Genotypic Characterisation of Indigenous *Rhizobium leguminosarum* bv. *viciae* Field Population in Croatia

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Summary

Genetic diversity of thirteen *Rhizobium legumnosarum bv. viciae* strains isolated from different field sites in continental part of Croatia was investigated. All rhizobial isolates were obtained either from plants grown in pots containing soil samples or from field grown plants. The strains were analyzed for DNA polymorphism using two DNA fingerprinting methods - randomly amplified polymorphic DNA (RAPD-PCR) and repetitive extragenomic palindromic-PCR (rep-PCR). Both methods resulted in very similar grouping of strains. Cluster analysis of rep- and RAPD-PCR profiles showed significant differences among *Rh. leguminosarum* bv. *viciae* isolates. The highest differences were detected among reference strains and all field isolates revealing considerable genetic diversity of rhizobial field populations. These results suggest the presence of adapted indigenous *Rhizobium leguminosarum* bv. *viciae* strains, probably with higher competitive ability, whose symbiotic properties have to be evaluated in further investigations.

Key words

field pea; indigenous strains; RAPD; rep-PCR; *Rhizobium leguminosarum* bv. *viciae*; symbiotic nitrogen fixation

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Introduction

Rhizobium is a genus of soil bacteria whose members are best known for their ability to establish symbiotic relationships with legumes of agricultural and environmental importance, in a process of biological nitrogen fixation (BNF) (Hirsh, 1992; Moschetti et al., 2005). The inoculation of cultivated leguminous plants with selected rhizobial strains is recommended in order to maximize the contribution of BNF to the nitrogen status of the host plant (de Oliveira et al., 1999). In the legume production, the application of high quality rhizobial inoculants substantially contributes to the N cost efficiency of farming systems through inputs from biological N fixation (Herridge et al., 2001; Peoples and Baldock, 2001).

Field pea (*Pisum sativum* L.) has a potential as high-yielding, short term crop with a high crude protein content (Fraser et al., 2001). It is used for seed, hay, pasture, sillage, and green manure and it is one of the best feed for animals and almost indispensable for efficient, economical livestock (Tekeli and Ates, 2003). Field pea may produce substantial yield without any requirement for nitrogenous fertilizers (Faulkner, 1985) due to symbiotic relations with nitrogen-fixing bacteria *Rhizobium legumnosarum* bv. *viciae*. In order to increase forage yield, the inoculation of field pea with appropriate rhizobial strains represents an agriculturally sustainable approach. However, the presence of indigenous rhizobia in soil may represent a barrier to efficient inoculation with commercially available strains because indigenous strains are often better adapted to the prevailing soil and climate conditions (de Oliveira et al., 1999). Therefore, the evaluation of diversity within indigenous rhizobial population is important for successfull inoculation, and for the screening of novel, highly effective inoculant strains (de Oliveira et al. 1999). However, no information is available about presence and diversity of *Rh. leguminosarum* bv. *viciae* field population in Croatia. The diversity of *Rh. legumnosarum* bv. *viciae* population has usually been determined by phenotypic and/or genotypic characterisation of strains isolated from legume root nodules (Martinez-Romero, 1994). A large numbers of molecular methods based on PCR reaction have been proposed to characterise the *Rh. leguminosarum* strains and to provide a high degree of differentiation of these strains (Laguerre et al., 1996; 2001; 2003; Moschetti et al., 2005). In this study, two PCR-based methods for evaluation of genetic diversity among *Rh. leguminosarum* bv. *viciae* field population were used. The chromosomal localization of rep sequences in *Rhizobium* species indicates phylogenetic relationships between different strains, and represents a relative simple but efficient tool for strain identification (Labes et al., 1996). Using RAPD–PCR method, characteristic fingerprints of complexed genomes can be obtained without any previous knowledge about the genomes being studied (Sikora et al., 1997). The fingerprints obtained by rep-PCR and RAPD-PCR are specific and reproducible and allow the distinction of bacteria at the (sub)species and strain level (Niemann, 1997). Many authors proposed these methods for identification and phylogenetic grouping of *Rhizobium* isolates (Labes et al., 1996; Sikora et al., 2000; 2003; Bradić et al., 2003).

The aim of present research was the assessment of genetic diversity and characterisation of indigenous *Rh. leguminosarum* bv. *viciae* strains isolated from different regions in Croatia.

Material and methods

Bacterial strains. Rh. leguminosarum bv. *viciae* isolates were obtained from pot grown plants (strains K10, K21, K32, K43, K54, K65, K98, K121) or from field grown plants (strains Z90, M11, M21, N23, V12) collected from different field sites in continental part of Croatia. The origin of *Rh. leguminosarum* bv. *viciae* isolates and main soil chemical characteristics are summarized in Table 1. The isolation of rhizobia from soil samples was performed by sowing surface-sterilized field pea seed directly into soil samples under controlled conditions. In full bloom stage, nodules from the field pea roots were collected. Strains were isolated from surface sterilized nodules following a standard protocol (Vincent, 1970). The ability of isolates to form nodules was checked by nodulation tests (Vincent, 1970). Thirteen isolates were used for further characterization. Four *Rh. leguminosarum* bv. *viciae* strains were also included in these investigations as reference and type strains. Reference strains 1045 and 1001 were obtained from the Institute of Grassland and Environmental Research in Dyfed, type strain 30132 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, and strain R397 from culture collection of Department of Microbiology, Faculty of Agriculture, University of Zagreb.

Fingerprinting of genomic DNA by rep-PCR and RAPD-PCR. Total genomic DNAs from field isolates and reference strains were extracted by using DNeasy Tissue kit (Quiagen), according to the manufacturer´s instructions. The concentration and the purity of DNA were estimated spectrophotometrically at 260 and 280 nm (Perkin Elmer, Lambda 12). Primers REP 1R and REP 2I, ERIC 1R and ERIC 2, and BOX A1R were used for rep-PCR fingerprinting analysis described by de Bruijn (1992). Four arbitrarily chosen primers (P3-P6) used for RAPD fingerprinting were 10 nucleotides in length and primers sequences as previously described (Sikora et al. 1997). All the primers used in this investigation were obtained from Microsynth (Balgach, Switzerland). Amplification reactions were performed in a

Table 1. Origin *of Rh. leguminosarum* bv. *viciae* isolates and chemicl properties of soil

Strain designation	Location	Field sites	Soil properties					
			pH (H_2O)	pH (KCl)	Humic (%)	Total N (%)	Available P (mg kg^{-1} soil)	Available K (mg kg^{-1} soil)
Z90	Zagreb	Maksimir	6.2	5.1	2.3	0.15	39.1	112.1
M11, M21*	Bjelovar	Visnjevac	6.5	5.2	1.9	0.24	29.7	94.6
N23	Nova Gradiška	Prvća	7.0	6.5	3.3	0.16	228.5	332.0
V12	Virovitica	Grabovac	7.0	6.2	2.8	0.16	218.0	353.7
K10	Koprivnica	Podravska Subotica	6.2	4.9	2.5	0.15	44.0	84.6
K21	Koprivnica	Podravska Subotica	6.5	5.4	4.0	0.22	25.0	120.4
K32	Koprivnica	Radeljevo selo	7.0	6.2	2.1	0.14	30.8	81.3
K43	Koprivnica	Reka	5.8	4.9	2.7	0.15	34.3	134.5
K54	Koprivnica	Široko selo	4.9	3.8	2.5	0.15	17.6	131.1
K65	Koprivnica	Novigrad Podravski	5.9	4.9	2.3	0.17	64.2	174.3
K98	Koprivnica	Drnje	6.1	4.9	3.0	0.14	145.6	257.3
K121	Koprivnica	Ždala	7.7	6.9	2.5	0.15	54.6	126.9

* isolates collected from same field sites but from nodules of two different field pea cultivars

25 µl volume, containing: 20 mmol L^{-1} Tris-HCl (pH 8.4), 50 mmol L^{-1} KCl, 2.5 mmol L^{-1} $MgCl_2$, 200 µmol L^{-1} each of dNTPs, 1 µmol L^{-1} primer, 40 ng of genomic DNA and 1.5 U Taq DNA polymerase (Life Technologies). The amplification reactions were performed in a GeneAmp, PCR System 2700 thermocycler (Applied Biosystems) with the following procedure: initial denaturation at 95 °C for 5 min, 35 cycles of 30 sec at 94 °C, 30 sec at 36 °C (RAPD) or 1 min at 40 °C (REP-PCR) or 30 sec at 50 °C and 1 min at 52 °C (ERIC-PCR) or 1 min at 53 °C (BOX-PCR), and 1 min at 72 °C followed by 7 min incubation at 72 °C. The amplified products were separated by horizontal gel electrophoresis on precast 6 % poly(NAT) gels run in SEA 2000 apparatus (Elchrom Scientific AG, Cham, Switzerland). A molecular size marker 1 kb DNA ladder (Invitrogen), was run in all gels. The restriction patterns were visualized under UV illumination and photographed with Polaroide type 667 positive film.

DNA fingerprinting analysis. All RAPD and rep-PCR fingerprints patterns were converted into a two-dimensional binary matrix (1, presence of a band; 0, absence of a band) and analysed by using the NTSYS-pc package (version 1.8; Exeter Software, Setauket, N.Y.). A simple matching (Sm) coefficient was calculated for each pair of strains. A UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

Results

rep-PCR analysis

The total genomic DNAs from all strains tested were used as a templates for PCR amplification with either REP, ERIC or BOX primers to produce DNA fingerprints. The DNAs amplified with specific rep-PCR primers produced an average of 5-15 bands per isolate depending on the primer used in amplification reaction. The highest number of bands per isolates were determined by BO X primers while REP-primers generated the lowest amount of polymorphic bands per strain (data not shown). The dendrogram derived from rep-PCR patterns (Fig. 1) showed that most strains were grouped within two major clusters which diverged at a similarity level of 0.67. Most of *Rh. leguminosarum* bv. *viciae* isolates were grouped within the first cluster together with reference strain R397. In the first cluster, the maximum similarity was determined between four isolates (K32, K54, K65, K98) obtained from the same region (location Koprivnica). The second major cluster comprised only two *Rh. leguminosarum* bv. *viciae* strains - reference strain 1045 and the isolate M11. The most genetically distant *Rh. leguminosarum* bv. *viciae* strains were reference strain 1001 and type strain 30132. These two strains significantly diverged from all strains tested and were separated from two major groups of strains.

RAPD analysis

RAPD fingerprinting was also used for studying genetic diversity among indigenous *Rh. leguminosarum* bv. *viciae* population. DNAs from all field isolates and reference strains were amplified with four different 10-mer primers which had G+C contents of 70%. The amplification patters obtained with primer P4 exhibited a higher level of polymorphism than the patterns obtained with other primers (data not shown). RAPD products generated by four oligonucleotide primers were used to produce a dendrogram since higher number of polymorphic bands enables more reliable grouping of strains. Cluster analysis of banding patterns generated from RAPD fingerprints (Fig. 2) showed that isolates were not closely related to the type strain 30132 neither to a reference strains 1045 and 1001. These three strains diverged from all tested strains at a similarity level of 0.6. The majority of the isolates was grouped in one cluster together with reference strain R397.

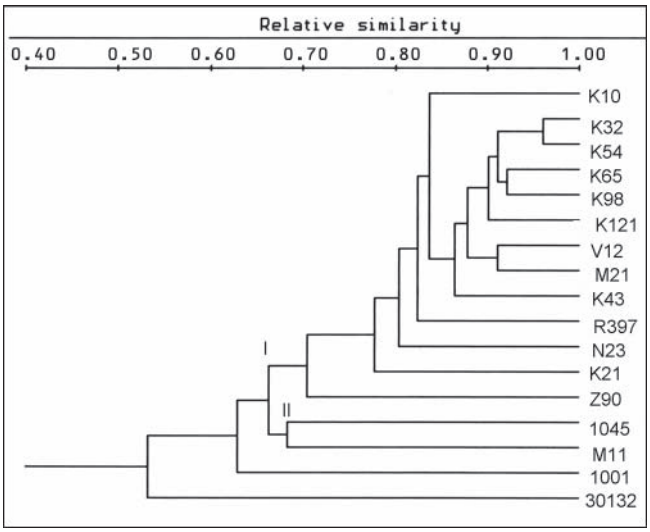

Figure 1.
Dendrogram of *Rhizobium leguminosarum* bv. *viciae* strains derived from rep-PCR fingerprints generated using REP, ERIC and BOX primers

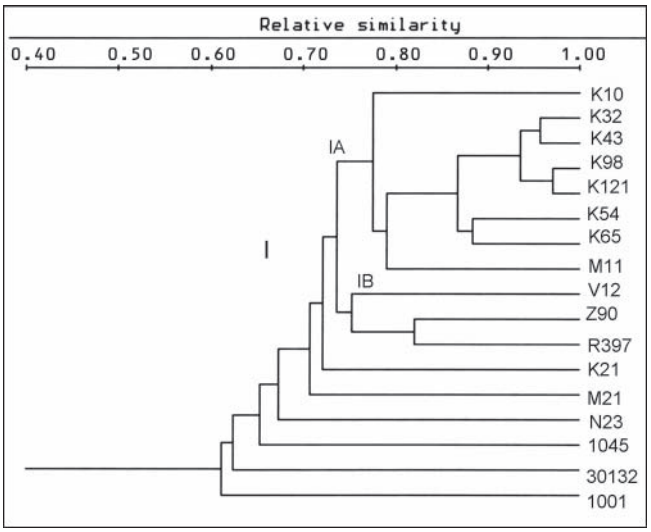

Figure 2.
Dendrogram of *Rhizobium leguminosarum* bv. *viciae* strains derived from RAPD fingerprints generated using four different primers (P3, P4, P5, P6)

This cluster comprised 11 strains and could be divided in two subgroups (IA and IB) that were related at a similarity level of 0.74. The first subgroup IA was formed from strain M11 and seven isolates from the same region (location Koprivnica). Second subgroup included two isolates V12, Z90 and reference strain R397.

Discussion

Genetic diversity of 13 *Rh. leguminosarum* bv. *viciae* strains isolated from different regions in Croatia was analysed. Eight strains were isolated from plants grown in pots containing soil samples and five strains were isolated from field grown plants. Soil samples and field pea plants were collected from arable soils, usually wheat/maize crop rotation from different regions of northwestern Croatia. Isolation of strains were performed from arable soils because diversity of *Rh. leguminosarum* bv. *viciae* population was higher in arable lands than in permanent grasslands, as it was concluded in some papers (Palmer and Young, 2000). The characterization of the indigenous rhizobial population plays an important role in better understanding of soil biodiversity and in the improving the contribution of biologically fixed nitrogen to field pea production. Genetic diversity and characterisation of indigenous *Rh. leguminosarum* bv. *viciae* isolates were determined using rep-PCR and RAPD-PCR fingerprinting methods. A high level of genetic diversity was found within *Rh. leguminosarum* bv. *viciae* populations, as each strain had a unique fingerprint. Both methods showed very similar, almost identical grouping of strains which demonstrated that these methods are reliable and suitable for rhizobial strain identification. Similar conclusions were obtained in previous study concerning characterisation of *Sinorhizobium meliloti* indigenous strains (Bradić et al., 2003) as well as *Bradyrhizobium japonicum* (Sikora et al., 2002). Cluster analysis of rep- and RAPD- PCR banding patterns showed that none of the isolates were closely related to the type strain 30132 or reference strain 1001 and 1045. The maximum similarity was obtained within group of strains isolated from pot-grown plants originated from the same location (Koprivnica) but from different field sites. Using both fingerprinting methods, higher level of genetic diversity was determined among rhizobia isolated from field grown plants. Several studies have reported the influence of the sampling effects on the assessment of genetic diversity of rhizobial populations. In the study of Alberton et al. (2005) rhizobia were isolated from nodules of plants grown under field conditions, in pots containing soil, or in Leonard jars receiving serially-diluted soil inoculum. Their results strongly confirmed that sampling methods affect the assesment of rhizobial diversity, both quantitatively and qualitatively. The differences reported in that study were attributed also to host-plant specificity, rhizobial competitiveness and population structure. Bala et al. (2001), using RFLP-PCR of the ITS, verified less diversity in higher soil dilutions used as inocula and suggested that more competitive but less abundant strains usually occupy the nodules, and in the same time with soil dilution less competitive but more numerous strains would be sampled. Results of comparisons of the relative abundance of indigenous rhizobial genotypes isolated from bulk soils, rhizo-

sphere, and nodules of host legumes, fava bean (*Vicia faba* L.), and pea (*Pisum sativum* L.) were reported in the study of Laguerre et al. (2003). These results revealed that the dominant genotypes in nodules, especially of pea plants, were not necesssarily dominant in bulk soil. The authors report that the frequently isolated strains in nodule population, reflect their abundance due to their ability to survive in the soil. Their results also emphasize the influence of the host plant on the diversity and the genetic structure of *Rh. leguminosarum* bv. *viciae* populations. The results of the present study also revealed the importance of host plants for assessment of rhizobial diversity. Two strains, M11 and M21, were isolated from the same field site but from different field pea cultivars. These strains produced RAPD and rep-PCR patterns that significantly differed (relative similarity of 0.7). These results indicate that the genotype of the host plant may have considerable influence on assessing diversity among indigenous rhizobia isolated from nodules. It has been also reported in other studies that host-plant specificity can greatly affect the spectrum of rhizobial genotypes selected from the soil (Handley et al., 1998; Mutch and Young, 2004; Vessey and Chemining'wa, 2005). In order to determine the correlation between distribution of indigenous strains and environmental factors, soil chemical properties were determined. According to Paffetti et al. (1996) chemical and physical differences between two soils may be at least partially responsible for genetic differences among rhizobial strains. Soil pH has been described as a factor with considerable influence on the degree of strain polymorhism between *Rhizobium leguminosarum* bv. *trifolii* natural populations (Harrison et al., 1989). The results of the present study indicated that there is no clear correlation between the characteristics of the isolation sites and genomic fingerprinting patterns of rhizobial isolates. For example, both fingerprinting methods grouped isolates K54 and K121 in the same main cluster although these strains were isolated from very different soil conditions regrading the soil pH level.

Conclusion

The results of the present study indicated the presence of indigenous population of *Rh. leguminosarum* bv. *viciae* in different field sites in Croatia. These indigenous strains were genetically diverse from reference and type strains used in this study. Symbiotic efficiency as well as other symbiotic properties of these strains should be further investigated. This study provides an ecological framework that can be used for selection of efficient rhizobial strains that are adapted to local environmental factors and could be used in production of high quality rhizobial inoculants.

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