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Original scientific paper

GENODIVERSITY OF DOMINANT *Rhizobium leguminosarum* bv. *trifolii* ISOLATED FROM 11 TYPES OF SOIL IN SERBIA

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Rhizobium leguminosarum bv. *trifolii* is microsymbiont *Trifolium pratense* and *Trifolium repens,* which are very important legumes in Serbia. The natural nodulating population of those bacteria was collected and estimated biodiversity distribution by monitoring dominant genotypes of these bacteria.

The population of *Rhizobium leguminosarum* bv. *trifolii* were colected from 50 marked locations of 11 types of soil in Serbia. 437 natural isolates, rescued from nodules of *Trifolium repens* or *Trifolium pratense*, were analysed by phenotypic approache. We obtained 156 different isolates on the basis of differences in their IAR - intrinsic antibiotic resistance (five antibiotics) and HMT- heavy methal tolerance (five heavy metals). We investigated 56 dominant isolates with more than

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three differences in IAR-HMT patterns by REP-PCR and RAPD fingerprinting (AP10 and SPH 1 primers). The results showed genodiversity of dominant *Rhizobium leguminosarum* bv. *trifolii* field isolates and offered the possibility to assess their changes on marked locations during time and under different environmental conditions and geographical distribution.

Key words: genodiversity of *Rhizobium leguminosarum* bv. *trifolii* indigenous population *Rhizobium leguminosarum* bv. *trifolii,* IAR, HMT RAPD fingerprinting, REP-PCR

INTRODUCTION

Bacteria grouped within the *Rhizobiaceae*, *Phyllobacteriaceae*, and *Bradyrhizobiaceae* families, collectively known as rhizobia, are able to establish nitrogen-fixing symbiosis with leguminous plants. Many of these organisms contain complex genomes, with one chromosome and one or more large plasmids ranging in size from 100 kb to two Mb. Rhizobia are difficult to isolate directly from the soil or rhizosphere. They are often isolated by virtue of their ability to modulate specific legumes *(*HARRISON *et al.* 1993). This ability provides rhizobia with the capacity to exploit a very exclusive ecological niche and some important advantages over a strictly saprophytic lifestyle.

Diversity of rhizobia are investigated by phenotypic and genotyping approache. The standard microbial techniques are supplemented with DNA-based typing methods to subdivide species into distinct types. Various methods based on the PCR have been proposed to characterize *Rhizobium* strains and to examine genetic relationships in *Rhizobium* groups. Direct sequencing of genes coding for 16S rRNA (16S rDNA) amplified by PCR (BOTH *et al.* 1991, EARDLY *et al*. 1992, OYAIZU *et al.* 1992, YOUNG *et al*. 1991) and RFLP analysis of these PCR amplified sequences (LAGUERRE *et al*. 1994) have been used to establish genetic relationships and to characterize *Rhizobium* strains at the species and higher levels. Sequences of 16S rDNA are known as highly conserved among eubacteria, and analysis of genetic variations in this region is not appropriate to differentiate strains within rhizobial species. In recent years the repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC)-PCR method and random amplified polymorphic DNA (RAPD) fingerprinting have been applied to taxonomic grouping of bacteria, including enterobacteria (VERSALOVIC *et al*. 1991, COCCONCELLI *et al*. 1995), lactic acid bacteria (WARD *et al.* 1999), and rhizobia (DE BRUIJN 1992, DOOLEY *et al.* 1993). Random primers have been proposed to fingerprint *Rhizobium* strains (COUTINHO *et al.* 1992, DOOLEY *et al.* 1993, HARRISON *et al.* 1993).

In this study, we investigated diversity of indigenous bacterial isolates obtained from nodules of *Rhizobium leguminosarum* bv. *trifolii* using the IAR-HMT sensitivity test, REP-PCR and RAPD methods.

MATERIAL AND METHODS

The population of *Rhizobium leguminosarum* bv. *trifolii* were colected from 50 marked locations of 11 types of soil in Serbia: 80 isolates from chernozem, 27 from hydromorphic black soil, 92 from pseudogley, 35 from sandy soil, 69 from eutric cambisol, 26 from smonitza, 26 from fluvisol, 37 from hipogley mineral, 18 from and 27 from different atypical soil type, to investigate the diversity level of rhizobial population. Those 437 natural isolates were rescued from nodules of *Trifolium repens* or *Trifolium pratense* according VINCENT (1970). Isolates were analysed by phenotypic approache using IAR and HMT methods. Five antibiotics (concentracions in μg/ml): Tet (3); Sm (4 i 5); Amp (30 i 40); Chl (40 i 50) and Gen (3 i 5) and five heavy metals (concentracions in μg/ml): Hg (2 and 3); Zn (100 and 120); Co (35 and 40); Ni (15 and 25); Cd(3) were added in YMA medium and 437 natural isolates inoculated in triplicate using 48 well replicator. The results were scored as binary results and compared.

Total DNA extraction and bacterial cell preparation for PCR amplification were done as previously described (CHEN and KUO, 1993). The PCRs were carried out with 50ng of pure genomic DNA. PCRs with random primers SPH1 and AP10 and REP primers were performed as described by DOOLEY *et al.* (1993), SELENSKA-POBEL *et al.* (1996), and DE BRUIJN (1992), respectively. All reaction mixtures were sealed with a thin layer of paraffin oil. RAPD primers were purchased from Sigma Genosys, *Taq* Gold polymerase and dNTP from Appllied Biosystems. PCR amplifications were performed by using a thermal cycler (Eppendorf Master Cycler Perssonel) with the following standard temperature profile for each primer as described in table 1. Electrophoresis was carried out on a horizontal 1.5% agarose gel in TBE electrophoresis buffer (89mM Tris-HCl, 89mM boric acid, 2.5 mM EDTA [pH 8.2]) at a constant voltage of 100V. The gels were stained with ethidium bromide and photographed under UV illumination. KiloBase DNA Marker (Amerscham Bioscience) and Gibco BRL 1kb plus marker were used. Similarity level between isolates was estimated by the score for the presence or absence of bands to derive simple matching coefficients (SNEATH and SOKAL, 1973) across fingerprint patterns of the isolates. Results were processed in Stat5 and NTSYS-pc programs. Matrices of simple matching coefficients were used in clustering analyses using the unweighted pair group arithmetic average method.

Table1. Oligonucleotid sequences of primers and amplification conditions

Primer	Oligonucleotid sequences	Denaturation	Anilling	Primer extension	No of cyclus
AP 10	5'-CAGGCCCTTC-3'	95°C 5min			1
		94°C 1min	36°C	72°C 2min	45
			1min	72°C 5min	1
SPH 1	5'-$(GAC)_5$ -3'	94°C 3min			1
		94°C 1min	36°C	72°C 2min	40
			1min	72°C 5min	1
REP IR-I	5'-IIIICGICGICATCIGGC-3'	95°C 2min.			1
		94°C 1min	40°C	65°C 8min	30
REP 2-I	5'-CGICTTATCIGGCCTAC-3'		1min	65°C16min	1

RESULTS AND DISCUSION

Using IAR-HMT sensitive patterns to estimate diversity of indigenous 437 isolates in natural population of rhizobia we compared data by cluster analysis and obtained 156 different patterns and frequency of the same isolates. The dendrogram of dominant isolates based on IAR-HMT pattern is shown in Figure 1.

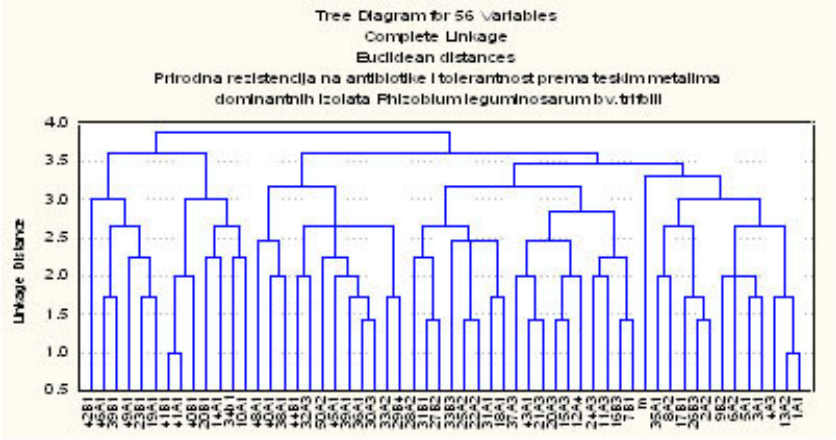
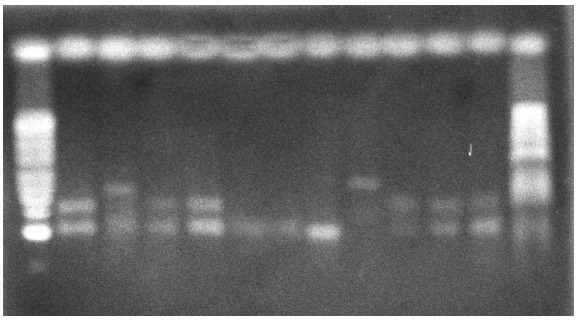


Figure 1. Dendrogram of dominant *Rhizobium leguminosarum* bv. *trifolii* isolates derived from IAR-HMT data.

Isolates from hydromorphic black soil was more sensitive on Hg, Chl and Sm then others isolates, but less sensitive and very tolerant on Co (Table 2.). Isolates from pseudogley was very tolerant on Ni 25 mg/ml, more than isolates from other soil types, but very sensitive on Gen. All isolates exhibited uniformly low level of tolerance to nickel (25mg/ml). The pattern of antibiotic resistance of *Rhizobium* isolates was a stable trait by which rhizobia could be recognized. IAR markers have been successfully used for strain differentiation as already reported (SHISHIDO and PEPPER, 1990; MOAWAD *et al*., 1998).

DNAs prepared from pure cultures of all isolates obtained from samples taken at different location were amplified by REP-PCR (Fig. 2.). Based on the amplification profiles, the bacterial strains were divided into four groups. Most of the isolates produced REP bands at 1000bp, since band at 1350bp showed four isolates, and band at 1500bp tree of them. Dendrogram of dominant *Rhizobium leguminosarum* bv. *trifolii* isolates derived from REP-PCR profiles is shown on Figure 3. Genetic distance ranged from 0 to 0, 57.



1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 2. Genodiversity of dominant isolates Rhizobium *leguminosarum* bv. *trifolii* on the basis of REP -PCR pattern. Isolates: 1. 1Kb plus marker Gibco BRL 2. *46A1*, 3. *44B1*, 4. *40A1*, 5. *3A1*, 6. and 7. *7B1*, 8. *9B2*, 9. *38A1*, 10. *28A2*, 11. *15A3*, 12. *19A1*, 13. Kb marker (Am.Biosc.)

The strains were further examined by PCR DNA fingerprinting using two different oligonucleotides as a single random primer, SPH1 and AP10. On average, the PCR products obtained with RAPD analysis primer SPH 1 generated fingerprints consisting fragments that varied in length from 440 to 1500bp (Figure 4.). PCR of representative isolates yielded multiple DNA products of molecular sizes ranging from 160 to 1200bp with AP10 primer (Figure 5.). Dendrogram of dominant *Rhizobium leguminosarum* bv. *trifolii* isolates derived from RAPD profiles generated SPH1 and AP10 primers is shown on Figure 6. Genetic distance ranged from 0 (for 15A3 and 19A1) to 0,57 (for 9B2 and 40A1; for 38A1 and 40A1).

Table 2. IAR-HMT of dominant R. leguminosarum bv. trifolii isolates

Soil type	tm/ant / μg/ml / Izolat	Hg		Zn		Co		Ni		Cd	Tet	Sm		Amp		Chl		Gen	
		2	3	100	120	35	40	15	25	20	3	4	5	30	40	40	50	3	5
chernozem	1A1	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	-	+	-
	2A2	+	-	-	-	+	+	+	-	+	+	+	+	-	-	+	-	+	-
	4A3	+	-	+	+	+	+	+	-	+	-	-	-	+	+	+	+	+	+
	5A1	+	-	-	-	+	+	+	-	+	-	-	-	+	+	+	+	+	-
	6A2	-	-	+	-	+	+	+	-	+	-	-	-	+	+	-	-	+	-
	7B1	+	-	+	+	+	-	+	-	+	-	+	-	+	+	-	-	-	-
	8A2	+	-	-	-	+	+	-	-	+	+	-	-	+	+	+	-	+	-
	10A1	-	-	+	+	-	-	+	+	-	+	+	+	+	-	+	+	+	-
	16B3	+	-	+	+	+	-	+	+	+	-	+	-	+	-	-	-	-	-
hydromorphic black soil	9B2	+	-	-	-	+	+	+	-	+	-	+	+	+	+	-	-	+	-
	11A3	+	-	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-	-
	12A4	+	-	+	-	+	-	+	+	+	+	-	-	+	+	-	-	+	-
sandy soil	13A2	+	-	+	+	+	+	+	-	-	-	-	-	+	+	+	-	+	-
	14A1	+	-	+	+	-	-	+	+	-	-	+	+	-	-	-	-	-	-
	15A3	+	-	+	+	+	-	+	-	-	+	-	-	+	+	+	-	+	-
	35A1	+	-	-	-	+	-	-	-	+	+	-	-	-	-	+	+	+	-
eugley vertic	46A1	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-
	48A1	-	-	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	-
cutric cambisol	17B1	+	-	+	-	+	+	+	+	+	+	+	-	-	-	+	-	+	-
	18A1	+	-	-	-	+	-	-	-	-	+	+	+	+	+	+	-	-	-
	19A1	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	-	+	-
	20A3	+	-	+	+	+	-	+	+	-	+	-	-	+	+	+	+	+	-
	20B1	+	-	+	+	-	-	-	-	-	-	+	+	-	-	+	-	+	+
	23B1	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	-	-
	25A2	+	-	+	+	-	-	-	-	-	+	-	-	+	+	+	-	+	-
	26B3	+	-	+	-	+	+	+	-	+	+	+	+	-	-	+	-	+	+
	27B2	-	-	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-
smonitza	28A2	-	-	+	+	+	-	-	-	-	-	+	-	+	+	-	-	-	-
	29B4	-	-	-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-
	45A1	-	-	-	-	+	-	+	+	-	+	+	-	-	-	+	-	-	-
pseudogley	21A3	+	-	+	+	+	+	+	+	+	-	-	-	+	-	+	-	-	-
	22A2	+	-	+	-	-	-	-	-	-	+	-	-	+	+	+	-	-	-
	31A1	+	-	+	+	+	-	-	-	-	+	+	-	+	+	+	-	-	-
	31B1	-	-	+	+	+	+	+	-	-	+	+	-	+	+	+	-	+	-
	32A3	+	-	+	-	+	+	+	+	+	+	+	+	+	-	+	+	-	-
	33A2	-	-	+	-	+	+	+	-	+	+	+	-	+	-	+	-	-	-
	33B3	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	+	-
	34B1	+	-	-	-	-	-	+	+	-	+	+	+	-	-	+	-	+	-
	36A1	+	-	-	-	+	+	+	+	-	+	+	-	-	-	-	-	-	-
	41A1	-	-	-	-	+	+	+	-	-	-	+	+	-	-	+	-	-	-
	41B1	-	-	-	-	+	+	+	-	-	+	+	+	-	-	+	-	-	-
	42B1	-	-	+	-	-	-	+	+	+	+	-	-	+	-	-	-	+	+
	44B1	+	-	-	-	-	+	+	+	+	+	-	-	+	-	+	-	-	-
fluvisol	24A3	+	-	+	+	-	-	+	-	-	-	-	-	+	-	-	-	+	-
	39A1	+	+	-	-	+	+	+	-	-	+	+	-	-	-	+	-	-	-
	39B1	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
	40A1	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	-	-
	40B1	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-
hipogley mineral	37A3	-	-	+	-	+	-	+	+	+	-	-	-	+	+	+	-	-	-
	38A1	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-
	49A1	-	-	-	-	-	-	+	-	+	+	-	-	+	+	-	-	-	-
	50A2	+	-	-	-	-	-	+	+	-	+	+	-	+	-	-	-	-	-
atypical soil type	3A1	+	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-	+	-
	30A3	+	-	-	-	+	+	+	+	-	-	+	-	-	-	+	-	-	-
	43A1	+	-	+	+	-	-	+	+	+	-	-	-	+	-	+	-	-	-

(-) no growth of bacteria; (+) good growth of bacteria

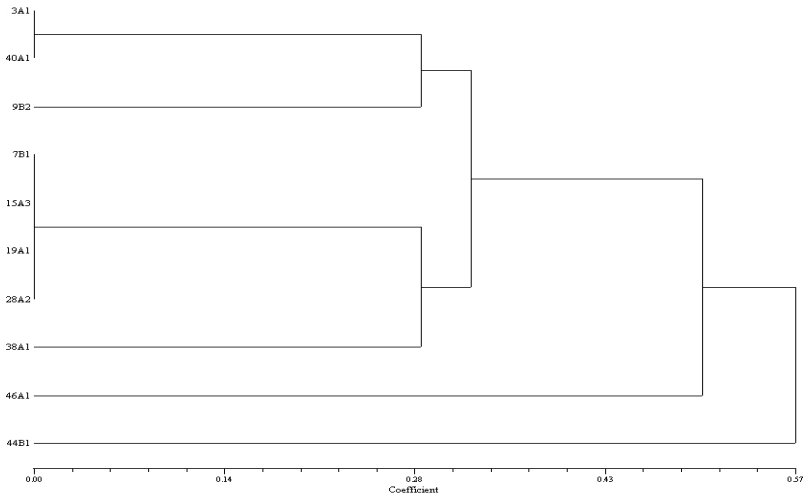
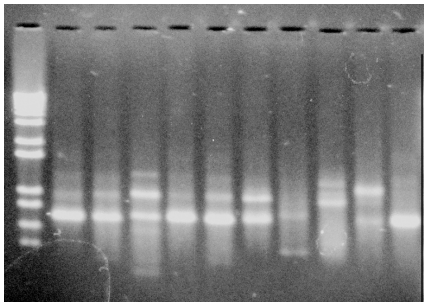


Figure 3. Dendrogram of dominant *Rhizobium leguminosarum* bv. *trifolii* isolates derived from REP-PCR profiles

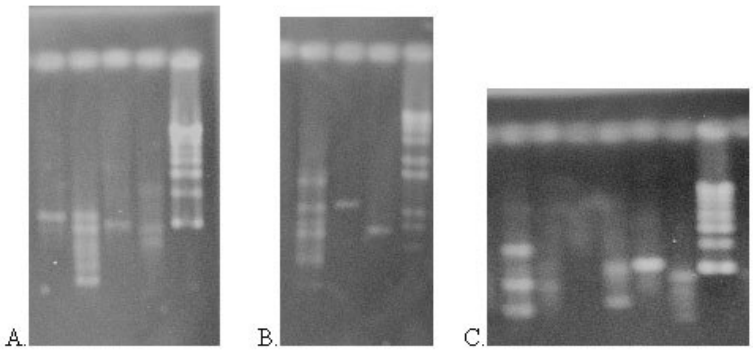
The results were in agreement with the classification of the strains into major REP and RAPD group (isolates: 15A3; 19A1; 28A2; 7B1), although isolates 41A1 and 44B1 were subdivided into distinct types according REP and, conversely, similar according RAPD. Our results were in agreement with previous published data. DE BRUIJN (1992) demonstrated the usefulness of DNA fingerprinting by PCR using REP and ERIC primers (REP- and ERIC-PCR) for the identification and classification of members of several *Rhizobium* species. These methods have been used to type several rhizobial strains with genetic distance similar to our results (JUDD *et al*. 1993; LEUNG *et al.* 1994).

HARRISON *et al.* (1992) have reported the use of RAPD for *Rhizobium leguminosarum* bv. *trifolii* direct from cell cultures and nodule tissue and recomended it for study of natural rhizobial populations. DOOLEY *et al.* 1993 proposed RAPD approach with SPH1 primer for identification and phylogenetic grouping of *Rhizobium* isolates in same species and same type – *R. leguminosarum*, but different biovar: *trifolii, phaseoli* and *viciae*. All amplified product for *Rhizobium leguminosarum* bv. *trifolii* were between 0,1 and 2 kb, which is in agreement with results for dominant isolates in Serbia (0,4 to 1,5 kb).



1 2 3 4 5 6 7 8 9 10 11

Figure 4.. Genodiversity of dominant isolates *Rhizobium leguminosarum* bv. *trifolii* on the basis of RAPD pattern derived by SPH1 primer. Isolates: 1. 1Kb plus marker Gibco BRL, 2. *3A1*, 3. *7B1*, 4. *9B2*, 5. *15A3*, 6. *19A1*, 7. *28A2*, 8. *38A1*, 9. *40A1*, 10. *44B1*, 11. *46A1*



1 2 3 4 5 1 2 3 4 1 2 3 4 5 6 7

Figure 5. Genodiversity of dominant isolates *Rhizobium leguminosarum* bv. *trifolii* on the basis of RAPD pattern derived by AP10 primer. Isolates: **A**. 1. *38A1,* 2. *40A1*, 3. *44B1*, 4. *46A1*, 5. Kb marker (Am.Biosc.) **B**. 1. *9B2*, 2./ 3. *3A1*, 4. 1Kb plus marker Gibco BRL **C**. 1. *7B1* 2. *15A3* 3./ 4. *28A2* 5. *19A1* 6. / 7. Kb marker (Am.Biosc.)

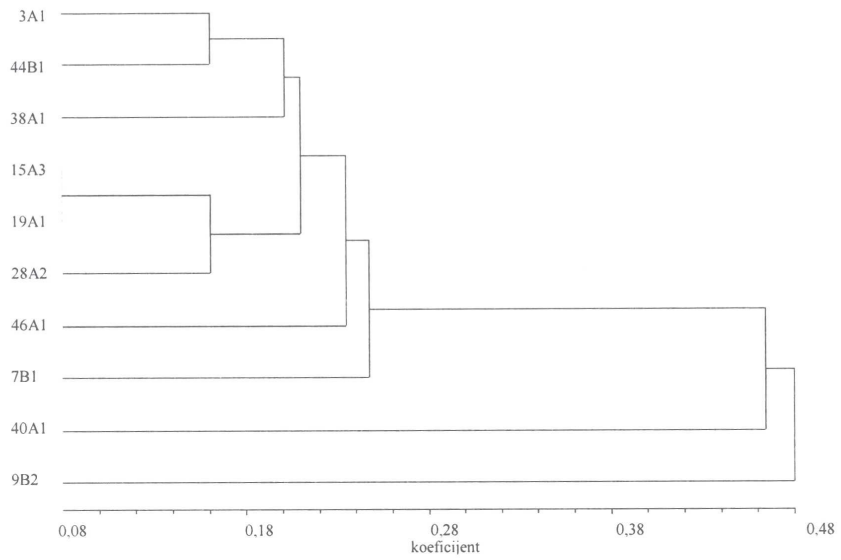


Figure 6. Dendrogram of dominant *Rhizobium leguminosarum* bv. *trifolii* isolates derived from RAPD profiles generated SPH1 and AP10 primers

The presented study showed high diversity level of indigenous dominant *Rhizobium leguminosarum* bv. *trifolii* isolates confirmed by PCR techniques and supplied information at the molecular level of isolates from different type of soils and geographic origin.

CONCLUSIONS

The results of the present study show the wide diversity of *R. l.* bv. *trifolii* field populations from 11 soil type with different IAR and HMT patterns. Different 156 isolates were obtained from investigated 437 isolates, and 56 of them were dominant.

REP and RAPD fingerprint patterns of *R.l.* bv. *trifolii* showed significant differences between isolates with similar IAR-HMT pattern. Those PCR methods are efficient means for rapidly typing a large number of strains and estimation of their diversity.

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GENODIVERZITET DOMINANTNIH *Rhizobium leguminosarum* bv. *trifolii* IZOLOVANIH IZ 11 TIPOVA ZEMLJIŠTA SRBIJE

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Rhizobium leguminosarum bv. *trifolii* je mikrosimbiont *Trifolium pratense* i *Trifolium repens,* veoma značajnih leguminoza u Srbiji. Kolekcionisana je prirodna populacija ovih simbiotskih azotofiksatora i procenjen nivo biodiverziteta analizom dominantnih izolata ovih bakterija.

Populacija *Rhizobium leguminosarum* bv. *trifolii* je prikupljena sa 50 markiranih lokacija sa 11 tipova zemljišta u Srbiji. 437 autohtonih izolata, izolovanih iz nodula *Trifolium repens* ili *Trifolium pratense*, analizirano je fenotpskim metodama. Dobijeno je 156 različitih izolata na osnovu razlika u osetljivosti na pet antibiotika (IAR - intrinsic antibiotic resistance) i tolerantnosti na pet teških metala (HMT- heavy methal tolerance). Utvrđeno je prisustvo 56 dominantnih izolata sa značajnim razlikama u IAR-HMT profilu i za reprezentativne izolate grupe urađen REP-PCR i RAPD fingerprinting (AP10 i SPH 1 prajmerima). Rezultati su pokazali visok stepen genodiverziteta dominantnih izolata *Rhizobium leguminosarum* bv. *trifolii* omogućili praćenje promena diverziteta i zastupljenosti na markiranim lokacijama tokom vremena i pri različitim uslovima životne sredine i geografske rasprostranjenosti.

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