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Original scientific paper

GENETIC DIVERSITY OF RED CLOVER CULTIVARS (*TRIFOLIUM PRATENSE* L.) BASED ON PROTEIN POLIMORPHISM

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Red clover is the second most important perennial forage legume. Based on morphological characters alone, it is difficult to distinguish accessories of red clover from each other because they have overlapping variations in terms of the major delimiting morphological and biological characters. The aim of this study was to analyze the genetic relationships of

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32 red clover cultivars collected from European gene banks, as well as created in Serbia, based on seed storage proteins and isozymes.
From 8 analyzed enzymic systems, there were no differences in zymograms for six enzymes only two enzymes were polymorphic. Two different allelic variants were found for enzyme shikimate dehydrogenase and three for phosphohexose isomeraze.

The seed proteins in the area of higher molecular high weight, 55-95 kDa, were identified as polimorphic. All the cultivars were placed into three clusters on the basis of Ward's distance range. The cluster pattern showed correlation between genetic diversity and geographic origin.

Genetic distance between cultivars based on protein fingerprint could be used as a tool to control and protect intellectual property right over plant breeding material.

Key words: breeding, genotypes, popping maize, sweet maize, white-seeded maize

INTRODUCTION

Red clover is the second most important perennial forage legume in Republic of Serbia, where it is planted on about 120.000 ha. The domestic market is predominated by cultivars developed in domestic research centers (Institute for Forage Crops, Kruševac, and Institute of Field and Vegetable Crops, Novi Sad). It is a cross-pollinated diploid (2n = 2x = 14) species with a gametophytic self-incompatibility system. The red clover populations are heterogeneous and showed high levels of genetic variation within and among populations (VASILJEVIĆ *et al*., 2000). The populations are represented by various biotypes that differ a lot in terms of productivity and the type of development, which is indicative of their great potential if used as starting materials for breeding (VASILJEVIĆ *et al.,* 2001).
Characteristically, legume seeds have large protein contents, ranging from 20% to as much as 40% of their dry matter, according to species, genotypes within species, and environments (BAUDOIN, 1991). The major storage proteins in legume seeds are the globulins which usually account for about 70% of the total protein. Glutelins (10–20%) and albumins (10–20%) make up the remainder. The principal storage globulins in most legumes are legumin and vicilin, the latter predominating in common bean (JANSMAN, 1996).

As specific gene products, proteins could indicate the genetic specificity of tested plant material, and therefore could be used as markers for characterization of varieties, for seed purity testing, or to resolve taxonomic relationships (NIKOLIĆ *et al*., 2007a, b; DRINIĆ-MLADENOVIĆ and KONSTANTINOV, 2001; NIKOLIĆ *et al*., 2008). They are stabile, uniform, reliable, reproducible and largely independent of environmental fluctuations (PANIGRAHI *et al*., 2007).

Isozymes have been used in taxonomic, genetic, evolutionary and ecological studies, cultivar and lines identification (ZLOKOLICA *et a, l.1996* a, b; LANGE and SCHIFINO-WITTMANN, 2000).

The aim of this study was to analyze the genetic diversity of red clover cultivars collected from European gene banks, as well as created in Serbia, based on seed storage proteins and isozymes.

MATERIALS AND METHODS

Seeds from thirty two red clover cultivars, from European gene banks (Leibniz Institute of Plant Genetics and Crop Plant Research - IPK, Germany, Nordic Gene Bank –NGB, Sweden, Departement plantengenetica en Veredeling – DvP, Belgium) and Serbian cultivars (Institute of Field and Vegetable Crops – IFVC, Novi Sad and Institute for Forage Crops – IFC, Kruševac) were collected and examined in the present study (Table 1).

Table 1. List of cultivars, origin, and ploidy

No.	Cultivar name	Institution/country of origin	Ploidy
1.	Kolubara	IFVC, Serbia	2n
2.	Una	IFVC, Serbia	2n
3.	Avala	IFVC, Serbia	2n
4.	Marino	IPK, Germany	2n
5.	Renova	IPK- Switzerland	2n
6.	Titus	IPK- Germany	4n
7.	Temara	IPK- Switzerland	4n
8.	Rotra	IPK- BELgium	4n
9.	Barfiola	IPK- Netherlands	4n
10.	Kora	IPK- Sweden	2n
11.	Vivi	NGB- Sweden	4n
12.	Silva	NGB- Sweden	4n
13.	Lucrum	IPK- Germany	2n
14.	Noe	IPK- France	2n
15.	Merviot	DvP- Belgium	2n
16.	Violetta	IPK- Belgium	2n
17.	Alpille	IPK- France	2n
18.	Britta	IPK- Sweden	2n
19.	Krano	IPK- Denmark	2n
20.	Sabtoron	IPK- Great Britain	2n
21.	Triton	IPK- Germany	4n
22.	Lutea	IPK- Germany	2n
23.	Bjorn	IPK- Sweden	2n
24.	K2	IFVC, Serbia	2n
25.	P1	IFVC, Serbia	2n
26.	P2	IFVC, Serbia	2n
27.	P3	IFVC, Serbia	2n
28.	P4	IFVC, Serbia	2n
29.	P5	IFVC, Serbia	2n
30.	P6	IFVC, Serbia	2n
31.	P7	IFVC, Serbia	2n
32.	K17	IFC, Serbia	2n

SDS PAGE electrophoresis

The bulked seeds of each accession were ground into fine powder. The protein content in the seed extracts was determined by the method described by BRADFORD (1976) using bovine serum albumin (96%, Sigma Chemical Co, St. Louis, MO, USA), as standard.

Fifty µl of the extract were mixed with 50 µl of SDS-sample buffer (0.15 M TRIS-HCl, pH 6.8, 3% w/v SDS, 5% v/v β-mercaptoethanol, 7% v/v glycerol and 0.03% Bromphenol Blue) and heated for 3 min in a boiling water bath. Solution was cooled to the room temperature and 12 µl of the sample was loaded onto each well.

Electrophoresis SDS-PAGE was carried out according to the procedures of Laemmli (LAEMMLI, 1970) in 1.5 mm thick gels with 12 % (w/v) separating gel and 5% (w/v) stacking gel in a vertical electrophoreses unit (Carl Roth, Germany).
SDS-PAGE was carried out at 90 mA per gel for 4 hours. After electrophoresis, the gels were stained for 2 h using 0.1% (w/v) Coomassie Brilliant Blue R-250. After staining, the gels were distained using a 10% (v/v) acetic acid solution until a clear background was achieved. A Page ruler presteined protein ladder (10-170 kDa) (Fermenats, Lithuania) was used as protein molecular weight ladder.

Analysis of isozymes

Stem tissues of 5 days old seedling homogenized in 50mMTrisHCl, pH 6, 8 in which 1% mercaptoetanol was added, was used for analysis. Isozyme systems: malate dehydrogenase (MDH), malic enzyme (ME), phosphohexose isomerase (PHI), phosphoglucomutase (PGM), shikimate dehydrogenase (SKDH), isocitrate dehydrogenase (IDH), acide phosphatase (ACP) and beta glucozidaze (ßGLU), were analyzed according to STUBER *et al*. (1988).

Statistical analysis

To generate a binary matrix for SDS PAGE data, the presence or absence of a band of each cultivar was marked as 1 or 0. The Ward's distance between each cultivar was calculated and the cultivars were clustered based on the sum of square variance of Ward's distances with the DPS2000 software (CHEN *et al.,* 2007).

RESULTS AND DISCUSSION

Based on morphological characters alone, it is difficult to distinquish accessories of red clover from each other because they have overlapping variations in terms of the major delimiting morphological and biological characters such as stem length, stem thickness, number of internodes, length and width of central lamina of medial leaflet, shape of of medial leaflet, color of leaf in the year of sowing, intensity of white marks on the leaftime of flowering, growth habit, yield of green mass per plant, yield of dry matter (VASILJEVIĆ *et al.,* 2007).

In this work the protein polimorphism of domestic cultivars and genotypes (Kolubara, Una, Avala, K2, P1, P2, P3, P4, P5, P6, P7) and European cultivars (Marino, Renova, Titus, Temara, Rotra, Barfiola etc.), which have been used as

standard for registration and protection of new cultivars according UPOV procedure, were analysed.

From 8 analyzed enzymic systems, there were no differences in zymograms for six enzymes only two enzymes, PHI and SKDH, were polymorphic. Two different allelic variants were found for enzyme shikimate dehydrogenase and three for phosphohexose isomeraze (Figure 1). The cultivars examined in this study represent the most red clover cultivars originated from Europe. The occurrence of dissimilar isozyme phenotypes among individuals of the same cultivars represents intravarietal variation (BAILEY, 1983). Using isozymes as genetic markers for cultivar identification in red clover would not be as straightforward as in homogeneous, selfing species because of high variation within cultivars.

Figure 1. Zimogram observed for phosphohexose isomerase within and between red clover cultivars

An extensive study on diversity of North American red clover cultivars, conducted by YU *et al*. (2001) showed that the most of the genetic diversity in red clover cultivars is within the cultivars and the isozyme variability in the 34 cultivars studied could be represented by a few cultivars of each of four groups. However, several European cultivars could not be distinguished using these isozymes, which is in accordance with our results. XIE and MOSJIDIS (2001) concluded that the low level of polymorphic was due to the narrow gene base of the populations from which the plants used in the crosses were derived and also it may be due to a sampling effect.

The high genetic diversity measured by isozymes at the species level and nearly twice as much variability among the wild populations as among the cultivars or landraces included in the core subset of red clover (MOSJIDIS and KLINGLER, 2006).

GUSTINE *et al*. (2002) reported genetic similarities among eight white clover populations, which might have indicated a common European origin. The analysis based on isozyme of 25 species of the genus Trifolium, represented by 134 accessions, showed similarity (MALAVIYA *et al.,* 2008).

Electrophoretic spectra of seed storage proteins

Genetic diversity among 32 red clover cultivars was investigated based on seed protein profiles produced by SDS-PAGE electrophoresis. The proteins in the area of higher molecular high weight, 55-95 kDa, were identified as polimorphic (Figure 2).

All the cultivars were placed into three clusters on the basis of Ward's distance range, and each cluster was further divided into 2 subclusters (Figure 3).

Almost all Serbian cultivars were collected in one cluster, Kolubra, Avala and Una were grouped in subcluster with Lutea at a Ward's distance of 1.49. Previous research based on morphological-biological characteristics according to UPOV protocol has been shown similarities of Serbian cultivars (VASILJEVIC *et al.,* 2006).

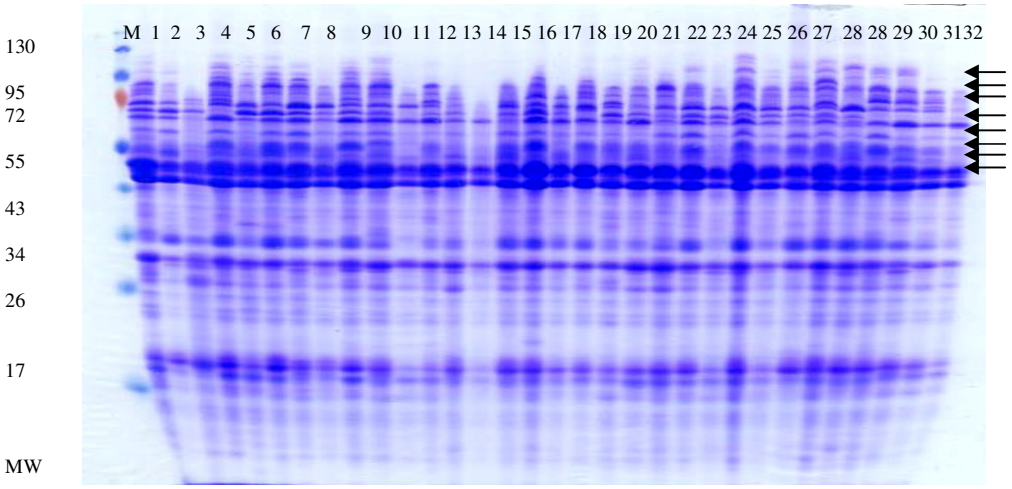

Figure 2. SDS PAGE of seed storage proteins of 32 red clover cultivars

The other subcluster were consist of genotypes labeled as P1, P2, P3, P4 and P7 which were similar with Renova, Britta and Crano, while K2 showed similarity with Violetta and Alpille, at distance of 1.12.

In the second clusted dominated tetraploid red clover cultivars from Northern Europe (Rotra, Barfiola, Vivi, Silva, and Triton) at distance at 1.16. The Serbian cultivar K17 showed similarity with Lucrum, Sabtoron, Bjorn. The cultivars Titus, Temara and Merviot were gruped in the third cluster, at distance of 1.21. The genotypes P5 and P6 were at genetic distance at 1.86 from all other cultivars. We have found that the cluster pattern showed correlation between genetic diversity and geographic origin.

Genetic distances, based on protein and RAPD markers were similar and in concurrence with the date on the origin of maize inbred lines (SRDIĆ *et al.,* 2007).

Obtained results are in agreement with GHAFOOR *et al.* (2002), who suggested the usefulness of SDS-PAGE specific bands for identifying red clover from mixed germplasm. Seed protein profile have been used to resolve phylogenetic relationships of different pepper varieties (ZEČEVIĆ *et al.,* 2000) or for the rapid classification of parents and confirmation of hybridization of their crosses (NAVEED *et al*., 2005), or to determining sectional, sub-sectional and delimitation of 20 species of *Trifolium* (BADR, 1995).

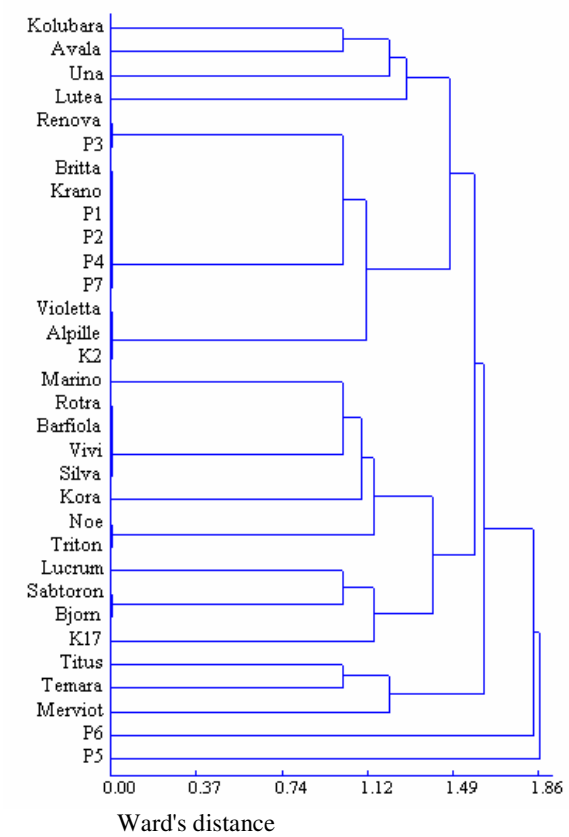

Figure 3. Dendogram based on SDS PAGE of 32 accessions of red clover by Ward's method

GREENE *et al.* (2004) reported no significant correlation between morphological characters and geographic distance and either morphological distance or RAPD marker distance.

For some crops, however, biochemical methods have not proven sufficiently discriminative. Due to their low polymorphism levels seed storage proteins and isozymes cannot be used for genetic identification of sunflower hybrids (NIKOLIĆ *et al.*, 2008). In the plant with a narrow genetic base in their pool, such as soybean, protein markers may not be sufficient for characterization and study of genetic diversity (NIKOLIĆ *et al.*, 2005).

In conclusion, electrophoresis (SDS-PAGE) of seed storage proteins can be economically used to assess genetic variation and relation in germplasm. It is

suggested that genotypes with similar banding patterns should be further characterized by 2-D electrophoresis.

Genetic distances between plants based on different types of fingerprint could be used, in cases where morphological comparisons can not distinguish cultivars, as a tool to control and protect intellectual property right over plant breeding material.

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GENETIČKA DIVERGENTNOST SORTI CRVENE DETELINE (*TRIFOLIUM PRATENSE* L.) NA OSNOVU PROTEINSKOG POLIMORFIZMA

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I z v o d

Crvena detelina je druga po značaju višegodišnja krmna leguminoza. U radu je proučavan proteinski polimorfizam sorti crvene deteline iz Srbije u poređenju sa sortama iz zapadne i severne Evrope koje se koriste kao standardni u procesu registracije i zaštite sorti poljoprivrednog bilja prema protokolu UPOV organizacije. Metodom horizontalne elektroforeze, na 11% skrobnom gelu, analizirani su enzimski sistemi: kisela fosfataza (ACP), šikimik dehidrogenaza (ShDH), malik enzim (ME), beta glukozidaza (ßGLU), izocitrat dehidrogenaza (IDH), malat dehidrogenaza (MDH), fosfoglukozo izomeraza (PGM), fosfoheksozo izomeraza (PHI). Polimorfizam proteina semena analiziran je 12% SDS PAGE elektroforezom iz homogenizovanog uzorka semena. Enzim PHI je imao tri, ShDH dve alelne varijante, dok su ostali enzimi bili monomorfni. Proteini semena su polimorfni, naročito proteini velikih molekulskih masa u opsegu 50-80 kDa. Multivarijacionom analizom (Ward's distance range) izdvojena su tri klastera, među kojima se posebno izdvaja podgrupa koja obuhvata NS sorte crvene deteline, što ukazuje na srodnost materijala koji je korišćen u stvaranju ovih sorti. U odnosu na nivo ploidnosti takođe se izdvaja zaseban podklaster u kojem dominiraju tetraploidne forme crvene deteline sa severa Evrope.

Polimorfizam proteina semena sorti crvene deteline bi mogao da se koristi kao dopunski pokazatelj u procesu njihove registracije i zaštite.

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