# Molecular characterization of red clover genotypes utilizing microsatellite markers

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## ABSTRACT

Genetic resources of red clover (*Trifolium pratense* L.) are the basis for the improvement of this important forage legume. The objective of this study was microsatellite characterization of the accessions from the collection of the Institute of Field and Vegetable Crops in Novi Sad, Serbia. Molecular evaluation of 46 red clover genotypes was performed by applying the set of 14 primer pairs of microsatellite markers. These primer pairs amplified a total of 187 alleles, with an average of 13.36 alleles per *locus* and average polymorphism information content (PIC) value was 0.306. The minimum values of Dice genetic distances based on polymorphism of microsatellite markers were found among genotypes NCPGRU2 and NCPGRU5 (0.311) and the highest values of genetic distances were determined for a couple of genotypes Violeta and BGR2 (0.933). The average genetic distance between all pairs of genotypes amounted 0.587. The results of the principal coordinate analysis (PCoA) were consistent with the results obtained on the basis of cluster analysis, except that the PCoA allocated another four genotypes. There was no relationship between groups of genotypes formed by the use of cluster analyses and PCoA with their geographical origin. Analysis of molecular variance of 46 red clover genotypes by the status and ploidy level was significant, but it also suggested a weak genetic differentiation of groups formed on the basis of those characteristics. Observed groups of genotypes, according to the cluster analyses and PCoA of microsatellite data, could be used in future breeding programs for the selection of germplasm.

**Key words:** AMOVA, cluster analysis, genetic diversity, microsatellite markers, PCoA analysis, *Trifolium pratense*.

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## INTRODUCTION

Red clover (*Trifolium pratense* L.) belongs to the *Fabaceae* family, the genus *Trifolium*. This genus comprises more than 250 species with about 10% of them being important in agriculture (Kiran et al., 2010). Red clover, as one of the most extensive species of *Trifolium* genus, can be found in nature or as a cultivated crop in pure stands or in grass-legume mixtures. Traditionally, benefits of growing red clover include also N fixation and soil improvement through legume-*Rhizobium* symbioses (Yates et al., 2014). High protein content and excellent yielding potential, with some varieties that can have higher fodder yields than alfalfa (Drobna and Jancovic, 2006), make red clover considerably used in silage production for livestock farming. Yield and protein content are the most valuable traits that were developed and upgraded in breeding programs, as well as persistence and resistance to various biotic and abiotic stress factors (Repkova et al., 2006). Genetic diversity at crop level in conjunction with biodiversity gains due to agronomic practices ensures achieving crop yield and quality, taking into account biotic and abiotic stress factors that are inevitably present in crop production (Finckh, 2008).

Therefore, for the further improvement of red clover and other economically important *Trifolium* sp. varieties, genetic resources are still having special importance.

Originally, red clover is a diploid species, with the default number of seven chromosomes ($2n = 2x = 14$). Today it is grown commercially diploid and tetraploid cultivars of red clover (Zuk-Golaszewska et al., 2010). Although the most common method for obtaining tetraploids in red clover is colchicine doubling, there are also other methods that could be used for inducing polyploidy, like $N_2O$ and sexual polyploidization through unreduced gametes. The induced tetraploid forms could exceed their diploid counterparts in many traits like increased disease resistance, persistence, winter hardiness and forage DM yield (Sattler et al., 2016).

Red clover as allogamous species is characterized with homomorphic gametophytic self-incompatibility (GSI) system (Riday and Krohn, 2010). Accordingly, red clover populations are heterogeneous and consist of heterozygous genotypes. As a result, there are high levels of genetic variation within and between populations (Tucak et al., 2009). Besides, perennial, outcrossing species in relation to annual self-pollinators have higher genetic diversity and less differentiation among populations (Tanhuanpaa and Manninen, 2012).

Analysis of intra-group and inter-group genetic variability is of fundamental importance for plant breeding and germplasm conservation. This is particularly important for cross-pollinating species such as red clover, in which inbreeding depression can be manifested. Development and breeding of new varieties of red clover and similar forage legumes such as alfalfa is a very slow and long process (Tucak et al., 2009). In addition, it is still insufficiently studied genetic variability of both natural populations and local populations of forage species, such as red clover, in which is a fairly widespread use of local populations (Kouame and Quesenberry, 1993; Dias et al., 2008).

The neutral DNA markers have proven useful in detecting diversity of genetic resources because they allow more precise identification of the individuals independently of the influence of environmental factors. Today there are a significant number of techniques based on the variability of DNA sequences which complement the researches of allozyme methods (Pagnotta et al., 2005). Compared with phenotypic markers, DNA marker technology based on scientific explorations in molecular biology or biotechnology can be of great use for improvement or development of new cultivars and molecular plant breeding (He et al., 2014).

Simple Sequence Repeats (SSR) or microsatellite markers are the arrays of DNA sequences, consisting of tandemly repeating mono-, di-, tri-, tetra-, penta-, and hexa-nucleotide motifs, and they are flanked by unique sequences (Xu et al., 2013). These markers are based on PCR-polymerase chain reaction, there are many of them, they are codominant, highly reproducible (He et al., 2009) and also among the most preferred types of molecular markers for their ubiquitous distribution (Zhao et al., 2011). SSRs have been widely used in the analysis of genetic diversity (Zhang et al., 2012).

Red clover genotypes that are presented in a collection of the Institute of Field and Vegetable Crops in Novi Sad so far were differentiated using morphological UPOV descriptors and weren't completely characterized by the use of microsatellite markers. In this respect, the objectives of this study were to: i) accomplish SSR molecular analysis of varieties and populations of red clover; ii) assess genetic similarities and relationships of red clover genotypes on the basis of microsatellites allelic diversity; iii) classify genotypes according to the results of SSR molecular analysis. Screening germplasm of 46 red clover accessions at the molecular level could be useful for management of the collection and for efficient exploitation of genetic resources in future red clover breeding programs.

## MATERIALS AND METHODS

### Plant material

The plant material that has been used for this research consisted of 46 varieties and populations of red clover (Table 1). The entire plant material is part of the collection of the Institute of Field and Vegetable Crops in Novi Sad. The analyzed genotypes of red clover were chosen so that they are genetically divergent and consisted of diploid and tetraploid genotypes originating from 17 different countries of the world, as well as local varieties and populations.

**Table 1. Names, origin, status and ploidy level of the genotypes of red clover.**

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

### DNA extraction and PCR allele detection

The experiment consisted of 46 plants, each coming from 46 genotypes, and genomic DNA isolation was performed from the leaves according to the protocol of Rogers and Bandich (1988). Molecular characterization of 46 red clover genotypes was done on the basis of selected set of 14 microsatellite markers. List of tested microsatellite *loci*, their positions on chromosomes, primer sequences, and repetitive motifs are given in Table 2.

**Table 2. Microsatellite markers, chromosomes on which they are located, their primer sequences and motif repeats.**

| Microsatellite markers | Linkage group | Sequences of left and right primers (5'-3' direction) | Motif |
|---|---|---|---|
| RCS0035 | 1 | CATTGTAGGTTATGTTTATCAGG<br>CCCAAAGCCTACAAGGAAAG | $(AC)_{18}$ |
| RCS0453 | 2 | TCGCCACAAGGTCTCTTTTT<br>CGCTCTCTCTCTCTGCTTCA | $(AAG)_{15}$ |
| RCS2860 | 2 | GAAGCAAAGCTGTGAAAGGG<br>GAGAATCTTGAGTGTGTGAAGGTT | $(AAT)_{21}$ |
| RCS0078 | 2 | ATTCCCCCAATTTCCATCTC<br>TGCCCTGAAACCAAAAATGT | $(AG)_{32}$ |
| RCS0894 | 3 | CCTCATCATCAAATTCATTCTCA<br>AGCCAGAACCAGAACCTGAA | $(AAG)_{70}$ |
| RCS1667 | 3 | CAGCAATCCAACGTTTCTGA<br>ATCATCACCAGCTTCAGCAC | $(AAC)_{15}$ |
| RCS1729 | 4 | ATGGCTTCCTTCTTCACCCT<br>TCGACTGGGAAATCGATAGG | $(AAG)_{19}$ |
| RCS2728 | 4 | GTCCATGAAGGCCGAAAATA<br>CAGAGGACCAGGAGGTGAAG | $(AAC)_{24}$ |
| RCS1225 | 5 | TGCAAACTCCGCTTTATGC<br>CTCGCTGAAGGAGGAAACAG | $(ATC)_{15}$ |
| RCS3681 | 5 | AAAGCACGTGAAGAAAATGGA<br>CCCTTCATCAATGGCTTTCT | $(ATC)_{15}$ |
| RCS0252 | 6 | GGTAGTTTCTGACTTTCCCGTGT<br>TACAAAAGGGACCTGCTGCT | $(ATC)_{15}$ |
| RCS0031 | 6 | CCTCCTTGCATCATCTTTTC<br>AAAACTCGTTCGAGAGAGTG | $(AAAG)_{19}$ |
| RCS0685 | 7 | TGTTGCTACAAGGCCAAAGA<br>AGCACTTTCGAACACAGCAA | $(GGT)_{21}$ |
| RCS0793 | 7 | CGCAATCTTTCTTCTCATTTCA<br>TTCAACATGCAGGCTAAGAAAA | $(AAG)_{20}$ |

PCR was accomplished in the reaction mixture that contained 10 $\mu$l volumes with approximately 25-50 ng of template DNA, 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.3), 1.5 mM $MgCl_2$, 0.15 mM of each primer, 0.25 mM dNTPs and 0.3 U Taq polymerase (Applied Biosystems). Thermal cycling conditions involved a denaturation step at 94 °C for 3 min, then 45 cycles at 94 °C for 1 min, 1 min at 55 °C, 2 min at 72 °C and a final extension step of 72 °C for 7 min. Two markers that were labeled by different ABI-dyes were simultaneously analyzed for fragment detection. The 36 cm capillary arrays were used for separation of samples which contained 0.5-1 $\mu$L PCR products of each marker, 1 $\mu$L internal size standard and 9 $\mu$L Hi-Di formamide. GeneScan/Genotyper software package of Applied Biosystems was used for detection of alleles.

## Statistical analysis

In order to characterize the SSR genetic diversity of red clover germplasm, the number of alleles, allele frequency, fragments size range and polymorphism information content (PIC) values were determined using PowerMarker software (Liu and Muse, 2005). Basic indicators of microsatellite genetic variability in red clover are presented in Table 3. Analysis of microsatellites data served to construct Dice genetic distance matrix (Dice, 1945), by the use of NTSYS software (Rohlf, 2009). Dice genetic distance matrix was further used as the basis for the analysis of the main coordinates (Principal Coordinate Analysis [PCoA]; synonymous with Multidimensional scaling [MDS]) and

**Table 3. Parameters of genetic diversity for 14 microsatellite *loci* of red clover.**

| *Locus* | Sample size | Number of alleles | Frequency of alleles | Fragment size range (bp) | Pic |
|---|---|---|---|---|---|
| RCS0031 | 46 | 22 | 0.092 | 139-188 | 0.169 |
| RCS0035 | 46 | 7 | 0.317 | 184-196 | 0.434 |
| RCS0078 | 46 | 35 | 0.062 | 161-229 | 0.116 |
| RCS0252 | 46 | 5 | 0.249 | 171-182 | 0.371 |
| RCS0453 | 46 | 14 | 0.144 | 190-220 | 0.243 |
| RCS0685 | 46 | 4 | 0.383 | 196-208 | 0.466 |
| RCS0793 | 46 | 16 | 0.172 | 198-268 | 0.284 |
| RCS0894 | 46 | 14 | 0.121 | 128-185 | 0.216 |
| RCS1225 | 46 | 18 | 0.144 | 221-275 | 0.246 |
| RCS1667 | 46 | 6 | 0.391 | 255-270 | 0.482 |
| RCS1729 | 46 | 13 | 0.219 | 236-276 | 0.339 |
| RCS2728 | 46 | 11 | 0.258 | 218-247 | 0.382 |
| RCS2860 | 46 | 15 | 0.100 | 105-158 | 0.180 |
| RCS3681 | 46 | 7 | 0.228 | 152-170 | 0.352 |
| Average | | 13.36 | 0.206 | | 0.306 |

PIC: Polymorphism information content.

grouping of red clover genotypes, based on a set of selected microsatellite markers. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) tree was constructed using MEGA software (Tamura et al., 2007). Analysis of molecular variance (AMOVA) was also performed, and by the use of its results the intra-population and inter-population diversity of red clover genotypes were estimated, on the basis of status (cultivar/population) and ploidy (2*n* or 4*n*). AMOVA was carried out using ARLEQUIN program (Excoffier and Lischer, 2010).

# RESULTS

## Genetic variability of microsatellites profiles

Fourteen primer pairs amplified a total of 187 alleles, with an average of 13.36 alleles per *locus*. The highest number of alleles had a marker RCS0078, while the lowest number of alleles was presented for the marker RCS0685. A third of the total number of *loci* had eight or more alleles. Allele frequencies for 14 microsatellite *loci* were in the range of 0.062 (RCS0078) to 0.391 (RCS1667). In analyzed red clover genotypes dominantly were present moderately frequent alleles (with frequencies from 0.05 to 0.50), while rare alleles with frequencies less than 0.05, as well as frequent alleles with frequencies exceeding 0.50 were not represented. The amplified DNA fragments were in different ranges for different SSR markers, wherein the least value range was for the marker RCS0252 (11 bp) and the highest was for the RCS0793 marker (70 bp). The Polymorphism Information Content (PIC) value is an indicator of the power of the specific markers to detect polymorphism in the population (Botstein et al., 1980). The highest PIC value (0.48) was present for the RCS1667 marker, and the lowest (0.12) was characteristic for the marker RCS0078. Markers with dinucleotide repeat motifs on average had higher polymorphism (0.43) then the markers with trinucleotide motifs (0.32), and RCS0031 marker with a tetranucleotide motif (0.17).

## Cluster analysis

Genetic distance for 46 red clover genotypes was evaluated based on polymorphism of microsatellite markers and Dice distance matrix was constructed (Figure 1). The minimum values of genetic distances were found among genotypes NCPGRU2 and NCPGRU5 (0.311). The highest values of genetic distances were determined for a pair of genotypes Violeta and BGR2 (0.933). The average distance between all pairs of genotypes amounted 0.587.

The cluster analysis of microsatellites data arranged red clover genotypes in the two groups (Figure 2). The first cluster contains following genotypes: NCPGRU2, NCPGRU5, NCPGRU3, Diana, Britta, Avala, Fertody, SA4, BGR3, NS-Mlava, Mercury, Vivi, Titus, Triton, Lutea, Bradlo, Sofia52, 89 E-0, Amos, Krano, Italia centrale, SA3, BGR1, Bolognino, Kora, Bjorn, Lemmon, Noe, Marina, Renova, Rotra, BGR2, Nemaro, Lucrum. The second cluster comprises genotypes SA1, Marino, Violetta, Quiñequeli, Cortanovci, 91 E-63, Nessonas, Una, 91 E-44, NCPGRU4, Violeta, Dicar.

Grouping of genotypes by the use of microsatellite markers was not in accordance with their geographical origin.

## Principal Coordinate Analysis (PCoA)

Figure 3 refers to the PCoA of 46 red clover genotypes based on microsatellite markers and it shows that the first and second axis explained 19.3% of the total genetic variability of the original data set. The grouping of genotypes based on microsatellites, reveals the following genotypes which are genetically different in their molecular data in relation to the majority of genotypes that were clustered around the axis of the central part of PCoA graphics: Čortanovci, 91 E-63, NCPGRU4, Violeta, Nessonas, Una, 91 E-44, Violetta, Marino. The results of PCoA analysis were consistent with the results obtained on the basis of cluster analysis, except that the PCoA allocated another four genotypes (Noe, Lemmon, BGR2, Italia centrale). There was no relationship between (PCoA) grouping of genotypes and their geographical origin, as with the cluster analysis.

## Analysis of molecular variance (AMOVA)

AMOVA was used to test genetic variation among and within three groups: the first group consisted of 18 diploid populations, the second group was comprised of 21 diploid varieties, and the third group consisted of seven tetraploid varieties. Applying AMOVA analysis based on microsatellite markers of 46 red clover genotypes that were grouped based on type (variety or population) and ploidy level (2*n* or 4*n*) (Table 4), showed significant ($p < 0.05$) intergroup differentiation. However, the variance between groups was much lower in regard to variations within analyzed groups, pointing to weak intergroup differentiation. AMOVA also

**Figure 1. Distance matrix of the 46 red clover genotypes calculated using the Dice coefficient of similarity based on microsatellite markers.**

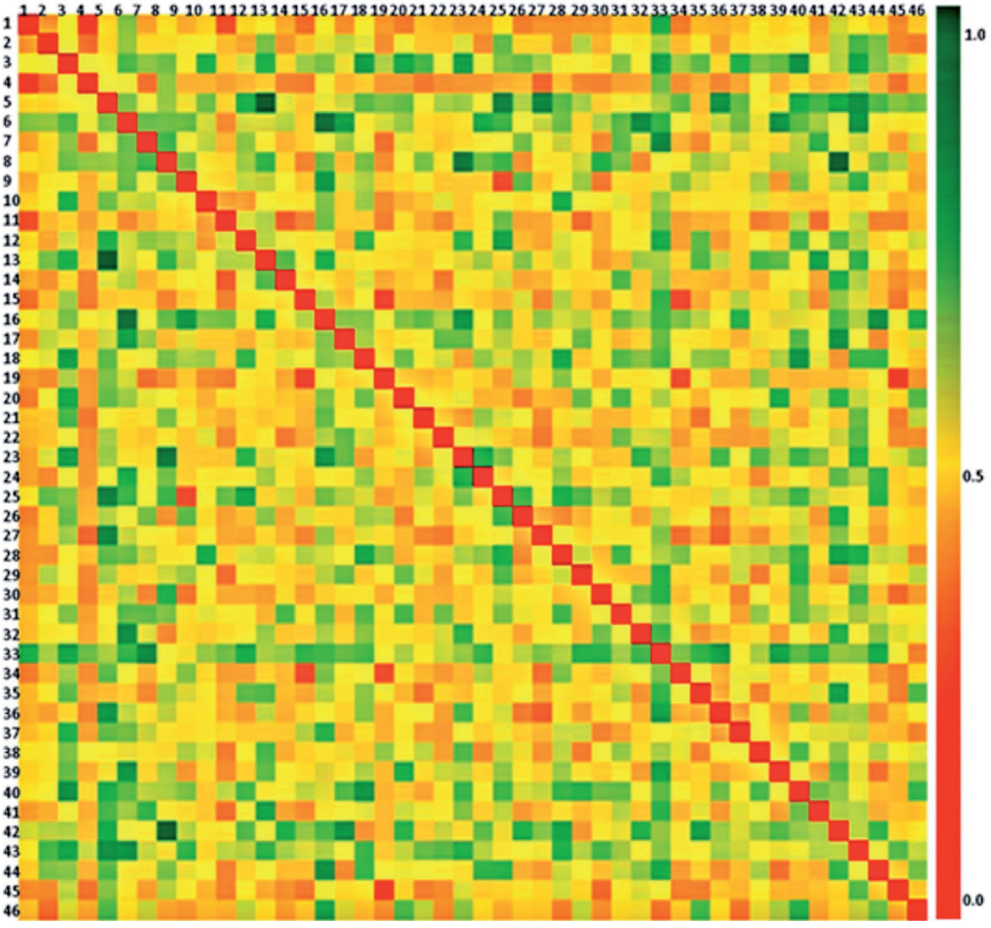

**Figure 2. Pair group method with arithmetic (UPGMA) dendrogram for 46 red clover genotypes based on Dice distance matrices of simple sequence repeats (SSR) data.**

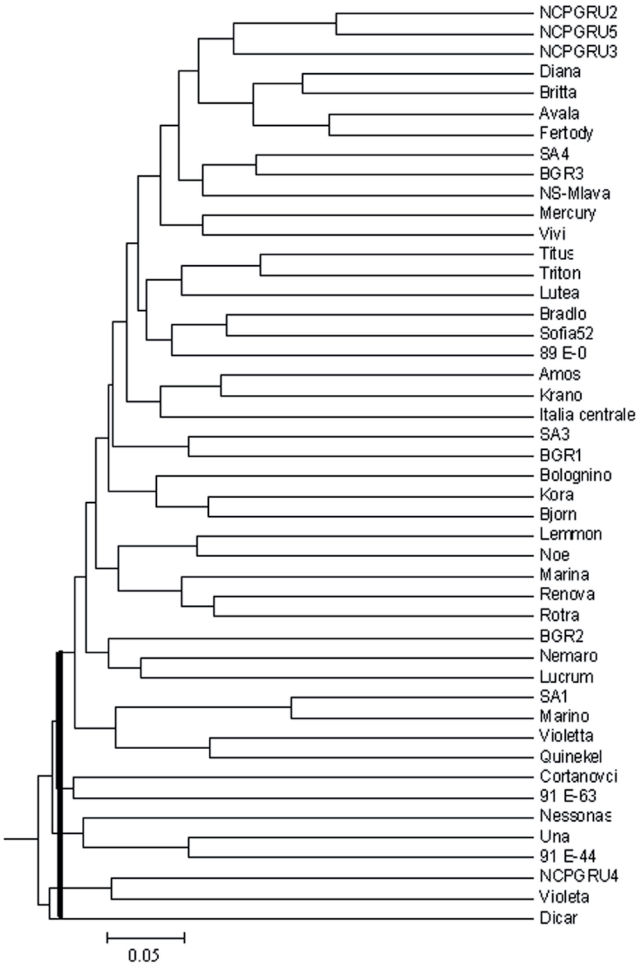


**Figure 3. Principal coordinate analysis (PCoA) of 46 genotypes based on 14 simple sequence repeats (SSR) markers.**

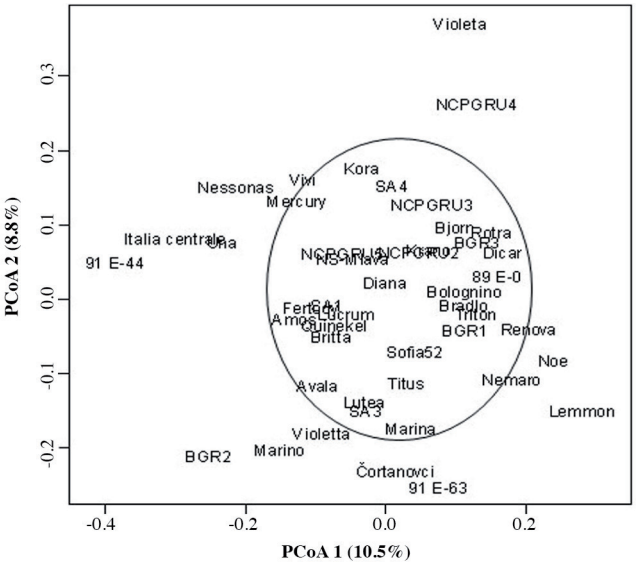


**Table 4. Analysis of molecular variance based on 14 simple sequence repeats (SSRs) of red clover genotypes grouped according to the type and ploidy.**

| Source of variation | df | Sum of squares | Variance components | % Total variance | $\Phi_{ST}$# | $P$# |
|---|---|---|---|---|---|---|
| Among groups | 2 | 0.729 | 0.00388 | 1.24 | 0.01236 | 0.04692 |
| Within groups | 43 | 13.319 | 0.30975 | 98.76 | | |
| Total | 45 | 14.048 | 0.3236 | | | |

#Calculated on the basis of the 1000 permutations.
$p < 0.05$ - significant
df: Degrees of freedom; $\Phi_{ST}$: index of genetic differentiation.

served for determining the index of genetic differentiation ($\Phi_{ST}$), which is a standardized inter-group genetic distance of the two geographic groups, and represents an indication of the correlation of genes of different individuals in a population (Chen and Nelson, 2005). Values of $\Phi_{ST}$ index according to Hartl and Clark (2007) are defined as follows: differentiation may be little ($\Phi_{ST} < 0.05$), moderate ($0.05 < \Phi_{ST} < 0.15$), great ($0.15 < \Phi_{ST} < 0.25$) and very great ($\Phi_{ST} > 0.25$). It can be observed that the genetic distance, expressed as $\Phi_{ST}$ index between groups defined on the basis of type and ploidy of 46 red clover genotypes, was low (0.01236), and pointed to a weak genetic differentiation between these three groups.

## DISCUSSION

An average number of alleles per *locus* in this study (13) was a significant indicator of the genetic diversity of investigated red clover genotypes, and it was higher than in the research studies of other authors who have also used SSRs in red clover. Sato et al. (2005) and Dugar and Popov (2013) showed that the average number of alleles per SSR *locus* in the red clover was 9. Dias et al. (2008) reported that the average number of SSR alleles in red clover was 9 in three population and 11 for 56 individuals, which represented 56 populations. Berzina et al. (2008) found that the average number of alleles for seven analyzed cultivars was lowest in Aria variety (11.3), and the highest in 'Priekuli' (19.2). Vymyslicky et al. (2012) reported that the number of alleles per *locus* in red clover ranged from 3 to 8, with an average of 4.4. Gupta et al. (2016) determined the lowest value of the average number of alleles per *locus* in the red clover, which amounted 3.18. PIC values determined in this study were somewhat lower when compared to the results of other authors. PIC values established by Sato et al. (2005) for the same SSRs that were used in this study were within the range of 0.54 to 0.83. PIC values determined in the work of Dias et al. (2008) were in the range of 0.64 to 0.85 for the three populations and in the range of 0.70-0.91 for 56 individuals of red clover. Gupta et al. (2016) reported SSR PIC values range from 0.301 to 0.719 for red clover genotypes, and Vymyslicky et al. (2012) found SSR PIC values to be in the range of 0.4 to 0.86.

AMOVA was used for the purpose of detailed consideration of genetic variability and differentiation of the studied 46 red clover genotypes.

The high within-population variability and high heterogeneity and heterozygosity of red clover are expected because of cross pollination of this species and its extraordinarily high level of gametophytic self-incompatibility (Rosso and Pagano, 2005).

Greater within-group variability in relation to among-group also was found by other authors. Dias et al. (2008) tested five clusters obtained on the basis of morphological characteristics of 56 individuals in relation to the SSR data and found that within-group variability was 98.1%, while the among-group variation was 1.9%. In the same research by the application of AMOVA in three populations of red clover, it was also found that the within-group variability was higher (83.6%) and among-group much lower (16.4%). Dugar and Popov (2013) have studied 15 Ukrainian red clover cultivars and found that the among-group genetic variability of SSR markers was low and that it amounted only 6.9% of the total variability. Gupta et al. (2016) analyzed a core collection of red clover which was established by (Kouame and Quesenberry, 1993), and dismantling of the genetic variance using AMOVA showed that most of the genetic diversity was contained within the population (91%), while 9% of genetic variation is accounted for among-group variability. Berzina et al. (2008) were studying seven diploid red clover cultivars based on six SSRs and found that inter-group variation represented only 2% of the total genetic polymorphism, as well as that $\Phi_{ST}$ values (0.006 to 0.043), indicated a low genetic differentiation between varieties.

## CONCLUSIONS

Considering our molecular results, it can be concluded that the application of 14 microsatellite markers on the selected set of 46 red clover genotypes in these research, detected a significant genetic variability, which is the basic precondition for the creation of new and improvement of existing varieties. In this respect, based on microsatellite markers, we observed two groups of genotypes, specifically 16 genotypes which were separated in relation to the larger number of the remaining 30 genotypes, which in addition to data on agronomically important qualitative and quantitative traits could be used in future breeding programs for the initial selection of germplasm.

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