American-Eurasian J. Agric. & Environ. Sci., 4 (5): 541-549, 2008
ISSN 1818-6769

Molecular Biodiversity and Identification of Free Living *Rhizobium* Strains from Diverse Egyptian Soils as Assessed by Direct Isolation Without Trap Hosts

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Abstract: Soils from six governorates in the middle of Delta Nile Valley and the Al Ismailyah region of Egypt were examined to determine the dominant, trap-host independent, species of *Rhizobium* by using a direct soil isolation technique. Thirty four Egyptian Free living Rhizobial Isolates (EFLRI) were directly isolated from these soils without the use of a trap host and species status was determined by using partial sequencing of *16S rDNA*. Sequences of *16S rDNA* and phylogenetic analyses revealed that 38.2% of strains were identified as *Sinorhizobium meliloti*, 29.4% were highly related to *Sinorhizobium medicae*, 23.5% were identified as *Agrobacterium tumefaciens* and 8.8% of the strains were taxonomically similar to *Rhizobium etli*. Amplified ribosomal DNA restriction analysis (ARDRA) revealed that strains of *Sinorhizobium medicae*, *Agrobacterium tumefaciens* and *Rhizobium etli* each belonged to two different genotypes while strains of *Sinorhizobium meliloti* belonged to one genotype that was identical to the standard strain *S. meliloti* 2011. The reminder of EFLRI strains, with the exception of the three *Rhizobium etli* strains, failed to give amplified fragment with *nodC* primers indicating that free *Rhizobium* strains existing in the soil without their legume hosts often lack the symbiotic genes. Results of ARDRA and sequence analysis of the 16S *rDNA*, *atpD* and *glnII* genes indicated that the most dominant species in these soils was *S. meliloti* and that this species can be commonly found in the rhizosphere of wheat plants.

Key words: Genetic diversity · *Rhizobium* · Egyptian soils · *atpD, glnII, nodC* · *16S rDNA genes*

INTRODUCTION

Rhizobium sp. strains have two different life-styles; they can exist as free-living soil saprophytes or as nitrogen-fixing endo-symbionts of legume host plants. While many studies have examined the genetic diversity of *Rhizobium* isolated from root nodules of legume hosts [1-3], only a limited number of studies have examined rhizobia directly isolated from soils [4,5]. This is chiefly due to a lack of reliable selection techniques. However, some protocols that allow the direct isolation of several species of rhizobia using selective media has been developed [6-8]. Graham [6] developed a specific medium to isolate strains of *S. meliloti* and *Rhizobium legminosarum* from soils. Similarly, Tong and Sadowsky [7] developed a selective medium that facilitates the isolation of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* strains directly from soils and inoculants. Pattison and Skinner [9] reported the formulation of a selective medium for rhizobia that contained pentachloronitrobenzene, brilliant green, sodium azide, crystal violet and penicillin. Barber, [10] subsequently modified the concentrations of these inhibitors to make the medium more selective for *R. (S.) meliloti*.

The majority of world soils contain indigenous rhizobial strains which are often more competitive for nodule formation than inoculant strains and some indigenous strains are ineffective in forming symbiosis leading to the failure of inoculation [11]. Several molecular tools have been readily adapted to examine biodiversity of rhizobia. These include, DNA fingerprinting using REP and BOX primers [12], amplified ribosomal DNA restriction analysis (ARDRA) of both16S and 23S rDNA [1] and phylogenetic analysis of *atpD* and *glnII* genes[13].

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At the initial stage of interaction between *Rhizobium* and its legume host there is an exchange of signals. Plants produce flavonoides which differed from plant to plant and the micro-symbionts respond by introducing a complex of lipo-polysaccharide (Nod factor) and nodulation genes which initiates nodule formation. The later compounds are produced via the action of nodulation (*nod*) gene which also differs between *Rhizobium* species. The type and sequence of *nod* gene can be used as a main characteristic to differentiate among rhizobial isolates, therefore *nod* gene targeting-PCR is a useful tool for identifying rhizobial isolates obtained directly from soil [14].

The objectives of this study were to assess the dominant species of free living *Rhizobium* strains from Egyptian soils, to study the genetic diversity of these natural *Rhizobium* populations using different molecular tools and to determine phylogenetic relationships of the dominant free-living *Rhizobium* strains from Egyptian soils.

MATERIALS AND METHODS

Soil samples were collected from different agricultural field sites in six Egyptian governorates, samples were grinded and dried at 30°C and kept at 4°C for five days prior to isolation of rhizobia. Ten gram of each soil samples were diluted in sterile water to 10^{-4}. From each soil dilution, a 100 µl aliquots of this dilution was plated out on selective yeast extract mannitol [15] YEM agar medium amended with bromothymol blue, 200 mg/l of cyclohexamimide, 100 mg/l pentachloronitrobenzene, 25 mg/l sodium benzyl pencillin, 10 mg/l chloromphenicol and 25 mg/l neomycin [6]. Plates were incubated for three days at 28°C. Fast growing yellow colonies were selected and were re-streaked on YEM agar containing 0.0025% Congo red. All isolates and standard strains were stored at -70°C, in yeast extract mannitol medium containing 50% (vol/vol) glycerol until further analysis.

Total genomic DNA from rhizobial strains was isolated as described by Ausubel *et al*. [16]. Primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used to amplify 16S *rDNA* genes [17], while primers nodCFu (5'-AYGTHGTYGAYGACGGCTC-3') and nodCI (5'-CGYGACAGCCANTCKCTATTG-3') were used to amplify the *nodC* gene as described by Laguerre *et al*. [18]. Primers atpD 255F (5'-GCTSGGCCGCATCMTSAACGTC-3') and atpD 782R (GCCGACACTTCMGAACCNGCCTG-3') were used for amplifying *atpD* gene while primers glnII 12F (5'-YAAGCTCGAGTACATYTGGCT-3') and glnII 689R (TGCATGCCSGAGCCGTTCCA-3') were used to amplify the *glnII* gene [13]. PCR reactions (50 µl) were performed in 1× PCR buffer, 1.5 mM $MgCl_2$, 5% dimethyl sulfoxide, 200 µM of each dNTP, 15 pmol of each primer, 1 U of Taq polymerase and 50 ng of purified DNA. The temperature program was as follows: initial denaturation at 95°C for 4 min; 35 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1 min and extension at 72°C for 2 min, except for the *glnII* where the annealing temperature was 58 °C. A final extension was conducted at 72°C for 7 min. PCR products were separated on 0.8% agarose gels for 30 min at 100 V followed by staining with ethidium bromide and detection under UV light. PCR products of 16S *rDNA* fragments were purified by using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA) according the manufacture's instructions and were digested with *CfoI*, *HinfI* and *MspI* as recommended by the manufacturer (Takara, Seoul, South Korea) [1].

DNA sequencing of 16S rDNA, atpD and glnII The amplified fragments of *16S rDNA*, *atpD* and *glnII* were purified using QIAquick PCR purification kit according the manufacture's instructions (QIAGEN, Valencia, CA, USA). Products were subjected to cycle sequencing as described elsewhere by Shamseldin *et al*... [1].

Phylogenetic analyses Construction of phylogentic tree has been done according to the method explained in details by Shamseldin *et al*... [19]. Sequences of 16S *rDNA* of 13 representative Egyptian free living rhizobial isolates EFLRI 60, 62, 70, 75, 78, 83, 88, 90, 94, 116, 123, 126 and 128 were used in a context with the sequences of 13 different standard rhizobial strains obtained from Genbank. The standard strains were *R. l.* bv. *viciae* USDA 2370 (U29386), *R. tropici* CIAT 899 (U89832), *R. lusitanum* CCBAU03301 (EU074200), *R. giardinii* H152 (U86344), *R. rubi* IFO 13261 (D14503), *R. radiobacter* NCPPB 2437 (D14500), *S. terangae* LMG 7834 (X68388), *S. medicae* A321 (L39882), *S. meliloti* LMG 6133 (X67222), *B. japonicum* LMG 6138 (X66024), *B. elkani* USDA 76 (U35000), *R. etli* CFN42 (U28916) and *Bradyrhizobium* sp. BTAil (NC_009485). The *glnII* sequences of two representative strains, EFLRI 88 and 128, were compared with six different standard rhizobial strains *S. meliloti* CCBAU 83493 (EF549447), *B. elkani* ICMP 13638 (AY494804), *Bradyrhizobium* sp. ICMP 14752 (AY494798), *R. tropici* CCBAU 41189 (EU170596), *R. lusitanum* P1-7 (EF639841) and *R. etli* bv. *phaseoli* GR-12 (AY929464).

The *atpD* sequences of two representative strains, EFLRI 70 and 116, were compared using the phylogenetic tree with six standard and different rhizobial species *R. tropici* CCBAU 41189 (EU170576), *R. lusitanum* P1-7 (DQ431671), *S. medicae* A 321 (AJ294401), *R. radiobacter* LMG 140 (AM418785), *B. elkani* ICMP 13638 (AY493446) and *Bradyrhizobium* sp. ICMP 14533 (AY493445).

Accession numbers 16S rDNA sequences for Egyptian free living rhizobial isolates (EFRLI) were deposited in GenBank under the accession numbers EU445236 to EU445269 and EU404161-EU404164 (*atpD* sequences) correspond to EFLRI 60, 70, 90 and 116 and EU404165-EU404166 (*glnII* sequences) correspond to EFLRI 88 and 128,respectively.

RESULTS

Strain Isolation and Identification: Samples from agricultural soils obtained from six Egyptian governorates in the middle of the Delta Nile valley and in the Al-Ismailyah region were used to isolate free-living rhizobial strains using a direct soil isolation approach (Table 1). A total of 34 free-living rhizobial isolates were directly isolated from these soil samples and the majority (13 of 34, 38.24%) were isolated from four distinct sites in the Al-Sharqiyah governorate and 7 isolates (20.59%) were from three different sites in the Al-Qalybiyah governorate. The remainder of the isolates was obtained from the Al-Minufiyah, Al-Byhayrah, Al-Ismailyah and

Table 1: Identification of Egyptian Free Living Rhizobial isolates (EFRLI) based on the partial sequencing of 16S rDNA

EFLRI no. and $(ntl)^{-1}$	Query coverage	Identity %	Identified species	origin of isolates	crop
54 (864 bp)	99	97% (852/873)	A. tumefaciens	Kafer El Dawar, Al Buhayrah	Clover
59 (659 bp)	98	96% (628/652)	S. medicae	Abu hammad, Al Sharqiyah	Clover
60 (481 bp)	96	97% (458/471)	A. tumefaciens	Kafer El Dawar, Al Buhayrah	Clover
62 (727 bp)	98	97% (701/719)	S. medicae	Kafer El Dawar, Al Buhayrah	Wheat
70 (936 bp)	99	98% (922/939)	A. tumefaciens	Abu hammad, Al Sharqiyah	Faba bean
71 (792 bp)	97	95% (748/781)	S. medicae	Abu hammad, Al Sharqiyah	Faba bean
72 (864 bp)	99	97% (845/869)	A. tumefaciens	Abu hammad,Al Sharqiyah	Faba bean
73 (936 bp)	99	98% (925/941)	A. tumefaciens	El tal El kibeer,Al Sharqiyah	Wheat
75 (599 bp)	98	98% (587/593)	S. meliloti	El tal El kibeer,Al Sharqiyah	Wheat
76 (912 bp)	99	99% (901/904)	S. meliloti	Benha, Al Qalyubiyah	Potato
78 (864 bp)	99	99% (854/862)	S. meliloti	Benha, Al Qalyubiyah	Wheat
79 (848 bp)	99	99% (840/842)	S. meliloti	Kassassin, Al Sharqiyah	Wheat
80 (955 bp)	98	99% (941/943)	S. meliloti	Kassassin, Al Sharqiyah	Clover
81 (864 bp)	99	99% (859/861)	S. meliloti	Kassassin, Al Sharqiyah	Faba bean
82 (746 bp)	71	97% (522/533)	S. meliloti	Benha, Al Qalyubiyah	Faba bean
83 (959 bp)	99	99% (954/959)	S. meliloti	Benha, Al Qalyubiyah	Wheat
84 (873 bp)	97	99% (849/856)	S. meliloti	Benha, Al Qalyubiyah	Wheat
88 (963 bp)	99	99% (955/958)	S. meliloti	Berket Elsabeh, Al Minufiyah	Wheat
89 (924 bp)	98	99% (908/913)	S. medicae	Itay El baroud, Al Buhayrah	Clover
90 (883 bp)	98	97% (857/875)	S. medicae	Abu Suwauer, Al Ismailyah	Faba bean
94 (960 bp)	99	99% (952/958)	S. meliloti	Tanta, Al Gharbiyah	Wheat
95 (919 bp)	98	99% (902/904)	S. meliloti	Tanta, Al Gharbiyah	Wheat
98 (975 bp)	99	99% (969/977)	S. meliloti	Az Zagazig, Al Sharqiyah	Wheat
100 (910 bp)	99	97% (885/905)	S. medicae	Berket Elsabeh, Al Minufiyah	Clover
116 (903 bp)	98	97% (878/896)	S. medicae	Benha, Al Qalyubiyah	Common bean
118 (911 bp)	98	97% (874/900)	S. medicae	El tal El kibeer, Al Sharqiyah	Wheat
120 (916 bp)	99	97% (894/916)	S. medicae	Az Zagazig,Al Sharqiyah	Wheat
121 (929 bp)	99	98% (910/928)	A. tumefaciens	Benha, Al Qalyubiyah	Clover
122 (932 bp)	99	98% (921/936)	A. tumefaciens	Berket Elsabeh, Al Minufiyah	Clover
123 (901 bp)	97	99% (878/881)	A. tumefaciens	Berket Elsabeh, Al Minufiyah	Clover
125 (897 bp)	99	97% (873/892)	S. medicae	Az Zagazig, Al Sharqiyah	Wheat
126 (849 bp)	98	96% (806/839)	R. etli	Abu Suwauer, Al Ismailyah	Wheat
127 (487 bp)	96	98% (463/471)	R. etli	Abu Suwauer, Al Ismailyah	Wheat
128 (858 bp)	98	97% (834/854)	R. etli	Ashmun, Al Minufiyah	Clover

ntl^{-1}: neuclotide length: A: Agrobacterium, S: Sinorhizobium, R: Rhizobium. Genebank accession numbers that used for comparison were (NC_003062, NC_009636, NC_003047 and NC_007761) belong to A. tumefaciens C58, S. medicae WSM419, S. meliloti 1021 and R. etli CFN 42 respectively

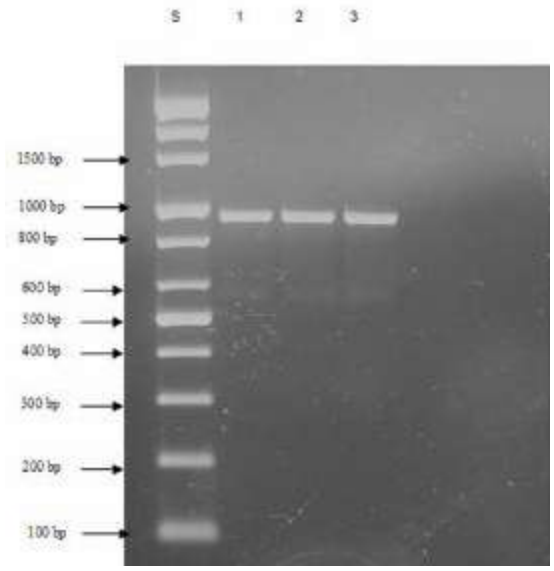

Fig. 1: nodC amplification 970 bp of R. etli strains, S: 100 bp ladder, 1: EFLRI 126, 2: EFLRI 127 and 3: EFLRI: 128 on respectively

Al-Gharbiyah governorates. PCR products of 16S rDNA from each isolate were sequenced and compared with those in Genbank and in RDPII using the BLASTN algorithm. Sequence analysis of 16S *rDNA* in Table 1 indicated that 13 strains had 97 to 99% similarity with *Sinorhizobium meliloti* strain 1021, 10 strains were classified as *Sinorhizobium medicae* with similarity level between 95 to 97% with *Sinorhizobium medicae* strain WSM 419, 8 strains were categorized as *Agrobacterium tumefaciens* with similarity level between 97 to 99% with strain *Agrobacterium tumefaciens* C58 and 3 strains shared maximum similarity (96 to 98%) with *Rhizobium etli* CFN 42 strain.

Presence of *nodC* in EFLRI Strains: All the EFLRI strains were examined for the presence of *nodC* by using PCR and primers nodCFu and nodCI [18]. With the exception of EFLRI 126, EFLRI 127 and EFLRI 128 (Fig. 1), the remainder of the tested isolates did not contain a *nodC* homolog that was revealed by PCR using the tested primers.

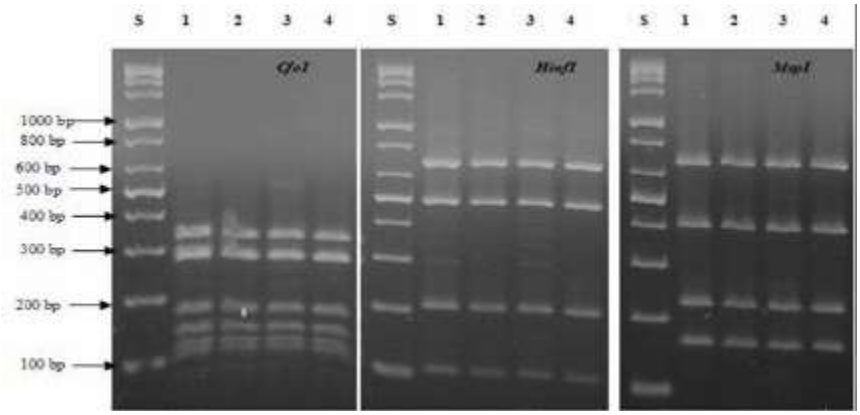

Fig. 2: ARDRA of Sinorhizobium meliloti like-isolates isolated directly from soils, S 100 bp ladder, 1: Sinnorhizobium meliloti 2011, 2: EFLRI 75, 3: EFRLI 83 and 4: EFRLI 88

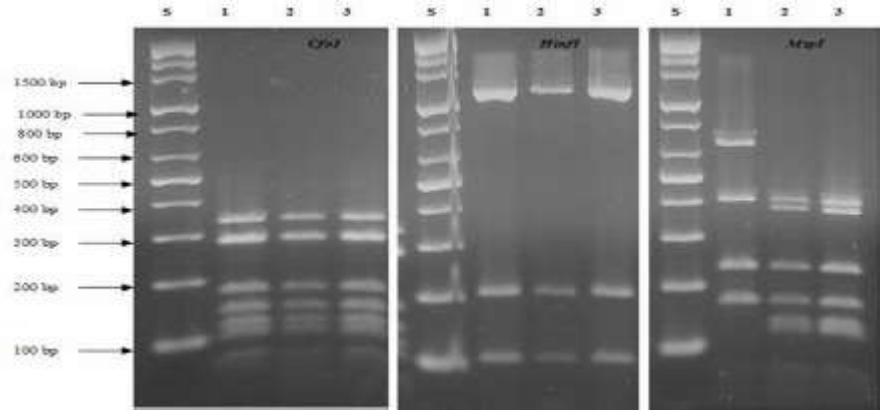

Fig. 3: ARDRA of Sinorhizobium medicae like-isolates isolated directly from soils, S: 100 bp ladder, 1: EFLRI 62, 2: EFLRI 90 and 3: EFLRI 116

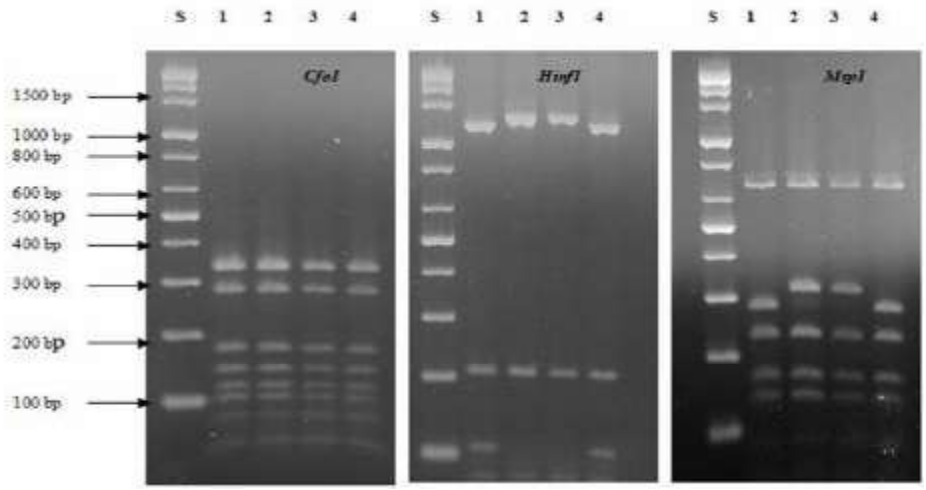

Fig. 4: ARDRA of Agrobacterium tumefaciens like-isolates isolated directly from soils, S: 100 bp ladder, 1: EFLRI 60, 2: EFLRI 70, 3: EFLRI 123 and Agrobacterium tumefaciens DSM 30150

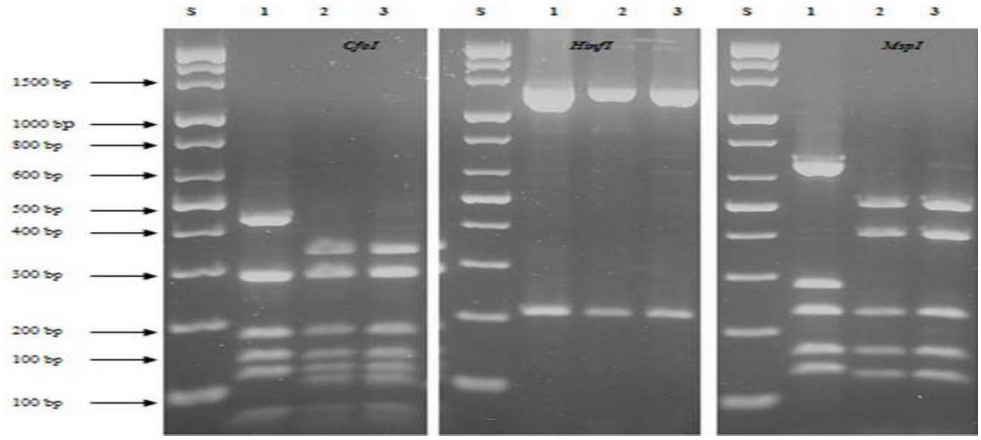

Fig. 5: ARDRA of Rhizobium etli like-isolates isolated directly from soils, S: 100 bp ladder, 1: EFLRI 126, 2: (EFLRI 127) and 3: Rhizobium etli CFN 42.

Genetic Diversity Based on ARDRA Results: The genetic diversity of the EFLRI was examined by ARDRA and the results in Fig. 2 show that the 13 *Sinorhizobium*-like isolates had identical ARDRA patterns with *Sinorhizobium meliloti* 2011 when the amplified fragment of 16S *rDNA* was digested with *CfoI*, *HinfI* and *MspI*.

ARDRA of the *Sinorhizobium medicae*–like isolates (10 strains) showed that the representative strains EFLRI 62, 90 and 116 were nearly identical, except for only one to *MspI* restriction site (Fig. 3).

Analysis of the *Agrobacterium*-like isolates revealed that 16S *rDNA* from strains EFLRI 60, 70 and 123 were similar with that of the standard *Agrobacterium tumefaciens* strain DSM 30150, when 16S rDNA was only digested with enzyme *CfoI*. In contrast the later two strains had one different restriction site than the standard strain with *HinfI* and *MspI* (Fig. 4).

ARDRA of *Rhizobium etli*-like strains indicated that the three strains (EFLRI 126, 127 and 128) consisted of two different genotypes (Fig. 5). The restriction profiles of 16S *rDNA* of strains EFLRI 127 and 128 were identical with the ARDRA of *Rhizobium etli* CFN 42, while strain EFLRI 126 was a unique genotype.

Phylogenetic Analysis: Partial sequences of 16S *rDNA* of strains EFLRI 75, 78, 83, 88 and 94 (as representative strains for *Sinorhizobium meliloti*-like isolates), strains EFLRI 62, 90 and 116 (as representative strains for *Sinorhizobium medicae*-like isolates), strains EFLRI 60, 70 and 123 (from the group of *Agrobacterium*-like isolates) and strains EFLRI 126 and 127 (from the group of

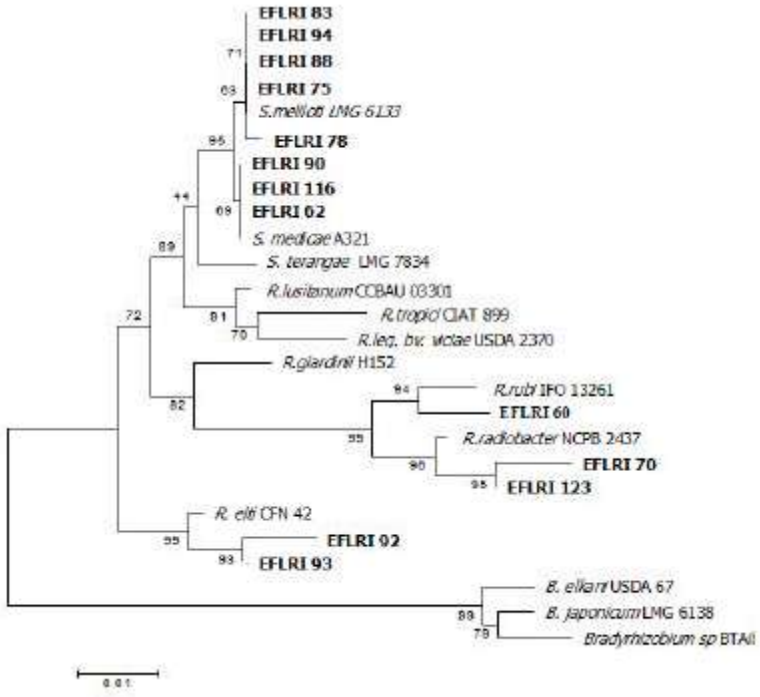

Fig. 6: Neighbor-joining phylogenetic analysis of 16S rDNA sequences (487 bp) of Egyptian Free living Rhizobial isolates compared with the sequence of standard strains. Bootstrap probabilities were indicated and the bar represents a 0.01% of nucleotide divergence

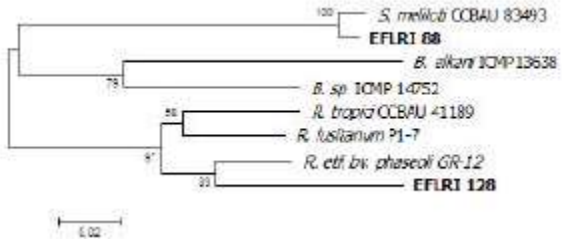

Fig. 7: Neighbor-joining phylogenetic analysis of *glnII* sequences (204 bp) of Egyptian Free living Rhizobial isolates compared with the sequence of standard strains. Bootstrap probabilities were indicated and the bar represents a 0.02% of nucleotide divergence

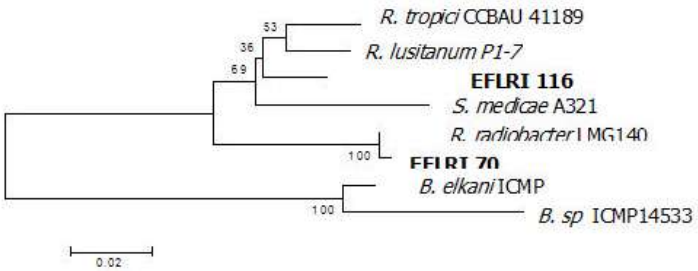

Fig. 8: Neighbor-joining phylogenetic analysis of *atpD* sequences (426 bp) of Egyptian Free living Rhizobial isolates compared with the sequence of standard strains. Bootstrap probabilities were indicated and the bar represents a 0.02% of nucleotide divergence

Rhizobium etli-like isolates) were used to construct phylogenetic tree using sequences of 16S *rDNA* available from Genbank (Fig. 6). The EFLRI sequences were compared to 13 rhizobial strains belonging to different species. Phylogenetic analyses indicated that strains belonging to the *S. meliloti* group were in the same ancestral clade as *S. meliloti* strain LMG 6133 with 71% bootstrap probability, strains belonging to *S. medicae* group were intermingled with strain *S. medicae* A 321 with 69% (Fig. 6). Two of *Agrobacterium*-like isolates, EFLRI 70 and 123 were in the same clade with *R. radiobacter* (formerly classified as *Agrobacterium tumefaciens*) with 96% probability, while EFLRI 60 that was identified as *Agrobacterium* strain (Table 1), shared the same clade as *R. rubi* (formerly classified as *Agrobacterium tumefaciens*) with 94% bootstrap probability. Strains EFLRI 126 and 128 formed genetic cluster with *Rhizobium etli* CFN 42 with 99% bootstrap probability.

For further confirmation of phylogenetic association, two other phylogenetic trees were constructed, one based on sequence alignments using the *glnII* gene and the other *atpD* gene. Strains EFLRI 88 and 128 were used as representative strain for *Sinorhizobium meliloti*-like isolates and *Rhizobium etli*-like isolates, respectively. DNA from these two strains served as templates for PCR, producing amplified fragment of about 650 bp with primers glnII 12F and glnII 689R (data not shown). Sequences of *glnII* fragment were used for constructing the phylogenetic tree shown in Fig. 7. Strain EFLRI 88 clustered with the phylogenetic lineage of *S. meliloti* strain CCBAU 83493 with 100% bootstrap probability, while strain EFLRI 128 shared the genetic branch with *R. etli* bv. *phaseoli* GR-12 with 83% probability.

PCR of DNA from EFLRI strains (116 and 70) representatives the *S. medicae* and the *Agrobacterium*-like isolates respectively, produced amplified fragment of 550 bp using primers atpD 255F and atpD 782R (data not shown). Results in Fig. 8 shows strain EFLRI 70 belonged to the ancestral branch of *R. radiobacter* LMG 140 confirming the previous results whereas strains EFLRI 116 clustered together *S. medicae* A 321 with a 65% similarity level.

DISCUSSION

To explore the genetic diversity of rhizobia without using a trap host, several selective media have been developed [6-8]. In this study we used a selective medium that developed by Graham, [6] to directly isolate rhizobia from six governorates in the middle of the Nile Valley Delta and from the Al-Ismailyah region of Egypt. Among 34 rhizobial isolates obtained, 26 strains were taxonomically identified as three different species of *Rhizobium* and 8 strains were identified as *Agrobacterium* based on sequencing analysis. The later strains are taxonomically related to the *Rhizobium rubi* or *Rhizobium radiobacter* [20]. Our results indicate that selective method employed in these studies was useful for directly isolating rhizobia from Egyptian soils, specially the fast growing *Sinorhizobium* strains. The medium facilitated the isolation of *Sinorhizobium meliloti*, *Rhizobium etli* and *Agrobacterium tumefaciens* strains. This is similar to results reported by Louverier *et al.* [21] who isolated the same three species of *Rhizobium* on MNBP selective medium that used the same chemical agents used in this study to inhibit the growth of fungi and actinomycetes. Our results are in agreement with those of Laguerre *et al.*. [22], Segovia *et al*. [23] and Soberon-Chaves and Najera [24] who reported that non symbiotic *Rhizobium* strains can survive in presence or absence of their legume hosts. While the origin of these non-symbiotic rhizobial strains is unknown, there are two possible explanations for their presence in soils, the non-symbiotic rhizobial strains may exist in the soil purely as saprophytic bacteria, or they released from dead or senescing nodulated legume roots [8]. On the other hand, results of *nodC* amplification revealed that with the exception of the three *R. etli* isolates, all the other EFLRI strains under study did not contain a *nodC* fragment using the conserved primer set. This may be due the fact that the cultivation of beans is a common in Egyptian agricultural fields under agricultural intensification system and beans frequently harvested before the second crop. In contrast, the inability of other rhizobial strains to give amplified fragment with *nodC* primers may be that crops associated with these rhizobia are not commonly grown in Egyptian soils leading to low populations of symbiotic bacteria. Our results are in agreement with those obtained by Neelawan *et al.* [4] who found that non-symbiotic *Bradyrhizobium* isolates isolated directly from soil failed to hybridize with both *nifH* and *nod* gene probes. These results are also confirmed by those obtained by Soberon-Chaves and Najera [24] who found that *Rhizobium leguminosarun* strains isolated directly from soil often lack the symbiotic properties. The restriction patterns of 16S rDNA of the *Agrobacterium*-like isolates revealed that strains EFLRI 70 and 123 differed in one restriction site than the standard strain to DSM 30150 (Fig. 4). Similarly *Sinorhizobium*-like strains and *Rhizobium etli* strains gave two different genotypes. This may be due to either lateral gene transfer or mutation that caused changes of ARDRA results. Lateral gene transfer was reported by Ueda *et al.* [25] and Herrera-Cervera *et al*. [2] and this supports the notion that

free bacterial populations in soils are dynamic. In contrast, results of *S. meliloti*-like isolates indicated that there is only one genotype dominant in the majority of examined strains in this study. The two phylogenetic trees constructed by the sequence of *glnII* and *atpD* genes (Fig. 7 and 8) confirmed the results and identification of strains by 16S rDNA sequences (Fig. 6). Although there are many different *Rhizobium* species have been reported worldwide [20], we were only able directly to isolate three different *Rhizobium* species from diverse Egyptian soils using this selective media. The most dominant species was *S. meliloti* and these results are in agreement with Graham, [6] who could isolate and recovering *S. meliloti* strains from soils and inoculants using this specific medium. The majority of *Sinorhizobium meliloti* strains were isolated from the rhizosphere of wheat plants (Table1) and these results supported by Hirsh, [26] who reported the increase of titre of this *Rhizobium* species around the root of wheat plant (25×10^{-4} g^{-1}). Beside contributing both to worldwide knowledge about soil biodiversity and to the utility of rhizobial collections, the assessment of rhizobial genetic diversity free in soils plays an important role in developing long-term strategies to increase the contribution of biological N_2 fixation to agricultural productivity. The assessment of genetic diversity of free-living non-symbiotic *Rhizobium* strains can provide valuable information about bacterial genotypes, dominance species, lateral gene transfer and the stability of symbiotic genes in these strains. This study reflects the importance of renewing the inoculation of legumes annually due to the absence of *nodC* gene in the majority of free living *Rhizobium* strains that isolated without the trap host.

ACKNOWLEDGEMENTS

The authors wish to thank H. Abdelsalam for his help to collect soil samples, E. Hafez for financial the collecting of soil samples. This work was supported by Korean Science and Engineering Foundation (KOSEF) through post doctoral fellowship for (A. Shamseldin) and a part by grant from the Biogreen 21 (20050401034793) to (C. S. An).

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