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Isolation and Characterization of *Rhizobium* Strains from Wild Vetch Collected from High Altitudes in Erzurum-Turkey

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Abstract

Recently, there has been a growing level of interest in environmental friendly sustainable agricultural practices and organic farming systems. 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 big importance in alleviating the deterioration of natural and environmental pollution. Besides, the assessment of rhizobial genetic diversity is contributing both to the worldwide knowledge of the biodiversity of soil microorganisms and to the utility of rhizobial collections. Particularly, in the last decades, the use of molecular techniques has been contributed greatly to enhance the knowledge of rhizobial diversity. This study was conducted in order to determine the phenotypic and genotypic characterization of Rhizobium leguminosarum subsp. viciae strains that were isolated from perennial wild vetch (Vicia cracca) collected from high altitudes (2000-2500 m) in mountains of Erzurum, Eastern Anatolia, Turkey. In this work, rep-PCR (ERIC-, REP- and BOX-PCR) fingerprinting method was used for the genotypic characterization of R. leguminosarum subsp. viciae strains isolated from perennial wild vetch. As a result, a high intraspecies diversity was observed in the rep-PCR (ERIC-, REP- and BOX-PCR) analysis with BOX, ERIC and REP primers between R. leguminosarum subsp. viciae strains.

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

Introduction

Legume plants are very significant not only ecologically but also agriculturally because they are responsible for major change of nitrogen from atmospheric N_2 to ammonia [1]. Worldwide, some 44-46 million tons of N_2 are fixed annually, providing approximately half of all N used in agriculture. [2].

Vetch *(Vicia sativa)* has an important potential in terms of fodder production, and it is grown under both arid and wet conditions in every region of Turkey. Additionally, it is produced as dry and wet grass, and it is used as grain fodder plant and as green fertilizer plant in soil improvement [3]. Vetch is nodulated by the strains of *Rhizobium leguminosarum* subsp. *viciae.* The same rhizobia also form nodules with other legumes grown in Turkey,

including vetch *(Vicia sativa* L.*),* lentil *(Lens culinaris* Medik*)* and the infrequently grown grass pea *(Lathyrus sativus* L.*)* [4]. Additionally, symbiotic rhizobia of naturally growing legumes successfully establish effective symbioses under these conditions [5]. Temperate climates are characterized by short growing seasons, which are exposing temperatures to below the optimal temperature for symbiotic nitrogen fixation [6]. Cool soil temperatures in temperate climates affect the competitiveness of rhizobia for nodulation, retard the infection of root hairs, decrease nodulation, and depress nodule activity [7]. However, the strain of *Rhizobium* plays an important role in determining the efficiency of nitrogen fixation at low temperatures [8], and a variation exists among *Rhizobium* strains in terms of the ability of nodulating under cool conditions [9]. Most of the bacteria isolated from cold environments are able to grow and to show earlier nodulation and higher nitrogenase activity at low temperatures [10].

The rhizobia widely used in agricultural systems, are represented by approximately 40 species in seven genera including *Alphaproteobacteria: Allorhizobium*, *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium* [11] and a species in the genus of *Methylobacterium* [12]. Recently, symbiotic nitrogen fixing species have also been defined among the genera *Burkholderia* and *Cupriavidus* within the beta subclass of Proteobacteria [13]. The design of the diversity of the rhizobia is however far from clear, particularly due to the large number of leguminous species and their wide geographical distribution [11].

Since rhizobia are taxonomically very diverse [14], efficient strain classification methods are needed to identify genotypes displaying, such as, superior nitrogen-fixation capacity [15]. Recent advances in molecular biology and gene technology are creating exciting possibilities for the rapid and accurate identification and characterization of microorganisms including rhizobia [16-17]. The polymerase chain reaction (PCR) and the use of primers corresponding to consensus repetitive sequences scattered in the Eubacteria genome, which are thought as enterobacterial repetitive intergenic consensus (ERIC) and enterobacterial repetitive sequences (BOX), can create highly characteristic patterns when distinguished in agarose gels, which are providing well separation on strain level [18]. ERIC sequences are highly protected among rhizobia genomes, and they were used to distinguish and classify different rhizobia strains in population works, and to evaluate the environmental effect in defined populations [17].

The objective of this experiment was to isolate and characterize the rhizobial populations naturally associated with wild vetch (*Vicia cracca*) originating from different ecological areas by a polyphase approach including the evaluation of phenotypic properties as well as genotypic characteristics.

Material and Methods

Morphological, physiological, and biochemical characterization of Rhizobial strains

The root nodules were obtained from wild vetch (host plants were shown in Table 1) grown 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 [19]. Single colonies were marked and checked for purity by repeated streaking on YEMA medium [19] 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 (mucoid), borders, transparency and elevation) and acid

/ alkaline reaction were evaluated on YEMA containing bromothymol blue (0.00125 mg kg^{-1}) as indicator [20] (Table 2). All strains were incubated at 28°C and stored at -20°C in 25% glycerol-YEM broth.

DNA Extraction from Pure Cultures

Total genomic DNA was extracted from bacteria samples using a modified method previously described by Adiguzel [18].

Genetic characterization

A total of 10 strains were subjected to rep-PCR genomic fingerprinting using primer sets corresponding to BOX, ERIC, and REP elements [21]. 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'-AAGTAAGTGACTGGGGGTGAGC-3') and BOX A1R (5'-CTACGGCAAGGCGACGCTGACG-3') were used to amplify putative REP-, ERIC- and BOX-like elements in bacterial DNA, respectively. Briefly, approximately 50ng of purified DNA was used as a template in a 30µl reaction mixture. 27 µl of reaction cocktail was prepared as follows: Gitschier Buffer 5µl, Dimethyl sulfoxide 2.5µl (100%, 20X), dNTPs (10mM) 1.25µl, Bovine serum albumin 1.25µl (20mg/ml), primer/primers (5µM) 3.0 µl, *Taq* polymerase (250unit) 0.3µl, water 10.7µl (for BOX PCR, 13.7µl). 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 strain for at least three times.

Electrophoresis

The PCR products (10µl) were mixed with 6X gel loading buffer (3µl) and loaded onto an agarose (1.5% w/v) gel electrophoresis in 0.5X TAE (Tris-Acetate- EDTA) buffer at 40 V for 200 min. Amplification products separated by gel was stained in ethidium bromide solution (2µl 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

Morphological, physiological and biochemical characterization

In the present study, 31 strains were isolated from the root nodules of species of wild vetch (*Vicia cracca*) collected from the different geographical and ecological areas of Erzurum province, Turkey (Table 1). Ten strains among 31 tested bacteria were found to be having 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°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 Manual [22] (Table 2).

Table 1. Isolate number, origin and altitudes from where *Rhizobium leguminosarum* subsp. *viciae* was collected

Isolate number	Origin	Altitude (m a.s.l.)
HF 20, HF 22, HF 147, HF 149, HF 157 and HF 163	Telsizler Mountain	2100
HF 201, HF 203, HF208 and HF209	Deveboynu locality	2000
HF 30 and HF 32	Kayakyolu locality	2250
HF 134, HF 135, HF 138, HF 140 and HF 143	Palandöken Mountains	2500
HF 160	Yıldırım Mountains	2350
HF 13 and HF 16	Köşk Mountain	2400
HF 62 and HF 74	Turnagöl Mountain	2250
HF 85, HF 86, HF 92 and HF 99	Alibaba Mountain	2350
HF 129, HF130 and HF 173	Hasanbaba Mountain	2100
HF 251	Eğerli Mountain	2300
HF 365	Çubuklu Mountain	2400

Table 2. Morphological and biochemical characteristics of *Rhizobium leguminosarum* subsp. *viciae* strains.

Isolate No.	Gram stain-reaction	Cell morphology	Colony color	Mucocity	Brom thymol blue with medium colony color	Congo red with medium colony color	Movement	Catalase test	Oxidace test
HF 22	-	bar	creamy	+	yellow	white	+	+	+
HF 134	-	bar	creamy	+	yellow	white	+	+	+
HF 140	-	bar	creamy	+	yellow	white	+	+	+
HF 157	-	bar	creamy	+	yellow	white	+	+	+
HF 160	-	bar	creamy	+	yellow	white	+	+	+
HF 163	-	bar	creamy	+	yellow	white	+	+	+
HF 201	-	bar	creamy	+	yellow	white	+	+	+
HF 203	-	bar	creamy	+	yellow	white	+	+	+
HF 208	-	bar	creamy	+	yellow	white	+	+	+
HF 209	-	bar	creamy	+	yellow	white	+	+	+
HF 13	-	bar	orange	-	white	red	-	-	-
HF 62	-	bar	yellow	-	white	red	-	-	-
HF 129	-	bar	orange	-	white	red	-	-	-
HF 147	-	bar	orange	-	white	red	-	-	-
HF 143	-	bar	yellow	-	white	red	-	-	-
HF 130	-	bar	white	-	white	red	-	-	-
HF 92	-	bar	yellow	-	white	red	-	-	-
HF 85	-	bar	orange	-	white	red	-	-	-
HF 16	-	bar	yellow	-	white	red	-	-	-
HF 32	-	bar	orange	-	white	red	-	-	-
HF 20	-	bar	white	-	white	red	-	-	-
HF 74	-	bar	orange	-	white	red	-	-	-
HF 30	-	bar	yellow	-	white	red	-	-	-
HF 99	-	bar	creamy	-	white	red	-	-	-
HF 86	-	bar	white	-	white	red	-	-	-
HF 149	-	bar	white	-	white	red	-	-	-
HF 135	-	bar	yellow	-	white	red	-	-	-
HF 365	-	bar	white	-	white	red	-	-	-
HF 138	-	bar	white	-	white	red	-	-	-
HF 173	-	bar	white	-	white	red	-	-	-
HF 251	-	bar	white	-	white	red	-	-	-

rep-PCR genomic fingerprinting analysis

The REP-PCR genomic fingerprints showed that bacterial strains have distinct patterns with 1–6 fragments in the size of 550–4000 bp and in frequently observed faint bands. The ERIC primer set generated a reproducible and differentiating fingerprints including 3–8 fragments of 200–4500bp. BOXA1R PCR fingerprint revealed 4–9 fragments ranged from 300 to 2700bp (Figures 1-3). A high level of genetic diversity was determined within *R. leguminosarum* bv. *viciae* populations with ERIC- and BOX-PCR analysis. Both methods (ERIC- and BOX-PCR) showed very similar, almost identical, grouping of strains which demonstrated that these methods are reliable and suitable for rhizobial strain identification. And, by the result of REP-PCR, HF209 and HF203 strains did not exhibited amplification, and a low polymorphism was observed between other strains. In general, the fingerprints, which were generated with the BOX derived DNA fingerprints, showed the highest genetic polymorphism with compared to ERIC- and REP- fingerprints.

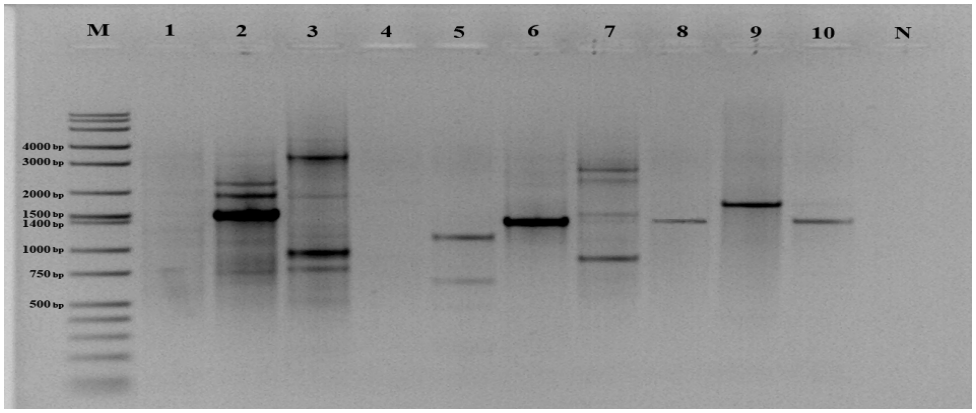

Figure 1. REP-PCR profile generated with the REP 1R and REP 2 primers. Lanes: 1) HF209; 2) HF208; 3) HF160; 4) HF203; 5) HF201; 6) HF163; 7) HF134; 8) HF59; 9) HF140; 10) HF22; Negative Control; M) Molecular Marker (10kb)

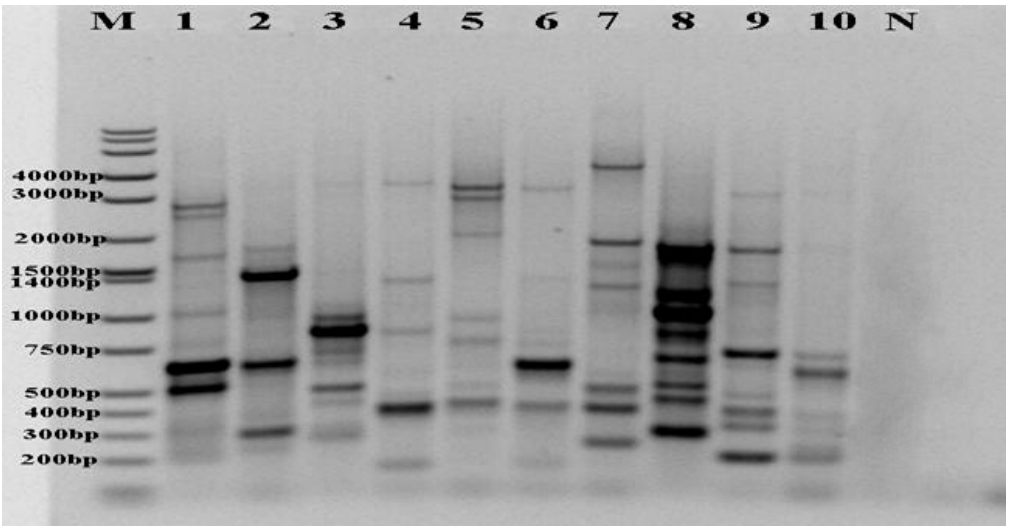

Figure 2. ERIC-PCR profile generated with the ERIC 1R and ERIC 2 primers. Lanes: 1) HF209; 2) HF208; 3) HF160; 4) HF203; 5) HF201; 6) HF163; 7) HF134; 8) HF59; 9) HF140; 10) HF22; N; Negative Control; M) Molecular Marker (10kb)

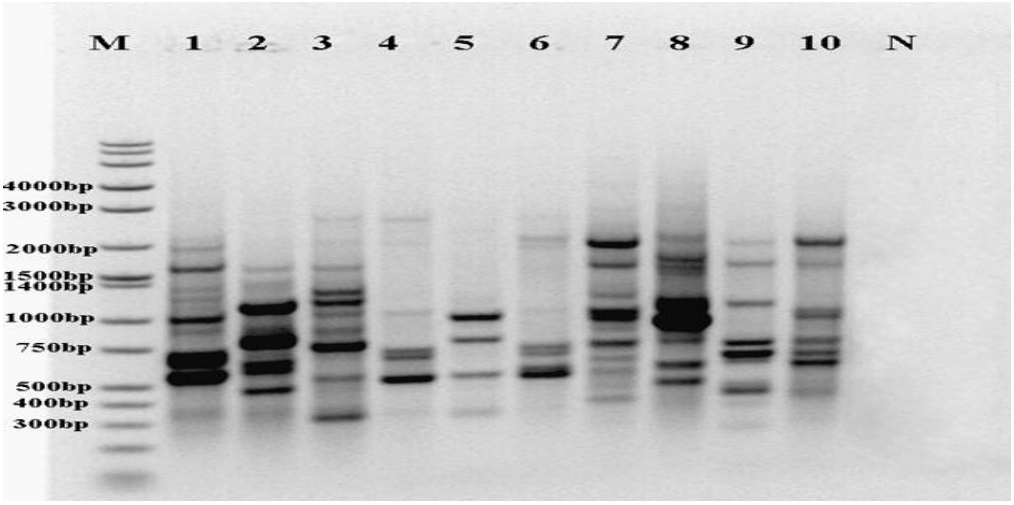

Figure 3. BOX-PCR profile generated with the BOX A1R primer. Lanes: 1) HF209; 2) HF208; 3) HF160; 4) HF203; 5) HF201; 6) HF163; 7) HF134; 8) HF59; 9) HF140; 10) HF22; N; Negative Control; M) Molecular Marker (10kb)

Similar conclusions were obtained in previous study concerning the characterization of *Sinorhizobium meliloti* indigenous strains [23] as well as *Bradyrhizobium japonicum* [24]. This finding is in agreement with Blazinkov *et al*. [25], who reported that rep-PCR performed with REP primers was less reliable than PCR performed with ERIC primers for differentiating among *R. legumnosarum bv. viciae* strains isolated from different field sites in continental part of Croatia and concluded from their data that the discriminatory efficacy of BOX-PCR was superior to REP-PCR. Overall, our results suggested that when primer BOX was used, the rep-PCR technique produced the highest number of polymorphic bands, which classified bacterial strains into 2 different clusters (Figure 4). The largest cluster, which are represented by eight strains (HF134, HF22, HF59, HF203, HF160, HF208, HF201, HF140) tested in this study, has 44-96% similarity. And, there are two strains (HF209, HF160) in the second cluster in total.

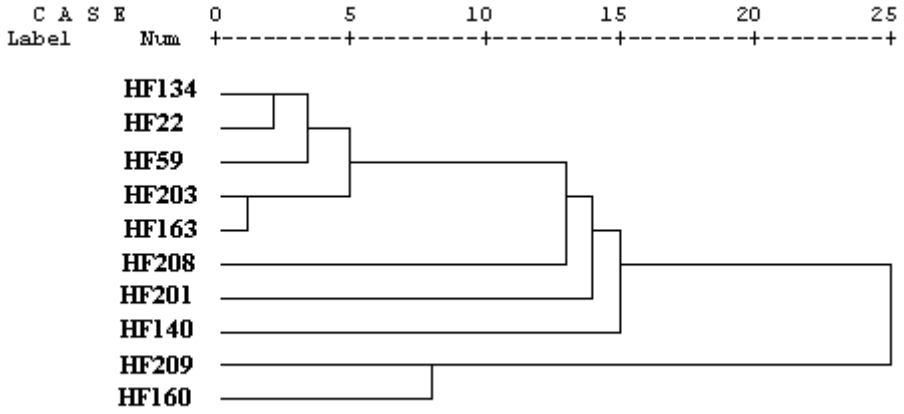

Figure 4. BOX-PCR Cluster Analyses

Our data supported the previous studies in which rep-PCR genomic fingerprinting is an adequate technique for differentiating rhizobial strains [26-29] and many other closely related sub (species) or strains and/or for determining phylogenetic relationship [30-33].

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

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