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Genotypic Characterisation of Indigenous Soybean Rhizobia by PCR-RFLP of 16S rDNA, rep-PCR and RAPD Analysis

Sanja Sikora and Sulejman Redžepović*

Department of Microbiology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25, HR-10000 Zagreb, Croatia

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Summary

The taxonomy of nitrogen fixing bacteria that form symbiotic associations with leguminous plants has been deeply changed in recent years. The use of very sensitive and accurate molecular methods has enabled the detection of large rhizobial diversity, particularly among natural field populations of these soil bacteria. The aim of the present investigation was to identify and characterise the indigenous soybean rhizobia isolated from different soil types in eastern Croatia that are regularly used for agricultural production. The actual composition and genetic diversity of natural field population was studied by using different PCR fingerprinting methods such as 16S rDNA PCR-RFLP, rep-PCR and RAPD analysis. Eighteen rhizobial strains, isolated from soybean nodules, were characterised and compared with reference and/or type strains representing *Bradyrhizobium japonicum*, *B. elkanii* and *Sinorhizobium fredii*. Cluster analysis of combined RFLP patterns obtained with six restriction endonucleases revealed that all soybean isolates differ significantly from *B. elkanii* and *S. fredii* type strains, while they were closely related to *B. japonicum* type strains. However, a considerable level of genetic diversity was determined among *B. japonicum* isolates. PCR-RFLP of 16S rDNA clearly showed the existence of two divergent groups among indigenous bradyrhizobia. After identification at the species level, all isolates were further characterised by RAPD and rep-PCR. Both RAPD and rep-PCR generated highly specific and reproducible patterns that enabled accurate strain differentiation. Among *B. japonicum* strains a high level of diversity was found with these two fingerprinting methods. Dendrograms derived from RAPD, REP and ERIC profiles showed that all indigenous strains could be divided into three main groups. The grouping of strains was consistent with all methods used in this study.

Key words: nitrogen fixation, rhizobia, soybean, indigenous strains, *Bradyrhizobium japonicum*, DNA fingerprinting, genetic diversity

Introduction

Rhizobia are usually defined as nitrogen-fixing soil bacteria capable of inducing the formation of root or stem nodules on leguminous plants in which atmospheric nitrogen is reduced to ammonia for the benefit of the plant. Due to their considerable agricultural and environmental significance, these legume symbionts have been extensively studied. During the last years, the assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention (*1–4*). Many attempts have been made

* Corresponding author; Phone: ++385 (0) 1 23 93 880; Fax: ++385 (0) 1 23 93 881; E-mail: ssikora@agr.hr

to determine the actual composition and characteristics of indigenous strains isolated from different cultivated legumes (*5,6*), and also from less explored legumes like different shrubs and herbaceous plants that have important role in certain ecosystems (*7–10*). The development of numerous molecular genetic methods has greatly contributed to these investigations. The availability of several sensitive and accurate PCR-based genotyping methods (*11–13*) has enabled the differentiation among closely related bacterial strains and the detection of a higher rhizobial diversity than previously considered (*14–16*).

Consequently, the taxonomy of root- and stem-nodulating bacteria has been deeply changed in the recent years. The rhizobial species are currently classified in the following genera: *Allorhizobium* (emended genus *Rhizobium*), *Mesorhizobium*, *Rhizobium*, *Sinorhizobium*, *Azorhizobium*, *Bradyrhizobium* and *Methylobacterium* (*17–20*).

As a valuable source of proteins and oil, used both in human and livestock nutrition, soybean [Glycine max L. (Meril)] is one of the most important legume crops in the world. Therefore, the nitrogen fixing microsymbionts of soybean are of great agricultural value. Revisions and additions to the taxonomy of soybean rhizobia have also been made both at the generic and species level. In 1982, Jordan revised the taxonomy of symbiotic nitrogen-fixing bacteria (*21*). Soybean microsymbiont, previously named *Rhizobium japonicum*, was allocated to the new genus *Bradyrhizobium* as *Bradyrhizobium japonicum*. Accordingly, the slow growing, alkali producing strains were separated from the fast growing, acid producing rhizobia. Since then, other nitrogen-fixing bacteria able to nodulate soybean roots like *Bradyrhizobium elkanii* (*22*) and *Bradyrhizobium liaoningense* (*23*) have also been described. In 1988, the genus *Sinorhizobium* was proposed for the fast-growing soybean rhizobia (*24*), with one species named *Sinorhizobium fredii.* Currently, this genus includes several rhizobial species which nodulate other legumes (*20*).

It has been recognised that an important requirement for agronomically useful rhizobium-soybean associations is the ability of inoculant strains to compete with very diverse indigenous rhizobial strains. Therefore, in order to improve the beneficial effect of soybean inoculation it is important to determine the characteristics of rhizobial field populations. The main aim of the present study was to identify and characterise the indigenous soybean rhizobia isolated from the most important soybean growing areas in Croatia, and to assess genetic diversity of natural field population by using different PCR-based methods.

Materials and Methods

Soybean rhizobial isolates and reference strains

All rhizobial isolates were obtained from different field sites in eastern Slavonia, which is the most important soybean production area in Croatia. Soil samples were taken at a depth of 0–30 cm from different soil types that are regularly used for agricultural production. Under bacteriologically controlled conditions in the greenhouse, surface sterilised soybean seeds (cv. Hrvatica) were sown directly into the soil samples collected from this region. Rhizobial strains were isolated from surface sterilised nodules following a standard protocol (*25*).

All field isolates were compared with reference and/or type strains representing *B. japonicum* (USDA 110, USDA 6^T), *B. elkanii* (USDA 76^T) and *S. fredii* (USDA 205^T). Two *B. japonicum* strains from a commercial inoculant were also included in these investigations as reference strains.

The origin and designation of rhizobial isolates as well as reference and type strains used in this study are presented in Table 1.

DNA preparation

Total genomic DNAs from all strains were isolated using standard phenol-chloroform-isoamyl extraction and ethanol precipitation in the presence of sodium acetate (0.3 mol/L). The pellets were washed with 70 % ethanol, dried and redissolved in 150 μL of TE buffer. The concentration and purity of DNA were estimated spectrophotometrically at 260 and 280 nm, respectively.

PCR-RFLP analysis of 16S rDNA

The universal primers fD1 and rD1 were used for PCR amplification of 16S rDNA (*26*). Amplification reactions were performed in a 25 μL volume, containing: 20 mmol/L Tris-HCl (pH=8.4), 50 mmol/L KCl, 2.0 mmol/L $MgCl_2$, 200 μmol/L of dNTPs, 1 μmol/L of each primer, 30 ng of genomic DNA and 1.5 U of Taq DNA polymerase (Life Technologies, Switzerland). The temperature profile was as follows: initial denaturation at 95 °C for 3 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; and final extension at 72 °C for 3 min. Amplified PCR products were digested with restriction endonucleases *Hinf*I, *Msp*I, *Cfo*I, *Nde*II, *Dde*I, *Rsa*I (Roche, Germany), as recomended by the manufacturer. The digests were separated by submerged gel electrophoresis on precast 6 % poly(NAT) gels run in SEA 2000 apparatus (Elchrom Scientific AG, Switzerland) for 2.5 h at 7 V/cm and 20 °C. The restriction patterns were visualised under UV illumination after staining with ethidium bromide and photographed with Polaroid film type 667. A 20 bp ladder (GenSura Laboratories, CA) was used as molecular size marker.

RAPD and rep-PCR genomic fingerprinting

REP and ERIC fingerprinting were performed with primers REP 1R and REP 2I, and ERIC 1R and ERIC 2, respectively (*27*). Six arbitrarily chosen primers (P1-P6) used for RAPD fingerprinting were 10 nucleotides in length and had a GC content of 70 %. Primer sequences were as previously described (*28*).

For both fingerprinting methods, the PCR reaction mixture was the same as described above for PCR-RFLP analysis of 16S rDNA.

The cycling programs for RAPD and rep-PCR fingerprinting differed only in the annealing temperature and time. The reaction mixtures were incubated for 5 min at 95 °C for initial denaturation, and then amplified

for 35 cycles consisting of 30 s at 94 °C, 30 s at 36 °C (RAPD), 1 min at 40 °C (REP-PCR), or 30 s at 50 °C and 1 min at 52 °C (ERIC-PCR), and 1 min at 72 °C followed by a 7-min incubation at 72 °C.

The amplification products were separated by gel electrophoresis on precast 6 % poly(NAT) gels and visualised as described above.

Data analysis

All restriction patterns as well as RAPD and rep--PCR fingerprints were coded in the binary form, and analysed using NTSYS-pc package (*29*). A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

Results

RFLP analysis of PCR-amplified 16S rDNA

The 16S rDNA region of 18 field isolates and six reference strains (Table 1) was PCR amplified with universal primers rD1 and fD1. The PCR products were individually restricted with endonucleases *Msp*I, *Hinf*I, *Cfo*I, *Nde*II, *Rsa*I and *Dde*I. Although each restriction enzyme produced polymorphic banding patterns, the most discriminative were those obtained with *Msp*I, *Hinf*I and *Cfo*I. The RFLP patterns of 16S rDNA obtained with *Msp*I are shown in Fig. 1. A number of well separated, sharp bands up to 500 bp were detected, clearly showing differences between all isolates and the type strains of *S. fredii* as well as *B. elkanii*. Moreover, among field isolates slightly different restriction patterns were detected. Besides *Msp*I, other restriction enzymes also produced different RFLP patterns among soybean rhizobia (Table 1).

Cluster analysis of combined RFLP patterns revealed that all field isolates obtained from soybean nod-

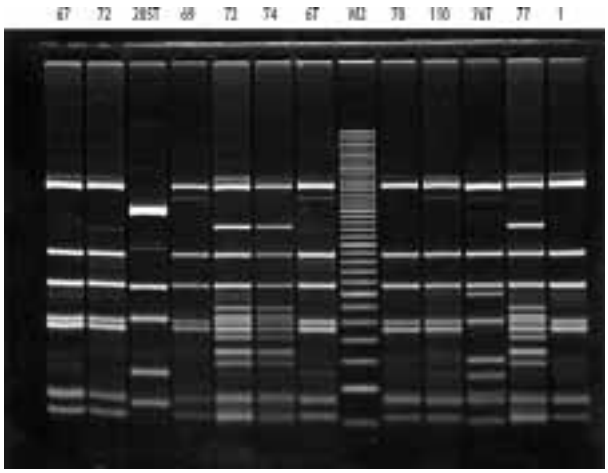

Fig. 1. Types of restriction patterns of PCR-amplified 16S rDNA digested with *Msp*I obtained with strains used in this study. Molecular size marker: M2, 20 bp ladder

Table 1. Origin, designation and 16S rDNA restriction patterns of the rhizobial isolates and reference strains used in this study

Location/Source	Isolation site	Reference/ type strain	Strain designation	16S rDNA pattern[1]
Osijek	PZC IPK, Čepin		66	aaaaaa
			67	abaabb
			68	aaaabb
Đakovo	Piškorevci		69	aaaabb
	Kešinci		70	aaaabb
	Slašćak		71	bcbbcc
	Drenje		72	bcbbcc
Našice	Lila		73	aaaabb
	Koška		74	bcbccc
	Budimci		75	bcbbcc
	Podgorač		76	aaaabb
			77	bcbbcc
			78/1	aaaaab
			78/2	aaaabb
Orahovica	Čačinci		79	aaaaaa
	Nelin Dvor		80	aacdbb
	Gutmanovci		81	bcdccc
	Zdenci		82	bcdbcc
Dpt. Microb.		*B. japonicum*	1	aaaabb
Fac. of Agric., Univ. of Zagreb			2	aaaebb
HAMBI Cult. Coll.		*B. japonicum*	USDA 6^T	aaaabb
Dpt. Microb., Univ. de La Laguna		*B. japonicum*	USDA 110	aaafab
DSMZ Cult. Coll.		*B. elkanii*	USDA 76^T	cdegdd
DSMZ Cult. Coll.		*S. fredii*	USDA 205^T	defhee

[1] Letters refer to the type of restriction pattern of 16S rDNA digested with *Hinf*I, *Msp*I, *Cfo*I, *Nde*II, *Dde*I and *Rsa*I

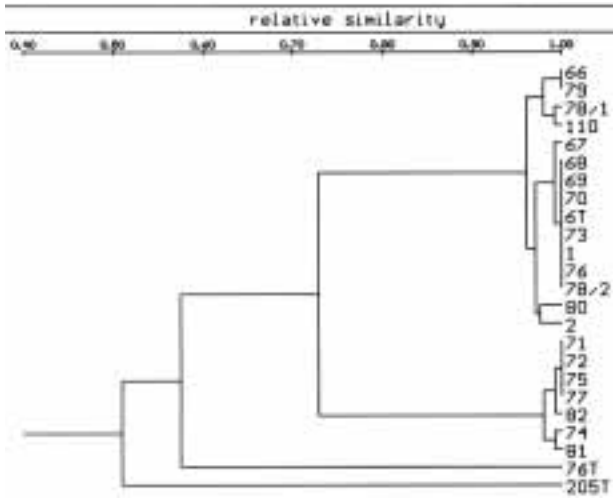

Fig. 2. Dendrogram of indigenous soybean rhizobia and representative strains of *Bradyrhizobium japonicum*, *B. elkanii* and *Sinorhizobium fredii* derived from combined *Msp*I, *Hinf*I, *Cfo*I, *Nde*II, *Dde*I, *Rsa*I restriction patterns of amplified 16S rDNA

characterised by extra slow growing rate and therefore, *B. liaoningense* was discarded from further investigation.

rep-PCR fingerprinting

Reproducible and specific banding patterns were obtained with both REP-PCR and ERIC-PCR (Figs. 3a and 3b). Sizes of the DNA fragments obtained by both fingerprinting methods were in a similar range (from 0.1 to 2.0 kb), while the total number of fragments depended on the primers used. In amplification reactions with all strains tested, the ERIC primer set produced, in all, 40 different fragments while the REP primer set yielded in total 63 fragments that were used for the construction of dendrogram. Thus, amplification reactions with both sets of primers generated a sufficient number of distinct polymorphic bands for reliable strain discrimination.

The dendrogram derived from REP-PCR fingerprints clearly showed the existence of three divergent groups among all the strains tested (Fig. 4a). The majority of soybean rhizobia were grouped within the first major cluster, while the second one comprised only

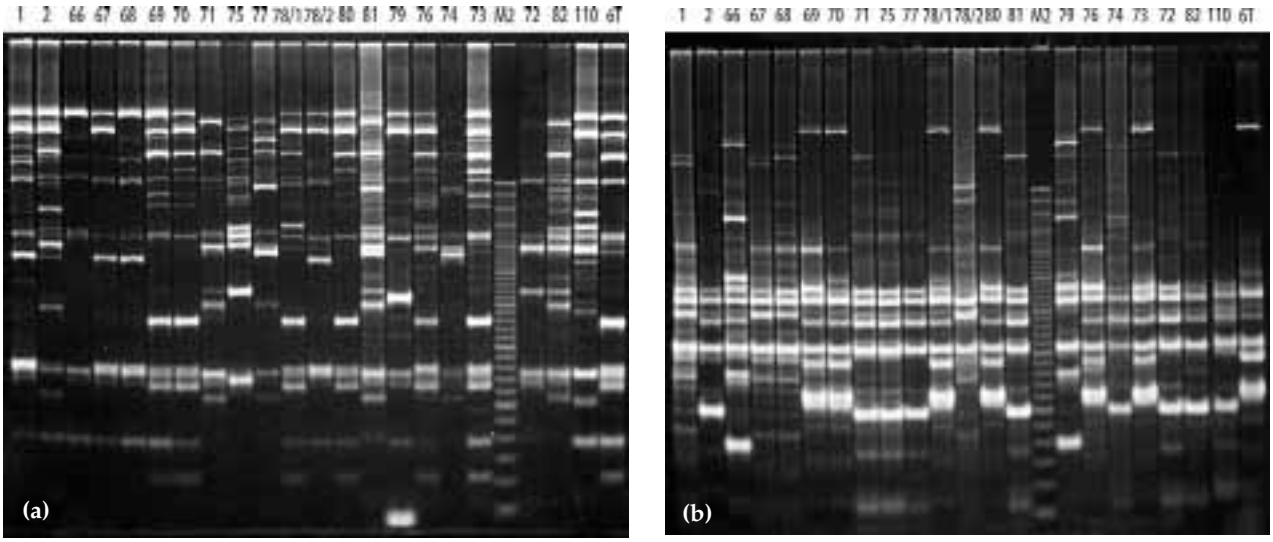

Fig. 3. REP (a) and ERIC (b) fingerprints of 18 field isolates and *Bradyrhizobium japonicum* reference strains. Molecular size marker: M2, 20 bp ladder.

ules significantly differed from representative strains of *S. fredii* (USDA 205^T) and *B. elkanii* (USDA 76^T), while they were closely related to the *B. japonicum* type strain (USDA 6^T). However, a considerable level of genetic diversity was also detected among *B. japonicum* isolates. The dendrogram derived from RFLP patterns clearly showed the existence of two divergent groups among indigenous bradyrhizobia (Fig. 2.) Most of the isolates were grouped together with *B. japonicum* reference and type strains into the first major cluster, whereas the second cluster consisted of only seven isolates, which differed from all other isolates at the similarity level of 0.73. *B. liaoningense* as one of the species that nodulate soybean, was also initially included in our investigation. The results revealed that 16S PCR-RFLP patterns of this species were identical to *B. japonicum* (data not shown). On the other hand, isolates used in this study were not

seven field isolates. The first cluster could be further subdivided into two groups that were related at a similarity level of 0.78. Two reference strains from commercial inoculant, strain USDA 110 and five isolates were clustered within the first subgroup, whereas the remaining isolates and *B. japonicum* type strain USDA 6^T formed another subgroup.

Cluster analysis of ERIC-PCR profiles resulted in a similar grouping of the strains (Fig. 4b). The only differences detected were in the position of the strain USDA 110 and the field isolates 66 and 79. The latter two isolates diverged from all other strains at a similarity level of 0.69, and they were separated from the main three groups of strains. However, slightly higher level of strain differentiation within the main groups was determined by REP-PCR as compared to ERIC-PCR.

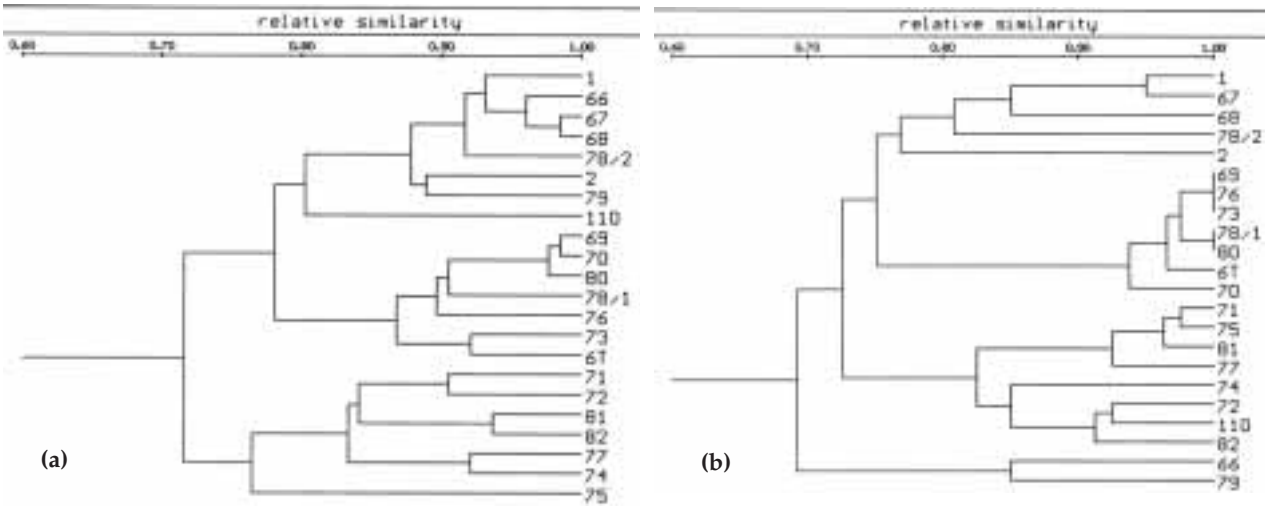

Fig. 4. Dendrograms of *B. japonicum* strains derived from REP-PCR fingerprints (a) and ERIC-PCR fingerprints (b)

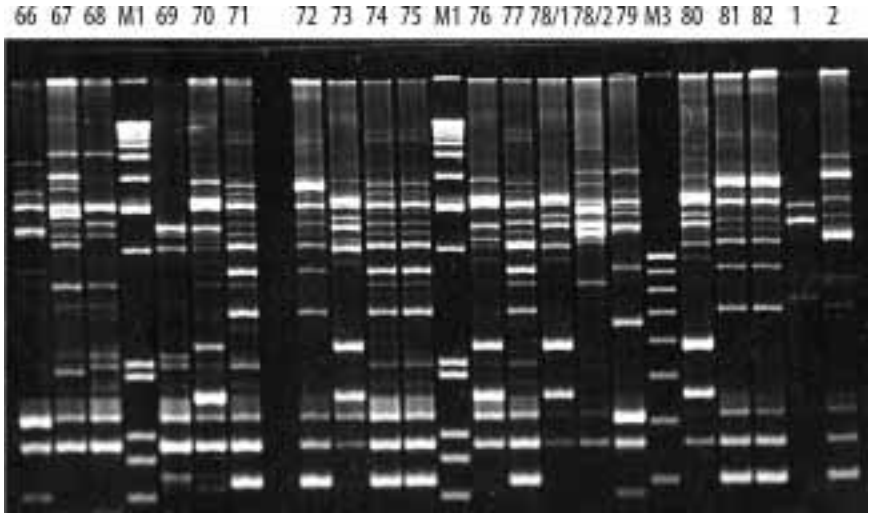

Fig. 5. Representative RAPD profiles of soybean isolates and *B. japonicum* reference strains. Amplification of the genomic DNA was performed using primer P5. The markers are 100 bp ladder (M3) and 1 kb ladder (M1).

RAPD fingerprinting

All soybean isolates as well as reference strains were further genotypically characterised by RAPD fingerprinting. Total genomic DNAs were amplified with six oligonucleotide primers. The amplification patterns revealed a high level of polymorphism. All primers produced multiple DNA products ranging in size from 0.15 to 2.8 kb. The primers produced up to 14 products per strain. An example of RAPD fingerprints is shown in Fig. 5. Compared with other fingerprinting methods used in this study, the highest level of genetic diversity among indigenous soybean rhizobia was detected by using several oligonucleotide primers in amplification reactions.

The dendrogram derived from RAPD profiles (Fig. 6) shows that all *B. japonicum* strains could be divided into two major clusters. Six isolates that formed the second major cluster diverged from the other strains at a similarity level of 0.63. Within the first major cluster, two very distinct groups of strains could be distinguished.

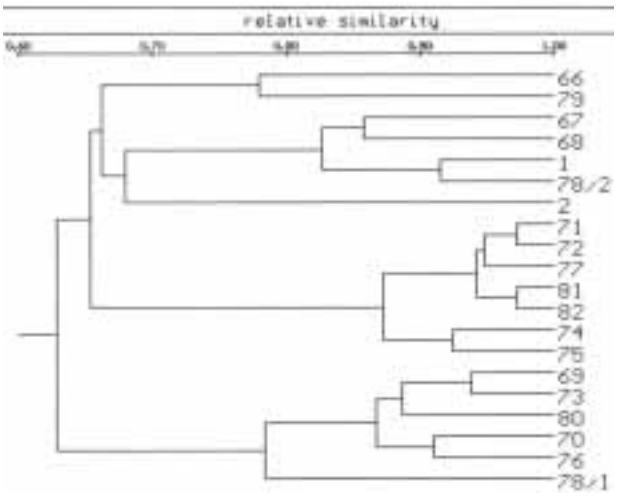

Fig. 6. Dendrogram of *B. japonicum* strains derived from RAPD fingerprints generated by using six different 10-mer primers

The first one comprised the two inoculant strains plus five field isolates that were related at a similarity level of 0.66. The other subgroup within the first major cluster was formed from the same seven isolates that were also grouped together based on PCR-RFLP and rep-PCR profiles.

Discussion

Soybean rhizobia, isolated from different field sites in eastern Croatia, were characterised using three DNA--based methods. The dendrograms derived from PCR--RFLP, rep-PCR and RAPD analysis were similar (Figs. 2, 4 and 6) and this consistency provided confidence that strain grouping reflected true relationships among rhizobial strains tested.

Initially, all field isolates and reference strains were analysed by RFLP of PCR amplified 16S rDNA. The considerable diversity observable among the RFLP patterns undoubtedly showed that all field isolates were very distinct from *B. elkanii* and particularly *S. fredii* type strains. Although most of the soybean isolates were closely related to the *B. japonicum* type strain, seven isolates formed a separate group of strains. Our results indicate that this divergent group should be considered as *B. japonicum* strains. However, in order to confirm this statement and to clarify the status of these isolates, further genetic study is needed. After the identification at the species level, the rhizobial strains were characterised by two other DNA fingerprinting methods. Both RAPD and rep-PCR profiles enabled strain differentiation and demonstrated a considerable degree of genetic diversity among soybean isolates. These findings are in agreement with the results obtained by other authors who studied diversity among natural rhizobial populations in different world regions (*1,3,30*).

All DNA fingerprinting methods used in this study revealed high level of genetic diversity among inoculant strains and field isolates. Although inoculant strains are usually applied in soybean production, most of the soybean isolates significantly diverged from inoculant strains. These results suggest that the indigenous rhizobial population remained predominant in most of the investigated soil types. Moreover, our results also strongly confirmed the other important feature of indigenous strains, their very close relation with environmental factors. Dendrograms derived from all DNA fingerprinting methods, including RFLP patterns of 16S rDNA, always grouped together the same seven field isolates. All these isolates originated from soil types with very low pH values (measured in KCl). Only one strain was isolated from soil with pH=5.93, while the remaining six strains were isolated from very acid soils with pH values ranging from 4.16 to 5.00 (data not shown). These unfavorable conditions enabled the survival of only well adapted indigenous strains. Most likely that is the reason why, despite the selection of high quality strains for soybean inoculation, the beneficial effect of that procedure can sometimes be considerably reduced. Inoculant strains are usually characterised by their high capacity for nitrogen fixation but very often by a low competitive ability for the nodulation site in comparison with indigenous soil strains. On the contrary, the major advantage of indigenous rhizobia with respect to inoculant strains is in their adaptability to diverse soil conditions. At the same time, nitrogen fixing capacity of indigenous strains is often low or unknown. The interaction between soybean and bacterial genotypes may also be considerable.

All indigenous strains used in this study will be further characterised for their symbiotic properties in order to select the highest quality and the most suitable strains for soybean inoculation under particular agroecological conditions.

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Genotipska karakterizacija autohtonih kvržičnih bakterija na soji primjenom PCR-RFLP 16S rDNA, rep-PCR i RAPD analize

Sažetak

U novije se vrijeme značajno promijenila taksonomija bakterija koje vežu dušik u simbioznim zajednicama s leguminoznim biljkama. Primjena vrlo osjetljivih i preciznih molekularnih metoda omogućila je utvrđivanje velike raznolikosti kvržičnih bakterija, a osobito unutar prirodnih populacija tih bakterija tla. Glavni je cilj ovih istraživanja bio identificirati i karakterizirati autohtone kvržične bakterije na soji, izolirane iz različitih tipova tla istočne Hrvatske koja se redovito koriste u poljoprivrednoj proizvodnji. Stvarni sastav i genetička raznolikost prirodne populacije proučavana je primjenom različitih PCR »fingerprinting« metoda, kao što su 16S rDNA PCR-RFLP, rep-PCR i RAPD analiza. Osamnaest sojeva, izoliranih iz sojinih kvržica, karakterizirani su i uspoređivani s referentnim i/ili tipskim sojevima *Bradyrhizobium japonicum*, *B. elkanii* i *Sinorhizobium fredii*. »Cluster« analiza zajedničkih RFLP profila, dobivenih primjenom šest restrikcijskih endonukleaza, pokazala je da se svi izolati bitno razlikuju od tipskih sojeva *B. elkani* i *S. fredii*, a vrlo su srodni s tipskim sojevima *B. japonicum*. Međutim, znatna je genetička raznolikost utvrđena između izolata *B. japonicum*. PCR-RFLP 16S rDNA jasno je pokazala postojanje dvaju različitih skupina između autohtonih bradirizobia. Nakon identifikacije na razini vrste, svi su izolati nadalje karakterizirani primjenom RAPD i rep-PCR. Obje metode dale su visokospecifične i reproducibilne profile koji su omogućili diferencijaciju sojeva. Između sojeva *B. japonicum* utvrđen je visoki stupanj raznolikosti primjenom tih dvaju PCR metoda. Dendrogrami dobiveni na osnovi RAPD, REP i ERIC profila pokazali su da se svi autohtoni sojevi mogu podijeliti u tri glavne skupine. Primjenom svih upotrijebljenih metoda omogućeno je konzistentno grupiranje sojeva.