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Phenotypic variability of autochthonous walnut (*Juglansregia* L) genotypes in northwestern Bosnia and Herzegovina

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Abstract: The main objective of this study was to examine the characteristics and the relationship between walnuts in the Una-Sana Canton based on phenotypic properties. This is the first study of preselection of walnut variability in this area. In this study, we evaluated the phenological and morphological characteristics of 75 selected genotypes during 2014 and 2015. Twenty-four phenotypic traits were monitored throughout 2 years on different sites. The results of the PCA analysis showed a very high heterogeneity of walnut seedlings on the examined sites, from the aspect of observed parameters. A limited number of superior genotypes were found, but individual genotypes with high nut and kernel weight, as well as the colour of the kernel were determined, which would classify them as high-ranked parents in hybridization programs. Certain number of genotypes with high values of traits such as: nut weight, kernel weight and a high percentage of kernel were selected for future studies. Amongst the selected trees, genotype G25 had the highest weight of nuts and kernel, as well as a satisfactory color of the kernel. This genotype could be used as a superior tree for further breeding programs in the future. A high level of phenotypic variability in this study revealed the existence of a rich genetics pool of nuts variation. The genetic diversity of walnuts obtained from our studies will represent a valuable source of information for breeding programs of Persian walnut in future.

Key words: Persian walnut, seedlings, PCA analysis, cluster analysis, phenotypic variability

1. Introduction

The walnut is an important species of edible nuts. The *Juglandaceae* family consists of seven genera, comprising of about 60 monoecious species. Persian walnuts, known as common or the English walnut (*Juglansregia* L.), are the only species that are grown for their edible nuts. It is extensively grown for the high quality of its nuts and timbers (McGranahan and Leslie, 2012). Generally, today's European walnut production still depends on trees grown from seeds rather than clonal trees. The characteristics of such production are inequality in the quality of fruit and irregular yielding which result in decreasing of the supply of walnuts to markets. Genetic variability in walnut populations has been observed very high and exists in various parts of the world (Polat et al., 2015; Toprak, 2019). Morphological characters are considered to be an option for selection and classification of promising germplasm. Morphological variation in nut sizes, thickness of shell, kernel percentage and yield of kernels in walnut trees from various geographical areas have been reported (Okatan, 2018; Gundesli et al., 2019).

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According to Forde and McGranahan (1996), walnut breeding programs focused mostly on the selection of genotypes with commercially important traits, such as high percentage of kernel, late leafing and flowering, and lateral habits of fruiting. Germplasm diversity is usually estimated using morphological descriptors (Bernard et al., 2018). Until recently, phenotypic evaluation was the first step for classifying and describing the germplasm and selecting superior walnut genotypes. So far, a large amount of research has been carried out on the evaluation of genotypes of walnuts in many countries of the world (Sharma and Sharma 1998, 2001; Kazankaya et al., 2001; Akca & Ozungun 2004; Asadian and Pieber 2005; Zeneli et al., 2005; Arzani et al., 2008; Ruiz-Garcia et al., 2011; Cosmulescu 2013; Ebrahimi et al., 2015; Rezaei et al., 2018). In recent years, more and more researchers in Bosnia and Herzegovina have become involved in researching autochthonous and wild varieties and fruit types, which points to an increasing importance of preservation and the possibility of exploiting these plant genetic resources in breeding (Skender et al., 2015, 2017a, 2017b,

Becirspahic et al. 2017a, 2017b). According to Ruiz-Garcia et al., (2011), accurate identification of genotypes is a basic requirement for the management and use of germplasm for practical purposes of breeding. According to Solar and Stampar (2011), morphological studies provide a direction for the selection of varieties suitable for specific conditions of growth. Due to their diversity and simple application, morphologic markers may be suitable for classifying walnut genotypes (Asadian and Pieber, 2005; Arzani et al., 2008). The aim of this study is to evaluate the morphological variability of genotypes of walnuts and to identify the superior and promising genotypes in the northwestern area of Bosnia and Herzegovina, as a center of cultivation.

2. Material and methods

2.1. Plant material and experimental site

Walnut genotypes that were used in this study were propagated by seeds in northwestern Bosnia and Herzegovina (latitude 45°00'47"-45°05'48"N; longitude 15°54' 29"-16°35'66"E) in an area of 80,000 km². The area has moderate-continental climate with cold and humid winters and long warm summers. The average annual temperature is 11 °C and the rainfall ranges from 880 to 1330 L/m². Based on the primary information obtained from local nutgrowers, 75 genotypes were selected, and 25 fruits and 10 leaves were taken from each tree. The genotypes (GT) were named using the abbreviation "Genotype" and were numbered from 1 to 75. The morphological data was monitored and researched throughout 2 experimental years.

2.2. Methods

The phenological characteristics (Table 1) were measured based on the following parameters: the period of vegetative growth, the beginning and end of flowering time for male and female flowers, dichogamy and maturation time (IPGRI, 1994). The period of vegetative growth was recorded for each selected genotype by the date when more than 50% of terminal buds had swelled, shed, and green leaflets had appeared in the bud. The beginning of flowering of male and female flowers implied the date when 50% of male flowers were open, when 50% of male flowers had reached pollen sprouting, or when 50% of female flowers had flourished with a highly waxy appearance, greenish in colour and the endings were orange and separated from each other by about 60°. Maturation time was recorded when the green exocarp was divided and 50% of the fruits began to fall to the ground. In this study, 11 quantitative and 13 qualitative traits (IPGRI, 1994; UPOV, 1999) were analysed (Table 1). The nut traits were measured using 25 nuts chosen randomly (McGranahan et al., 1992). In order to determine the similarity or diversity of the analysed genotypes of the observed population based on the measured properties of the fruit, we used multivariate statistical methods (Chatfield and Collins 1995): the cluster analysis and principal component analysis (PCA). The conducted cluster analysis provides a hierarchical tree, where clustering uses the UPGMA method, and the Euclidean distance to define the distance between investigated objects (Hanschke et al., 1972). In order to determine which features best discriminate groups obtained by cluster analysis, PCA analysis was used (Hotelling, 1936). This gives the best evidence

Table 1. Analysed traits of 75 walnut trees.

Phenological and flowering traits	Quantitative traits	Qualitative traits
Beginning of vegetation	Leaf length	Leaflet shape
Beginning of flowering of male flowers	Leaf width	Leaflet margin
Beginning of flowering of female flowers	Leaflet length	Nut shape
Ending of flowering of male flowers	Leaflet width	Nut: shape of base perpendicular to suture
Ending of flowering of female flowers	Nut width	Nut: shape of apex perpendicular to suture
Maturation time	Nut thickness	Nut: position of pad on suture
	Nut height	Nut: prominence of pad on suture
	Inshell nut weight	Nut: width of pad on suture
	Nut weight	Shell texture
	Kernel weight	Shell colour
	Kernel percentage	Shell strength
		Kernel colour
		Number of leaflets

of existing phenotypic variability between invented genotypes. For the processing and analysis of the obtained phenotypic data (phenological and morphological-pomological properties of walnuts), the following was applied: descriptive, univariate and multivariate statistics – analysis of main components (PCA) (Hotelling, 1936). The abovementioned analyses of the obtained data set are processed in statistical programs PAST, XLSTAT 13 and SPSS 21 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Phenological and flowering traits

Table 2 shows the research results of Phenological and flowering traits. In the beginning of vegetation in observed genotypes ranged from 13.3. (GT-20) to 25.3. (GT-90). The earliest beginning of flowering of the male flowers was observed in genotype GT-58 and it is 27.3, and the latest was 29.4 in genotype GT-80. The wide range of budbreak between the different genotypes is the result of the sensitivity of each variety to high temperatures (Germain 1997). The end of flowering of male flowers was recorded from 6.4. (GT-47 and GT-75) to 7.5. (GT-80). Such a large range can be explained by genetic conditioning and ecological factors. During this period, frosts occurred often, and this is the reason of the ecological sterility of walnuts in this area. The longest period of male flowering was observed in genotype GT-78 (23 days). The beginning of flowering of female flowers was recorded from 29.3. (GT-52 and GT-57) to 8.5. (GT-86). The end of female flowering was recorded from 16.4. to 18.5. The longest

flowering time was observed in genotype GT-57 and lasted for 22 days. Together, later budbreak and flowering period create more opportunity to pistillate flowers that survive the late spring frosts (Forde 1979). A complete dichogamy (protandry) was found in 23 of the genotypes examined, where the phenophases of flowering of male and female flowers did not overlap in a single day. The best rated genotypes in the number of overlapping days of male and female flowering are: GT-76 (13 days), GT-53(12 days), GT-62 (12 days), GT-61 (11 days), GT-39 (11 days) and GT-57 (11 days). Protogyny did not occur in examined genotypes. Protogyny is a useful property in the selection of pollenizers for the main cultivated varieties that usually shed their pollen before their peak of pistillate receptivity (Germain 1997). Maturation time varied from 1.9. (GT-52) to 20.9. (GT-33). The largest number of genotypes (62%) had mature fruits in the first week of September. In the second week of September, maturation of fruits was recorded in 34.66% of genotypes. Only two genotypes (GT-50 and GT-33) matured in the third week of September (2.66%). Genotypes that have a late flushing and early end to the growing period are desirable (Korac et al., 1997).

3.2. Quantitative properties

Table 3 presents the research results of quantitative fruit properties. It can be seen from Table 3 that the average nut thickness is in range of 23.27–37.15 mm, nut width 22.47–31.18 mm, nut height 24.71–43.30 mm, nut weight 5.86–16.25 g, kernel weight 1.66–5.07 g and kernel percentage 26.96%–48.25%. The largest nut weight and

Table 2. Data on phenological and flowering analysis.

Beginning of vegetation	13th March – 25th March
Beginning of flowering of male flowers	27th March – 29th April
Beginning of flowering of female flowers	29th March – 8th May
Ending of flowering of male flowers	6th April – 7th May
Ending of flowering of female flowers	16th April – 18th May
Maturation time	1st September – 20th September

Table 3. Minimum, maximum and mean for 11 quantitative properties of 75 walnut trees.

	LL (cm)	LW (cm)	LTL (cm)	LTW (cm)	NWI (mm)	NT (mm)	NH (mm)	INW (g)	NWE (g)	KW (g)	KP (%)
Min	22.75	15.0	8.34	2.42	22.47	23.27	24.71	1.82	5.04	1.66	26.96
Max	42.00	34.33	13.40	6.37	31.18	37.15	43.30	5.29	16.25	5.07	48.25
Mean	31.67	23.69	10.24	4.41	27.03	27.51	33.26	3.51	8.34	3.29	39.57

LL: Leaf length, LW: Leaf width, LTL: Leaflet length, LTW: Leaflet width, NWI: Nut width, NT: Nut thickness, NH: Nut height, INW: Inshell nut weight, NWE: Nut weight, KWE: Kernel weight, KP: Kernel percentage.

kernel weight was recorded in GT 25 (16.25 g; 5.07 g), and the largest kernel percentage of kernel in the total nut weight was 48.25% recorded in GT-51. The highest value for nut weight among our evaluated genotypes (16.25 g) was less than the corresponding data reported by Sen and Tekintas (1992); Atefi (1997); Sharma and Sharma (1998); Yarilgac et al., (2001); Sharma and Sharma (2001); Zeneli et al. (2005); Cosmulescu and Botu (2012); Cosmulescu (2013); Khadivi-Khub and Ebrahimi (2015) and Rezaei (2018). The highest value for nut weight among our evaluated genotypes was higher than the reported values by Akca and Ozungun (2004), Aslantas (2006), Arzani et al., (2008), Becirspahic et al., (2017a) and Keles (2014). According to McGranahan and Leslie (1990), it is desirable for nut weight and kernel weight in superior genotypes to be 12–18g and 6–10 g, respectively, i.e. the kernel mass should be at least 50% of the total fruit mass and the kernel should be light in colour. According to Germain (1997) and Korac et al. (1997), in breeding programs, genotypes with a kernel ratio of more than 48%–50% are desirable. In this study, only three genotypes had values approximate to the aforementioned – the average fruit mass being 12.37g (GT-86), 12.41g (GT-73) and 16.25g (GT-25), whereas kernel weight was not recorded in this range in any of the 75 genotypes, and only one genotype had a kernel share in this interval (48.25%). Genotypes GT-25 and GT-51 can be called superior because the characteristics of their nuts are similar to commercial cultivars (Chandler and Franquette). If these genotypes were planted in orchards, yields would be highly increased.

3.3. Qualitative properties

Table 4 presents the research results of qualitative fruit properties. Data on leaf analysis indicates that the form of leaflets in all genotypes is broadly elliptic, the leaflet

margin is whole, and the number of leaf blades in the largest number of genotypes is 7 and 5. When it comes to nut shape, it is obvious that a round shape pattern is dominative, with fewer fruits of the egg shape, broad elliptical shape and elliptical shape. As part of the qualitative fruit properties, the analysis of a vertically seamed fruit base shape was conducted. The shortened shape of the base of the fruit has been recorded in the largest number of genotypes, slightly less of those with round shape, and the smallest number of those with the wedge shape of the vertically seamed fruit base. The shape of the vertically seamed nut top can be pointed, rounded, shortened, and cut-in. The rounded top was recorded as highly dominant in the examined genotypes, with very few nuts having a shortened and pointed shape of the fruit top. When it comes to the seam pads position, it is mostly found on the upper half of the fruit and on the upper two third of the nut, and a significantly smaller number of fruits has pads over the entire fruit length. The prominence of the pad at the fruit seam in all genotypes is of medium intensity. The width of pad on suture is highly dominated. The properties of walnut shells also affect its production value. The most expensive are nuts with a shiny, light and smooth shell that is not difficult to break to extract the kernel (McGranahan and Leslie 1990). In this study, the following shell properties were analysed: texture, colour, and thickness. The examined genotypes had a smooth and intermediate pored shell. No genotypes were observed having highly smooth or rough shells. All genotypes had nuts with a moderately dark shell colour. Medium weak shell thickness was highly dominated, few genotypes had weak thickness, and only one genotype had a strong shell thickness. The kernel colour in the examined genotypes is bright and light amber, and only the genotype (GT-87)

Table 4. Data on qualitative traits of 75 walnut trees.

Leaflet shape	Broadly elliptic
Leaflet margin	Whole
Nut shape	Rounded
Nut: shape of base perpendicular to suture	Shortened
Nut: shape of apex perpendicular to suture	Rounded
Nut: position of pad on suture	On the upper half of the nut
Nut: prominence of pad on suture	Medium intensity
Nut: width of pad on suture	Narrow
Shell texture	Smooth and intermediate pored
Shell colour	Moderately dark
Shell strength	Medium weak
Kernel colour	Bright and light amber
Number of leaflets	7 and 5

had an extra bright colour of kernel, which is also the most desirable.

3.4. PCA analysis

Table 5 presents eigenvalues, variance and cumulative variance of the first five principal components (PC) estimated from the correlation matrix with 24 variables in 75 genotypes. In PCA analysis, the first five out of total 24 variables were analysed in greater detail regarding the values of their properties and the percentage that these variables had in the overall variance. The analysis of major components explains over 90% of phenotype diversity using the first five components. The first two main components, obtained by analysis of 75 genotypes of walnut, contained 61.14% of the total variance of the experiment. Ebrahimi et. al. (2015) examined the pomological properties of superior genotypes of walnuts in Iran, where the first two major components contained 28.96% of the total variance of the experiment. In the research by Iranian scientists Arzani et. al. (2008), in which fruit and kernel properties were investigated, the first two major components contained 65.88% of the total experimental variance. Somewhat bigger values were published as a result of research by Cosmolescu and Botu (2012) of the fruits and kernel properties in Romania, with the first two components containing 94.67% of the total variance of the experiment. The Principal Component (PC) represents the maximum share of total variables, i.e. 37.31% of the total variance in the experiment (Table 5). Table 6 presents Principal component (PC) coefficients of 24 variables. The most important features of the first component are the leaf length, leaf width, nut height and nut thickness. The highest values of the characteristic vectors were the leaf width (0.5932) and leaf length (0.5330). The lowest value of the characteristic vectors was the property of the number of leaflets (0.0246). The contribution of the second major component in total variability is 23.83% (Table 5), and most of the high value characteristics for the vector are related to the pomological properties of the nut (Table 6). Of the aforementioned, the highest value of the characteristic vectors were the nut height, the nut thickness, the nut weight and the nut width (0.5063; 0.4051; 0.3082 and 0.2539). The variables with the biggest characteristic vectors in the third, fourth

and fifth major component are the following: PC3 – kernel percentage (0.7548); PC4 – the nut shape (0.6901); PC5 – the leaf length (0.5353). The distribution of the 75 genotypes of walnuts obtained using the first two major components (representing 61.14% of total variability) that have been calculated using the correlation matrix indicates a high dispersion of the analysed genotypes. Such dispersion points to a more pronounced phenotypic or genetic variability of the examined walnut genotypes in the analysed nut group (Figure 1). Also, certain genotypes (GT-56, GT-25, GT-55 and GT-85) have been distinguished in special groups outside the ellipsis that represent 95% of the reliability limit for belonging to a particular group. Figure 1 shows that the GT-56 genotype stood out with the biggest leaf length and blade length, while the isolated genotype GT-85 had the smallest blade length measured. The GT-55 genotype stood out with the biggest fruit height, while the GT-25 genotype was distinguished by more than one trait: a dark-coloured shell, a wide-seamed pads width, maximum width and thickness of the fruit, and very important agronomic properties such as the highest measured mass of fruit and the largest measured mass of kernel. Figure 2 shows the grouping and correlations of 11 quantitative variables analysed in 75 genotypes of walnut vis-à-vis the first two main components (PC1 and PC2). Figure 2 reveals a strong positive correlation between the nut width, the nut height, the nut weight, the kernel weight, inshell nut weight (the weight of kernel with primary and secondary septum), kernel weight, and somewhat weaker correlation of said parameters with the thickness of the fruit. The obtained results have a fairly logical explanation, the weight of the nut and the weight of the kernel, as well as all the other pomological properties are proportionally increased with the growth of the nut. A strong positive correlation was also observed in the observed morphological properties of the leaf width, the leaf length, the leaflet length and the leaflet width.

3.5. Cluster analysis

The grouping of walnut genotypes by degree of similarity was performed using a hierarchical cluster analysis on factor analysis of combined data as presented in Figure 3. The hierarchical cluster analysis of all autochthonous

Table 5. Eigenvalues, variance and cumulative variance on first five principal components (PC) estimated from the correlation matrix with 24 variables in 75 genotypes.

Variables	PC1	PC2	PC3	PC4	PC5
Eigenvalues	31.28	19.98	14.30	6.30	5.12
Variance (%)	37.31	23.83	17.06	7.51	6.10
Cumulative variance (%)	37.31	61.14	78.20	85.71	91.81

Table 6. Principal component (PC) coefficients of 24 variables.

Variables	PC 1	PC 2	PC 3	PC 4	PC 5
Leaf length	0.5330	-0.2017	-0.5178	-0.3320	0.5353
Leaf width	0.5932	-0.2594	-0.1386	0.3368	-0.6345
Number of leaflets	0.0246	-0.0213	-0.0090	-0.0363	-0.0372
Leaflet length	0.1231	-0.0436	-0.0637	0.1088	-0.0553
Leaflet width	0.0603	-0.0240	-0.0729	0.0243	-0.0106
Leaflet shape	-0,0000	0,0000	-0,0000	0,0000	0,0000
Leaflet margin	-0,0000	-0,000	0,0000	-0,0000	-0,0000
Nut shape	-0.0442	0.0059	-0.0304	0.6901	0.4092
Nut: shape of base perpendicular to suture	-0.0174	-0.0030	0.0267	-0.1055	-0.0539
Nut: shape of apex perpendicular to suture	-0.0143	-0.0244	0.0166	-0.0250	0.0313
Nut: position of pad on suture	-0.0046	-0.0267	-0.0101	0.0218	0.0420
Nut: prominence of pad on suture	-0.0028	-0.0195	0.0103	0.0252	-0.0011
Nut: width of pad on suture	0.0145	0.0719	0.0236	-0.1391	-0.0071
Shell colour	0.0244	0.0174	-0.0074	-0.0178	0.0268
Shell texture	0.0253	0.0745	0.0192	-0.0062	0.0160
Nut height	0.3972	0.5063	0.2250	0.3445	0.2546
Nut width	0.1904	0.2539	0.1799	-0.2226	-0.0405
Nut thickness	0.2133	0.4051	0.1652	-0.2521	-0.1067
Nut weight	0.1638	0.3082	0.0659	-0.0942	-0.0524
Shell strength	-0.0110	-0.0035	-0.0026	-0.0008	0.0271
Kernel color	0.0259	-0.0087	0.0130	-0.0207	-0.0106
Inshell nut weight	0.0855	0.0648	0.0902	-0.0488	-0.0061
Kernel weight	0.0803	0.0603	0.0867	-0.0401	0.0027
Kernel percentage	0.2447	-0.5371	0.7548	-0.0939	0.2252

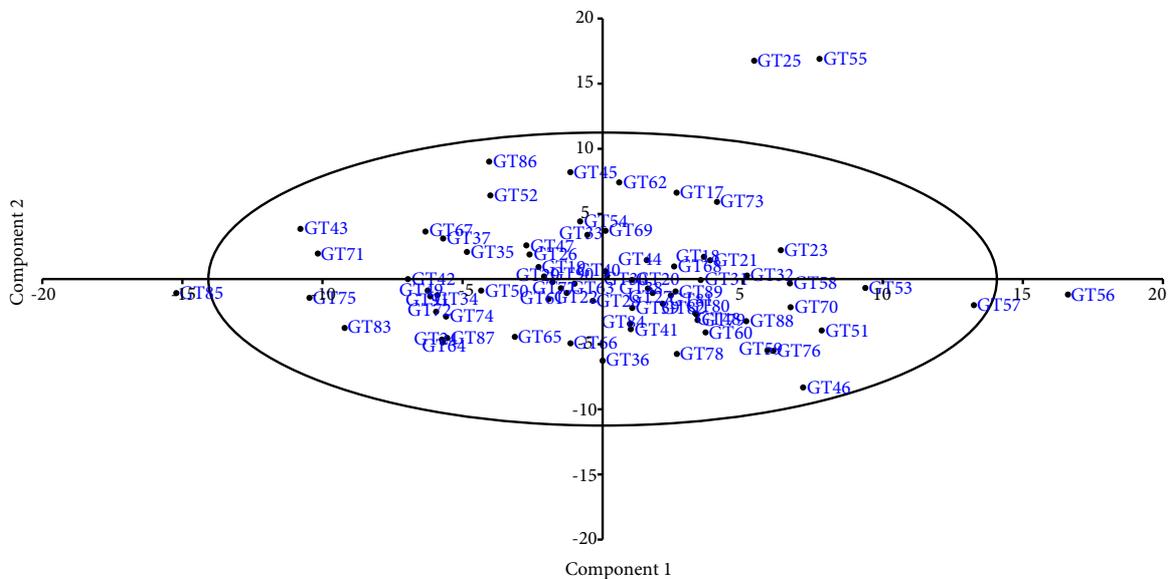


Figure 1. Scatter plot of the 75 selected genotypes on the first two principal components. Ellipse around the group represents 95% of the reliability boundary for a separate group affiliation.

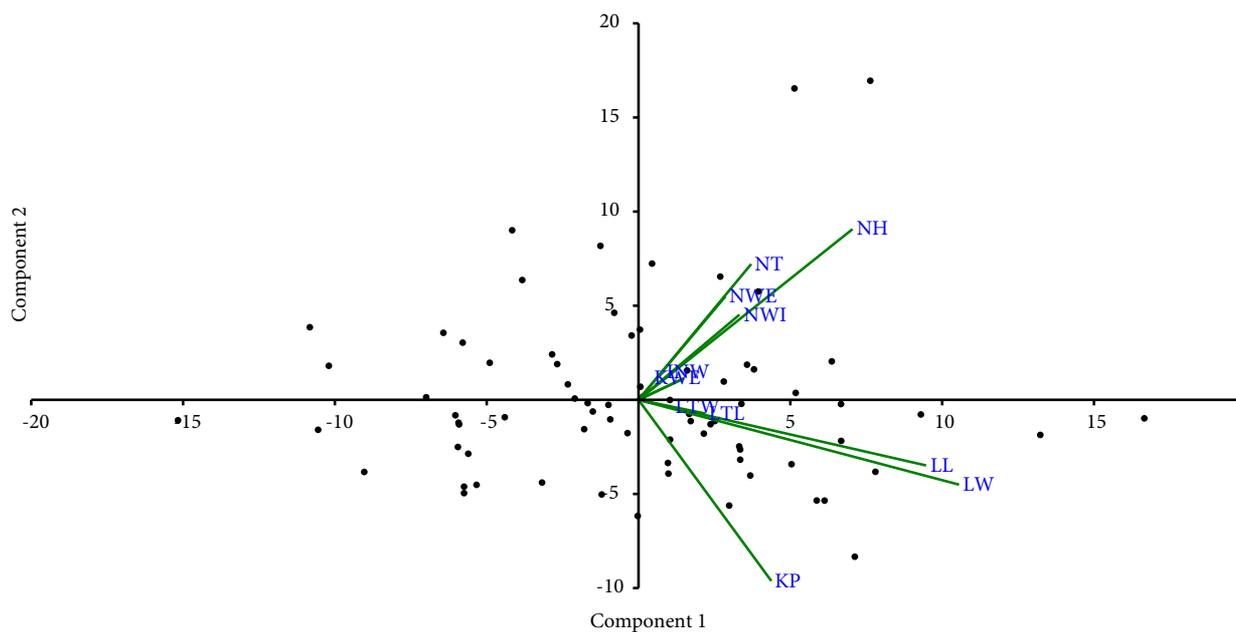


Figure 2. Grouping and interrelations of 11 quantitative variables analyzed on the 75 walnut genotypes on the basis of the first two principal components.

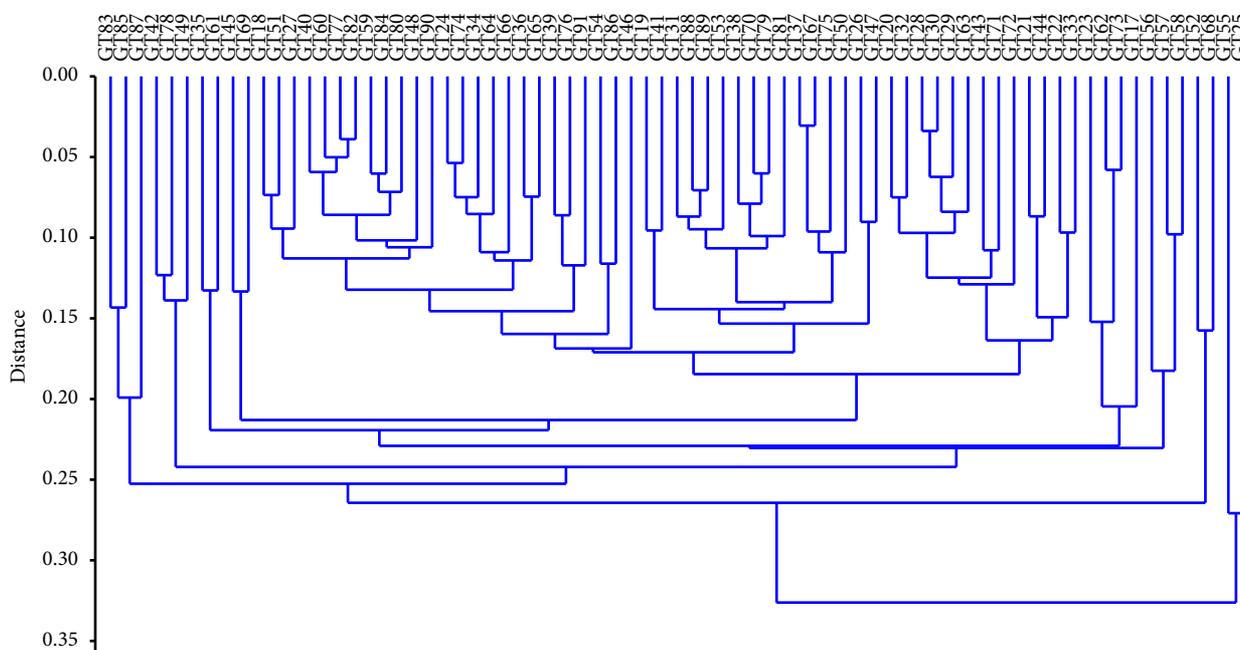


Figure 3. Cluster analysis of the 75 selected genotypes on the basis of 11 quantitative and 13 qualitative characteristics.

genotypes was performed based on 24 phenotypic properties. The cluster analysis results largely confirm the results of principal component analysis (PCA) on genotypes. Hence, hierarchical cluster analysis clearly distinguished the two most divergent genotypes vis-à-vis the pomological properties of the GT-55 and GT-25 genotypes. The GT-25 genotype stood out with a high

value of the fruit mass and kernel mass, and the GT-55 genotype had the highest average width and height of the fruit. This analysis showed divergence of the examined genotypes.

In conclusion, this study confirmed the hypothesis about the high degree of phenotypic variability of walnuts in the examined area, which proves the existence of a rich

genetic pool of this fruit species. The results indicate a very high heterogeneity of walnut on the examined sites, regarding the observed parameters. No large number of superior genotypes was found, but individual superior properties were determined, which would classify them as well-rated parents in hybridization programs. In a certain number of genotypes, high values of important agronomically valuable properties have been noted: the

nut weight, the kernel weight, and kernel percentage. The genotype GT-25 particularly stood out with the highest weight of nut and kernel, as well as the satisfactory colour of the kernel. This genotype could serve as a selection for further breeding programs, when it comes to walnuts. Data on the agronomically significant properties observed during this study will present a valuable set of information for future breeding programs of this species.

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