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Original paper

Characterization of root-associated bacterial species of Leklek variety of common bean (Phaseolus sp.)

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Abstract The common bean is a valuable food source in the human diet. Leklek is a local variety of common bean (*Phaseolus sp*.) widely grown in Mersin's Gülnar district, but little is known about this variety. In the present study, bacterial species from root nodules of this common bean variety were identified by PCR-amplified 16S ribosomal RNA (rRNA) gene and 16S-23S ribosomal RNA (rRNA) Internal Transcribed Spacer (ITS) region and sequencing. The partial 16S rRNA gene and 16S-23S rRNA ITS region sequences were submitted to the NCBI database (accession numbers MT967369, MT968518, respectively). Amplified sequences were used to construct a phylogenetic tree. Phylogenetic analysis based on the identified sequences showed that the isolate belonged to the genus *Microbacterium* and was closely related to *Microbacterium paraoxydans.* The findings presented here will provide a clue for understanding this bacterium's role in nodule formation in *Phaseolus sp*. (variety Leklek).

Keywords Leklek, Mersin, *Phaseolus sp*., 16S rRNA, 16S-23S rRNA ITS

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Introduction

A significant source for the human diet and the world's primary source of protein and calories is beans. Beans are commonly considered a legume that fixes nitrogen (N) in a minimal amount; however, some studies have shown its positive ability to fix nitrogen (LUCAS GARCIA et al., 2005; REMANS et al., 2008). Due to its capacity to modify atmospheric nitrogen appropriately in a process known as biological nitrogen fixation (BNF), the linkage between root-nodulating bacteria and legume plants is advantageous. Molecular advances in microbiology and the isolation of rhizobia from formerly uninvestigated legumes have contributed to significant rhizobial nomenclature changes. Symbiotic and non-symbiotic bacteria from the root nodules of a broad range of legumes have been isolated to date. Plant growth-promoting rhizobacteria (PGPR) are beneficial native soil bacteria, and PGPRs colonize the roots of plants and stimulate the growth of plants (REMANS et al., 2008). The efficiency of symbiosis can also be strongly impacted by genetic variation in beans and Rhizobium-plant varieties' efficiency.

Leklek is a local variety of common bean widely grown in the Gülnar district of Mersin, but little is known about this variety. Leklek bean is one of the geographical indicators of agricultural products that have received registration in Turkey (EROGLU PEKTAŞ et al., 2018). There have been no investigations of which bacterial species are available in the root of the Leklek variety to date. Partial 16S rRNA and 16S-23S rRNA ITS sequencing have been performed for the identification of bacterial isolates. This research was primarily based on the Leklek variety and describes the application of 16S rRNA gene and 16S-23S rRNA ITS region sequencing in defining *Microbacterium* sp. associated with nodule formation in Leklek variety.

Materials and Methods

Plant material and bacterial isolation

In this research, Leklek's seeds were obtained from the local farmer in the Gülnar district of Mersin in Turkey via the Republic of Turkey Ministry of Agriculture and Forestry Mersin Directorate of Provincial Agriculture and Forestry. The coordinates of the seed points are 36° 19′ 59.9124"N, 33° 24′ 35.6004" E, and 952 m. The seeds were sown in a viol filled with coconut shell and turf (1:1) in the greenhouse of Mersin University, Mersin, Turkey, for germination. The plantlets were then transferred to a plastic pot filled with garden soil and maintained until use. Plants were grown in the greenhouse under natural light at 80% relative humidity and 24-28°C. Roots of a single plant of Leklek were harvested after two months of planting, and nodules were extracted from the root of Leklek for bacterial isolation. Extracted nodules were directly taken into a sterile falcon tube containing sterile distilled water and brought to the laboratory. Isolated nodules were taken in 0.5 M NaOH for 10 min., rinsed three times with sterile distilled water, and then taken in solid Luria-Bertani (LB) medium containing 5 g L^{-1} of yeast extract, 10 g L^{-1} of tryptone, and 10 g L^{-1} of NaCl and incubated at 28°C for two days. Bacterial suspensions in a liquid medium were diluted to 1 in 10 serial dilutions and incubated on a solid medium to obtain single colonies.

The isolation of genomic DNA

According to the manufacturer's protocol, genomic DNA was extracted from a single colony of isolated bacteria using the EcoPure Genomic DNA Kit (EcoTech Biotechnology, Turkey). NanoDrop ® ND-1000 Spectrophotometer was used to determine the concentration and purity of gDNAs isolated.

PCR analysis and agarose gel electrophoresis

16S rRNA and 16S-23S rRNA markers were used in this study. According to the manufacturer's protocol, PCR was performed using the MyTaq™ Direct PCR kit (meridian BioScience, USA). PCR amplifications were conducted in a 25 µL reaction mixture containing 5 ng DNA and 0.8 µM of each primary (forward and reverse). PCR conditions were 5 min at 94 ° C; 35 cycles were 1 min at 94°C; 30 seconds were at 50°C; 72°C was 30 seconds; final extension was 72°C for 7 minutes. A thermocycler (MiniAmp Thermal Cycler, ThermoFisher Scientific, UK) was used for the amplification. 16S rRNA genes were amplified using the forward primer 5' AGAGTTTGATCCTGGCTCAG -3' (D1F) and the reverse primer 5' AAGGAGGTGATCCAGCC -3' (D1R). 16S-23S rRNA ITS region was identified using the forward primer 5' TGCGGCTGGATCCCCTCCTT -3' (FGPS1490-72) and the reverse primer 5' CCGGGTTTCCCCATTCGG -3' (FGPL132-38). The agarose gels (2%) had PCR products isolated and visualized. DNA samples combined with DNA loading dye were loaded onto the gel at 100V and 70 mA for 45 min in 0.5X TBE. For the visualization of the DNA bands, the Biorad Imager (Bio-Rad Laboratories, Segrate (Milan), Italy) was used. DNA bands of approximately the predicted size were removed from the agarose gel and extracted using the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, USA) procedure.

Sequencing and sequence analysis

With the same primers as those used for PCR amplification, isolated DNA bands from the agarose gel were submitted for sequencing. The sequencing service was commercially provided by Sentebiolabs**,** Ankara, Turkey (https://sentebiolab.com.tr/). Raw DNA sequence data were analyzed manually, and forward and reverse complement sequences were matched using the EMBL-EBI Pairwise Sequence Alignment (Nucleotide) Tool https://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Phylogenetic analysis

The consensus sequence was used to search for similar sequences in GenBank at the National Center for Biotechnology Information (NCBI), Bethesda, USA, using the BLAST (Basic Local Alignment Search Tool; https://blast.ncbi.nlm.nih.gov/Blast.cgi) search program (ALTSCHUL et al., 1990). The MEGA7 software (https://www.megasoftware.net/) was used to generate a phylogenetic tree (KUMAR et al., 2016). The evolutionary

history was inferred by using the Maximum Likelihood method. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

NCBI GenBank data transfer

The determined Leklek 16S rRNA and 16S-23S rRNA partial sequences were deposited in the NCBI GeneBank using the submission portal (https://submit.ncbi.nlm.nih.gov/subs/genbank/).

Results and Discussion

The seeds of Leklek were cultivated in the greenhouse at Mersin University, Mersin, Turkey. A week later, seed germination was observed (Figure 1-A), and plantlets were obtained on the 15th day of cultivation (Figure 1-B).

For the isolation of bacteria, Leklek nodules were used (Figure 2). Nodules were nearly 1.0 mm in diameter and brownish.

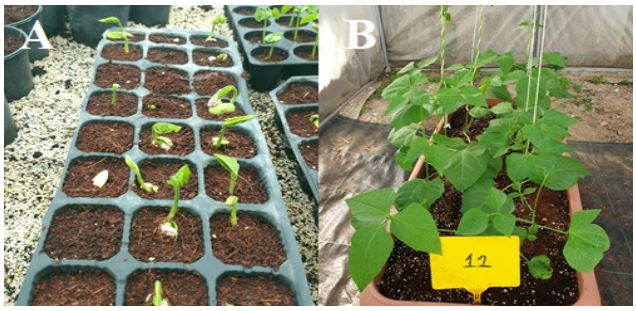

Figure 1. General view of growing conditions of Leklek from the seeds (A) germinated seeds, (B) Leklek plantlet

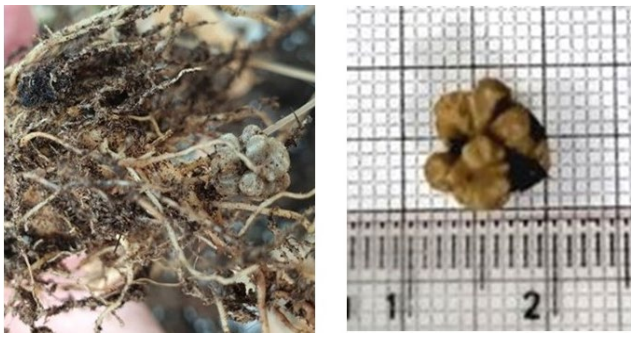

Figure 2. The root nodule picture. The left-to-right image is centered.

Several bacterial species (rhizobia and endophytic) were isolated from Leklek root nodules and sterilized to remove nodule surface rhizobacteria. The morphologically related endophytic bacterial colonials formed on the medium of LB medium (Figure 3), and since all of them were all white, rod-shaped, and opaque colonies, out of total isolates, four isolates were selected for further study. In general, a single form of the colony was extracted from a single nodule. In order to acquire bacterial cultures, single colonies have been purified. Single colonies were observed from the nodule samples at dilutions up to 10^{-10}.

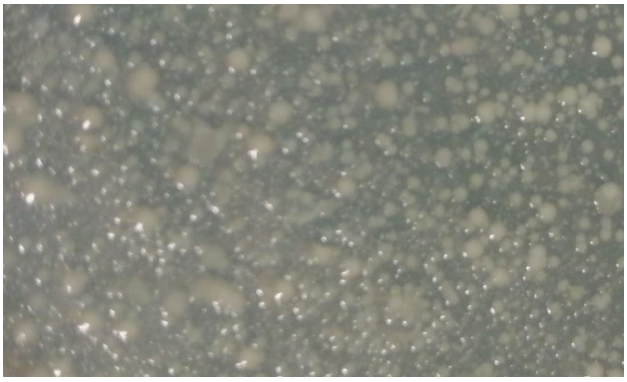

Figure 3. Bacterial colonies developed on the LB agar from root-nodules

Genomic DNAs were isolated from the bacterial isolates extracted from the root nodule of Leklek, which were maintained in the greenhouse. The quality of isolated DNAs was determined using agarose gel (1%) electrophoresis (Figure 4) with a high-quality level of 1.8 to 2.0 at a 260/280 ratio.

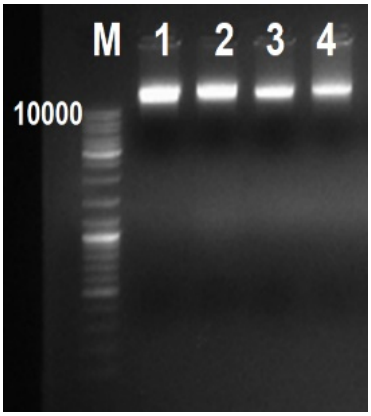

Figure 4. Agarose gel (1%) electrophoresis results of genomic DNAs of bacteria isolated from the root-nodule of Leklek

PCR products were obtained using gDNAs of the bacteria isolated from the root nodules of the Leklek variety. To identify the partial sequences of the 16S rRNA gene and 16S-23S rRNA ITS in isolated bacteria, purified PCR products of gDNAs were amplified by conventional PCR with the primers indicated in the Material and Methods section (Figure 5). The Sanger method was performed to sequence the isolated PCR products directly. One of the main measures for successfully recovering any desired gene is high-quality sequence data (DONG et al., 2014). The integrity of isolated DNAs with 16S rRNA and 16S-23S rRNA ITS bands was determined using agarose gel electrophoresis and was found to be of acceptable quality (Figure 5).

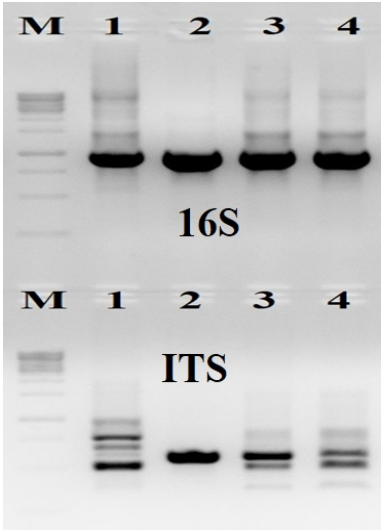

Figure 5. Agarose gel electrophoresis results in which 16S and ITS primers were used; upper gel indicates the results of the samples studied with the primer 16S and below gel determines the ITS gene regions of bacterial isolates that were excised and used for gel extraction

Bacterial isolation was made from the root nodule of the Leklek bean genotype, of which there is very little information in the literature. In the present study, four bacterial isolates were screened and identified by 16S rRNA and 16S-23S ribosomal RNA intergenic spacer sequencing. It was determined that the isolated bacteria were *Microbacterium paraoxydans.* A 664 bp putative 16S rRNA homolog for the isolate PvL1 was obtained. Similarly, a confirmed 517 bp sequence was derived from the PCR product in which FGPS1490-72 and FGPL132-38 were used as a primer pair and considered a putative homolog of the 16S-23S rRNA ITS. The 16S rRNA gene and ITS gene region sequences were registered in the NCBI database with accession numbers MT967369 and MT968518. The species belonged to the genera *Microbacterium* are yellow-pigmented, coryneform Gram-positive rods, and they can be isolated from diverse habitats such as soil and sewage (YADEGARI et al., 2010). PCR-amplified 16S rRNA gene is beneficial for the characterization of several bacteria, including *Microbacterium* species, since it is strongly conserved within species of the same genus (LAU et al., 2002).

The 16S rRNA and 16S-23S ITS sequences of the isolated bacterium were linked to those found in the GenBank databases. The 16S rRNA analysis results showed that the isolate was *Microbacterium paraoxydans* and closely phylogenetically related to *M. paraoxydans strain M59 and M. Paraoxydans strain* M8S2B3, showing 99% similarity in their 16S rRNA sequencing (Figure 6). Besides, 16S-23S ITS analysis showed that the dominant genus was *Microbacterium* in the isolated bacterium. The strain PvL1 was also phylogenetically closely related to *M. oxydans* due to 16S-23S ITS analysis (Figure 7). The phylogenetic tree of both 16S rRNA and 16S-23S ITS genes showed that *M. paraoxydans* is closest to the other *Microbacterium* sp. and was well separated from *Agrobacterium tumefaciens* (Figures 6 and 7). The Maximum Likelihood analysis of the region of 16S-23S ITS revealed a cluster of the genus *Agrobacterium* and *Clavibacter* at distinct nodes.

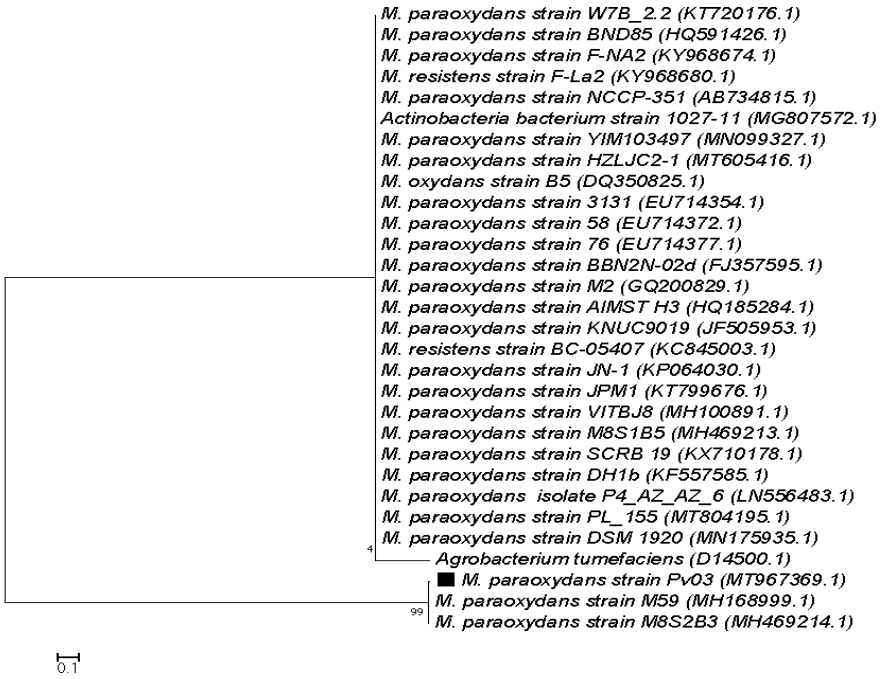

Figure 6. The evolutionary history was inferred using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (HASEGAWA et al., 1985). The tree was inferred from the partial sequence data from this study for the isolate (Pv03). The analysis involved 30 nucleotide sequences. Bootstrap values expressed as a percentage of 1.000 replications are given at the nodes. Bar equals 0.1% sequence variation. As stated in the NCBI GenBank, names and accession numbers are provided.

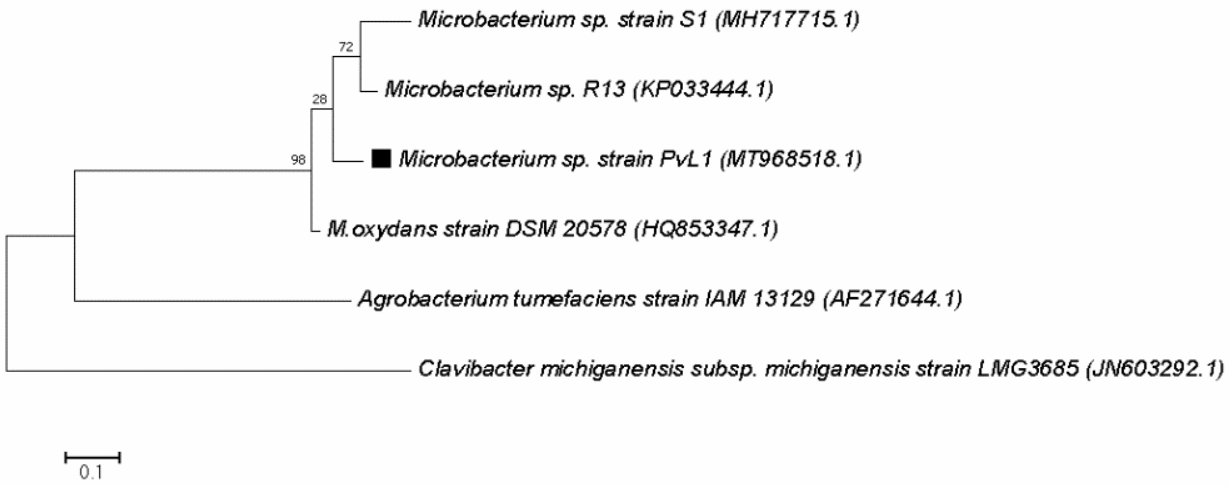

Figure 7. The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (KIMURA, 1980). The tree was inferred from the partial sequence data from this study for the isolate (PvL1). The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved six nucleotide sequences. There were a total of 237 positions in the final dataset. Bootstrap values expressed as a percentage of 1.000 replications are given at the nodes. Bar equals 0.1% sequence variation.As stated in the NCBI GenBank, names and accession numbers are provided.

The plant rhizosphere is a complex and adaptable ecological environment of intense interactions between microbes and plants to harness essential micro and macronutrients from the smaller amount of nutrients. Helpful plant-associated bacteria play a significant role in enhancing plant growth and development (BHATTACHARYYA & JHA, 2012). The Rhizosphere bacteria referring to the PGPR include the *Azospirillum* and *Azotobacter* sp. and various other bacteria, namely *Acetobacter*, *Alcaligenes*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Herbaspirillum*, *Klebsiella*, *Pseudomonas*, and *Xanthomonas* (BAL et al., 2013). Microbacterium species with PGPR behaviors have been recorded earlier (KAUR et al., 2011). *M. paraoxydans* has been isolated from various sources such as wastewater (MISHRA et al., 2020) and the rhizosphere soil (BAL et al., 2013). In the literature, it is mentioned that *M. paraoxydans* can ease phosphate uptake for plants by solubilizing it (KAUR et al., 2011). Phosphate solubilization bacteria are known to increase plant growth due to biosynthesis and action on releasing available phosphorous (PONMURUGAN & GOPI, 2006). Phosphate solubilizing microorganisms that solubilize phosphate are documented to dissolve insoluble phosphates through the processing of inorganic or organic acids, resulting in a decrease in pH.

Several factors are recorded to impact microbial species' existence and distribution in the soil and rhizosphere, including root morphology, root exudates, plant growth stage, and physicochemical soil properties. The synthesis of siderophores of *M. paraoxydans* was reported in previous research (BAL et al., 2013). The ion that does not get into available forms for the plants, probably chelated by bacterial siderophores. Increased secretion of siderophores by *M. paraoxydans* can be essential for iron uptake in Leklek.

Conclusions

Possible unique relationships between *M. paraoxydans* and Leklek have not yet been examined. More such research into the effect of *Microbacterium* on Leklek should be conducted.

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