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Original scientific paper

CHARACTERIZATION AND MOLECULAR DIVERSITY OF IRANIAN RHIZOBIA ISOLATED FROM FABA BEAN

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The diversity and phylogeny of 30 rhizobia isolated from nodules of faba bean plants grown on 5 geographic regions located in the East Azerbaijan province of Iran were examined using rep-PCR fingerprinting, sequence analysis of 16S rRNA accompanied with *nodC* genes. Based on cluster analysis of rep-PCR fingerprints, faba bean rhizobia isolates were differentiated into five clusters (A to E) at 80% similarity level. The cophenetic correlation coefficient for the dendrogram obtained from the combined dataset of BOX and ERIC primers was 0.942. The percentage of polymorphic loci was 59.2% using the BOX-PCR primer and 67.3% using the ERIC-PCR primers. The data obtained by rep-PCR fingerprinting showed high apparent correlation between genetic diversity and geographical origin of the isolates. The phylogenetic analysis based on 16S rRNA and *nodC* sequences showed that representative isolates were closely related to *R. leguminosarum* bv. *viciae* and *R. fabae*. To the best of our knowledge, this is first report of isolation and characterization of *R. fabae* from Iran.

Key words: Faba bean, NodC gene, Rep-PCR, 16S rRNA

INTRODUCTION

Members of the *Leguminosae* (*Fabaceae*) comprise 17,000 to 19,000 species and play an important ecological role, with representatives in nearly every terrestrial biome on Earth (LEWIS *et al*., 2005). These plants are best characterized by their ability to establish N2-fixing symbiotic associations with Alphaproteobacteria of the genus *Azorhizobium*, *Bradyrhizobium*,

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Mesorhizobium, *Rhizobium*, and *Ensifer* (RIVAS *et al*., 2009; VELAZQUEZ *et al*., 2010), collectively referred to as rhizobia. Other non-rhizobial genera have been shown to nodulate legumes (VALVERDE *et al*., 2005; ZURDO-PINEIRO *et al*., 2007), which can also be nodulated by Betaproteobacteria, specifically, the genera *Burkholderia* and *Cupriavidus* (ANGUS and HIRSCH, 2010; GYANESHWAR *et al*., 2011).

Vicia faba, considered to be one of the nine founder crops (or primary domesticates), is probably native to southwestern Asia (TANNO and WILLCOX, 2006). The amounts of N2-fixed by V. *faba* is estimated to be between 250 and 350 kg ha^{-1} (HERRIDGE *et al*., 2008), with percentage efficiency (66% Ndfa; Nitrogen derived from the air) and fulfills 80% of its nitrogen requirements (ZAPTA *et al*., 1987).

Rhizobia require several classes of specific genes for formation an effective symbiosis. These include *nod* genes, encoding the production of Nod factors, which stimulate the plants to produce symbiotic nodules, and *nif* genes, which produce the nitrogen-fixing nitrogenase enzyme. The *nod* genes are unique to rhizobia, and the *nodA*, *nodB* and *nodC*, which are found in all rhizobia, are all functionally similar among rhizobial species. These genes are involved in the very early stages of symbiosis and all three genes are necessary for nodulation (TRIPLETT and SADOWSKY, 1992). As a result of conservation and ubiquity, *nodC* gene has been used for phylogenetic studies of rhizobia (PEIX *et al*., 2015; ROUHRAZI *et al*., 2016). Until recently, *Vicia*-nodulating rhizobia were presumed to be rather host specific, with two described rhizobial species, *Rhizobium leguminosarum* (KUYKENDALL, 2005) and *R. fabae* (TIAN *et al*., 2008). However, in recent years, several unclassified strains were isolated from different *Vicia* species in different continents (SANTILLANA *et al*., 2008; TIAN *et al*., 2008, 2010; SAIDI *et al*., 2014).

The genetic diversity of *Vicia* rhizobia has been addressed in a number of studies (LAGUERRE *et al*., 2003; TIAN *et al*., 2007, 2010; SAIDI *et al*., 2013). However, our knowledge about the phenotypic and genotypic features of *Vicia* root-nodulating bacteria in Iran is very poor (SERAJZADEH and KHODAKARAMIAN, 2014). Genomic fingerprinting by PCR amplification, with primers specific to the highly conserved, repetitive elements such as, enterobacterial repetitive intergenic consensus (ERIC) and BOX, was used successfully to characterize a large number of bacteria and differentiate closely related strains of bacteria (VERSALOVIC *et al*., 1991, 1994). Repetitive DNA polymerase chain reaction based fingerprinting (rep-PCR) is a rapid, low-cost, and reliable diagnostic method that has been extensively used to assess the genetic diversity of rhizobia strains (GRANADA *et al*., 2014; ROUHRAZI and KHODAKARAMIAN, 2015; ROUHRAZI *et al*., 2016). The identification of indigenous rhizobial strains well adapted to local environmental stresses and edaphic characteristics with the potential to serve as inoculants of *V. faba* in Iranian soils could have a considerable economic and environmental impact. Therefore, the aims of this study were: (1) the isolation of rhizobial strains from nodules of *V. faba* growing in East Azerbaijan province of Iran; (2) the analysis of their diversity based on rep-PCR patterns; (3) the analysis of their phylogenetic diversity based on sequencing of 16S rRNA and *nodC* genes.

MATERIALS AND METHODS

Isolation of root-nodule bacteria

Nodules from *Vicia* roots were collected from five geographic regions (Maragheh, Bonab, Malekan, Ajabshir, Azarshahr) belonged to East Azerbaijan province of Iran in year 2016. The nodules were first rehydrated for 30 min in sterile distilled water, followed by surface sterilization by 2 min immersion in 3 % NaClO. They were then washed for 2 min by immersion

and vortexing in sterile distilled water. For the isolation of bacteria from nodules, a single nodule was crushed in 50 µl of sterile water using a homogenizer. A loop of the suspension was streaked on yeast-extract mannitol (YEM) agar plates (VINCENT, 1970) and the plates incubated at 28 °C for 3–5 days. Two colonies of each morphological type were selected for further testing. The bacteria were purified by repeated streaking on YEM agar plates. Single colonies were preserved either on agar slants at 4°C or frozen in YEM broth with 30% glycerol at −80°C until further analysis. In this study, the strains *Rhizobium leguminosarum* USDA 2370 and *R. fabae* CCBAU 33202, kindly provided by Prof. Dr. Encarna Velázquez, University of Salamanca, Spain, were used as references.

Nodulation test

All bacterial isolates were tested for nodule formation with *V. faba* L. cv. Barekat seeds. Seeds were surface sterilized in 70% ethanol (1 min) and 3% NaClO (3–5 min) and washed at least six times with sterile distilled water in order to completely remove the disinfectant. After imbibing 3 h in sterile water, seeds were transferred aseptically to 1% water agar plates and allowed to germinate for 2 days at room temperature in the dark. Three seedlings were transferred into each pot filled with a mixture of autoclaved sand and perlite (2:1, v:v), and each seedling was inoculated with 1 mL of bacterial culture grown in YEM broth to the exponential phase. The seedlings were grown in a growth chamber under a 12-h day/12-h night regime. Un-inoculated plants served as negative controls. Plants were watered alternatively with sterilized Jensen's nitrogen-free medium and distilled sterilized water according to the procedure described by VINCENT (1970). After 8 weeks, plants were uprooted and the nodulation status of roots and appearance of the plants checked.

Template DNA preparation

Bacteria were grown on nutrient agar at 27°C for 48 h. Bacterial suspensions (10^8 CFU mL^{-1}) were lysed in sterile distilled water by the addition of 1:10 volume of 3% KOH and subsequent heating at 95°C for 2 min, followed by cooling on ice. The lysates were centrifuged at 8000 g for 2 min, and the supernatants were used directly for the PCR assays or stored at −20°C until further analysis (ROUHRAZI *et al*., 2016).

BOX and ERIC- PCR pattern analyses

Repetitive element sequence-based PCR (rep-PCR) was performed using the BOX primer (BOXA1R) and the ERIC primers (ERIC-1R/2) (VERSALOVIC *et al*., 1991, 1994). PCR cycling was performed in a final volume of 25 µl containing 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, 2.5 µl of 10× buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.4), 1.25 U Taq DNA polymerase (CinnaGen, Tehran, Iran), and 3 µl of template DNA. The PCR amplification program consisted of an initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C or 51°C for 1 min with the ERIC and BOX primers, respectively, and extension at 72°C for 1 min, with a final elongation for 10 min at 72°C. All isolates were used in PCR tests, and each reaction was repeated at least twice. The ERIC- and BOX-PCR-amplified products were electrophoresed in 3% agarose gels in TEB buffer (90 mM Trisbase, 2 mM EDTA, 90 mM boric acid, pH 8.3) at 85 V and stained with ethidium bromide (0.5 µg mL^{-1}). Gels were photographed under UV light.

Cluster analyses of the results of the ERIC- and BOX-PCR fingerprinting assays were performed. For each isolate, the presence or absence of each band was determined and designated (1) if present or (0) if absent in order to obtain binary banding data. Similarity matrices from the binary banding data of each of the three primers were derived with the Similarity for Qualitative Data Program (SIMQUAL) in the Numerical Taxonomy and Multivariate analysis System for personal computer (NTSYS-pc) version 2.0 (ROHLF, 1993). Estimates for similarity were based on Jaccard's coefficient (SNEATH and SOKAL). Matrices of similarity were analyzed using the un-weighted pair group method with arithmetic averages clustering method using NTSYS software.

Sequence analyses of 16S rRNA and nodC genes

For amplification of 16S rRNA in the nuclear ribosomal DNA of rhizobia, primers used in this study were fD1 and rP2, which are broad-range 16SrRNA primers designed by WEISBURG et al. (1991). PCR was performed with a total volume of 50 μL containing 1 μL of 30 ng DNA, 2.5 mL of 2.5 mM dNTP, 1 μL of 100 pmol of each primer, 5 μL of 10X buffer (500 mM KCl, 100 mM Tris-HCl (pH 8.0)), 0.5 μL of 1.5 U Taq polymerase (Cinagene, Iran), and 40 μL of double distilled H_2O. The amplification was performed as follows: an initial denaturation step of 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min. After 30 cycles were completed, the samples were incubated at 72°C for 10 min.

The *nodC* gene was partially amplified using the primer pair nodCF–nodCI, according to the PCR procedure described by LAGUERRE *et al*. (2001). The PCR mixture employed was the same as described for the amplification of 16S rDNA in 50 μL volumes. The sequences obtained were compared with those from GenBank, using the BLASTN program (ALTSCHUL *et al*., 1990), and EzTaxon-e server (KIM et al., 2012). These sequences and those of related bacteria were aligned using the Clustal W program (THOMPSON *et al*., 1997). The distances were calculated according to Kimura's two-parameter model (KIMURA, 1980). The phylogenetic trees were inferred using the neighbour joining model and MEGA5.0 (Mega, Molecular Evolutionary Genetics Analysis, Pennsylvania, USA) (TAMURA, *et al*., 2011) was used for all the phylogenetic analyses.

RESULTS

Isolation of rhizobia

A total of 30 rhizobia were isolated from five sites located mainly in northwestern of Iran. The bacterial colonies grown in YEM agar were gummy, translucent, circular and convex with entire smooth margins. The cells were Gram negative and rod-shaped under the light microscope. All isolates were able to induce effective nodules in *V. faba* cv. Barekat.

Diversity of the strains based on BOX and ERIC patterns

Banding patterns of isolates were generated using the BOXA1R and ERIC primers (Fig. 1). The bands amplified in the ERIC-PCR ranged in length from 200 to 3000 bp and those amplified in the BOX-PCR ranged from 100 to 2750 bp. Reproducible fingerprint profiles were generated with each technique upon repetition of the procedures. *Vicia* rhizobia isolates were differentiated into five clusters (A–E) at the 80 % similarity level (Fig. 1). Cluster A includes seven isolates from the Ajabshir region (A), cluster B includes three isolates (B) from the Bonab region, cluster C includes 3 nodulating isolates (Mi) from the Malekan region, cluster D consists

of 15 isolates (M) from the Maragheh region in addition to *R. leguminosarum* USDA 2370, cluster E includes 2 isolates (Az1 and Az2) from the Azarshahr region in addition to *R. fabae* reference strain CCBAU 33202.

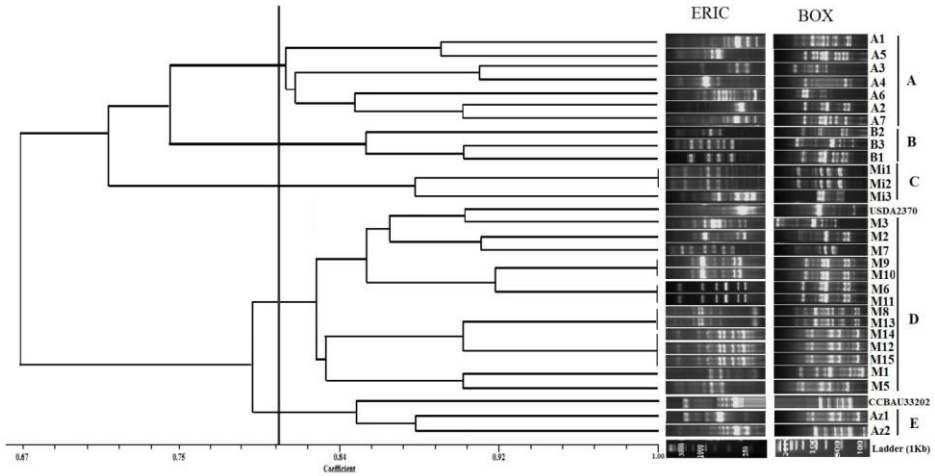

Figure 1. Dendrogram obtained for the isolates of *V. faba* isolated in Iran using Jaccard's coefficient and UPGMA analysis of the ERIC and BOX profiles.

The cophenetic correlation coefficient for the dendrogram obtained from the combined dataset was 0.942. The percentage of polymorphic loci was 59.2 % using the BOX-PCR primers and 67.3 % using the ERIC-PCR primers. These results demonstrated the existence of a considerable genetic diversity among root-nodulating bacteria isolated from *V. faba* in northwest of Iran.

16S rRNA gene sequencing and analysis

The current classification of the rhizobia is based on their 16S rRNA gene sequences (KUYKENDALL, 2005) and thus, they were obtained from a representative strain of each BOX and ERIC groups and compared with those held in GenBank. After comparing with the available sequences of other related taxa representatives, a dendrogram generated by neighbor-joining was created (Fig. 2). The results showed that the closest relative of the strains A1, B2, Mi2 and M1, representing ERIC and BOX clusters A, B, C and D, respectively, was *R. leguminosarum* USDA2370 with 99.9% similarity. The 16S rRNA gene of the strains Az1 and Az2 showing ERIC and BOX type E was identical to that of *R. fabae* CCBAU 33202. The sequences have been deposited in the GenBank database under the accession numbers from KY038064 to KY038069.

Analysis of nodC gene

Based on ERIC and BOX clusters, 6 isolates were selected (i.e. M1, B2, A1, Mi2 Az1, and Az2) and the 850 bp length of their *nodC* sequence was determined. The sequences have

been deposited in the GenBank database under the accession numbers from KY056641 to KY056646. After comparing with the available sequences of other related taxa representatives, a dendrogram generated by neighbor-joining was created (Fig. 3). Representative isolates A1, B2, Mi2 and M1 were phenogenetically very close to *R. leguminosarum* bv. *viciae* and Az1 and Az2 were close to *R. fabae.*

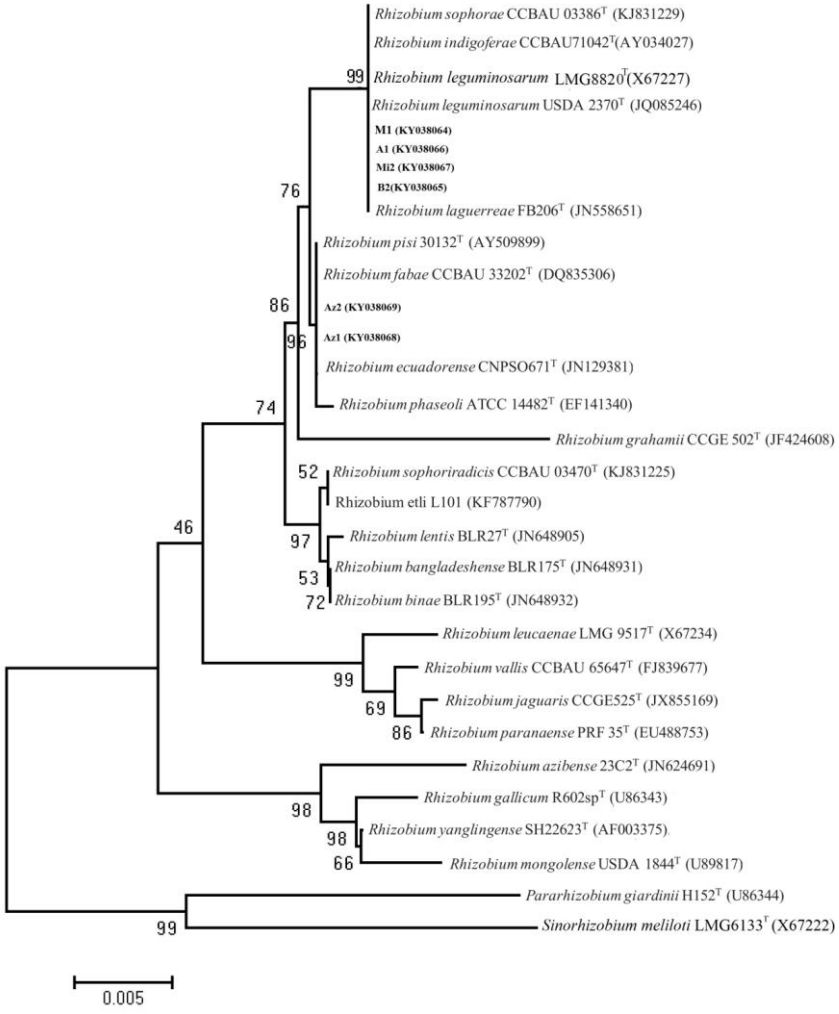

Figure 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences of the representative rhizobia isolated in this study, their phylogenetically related species and strains nodulating *V. faba* in other countries. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 1000 nt. Accession numbers from GenBank are given in brackets.

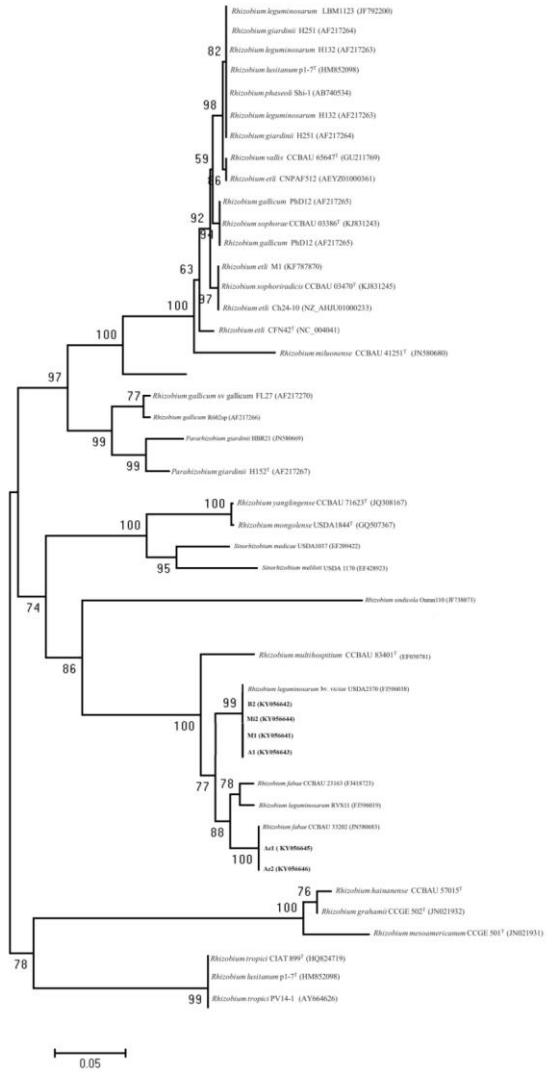

Figure 3. Neighbour-joining phylogenetic tree based on *nodC* gene sequences of the representative rhizobia isolated in this study, their phylogenetically related species and strains nodulating *V. faba* in other countries. Bootstrap values calculated for 1000 replications are indicated. Bar, 5 nt substitution per 100 nt. Accession numbers from GenBank are given in brackets.

DISCUSSION

Faba bean is a cultivated legume from tribe *Viciae* that constitutes an important crop in all continents and which fixes atmospheric nitrogen in symbiosis with fast-growing rhizobial species from the genus *Rhizobium* (KUYKENDALL, 2005). To date, only limited studies have been

carried out in Iran on the characterization of *Vicia* nodulating bacterial isolates (SERAJZADEH and KHODAKARAMIAN, 2014). The aim of this study was, therefore, to expand our knowledge of the characterization and assessment of genotypic diversity of *Vicia* root nodulating bacteria in Iran. In this study, root nodules of faba bean were collected from different geographic regions of Northwestern Iran, including the Maragheh, Bonab, Malekan, Azarshahr and Ajabshir. All the strains showed positive nodulation under sterilized conditions indicating that all the isolates are rhizobia.

Rep-PCR profiles may be very useful for strain identification (LAGUERRE *et al*., 1996), and in this study, we examined the applicability of the ERIC and BOX-PCR methods for the characterization of root-nodule bacteria isolated from faba bean plants growing in Iran. Based on the combination data set of both BOX-PCR and ERIC-PCR, the majority of isolates i.e. 50% were present in cluster D followed by 23.3% in cluster A. These clusters were further subdivided into subclusters. These results indicated a considerable genetic diversity of Iranian rhizobia isolated from faba bean. Similarly, large number of groups and sub-clusters of rhizobia infecting faba bean have been discovered using random BOX profiles in China (TIAN, *et al*., 2007).

Banding patterns of all 30 faba bean rhizobial isolates showed the presence of 16 and 13 polymorphic bands by ERIC and BOX-PCR methods, respectively. The reproducibility of each PCR amplification profile was confirmed by repeating the amplification at least two times. The data obtained from the rep-PCR fingerprinting showed high apparent correlation between genetic diversity and geographical origin of the isolates. That the factors of the geographical origin affect the distribution and diversity of rhizobia is the physical and chemical properties. The soil pH of different regions of Iran is not the same. Therefore, the rhizobia population showed evident difference.

In this study based on sequence analysis of *nodC* and 16S rRNA genes, representative isolates M1, A1, Mi2 and B2 were phenogenetically very close to *R. leguminosarum* and Az1, Az2 were close to *R. fabae*. In previous study, SERAJZADEH and KHODAKARAMIAN (2014) showed that soils of Iran contained very effective or ineffective isolates of faba bean nodulating rhizobia with a great genetic diversity based on 16S rRNA gene analysis. They reported, to better evaluate the diversity of faba bean rhizobia and the phylogeny of strains isolated from Iran, further studies are required.

The amplification of *nodC* gene by using specific PCR primers confirms that this approach is a reliable and fast method for the identification of rhizobial strains belonging to biovar *viciae*, as previously reported (MOSCHETTI *et al*., 2005; VENTORINO *et al*., 2007). The nodulation genes have clearly been transferred many times between sublineages of *Rlv* and also between this species and *R. fabae*; in this respect, they are typical representatives of the bacterial accessory gene pool (YOUNG *et al*., 2006). The consequence is that the phylogeny of the nodulation genes is almost independent of that of the chromosomal markers (KUYKENDALL, 2005). LAGUERRE *et al*. (2001) used the *nodC* gene, a common nod gene essential for nodulation in all rhizobial species, to characterize a collection of 83 rhizobial strains which represented 23 recognized species distributed in the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Bradyrhizobium*.

In this study based on phenotypic and genotypic characteristics, 28 and 2 Iranian strains were identified as *R. leguminosarum* and *R. fabae*, respctively. To the best of our knowledge, this is the first report of isolation and characterization of *R. fabae* from Iran. Dominant presence of *R. leguminoarum* was also reported in Europe (LAGUERRE *et al*., 2003; VENTORINO *et al*.,

2007), Peru (SANTILLANA *et al.*, 2008), Jordan (MUTCH *et al*., 2003), China (TIAN *et al*., 2010) and Tunisia (SAIDI *et al*., 2013).

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KARAKTERIZACIJA I MOLEKULARNA RAZNOVRSNOST SOJEVA RHIZOBIA IZOLOVANIH IZ IRANSKOG BOBA

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Izvod

U radu je proučavana raznovrsnost i filogenija 30 sojeva Rhizobia izolovanih iz kvržica biljaka boba, gajenih u 5 geografskih regiona u provinciji Istočni Azerbejdžan u Iranu, upotrebom rep-PCR-a, analize sekvenci 16S rRNA zajedno sa *nodC* genima. Na osnovu klaster analize, izolati su se diferencirali u pet klastera (A to E) sa 80% sličnosti. Zastupljenost polimorfnih lokusa bila je 59,2% korišćenjem prajmera BOX-PCR i 67,3% sa prajmerom ERIC-PCR. Dobijeni podaci pokazuju visoku korelaciju između genetičkog diverziteta i geografskog porekla izolata. Filogenetska analiza zasnovana na 16S rRNA i *nodC* sekvencama pokazala je da su reprezentativni izolati veoma srodni *R. leguminosarum* bv. *viciae* i *R. fabae*. Prema našim saznanjima, ovo je prvi primer izolovanja i karakterizacije *R. fabae* iz Irana.

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