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Characterization and diversity of the nitrogen fixing microbiota from a specific grassland habitat in the Ciuc Mountains

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Abstract

This study presents the result of a screening of symbiotic nitrogen fixing bacteria from a specific mountainous grassland habitat lying at 1200 m above sea level in the Ciuc Mountains. A number of 50 bacterial isolates from rhizosphere and from the nodules of 14 specific leguminous plant species are presented and characterized. Pure cultures were obtained on selective media. The characterization of the isolated pure cultures through colony morphology analysis, cellular morphology and biochemical properties are discussed. From the 50 chosen bacteria isolates 20 possess morphologic, culture and physiologic properties systematically characteristic of Rhizobia and 16S rDNA gene characterization showed a great genetic diversity of our isolates.

Keywords: biological N_2 fixation, leguminous plants, Rhizobia, isolates, diversity

Introduction

Soil microorganisms constitute the world's largest reservoir of biological diversity and are crucial to the functioning of terrestrial ecosystems. The plant diversity significantly enhances the rates of microbial processes that mediate ecosystem C and N cycling, this effect being more strongly dependent on plant production than on species richness [1].

Rhizobia, through their ability to fix N_2 in symbiosis with legumes, play a central role in the N supply of most soil ecosystems. They are relatively unique among the majority of soil microorganisms in that they have an extensive soil phase as free-living, saprotrophic i.e. heterotrophic microorganisms, yet in conjunction with leguminous plants they have the ability to form species-specific N_2-fixing symbiotic associations. Rhizobium sensu lato are representatives of the following genera: *Allorhizobium, Azorhizobium, Blastobacter, Bradyrhizobium, Burkholderia, Devosia, Ensifer, Mesorhizobium, Ralstonia, Rhizobium, Sinorhizobium* [2,3]. They are relatively robust, ubiquitous, aerobic bacteria with the ability to utilize many different substrates. More mobile strains are more competitive and they develop nodules faster. Strains with intensive extracellular polysaccharide production tend to have the ability of free living N_2-fixation just like facultative anaerobe ones [4]. The capacity to respond to variations in nutrient availability enables the persistence of rhizobial species in soil, and consequently improves their ability to colonize and to survive in the host plant. Rhizobia, like many other soil bacteria, persist in nature most likely in sessile communities known as biofilms, which are most often composed of multiple microbial species [5].

The practical importance of these microorganisms is evident from the fact that although a total of about 100 million metric tons of synthetic nitrogen fertilizers are produced per year, nitrogen-fixing microorganisms convert yearly about 200 million tons of nitrogen to ammonia, and the major portion of this biological nitrogen fixation is carried out by the symbiotic nitrogen fixers such as *Rhizobium* [6].

Related to biological N_2 fixation, grasslands represent the principal source of fixed nitrogen [7]. The root-nodule bacteria of legumes are multipurpose bacteria with very interesting characteristics. The majority of these bacteria have wide host range, a characteristic which offers to the legumes ecological advantages. Many Rhizobia can form nodules with several wild or crop legumes, and can also be a source for genetic information to improve symbiotic characters of other Rhizobia [8].

The significance of Rhizobia to legumes are not restricted to their symbiotic nitrogen fixation activity or to several decomposition activities in the soil, which eventually improves soil fertility and plant productivity, but some strains of Rhizobia produce plant hormones, antimicrobial compounds etc. Beside its importance in plant productivity these activities may be used in biotechnologies, for example in the production of polysaccharides, enzymes and antibiotics [8].

Highly conserved in structure and presumed function throughout the evolution, the rRNAs, and particularly the small subunit rRNA, have become the most commonly used markers for establishing phylogenetic relationships between organisms [9]. The RFLP analysis of 16S rDNA gene is a widely used technique for studying the phylogenetic homology and grouping of the nitrogen fixing bacterial isolates, it has been used earlier in the case of rhizobial isolates from bean [10], from soybean [11], or from *Sesbania* sp. plant nodules [12].

The aim of this present study was the isolation, characterization and evaluation of the diversity of nitrogen fixing bacteria from the rhizosphere and especially from the nodules of leguminous plants, inhabiting alpine and subalpine meadows characteristic to the Ciuc Mountains.

In this work, we have characterized 18 bacterial isolates from nodules of wild leguminous plants and soil samples from the Ciuc Mountains. 16S rDNA gene characterization showed a great genetic diversity of our isolates.

Materials and Methods

Isolation of nitrogen fixing bacteria

With the aim of obtaining new N_2 fixing strains, competitive under natural conditions and adapted to different types of soils as well as of plant, the first step was isolation and cultivation of these microorganisms on selective medium. Isolation of the Rhizobia was performed from the nodules of leguminous plants but also from rhizosphere soils. The leguminous plants were collected from a meadow, situated at 1200 m altitude in the Mountains of Ciuc. The studied plant species were *Anthyllis vulneraria* L., *Lotus corniculatus* L., *Medicago lupulina* L., *Trifolium repens* L., *Trifolium montanum* L., *Onobrychis montana ssp. transilvanica* Simk., *Trifolium pannonicum* L., *Trifolium alpestre* L., *Trifolium medium* Grufb., *Vicia cracca* L., *Vicia sepium* L., *Lathyrus transsilvanicus* (Spr.) Rchb., *Cytisus hirsutus* L., *Tetragonolobus maritimus* (L.) Roth.

The nutrient medium used for isolation of the Rhizobia was yeast extract mannitol (YEM) agar, with the following composition: mannitol 10.00 g, $MgSO_4 \cdot 7H_2O$ 0.20 g, NaCl 0.10 g, K_2HPO_4 0.50 g, $CaCl_2 \cdot 2\ H_2O$ 0.20 g, $FeCl_3 \cdot 6\ H_2O$ 0.01 g, yeast extract 1.00 g, agar 20.00 g, distilled water 1000 ml (pH=6.7-7). The followings were also added to the medium: cycloheximide (20.0 μg/ml) to reduce fungal contamination and bromthymol blue (25.0 μg/ml) to facilitate selection of rhizobia. Colonies characteristic for the Rhizobia that developed on the medium, slimy with yellow halo, were picked and purified by single-colony streaking on solid media. Pure strains were maintained on YMA medium (yeast extract 0.4 g, mannitol 10.0 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7\ H_2O$ 0.2 g, NaCl 0.1 g, agar 20.0 g, distilled water

1000 ml) or Nutrient agar (meat extract 1.0 g, yeast extract 2.0 g, peptone 5.0 g, NaCl 5.0 g, agar 20.0 g, distilled water 1000 ml).

Morphological and physiological characterization

Strains were characterized by cell (Gram stain, determination of the presence of spores), colony (form, elevation, margin, appearance, optical property, pigmentation, texture), morphological and cultural characteristics (gelatin liquefaction, growth in thioglycolate agar) and biochemical tests (glucose, lactose utilization, oxidase test, nitrate reduction) [13].

Extraction of DNA from strains

A total of 1 ml of strain culture grown for 24 hour in liquid YMA medium at 30°C, 150 rpm, was centrifuged at 2370 g for 15 min. The pellet was resuspended, and the DNA was extracted using DNA extraction kit (Wizard Genomic DNA purification kit, Promega).

PCR amplification

The oligonucleotides used were the universal 27f 5' AGAGTTTGATCMTGGCTCAG 3'and 1492r 5'TACGGYTACCTTGTTACGACTT3' primers flanking the bacterial 16S rDNA region.

The PCR reaction was set up to a 50 µl final volume and contained the following reaction mix: 5 µl 10x PCR buffer, 5 µl $MgCl_2$ (2.5 mM), 4 µl dNTP (0.2 mM per nucleotide), 1-1 µl of each primer (10 pmole), 0.25 µl Taq Polymerase (1 U), 1-3 µl DNA in ultrapure water.

Amplifications were carried out in an ESCO Swift mini thermocycler with the following temperature profile: 5 min at 94°C, 30 cycles of denaturation (30 s at 94°C), annealing (30 s at 55°C), extension (1 min at 72°C) and a final extension for 7 min at 72°C. Negative controls were included to check for the presence of false positives due to reagent contamination. Amplified products were separated on 1% agarose gels in 1x TAE buffer at 10 V cm^{-1} for 50 minutes. Amplification products were stained with ethidium bromide, observed with a BioRad UV transilluminator and documented with GelDocXR software.

Restriction fragment analysis

Restriction fragment analysis of PCR-amplified 16S rDNA was performed for 19 isolates. The following enzymes were used: *TaqI, MspI, AluI* and *HaeIII*. The enzymes were incubated for 3 h at 37°C for *MspI, AluI* and *Hae III*, and the TaqI reactions were incubated at 65°C for the same time. Each reaction contained, in a final volume of 20 µl, 5 U of restriction enzyme, 2 µl of 10x reaction buffer (according to each enzyme requirements) and 10 µl of the PCR products.

Agarose gel electrophoresis, using 1.5% gel was carried out to separate the digestion fragments. Gels were run with 1x TAE at 10 V cm^{-1} for 60 minutes. A DNA marker pUC Mix 8 (Fermentas) was used, and fragment size determination was made using the Gel Doc XR software (Bio Rad). The data were statistically evaluated using the PAST software.

Results and discussion

As a result of isolations on the selective medium, 65 strains were obtained, deriving from nodules of the following plant species: *Anthyllis vulneraria* L., *Lotus corniculatus* L., *Trifolium montanum* L., *T. pannonicum* L., *T. alpestre* L., *T. medium* Grufb., *Onobrychis montana ssp. transilvanica* Simk., *Vicia cracca* L., *V. sepium* L., *Tetragonolobus maritimus* (L.) Roth., and 70 strains from the rhizosphere of the species: *Medicago lupulina* L., *Trifolium repens* L., *T. montanum* L., *T. pannonicum* L., *T. alpestre* L., *Onobrychis montana ssp. transilvanica* Simk., *Lathyrus transsilvanicus* (Spr.) Rchb., *Cytisus hirsutus* L., *Tetragonolobus maritimus* (L.) Roth.

From the 135 strains, 50 were selected based on morphological and biochemical properties of the cells and cultures, characteristic of the Rhizobia. Considering cell morphological characteristics, Rhizobia are Gram negative bacilli and they do not form endospores. *Rhizobium, Mesorhizobium, Sinorhizobium* and *Allorhizobium* strains generally produce moist, gummy colonies on YEM medium that are 4-6 mm in diameter after 7 days incubation. On medium containing bromthymol blue, the colonies and surrounding medium are yellow due to acid production by the microorganisms. Slower growing *Bradyrhizobium* strains produce smaller colonies, usually only 1-2 mm diameter after 7-10 days incubation, which are raised and mucoid. Most nodule isolates produce white or cream colored colonies. Rhizobia produce copious quantities of capsular- and exo-polysaccharides in YEM medium. In case of 36 strains the characteristic cultural properties of the Rhizobia could be observed.

The strain designations are composed as follows: the numbers before the letters indicating the nodules (G) or the rhizosphere soil samples (T) refer to the plant species: 1-*Anthyllis vulneraria*, 2-*Lotus corniculatus*, 3-*Medicago lupulina*, 4-*Trifolium repens*, 5-*Trifolium montanum*, 6-*Onobrychis montana ssp. transilvanica*, 7-*Trifolium pannonicum*, 8-*Trifolium alpestre*, 9-*Trifolium medium*, 10-*Vicia cracca*, 11-*Vicia sepium*, 12-*Lathyrus transsilvanicus*, 13-*Cytisus hirsutus*, 14-*Tetragonolobus maritimus*. The other numbers refer to the isolation characteristics of the strain.

According to the principal analysis component, based on colony morphology properties, the majority of the bacteria isolated from the nodules and the soil samples belong to the same group. The morphological properties within both groups, with the exception of a few strains, were the same or very similar (Fig 1). The shape of the colonies in most of the cases is round, one of the colonies presents irregular shaped colony (14T101), while one of the isolates obtained from a nodule is punctiform (14G/2). The elevation of the colonies is raised with the exception of one bacterium from a nodule, which presents a flat colony (9G/2). The margins are entire, only two colonies presented undulated margins (12T103/3, 11G). In most of the cases the middle of the colonies is yellow and the margins are creamy. In the case of some nodule bacteria, the colonies are yellow, except for one colony which is creamy (8G/4). With the exception of one (11G) of the chosen 50 isolates, the surface of the colonies is glistening. Most frequently the density of colonies in the middle is opaque and in the margins transparent, while in the case of bacteria originating from 4 different nodules is opaque (10G/5, 6G/1, 14G/2, 14G/8).

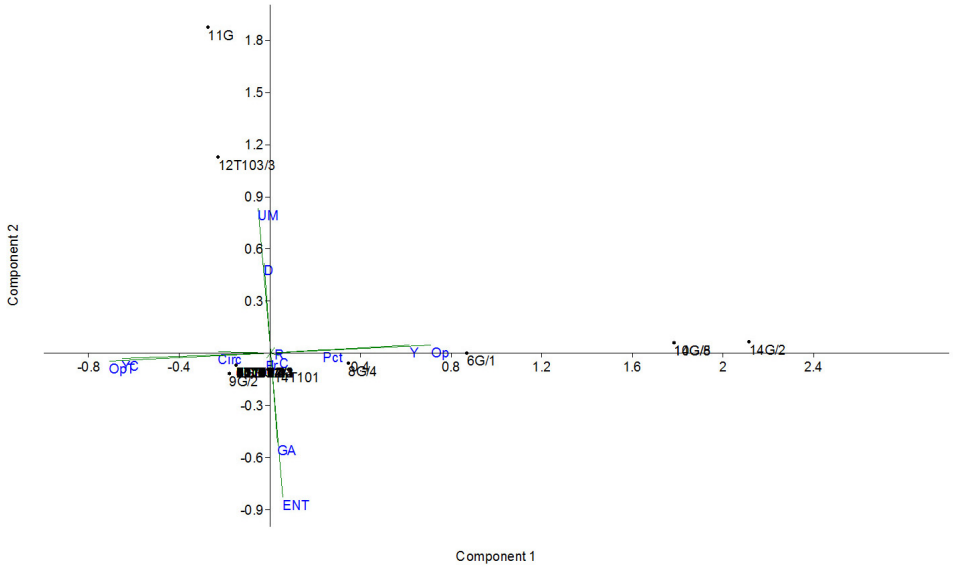

Figure 1. Principal component analysis of bacterial isolates based on morphological characteristics

The explanation of the used notations: Circ- circular form; Ir- irregular form; Pct- punctiform; R- elevation-raised; F- elevation-flat; ENT- margin-entire; UM- margin-undulate; YC- middle yellow, round cream; Y-yellow; C- cream; GA-surface-glistening; D- surface-dull; OpT- middle opaque, round translucent; Op- opaque.

Rhizobia are aerobic bacteria. On the basis of data obtained by cultivation of strains in thioglycolate medium, the majority of the strains proved to be strict aerobe and 12 were facultative anaerobes.

Most species of heterotrophic bacteria present the property of decomposing glucose producing acids, aldehydes and gases. Rhizobia utilize glucose (while producing acid), but they do not utilize lactose. On the basis of results obtained after examination of sacharolitic properties in peptone water with bromthymol blue, 43 strains oxidized glucose and only 7 isolates presented negative results. In case of the lactose, 45 strains were negatives. Gelatin is not hydrolyzed by the Rhizobia or after a long incubation it is slightly hydrolyzed only. The majority of the studied strains corresponded to these criteria, but 13 isolates produced gelatinase, giving a characteristic liquefaction of the gelatin.

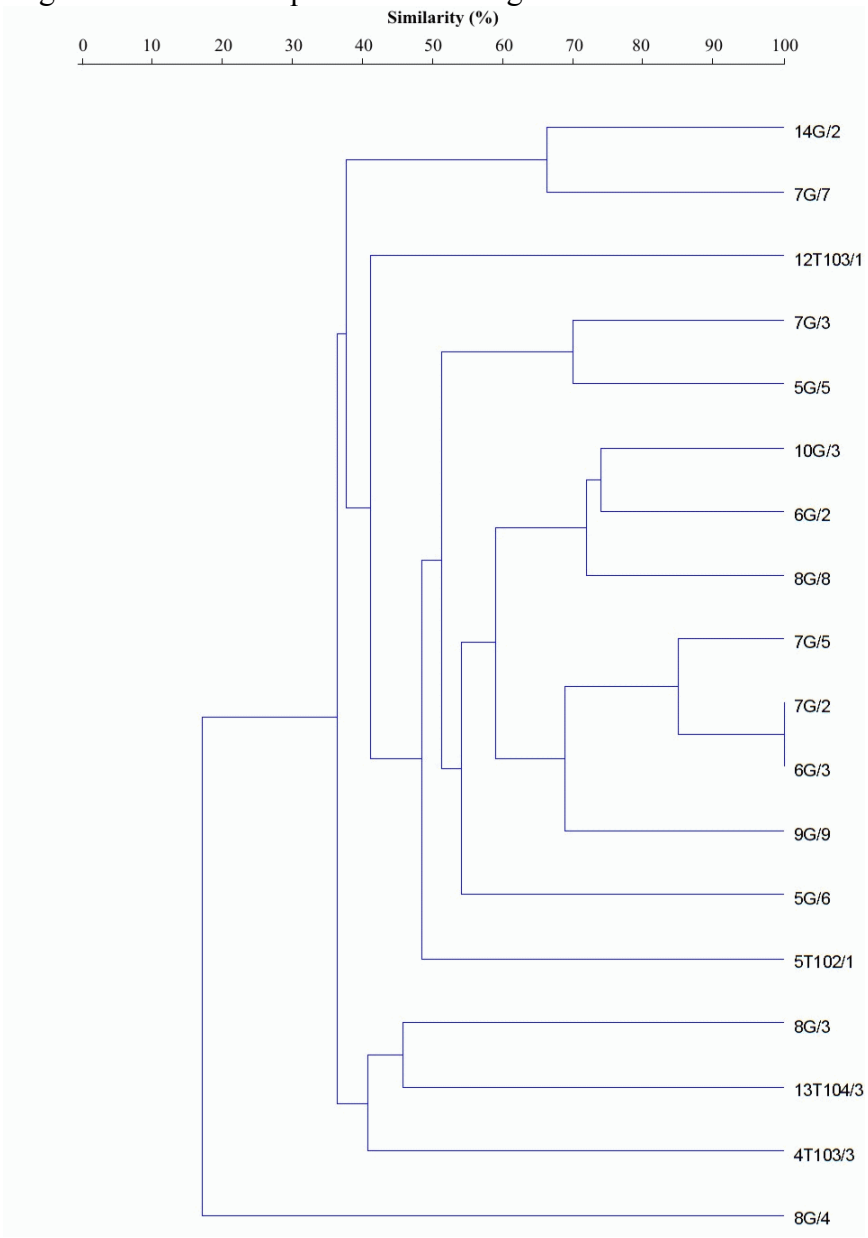

Figure 2. Cluster analysis of bacterial strains based on their physiological characteristics

Among the tests used with the aim of differentiating of certain bacterial groups, based on their respiratory system, one of the most conclusive is the production of oxydases. Rhizobia are oxydase negatives. The majority (42) of the studied strains were oxydase negatives and only 8 isolates presented positive test results.

Many bacteria are capable to utilize nitrates as electron acceptors to grow anaerobically. Rhizobia can reduce nitrates to nitrites. This characteristic was presented by 30 of the studied isolates.

Based on the biochemical properties, a cluster analysis has been performed. After their growth in thioglycolate agar two major strain groups, facultative anaerobes (12 strains) and aerobes (38 strains) can be differentiated (Fig 2), some of them being able to ferment. Within the group including the 38 strains, 2 other subgroups, different in their capability of nitrate reduction can be observed: 13 isolates are able to reduce nitrates to nitrites, whereas the other 7 are not. The two subgroups consisting of 4-4 strains can be differentiated on the basis of the presence or lack of capacity of liquefying gelatin. Within the group of facultative anaerobe strains soil and nodule originating, ones clearly separated. In the group consisting of 13 strains, bacteria (9 isolates) originating from the nodules of *Trifolium* species and from the soil of *Trifolium* rhizosphere dominated.

We performed 16S rDNA characterization with 18 of the strains possessing morphological, cultural and biochemical characteristics typical for Rhizobia. PCR amplification of the bacterial 16S rDNA gave a 1.4 kb fragment, which was subjected to RFLP analysis. The four enzymes used had different discriminating abilities; *Alu*I distinguished 16 types of restriction profiles, *MspI* 14 types, *TaqI* 5 types and *Hae*III 4 types. The restriction patterns obtained by the most discriminatory enzymes are depicted in Fig 3. and Fig 4.

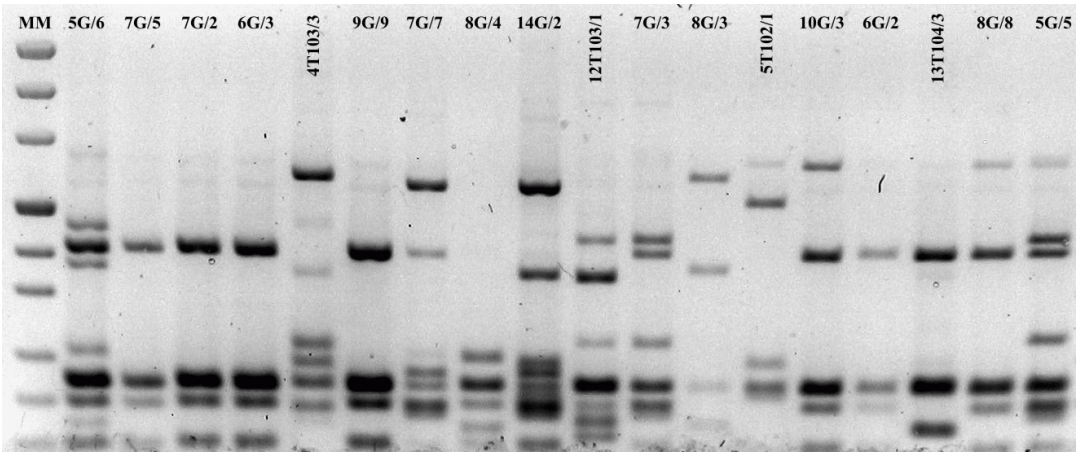

Figure 3. Restriction patterns of the 16S rRNA region of selected strains after digestion with *AluI.*

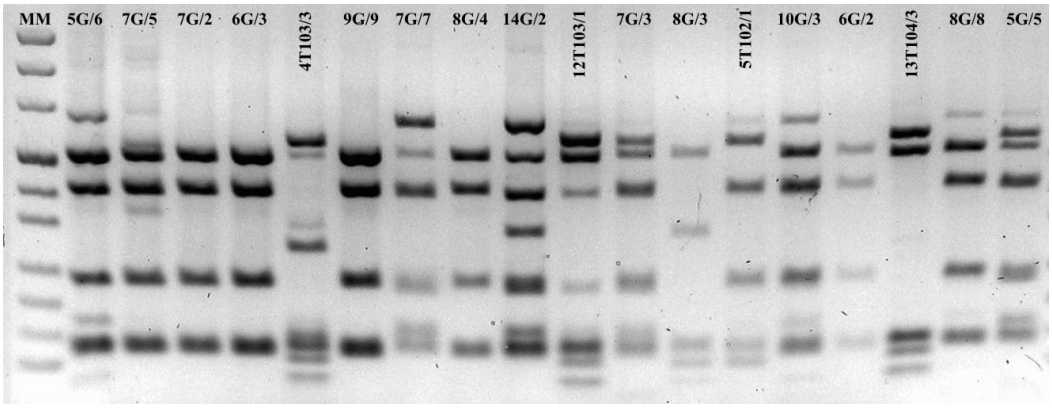

Figure 4. Restriction patterns of the 16S rRNA region of selected strains after digestion with *Msp*I

By combining the results obtained from the four enzymes, it was possible to distinguish 5 loose groups of divergent lineages (Fig 5). The relatively high genetic divergence of the strains is by no means a surprise, since the 18 strains investigated were isolated from 10 different plants (nodules) or rhizosphere soils. However isolates from the same nodule (like 5G/5 and 5G/6 from *Trifolium montanum*) showing uniform morphological and biochemical characteristics differed genetically, whereas in other cases strains isolated from different plant species nodules clustered together. According to the grouping only 2 isolates (7G/2, 6G/3) are in all respect similar. The morphological, just like biochemical properties of the two strains are corresponding, with one exception of the strain (7G/2) isolated from the nodules of *Trifolium pannonicum* which is able to reduce nitrates to nitrites, while the strain isolated from *Onobrychis montana ssp. transilvanica* nodules is not. In the case of two of the groups (1: 14G/2, 7G/7; 2: 10G/3, 6G/2, 8G/8), the strains belong to the same group even according to their physiological characteristics. For the identification of the isolated and characterized strains the sequence analysis of the 16S ribosomal DNA would be necessary.

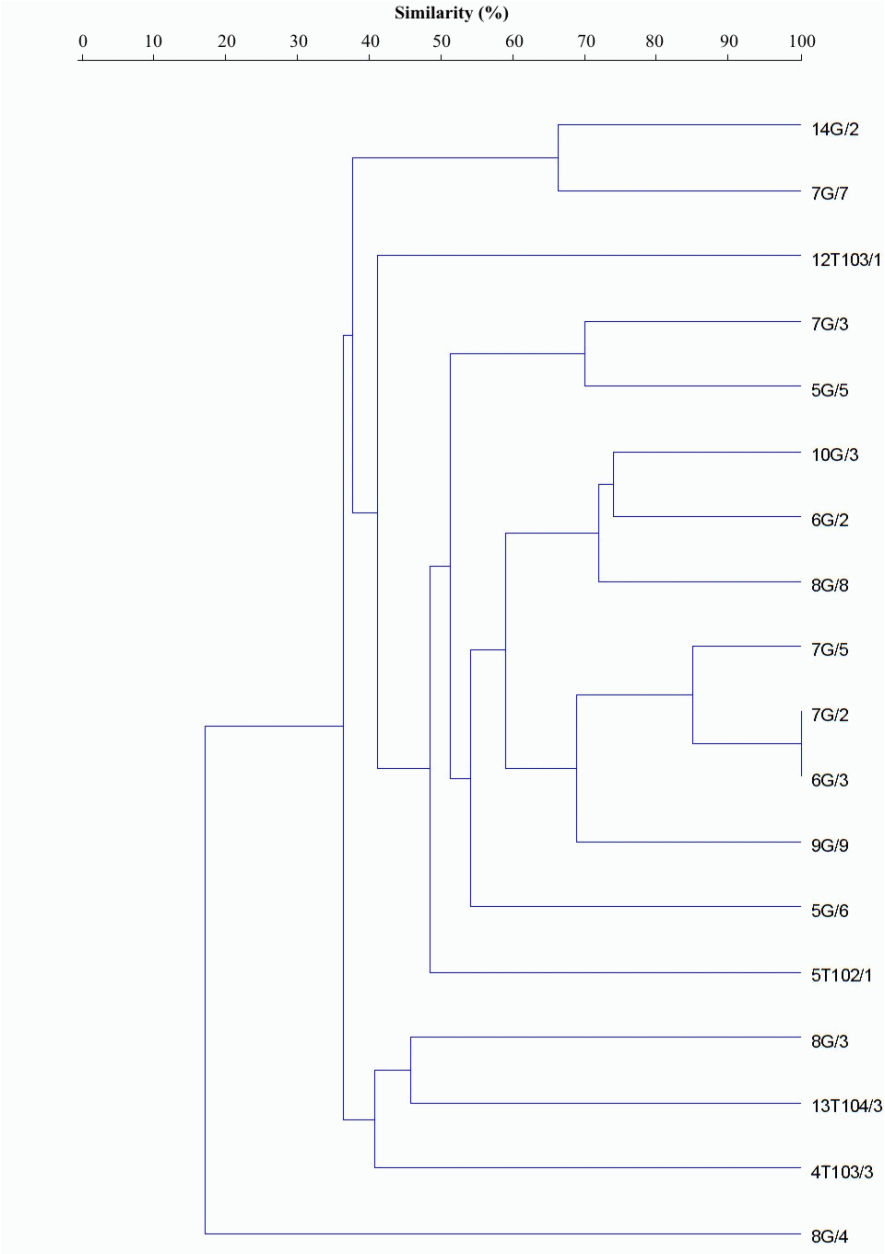

Figure 5. Genetic divergence of the isolated bacterial strains based on 16S ribosomal DNA sequence RFLP analysis.

Conclusion

Many various strains were found during the isolation and characterization of bacteria originating from the nodules of several leguminous plants and from the soil of the rhizosphere. These strains probably represent many species and between these we can found some new species with biotechnological and ecological agricultural importance. The significance of our research consists in the examination for the first time in a natural mountainous habitat of Rhizobia associated with leguminous plants which were not cultivated.

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