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Original scientific article

USING MOLECULAR MARKERS IN THE IDENTIFICATION OF DIFFERENT GENOTYPES OF LUCERNE (*Medicago sativa* L.)

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In order to have successful breeding, it is necessary to introduce new breeding material constantly and to use it through various types of hybridisation to increase the existing variability. Ten (10) lucerne varieties of different geographic origin were used in the study. Six varieties originated from the Republic of Serbia (Kruševačka 22, Kruševačka 28, NS-Banat ZMS II, NS-Mediana ZMS V, Zaječarska 83 and Čačanka 10), three varieties originated from the Republic of Croatia (Osječka 66, Osječka 88 and Osječka 99) and one variety originated from the Republika Srpska (Banjalučanka). A total of 100 seeds per each of 10 (ten) lucerne varieties were placed in Petri dishes to germinate. The dishes were placed in the seed germination chamber with the altering temperature of 20 °C in the dark for 16 h and 30 °C in the light for 8 h for seven days. The first green leaflets of lucerne seedlings (cotyledons) were used for the DNA extraction. The first and the second axes from the principal coordinates analysis accounted for a total of 63.1% of genetic variation, contained in the original dataset. It is clearly observed that the genotype Zaječarska 83 is genetically most distant from other studied lucerne genotypes. These studies confirmed that the observed collection of lucerne varieties is variable enough for the successful breeding process. Using an appropriate breeding model it is possible to breed varieties for certain purposes.

Key words: lucerne, molecular markers, PCoA, RAPD, variety

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INTRODUCTION

Lucerne (*Medicago sativa* L.) is one of the eldest fodder crops and was known as far back as 6000 BC. It originates from the territory of the today's Iran and Arabia, although species of the genus *Medicago* can be found throughout Asia (MICHAUD *et al*., 1988). Lucerne is widely grown on all continents in more than 80 countries, from moderately cold to tropical areas. The wide geographical distribution of lucerne was achieved by its increased adaptability to different climatic and soil conditions (JULIER *et al*., 1995). Lucerne is a natural tetraploid, 2n=32 (stanford and CLEMENT, 1958). Taxonomically lucerne belongs to the family *Fabaceae* (legumes), genus *Medicago*, which includes more than 60 different species, annual and perennial diploids, tetraploids and hexaploids with a haploid chromosome number n = 8.

Regarding the evolution of the genus *Medicago*, it has developed from diploid and tetraploid to hexaploid populations through natural hybridisation (IVANOV, 1980).

Lucerne has a special role in the crop rotation. The ability to fix atmospheric nitrogen in the symbiosis with the bacterium *Rhizobium meliloti* greatly reduces the needs for the application of nitrogen mineral nutrients both in lucerne fields and in crops grown after the ploughing up of lucerne fields, due to which this species gains ecological significance. The amount of fixed nitrogen depends on a large number of factors and ranges from 50 to 463 kg ha^{-1} per year (DELIĆ *et al*., 2016).

Yield characteristics of alfalfa have been reported as follows; green herbage yield 3520-11660 kg ha^{-1}, dry matter yield 1780-3230 kg ha^{-1} and crude protein yield 246.4-321.3 kg ha^{-1} (KAVUT and AVCIOGLU, 2015). In addition to high yields, lucerne is characterised by exceptional quality of dry matter, particularly in terms of the protein content, which ranges from 18 to 22 % depending on the stage of the development of plant vegetative organs. Lucerne proteins are of high biological values, especially in regard to the amino acid composition, because the proportion of essential amino acids is very pronounced. Besides proteins, lucerne has a significant content of other organic compounds, such as: cellulose, lipids, various sugars, etc. The content of mineral substances is also significant, especially of phosphorus, calcium, potassium, sulphur, magnesium, chlorine, etc., which are necessary in the animal nutrition (MARKOVIĆ *et al*., 2007). In addition to the above mentioned, lucerne contains a number of organic stimulants such as vitamins A, B_1, B_2, B_3, C, D, E, K, PP, then hormones and various organic acids (citric, malic and malonic).

Lucerne is, after maize, the most important fodder species in Serbia, due to the favourable chemical composition, increased content of proteins, high yields and good biological traits. This crop can be used in animal nutrition as green matter, hay or ensiled in the combination with other fodder plants (DJORDJEVIĆ and DINIĆ, 2007).

In order to have successful breeding, it is necessary to introduce new breeding material constantly and to use it through various types of hybridisation to increase the existing variability (FALCONER and MACKAY, 1996). The determination of the genetic distance of varieties is a very important step in lucerne breeding prior to selecting the appropriate parents. Moreover, the identification of groups with similar genetic diversity is of essential importance in preserving the gene pool (ŽIVKOVIĆ *et al*., 2012). Lucerne as a fodder plant species has such a great importance in the world and in Serbia. According to that, huge attention was paid to it

with the aim of obtaining the highest possible yield of biomass and seeds both in terms of quantity and quality. The objective of this study was to determine the variability and grouping of related lucerne genotypes using *RAPD* molecular markers by observing ten different lucerne varieties.

MATERIALS AND METHODS

Ten (10) lucerne varieties of different geographic origin were used in the study. Six varieties originated from the Republic of Serbia (Kruševačka 22, Kruševačka 28, NS-Banat ZMS II, NS-Mediana ZMS V, Zaječarska 83 and Čačanka 10), three varieties originated from the Republic of Croatia (Osječka 66, Osječka 88 and Osječka 99) and one variety originated from the Republika Srpska (Banjalučanka). Seeds of all lucerne varieties were harvested in 2013. In order to realize this research, the experiments were conducted in the accredited laboratories of the Institute for Plant Protection and Environment in Belgrade in 2016.

DNA extraction: 100 seeds per each 10 lucerne varieties were placed in Petri dishes to germinate and then the dishes were placed in the seed germination chamber with the altering temperature of 20℃ in the dark for 16 h and 30℃ in the light for 8 h. Seeds were germinated for seven days. The initial green leaflets of lucerne seedlings (cotyledons) were used for the DNA extraction. The DNA extraction from the plant material was performed by the modified *CTAB* method (DOYLE and DOYLE, 1990). Plant tissue (0.1g) was ground to a fine powder under liquid nitrogen using a mortar and pestle.

The amplification of DNA fragments by the *PCR* method: the *PCR* reaction mix of each lucerne variety, 25 μl in volume, contained: 1 x PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]) 1 μl extracted DNA; 0.5 μM primer; 200 μM dNTP mix (dATP, dCTP, dGTP, dTTP); 1.5 mM $MgCl_2$; 0.025 U Taq polymerase (Fermentas, Lithuania). The *PCR* reaction mix to which 1 μl of sterile water was added instead of DNA was used as a negative control. The amplification of DNA fragments was performed in the *PCR* device (Eppendorf Mastercycler, Hamburg, Germany).

Table 1. Primers used in the study

Primer name	PCR-method	Primer sequence	Reference:
OPA 01	RAPD	CAGGCCCTTC	(MORETTI *et al.*, 2004)
OPA 02	RAPD	TGCCGAGCTG	(MORETTI *et al.*, 2004)
OPA 13	RAPD	CAGCACCCAC	(MORETTI *et al.*, 2004)
OPB 10	RAPD	CTGCTGGGAC	(MORETTI *et al.*, 2004)

The obtained *PCR* products were separated by electrophoresis on the 1 % agarose gel in the TE buffer. Mixtures of 5 μl per sample and 3 μl of loading dye (Fermentas, Lithuania) were applied to the gel. Electrophoresis was run at a constant voltage of 95 V. Gel staining was performed by immersion into the solution of ethidium bromide (0.2 mg/l) for 15 minutes, while the visualisation of the amplified products was performed on the UV transilluminator (TFP-

M/WL 312, Vilber Lourmat, France). After that, gels were photographed with the camera DOC PRINT DO-001, FDC.

Based on the DNA markers (100 bp GeneRulerTM DNA Ladder Mix, Fermentas, Lithuania), the approximate molecular weight of the PCR products was determined. Primers used in this study are presented in Table 1.

RAPD analysis

The following four primers were used in the analysis: OPA 01, OPA 02, OPA 13 and OPB 10 (Table 1). The amplification of DNA fragments was performed in 25 µl of the reaction mixture that contained the DNA buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3]), 1.5 mM $MgCl_2$, 200µl dNTPs, 0.5 mM of primers, 0,025 U *Taq* polymerase and 1 µl of the sample. Sterile distilled water was used as a control. The programme used for the PCR analysis is presented in Table 2.

Table 2. Programme for PCR (RAPD analysis)

Programme for PCR	Duration (min.)	Temperature (°C)
Initial denaturation	5	94
45x		
Denaturation	1	94
Primer hybridisation	1	37
Elongation	2	72
Final elongation	5	72

Statistical programs for the population genetics data analysis were used to analyse data obtained by molecular characterisation. The position of the bands were observe through the binary data system and then converted into genetic distances by the Restdist program according to the method developed by Nei and Li, 1979, all within the FILIP software package (Felsenstein, 1993). The data were processed in the program STATISTICA 8.0 (StatSoft Inc, Tulsa, OK, USA).

RESULTS AND DISCUSSION

It was noticed that the repetitive sequences, that account a substantial portion of the genetic material, could be observed in the *RAPD* analysis of plant genomes. The repetitive-sequence-based polymerase chain reaction (*RAPD-PCR*) was used to establish genetic diversity among various lucerne varieties. Primers OPA 01, OPA 02, OPA 13 and OPB 10 were used to produce different DNA profiles (Figures 1-4). Based on *RAPD* DNA profiles, fractioning of DNA fragments of different sizes from 100 bp to 3 kb was observable. Differences among fragments were assessed visually based on their position on the gel. Whether the DNA profiles obtained by primers OPA 01, OPA 02, OPA 13 and OPB 10 were identical or dissimilar/diverse among observed lucerne varieties can clearly be distinguished.

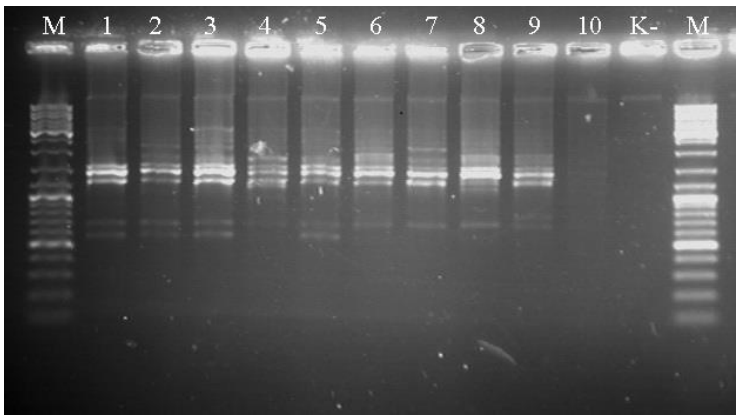

Figure 1. Gel containing lucerne varieties with the primer OPA 01 (M-Marker; K-Negative control; 1. NS-Banat; 2. OS-99; 3. OS -88; 4. ZA-83; 5. K-22; 6. Čačanka 10; 7. Banjalučanka; 8. NS-Mediana; 9. OS -66; 10. K-28).

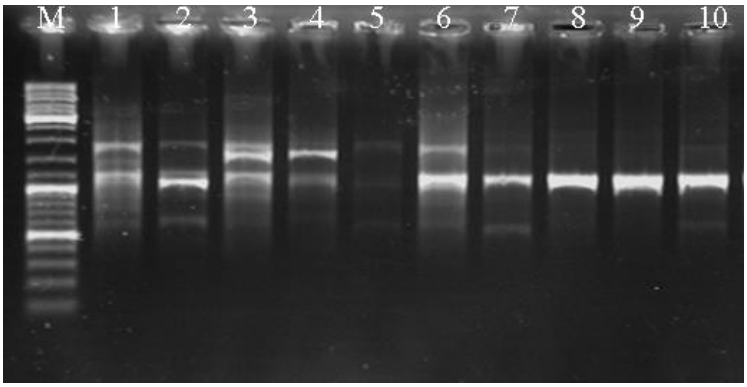

Figure 2. Gel containing lucerne varieties with the primer OPA 02 (M-Marker; 1. NS-Banat; 2. OS-99; 3. OS -88; 4. ZA-83; 5. K-22; 6. Čačanka 10; 7. Banjalučanka; 8. NS -Mediana; 9. OS -66; 10. K-28).

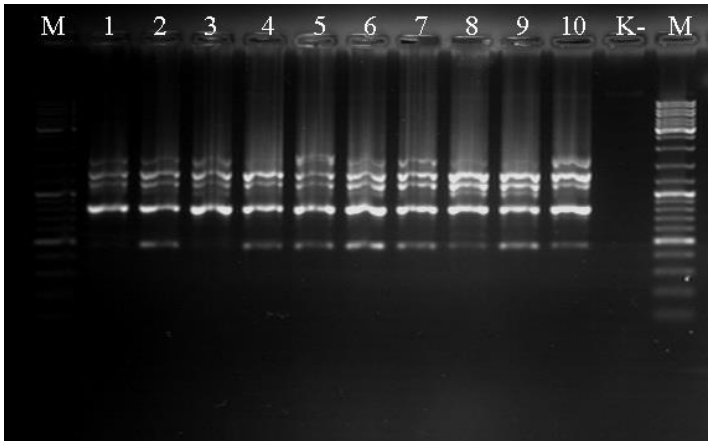

Figure 3. Gel containing lucerne varieties with the primer OPA 13 (M-Marker; K-Negative control; 1. NS-Banat; 2. OS-99; 3. OS -88; 4. ZA-83; 5. K-22; 6. Čačanka 10; 7. Banjalučanka; 8. NS -Mediana; 9. OS -66; 10. K-28).

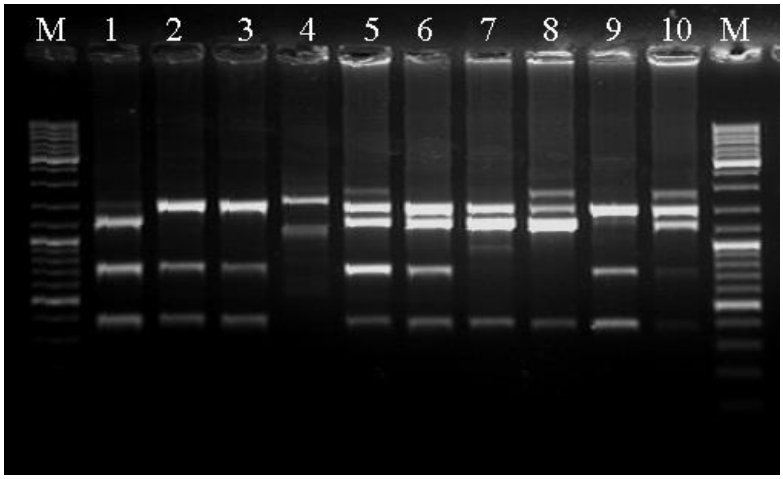

Figure 4. Gel containing lucerne varieties with the primer OPA 10 (M-Marker; 1. NS-Banat; 2. OS-99; 3. OS -88; 4. ZA-83; 5. K-22; 6. Čačanka 10; 7. Banjalučanka; 8. NS -Mediana; 9. OS -66; 10. K-28).

It is clearly observable that the lucerne variety Zaječarska 83 in the column 4 differs from remaining lucerne varieties. The existence of the common bands of 300 bp is also observed in all varieties but Zaječarska 83. Varieties Osječka 99, Osječka 88 and Osječka 66 (columns 2, 3 and 9) show a high level of similarity, as well as the varieties Kruševačka 22 and Kruševačka 28 (columns 5 and 10), which was expected given that the stated varieties have been bred in the same breeding institutions.

The cluster diagram obtained by the analysis of all observed molecular makrers consists of four groups of clusters (Figure 5). The vairieties NS-Banat, Kruševačka 22, Čačanka 10, Osječka 99 and Osječka 88 stand out in the first group. There are two subgroups within the first group. The first subgroup encomapasses varieties NS-Banat and Kruševačka 22, while the second subgroup includes varieties Čačanka 10, Osječka 99 and Osječka 88. The vairieties Kruševačka 28 and Osječka 66 belong to the second group, while varieties NS-Mediana and Banjalučanka are included into the third group. In the fourth group, the variety Zaječarska 83 stands out as the most genetically distant variety from remaining varieties.

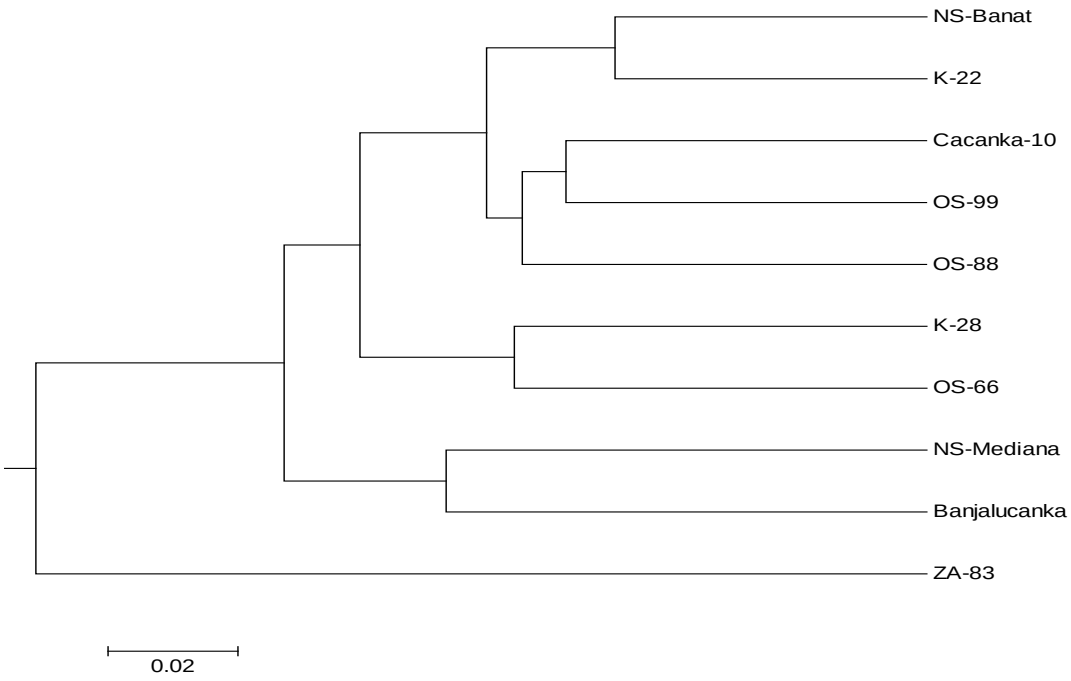

Figure 5. Cluster diagram of observed lucerne varieties based on all studied molecular markers

Principal Coordinates Analysis (PCoA)

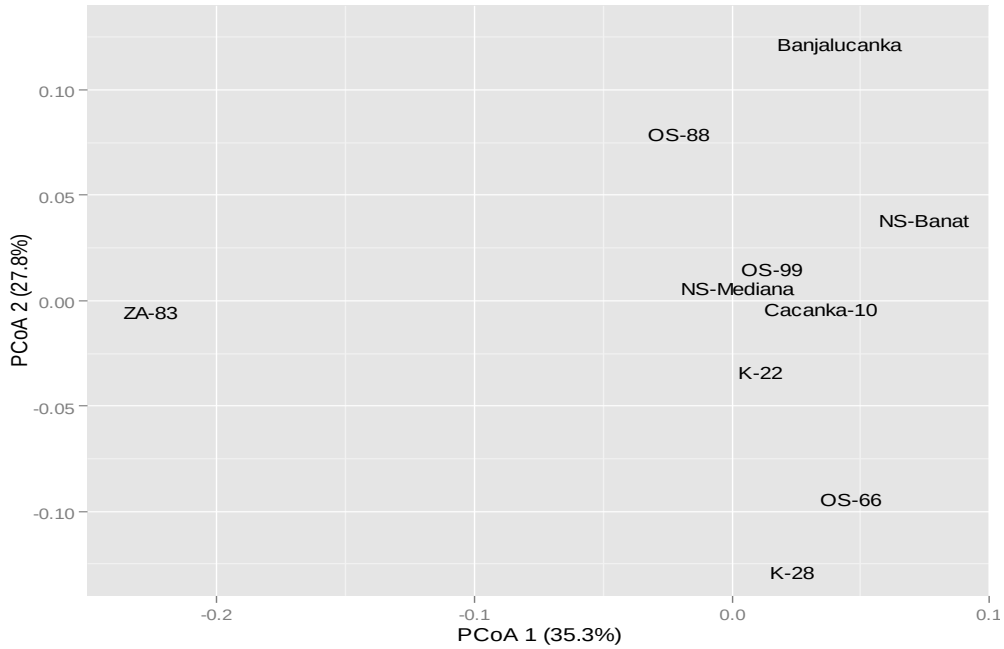

Figure 6. Plot of the first two axes from Principal Coordinates Analysis (PCoA) of genetic structure of 10 lucerne genotypes based on molecular markers

The Principal Coordinates Analysis (PCoA) is used to identify multidimensional relationships that describe portions of genetic variability of given data. The plot of the first and the second axes from the principal coordinates analysis (Figure 6) account for 63.1 % of the genetic variations contained in the original dataset. Figure 6 shows that the genotype Zaječarska 83 is genetically the most distant from remaining studied lucerne genotypes.

The grouping of data on the basis of the Principal Coordinates Analysis showed similarity with the grouping model based on the cluster analysis (Figure 5).

The plot of the first and the third axes from the Principal Coordinates Analysis (Figure 7) accounts for 58.0 % of the genetic variation contained in the original dataset. Figure 7 shows that the grouping of genotypes was similar to that of the first two axes.

Genotypes NS-Mediana and Zaječarska 83 are genetically most distant from remaining genotypes, while genotypes Osječka 99 and Osječka 88 are the closest, which points out to the similarity of the genetic material used in the development of these varieties because they originate from the same breeding institution.

Zaječarska 83 it was created by individual selection from domestic alfalfa populations, mainly from the area of Eastern Serbia (a region with arid climatic conditions). According to its botanical affiliation, it is *Medicago sativa* L. It is a medium early variety. Very tolerant to

drought and low temperatures. It was recognized by the Federal Commission for the Recognition of Varieties in 1984.

The application of population genetics to *SSR* loci can be used to assess differences among varieties, i.e. populations, whether they are used only for differentiation or for the management of genetic resources (FLAJOULOT *et al*., 2005).

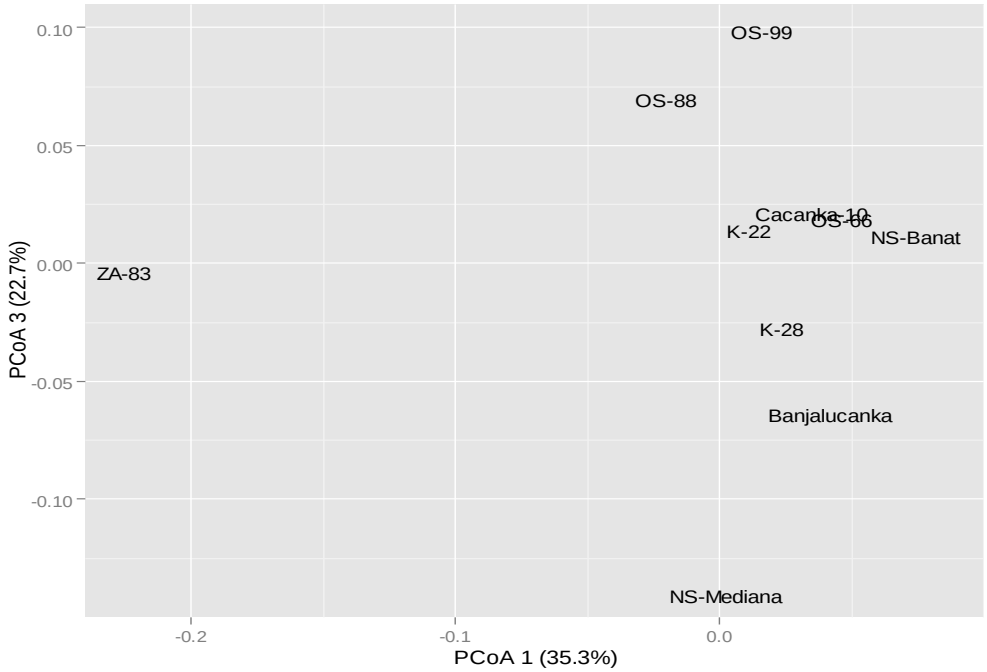

Figure 7. Plot of the first and the third axes from Principal Coordinates Analysis (PCoA) of genetic structure of 10 lucerne genotypes based on molecular markers

The genetic characterisation of plants has been used in many studies (FLAJOULOT *et al*., 2005; KUBIK *et al*., 2001; TUCAK *et al*., 2008; YANG *et al*., 2010; ŽIVKOVIĆ *et al*., 2012). The determination of genetic diversity among observed lucerne varieties has been successfully performed using *RAPD-PCR* and *ISSR-PCR* methods (XAVIER *et al*., 2011; ŽIVKOVIĆ *et al*., 2012).

Different genetic profiles obtained using *RAPD* and *ISSR* primers showed genetic diversity derived from various lucerne varieties.

DIWAN *et al*. (1997) were the first to develop *SSR* molecular markers on the species of the genus *Medicago*. These authors presented the method of using *SSR* molecular markers in the description of genetic diversity and analyses of genetic linkage among lucerne genotypes.

TUCAK *et al*. (2010) determined that *RAPD* molecular markers had been efficient in the assessment of genetic diversity among observed lucerne varieties. In addition, the obtained

results suggested that *RAPD* molecular markers could be useful for grouping germplasm with a similar genetic base and for pre-screening of potential heterotic groups in breeding programmes.

The application of *RAPD* and *ISSR* molecular markers on 25 species of the genus *Medicago,* collected from the Leh valley in the Himalayan region, showed that the level of genetic variation was relatively high, as indicated by the percentage of allelic polymorphysm (P=96.54 %) and the index of total genetic diversity (Ht=0.285).

A very pronounced level of genetic variation indicates that the species of the genus *Medicago* are suitable for further genetic studies and that there is a great potential for breeding lucerne to improve already existing varieties (XAVIER *et al*., 2011).

RUIZ-CHUTAN *et al*. (2019) used RAPD markers as a suitable tool for the identification of a high level of polymorphism among sorghum genotypes, through the visualisation of a high percentage of polymorphic bands, thus the existence of genetic diversity is evident.

Genetic diversity based on different markers plays a key role in breeding programmes and is one of the most important criteria for the selection of parents in the process of breeding. Using eight primers of *ISSR* molecular markers in 18 lucerne genotypes, the difference among observed genotypes was determined. The smallest, i.e. highest number of bands refers to the primer IS1, i.e. IS16, respectively (HABIBI *et al*., 2012).

The aim of the principal components analysis (PCoA) based on quantitative data is to check the significance of different traits in explaining multivariate polymorphism and is the most common tool used for screening, and thus leads to a further selection of parents for hybridisation (CHOZIN, 2007).

According to AHSYEE (2013) who studied 40 various red clover accessions of different geographical origin, the first and the second axes of the principal components analysis accounted for 83.7 % of genetic variation contained in the original dataset.

In the principal components analysis of 90 soya bean genotypes based on *SSR* markers, the first and the second axes accounted for a total of 48.8 % of genetic variation in the original dataset (PERIĆ, 2015).

ŠTRBANOVIĆ *et al*. (2017), studied 15 different lucerne varieties originating from Europe and America, and found out that based on their variability, lucerne varieties could be selected for the conventional cultivation or for selection and breeding of varieties with good potential for high dry matter yield and forage quality (ŠTRBANOVIĆ *et al*., 2017).

According to PRIOLLI *et al*. (2013), modern breeding provides the exchange of the material used in different breeding programmes, which results in a greater proportion of variation that is caused more by differences within the group than by differences between/among observed groups.

CONCLUSIONS

The lucerne variety Zaječarska 83 differs from all other Lucerne varieties. The existence of common bands of 300 bp is also observed in all varieties except for the variety Zaječarska 83. Varieties Osječka 99, Osječka 88 and Osječka 66 expressed a high level of similarity, as well as varieties Kruševačka 22 and Kruševačka 28. The first and the second axes from the principal coordinates analysis accounted for 63.1 % of genetic variation contained in the original dataset. It is clearly observed that the genotype Zaječarska 83 is genetically the most distant from

remaining studied lucerne genotypes. These studies confirmed that the observed collection of lucerne varieties is sufficiently variable for a successful breeding process. The breeding material can be used for new varieties and with the selection of an appropriate breeding model it is possible to breed varieties for certain purposes.

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PRIMENA MOLEKULARNIH MARKERA U IDENTIFIKACJI RAZLIČITIH GENOTIPOVA LUCERKE (*Medicago sativa* L.)

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Izvod

Za uspešnu selekciju je neophodno stalno uvoditi novi selekcioni materijal i koristiti ga preko raznih vidova hibridizacije za povećanje postojeće varijabilnosti. Kao proučavani materijal koristilo se deset (10) sorata lucerke različitog geografskog porekla. Šest sorata je poreklom iz Republike Srbije (Kruševačka 22, Kruševačka 28, NS-Banat ZMS II, NS-Mediana ZMS V, Zaječarska 83 i Čačanka 10), tri sorte su poreklom iz Republike Hrvatske (Osječka 66, Osječka 88 i Osječka 99) i jedna sorta je poreklom iz Republike Srpske (Banjalučanka). Po 100 semena svih 10 (deset) sorata lucerke postavljeno je na naklijavanje u Petri šolje i stavljeno u klijalište sa naizmeničnom temperaturom od 20 °C u tami u trajanju od 16 h i 30 °C na svetlu u trajanju od 8 h. Seme je naklijavano sedam dana. Za ekstrakciju DNK iz biljnog materijala korišćeni su prvi zeleni listići klijanaca lucerke (kotiledoni). U analizi glavnih koordinata prve i druge ose objasnile su ukupno 63,1 % genetičke varijabilnosti sadržane u originalnom setu podataka. Jasno se može videti da je genotip Zaječarska 83 genetički najudaljeniji od ostalih proučavanih genotipova lucerke.

Ova istraživanja su potvrdila da proučavana kolekcija sorti lucerke poseduje varijabilnost neophodnu za uspešan selekcioni proces. Oplemenjivački materijal poseduje poželjne osobine za oplemenjivanje novih sorti i uz izbor odgovarajućeg modela oplemenjivanja moguće je selekcionisanje sorti za određene namene.

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