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VARIABILITY OF RED CLOVER GENOTYPES ON THE BASIS OF MORPHOLOGICAL MARKERS

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Red clover (*Trifolium pratense* L.) is an important forage legume of temperate regions dominantly used as a source of animal food. The present research aimed at assessment of genetic diversity based on morphological markers, through the analyses of five morphological markers in a collection of 46 red clover genotypes. These morphological markers were screened according to the UPOV descriptor (2001) in the trial laid out in a randomized complete block design with three replications. The traits analyzed and investigated were: time of flowering, growth habit, density of hairs, leaf color and intensity of white marks. The average value of Shannon's diversity index for five morphological markers amounted 0.711. Homogeneity analysis (HOMALS) of the same five descriptors accounted for 71.2% of the total variation of the standardized data, with the first and second axis explaining 38.4% and 32.8% of the morphological variability, respectively. Based on this analyses all red clover genotypes were grouped into seven homogeneous groups in two-dimensional space, thus providing visualization of genotypes diversity based on their morphological traits. UPGMA cluster analysis of the same morphological markers allowed the description of four groups with genetic

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distances represented by the simple matching coefficient of similarity ranging from 0.00 to 1.00. Observed results achieved by these two analyses were compared and although both of analyses were successful in grouping and discrimination of red clover genotypes with some similarities and differences, priority in future breeding programs was given to the HOMALS.

Keywords: red clover, variability, morphological markers, homogeneity analysis, cluster analysis

INTRODUCTION

Red clover (*Trifolium pratense* L.) is an important forage legume in nature or cultivated crop of temperate regions, and it can be grown as a pure crop or in grass-legume mixtures. Traditionally, its importance is reflected in being the source of nitrogen in crop rotation on farming systems and also as a feed resource for pollinating insects (SMYKAL *et al.*, 2015). It is forage with a high protein content that can be used in silage production for winterfeed in livestock agriculture (RAVAGNANI *et al.*, 2012).

It is thought that red clover originated from South East Europe and Asia, from the Mediterranean area (MUKHINA *et al.*, 1993). It represents native species in Europe, the Middle East, North Africa and Central Asia, while in other parts of the world, was introduced. Red clover along with alfalfa is forage plant with the longest history of cultivation (BOLLER, 2010). It is uncertain when was beginning of an active cultivation of red clover, but traces of red clover in the pastures of Europe are dating back to the Bronze Age, and the first written evidence of its agricultural use and cultivation originated from the 12th century (RIDAY, 2010).

Genetic resources are the basis for the development of modern high-yielding varieties worldwide. Their practical use in breeding programs is possible only after prior characterization and evaluation of samples that are in the collections of germplasm. Characterization and evaluation of genotypes traits include extensive and time-consuming work, and the estimates and measurement of characteristics are often carried out on core collection, instead of the entire collection of germplasm (GUARINO *et al.*, 2002). By introducing the concept of core collection (FRANKEL and BROWN, 1984), the study of genetic diversity and variability becomes particularly important, and the use of core collection did not help to fully characterize the genetic diversity and assess its most effective use in plant breeding (SMYKAL *et al.*, 2015). Characterization and evaluation of accessions in germplasm collections relate to the descriptors, which include qualitative and quantitative traits. Qualitative descriptors include morphological, physiological, and molecular (DNA and biochemical) traits, while quantitative descriptors subject to the functioning of environmental factors, such as yield and yield components, tolerance to stressful environmental conditions, etc. (ORTIZ RÍOS, 2015).

Use of markers in plant breeding has started a long time ago, with the fact that they were used in the selection of plants. In the early stages of plant breeding, these markers were generally implied as visible traits. These morphological markers usually represent genetic polymorphisms that can easily be detected and which can be useful for breeding. Some of these markers are in conjunction with agronomic traits and could be used for indirect selection in practical breeding (JIANG, 2015).

In addition to diploid varieties, today in commercial production are presented also tetraploid varieties of red clover, and these two forms are characterized by particular morphological and agronomical traits (ZUK-GOLASZEWSKA *et al.*, 2010). The primary genetic

pool of red clover includes four sub-pools: 1) the varieties represented in the production, new and old varieties, 2) selected populations or breed populations, 3) local populations, and 4) uncultivated, wild germplasm (MORRIS *et al.*, 2009). Besides, red clover is allogamous species, which also leads to the fact that there is meaningful genetic variability at the crop level that could be a good basis for the red clover plant breeding.

Homogeneity analysis (HOMALS), also known as Multiple correspondence analysis (MCA), enables to study the integration of a large number of qualitative variables and refers to the categorical nominal data. It is analogous to the Principal Component Analysis (PCA), which refers to the study of quantitative, numerical variables (POLLET, 2005). In relation to the Correspondence Analysis (CA) which applies to the two categorical variables, multiple correspondent analysis (MCA) can be regarded as an extension of the correspondent analysis (CA) and it can be applied to the research with a larger number of categorical variables (SOURIAL *et al.*, 2010). The main objectives of multiple correspondent analysis are examining the relationship between categories of variables and grouping of genotypes with similar profiles.

Genetic distances analysis is very present and very useful in breeding programs because it provides information concerning the use of available genetic resources (VIEIRA *et al.*, 2007). Measures of genetic distances may be based on phenotypic traits as well as molecular markers, and multivariate analysis allows tracking a large number of variables within a single analysis. Studies of genetic distances, with any plant species usually include the selection of genotypes that will be analyzed, obtaining and organizing data, the selection of distance or measure to be used for evaluation, selection of grouping procedure, analysis of the degree of distortion (deviation) due to the use of a certain type of grouping and interpretation of the data. Results of the analysis will be effective if all steps are strictly complied (BERTAN *et al.*, 2007).

Genetic variability at the morphological level, need to be properly accessed and interpreted, in order to be related to new molecular tools which only complement classical breeding methods.

The objectives of this study relate to red clover collection of the Institute of Field and Vegetable Crops in Novi Sad in terms of focusing on the goals which could help in future breeding programs. These objectives include: i) phenotypic evaluation of varieties and populations of red clover using UPOV descriptors and characterization of morphological traits; ii) estimation of genetic similarity and relationship of red clover genotypes based on morphological divergence; iii) grouping of genotypes according to the results of the assessment of morphological markers.

MATERIALS AND METHODS

Plant material in this study comprised 46 red clover varieties and populations, which were diploid or tetraploid genetic constitution, belonging to the collection of the Institute of Field and Vegetable Crops in Novi Sad, Republic of Serbia (Table 1). Selected red clover genotypes came from different countries of the world and included also local varieties and populations. The plant trial with red clover genotypes was set using a randomized block design with three replications (10 plants per repetition). Sowing of selected red clover collection is done by row spacing of 80 × 80 cm, at depth of 2.5 cm. Investigation of the morphological characteristics of red clover genotypes included characterization according to the protocol of UPOV (2001) for following traits: time of flowering (TFL), growth habit (GHA), density of hairs (DOH), leaf color (LCO), intensity of white marks (IWM). The trial was performed during 2011 year.

Table 1. List of 46 red clover genotypes used in this study, their origin, status and ploidy levels

Number	Genotypes	Origin	Status of genotypes	Ploidy level
1	NCPGRU2	Ukraine	population	2n
2	NCPGRU3	Ukraine	population	2n
3	NCPGRU4	Ukraine	population	2n
4	NCPGRU5	Ukraine	population	2n
5	Violeta	Bolivia	variety	2n
6	Nessonas	Greece	variety	2n
7	Mercury	Belgium	variety	2n
8	Lemmon	Belgium	variety	2n
9	SA1	Australia	population	2n
10	SA3	Australia	population	2n
11	SA4	Australia	population	2n
12	BGR1	Romania	population	2n
13	BGR2	Romania	population	2n
14	BGR3	Romania	population	2n
15	Diana	Hungary	variety	2n
16	Dicar	France	variety	4n
17	Nemaro	Germany	variety	4n
18	Una	Serbia	variety	2n
19	Avala	Serbia	variety	2n
20	Marina	Serbia	variety	2n
21	Amos	Denmark	variety	4n
22	NS-Mlava	Serbia	variety	2n
23	Italia centrale	Italia	population	2n
24	Bolognino	Italia	population	2n
25	Marino	Germany	variety	2n
26	Renova	Switzerland	variety	2n
27	Titus	Germany	variety	4n
28	Rotra	Belgium	variety	4n
29	Kora	Sweden	variety	2n
30	Vivi	Sweden	variety	4n
31	Lucrum	Germany	variety	2n
32	Noe	France	variety	2n
33	Violetta	Belgium	variety	2n
34	Britta	Sweden	variety	2n
35	Krano	Denmark	variety	2n
36	Triton	Germany	variety	4n
37	Abstract	Germany	variety	2n
38	Bjorn	Sweden	variety	2n
39	Bradlo	Slovakia	population	2n
40	Čortanovci	Serbia	population	2n
41	89 E-0	Bulgaria	population	2n
42	91 E-44	Bulgaria	population	2n
43	91 E-63	Bulgaria	population	2n
44	Sofia52	Bulgaria	population	2n
45	Fertody	Hungary	variety	2n
46	Quinekel	Chile	variety	2n

Shannon diversity index (SHANNON and WEAVER, 1949) was determined based on visual assessment of morphological characteristics using the following formula:

$$H' = -\sum_{i=1}^{n} -P_i \log_2 P_i$$

where n is the number of nominal classes of traits and P_i is the proportion of accessions in the i_{th} class of a trait. Normalization of H' values was performed by dividing each H' value with its maximum value, thus making H' values in the range of 0-1 (PERRY and MCINTOSH, 1991). Higher index value means a greater diversity and vice versa.

In order to determine genetic relationships among red clover genotypes, morphological markers were processed by HOMALS (multiple correspondence analysis-MCA). The red clover genotypes were positioned in two-dimensional space, thus providing visualization of diversity and discrimination of genotypes based on their estimated morphological traits.

A distance matrix for pairs of genotypes based on their morphological markers was generated by the employing simple matching coefficient (SOKAL and MICHENER, 1958). This distance matrix was further used for clustering of genotypes following Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. Statistical analysis of morphological traits for 46 red clover genotypes was performed using the program R (R CORE TEAM, 2015).

RESULTS

Visual characterization of five morphological traits of 46 genotypes of red clover (TFL-time of flowering, GHA-growth habit, DOH-density of hairs, LCO-leaf color, IWM-intensity of white marks) is given as a supplementary material.

The descriptors, the number of genotypes by categories of morphological markers, as well as Shannon's diversity index values (H') for each of the marker are summarized in Table 2.

Based on the homogeneity analysis (HOMALS) of five morphological traits assessed in 46 genotypes of red clover, HOMALS chart was designed (Figure 1), with the first axis explaining 38.4% and the second 32.8% of the total variability of morphological data. The distance between two genotypes on the chart reflects the similarity of their profiles (HSIEH, 2007), and analyzed red clover genotypes were grouped into seven homogeneous groups on the basis of morphological similarities of their profiles.

I group-genotypes of very early time of flowering, with the absent or very weak intensity of white marks, with the low density of hairs and medium green leaf color. In terms of growth habit, genotypes 91 E-63 and E-91 44 were with an intermediate form of the trait, and genotypes Bradlo and Čortanovci with a *prostratum* form of growth habit.

II group-genotypes of very early time of flowering, with the weak intensity of white marks or absent or the very weak intensity of white marks (Bolognino). According to the leaf color in the group II represented genotypes of medium green leaf color, as well as genotypes of dark green leaf color. Among genotypes of this group, based on growth habit, there was domination of genotypes with intermediate growth habit while SA3 and Sofia52 were with semi-*prostratum*, and Amos with a semi-*erectum* growth habit.

III group-genotypes of early time of flowering, with absent or the very weak intensity of white marks or weak intensity of white marks (89 E-0). Among the genotypes of the third group medium green leaf color or dark green leaf color were included. Most of the genotypes were characterized by semi-*erectum* growth habit, while the genotype Renova was with a semi-*erectum*, and genotype 89 E-0 with a semi-*prostratum* form of growth habit.

IV group-genotypes of medium time of flowering and only Violeta was with early flowering time, with the weak intensity of white marks. With respect to growth habit, genotypes of IV group had intermediate growth habit form or a semi-*erectum* growth habit. Medium green leaf color was present in five genotypes, while the other five genotypes had dark green leaf color.

V group-genotypes of early time of flowering, with medium intensity of white marks, medium density of hairs and dark green leaf color. For the genotypes of V group, intermediate growth habit was predominant, while the genotypes Diana and Titus were with semi-*erectum* growth habit.

VI group-genotypes of very late time of flowering, with medium intensity of white marks, with very low density of hairs and medium green leaf color. Genotypes NCPGRU5 and Bjorn had semi-*prostratum* growth habit and SA4 semi-*erectum* growth habit.

VII group- genotypes of medium time of flowering, with the weak intensity of white marks. NCPGRU4 and Lutea had intermediate growth habit, unlike genotype Lemmon which had a semi-*erectum* growth habit. In terms of leaf color, Lemmon and Lutea were characterized by dark green leaves, unlike the genotype NCPGRU4 which had a medium green leaf color.

Table 2. Descriptors, descriptors designations and categories, distribution of genotypes by descriptors categories and Shannon's diversity indices

Descriptor	Descriptor designation	Categories	Number of genotypes	Proportion of genotypes (%)	H’
Time of flowering	TFL	1- very early	12	26	0.721
		3- early	24	52	
		5- medium	8	17	
		7- late	1	2	
		9- very late	1	2	
Growth habit	GHA	1- *erectum*	1	2	0.752
		3- semi-*erectum*	10	22	
		5- intermediate	25	54	
		7- semi-*prostratum*	7	15	
		9- *prostratum*	3	7	
Density of hairs	DOH	1- very low	12	26	0.642
		3- low	30	65	
		5- medium	3	7	
		7- high	1	2	
		9- very high	0	0	
Leaf color	LCO	3- light green	1	2	0.712
		5- medium green	23	50	
		7- dark green	22	48	
Intensity of white marks	IWM	1- absent or very weak	15	33	0.728
		3- weak	23	50	
		5- medium	7	15	
		7- strong	0	0	

	9- very strong	1	2	
Average				0.711

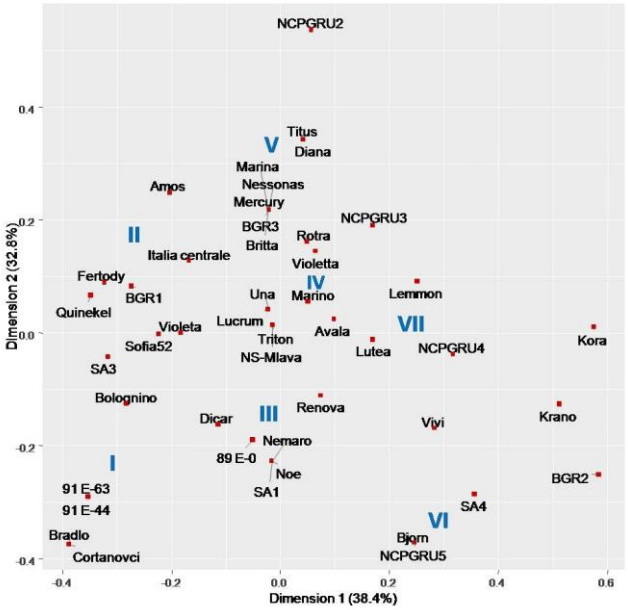

Figure 1. Grouping of red clover genotypes based on homogeneity analysis of morphological traits

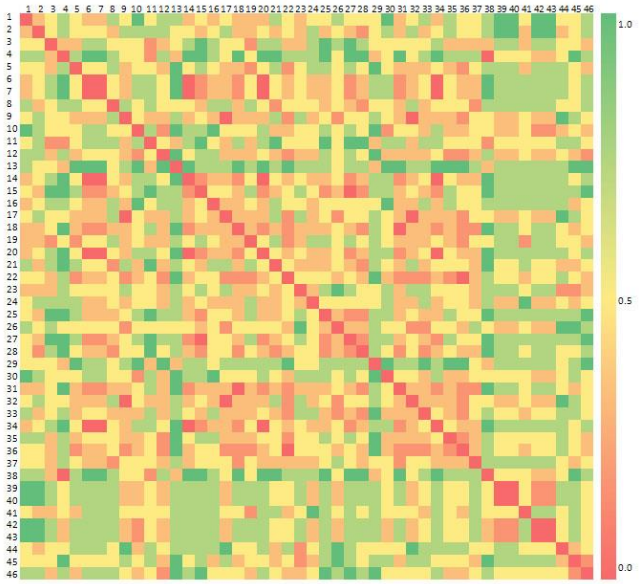

Figure 2. Genetic distances matrix for the 46 genotypes of red clover calculated using the simple matching coefficient of similarity of morphological markers (genotypes of red clover are numbered from 1 to 46)

Distance matrices between pairs of genotypes, calculated on the base of the simple matching coefficient of similarity (SOKAL and MICHENER, 1958) are displayed in the Figure 2. The smallest genetic distance was 0.000 and was present in a large number of pairs of genotypes, and the maximum distance amounted 1.000 and was also present in a large number of pairs of genotypes. The average genetic distance of all pairs of genotypes was 0.587.

Based on cluster analysis, using Unweighted Pair-Group Arithmetic Mean method (UPGMA) method of grouping, all genotypes of red clover are classified into four groups (Figure 3), primarily on the basis of a combination of the two morphological traits (time of flowering and leaf color).

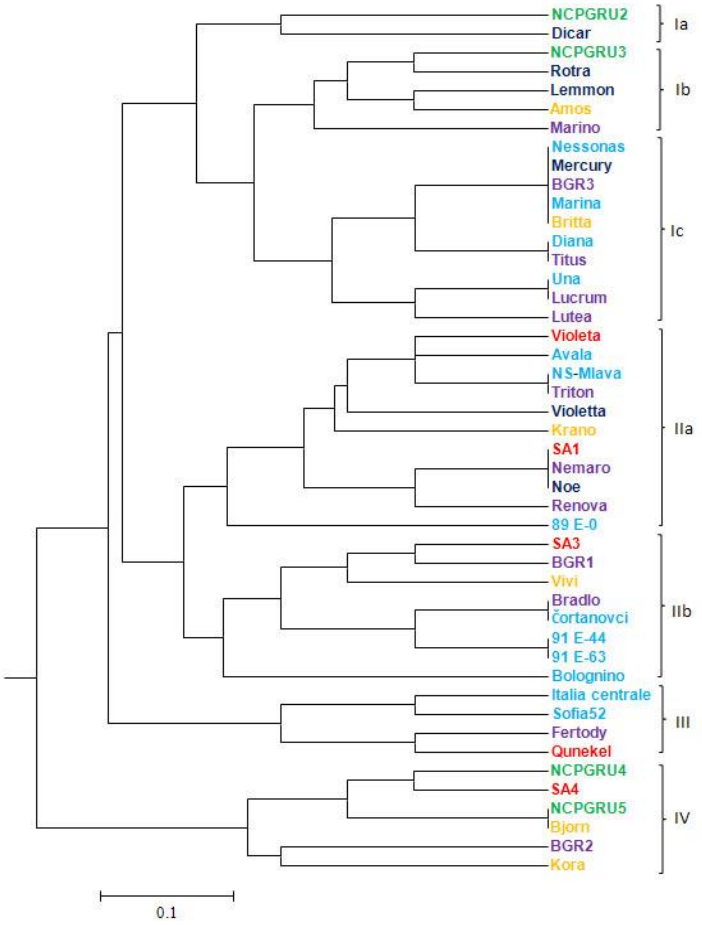

Figure 3. Dendrogram of red clover genotypes obtained by UPGMA cluster analysis of morphological traits (color tags for genotypes according to the geographical origin: red-Australia and South America, green-Eastern Europe, bright blue-Southern Europe, purple-Central Europe, yellow-Northern Europe; dark blue-Western Europe).

The first cluster included genotypes with dark green leaf color and early time of flowering. Also, this cluster included the three genotypes with a dark green leaf color, with a medium time of flowering (Lemmon and Lutea) and with a very early time of flowering (Amos). Genotypes of the I cluster are additionally split on the basis of growth habit into the clearly visible sub-clusters: Ib semi-erectum growth habit and Ic intermediate growth habit (with the exception of Diana and Titus which had semi-erectum growth habit type), while the third sub cluster Ia, was with only two genotypes (NCPGRU2, Dicar) allocated on the basis of intensity of white marks.

In the second cluster genotypes with medium green leaf color were grouped and they were divided into sub clusters according to time of flowering: IIa with early time of flowering (with the exception of Krano which had very late time of flowering) and IIb with very early time of flowering (with the exception of Vivi which had late time of flowering).

The third cluster encompassed genotypes with dark green leaf color and very early time of flowering (with the exception of Quinekel with a very early time of flowering and medium green leaf color), and also with a weak intensity of white marks.

In the fourth cluster genotypes of medium green leaf color and medium time of flowering (with the exception of BGR2 and Kora, which although had medium time of flowering, also deviated by the leaf color; BGR2-had light green leaf color and Kora-dark green leaf color) were grouped. All genotypes within this cluster were with very low density of hairs.

DISCUSSION

Diversity indices are mathematical functions that combine the wealth (richness) and uniformity (evenness) of sampling data. There are various diversity indices, including frequently used Shannon's index (H'), also referred to as the Shannon-Wiener index (COLWELL, 2009). Diversity indices of the observed descriptors were with high values, mainly due to a larger number of categories for each trait, despite more or less uneven distribution of genotypes for these categories. Average Shannon's diversity index was 0.711, indicating a high level of variability of investigated morphological traits of the red clover genotypes. Besides, high diversity index indicates that the observed descriptors (time of flowering, growth habit, density of hairs, leaf color, and intensity of white marks) are good morphological markers for successful identification, differentiation, and classification of red clover genotypes.

Based on the cluster analysis of morphological traits of 46 red clover genotypes, it could be noticed that grouping of red clover genotypes was not in accordance with their geographical origin. The same conclusions can be drawn when it comes to homogeneity analysis. It also can be observed that the grouping of genotypes using homogeneity analysis was different from the grouping of red clover genotypes by the implementation of UPGMA hierarchical cluster analysis. This discrepancy is the result of the differences of statistical methods themselves that were applied in the analysis of the same data values of morphological traits. Homogeneity analysis of five morphological traits grouped red clover genotypes into seven moderately homogeneous groups, based on the characteristics of time of flowering, the intensity of white marks and to some extent based on the characteristic of the density of hairs. UPGMA

cluster analysis performed grouping of red clover genotypes based on the time of flowering and leaf color (with some contributions of other traits), into the four clusters, wherein the first and second cluster had two sub clusters. What is in common in both used methods is that the time of flowering was the basic discriminatory trait. Homogeneity analysis clearly highlighted discriminatory traits that largely contribute to the differentiation of genotypes, while with the UPGMA cluster analysis, this is not the case. On the UPGMA dendrogram, there are exceptions i.e. genotypes that were often classified into certain clusters although they had discrepancies in the fundamental discriminatory features (time of flowering and leaf color) in relation to other genotypes of the same cluster. In the grouping of those genotypes, there was also a substantial contribution of other traits. Because of this, it could be argued that the homogeneity analysis method was more informative than cluster method and with a clearer display of morphological variability of tested material.

Different authors also performed a grouping of genotypes of red clover genotypes and found significant genetic variation by studying the morphological traits (PAGNOTTA *et al*., 2011; ASCI, 2011; GREENE *et al*., 2004; ROSSO and PAGGANO, 2005; DIAS *et al*., 2008).

CONCLUSIONS

The analyzed morphological traits (time of flowering, growth habit, density of hairs, leaf color and intensity of white marks) had significant discriminatory power, making them good markers for characterization of red clover genetic resources. Although implemented homogeneity and cluster analyses based on morphological variability of 46 red clover genotypes, could not clearly link groups of genotypes with their geographical origin, both analyses were sufficiently informative in explaining morphological variability of examined genotypes, with certain advantages of the outcomes of HOMALS analyses. Agronomically important traits and modern molecular-genetic markers, associated with morphological markers, could help breeders in their selection, crossing programs and gene bank conservation strategies.

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VARIJABILNOST GENOTIPOVA CRVENE DETELINE NA OSNOVU MORFOLOŠKIH MARKERA

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Izvod

Crvena detelina (*Trifolium pratense* L.) je značajna krmna leguminoza umerenih regiona koja se dominantno koristi kao izvor stočne hrane. Cilj ovog istraživanja bila je procena morfološkog diverziteta kolekcije sačinjene od 46 genotipova crvene deteline, na osnovu analize pet morfoloških markera. Morfološki markeri su ocenjivani na osnovu UPOV deskriptora (2001) u ogledu postavljenom po potpuno slučajnom blok sistemu sa tri ponavljanja. Analizirane su sledeće osobine: vreme cvetanja, forma rasta, maljavost stabla, boja lista i intenzitet obojenosti pege na listu. Prosečna vrednost *Shannon*-ovog indeksa diverziteta pet morfoloških markera iznosila je 0.711. Analizom homogenosti ovih deskriptora objašnjeno je 71.2% ukupne varijabilnosti standardizovanih podataka, pri čemu je prvom osom objašnjeno 38,4%, a drugom 32,8% morfološke varijabilnosti. Analiza homogenosti je grupisala genotipove crvene deteline u sedam homogenih grupa u dvodimenzionalnom prostoru, čime je omogućena vizualizacija genotipskog diverziteta na osnovu ostvarenih kategorija morfoloških osobina. UPGMA klaster analiza morfoloških markera je omogućila je deskripciju četiri grupe genotipova sa genetičkim distancama zasnovanim na "simple matching" koeficijentu sličnosti. koji je bio u rasponu od 0.00 do 1.00. Poređenjem rezultata dobijenih primenom ove dve analize uočava se da su obe analize bile uspešne u grupisanju i diskriminaciji genotipova crvene deteline uz izvesne sličnosti i razlike, s tim što je prioritet u smislu sveobuhvatnosti potrebno dati HOMALS analizi.

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