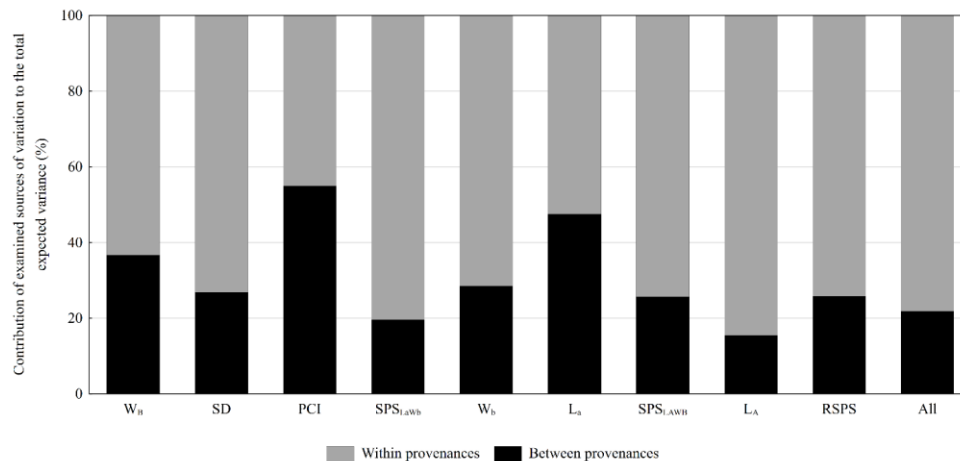


Figure 2. Contribution of examined sources of variation to the total expected variance for measured parameters of examined European beech provenance

Correlation analysis of stomatal parameters showed a positive linear association in less than half of the cases (Table 4). These results suggest the existence of multicollinearity within the examined set of parameters. However, PCI and RSPS parameters had significant correlations only between themselves and with SD, while SSC had only significant correlation with La, suggesting that they contain unique information on the differences between provenances. A positive correlation was observed between derived parameters and the original ones, from which they had been calculated. An inverse relationship was found to exist between SD and $L_a$ (r = -0.573, p< 0.05), which is consistent with results obtained by other authors (NIKOLIĆ *et al.*, 2003; ORCEN *et al.*, 2013). According to MISKIN *et al.* (1972) this negative association of the two mentioned parameters is probably balancing the correspondence of stoma, which tends to conserve constant total pore area. FRANKS *et al.* (2009) explained this occurrence as a tendency for higher maximum stomatal conductance of water vapor to be attained, based on a decrease of stomatal size and an increase in its density. Smaller, densely arranged, stomata react faster to environmental changes allowing for a better adaptation of the species (FRASER *et al.*, 2009; DRAKE *et al.*, 2013). RAVEN (2014) noted that a large number of small stomata is of primary importance for forthcoming climate changes, as they can quickly modify their area and regulate stomatal conductance in order to optimize water-use efficiency.

*Table 4. Correlation coefficients based on mean populations values for measured parameters of examined beech provenances*

|  | SD | $L_A$ | $W_B$ | $L_a$ | $W_b$ | $SPS_{LAWB}$ | $SPS_{LaWb}$ | SSC | PCI | RSPS |
|---|---|---|---|---|---|---|---|---|---|---|
| **SD** | - | -0.461 | -0.161 | -0.643* | -0.253 | -0.301 | -0.511 | 0.469 | 0.875** | 0.812** |
| **$L_A$** |  | - | 0.627* | 0.797** | 0.552* | 0.852** | 0.769** | -0.334 | 0.025 | -0.008 |
| **$W_B$** |  |  | - | 0.471 | 0.798** | 0.942** | 0.705** | 0.275 | 0.168 | 0.300 |
| **$L_a$** |  |  |  | - | 0.557* | 0.657* | 0.896** | -0.558* | -0.305 | -0.147 |
| **$W_b$** |  |  |  |  | - | 0.772** | 0.867** | 0.378 | 0.015 | 0.308 |
| **$SPS_{LAWB}$** |  |  |  |  |  | - | 0.803** | 0.042 | 0.130 | 0.203 |
| **$SPS_{LaWb}$** |  |  |  |  |  |  | - | -0.132 | -0.167 | 0.086 |
| **SSC** |  |  |  |  |  |  |  | - | 0.362 | 0.477 |
| **PCI** |  |  |  |  |  |  |  |  | - | 0.903** |
| **RSPS** |  |  |  |  |  |  |  |  |  | - |

Labels concerning significance of t-test: *$p < 0.05$; **$p < 0.01$;

According to PCA (Table 5), the first three principal components accounted for 49.7%, 32.5% and 11.3% of the diversity between provenances, respectively (cumulative value was 93.5%). In order to analyze the relationship between the examined traits, the varimax rotation (based on principle of maximization of variation of loadings within principal components) on the first three principal components was performed. Based on loadings with rotated principal components, the traits that had their highest loading, with the same principal component were grouped into the same group. As there is no correlation between principal components, a high correlation characterizes traits within the same group and a low correlation traits from different groups.

*Table 5. Eigenvalues for extracted principal components and their relation to the total variance in examined European beech provenances*

| Principal component | Eigenvalue | Contribution to the total variance (%) | Cumulative variance | Cumulative contribution to the total variance |
|---|---|---|---|---|
| **1** | 4.974 | 49.740 | 4.974 | 49.740 |
| **2** | 3.245 | 32.451 | 8.219 | 82.191 |
| **3** | 1.127 | 11.275 | 9.347 | 93.466 |
| **4** | 0.475 | 4.746 | 9.821 | 98.211 |
| **5** | 0.178 | 1.779 | 9.999 | 99.990 |
| **6** | 0.000 | 0.004 | 9.999 | 99.994 |
| **7** | 0.000 | 0.003 | 10.000 | 99.997 |
| **8** | 0.000 | 0.002 | 10.000 | 99.999 |
| **9** | 0.000 | 0.001 | 10.000 | 100.000 |
| **10** | 0.000 | 0.000 | 10.000 | 100.000 |

A high value of maximum loadings (over 0.70) were recorded for all examined parameters after the rotation (Table 6). These results confirm relations between traits based on a correlation matrix. Six stomatal parameters ($L_A$, $W_B$, $L_a$, $W_b$, $SPS_{LAWB}$ and $SPS_{LaWb}$) had the

highest loadings with the first rotated principal component (RC1), forming the first group. The highest loadings were detected for $SPS_{LAWB}$ (0.942) and $SPS_{LaWb}$ (0.936). Parameters that had their highest loadings with RC2 were SD (0.900), PCI (0.984) and RSPS (0.930), while SSC (0.928) was the only parameter that had its highest loading with RC3. STOJNIĆ *et al*. (2015) examined the variation of stomatal traits in five European beech provenances using PCA analysis without varimax rotation, and detected the highest loadings of SD and PCI with RC1, $W_b$ and $SPS_{LAWB}$ with RC2, and no stomatal feature with its highest loading with RC3. The above-mentioned stomatal parameters showed the same grouping trend, while the dissimilarity in their ranking suggests differences in the discriminating power of examined traits between the two studies.

*Table 6. Loadings between measured parameters of European beech provenances and the first three principal components after their rotation by varimax method*[*]

| Examined traits | RC1 | RC2 | RC3 |
|---|---|---|---|
| SD | -0.368 | <u>0.891</u> | 0.254 |
| $L_A$ | <u>0.812</u> | -0.017 | -0.466 |
| $W_B$ | <u>0.898</u> | 0.131 | 0.206 |
| $L_a$ | <u>0.747</u> | -0.274 | -0.551 |
| $W_b$ | <u>0.910</u> | -0.009 | 0.327 |
| $SPS_{LAWB}$ | <u>0.952</u> | 0.087 | -0.063 |
| $SPS_{LaWb}$ | <u>0.932</u> | -0.160 | -0.158 |
| SSC | 0.078 | 0.303 | <u>0.938</u> |
| PCI | 0.027 | <u>0.991</u> | 0.048 |
| RSPS | 0.212 | <u>0.922</u> | 0.208 |
| Explained variation | 4.814 | 2.844 | 1.689 |
| Proportion of the total variation | 0.481 | 0.284 | 0.169 |

[*] - The underlined values represent the highest loading of particular examined trait

In addition, information on the relationship between the examined beech provenances was presented by the first three rotated principal components' scores (Figure 3). One distinct group could be determined, containing provenances: BA30, HR42, HR24, RS36, HR25, RO64, BA61, BA59, which differed mainly according to RC2. Other provenances appeared to be clearly separated from this group, widely dispersed according to all three rotated principal components.

Statistics for the estimation of the significance of influence of the measured stomatal parameters to the discrimination of examined provenances did not show a statistically significant contribution for any of the examined traits (Table 7). In order to determine the relationship between measured stomatal parameters for the fourteen examined beech provenances, canonical discriminant analysis was performed. Results of $\chi^2$ test statistics showed that the first three canonical variables are significantly related to the variation between provenances (Table 8). The highest discriminative loadings, for the first canonical variable, were determined for parameters $W_B$ (-0.914) and $SPS_{LAWB}$ (-0.754), suggesting their high importance in the discrimination of examined provenances (Table 9). The values of SD (0.855), PCI (0.634) and RSPS (0.587) were

found to be highly correlated to the second canonical variable. Traits $L_A$ (0.437) and $W_b$ (-0.420) showed high loadings with the third canonical variable, but their highest loadings were with the forth canonical variable. These findings are consistent with results from the PCA analysis, except for $L_A$, $L_a$, $W_b$ and $SPS_{LaWb}$ which were grouped in the first group by PCA, but did not have the highest discriminative loadings with the first canonical variable as $W_B$ and $SPS_{LAWB}$. According to KOVAčEVIĆ (2014), the difference in the relationship among the examined provenances, between these two multivariate methods could be partially attributed to the fact that PCA analysis was based on provenances means and did not include information on intra-population variability, as discriminant analysis does.
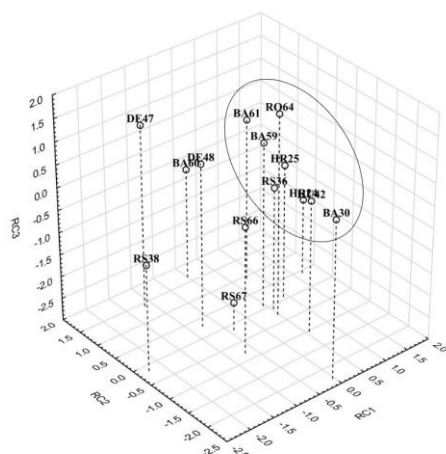


Figure 3. Relationship between examined *Fagus sylvatica* L. provenances based on the first three principal components after their rotation by varimax method

*Table 7. Statistics for the evaluation of the significance of contribution of measured parameters to the discrimination of examined beech populations*

| Examined traits | Wilks' lambda | Partial Wilks' lambda | F-remove | Tolerance |
|---|---|---|---|---|
| SD | 0.081 | 0.907 | 0.914 | 0.007 |
| LA | 0.082 | 0.893 | 1.074 | 0.002 |
| $W_B$ | 0.086 | 0.847 | 1.612 | 0.003 |
| La | 0.081 | 0.904 | 0.944 | 0.002 |
| Wb | 0.081 | 0.901 | 0.977 | 0.002 |
| $SPS_{LAWB}$ | 0.086 | 0.853 | 1.532 | 0.001 |
| SPSLaWb | 0.083 | 0.877 | 1.253 | 0.001 |
| SSC | 0.076 | 0.963 | 0.343 | 0.006 |
| PCI | 0.086 | 0.846 | 1.619 | 0.003 |
| RSPS | 0.082 | 0.894 | 1.054 | 0.004 |

Labels concerning significance of F-test: *p < 0.05

The relationship between European beech provenances based on the first three canonical variables can be observed in Figure 4. Provenances RS36, DE47, DE48, BA59, HR24, RO64, BA60 and BA61, form one group, while the remainder of the examined provenances is widely dispersed among this group according to all three canonical variables. As determined through the first three rotated principal components, in this case too, provenances RS38, RS66 and RS67 are considerably apart from the others.

*Table 8. Canonical analysis statistics for successive canonical variables*

| Roots removed | Eigenvalue | Canonical coefficient | Wilks' lambda | $\chi^2$-test | degrees of freedom |
|---|---|---|---|---|---|
| **0** | 1.593 | 0.784 | 0.073 | 329.671*** | 130 |
| **1** | 0.870 | 0.682 | 0.189 | 209.624*** | 108 |
| **2** | 0.393 | 0.531 | 0.354 | 130.742** | 88 |
| **3** | 0.276 | 0.465 | 0.494 | 88.950 | 70 |
| **4** | 0.206 | 0.413 | 0.630 | 58.198 | 54 |
| **5** | 0.143 | 0.353 | 0.760 | 34.575 | 40 |
| **6** | 0.081 | 0.273 | 0.868 | 17.786 | 28 |
| **7** | 0.039 | 0.194 | 0.938 | 8.009 | 18 |
| **8** | 0.020 | 0.139 | 0.975 | 3.162 | 10 |
| **9** | 0.006 | 0.074 | 0.994 | 0.697 | 4 |

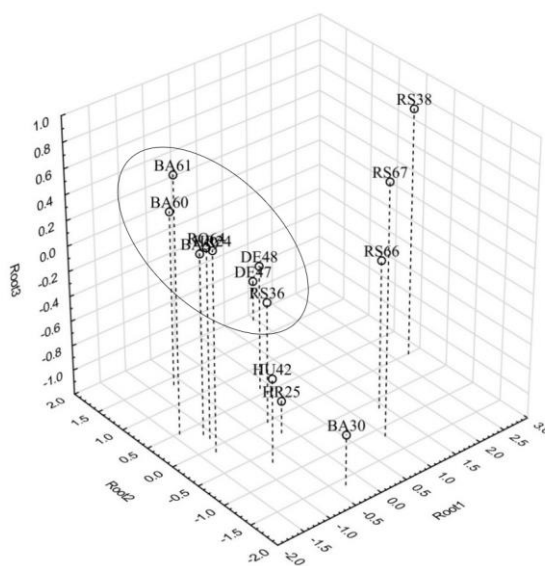Labels concerning significance of $\chi^2$-test: **p < 0.01, ***p < 0.001



Figure 4. Relationship between examined *Fagus sylvatica* L. provenances based on the first three canonical variables (roots)

*Table 9. Discriminative loadings between measured and canonical variables (roots)* [*]

| Examined traits | Root 1 | Root 2 | Root 3 | Root 4 | Root 5 | Root 6 | Root 7 | Root 8 | Root 9 | Root 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| SD | 0.157 | <u>0.855</u> | 0.113 | 0.050 | 0.009 | 0.340 | 0.100 | 0.205 | -0.191 | 0.157 |
| $L_A$ | -0.349 | -0.276 | 0.437 | <u>-0.629</u> | -0.367 | 0.254 | 0.088 | 0.085 | -0.034 | -0.011 |
| $W_B$ | <u>-0.914</u> | 0.101 | 0.013 | -0.234 | 0.113 | -0.206 | -0.033 | -0.076 | 0.007 | 0.194 |
| $L_a$ | -0.220 | -0.355 | -0.108 | <u>-0.738</u> | -0.062 | 0.143 | 0.226 | 0.303 | -0.233 | 0.218 |
| $W_b$ | -0.466 | -0.017 | -0.420 | -0.218 | <u>-0.612</u> | 0.022 | -0.245 | 0.349 | 0.043 | 0.001 |
| $SPS_{LAWB}$ | <u>-0.754</u> | -0.108 | 0.291 | -0.544 | -0.166 | 0.019 | 0.022 | 0.021 | -0.025 | 0.104 |
| $SPS_{LaWb}$ | -0.382 | -0.244 | -0.292 | <u>-0.633</u> | -0.347 | 0.113 | -0.001 | 0.375 | -0.127 | 0.139 |
| SSC | -0.128 | 0.351 | -0.202 | <u>0.576</u> | -0.401 | -0.144 | -0.403 | -0.015 | 0.330 | -0.189 |
| PCI | -0.050 | <u>0.634</u> | 0.356 | -0.255 | -0.183 | 0.459 | 0.101 | 0.247 | -0.230 | 0.186 |
| RSPS | -0.110 | <u>0.587</u> | -0.078 | -0.326 | -0.245 | 0.360 | 0.091 | 0.394 | -0.295 | 0.302 |
| Explained variation | 1.593 | 0.870 | 0.393 | 0.276 | 0.206 | 0.143 | 0.081 | 0.039 | 0.020 | 0.006 |
| Proportion of the total variation | 0.439 | 0.679 | 0.788 | 0.864 | 0.921 | 0.960 | 0.982 | 0.993 | 0.999 | 1.000 |

[*] - The underlined values represent the highest loading of particular examined trait

*Table 10. Statistics for forward stepwise discrimination analysis*

| Examined traits | Wilks' lambda | Partial Wilks' lambda | F-remove | Tolerance |
|---|---|---|---|---|
| $W_B$ | 0.090 | 0.840 | 1.710 | 0.003 |
| SD | 0.084 | 0.901 | 0.991 | 0.007 |
| PCI | 0.090 | 0.843 | 1.675 | 0.003 |
| SPSLaWb | 0.087 | 0.876 | 1.276 | 0.001 |
| $W_b$ | 0.087 | 0.876 | 1.277 | 0.004 |
| $L_a$ | 0.086 | 0.883 | 1.188 | 0.002 |
| $SPS_{LAWB}$ | 0.090 | 0.847 | 1.627 | 0.001 |
| $L_A$ | 0.085 | 0.890 | 1.111 | 0.002 |
| RSPS | 0.085 | 0.896 | 1.041 | 0.004 |

Labels concerning significance of F-test: * $p < 0.05$; **$p < 0.01$

In order to further determine the importance of the examined stomatal parameter on the discrimination of examined provenances, forward stepwise discriminant analysis was performed. The first included parameter was $W_B$, and the model, with this trait alone, achieved correct allocation of around 25% (Table 10, Figure 5). The model that included parameters SD, PCI, $SPS_{LaWb}$ and $W_b$ achieved jointly a correct allocation of around 40%, which is close to the correct allocation percentage of the model that included all for the examined traits. Therefore, the group of traits used for the discrimination of the examined provenances could be reduced to these five traits, probably as the result of multicollinearity. Although $L_a$, $SPS_{LAWB}$, $L_A$ and RSPS were included in the model with stepwise discriminant analysis, their contribution to discrimination power, according to the percentage of correct allocation, was not important. Likewise, the unique contribution of SSC to the discrimination between provenances appeared to be insignificant as it was not included in the model by stepwise discrimination analysis, at all. PCI and $SPS_{LaWb}$, the

two derived parameters included in the third and fourth step, made a considerable contribution to the percentage of correct allocation of the model, which justifies their use in further research. The fact that the first five parameters included by forward stepwise discriminative analysis, were grouped in groups defined by the first two rotated principal components, suggests that the traits were not selected as traits closely related to differences between provenances, but as loosely associated with previously selected traits. Interestingly, neither of the stomatal traits selected by stepwise discriminant analysis achieved maximum loading with the third rotated principal components. The reason for this could be the low proportion of RC3 in the total variation. As with PCA, the first five included parameters were also distributed among four different groups, defined with canonical variables by the loadings.
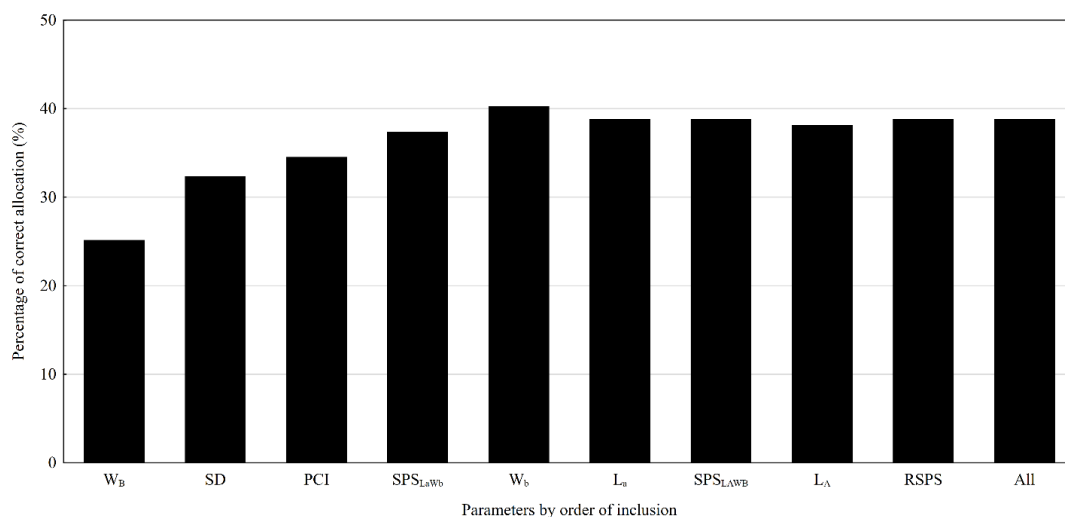


Figure 5. The percentage of correct allocation for models formed by forward discrimination analysis

The agglomeration of examined European beech provenances, in terms of examined stomatal parameters, was analyzed using Hierarchical cluster analysis, based on the Euclidian distances between provenances and the unweighted pair-group average method of agglomeration (UPGMA) (Figure 6). According to the amalgamation schedule (Scree test) and a dendrogram of cluster analysis, ten clusters were determined at a linkage distance of 2.5 (Figure 6). The first cluster was formed from an agglomeration of two provenances originating from Bosnia and Herzegovina (BA59, BA61) and one from Romania (RO64). The second cluster contained provenances from Croatia (HR25), one from Serbia (RS36), and one from Hungary (HU42), while, most of the provenances formed separate clusters (HR24, BA60, DE48, RS66, BA30, RS67, RS38, DE47). A cluster analysis, based on Euclidian distances, revealed that provenances RS38, and DE47 are noticeably apart from the others, which are in line with results from the PCA but not in line with canonical discriminant analysis, where DE47 appeared to be included in the main group. According to the cluster, provenance RS66, appears not to be as distinct from the other provenances, as it was found to be in two previous multivariate methods.

In this study, we did not find a correlation between provenances and geographic distribution. This finding showed the presence of genetic diversity, in all beech provenances from the different geographic origins. Although, certain, mostly phenology related studies, reported the existence of a clinal pattern of genetic variability, from east to west (ROBINSON *et al*., 2013; VITASSE *et al*., 2009), while a number of other studies, focusing on various functional traits (MÀTYÀS *et al*., 2009; STOJNIĆ *et al*., 2015; IVANKOVIĆ *et al*., 2011; ŠIJAČIĆ-NIKOLIĆ *et al*., 2012; JAZBEC *et al*., 2007) showed an ecotypic (unexplained, random) pattern of genetic variability related to seed origin, which effected leaf stomatal traits in *F. sylvatica* provenances. The results from the present study support findings on ecotypic variation. Ecotypes are the result a of populations adaption, not only to various selective pressures within a species natural range (e.g. altitude, climate, soil conditions, etc.) (BOGDAN *et al*., 2007), but also to anthropogenic factors that have shaped its natural habitat over an extended period of time (SUŁKOWSKA, 2010).

*F. sylvatica* survived the last glacial period in multiple refuge areas, located in different parts of Europe (i.e. southern France, the western Alps, eastern Alps-Slovenia-Istria and southern Moravia-southern Bohemia), and these populations were the initial gene pools for all present beech populations (MAGRI *et al*., 2006). Growing in numerous refugia, the population's genetic structure was shaped under the influence of different climate conditions and selective pressures. As a result of this phenomenon, genetically different beech populations were expanded across extended region during the post-glacial colonization of Europe. Similarly, human activities, such as artificial reforestations, conversions of broadleaved into coniferous stands, landscape fragmentation and selective logging were reported to affect both ecological and genetic processes (FARWIG *et al*., 2008; SUŁKOWSKA, 2010).
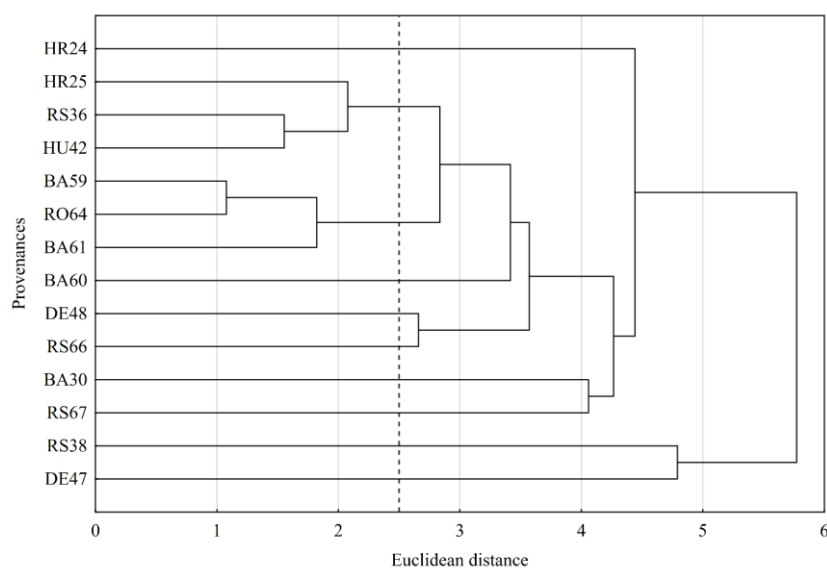


Figure 6. Dendrogram of cluster analysis of examined *Fagus sylvatica* L. provenances agglomerated by UPGMA method based on standardized measured parameters

CONCLUSION

The present study has provided significant insight into the genetic diversity and differentiation of European beech provenances and confirmed high genetic variability at the inter-provenances level, across all ten measured stomatal parameters. Out of all of the examined provenances, RS38 and RS67 were found to differ the most across all implemented multivariate methods, and based on the examined stomatal traits. PCA analysis revealed an ecotypic pattern of genetic variability, due to the absence of exclusively clustered provenances originating from the same geographical region. The result of forward discriminative analysis showed that the model which included $W_B$, SD, PCI, $SPS_{LaWb}$ and $W_b$ traits reached the same ability of discrimination as the model with all of the examined stomatal parameters. This analysis also detected a considerably small correct allocation of model that included all of the examined traits (38.85%), proving the importance of using other anatomical, morphological and molecular markers in order to entirely differentiate the examined beech provenances.

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# VARIJABILNOST STOMATALNIH KARAKTERISTIKA LISTA UNUTAR I IZMEĐU ČETRNAEST PROVENIJENCIJA EVROPSKE BUKVE (*Fagus sylvatica* L.)

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Izvod

Rad obuhvata istraživanje varijacije stomatalnih karakteristika lista između i unutar četrnaest provenijencija evropske bukve poreklom iz šest država (Bosna i Hercegovina, Hrvatska, Nemačka, Mađarska, Rumunija i Srbija). Ispitivano je deset stomatalnih karakeristika (broj stoma po mm$^2$ (SD), dužina (L$_A$) i širina (W$_B$) ćelija zatvaračica, dužina (L$_a$) i širina (W$_b$) stomatalnog otvora, specifični oblik stome (SPS$_{LAWB}$), specifični oblik otvora stome (SPS$_{LaWb}$), koeficijent oblika stome (SSC), potencijalni indeks propustljivosti stome (PCI) i relativna površina stomatalnog otvora (RSPS) primenom metoda univarijacione (analiza varijanse, Takijev test) i multivarijacione analize (analiza glavnih komponenata, diskriminaciona i klaster analiza). Rezultati istraživanja pružaju značajan uvid u genetički diverzitet i diferencijaciju ispitivanih provenijencija evropske bukve i potvrđuju da postoji značajna genetička varijabilnost kako unutar, tako i između provenijencija prema ispitivanim svojstvima stoma. Razlike unutar provenijencija su pokazale značajniji uticaj na većinu ispitivanih parametra (70-80% ukupne varijacije), nego razlike između provenijencija, koje su odgovorne za svega 20-30% ukupne očekivane varijacije. Primenom analize glavnih komponenata utvrđen je ekotipski obrazac genetičke varijabilnosti među ispitivanim provenijencijama, koji su uticali na stomatalne parametre lista. Diskriminaciona analiza je pokazala da model koji uključuje samo parametre W$_B$, SD, PCI, SPS$_{LaWb}$ i W$_b$ razlikuje ispitivane provenijencije u istoj meri kao model sa svim parametrima zajedno. Dobijeni rezultati analize potvrđuju nizak nivo tačnosti alokacije (38.85%) modela koji uključuje sve ispitivane parametre, što ukazuje na potrebu uključivanja dodatnih fenotipskih parametara (npr. morfološke, fiziološke, itd.) u cilju potpune diskriminacije ispitivanih provenijencija evropske bukve.