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Diversity of Rhizobia Associated With *Lablab purpureus* Isolated from Algeria by PCR Amplification of the 16S rDNA PCR / RFLP

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ABSTRACT

The objective of this study was determination of the taxonomic position of these isolates and the evaluation of the level of approximation or divergence between these strains and the reference strains belonging to different genus of rhizobia. Amplification of the ribosomal 16S rDNA gene (PCR / RFLP of 16S rDNA) was digested with four different restriction enzymes: *Msp* I, *Hinf* I, *Hha* I and *Taq* I. The results of different electrophoretic profiles of fragments obtained shown the selection of the most discriminating enzymes *Msp* I and *Hinf* I. The length polymorphism of the restriction fragments (RFLP) analysis of PCR amplified 16S rDNA was compared with those of reference strains. Numerical analysis of molecular characteristics showed that 20 strains studied were divided into three distinct groups; we noted that three isolates only *Lablab purpureus* have a high level of similarity with the reference strain "*Bradyrhizobium",* while 17 isolates did not exhibit precise taxonomic status and therefore their exact phylogenetic classification is to be determined. The nearly complete sequence of the 16S rRNA gene from a representative strain of each REP-PCR pattern showed that the strains were closely related to the members of the family *Bradyrhizobium.*

Keywords**:** *Lablab purpureus*, PCR/RFLP, numerical analysis, genetic diversity, repetitive extragenic palindromic (REP).

Introduction

Many legumes plants with grains, forage as *Lablab purpureus* and pasture legumes form symbiotic associations with a group of bacteria, generally called as rhizobia (Harrier *et al*., 1995, Yue li *et al*., 2011). With the advancement of bacterial phylogenetics based on the sequences of the small conserved subunit of 16S ribosomal RNA (Day *et al*., 1965, Diouf *et al*., 2010), the taxonomy of rhizobia is rapidly changing. However, it is not only the taxonomy of rhizobia which is changing from time to time. The selection of appropriate rhizobial microsymbionts is becoming a complex procedure due to the fact that several legumes species can be nodulated by single rhizobia (Bringer *et al*., 1992). Yet, the symbiotic association between the legumes and their microbial symbionts play a significant role in agriculture worldwide by reducing ca. 100 million metric tons of atmospheric nitrogen saving US$ 8 billion/year on fertilizer N (Burnie *et al*., 2006, Nera *et al*., 2009).

The association between rhizobia and the members of the family Leguminosae accounts for 80% of biologically fixed nitrogen and contributes 25-30% of the 'protein intake in the world (Vance *et al*., 1997). To date, more than 98 species have been described for legume-associated symbiotic nitrogen-fixing bacteria within the genus *Rhizobium, Mesorhizobium, Ensifer, Bradyrhizobium, Burkholderia, Phyllobacterium, Microvirga, Azorhizobium, Ochrobacterium, Methylobacterium, Devosia,* and *Shinella* in the *Alphaproteobacteria* group, as well as *Burkholderia* and

Cupriavidus in the *Betaproteobacteria* group (http://www.bacterio.cict.fr). Rhizobia were characterized from wild and tree legumes, and several novel taxa have been proposed on the basis of these studies (Wolde- Meskel *et al*., 2005, Yan *et al*., 2007, Shetta *et al*., 2011). The isolation and characterization of new Rhizobium isolates from different legumes species is an interesting field of work that helps to understand the diversity and evolution of rhizobia.

Considering the potential value of *Lablab purpureus* for sustainable agriculture, agroforestry, and the lack of studies on the diversity of rhizobia associated with these plants, we aimed to collect and characterize rhizobia associated with this plant in Algeria.

The aim of the present study was to assess the diversity of *Lablab* micro-symbiotes and molecular characterization of rhizobia associated with this legume by using PCR/RFLP of 16S rDNA in Algeria.

Materials and methods

Authentication of isolates

All the rhizobia isolates were evaluated as pure cultures that can serve as nodules on their respective host plants. The seeds of the leguminous plants were previously germinated in petri-dishes after scarification with conc. H_2SO_4. The pre-germinated seeds have been planted in growth pouches containing N-free nutrient solution (Somasegaran *et al*., 1994). Seven days after planting, the growth pouches were inoculated with 1 ml broth YEM culture of each isolate with each treatment replicated four times. Uninoculated pouches have served as control. The pouches were placed in racks and kept in the green house. Plants were harvested 12 weeks after planting and their roots assessed for the presence of nodules. The results obtained after two months of culture have revealed that the 20 isolates (100% of the isolates) are able to nodulate their host plants.

Bacterial strains and culture medium

The colonies obtained on the solid YEM medium in each of the 20 pure isolates culture were collected and cultivated on TY medium (tryptone-yeast extract) (Bringer *et al*., 1992) diluted in half.

Extraction of genomic DNA

DNA preparation: Total genomic DNAs from all strains were isolated using standard phenol-chloroform-isoamyl extraction and ethanol precipitation in the presence of sodium acetate (0.3 mol/L). The pellets were washed with 70% ethanol, dried and re-dissolved in 150 µL of TE buffer.

The concentration and purity of DNA have been estimated spectrophotometrically at 260 nm and 280 nm, respectively. From the bacteria grown on TY medium for two days at 28 °C, a multi-well-formed colony were picked and suspended in 25 µl of sterile double distilled water.

PCR amplification PCR amplification of the 16S rDNA PCR/RFLP

The amplification reactions were performed using a protocol optimization initially described by Bruijn *et al*., (1992). Amplification reactions were performed in a total volume of 25 µl and contain the following: 1× reaction buffer (10 mM Tris-HCl, 50 mM KCl) with 1.5 mM $MgCl_2$, 2.5 units *Taq* polymerase, 200 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 5 pmol of each forward and reverse primer and 100 ng of genomic DNA. The temperature profile was as follows: Initial denaturation at 95°C for 3 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 3 min. The amplified products were kept at a temperature of 4°C. All amplifications were carried out in Thermocycler. The PCR product was run on a 1% agarose gel stained with ethidium bromide.

Digestion of the amplification products with restriction enzymes

The universal primers FGPS 6 (5' GGA GAG TTA GAT CTT GGC ATT G 3 ') and FGPS 1509 (5'AAG GAG GGG CAG ATC CGC CA CAC 3') developed by Norman *et al*. (1996).

PCR products were separately digested with each of the following restriction endonucleases *Msp* I, *Hinf* I, *Hha* I and *Taq* I. The restriction fragment length polymorphism (RFLP) patterns were resolved by gel electrophoresis on 1.8% agarose for 4h at 120 mV.

Statistical Analysis

The results of the different profile of restriction have been treated by the UPGMA method with the Statistica software. The similarities between the various strains tested were evaluated by comparing the profile of restriction taken in pair's.

PCR amplifications

Repetitive extragenic palindromic (REP)-polymerase chain reactions (PCR) were performed using primers REPIR-I and REP2-I, according to Bruijn *et al*. 1992. PCR amplifications of 16S rRNA gene fragments were carried out using the two opposing primers 41f and 1488r as previously

reported (Wang *et al*., 2003). Amplification products were purified using the Qiagen PCR product purification system and subjected to cycle sequencing using the same primers as for PCR amplification, with ABI Prism dye chemistry. The products were analyzed with a 3130 × l automatic sequencer at the sequencing facilities of Estación Experimental del Zaidin, CSIC, Granada, Spain. The obtained sequences were compared to those in the GenBank database using the BLAST program (Ando *et al*., 1999) and with the sequences held in the EzTaxon-e server (Niemann *et al*., 1997). The sequences were aligned using Clustal W software (Bontemps *et al*., 2015). The distances were calculated according to Kimura's two-parameter model (Gyaneshwar *et al*., 2011). Phylogenetic trees were inferred based on the maximum likelihood (ML) method (Mohammed *et al*., 1997), using MEGA 5.0 software.

Results

PCR amplification of the 16S rDNA PCR/RFLP

RFLP Aanalysis of PCR amplification of 16S rDNA PCR/RFLP genes of almost all the 20 rhizobia isolates of *Lablab purpureus* has produced a single band 1500 bp representing the 16S rDNA PCR /RFLP gene amplified in all the *Lablab purpureus* rhizobial strains.

All the restriction enzymes tested produced polymorphic patterns. The most discriminative were those obtained with MSP I (Figure 1).

Numerical Analysis of phylogenetic groups established by the UPGMA

The results of the different restriction patterns were treated by UPGMA. The dendrogram derived from this analysis is shown in (Figure 2, Table 1). At a level of 83% similarity yields three clusters:

The first cluster (A) the strains of Rhizobium genus to a level of 75% similarity, the second cluster (B) includes *Mesorhizobium* strains to a level of 69% similarity; and the third cluster (C) groups of *Bradyrhizobium s*trains to a level of similarity 89%.

Comparing our isolates with reference strains, we noted that only three isolates of *Lablab purpureus* have a high level of similarity with the reference strain *"Bradyrhizobium"*. These isolates 2007, DLB (DLB 2008 and 2009) that form one and the same lineage with the reference strain *Bradyrhizobium.*

The most interesting results derived from the analysis by PCR/RFLP of the rDNA 16S is that isolates *Lablab purpureus* studied are totally distinct from *Bradyrhizobium s*trains.

In addition, 17 isolates did not present a specific taxonomic status, therefore their exact phylogenetic classification is to be determined.

Discussion

In this study, we performed molecular characterization by PCR RFLP 16S of 20 symbiotic bacteria isolated roots of *Lablab purpureus*. The amplification of the 16S rRNA gene of almost all the rhizobia isolates used in this study resulted in a single band 1.5 kb in size. This band size corresponds to the expected size reported earlier by Weisburg *et al*., (1991).

Polymorphism of lengthof the restriction fragments (RFLP) analysis of PCR amplified 16S rDNA were compared with those of reference strains. Numerical analysis of the molecular characteristics showed that 20 strains studied fall into three distinct groups, we noted that three isolates only of *Lablab purpureus* have a high level of similarity with the reference strain *"Bradyrhizobium",* while 17 isolates did not exhibit precise taxonomic status and therefore their exact phylogenetic classification is to be determined.

REP-PCR fingerprinting was used to group the strains. This technique has been extensively used to cluster bacteria at the subspecies or strain level (Jensent *et al*., 1968, Walkley *et al*., 1934) and is known to be a powerful tool for studies on microbial ecology and evolution (Ishii *et al*., 2009).

The combined restriction of the 16S rRNA genes of the rhizobia isolates with four endonucleases distinguished clearly different combinations of patterns or fingerprints at 80% similarity level which represents three distinct 16S rRNA genotypes among the isolates. This finding indicates great variations among the isolates and suggests that the soils harbour populations of highly diverse strains that nodulates the legume. This finding is in agreement with the results obtained in other parts of the world (Bremmer *et al.* 1967, Yue Downer *et al*., 2017).

These results, however, agree with those previously published, in which Yue *et al*., (2011) have shown in a study on Five strains isolated from root nodules of *Lablab purpureus* and *Arachis hypogaea* grown in the Anhui and Sichuan provinces of China were classified as members of the genus *Bradyrhizobium*. These strains had identical 16S rRNA gene sequences which shared 99.48%, 99.48% and 99.22% similarity with the most closely related strains of *Bradyrhizobium jicamae*, respectively. Parallel to our results in three distinct groups, we noted that three isolates only of *Lablab purpureus* have a high level of similarity with the reference strain *"Bradyrhizobium*".

Conclusion

We have focused our investigation on the genetic study using PCR/RFLP of 16S rDNA gene from 20 strains resulted in three groups, the first group includes the genus *Rhizobium* strains to a level of 75% similarity, the second combines the *Mesorhizobium* strains a level of similarity of 69% and the third groups of *Bradyrhizobium* strains to a level of similarity of 89%. Statistical Analysis of phylogenetic groups established by the UPGMA statistical software shows that among the twenty strains studied, 17 strains of the species described in the literature could be new species; this needs to be confirmed first by the complete sequencing rDNA16S.

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Table 1. Phylogenetic classification of bacterial strains.

Strains	REP-PCR pattern	Closest related genus	Family
DLB5011	1	*Rhizobium*	Rhizobiaceae
DLB5020	2	*Rhizobium*	Rhizobiaceae
DLB4012	3	*Rhizobium*	Rhizobiaceae
DLB4016	4	*Rhizobium*	Rhizobiaceae
DLB5017	5	*Rhizobium*	Rhizobiaceae
DLB5018	6	*Rhizobium*	Rhizobiaceae
DLM1111	7	*Mesorhizobium*	Rhizobiaceae
DLM1114	8	*Mesorhizobium*	Rhizobiaceae
DLM1120	9	*Mesorhizobium*	Rhizobiaceae
DLB4020	10	*Mesorhizobium*	Rhizobiaceae
DLM1121	11	*Bradyrhizobium*	Rhizobiaceae
DLM1123	12	*Bradyrhizobium*	Rhizobiaceae
DLM1122	13	*Bradyrhizobium*	Rhizobiaceae
DLb2006	14	*Bradyrhizobium*	Rhizobiaceae
DLb2005	15	*Bradyrhizobium*	Rhizobiaceae
DLb2004	16	*Bradyrhizobium*	Rhizobiaceae
DLb2007	17	*Bradyrhizobium*	Rhizobiaceae
DLb2008	18	*Bradyrhizobium*	Rhizobiaceae
DLb2009	19	*Bradyrhizobium*	Rhizobiaceae
DLB5015	20	*Bradyrhizobium*	Rhizobiaceae

Figure 1. Types of restriction patterns of PCR-amplified 16S rDNA digested with *Msp*I obtained with strains used in this study. Molecular size marker: M2, 20 bp ladder.

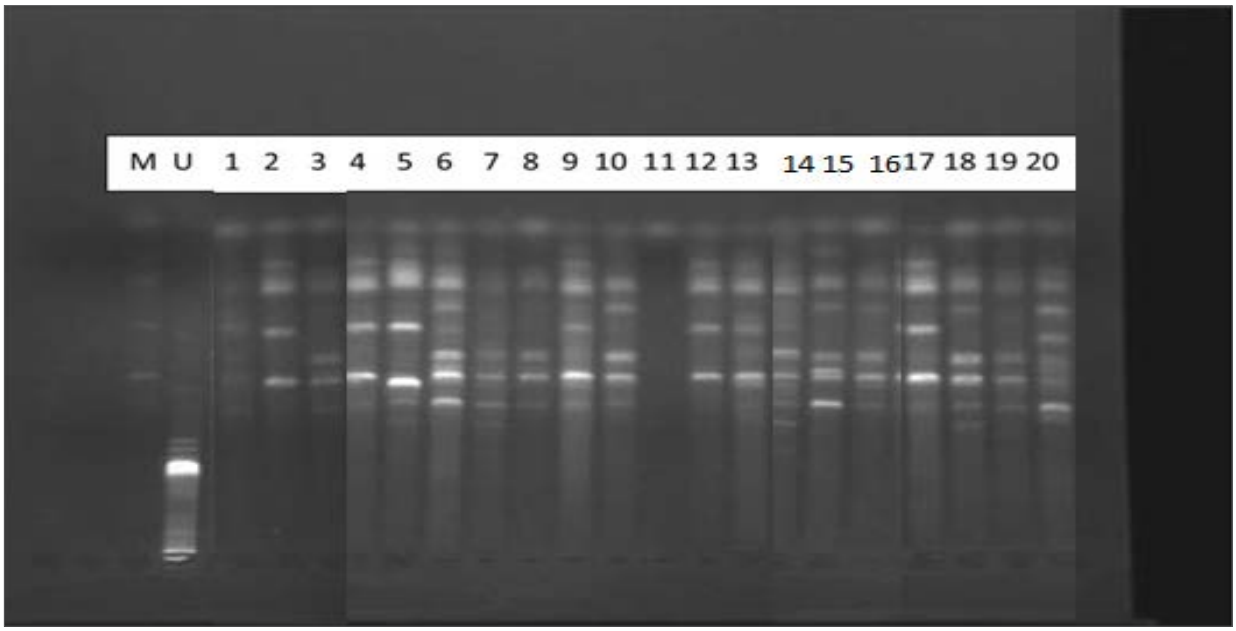
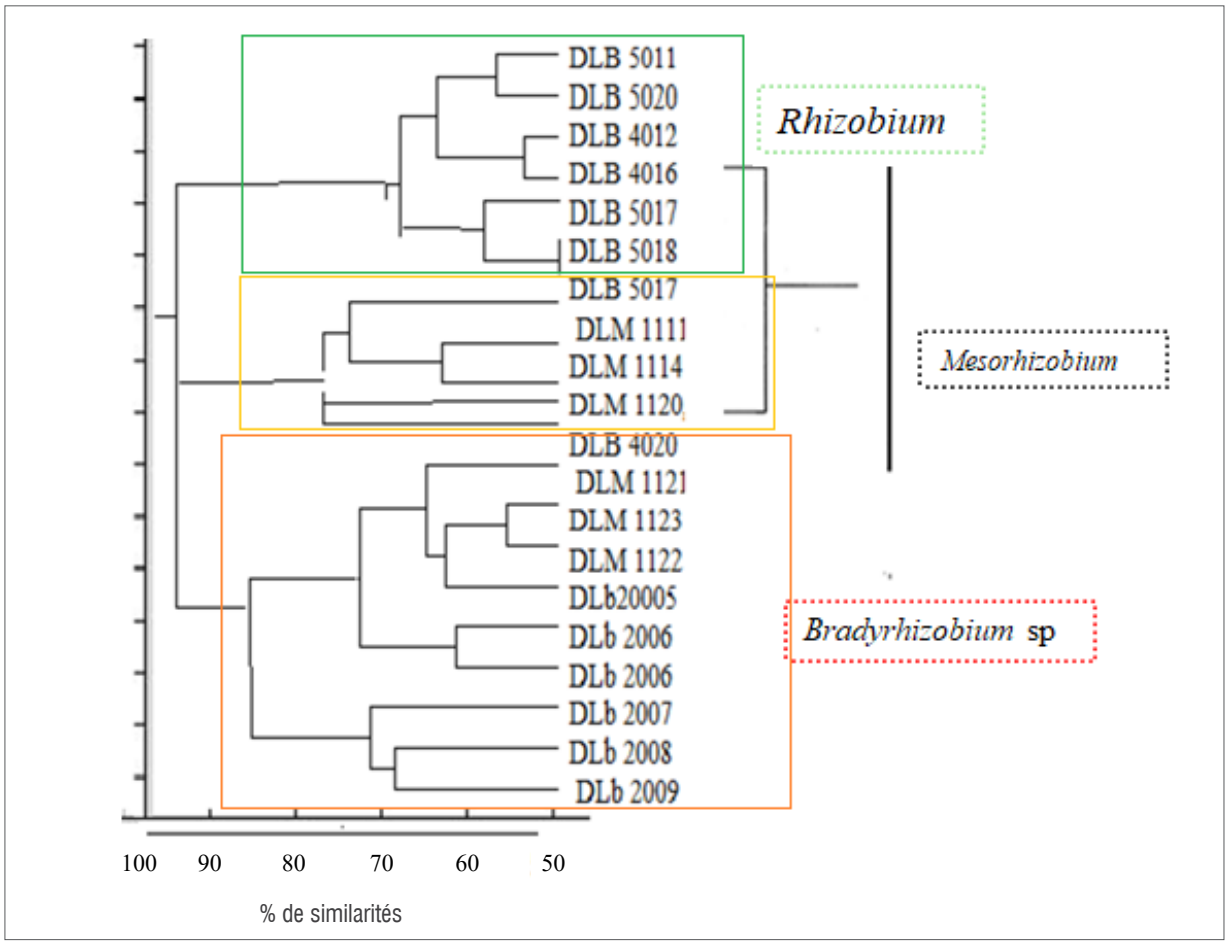


Figure 2. Dendrogram constructed by UPGMA indicating the phylogenetic position strains isolated from the legume plant *Lablab purpureus* compared to the reference strains used on the basis of the PCR / RFLP of 16S rDNA.



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