Acta Biologica Hungarica 66(3), pp. 316–325 (2015)
DOI: 10.1556/018.66.2015.3.7

RAPD-INFERRED GENETIC VARIABILITY OF SOME INDIGENOUS *RHIZOBIUM LEGUMINOSARUM* ISOLATES FROM RED CLOVER (*TRIFOLIUM PRATENSE* L.) NODULES

Andrei Stefan,[1] Craita M. Rosu,[2] Catalina Stedel,[2]
Lucian D. Gorgan[1] and Rodica C. Efrose[2]*

[1]Faculty of Biology, "Alexandru Ioan Cuza" University of Iaşi, Iaşi, Romania
[2]Department of Experimental and Applied Biology, NIRDBS-Institute of Biological Research Iaşi, Iaşi, Romania

(Received: January 9, 2015; accepted: March 5, 2015)

The application of commercial rhizobial inoculants to legume crops is proving to be an alternative to synthetic fertilizer use. The challenge for sustainable agriculture resides in the compatibility between crop, inoculants and environmental conditions. The evaluation of symbiotic efficiency and genetic diversity of indigenous rhizobial strains could lead to the development of better inoculants and increased crop production. The genetic variability of 32 wild indigenous rhizobial isolates was assessed by RAPD (Random Amplified Polymorphic DNA). The strains were isolated from red clover (*Trifolium pratense* L.) nodules from two distinct geographical regions of Northern and Eastern Romania. Three decamer primers were used to resolve the phylogenetic relationships between the investigated isolates. Cluster analysis revealed a high diversity; most strains clustered together based on their geographical location.

Keywords: RAPD – red clover – indigenous strains – *Rhizobium leguminosarum* – genetic variability

INTRODUCTION

Rhizobium leguminosarum is a rod-shaped Gram-negative soil alphaproteobacteria capable of nodulating members of the *Fabaceae* family. Some free-living proteobacteria can form symbioses with legumes and the non-legume *Parasponia* [13]. In this symbiotic relationship they are able of fixing atmospheric nitrogen and are collectively known as rhizobia. The association between rhizobia and legumes is considered as the most important nitrogen fixing systems in agriculture [7]. As food demand increases, the use of synthetic fertilizers and the application of commercial inoculants are alternative means of increasing crop production. Castro-Sowinski et al. [1] reported that there are millions of hectares of crops inoculated with a variety of rhizobial strains in South America. The inoculants are comprised of commercial strains, which are not adapted to local pedo-climatic conditions compared with indigenous rhizobial strains [11]. The isolation and characterization of native rhizobia could lead to improvement in the manufacture of commercial inoculants and crop production. Rhizobial strains can also be used as co-inoculants with plant growth rhizobacteria [8, 9] and for bioremediation purposes [18, 22, 25].

*Corresponding author; e-mail address: rodica.efrose@icbiasi.ro

Random Amplified Polymorphic DNA (RAPD) is a type of PCR by which random portions of the genome are amplified using arbitrarily chosen primers [26], usually 10 bp in length. This fast and relatively cheap method has been widely used in genetic variability studies of rhizobial species [10, 16–17, 20, 27]. An increasing emphasis is placed on genetic diversity of local, indigenous rhizobial strains, several more in-depth methods being employed [2, 4–5, 23].

Clovers are important forage and fodder crop and represent the second largest legume crop cultivated in Romania, after alfalfa (The Romanian Statistical Yearbook, 2012). In this study we assessed the genetic diversity and phylogenetic relationships between 32 *Rhizobium leguminosarum* isolates from wild-grown red clover (*Trifolium pratense* L.) nodules by the use of RAPD method.

MATERIALS AND METHODS

Cell culture

Thirty-two bacterial isolates were used in the present study. The indigenous rhizobial strains belong to the culture collection of the Institute of Biological Research Iaşi (Romania) and are denoted by the letter 'R' followed by a number. Bacterial strains

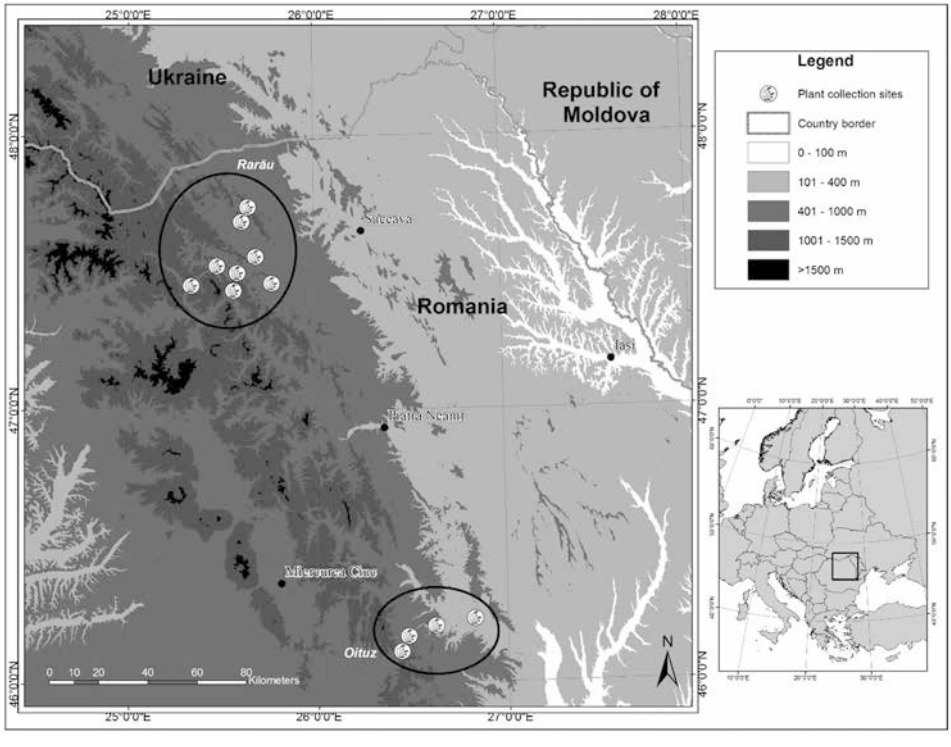

Fig. 1. Plant collection sites in North-Eastern Romania. The groups Rarau and Oituz are highlighted

were isolated from intact nodules of red clover (*Trifolium pratense* L.) plants, collected from two distinct natural ecosystems of North-Eastern Romania, Rarau and Oituz (Fig. 1). Stocks obtained from pure bacterial culture are stored at –80 °C in 20% (v/v) glycerol. As references, the rhizobial strains *Bradyrhizobium japonicum* LMG 4252, *Ensifer meliloti* LMG 6133 and *Rhizobium leguminosarum* LMG 8820 were used. These previously classified strains were obtained from the BCCM/LMG Bacterial Collection (Faculty of Sciences, Ghent University, Belgium). Rhizobial strains were grown in liquid YMB medium and incubated at 28 °C for three days on a rotary shaker [24]. Equal aliquots of each liquid culture were centrifuged in order to pellet the cells to be used for genomic DNA isolation.

DNA isolation

Genomic DNA was isolated using the CTAB technique according to standard protocols [17]. The bacterial cells were incubated in Tris-EDTA buffer, lysozyme (10 mg/ml), SDS (10% w/v) and proteinase K (10 mg/ml) at 37 °C for 1 hour. Salt (5 M NaCl) was added and mixed well. Samples were then incubated in CTAB-NaCl (274 mM CTAB; 700 mM NaCl), at 65 °C for 30 minutes. One volume of phenol:chloroform (1:1) was added after the incubation and the tubes were centrifuged for 10 minutes at 14,000 rpm. The aqueous upper phase was transferred to new tubes and 1 volume of chloroform was added to wash off any trace of phenol. The tubes were gently mixed and centrifuged for 10 minutes at 14,000 rpm. The aqueous upper phase was transferred to new tubes and 1 volume of ice cold isopropanol was added. After DNA precipitation occurred, the tubes were centrifuged for 10 minutes at 14,000 rpm, the supernatant was discarded and the DNA pellet was washed with 70% ethanol. The DNA pellet was vacuum-dried and rehydrated in TE buffer.

DNA amplification by RAPD

DNA amplification was carried out in 200 µl tubes in a 25 µl reaction volume. The reaction mix contained GoTaq® Green Master Mix (Promega, Madison WI, USA), 10 µM decamer primer, 50 ng/µl DNA and nuclease-free water. The primers used were OPA-18 (AGGTGACCGT), OPC-02 (GTGAGGCGTC), and S-17 (AGGGAACGAG) (Eurogentec, Liege, Belgium) with a GC content of or above 60%. RAPD was performed in a Px2 thermocycler (Thermo Electron Corporation, Waltham MA, USA) according to the following conditions: an initial denaturation step at 95 °C for 3 minutes followed by 40 cycles of DNA denaturation at 95 °C for 30 seconds, primer annealing at 36 °C for 30 seconds and extension at 72 °C for 1 minute. A final extension step was performed at 72 °C for 10 minutes. The amplified products were visualized in 1.5% agarose gel stained with ethidium bromide, under UV light and recorded on a High Performance Ultraviolet Transilluminator (Upland CA, USA).

Data analysis

Based on the RAPD pattern, binary matrices were constructed in which intense, reproducible bands were scored as 1 and the absence of bands was scored as 0. The 100 bp DNA Step Ladder (Promega) and the RAPD pattern of strains LMG 4252, LMG 6133 and LMG 8820 were used as references. The matrices were created with the help of PyElph 1.4 software [15] and were assembled in Microsoft Excel.

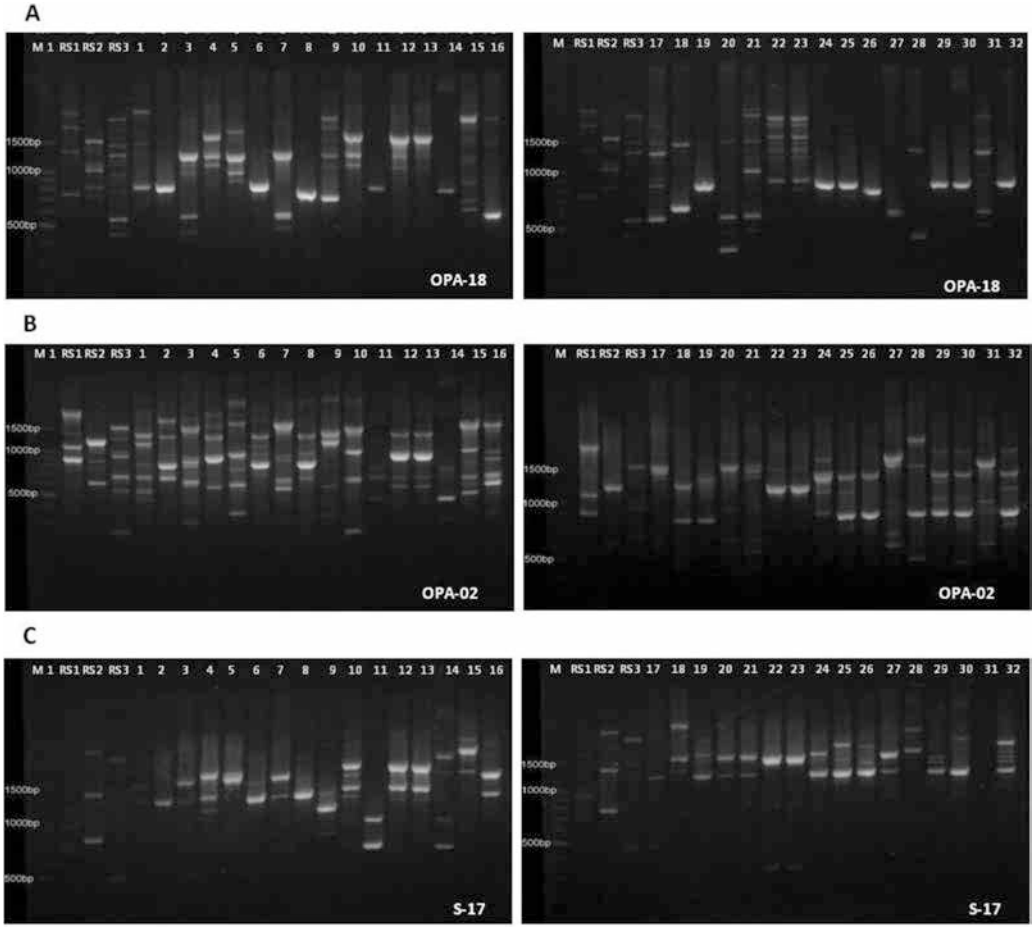

Fig. 2. RAPD-profiles of indigenous rhizobial isolates and reference strains produced by OPC-18 (A), OPC-02 (B) and S-17 (C) primers: lane M – 100 bp DNA step ladder, lane RS1 – *Bradyrhizobium japonicum* LMG 4252, lane RS2 – *Ensifer meliloti* LMG 6133, lane RS3 – *Rhizobium leguminosarum* LMG 8820; lane 1 – R1, lane 2 – R7, lane 3 – R9, lane 4 – R11, lane 5 – R16, lane 6 – R19, lane 7 – R21, lane 8 – R25, lane 9 – R36, lane 10 – R41, lane 11 – R45, lane 12 – R47, lane 13 – R50, lane 14 – R52, lane 15 – R55, lane 16 – R58; lane 17 – R69, lane 18 – R73, lane 19 – R79, lane 20 – R82, lane 21 – R85, lane 22 – R87, lane 23 – R89, lane 24 – R91, lane 25 – R106, lane 26 – R108, lane 27 – R117, lane 28 – R121, lane 29 – R124, lane 30 – R125, lane 31 – R127, lane 32 – R130

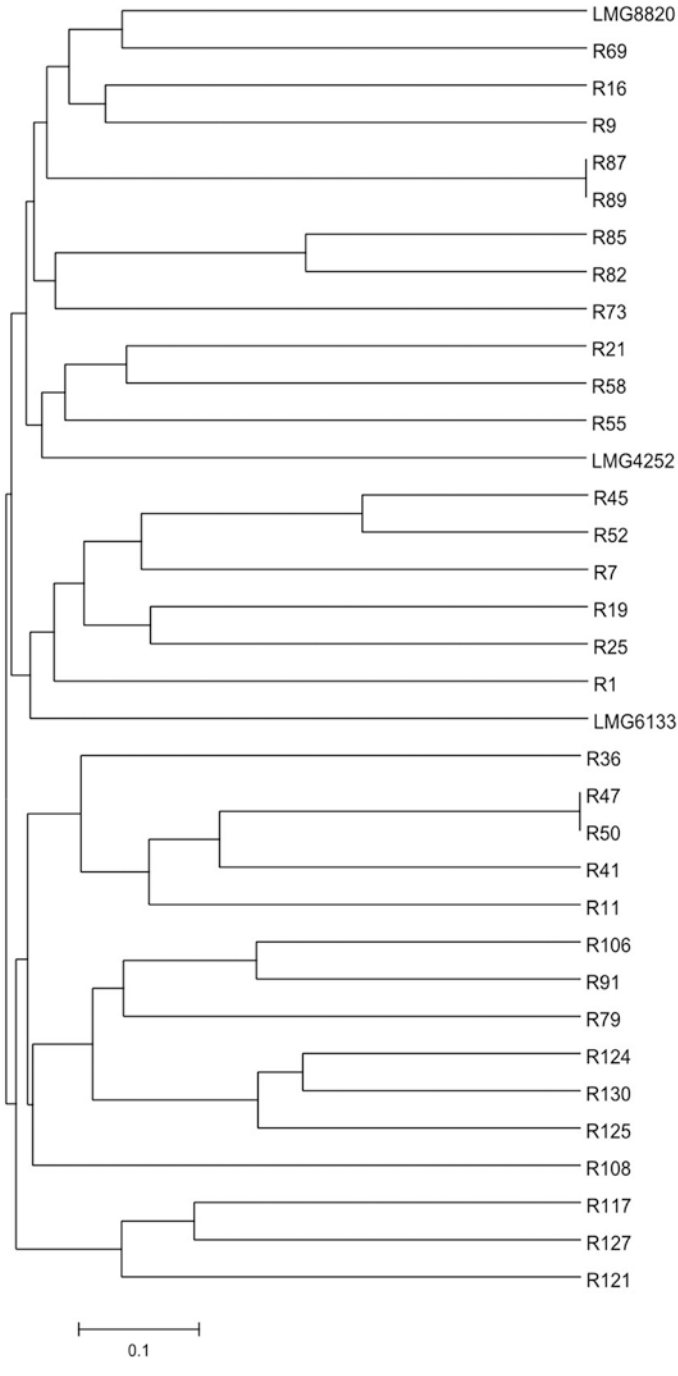

Fig. 3. Cladogram constructed based on the similarity index between the isolates. The groups Rarau and Oituz are shown

An UPGMA (Unweighted Pair Group Method with Arithmetic Mean [21]) dendrogram was constructed based on a Jaccard's similarity coefficient into FreeTree 0.9.1.50 [6]. Statistical analyses were conducted in FAMD 1.3 (Fingerprint Analysis with Missing Data) software [19].

RESULTS

In the present work, thirty-two *Rhizobium leguminosarum* strains isolated from two major geographic locations from North-Eastern Romania were analyzed. Three reference strains previously characterized were also included. In order to obtain useful RAPD patterns, three decamer primers were used: OPA-18, OPC-02 and S-17. The electrophoretic profiles scored with the selected RAPD-primers are shown in Figure 2. Cluster analysis was performed based on banding pattern. The primers produced multiple amplified DNA fragments, ranging from approximately 280 bp to 3000 bp in length. Without taking into consideration the number of bands produced on the reference strains, primer OPA-18 produced 89 bands (Fig. 2, panel A), with an average of 2,78 bands per strain, a maximum of 7 bands for isolates R87 and R89 and a minimum of one band for eleven strains. Primer OPC-02 produced 131 bands (Fig. 2, panel B), with an average of 4.09 bands per strain, a maximum of 7 bands for isolate R16 and a minimum of 2 bands for strains R69, R87, R89 and R108. Primer S-17 produced 86 bands (Fig. 2, panel C), with an average of 2.68 bands per strain, a maximum of 5 bands for isolates R106 and R130 and a minimum of zero bands for isolate R127. Isolates R47, R50 and R87, R89 had a similarity coefficient of 1, these isolates showing an identical RAPD pattern for all primers used.

Table 1
Band presences per strain for each group and the number of polymorphic bands

Parameter	Rarau group	Oituz group
Mean	9.68	9.14
Variance	5.06	1.47
Standard deviation	2.24	1.21
No. of polymorphic bands in group	93	37

Table 2
Analysis of molecular variance of the rhizobial isolates

Source of variation	Degrees of freedom	Variance components	Total variance (%)	Φ_{ST}
Among groups	2	0.0347	7.64	0.0764
Within groups	32	0.4203	92.35	
Total	34	0.4550		

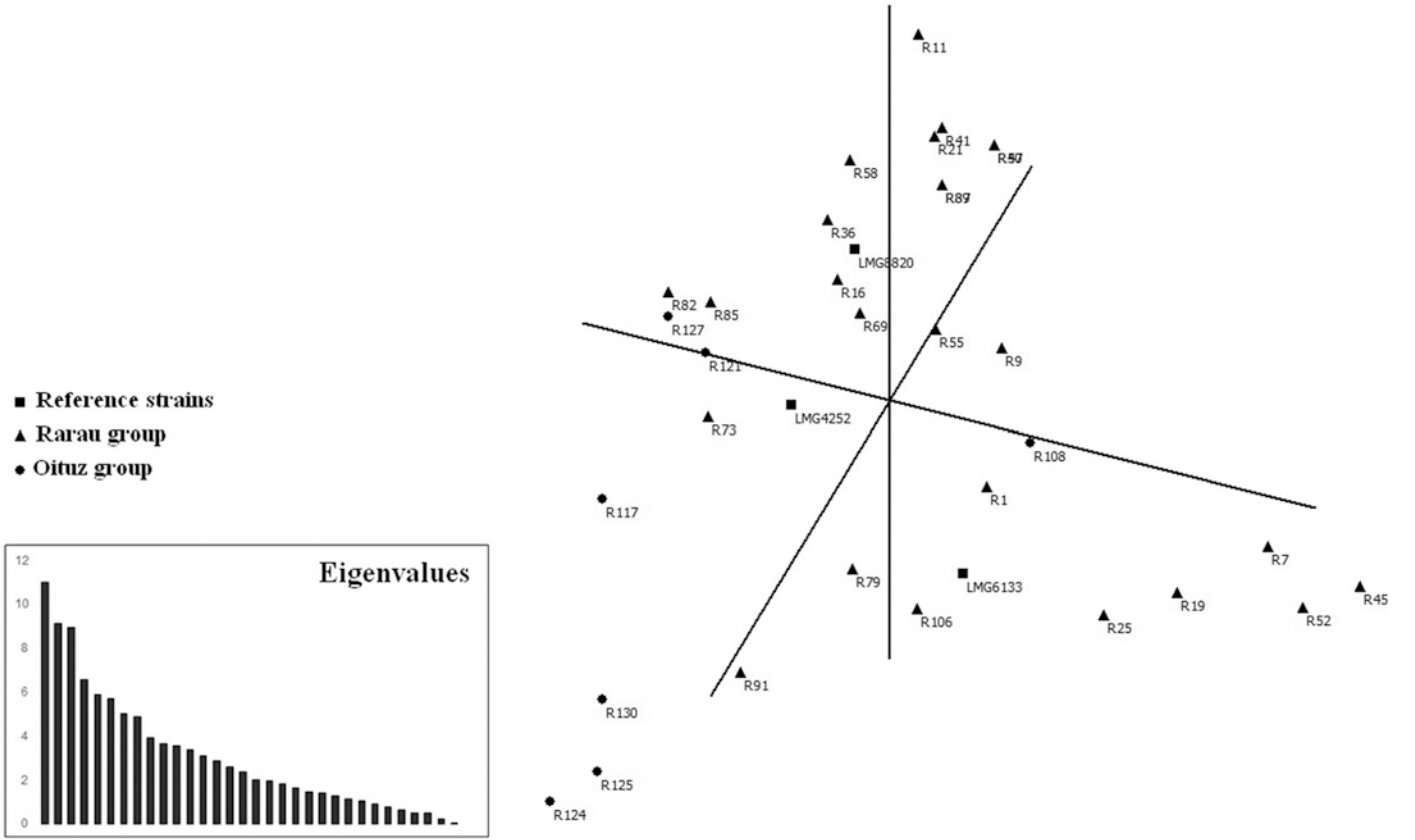

Fig. 4. PCoA analysis of the rhizobial isolates. Eigenvalues are shown in the lower left corner of the image and are given as percentages of the sum of positive Eigenvalues

The presence of bands per rhizobial isolate and the numbers of polymorphic bands obtained for each group are presented in Table 1. The difference in number of polymorphic bands between the two groups was due to the unequal size of the samples (25 isolates from Rarau vs. 7 isolates from Oituz). We found no monomorphic bands (bands present in all samples) for either one of the primers used.

Genetic distance was estimated by the variance among and within the two groups using AMOVA (Analysis of Molecular Variance, Table 2). The proportion of total diversity was 7.64% between the two groups and 92.35% within each group. The value of Φ_{ST} between groups shows that they are not different ($p < 0.0001$, significance test from 1000 permutations).

The genetic relationships among the isolates were also investigated by means of principal coordinates analysis (PCoA). In accordance with the PCoA results, the rhizobial strains were plotted on 3 principal coordinates accounting for 10.98%, 9.12% and 8.93% of variation. The graph generated by PCoA (Fig. 4) corresponded to the dendrogram generated in Figure 3, with slight differences.

DISCUSSION

The primers used in this study yielded reproducible and polymorphic bands for all the strains, except for isolate R127 which showed no amplification products for primer S-17. Based on the RAPD data, a dendrogram was generated in which the strains were apparently clustered based on their geographical location (Fig. 1). The commercial strains clustered together with the Rarau group and the isolate R69 was the closest to *Rhizobium leguminosarum* LMG 8820. The association of strains into smaller clusters inside the two major groups was not based entirely on their geographic location. The strains displayed a heterogeneous distribution, although there were some strains that cluster together with other strains from the same location or from neighboring locations. This is the case of isolates R16 and R9, R87 and R89, R47 and R50 that were collected from the same locations. An intriguing aspect was the fact that strains R106, R91 and R79 isolated from distinct locations within the Rarau group clustered together with isolates from Oituz group. There is no physical way soil bacteria could move across a hydrologicaly-fragmented forest-covered mountainous region. This similarity between strains from different geographical locations and their variability could stem from some intrinsic characteristic. Their diversity could also be the result of soil type, altitude and local climate.

The AMOVA results showed a small degree of genetic variation between the two groups as we expected because the rhizobial isolates belong to the same bacterial species and there is a high degree of genetic variation within the groups. The occurrence of a wide variety of strains increases the chance for a legume host to find a compatible rhizobium in any soil. All the commercial strains originate in indigenous rhizobial pools which represent an important resource to isolate and characterize new local rhizobial strains that could lead to the improvement of commercial strains [11].

Our AMOVA results are consistent with those obtained by Paffetti et al. [14] who also found a high level of genetic diversity within a population of *Sinorhizobium meliloti* isolated from root nodules of alfalfa and characterized by RAPD analysis. Similarly, Moschetti et al. [12] reported a high intraspecific genetic diversity among *Rhizobium leguminosarum* bv. *viciae* isolates and found no relationship between strains and their geographical origin. Some strains isolated from the same site were classified in different clusters. According to de Oliveira et al. [3] isolates of *R. tropici* and *R. leguminosarum* bv. *phaseoli* clustered together in relation to the soil characteristics of each sample site. Rajasundari et al. [17] also indicated that RAPD is a discriminative and efficient method for differentiating and studying genomic diversity of *Rhizobium* strains [17].

In this study we showed that RAPD had enough discriminatory power to distinguish between *Rhizobium leguminosarum* isolates from red clover nodules and that there is a high degree of genetic diversity between the isolates of the same group.

ACKNOWLEDGEMENTS

This work was supported by the strategic grant POSDRU/159/1.5/S/133652, co-financed by the European Social Fund within the Sectorial Operational Program Human Resources Development 2007–2013 and by the PN-II-ID-PCE-2011-3-1011/292/05.10.2011 grant financed by the Executive Agency for Higher Education, Research, Development and Innovation Funding (UEFISCDI)

REFERENCES

1. Castro-Sowinski, S., Herschkovitz, Y., Okon, Y., Jurkevitch, E. (2007) Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. *FEMS Microbiol. Lett. 276,* 1–11.
2. De Meyer, S. E., Van Hoorde, K., Vekeman, B., Braeckman, T., Willems, A. (2011) Genetic diversity of rhizobia associated with indigenous legumes in different regions of Flanders (Belgium). *Soil Biol. Biochem. 43,* 2384–2396.
3. De Oliveira, I. A., Vasconcellos, M. J., Seldin, L., Paiva, E., Vargas, M. A., de Sá, N. M. H. (2000) Random amplified polymorphic DNA analysis of effective *Rhizobium* sp. associated with beans cultivated in brazilian cerrado soils. *Braz. J. Microbiol. 31,* 39–44.
4. Durán, D., Rey, L., Sánchez-Cañizares, C., Navarro, A., Imperial, J., Ruiz-Argueso, T. (2013) Genetic diversity of indigenous rhizobial symbionts of the *Lupinus mariae-josephae* endemism from alkaline-limed soils within its area of distribution in Eastern Spain. *Syst. Appl. Microbiol. 36,* 128–136.
5. Giongo, A., Ambrosini, A., Vargas, L. K., Freire, J. R. J., Bodanese-Zanettini, M. H., Passaglia, L. M. P. (2008) Evaluation of genetic diversity of bradyrhizobia strains nodulating soybean [*Glycine max* (L.) Merrill] isolated from South Brazilian fields. *Appl. Soil Ecol. 38,* 261–269.
6. Hampl, V., Pavlícek, A., Flegr, J. (2001) Construction and bootstrap analysis of DNA fngerprinting-based phylogenetic trees with the freeware program FreeTree: application to trichomonad parasites. *Int. J. Syst. Evol. Micr. 51,* 731–735.
7. Herridge, D. F., Peoples, M. B., Boddey, R. M. (2008) Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil 311,* 1–18.
8. Iqbal, M. A., Khalid, M., Shahzad, S. M., Ahmad, M., Soleman, N., Akhtar, N. (2012) Integrated use of *Rhizobium leguminosarum,* plant growth promoting rhizobacteria and enriched compost for

improving growth, nodulation and yield of lentil (*Lens culinaris* Medik.). *Chil. J. Agr. Res. 72,* 104–110.
9. Joseph, B., Ranjan Patra, R., Lawrence, R. (2012) Characterization of plant growth promoting rhizobacteria associated with chickpea (*Cicer arietinum* L.). *Int. J. Plant Prod. 1,* 141–152.
10. Karthikeyan, K. A. M., Sasireka, G., Suresh, P. (2013) Genetic diversity of *Rhizobium leguminosarum* isolates as revealed by Random Amplified polymorphic DNA analysis. *J. Biosci. Res. 4,* 15–20.
11. Lindström, K., Murwira, M., Willems, A., Altier, N. (2010) The biodiversity of beneficial microbe-host mutualism: the case of rhizobia. *Res. Microbiol. 161,* 453–463.
12. Moschetti, G., Peluso, A., Protopapa, A., Anastasio, M., Pepe, O., Defez, R. (2005) Use of nodulation pattern, stress tolerance, *nodC* gene amplification, RAPD-PCR and RFLP–16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar *viciae*. *Sys. Appl. Microbiol. 28,* 619–631.
13. Op den Camp, R. H., Polone, E., Fedorova, E., Roelofsen, W., Squartini, A., Op den Camp, H. J., Bisseling, T., Geurts, R. (2012) Nonlegume *Parasponia andersonii* deploys a broad rhizobium host range strategy resulting in largely variable symbiotic effectiveness. *Mol. Plant Microbe In. 25,* 954–963.
14. Paffetti, D., Scotti, C., Gnocchi, S., Fancelli, S., Bazzicalupo, M. (1996) Genetic diversity of an Italian *Rhizobium meliloti* population from different *Medicago sativa* varieties. *Appl. Environ. Microb. 62,* 2279–2285.
15. Pavel, A. B., Vasile, C. I. (2012) PyElph – a software tool for gel images analysis and phylogenetics. *BMC Bioinformatics 13,* 9.
16. Pinto, P. P., Paiva, E., Purcino, H., Passos, R. V. M., de Sá, N. M. H. (2004) Characterization of rhizobia that nodulate *Arachis pintoi* by RAPD analysis. *Braz. J. Microbiol. 35,* 219–223.
17. Rajasundari, K., Ilamurugu, K., Logeshwaran, P. (2009) Genetic diversity in rhizobial isolates determined by RAPDs. *Afr. J. Biotechnol. 8,* 2677–2681.
18. Ruiz-Díez, B., Quiñones, M. A., Fajardo, S., López, M. A., Higueras, P., Fernández-Pascual, M. (2012) Mercury-resistant rhizobial bacteria isolated from nodules of leguminous plants growing in high Hg-contaminated soils. *Appl. Microbiol. Biotechnol. 96,* 543–554.
19. Schluter, P. M., Harris, S. A. (2006) Analysis of multilocus fingerprinting data sets containing missing data. *Mol. Ecol. Notes 6,* 569–572.
20. Sikora, S., Redžepović, S., Pejić, I., Kozumplik, V. (1997) Genetic diversity of *Bradyrhizobium japonicum* field population revealed by RAPD fingerprinting. *J. Appl. Microbiol. 82,* 527–531.
21. Sokal, R. R., Michener, C. D. (1958) A statistical method for evaluating systematic relationships. *Univ. Kans. Sci. Bull. 28,* 1409–1438.
22. Suominen, L., Jussila, M. M., Mäkeläinen, K., Romantschuk, M., Lindström, K. (2000) Evaluation of the *Galega-Rhizobium galegae* system for the bioremediation of oil-contaminated soil. *Environ. Pollut. 107,* 239–244.
23. Torres, A. R., Cursino, L., Muro-Abad, J. I., Gomes, E. A., de Araújo, E. F., Hungria, M., Cassini, S. T. A. (2009) Genetic diversity of indigenous common bean (*Phaseolus vulgaris* L.) rhizobia from the state of Minas Gerais, Brazil. *Braz. J. Microbiol. 40,* 852–856.
24. Vincent, J. M. (1970) *A Manual for the Practical Study of Root-nodule Bacteria*. IBP Handbook no. 15. Blackwell Scientific Publications, Oxford, England.
25. Wani, P. A., Khan, M. S. (2012) Bioremediation of lead by a plant growth promoting *Rhizobium* species RL9. *Bacteriol. J. 2,* 66–78.
26. Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res. 18,* 6531–6535.
27. Young, C. C., Cheng, K. T. (1998) Genetic diversity of fast-and slow-growing soybean rhizobia determined by random amplified polymorphic DNA analysis. *Biol. Fert. Soils 26,* 254–256.