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# Molecular Identification and Phylogenetic Relationships of Rhizobial Strains Nodulating Some Leguminous Crops

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## ABSTRACT

Isolation, molecular identification and phylogenetic relationships of Rhizobial isolates nodulated some leguminous crops grown in Sohag governorate, were conducted in this present study. According to the 16S rDNA sequencing analysis, all of isolates were belong to genus Rhizobium which is commonly colonizing root nodules of different leguminous crops. Among of all the 5 bacterial isolates only EMR2 gave 100% of similarity with *Rhizobium pusense* strain NRCPB10 NR_116874.1 while, EMR1 isolate exhibited 99.58% of similarity with *Rhizobium binae* strain BLR195 NR_137242.1. The two isolates EMR4 and EMR5 showed (97.35 and 97.14%, respectively) similarity with the two bacterial strains *Rhizobium aegyptiacum* strain 1010 NR_137399.1 and *Rhizobium bangladeshense* strain BLR175 NR_137241.1. Finally the isolate EMR3 was similar to *Rhizobium bangladeshense* strain BLR175 NR_137241.1 with 96.88%. It was found that the 2 rhizobial species (*Rhizobium bangladeshense* and *Rhizobium aegyptiacum*) of Egyptian clover were shared the same clade. As well as it also shared *Rhizobium binae* of Lupine as a common ancestor. Finally, it was found that *Rhizobium pusense* of Peanut was the common ancestor with all other rhizobial isolates. These findings revealed that the conserved gene of these 4 Rhizobium isolates was derived from *Rhizobium pusense*. The obtained results showed that the genetic diversity of the rhizobial isolates that nodulate the leguminous crop at the chosen sites in the Sohag governorate is very low, which may be cause their low host specificity and ability to increase nodules formation in different leguminous crops, making them a promising bio-fertilizer for Egypt's sustainable crop production.

**KEYWORDS:** Genetic diversity, Rhizobium, 16S rRNA, Phylogenetic tree, Root nodules

## 1. INTRODUCTION

Rhizobia are soil born gram-negative bacteria, endophytes and also able to colonize rhizospheric rejoins of legumes and many other crops (López-Guerrero *et al.*, 2013). They able to fix the atmospheric nitrogen with various leguminous plants and therefore play a

significant role in plant nutritional process throw enhancing nitrogen fixation and increasing crop production (Stocker *et al.,* 2008 and Toro *et al.,* 1997).

The symbiotic relationships between rhizobial bacteria and leguminous host is resulted from exchanging certain molecular signals that determine host-specificity whereas, seeds and roots of legume secrete many of organic compounds such as flavonoids, which change between different species (Wadhwa *et al.,* 2011).

According to the huge variation and the wide speared of the legumes, various rhizobial colonies were founded and their genetic diversity became obvious (Drew and Ballard, 2010). This diversity is coming from the actions of rhizobia, host legumes and the environmental biotic and abiotic factors (Yan *et al*., 2014). Studying the biodiversity of rhizobia could be helps us to isolate a promising isolates from wild legumes plants that might be used in bacterial improving programs. As well as, it`s considered a good method for improving leguminous crops quality and productivity when inoculated with such new isolates (Zahran *et al.,* 2012).

Using the molecular techniques to identify bacterial isolates were increased because the traditional microbiological characterization did not gave clear or true evolutionary relationship (Giller, 2001). Recently, molecular methods have been developed to determine different microbial genera, species and strains (Ismail *et al.,* 2013). Among of these techniques, the 16S rRNA genes sequencing, was an efficient in bacterial identification at both genera and species levels (Romdhane, *et al.,* 2006 and Lafay and Burdon, 2001). Due to, their highly conservative which supports well the establishment of rhizobia classification into genera and species (Sun *et al.,* 2010). The 16S rRNA genes sequence (1500 bp) is presented in almost all bacteria, and large enough to use in the informatics studies (Schröder, 2014 and Patel, 2001). So, it is use a lot in the bacterial phylogenetic relationships studies due to their stability and their vital functions in the bacterial cell (Pulawska *et al.,* 2000).

16S rRNA gene sequencing technique have been used in numerous of microbial phylogeny investigations (Kolbert and Persing, 1999, Ismail *et al.,* 2013 and El-Zanaty *et al.,* 2014). So, isolation, molecular identification and phylogenetic relationships of *Rhizobium* bacterial isolates nodulated different leguminous crops grown in Sohag governorate, were conducted in this present investigation.

## 2. MATERIALS AND METHODS

This study was conducted in Microbial Genetics Laboratories, Genetics Department, Faculty of Agriculture, Sohag University, Egypt.

### 2.1.Samples collection

Five bacterial strains were isolated from healthy root nodules of three leguminous crops Lupine (*Lupinus termis* L.), Peanut (*Arachis hypogaea* L.) and Egyptian clover (*Trifolium alexandrinum* L.) cultivated at several locations within Sohag Governorate as shown in, Table 1. These isolates were symbolized as EMR1, EMR2, EMR3, EMR4 and EMR5

**Table 1. Symbols, host plant and locations of rhizobial isolates used in the present work.**

| No. | Isolate symbol | Host plant | Locations |
|---|---|---|---|
| **1** | EMR1 | Lupine | Nazaa |
| **2** | EMR2 | Peanut | Tahta |
| **3** | EMR3 | Egyptian clover | Tema |
| **4** | EMR4 | Egyptian clover | Sohag University |
| **5** | EMR5 | Egyptian clover | Awlad salem, Dar El Salam |

### 2.2. Rhizobia isolation

According to Vincent, 1970, active and pink nodules, were washed many times with sterilized water ($H_2O$), surface sterilization were done for 3 min by 95% (v/v) ethanol and then washed thoroughly with sterile $H_2O$ for five times. The separated nodules were added on Petri dishes in a drop of sterile double distilled water ($H_2O$), and by sterile scalpel were crushed to obtain the nodule exudates then , streaked onto Yeast Extract Mannitol media (YEM) (Mannitol, 10 g; $K_2HPO_4$, 0.5 g; $MgSO_4$. $7H_2O$, 0.2 g; NaCl, 0.1 g; Yeast

extract, 3.0 g; Agar, 20.0 g; and Water to 1,000 ml. pH 6.8). After incubation for 3 days of at 28 °C, the selected bacterial single colonies were purified on YEM media for several times due to their growth and morphological characteristics. The purified single colonies were stored on slant agar of YEM at 4°C.

### 2.3. Morphological characterization

The bacterial isolates were tested for Gram staining reaction, cell shape, motility, growth in aerobic and un-aerobic conditions and presence of gummy substances as reported by Cowan and Steel (1975). Purified bacterial cultures, at log phase were observed microscopically according to Aneja (2006). Gram's staining was done according to Hucker and Conn (1923) to determine the Rhizobial staining ability (gram negative or gram positive). Motility test were done according to Elbeltagy *et al.* (2000) in a semi-solid nutrient agar plates (0.2% agar). Absorption of Congo red stain was also observed in YEM medium with 0.05% (w/v) stain as mentioned by Zuvenhuizen *et al*., (1986).

### 2.4. Molecular identification of rhizobial isolates

#### 2.4.1. Genomic DNA isolation

Genomic DNA were isolated from the fresh colonies of the five isolates by CTAB protocol according to Porebski *et al.* (1997). Some additional requirements were used such as, freshly phenol: chloroform: isoamyl alcohol (25: 24: 1), 96%~100% Ethanol and RNase (50mg/ml). The obtained DNA was dissolved in 100 $\mu$l TE buffer and used as the template for the PCR reactions.

### 2.5. Amplification and analysis of 16S rRNA gene

The amplification of 16S-rRNA gene sequence was done according to (Hilario *et al.*, 2004) by using the universal bacterial primers 16S-1F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1509R (5′-ACGGCTACCTTGTTACGACTT-3′). The PCR reaction mixture were done in (50 $\mu$l) of the final volume by mixing (4$\mu$l) of the DNA template with (2$\mu$l) from each primer and (17$\mu$l) of sterilized double distilled water then complete final volume by adding (25$\mu$l) of 10 X Dream TaqTM Green Buffer as a Master Mix. PCR reaction conditions was carried out as followed: Initial denaturation at 94$^{o}$C for 3 minutes, denaturation at 94°C for 30 seconds, primer annealing at 57oC for 30 seconds, chain extension at 72$^{o}$C for 2 minutes and a final extension at 72°C for 10 minutes. Denaturation, annealing and extension cycles were repeated for 30 cycles. The PCR products were sequenced by using ABI 3730xl DNA sequencer at GATC Company (GATC Biotech Ltd. – The London BioScience Innovation Centre - London, United Kingdom). The obtained sequences were checked for their similar sequences in the public database using Basic Local Alignment Search Tool (BLASTn) on the website *http://www.ncbi.nih.gov* (Shayne *et al.*, 2003).

All the 16S rRNA gene sequences of the bacteria isolates were deposited at the database of National Center for biotechnology Information (NCBI) and had accession numbers OM014203 to OM014207. The phylogenetic tree was constructed by MEGA X software package program (neighbor-joining NJ) method (Kumar *et al.*, 2018).

## 3. RESULTS AND DISCUSSION

The present investigation was conducted to isolate and characterize rhizobial isolates from different leguminous crops such as: Lupinus, Peanut and Egyptian clover cultivated at several locations within Sohag Governorate by traditional and molecular methods as well as, to determine the phylogenetic relationships among them.

### 3.1. Morphological characterization

According to their phenotypic characteristics corresponding to Rhizobia, 5 pure isolates of putative *Rhizobium* strains were isolated from root nodules of different leguminous crops (Lupine, Peanut and Egyptian clover) cultivated at several locations within Sohag Governorate. The phenotypic and growth features were further studied in order to determine their diversity and relatedness. Results revealed that all isolates grew rapidly on YEM medium and formed visible colonies after 3–5 days like many of *Rhizobium* species.

Their doubling time was found to be between 10 and 14 h. All bacterial isolates were negative to Gram stain, whitish color,

colonies had a round convex with regular margins and their extracellular gum were abundant on the surface.

Moreover, all of the isolated strains were motile, aerobic, rod shaped, heterotrophic, and non-spore forming bacteria. As well, all of the 5 isolates produced white colonies on YEM medium supplemented with 0.05 % Congo red. These results are in accordance with the results of Vincent (1970) and Dye (1980) who's identified that Rhizobia is a weakly absorb Congo red dye or presented in a white colonies, not similar to their so closely related *Agrobacterium* spp., which take up the dye strongly. It was also reported by (Hahn, 1966) that rhizobia species presented as a white colonies on nitrogen free or synthetic nitrate-containing medium supplemented with 0.0025% Congo red, and this could be used to differentiated it from the other colored colonies of the rhizospheric bacteria.

### 3.2. Molecular identification of bacterial isolates

Nowadays, the applications of 16S rRNA gene sequencing technology become wide spread and powerful tool for identification and classification of bacteria at species level. It is become a superior technique to characterize the un-known, rarely isolated, or the bacterial strains with the abnormal phenotypic shape (Clarridge, 2004). The PCR product fragments of 16S rRNA gene of the 5 bacterial isolates were given a band of 1500 base pairs (bp) (Figure 1).


**Figure 1. 16S rRNA gene banding pattern of 5 bacterial isolates. M: 100 bp ladder marker and lanes EMR1 through EMR5 refer to bacterial isolates.**

The similarity percentages of the obtained 5 bacterial isolates sequences and their closest related NCBI strain(s) in Genbank database, were shown in (Table 2).

**Table 2. Identification of bacterial isolates based on 16S rDNA sequence.**

| Isolates | accession No. | % Similarity | Closest strain in NCBI and their accession No. | Class |
|---|---|---|---|---|
| **EMR1** | OM014203 | 99.58 | *Rhizobium binae* strain BLR195 NR_137242.1 | *α*- Proteobacteria |
| **EMR2** | OM014204 | 100 | *Rhizobium pusense* strain NRCPB10 NR_116874.1 | *α*- Proteobacteria |
| **EMR3** | OM014205 | 96.88 | *Rhizobium bangladeshense* strain BLR175 NR_137241.1 | *α*- Proteobacteria |
| **EMR4** | OM014206 | 97.35 | *Rhizobium aegyptiacum* strain 1010 NR_137399.1 | *α*- Proteobacteria |
| **EMR5** | OM014207 | 97.14 | *Rhizobium bangladeshense* strain BLR175 NR_137241.1 | *α*- Proteobacteria |

Data in Table (2) showed that all of bacterial isolates were belong to genus *Rhizobium* which is commonly colonizing root nodules of different leguminous crops. Among of all the 5 bacterial isolates only EMR2 gave 100% of similarity with *Rhizobium pusense* strain NRCPB10 NR_116874.1 while, EMR1 isolate exhibited 99.58% of similarity with *Rhizobium binae* strain BLR195 NR_137242.1. The two isolates EMR4 and EMR5 showed (97.35 and 97.14%, respectively) similarity with the two bacterial strains *Rhizobium aegyptiacum* strain 1010 NR_137399.1 and *Rhizobium bangladeshense* strain BLR175 NR_137241.1. Finally the isolate EMR3 was similar to *Rhizobium bangladeshense* strain BLR175 NR_137241.1 with 96.88%. There was a symbiotic relationship between *Rhizobium* species and roots of different leguminous plants (Abdel-Lateif *et al.*, 2016).

The phylogenetic tree of the 16S rRNA gene sequence from each of the current bacterial isolates and their relatives in the GenBank database was generated using the neighbor-joining method. For all of the phylogenetic trees, the greatest composite likelihood technique was utilized to calculate branch lengths. The scale bar refer to base substitutions per location. The percentage of duplicate trees in which the relevant taxa clustered together in the bootstrap test is shown next to each branch (1000 replicates). Sequence from tested bacterial isolate is marked by red rhombus. *Azorhizobium caulinodans* strain LMG 6465 was used as outgroup. The phylogenetic tree (Figure 2) indicated that isolate EMR1 of Lupine had the closest relationship with *Rhizobium binae strain BLR195. (GeneBank accession Nos. NR_137242.1* which are found in the same clade.

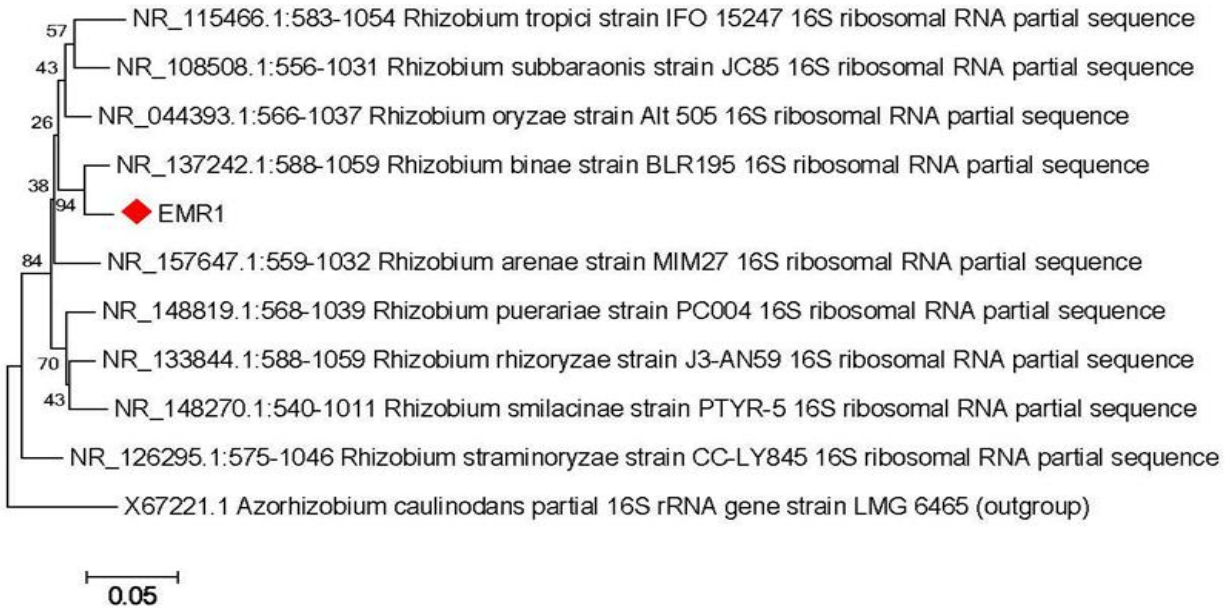


**Figure 2. Neighbor-joining phylogenetic dendrogram showing the genetic relationships between EMR1 isolate and reference their strains based on 16S rRNA gene sequence.**

Concerning isolate EMR2 of Peanut, the phylogentic tree in Figure 3 showed that it was close relaed to the two stains *Rhizobium pusense* strain NRCPB10 NR_116874.1 and *Agrobacterium fabrum* strain C58 but it showed 100% simalarity with *Rhizobium pusense* strain NRCPB10.

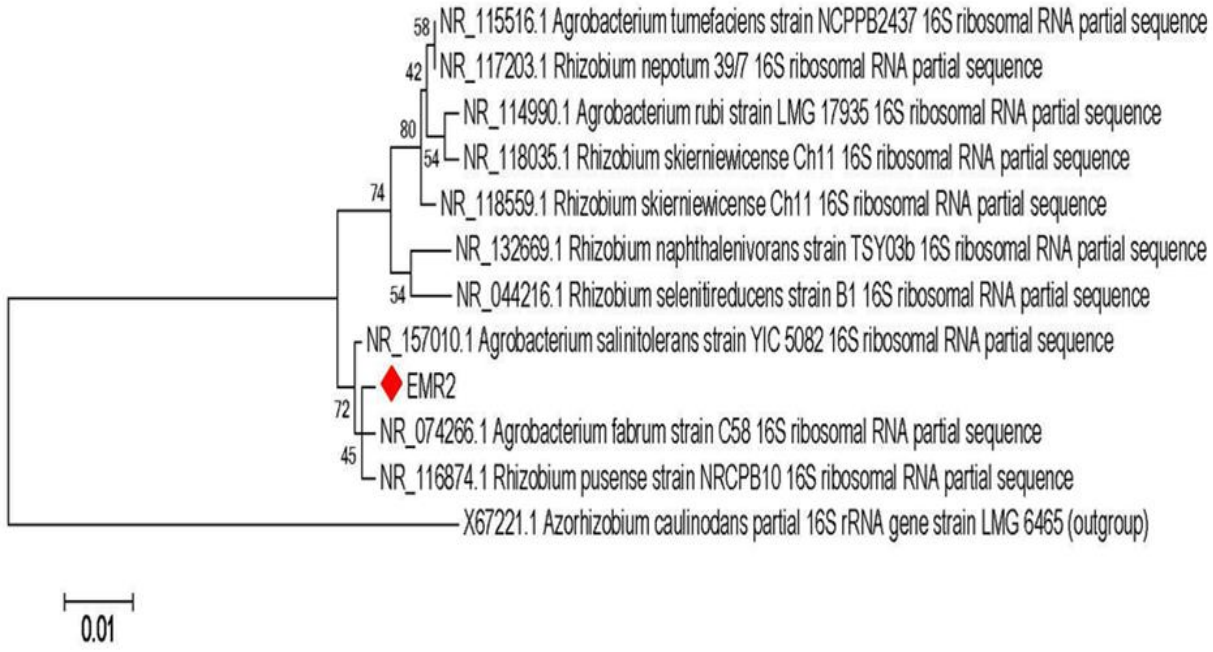


**Figure 3. Neighbor-joining phylogenetic dendrogram showing the genetic relationships between EMR2 isolate and their reference strains based on 16S rRNA gene sequence.**

The phylogenetic trees of the two isolates EMR3 and EMR5 of Egyptian clover (Figure 3 and 4 ,respectively) showed that they are close related to the same isolates *Rhizobium bangladeshense* strain BLR175 NR_137241.1 which engaged the same clade.

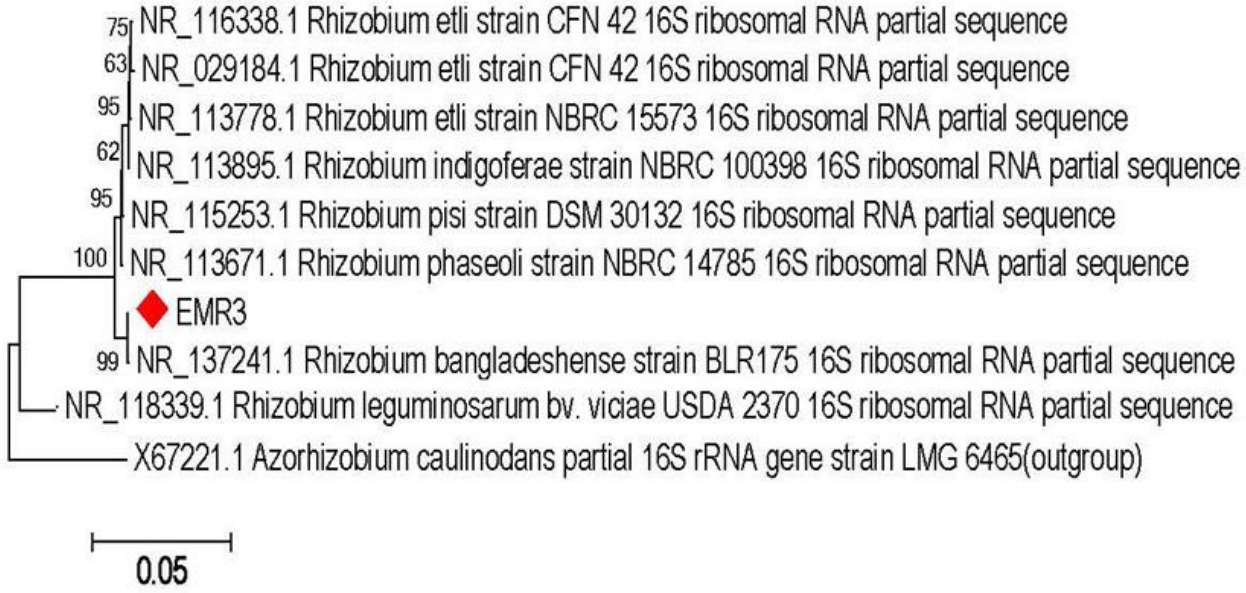


**Figure 4. Neighbor-joining phylogenetic dendrogram showing the genetic relationships between EMR3 isolate and their reference strains based on 16S rRNA gene sequence.**

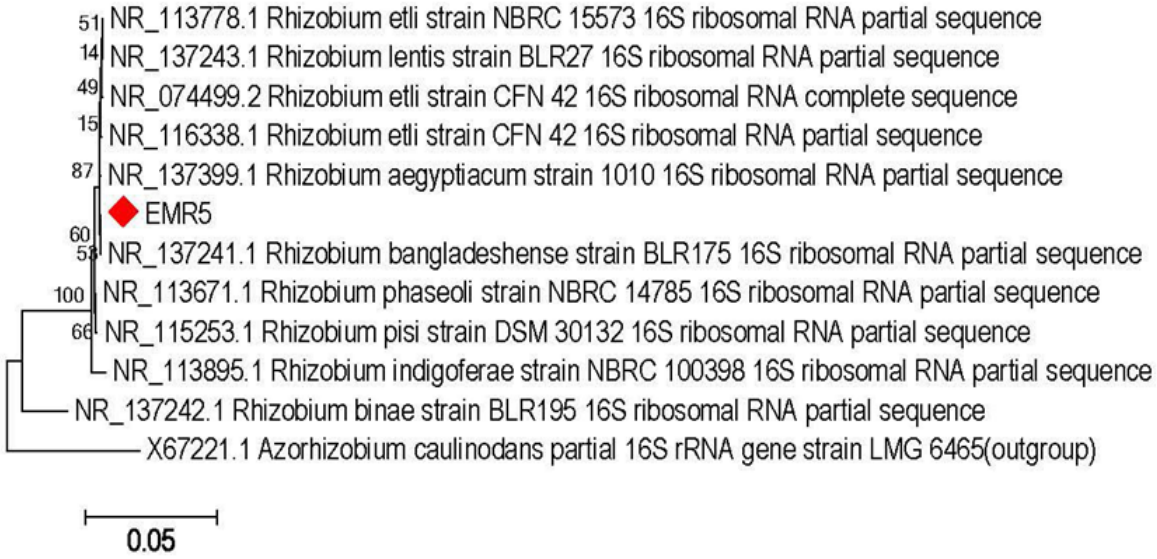


**Fig 5. Neighbor-joining phylogenetic dendrogram showing the genetic relationships between EMR5 isolate and their reference strains based on 16S rRNA gene sequence.**

Finally, the constructed phylogenetic tree (Figure 6) of isolate EMR4 and their related reference strains in database showed that it had the same clade with *Rhizobium aegyptiacum* strain 1010 NR_137399.1.

The phylogenetic tree of all 5 rhizobial isolates was presented in Figure (7). It was found that the 2 rhizobial species (*Rhizobium bangladeshense* and *Rhizobium aegyptiacum*) of Egyptian clover were shared the same clade. As well as it also shared *Rhizobium binae* of Lupine as a common ancestor. Finally, it was found that *Rhizobium pusense* of Peanut was the common ancestor with all other rhizobial isolates. These findings revealed that the conserved gene of these 4 *Rhizobium* isolates was derived from *Rhizobium pusense*.


**Figure 6. Neighbor-joining phylogenetic dendrogram showing the genetic relationships between EMR4 isolate and their reference strains based on 16S rRNA gene sequence.**

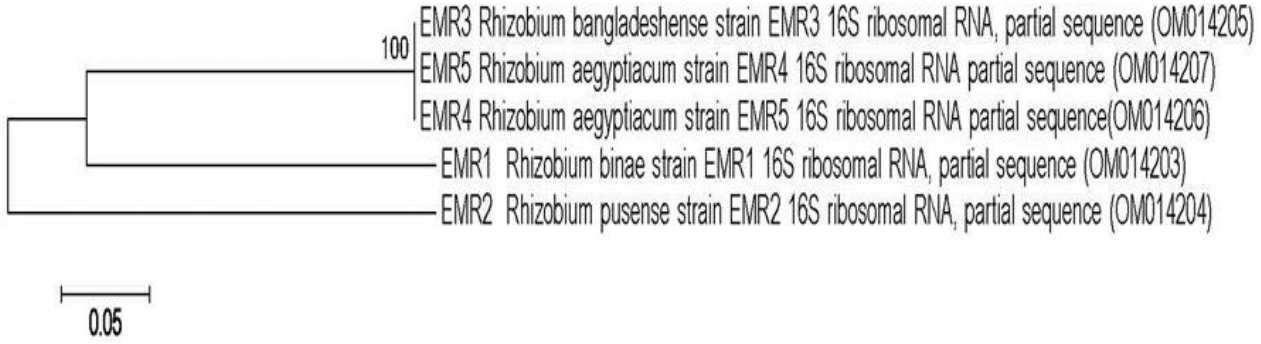


**Figure 7. Neighbour-joining phylogenetic dendrogram showing the genetic relationships of the 5 rhizobial isolates of three different leguminous crops based on the 16S rRNA gene sequence.**

It is necessary to know the genetic variation among *Rhizobium* species and its reflection on the fertility of soil and productivity of crops (Smalla, *et al.,* 2007). Using different *Rhizobium* inoculates as a valuable biofertilizers could be help to improve the production of legume crops (Binde *et al.,* 2009).

Our results were similar to these of Rashid *et al.,* 2015 and Shamseldin *et al.,* 2016 who found that *Rhizobium* strains that nodulate Egyptian clover (*Trifolium alexandrinum* L.) divided into two clusters, according to 16S rRNA, atpD and recA genes analyses. The first cluster strains were known as *Rhizobium aegyptiacum* sp, and the second cluster strains were named as *Rhizobium bangladeshense*.

On the other hand, the other *Rhizobium* species (*Rhizobium binae* and *Rhizobium pusense*) were isolated from many legumes crops such as *Trifolium alexandrinum* L., *Cicer arietinum* L. and *Pisum sativum* L. (Rashid *et al.,* 2015, Panday *et al.,* 2011 and Rahi *et al.,* 2020). The genes of nodulation in *Rhizobium* spp. are carrying on plasmids which could be easy moved between different rhizobial species (Kuykendall *et al.,* 2004). So it is possible to find several rhizobial stains attack more than one legume genus and forming nodules, there seem to be processes that give the rhizobium-legume relationships some specificity (Kuykendall *et al.,* 2004).

The obtained results showed that the genetic diversity of the rhizobial isolates that nodulate the leguminous crop at the chosen sites in the Sohag governorate is very low, which may be cause their low host specificity and ability to increase nodules formation in different leguminous crops, making them a promising bio-fertilizer for Egypt's sustainable crop production.

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## الملخص العربي

# التعريف الجزيئي وعلاقات القرابه لعزلات رايزوبيم العقد الجذريه فى بعض المحاصيل البقوليه

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في دراسه اجريت علي بكتريا الريزوبيوم التكافليه بعزلها من مجموعه مختلفه من النباتات التي تتكافل معها في نطاق محافظه سوهاج قد تم التفرقه بين العزلات اعتمادا علي التوصيف الجزيئي لها باستخدام تفاعل البلمره المتسلسل اعتمادا علي منطقه 16S rDNA وعمل تحليل لتتابعات القواعد النيتروجينيه للقطع الجينيه الناتجه من كل سلاله.وقد ثبت ان كل العزلات هي عزلات لبكتريا الريزوبيوم التكافليه. من بين العزلات الخمسه التي تم عمل تتابع للقواعد النيتروجينيه بها وجد ان العزله EMR2 قد اعطت تماثلا تاما مع السلاله البكتريه *Rhizobium pusense* strain NRCPB10_ والمحفوظه برقم مرجعي NR_116874.1 بينما كانت العزله EMR1 تتماثل بنسبه٩٩,٥٨% مع السلاله *Rhizobium binae* strain BLR195 ذات الرقم المرجعي NR_137242.1. اظهرت العزلتان EMR4 and EMR5 تماثلا بنسب٩٧,٣٥ %97.14 and علي الترتيب مع السلالتين *Rhizobium aegyptiacum* strain 1010 NR_137399.1 and *Rhizobium bangladeshense* strain BLR175 NR_137241.1. تواليا واخيرا اظهرت العزله EMR3 اقل نسب التماثل بنسبه ٩٦,٨٨%. مع السلاله *Rhizobium bangladeshense* strain BLR175 NR_137241.1. وجد أن العزلتان المعزولتين من البرسيم المصري لهما نفس الاصل المشترك بينما تتشاركان مع عزلات الريزوبيوم المعزوله من الترمس في نفس الفرع بينما كان الريزوبيوم المعزول من الفول السوداني عاملا مشتركا لكل اسلاف العزلات الاربعه. كشفت هذه النتائج ايضا أن هذا الجين المحفوظ لعزلات Rhizobium الأربعة مشتق من *Rhizobium pusense*.

أظهرت النتائج المتحصل عليها أن التنوع الوراثي للعزلات المعزوله من العقد الجذرية من المحاصيل البقوليه في المواقع المختارة بمحافظة سوهاج منخفض للغاية ، مما قد يتسبب في انخفاض خصوصية العائل والقدرة على إحداث تكوين عقد جذريه متخصصه لكل جنس نباتي كل علي حده مما يجعل امكانيه استخدام هذه العزلات كمخصب حيوي عام غير متخصص يصلح للمحاصيل المستدامة في مصر أمر ممكن الحدوث.