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ENETIC DIVERSITY IN RED CLOVER (*Trifolium pratense* L.) USING SSR MARKERS

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Red clover (*Trifolium pratense* L.) is one of the main forage species from temperate regions and it's originated in Eurasia where wild-type populations are found in the Caucasus Mountains. Considerable variation for this crop has been recorded for few molecular variations. The identification and understanding of molecular genetic diversity in red clover accessions will help in effective genetic conservation along with efficient breeding programs in this crop. This study has shown molecular diversity using microsatellite markers in red clover accessions from around of the world. In this investigation, 40 genotypes have been selected and analyzed using 15 SSR primers. These primer pairs amplified 1146 polymorphic loci among the genotypes screened. The number of fragments amplified by each SSR primer combination varied from 24 for RCS3681 to 109 for RCS1729 with an average value of 80.78 per primer combination. The SSR marker data was further analyzed using cluster algorithms and Principal coordinates analysis (PCoA). The results indicated that the considerable genetic variations were discovered among the analyzed genotypes. The SSR based clustering could identify the putative pedigree types of the present red clover types of diverse origins

Key words: cluster analysis, genetic distance, genetic variation, principal coordinates analysis, red clover, SSR molecular markers

INTRODUCTION

Red clover (*Trifolium pratense* L.), is one of the main forage species from temperate regions and it's originated in Eurasia where wild-type populations are found in the Caucasus

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Mountains. In its natural habitat it is found in meadows, forest margins, and field borders (GILLETT and TAYLOR, 2001; HERRMANN *et al.,* 2006).

It is one of the most important legumes in the world because of its adaptation to a wide range of soil types (pH levels from 6.0-7.5), environmental and management conditions (TAYLOR, 2008).

Red clover can be used as hay or as pasture in crop rotations and has high productivity and rapid regeneration after harvest. It is especially suitable for cattle, because it has good forage quality and high nutritive value of hay (AÇIKGÖZ, 2001).

In recent years, the use of natural antioxidants found in red clover has attracted interest due to their presumed nutritional and therapeutic value (VLAISAVLJEVIC *et al*, 2014). Red clover as an allogame plant is characterized by high variability because of its heterozygosis, fixed over the time by repeated open crossing. It is an outcrossing species, diploid genome is 2n = 2X = 14 (SATO, 2005) and it has a gametophytes self-incompatibility system (BOLLER *et.al*, 2010). Red clover and other legumes, in symbiosis with Rhizobial species are able to fix nitrogen from the atmosphere and convert it into a form of nitrogen that plants can utilize.

In comparison with other legumes its advantage is based on the following characteristics: high seeding vigor, rapid growth, tolerance to acid and humid conditions, nitrogen fixing ability, and high nutritive value for ruminants (LETO *et al.,* 2004; SATO *et al.,* 2005; VOIGT and MOSJIDIS, 2002).

In Serbia, red clover is the second most important perennial forage legume, where it is planted on about 120.000 ha. It is grown the most in the hilly and mountainous parts of central Serbia with an acid soil reaction and humid climate. The domestic market is predominated by cultivars developed in domestic research centers (Institute of Field and Vegetable Crops, Novi Sad and Institute for Forage Crops, Kruševac).

In genetics, molecular genetic markers are defined as differences at the genotype level that can be used to answer and explain questions of genetics (LOKKO *et al.,* 2005). SSR (simple sequence repeats) also known as microsatellites, consist of di-, tri- or tetra-nucleotide motifs repeated several times [for instance, (CA) n, (GAT) n or (AGCT) n] which are a common feature of most eukaryotic genomes. In comparison with other molecular marker techniques, SSR markers are highly polymorphic and informative, technically simple, quick, codominant, reproducible and relatively inexpensive when primer information is available, and efficient technique. It has high reproducibility. The use of radioactivity is not essential. Therefore they are widely used in genetic research of plants, cultivar characterization, plant breeding, paternity tests and phylogenetic studies (REAL *et al.,* 2007; DIAS *et al.,* 2008; SANTOS *et al.,* 2010; ZHANG *et al.,* 2010). Microsatellites, as a significant source of genetic markers, exhibit high mutation rates (VIGOUROUX *et al.,* 2002), preferential association with non-repetitive regions of the genome (MORGANTE *et al.,* 2002). Compared to other molecular markers, microsatellites show a much higher level of information given to study the genome of red clover. A limited number of SSR markers have also been developed and used in studies of red clover genetic variation (KOLLIKER *et al.,* 2001; KOLLIKER *et al.,* 2006; DIAS *et al.,* 2008).

Genetic resources are the foundation of any crop imrovement program. Twenty years ago, the Institute of Field and Vegetable Crops began to collect red clover accessions in order to obtain as much as possible genetic variability for the subsequent breeding of this crop. Domestic local populations (early type - medium type) from Serbia and the Republic of Srpska were collected first. More recently, the Institute's red clover collection has been significantly enlarged

through international collaborations with the world's leading gene banks and institutes maintaining reference collections (Plant Research International, Vageningen (the Netherlands), IPK-Gatersleben (Germany); Nordic Gene Bank (Sweden), Federal Centre for Breeding Research on Cultivated Plants- Braunschweig (Germany); Saatzucth Steinach GmbH (Germany), N.I. Vavilov Research Instiute of Plant Industry (VIR), St. Petersburg (Russia): USDA/ARS Western Regional Plant Introduction Station, Washington State University (USA), Institute of Plant Genetic Resources (IPGR), Sadovo (Bulgaria), Research Instiute of Plant Production, Piešt`ani (Slovakia), National Center for Plant Genetic Resources of Ukraine (NCPGRU), Kharkiv (Ukraine), Australian Medicago Genetic Resource Centre (South Australia), GKI - Szeged, (Hungary); OKI – Szarvas, (Hungary), Departement plantengenetica en Veredeling, (Belgie); Banca de Resurse Genetice Vegetale – Suceava (Romania); Jogeva Plant Breeding Institute (Estonia), Universidad Mayor de san Simon, Facultad de Sciencias Agricolas (Bolivia), International Center for Agricultural Research in the Dry Areas (Syria); National Agricultural Research Foundation Fodder Crops and Pastures Institute (Greece), National Agriculture Research Center for Hokkaido Research (Japan), Practical Centre of the NAS of Belarus for Arable Farming (Belarus), CRA, Lody (Italy).

Red clover collection of the Institute of Field and Vegetable Crops in Novi Sad currently has about 664 red clover accessions. Research of the current Institute's red clover collection had been started on the characterization and evaluation by morphogical and isozymes markers (NIKOLIC *et al*., 2010)

The objective of the current study was to assess the genetic diversity in some part of the institute's collection with 40 cultivars and populations of red clover by means of SSR markers.

Our intent was to understand better the level and pattern of genetic diversity within and among the red clover accessions. The generated information could improve: the efficiency of collection, conservation and exploitation of red clover genetic resources, as well as assisting plant breeders in locating parent germplasm with specific characteristics for their breeding programs.

MATERIALS AND METHODS

Plant material

Forty red clover accessions from 17 different countries around the world (Table 1) were evaluated in this study. The seed samples of foreign materials were obtained from the Institute of Field and Vegetable Crops, Novi Sad, Republic of Serbia. Ten plants per accessions were randomly selected, and green healthy leaves from each plant were collected for DNA extraction.

SSR primers

In this SSR experimental study, fifteen primer pairs were used for genotyping red clover accessions (Table 2). The SSR markers used are distributed widely across the red clover linkage groups thus giving the comprehensive coverage of the red clover genome. DNA was extracted from fresh plant material (leaf tissue).

Table 1. List of 40 red clover accessions used in this study.

Number	Accessions	0rigin	Status of accessions	Ploidy level
1	NCPGRU1	Ukraine	population	2n
2	NCPGRU6	Ukraine	population	2n
3	Keland-Bo	Bolivia	variety	2n
4	SA 2	Australia	population	2n
5	Kolubara	Serbia	variety	2n
6	Kohler	Switzerland	population	2n
7	Moser	Switzerland	population	2n
8	Changins	Switzerland	variety	2n
9	Bombi	Sweden	variety	2n
10	Fanny	Sweden	variety	4n
11	Pelly	Sweden	variety	4n
12	Atelo	Germany	variety	2n
13	Jubilatka	Germany	variety	4n
14	Matri	Germany	variety	4n
15	NfgMekra	Germany	variety	2n
16	Remy	Germany	variety	2n
17	Krano	Denmark	variety	2n
18	Crop	France	variety	2n
19	Pales	France	variety	2n
20	Skrzeszowicka	Poland	variety	2n
21	Viola,Hruszowska	Poland	variety	2n
22	Slovenska B	Czech Republic	variety	2n
23	Mir	Russia	variety	2n
24	Aberystwyth	UK	variety	2n
25	Rotonde, Kuhn	Holland	variety	2n
26	Teroba	Holland	variety	4n
27	Gkt Junior	Hungary	variety	2n
28	NS-Ravanica	Serbia	variety	2n
29	NCPGRU 8	Ukraine	population	2n
30	Valente	Italia	population	2n
31	Longevo	Italia	population	2n
32	Perseo	Italia	population	2n
33	Cremonese	Italia	population	2n
34	Brezova	Slovakia	population	2n
35	Rozbehi	Slovakia	population	2n
36	E-86	Bulgaria	population	2n
37	E-97	Bulgaria	population	2n
38	E-128	Bulgaria	population	2n
39	E-26	Bulgaria	population	2n
40	E-28	Bulgaria	population	2n

Table 2. Sequences of fifteen primer pairs of microsatellite markers tested in 40 accessions of red clover.

Primer	Size	SSR motif	Forward primer (5`-3`)	Reverse primer (3`-5`)
RCS005	162	AC	CATTGTAGGTTATGTTTATCA	CCCAAAGC
RCS043	189	AAG	TCGCCACAAGGTCTCTTTTT	CGCTCTCTC
RCS280	93	AAT	GAAGCAAAGCTGTGAAAGGG	GAGAATCT
RCS008	178	AG	ATTCCCCCAATTTCCATCTC	TGCCCTGA
RCS084	156	AAG	CCTCATCATCAAATTCATTCT	AGCCAGAA
RCS167	238	AAC	CAGCAATCCAACGTTTCTGA	ATCATCAC
RCS179	224	AAG	ATGGCTTCCTTCTTCACCCT	TCGACTGG
RCS278	202	AAC	GTCCATGAAGGCCGAAAATA	CAGAGGAC
RCS125	200	ATC	TGCAAACTCCGCTTTATGC	CTCGCTGA
RCS361	141	ATC	AAAGCACGTGAAGAAAATGG	CCCTTCATC
RCS022	153	ATC	GGTAGTTTCTGACTTTCCCGT	TACAAAAG
RCS001	135	AAG	ACCTCCTTGCATCATCTTTTC	AAAACTCG
RCS048	175	ATC	GAATGCCAAGACACCTGTGA	TCTCATCA
RCS065	178	GGT	TGTTGCTACAAGGCCAAAGA	AGCACTTT
RCS073	199	AAG	CGCAATCTTTCTTCTCATTTCA	TTCAACAT

PCR conditions and allele detection

PCR reactions were performed in a final volume of 10 µl containing approximately 25-50 ng of template DNA, 1 x 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 for each deoxynucleotide (dATP, dCTP, dGTP and dTTP), and 0.3 U of Taq polymerase (Applied Biosystems). Amplifications were run in Thermal Cycler set started with a denaturation step for 3 minutes at 94 °C followed by 45 cycles of 1 minute at 94 °C, 1 minute at 55 °C, 2 minutes at 72 °C and stopped after a final extension step of 72 °C for 7 minutes.

The fragment analysis was performed as a multi-loading assay analysing two markers simultaneously that was labelled by different ABI-dyes. Samples containing 0.5-1 µl PCR products of each marker, 1 µl internal size standard and 9 µl Hi-Di form amide were separated using 36 cm capillary arrays. Alleles were detected using the GeneScan/Genotyper® software package of Applied Biosystems.

Statistical analyses

The genetic associations between accessions were evaluated by calculating the Jaccard's similarity coefficient for pair wise comparisons based on the proportions of shared bands produced by the primers (JACCARD, 1908). Similarity matrix was generated using the MEGA 4.0 software (using matrices from R software). The similarity coefficients were used for cluster analysis and dendrogram was constructed by the Unweighted Pair-Group method (UPGMA).

Principal coordinate analysis (PCoA) of the traits was employed to examine the percentage contribution of each trait to total genetic variation, and performed to show the distribution of the genotypes in a scatter plot.

We carried out analysis of molecular variance (AMOVA) to partition the genetic variance between level of polyploidy, and status of cultivar.

RESULTS AND DISCUSSION

Genetic information of SSR markers

A total of 1146 polymorphic fragments were amplified between the different red clover accessions using fifteen SSR primer combinations. Number of fragments amplified by each SSR primer combination varied from 24 for RCS3681 to 109 for RCS1729 with an average value of 76.4 per primer combination.

High variation of amplification products within cultivars were observed from 18 for accessions: Kohler, NfgMekra, Cremonese to 43 for red clover accessions Teroba and E-86.

The details of SSR primers used for assessing the molecular diversity among 40 red clover accessions are given in Table 2. The PCR product size obtained by the amplification of SSR primers ranged from 105 to 270 bp.

SSR genetic distance, similarity and cluster analysis

The computed Jaccard's similarity coefficients based on 15 SSR markers ranged from 0.50 to 0.90 among the 40 accessions studied. The results of genetic diversity in this study is in accordance with previous reports that used RAPD markers (CAMPOS-DE-QUIROZ and ORTEGA-KLOSE, 2001; ULLOA *et al.,* 2003) for studied red clover populations as well as for alfalfa (AHSYEE *et al.,* 2013) and AFLP markers (KOLLIKER *et al.,* 2003; HERRMANN *et al.,* 2005). The lowest dissimilarity (0.520) was observed between Valente and Cremonese and the highest (0.918) was between SA 2 and Moser, with mean value of 0.769.

The measures of relative genetic distances among populations did not completely correlate with geographical distances of places of their origin. For instance, NCPGRU1 and Viola Hruszowska with high geographical distances grouped together in Cluster I; also geographically distant populations E-86, E128 and Aberystwyth grouped in Cluster II. The same results was obtained on *Medicago sativa* (TUCAK *et al.,* 2008; TOUIL *et al.,* 2008), *Bunium persicum* (PEZHMANMEHR *et al.,* 2010)*, Daucus carota* (BRADEEN *et al.,* 2002), *Phaseolus vulgaris* (MARTINS *et al.,* 2006), *Matricaria chamomilla* (SOLOUKI *et al.,* 2008) and grapevine (THEOCHARIS *et al.,* 2010).

The cluster analysis using UPGMA based on Jaccard similarity coefficients was done to resolve the close genotypic relationships between the analyzed red clover genotypes (Figure 1). The genetic similarity within the accessions was very high, with very few values lower than 0.6. The dendrogram produced ten distinct clusters (Figure 1). The cluster size varied from 14 (Cluster 1) to 1 (Clusters 9, 10).

Cluster I comprised of 14 including accessions and further it was divided into two subclusters. The subcluster I-a comprised NCPGRU 1 (Ukrania), Skrzeszowicka (Poland), Viola Hruszowska (Poland) and Teroba (Holand). Subcluster I-b contained ten accessions, three from Germany (Matri, Atelo, Remy), three from Italia (Valente, Cremonese, Perseo), one from Swedan (Pelly), one from Denmark (Krano), one from Czech Republic (Slovenska B) and one from Ukrania (NCPGRU8). Cluster II contained three accessions, two from Bulgaria (E-86 and

E128), and one from UK (Aberystwyth). Cluster III comprised of three accessions: Crop (France), E-26 (Bulgaria) and Rozbehy (Slovakia). Accessions E-97 and E-28 that originated from Bulgaria were clustered closely together with accessions Bombi (Sweden), NfgMekra (Germany) and Mir (Russia) in cluster IV. Cluster V include accessions: Gkt Junior, Longevo from Hungaria and Italia respectively. Cluster VI contain seven accessions two from Serbia (Kolubara, NS-Ravanica),one from France (Pales), one from Switzerland (Moser), one from Sweden (Fanny), one from Germany (Jubilatka) and one from Holland (Rotonde Kuhn). Cluster VII consist of two accessions: NCPGRU 6 from Ukraine and Kenland-Bo from Bolivia. Cluster VIII comprised of two accessions: Kohler, Brezova from Switzerland and Slovakia respectively. Cluster IX includes one accession (Changins) from Switzerland. Cluster X consists of one accession (SA-2) from Australia.

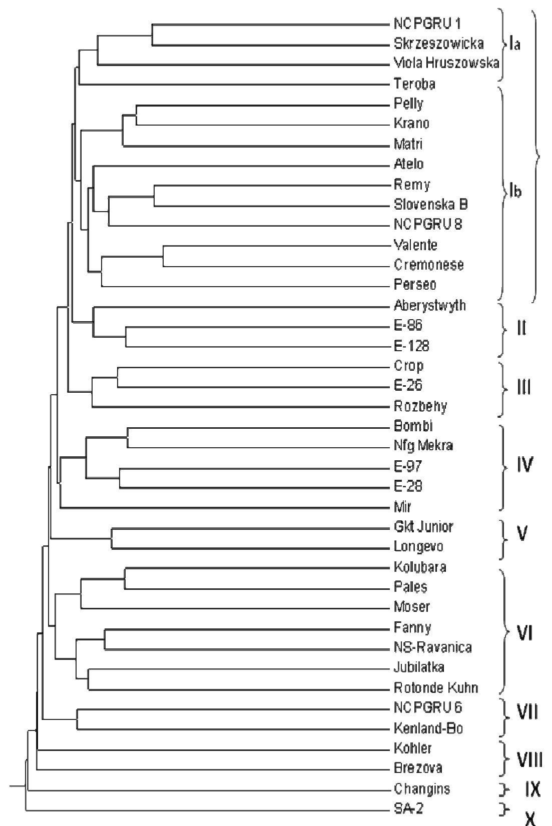

Figure 1. Dendrogram of 40 red clover accessions revealed by UPGMA cluster analysis based on SSR markers data.

Principal coordinate analysis using SSR markers

PCoA was used to identify multidimensional relationships that describe portions of the genetic variance in a data set. The genetic relationships among 40 red clover genotypes were also determined using Principal coordinate analysis PCoA of genetic distance estimates and are presented in Figure 2 and Figure 3. According to full accessions name and origin grouping respectively, the first (PCoA 1) and the second (PCoA 2) principal coordinate axes accounted for 5.5% and 5% of the total variation. So the first two components of the PCoA between red clover genotypes represented 10.5% of the variance, and as a graphical representation it summarizes the microsatellite data.

The PcoA biplot (Figure 2 and Figure 3) separated accessions comprehensively with regard to the dendrogram groups (Figure 1) which are based on accessions genetic similarity. The present study couldn`t established clear relationship between genetic diversity assessed by SSR and geographical origin, same as reported by DIAS *et al.* (2008) for T. *pratense* and ZHANG *et al.* (2010) for *Trifolium repens.*

Figure 2. First and second Principal Coordinate Analysis (PCoA) (accessions grouping). Genotypes accessions data are presented in Table 1.

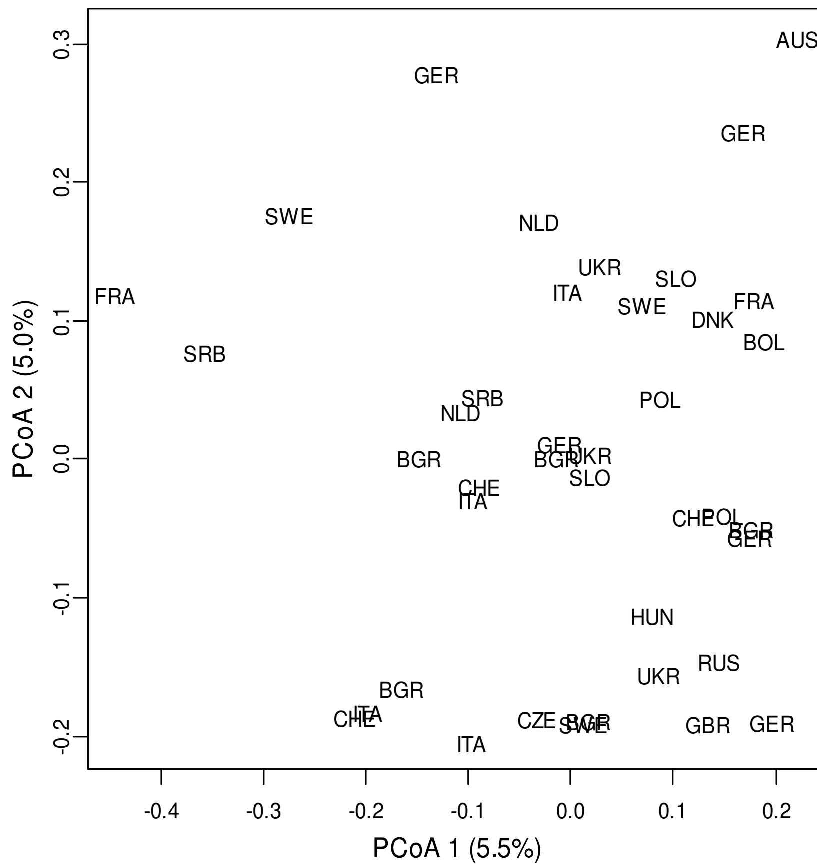

Figure 3. First and second principal coordinate analysis (PCoA) (country grouping). Genotypes origin data are presented in Table 1.

Analysis of molecular variance (AMOVA)

The AMOVA (Analysis of Molecular Variance) using the SSR data according level of polyploidy revealed that the total observed variation was high and accounted for 99.91% between groups and 0.09% among groups (Table 3). The overall (F_{ST} = 0.0009; P = 0.449) demonstrated the existence of high genetic differentiation among red clover accessions (Table 3).

The AMOVA analysis using SSR molecular markers revealed more of the variation between red clover groups than among groups.

The total amount of genetic variation was also partitioned by AMOVA into components according to the status of cultivar. Based upon the analysis of the accessions structure, the AMOVA results (Table 3) showed significant variation between groups component which

accounts for 99.41% of the total variance and the remnant amounts of the total genetic variation among groups was found to be 0.59%.

Table 3. Analysis of molecular variance (AMOVA).

Source of variation	df	Variance component	Percentage of variation	F_{ST}	P
		Level of polyploidy			
Among groups	1	0.00036	0.09	0.00092	0.449
Between groups	38	0.38454	99.91		
Total	39	0.38490			
		Status of cultivar			
Among groups	1	0.00226	0.59	0.00585	0.137
Between groups	38	0.38350	99.41		
Total	39	0.38576			

Other studies in red clover (KOLLIKER *et al.,* 2003) and in other forage crops (KOLLIKER *et al.,* 2001) found an extensive genetic diversity within population also when using different types of molecular markers (CAMPOS-DE-QUIROZ and ORTEGA-KLOSE 2001; ULLOA *et al.,* 2003; KOLLIKER *et al.,* 2003). The variation for within-population diversity based on SSR markers (MENGONI *et al.,* 2000) AFLPs (HERRMANN *et al.,* 2005) and RAPDs (ULLOA *et al.,* 2003) ranged from 68.3% to 99.5% in alfalfa and red clover. In addition, analysis of the seed yield of 11 *Medicago sativa* L. populations showed that the among-population variance accounted for 5% to 31% of the total genetic variance for seed yield components, while the within-population variance explained 69% to 95% (BOLANOS-AGUILAR *et al.,* 2000).

CONCLUSION

15 primer pairs applied over 40 red clover genotypes amplified 1146 polymorphic loci. The number of fragments amplified by each SSR primer combination varied from 24 for RCS3681 to 109 for RCS1729 with an average value of 80.78 per primer combination. Cluster algorithms and Principal coordinates analysis (PCoA) results indicated that genetic similarity within the accessions was very high, with very few values lower than 0.6. The dendrogram produced ten distinct clusters. The cluster size varied from 14 (Cluster 1) to 1 (Clusters 9, 10). The SSR based clustering could identify the putative pedigree types of the present red clover types of diverse origins. The PcoA biplot clustered accessions similarly to the dendrogram based on their genetic similarity with some differences.

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GENETIČKI DIVERZITET CRVENE DETELINE (*Trifolium pratense* L.) NA OSNOVU SSR MARKERA

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Izvod

Crvena detelina (*Trifolium pratense* L.) je jedna od glavnih krmnih vrsta umerenih regiona, poreklom je iz Evroazije, a divlje populacije ove vrste su nađene na Kavkazu. Značajno variranje na molekularnom nivou je zabeleženo kod ovog useva. Identifikacija i razumevanje molekularnog genetičkog diverziteta uzoraka crvene dateline je od pomoći za efikasno očuvanje i efikasno oplemenjivanje ove vrste. U ovom radu je prikazan molekularni diverzitet na osnovu mikrosatelitskih markera kod uzoraka crvene dateline poreklom iz čitavog sveta. Za istraživanje je odabrano i analizirano 40 genotipova pomoću 15 SSR prajmera. Na osnovu ovih prajmera je amplifikovano 1146 polimorfnih lokusa kod ispitivanih genotipova. Broj amplifikovanih fragmenata pomoću prajmer kombinacija je varirao od 24 za RCS3681 do 109 za RCS1729 sa prosečnom vrednosti od 80.78 po prajmer kombinaciji. Ovi SSR marker podaci su dalje analizirani pomoću klaster analize i analize glavnih koordinata (PCoA). Dobijeni rezulatiti ukazuju na značajno variranje koje je ustanovljeno među analiziranim genotipovima. Klaster analiza na osnovu SSR markera bi mogla omogućiti da se identifikuju pretpostavljeni tipovi pedigrea prisutni kod uzoraka crvene dateline različitog porekla.

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