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Molecular Characterization of *Rhizobium* Strains Isolated from Wild Chickpeas Collected from High Altitudes in Erzurum-Turkey

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Abstract

The fixation of N_2 by legumes plays key role in agricultural sustainability. Moreover, the further assessment of rhizobial genetic diversity is contributing both to the worldwide knowledge of biodiversity of soil microorganisms and to the usefulness of rhizobial collections, and it is developing long-term strategies to increase contributions of legume-fixed to agricultural productivity. In the last decades, the use of molecular techniques has been contributed greatly to enhance the knowledge of rhizobial diversity. This study was conducted to determine the phenotypic and genotypic differences in Rhizobium leguminosarum subsp. ciceri strains isolated from perennial wild chickpeas (Cicer anatolicum) from high altitudes (2000-2500 m) in mountains of Erzurum, Eastern Anatolia, Turkey. In this study, rep-PCR (ERIC-, REP- and BOX-PCR) fingerprinting methods were used for the genotypic characterization and phylogenetic analysis of Rhizobium leguminosarum subsp. ciceri strains isolated from perennial wild chickpeas. The results showed a high intraspecies diversity among the strains in terms of rep-PCR (ERIC-, REP- and BOX-PCR) profiles.

Keywords: *Rhizobium leguminosarum* subsp. *ciceri*, phenotypic characterization, genotypic characterization, REP-PCR, ERIC-PCR, BOX-PCR

Introduction

Legumes play an important role in sustainable management of dry arid. Rhizobia have been widely used in agricultural systems for enhancing the ability of legumes to fix atmospheric nitrogen [1].

Nitrogen was known to be an essential nutrient for plant growth and development. Intensive farming practices that accomplish high yields need chemical fertilizers, which are not only cost effective but also may create environmental problems. The extensive use of chemical fertilizers in agriculture is currently under debate due to environmental concern and fear for consumers' health. Consequently, there has recently been a growing level of interest in environmentally friendly sustainable agricultural practices and organic farming systems [2,3]. Increasing and extending the role of biofertilizers such as *Rhizobium* would decrease the need for chemical fertilizers and reduce adverse environmental effects. Thus, in the

development and implementation of sustainable agriculture techniques, biofertilization is of major importance in alleviating environmental pollution and the deterioration of nature [4].

The rhizobia, which are widely used in agricultural systems, are represented by 7 genera containing about 40 species as *Alphaproteobacteria: Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* [5] and a species in the genus *Methylobacterium* [6]. Recently, symbiotic nitrogen fixing species have also been defined among the genera *Burkholderia* and *Cupriavidus* within the beta subclass of proteobacteria [7]. The design of the diversity of the rhizobia is however far from clear, particularly thinking the large number of leguminous species and their wide geographical distribution [5].

Since rhizobia are taxonomically very diverse [8], efficient strain classification methods are needed to identify genotypes displaying, such as, superior nitrogen-fixation capacity [9]. Molecular techniques have helped to develop easy and quick methods to microbial characterization including works distinguish genera, species and even strains [10,11]. The polymerase chain reaction (PCR) and the use of primers corresponding to consensus repetitive sequences scattered in the eubacteria genome, thought as enterobacterial repetitive intergenic consensus (ERIC) and enterobacterial repetitive sequences (BOX) can create highly characteristic patterns when distinguished in agarose gels, providing well separation on strain level [12]. ERIC sequences are highly protected among rhizobia genomes and they were used to select and classify different rhizobia strains in population works and to evaluate the environmental effect in defined populations [11].

Recently, wild legumes and their symbionts have drawn the attention of ecologist because of their tolerance to extreme environmental conditions such as severe drought, salinity and elevated temperatures. Addition, symbiotic rhizobia of naturally growing legumes successfully establish effective symbioses under these conditions [13].

The objective of this study was to isolate and characterize the rhizobial populations naturally associated wild legumes originating from different ecological areas by a polyphasis approach including the evaluation of phenotypic properties as well as genotypic characteristics.

Material and Methods

Reference Strain

One reference strain (IFO 14778) was obtained from Institute of Fermentation, Ojaka, Japan, and used in the present study.

Isolation of Rhizobial and morpho-physiological, biochemical characterization of isolates

The root nodules were sum from wild chickpea (host plants were shown in Table 1) in several regions at high altitudes (2000-2500) in Erzurum province, Turkey. From each plant sampled, three to six nodules were at random excised and surface sterilized with ethanol and hydrogen peroxide. Rhizobia were isolated on yeast-extract mannitol agar (YEMA) using standard procedures. Single colonies were marked and checked for purity by repeated streaking on YEMA medium [14] and verifying a single type of colony morphology, absorption of Congo red (0.00125 mg kg^{-1}) and a uniform Gram-stain reaction. Colony morphology (color, mucosity, borders, transparency and elevation) and acid / alkaline reaction were evaluated on YEMA containing bromthymol blue (0.00125 mg kg^{-1}) as indicator [15]. All isolates were incubated at 28^{o}C and stored at -20^{o}C in 25 % glycerol-YEM broth.

DNA Extraction from Pure Cultures

Total genomic DNA was extracted from bacteria samples using a modified method described by Khoodoo *et al*. [12,16].

Genetic characterization

A total of 18 isolates were subjected to rep-PCR genomic fingerprinting using primer sets corresponding to BOX, ERIC, and REP elements [17]. The 18-mer primer pair REP 1R (5'-IIIICGICGICATCIGGC-3') and REP 2 (5'-ICGICTTATCIGGCCTAC-3') (where I is Inosine); ERIC 1R (5'-ATGTAAGCTCCTGGGGAT-3') and ERIC 2 (5'-AAGTAAGTGACTGGGGGT GAGC-3') and BOXA1R (5'-CTACGGCAAGGCGACGCTG

ACG-3') were used to amplify putative REP-, ERIC- and BOX-like elements in bacterial DNA, respectively. Briefly, approximately 50 ng of purified DNA was used as a template in a 30 µl reaction mixture. Twenty seven µl of reaction cocktail was prepared as follows: Gitschier Buffer 5 µl, Dimethyl sulfoxide 2.5 µl (100%), dNTPs (10mM) 1.25 µl, Bovine serum albumin 1.25 µl (20 mg/ml), primer/primers (5µM) 3.0 µl, taq polymerase (250unit) 0.3 µl, water 10.7 µl (for BOX PCR, 13.7 µl). PCR amplification reactions were performed with a Corbett Research Palm Cycler (Corbett CG1-96 AG, Australia) using the following conditions: an initial denaturation at 95°C for 7 min; 30 cycles consisting of 94°C for 1 min and annealing at 40, 52 or 53°C for 1 min with either REP, ERIC or BOX primers, respectively; extension at 65°C for 8 min; and a sing1e final polymerization at 65°C for 15 min before cooling at 4°C.

To ensure consistency in results, PCR was repeated for each isolate for at least three times.

Electrophoresis

The PCR products (10 µl) were mixed with 6xgel loading buffer (3 µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5XTAE (Tris-Acetat- EDTA) buffer at 40 V for 200 min. Amplification products separated by gel was stained in ethidium bromide solution (2 µl EtBr/100ml 1xTAE buffer) for 40 min. The amplified DNA product was detected by using the DNR-Imaging System with UV-soft analysis package (Israel).

Data Analysis

PCR products were scored as presence (1) and absence (0) of band for each of the 6 accessions analyzed. Data were used to calculate a Jaccard (1908) similarity.

All of the experiments in this study were repeated at least twice.

Results and Discussion

Morpho-physiological, biochemical characterization

In the present study, seventeen rhizobial strains (Table 1) were isolated from root nodules of species of wild chickpea (*Cicer anatolicum*) collected from different geographical and ecological areas of Erzurum province, Turkey. All strains tested were found to have circular colonies with regular borders, flat in elevation, creamy in color, showing intermediate to high production of mucus. After 3 to 5 day of growth on YMA at 28^{o}C, all of strains acidified the medium (as indicated by the bromothymol blue) and colony diameter ranged from 2-5mm as informed in Bergey's Manuel [15,18] (Table 2).

Table 1. Isolates, sites and altitudes from where *Rhizobium leguminosarum* ssp. *ciceri* was collected

Isolate number	Locations of isolation	Altitude (m a.s.l.)
HF 2 and HF 4	Telsizler Mountain	2000
HF 10 , AK5, AK6, AK8, AK9, P2	Telsizler Mountain	2250
R.C	Rabat Mountain	2350
HF 176	Hasanbaba Mountain	2350
HF 269, HF 270, HF 274, HF 281, HF 282 ,HF 286 and HF 288	Palandöken Mountains	2500

Table 2. Morphological, biochemical traits of isolates of *Rhizobium leguminosarum* sp. *ciceri*

Isolate No.	Gram stain-reaction	Colony morphology	Colony color	Mucocity	Brom thymol blue with medium colony color	Congo red with medium colony color	Movement	Catalase test	Oxidase test
HF 2	-	bar	creamy	+	yellow	White	+	+	+
HF 4	-	bar	creamy	+	yellow	white	+	+	+
HF 10	-	bar	creamy	+	yellow	white	+	+	+
R.C	-	bar	creamy	+	yellow	white	+	+	+
HF 176	-	bar	white	+	yellow	white	+	+	+
HF 269	-	bar	creamy	+	yellow	white	+	+	+
HF 270	-	bar	white	+	yellow	white	+	+	+
HF 274	-	bar	creamy	+	yellow	white	+	+	+
HF 281	-	bar	creamy	+	yellow	white	+	+	+
HF 282	-	bar	creamy	+	yellow	white	+	+	+
HF 286	-	bar	creamy	+	yellow	white	+	+	+
HF 288	-	bar	creamy	+	yellow	white	+	+	+
AK5	-	bar	creamy	+	yellow	white	+	+	+
AK6	-	bar	creamy	+	yellow	white	+	+	+
AK8	-	bar	creamy	+	yellow	white	+	+	+
AK9	-	bar	creamy	+	yellow	white	+	+	+
P2	-	bar	creamy	+	yellow	white	+	+	+

Rep-PCR genomic fingerprinting analysis

The rep-PCR genomic fingerprints showed that bacterial strains have distinct patterns with 4–9 fragments in the size of 300–4500 bp and faint bands frequently observed. The ERIC primer set generated a reproducible and differentiating fingerprints including 5–14 fragments of 300–4000 bp. BOXA1R PCR fingerprint revealed 4–11 fragments ranged from 400 to 2500 bp (Figures 1-3). In general, the fingerprints generated with the ERlC derived DNA fingerprints showed the highest genetic polymorphism with compared to REP- and BOX-fingerprints. Similar data reported in other studies showing that rep-PCR performed with REP primers was less reliable than PCR performed with enterobacterial repetitive intergenic consensus (ERIC) primers for differentiating among *E. coli* strains from various sources [19]. Overall, our results suggested that when primer ERIC was used, the rep-PCR technique produced the highest number of polymorphic bands, which classified bacterial strains into 5 different clusters (Figure 4). The largest cluster represented by fourteen strains tested in this study has 86-96% similarity. The remaining each of four clusters is represented by only one strain.

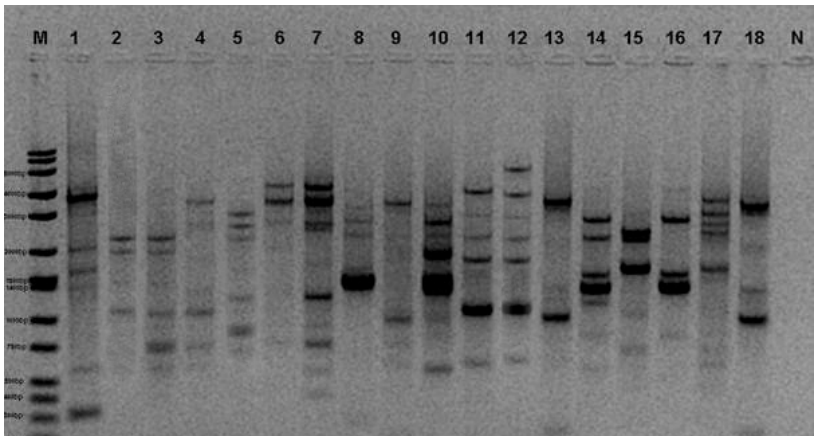

Figure 1. REP-PCR profile generated with the REP 1R and REP 2 primers. Lanes: 1) AK9; 2) AK5; 3) P2; 4) HF288; 5) AK8; 6) AK6; 7) HF4; 8) HF270; 9) HF269; 10) HF274; 11) HF286; 12) HF2; 13) HF10; 14) HF176; 15) HF282; 16) HF281; 17) R.C; 18) IFO; N; Negative Control; M) Molecular Marker (10kb)

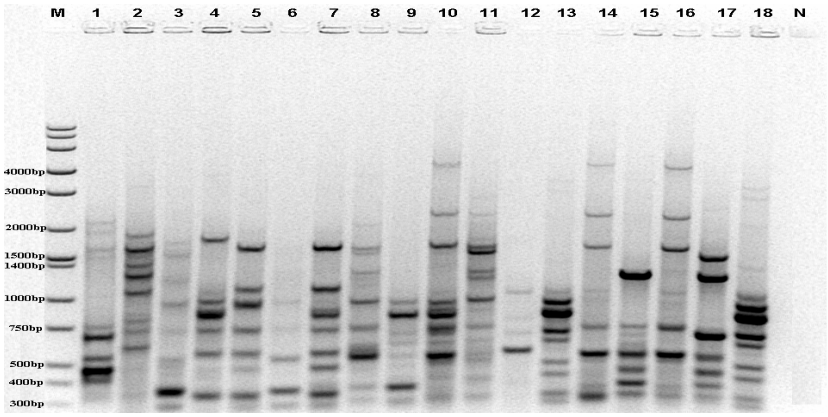

Figure 2. ERIC-PCR profile generated with the ERIC 1R and ERIC 2 primers. Lanes: 1) AK9; 2) AK5; 3) P2; 4) HF288; 5) AK8; 6) AK6; 7) HF4; 8) HF270; 9) HF269; 10) HF274; 11) HF286; 12) HF2; 13) HF10; 14) HF176; 15) HF282; 16) HF281; 17) R.C; 18) IFO; N; Negative Control; M) Molecular Marker (10kb)

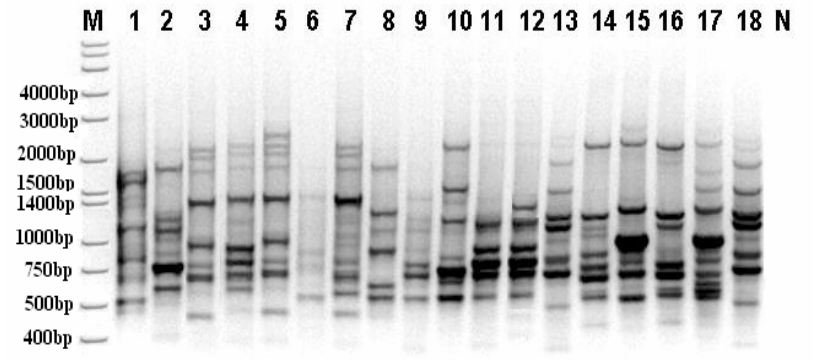

Figure 3. BOX-PCR profile generated with the BOX A1R primer. Lanes: 1) AK9; 2) AK5; 3) P2; 4) HF288; 5) AK8; 6) AK6; 7) HF4; 8) HF270; 9) HF269; 10) HF274; 11) HF286; 12) HF2; 13) HF10; 14) HF176; 15) HF282; 16) HF281; 17) R.C; 18) IFO; N; Negative Control; M) Molecular Marker (10kb)

With respect to the cluster analysis data, 70-96% of similarity ratio was found between the reference strain (IFO 14778) and *Rhizobium leguminosarum* subsp. *ciceri* strains tested in this study (Figure 4). Our data supported the previous studies in which rep-PCR genomic

fingerprinting is an adequate technique for differentiating rhizobial strains [20-22], and many other closely related sub(species) or strains and/or determining phylogenetic relationship [23,24].

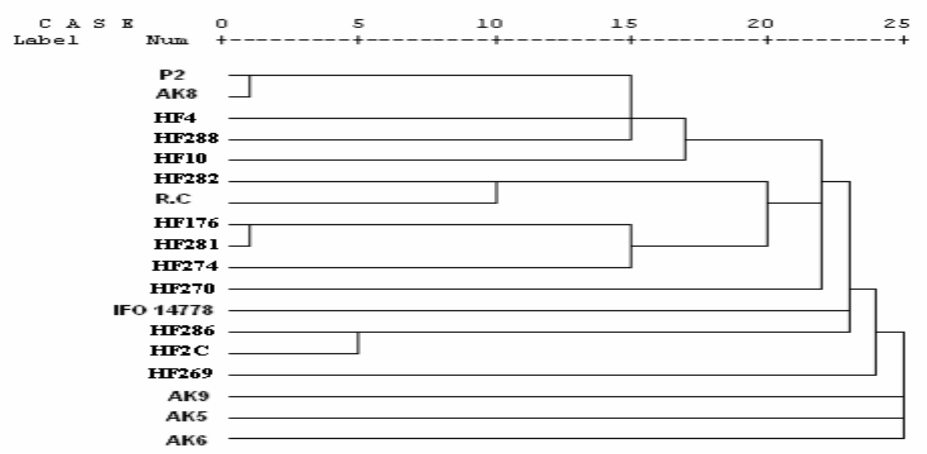

Figure 4. ERIC-PCR Cluster Analyses

The results in the present study demonstrated that all REP PCR fingerprints performed with ERIC-, REP-, and BOX primers are sensitive and reliable for identification and characterization of *Rhizobium leguminosarum* subsp. *ciceri* strains isolated from the chickpea plant species. ERIC-PCR was confirmed to be the best fingerprinting method for determination of genomic diversity among *Rhizobium leguminosarum* subsp. *ciceri* strains. Therefore, rep-PCR (ERIC-, REP- and BOX-PCR) fingerprinting methods could be a good choice for the genotypic characterization and phylogenetic analysis of *Rhizobium leguminosarum* subsp. *ciceri* strains isolated from perennial wild chickpeas.

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