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Full Length Research Paper

Genetic identification and symbiotic efficiency of *Sinorhizobium meliloti* indigenous to Saudi Arabian soils

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Soil bacteria *Sinorhizobium meliloti* are of enormous agricultural value, because of their ability to fix atmospheric nitrogen in symbiosis with an important forage crop legume-alfalfa. The main aim of this study was (i) to isolate indigenous *S. meliloti* strains from different field sites in Saudi Arabia, (ii) to assess genetic diversity and genetic relationships amongst strains of natural populations and (iii) to provide information about nodulation and symbiotic efficiency of indigenous *S. melitloti* strains. Nineteen strains isolated from alfalfa nodules collected from different field sites and one reference strain were analyzed. Genetic characterization by rep-PCR and RAPD-PCR was applied to study the status of *S. melitloti* populations inhabiting nodules of alfalfa. Cluster analysis of rep-PCR profiles showed significant differences among *S. meliloti* isolates. Both methods resulted in almost identical grouping of strains. Among indigenous strains two divergent groups could be determined. The biggest differences were detected among the reference strains along with two local isolates and all field isolates. Quantitative expression of symbiotic efficiency was evaluated by measurement of total nitrogen in plants and dry matter yield of plants. All strains nodulated alfalfa cultivar CAF 101 but with different efficiency. Significant differences in dry mater yield of alfalfa as well as total nitrogen content were determined depending on the strain used. The results indicate that sixty one percent of indigenous *S. meliloti* strains can be characterized as low efficient strains.

Key words: Alfalfa, *Sinorhizobium meliloti*, symbiotic efficiency, genetic diversity.

INTRODUCTION

Soil bacteria forming nitrogen-fixing nodules on legumes are divided into five genera *Rhizobium, Mesorhizobium, Bradyrhizobium, Azorhizobium,* and *Sinorhizobium*. However, the continued application of molecular approaches has led to their division into additional genera (Martinez-Romero and Caballero-Mellado, 1996). The economical significance of the *Rhizobium*-legume association has resulted in extensive research designed to improve the efficiency of the symbiosis. Many attempts have been made to select naturally occurring strains that are both symbiotically effective and able to persist in the environment.

The perennial species *Medicago sativa* is the most widely cultivated species of alfalfa in the world. Symbiotic strains isolated from this legume have been studied intensively and have been included in many genetic and taxonomic studies of the *Rhizobiaceae*. *M. sativa* is well adapted and native to different areas of Saudi Arabia (Al-Asmary, 2007)). Alfalfa is the most important routinely cultivated legume in Saudi Arabia. This forage crop occupies more than 30% of the completely cultivated area. Alfalfa is of great importance in Saudi Arabia soils for many years, therefore, it is expected that Saudi Arabian soils contain high densities of rhizobia capable of nodulating this widely cultivated legume. To take advantage of biological nitrogen input in semi-natural systems of economic and environmental importance, suitable rhizobia must be present in the soil capable of establishing an efficient symbiosis with *M. sativa*. Thus, it is

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essential to characterize the native *Sinorhizobium* population and the factors which influence their composition and population dynamics.

Various tools have been used to explore the phenotypic and genotypic variation among rhizobial populations and to assess their diversity. Phenotypic characterizations of *Sinorhizobium meliloti* strains indicated that the composition of field population varies greatly between soils of different geographical areas. However, the *S.meliloti* population obtained from nodules is not representative of the total population present in the soil (Bromfield et al., 1995).

The efficiency of sinorhizobial nodulation and symbiotic nitrogen fixation is determined by their individual genotype. In order to identify genotypes displaying, for instance, superior nitrogen fixing capabilities, it is essential to characterize the natural population of competitors that are well adapted to a certain environment. Rapid and reliable molecular methods have been used for differentiation of rhizobial strains (Terefework et al., 1998; Nick et al., 1999; Olive and Bean, 1999; Zhang et al., 1999; Doignon-Bourcier et al., 2000; Gao et al., 2001). A large number of molecular methods based on polymerase chain reaction have been proposed to characterize *Sinorhizobium* strains and to provide a high degree of differentiation among closely related bacterial strains. The random amplified polymorphic DNA (RAPD) fingerprinting method is based on the use of short primers which hyberdise with sufficient affinity to chromosomal DNA sequences at low annealing temperatures in a way that they can be used to initiate amplification of regions of the bacterial genome (Williams et al., 1990). The number and location of these random sites vary for different strains of a bacterial species. In many studies, RAPD-PCR has been proposed for identification and phylogenetic grouping of *Sinorhizobium* isolates (Sikora et al., 1997; Niemann et al., 1999). DNA fingerprints can also be generated by using pairs of primers derived from repetitive extragenic palindromic (REP) and enterobacterial repetitive intrergenic consensus (ERIC) sequences and the BOX element (Versalovic et al., 1994). It has been shown that REP-and ERIC-like sequences are present in rhizobia and in other Gram-negative soil bacteria and that they can be used for bacterial taxonomy (Niemann et al., 1997; Di Giovanni et al., 1999; Sikora et al., 2002). The usefulness of DNA finger-printing by PCR using REP and ERIC primers for identification and classification of the members of several *Sinorhizobium* species was demonstrated (de Brujin, 1992). The fingerprints obtained by rep-PCR and RAPD are specific and reproducible and allow the distinction of bacteria at the (sub) species and strain level (Niemann et al., 1997).

It is also important to evaluate the most effective *S. meliloti* strains for alfalfa inoculation in agricultural production. Dry matter analyses of plants provide a useful measurement of the efficiency of symbioses formed between the crop and the rhizobia (Gandee et al., 1999). The term "symbiotic efficiency" is used to describe the ability of nodulated plants to fix nitrogen, and a quantitative expression is dependent on comparisons of dry weights (Gandee et al., 1999). Besides the measurement of dry weight, quantitative expression of symbiotic efficiency could be evaluated by the measurement of content of proteins, content of total nitrogen in plants and green mass yield of plants.

The aim of the present investigation was to assess the biodiversity of field populations of sinorhizobia nodulating the common *M. sativa* in Saudi Arabia by means of plasmid profiling, rep-PCR, RAPID-PCR, examining symbiotic effectiveness, and by carrying out phenotypic tests. Therefore, comparisons were made between different methods and their ability to demonstrate the characteristics and symbiotic effectiveness of strains. The final goal of this study was to develop a collection of appropriate strains that are adapted to alfalfa species (CAF 101 variety) and soils stress environment.

MATERIALS AND METHODS

Bacterial strains

Nineteen strains of *S. meliloti* were isolated from nodules of alfalfa (*M. sativa*) plants from different geographical sites throughout Saudi Arabia (Table 1). After rinsing in 95% ethanol, nodules were surface sterilized using 0.1% acidified mercuric chloride (Vincent, 1970), squashed in 0.2 ml sterile water, and then streaked onto yeast extract mannitol agar plates. Single colonies of *S. meliloti* were isolated. The symbiotic ability of isolates was checked by nodulation tests (Vincent, 1970).

NaCl tolerance

The tolerance of isolates was tested by using YEM agar containing 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, and 15.0% NaCl. The standard YEM medium with 0.1% NaCl was used as control.

Temperature tolerance

The tolerance of isolates was tested by using YEM agar and incubated on different temperature 30, 35, 40, 45, 50, and 55°C. The standard YEM medium was also incubated at 28°C and used as control.

Plant assay

A total of 19 *S. meliloti* isolates were tested for symbiotic effectiveness using sterile modified Leonard jars (Leonard, 1943) containing a 3:1 mixture of vermiculite and perlite as plant growth medium. Seeds were surface sterilized (Vincent, 1970) before planting and 5 seeds were sown in each jar. Each jar was inoculated with 5 mL of broth culture containing 10^9 cells/ml at planting time. Plants were watered with N-free plant growth medium (Somasegran and Hoben, 1985). A set of jars fertilized with combined nitrogen and controls with inoculation were included. Control treatments received totals of 20, 60, 100 mg NO_3- N/pot in three equal doses 10, 20, 30 days after planting. Planting were randomized in the green house. At harvest (50 days after planting), shoots were removed and dried at 70°C to constant weight. Dried plant material was ground in a Wiley mill, and analyzed for N-con-

Table 1. Isolates of *Sinorhizobium meliloti* from different geographical regions of Saudi Arabia.

Geographical Region	Sites	Number of isolates	Code number of isolates
Middle area	Alkharj	1	121
	Qassim	4	16, 30, 41, and 69
	Wadi- Aldawaser	4	140, 153, 154, and 156
North area	Hail	5	73, 75, 76, 100, and 229
East area	Al hasaa	3	167, 175, and 176
West area	Al- Madina	2	185 and 188

tent after Kjeldahl digestion. From comparisons with N-controls, three classes of effectiveness were defined; highly effective if the isolates produced plant dry weight equal to plants given 100 mg N/jar, moderately effective if they produced dry weight equal to plants given 60 mg N/jar, or poorly effective if plant dry weight were equal to plants given 20 or less mg N/jar. Statistical analyses were performed using the analysis of variance (ANOVA) procedure of SAS.

Plasmid profiles

Using agarose gel electrophoresis, 19 isolates were analyzed for plasmid content as described by Hashem and Kuykendall (1994).

DNA isolation

Total genomic DNAs from nineteen field isolates and one reference strain USDA 1037 were isolated using GenElute Bacterial Genomic DNA Kit, Mini (SIGMA) according to the protocol of Sambrook and Russell (2001). The concentration and the purity of DNA were estimated spectrophotometrically at 260 and 280 nm.

Fingerprinting of genomic DNA by rep-PCR and RAPD-PCR

Primers REP1R-I and REP2-I were used for rep-PCR fingerprinting analysis as described by de Bruijn (1992). Five arbitrarily chosen primers used for RAPD fingerprinting were 10 nucleotides in length. Primer sequences were as follows: 5'-GATCGGACCG, 3'(PI), 5'-TCGCCAGCCA-3'(P16), 5'-GATCCTGCCG-3'(P5), 5'-GATCGGACGG-3'(P2), 5'-GATCCCAGCG-3'-(P4). All the primers used in this investigation were obtained from Aldrich (Pharma, Germany). The cycling conditions for RAPD and rep-PCR differ in annealing time and temperature. The reaction mixtures were overlaid with two drops of mineral oil, incubated at 95°C for 5 min for initial denaturation, 35 cycles in intervals of 30 s at 94°C, 30 s at 36°C (RAPD) or 1 min at 40°C (REP-PCR) and 1 min at 72°C followed by 7 min of incubation at 72°C. PCR-amplified DNA fragments were separated by horizontal gel electrophoresis on 1.5% agarose gels. A molecular size marker, a 1 kb λ Hind III digested DNA ladder (life Technologies), was run in all gels. Electrophoresis was carried out at 7 V/cm and 25°C during 2 h and 45 min. The restriction patterns were visualized using the Gel Documentation System.

DNA fingerprinting analysis

All RAPD and rep-PCR fingerprints and restriction patterns were converted into a two-dimensional binary matrix (1, presence of a band; 0, absence of a band) and analysed by using the NTSYS-pc package (version 1.8; Exeter Software, Setauket, N.Y.). For each pair of strains, a simple matching (Sm) coefficient was calculated, and a UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

RESULTS

Bacterial collection

A collection of 19 rhizobial isolates were obtained from different ecological area of Saudi Arabia from 12 different sites grown with alfalfa *M. sativa* variety CAF. 101.

Phenotypic characteristics of isolates

All of the 19 isolates studied were rod-shaped bacteria. On YEM medium, they were mucous, and they acidified the medium, changing the pH to acidity. The generation time ranged between 3 and 5 h. All of the isolates grew at 28°C.

Salt tolerance of collected local *S. meliloti* isolates

Great variations were recorded among the different *S. meliloti* isolates in relation to their salt tolerance. None of the isolates was able to grow in more than 12.5% NaCl. All isolates were able to grow on the lowest concentration of NaCl (1.0%). On the other hand, 16 isolates grew on the concentration of 2.5% NaCl. These isolates represent about 84% from total isolates. This means that, large native population of *S. meliloti* presents in Saudi Arabian soils appeared to be sensitive to ecological factor of salt stress. However, only one isolate (isolate No. 185) was capable to grow on high concentration of NaCl (12.5%).

High temperature tolerance of collected local *S. meliloti* isolates

Variations among the different *S. meliloti* isolates in relation to their resistant to high temperature were observed. All isolates were able to grow on the temperature of 35°C. Eighteen isolates were not able to grow in higher tempe-

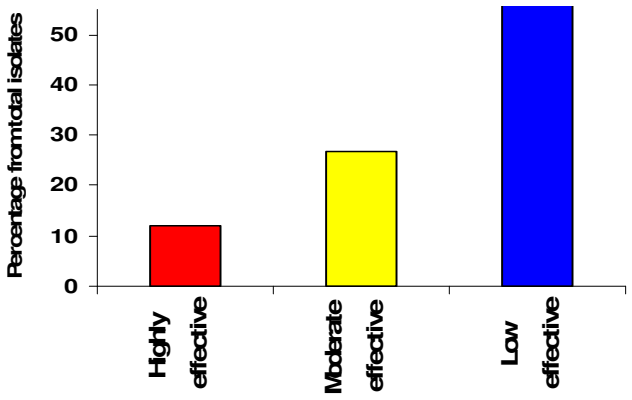

Figure 1. Effectiveness of *Sinorhizobium meliloti* isolated from Saudi Arabian soils.

rature than 35°C. Only one isolate of *S. meliloti* (isolate No. 156) was tolerant to high temperature of 50°C. This clearly showed that, the majority of native population of *S. meliloti* appeared to be sensitive to ecological factor of high temperature more than 35°C.

Symbiotic effectiveness of indigenous *S. meliloti*

Knowledge of the symbiotic characteristics of the indigenous population is required to predict the outcome of inoculation (Brockwell et al., 1988). The symbiotic effectiveness of indigenous isolates from alfalfa legume is presented in Figure 1. Plant dry matter production was strongly correlated with total plant nitrogen; therefore, the biomass production was used as criterion strain effectiveness in N_2 fixation. About 12% of rhizobial isolates were highly effective whereas 27 and 61% of the isolates were moderately and poorly effective, respectively.

Plasmid profiles

The 19 isolates were analyzed for their plasmid content by the procedure of Hashem and Kuykendall (1994). The results showed remarkable plasmid heterogeneity, and 2 to 5 bands were detected in each isolate (Table 2). However, 2 bands of all isolates were common.

DNA fingerprinting by rep-PCR and RAPD

Total genomic DNAs from all isolates and the reference strain USDA 1037 were used as templates in amplification reactions with REP and RAPD sets of primers. The PCR products from the different isolates were separated by horizontal gel electrophoresis on 1.5% agarose gels. The primers yielded multiple DNA products ranging in size from 1444 to 23130 base pair (bp) with Rep-PCR and from 736 – 23130 bp with RAPD- PCR method. The

Table 2. Plasmid numbers in different sinorhizobial field isolates.

Isolates	Plasmids	Isolates	Plasmids
16	4	153	4
30	2	154	4
41	4	156	5
69	2	167	3
73	4	175	4
75	4	176	3
76	4	185	5
100	4	188	4
121	3	229	4
140	3	USDA1037	4

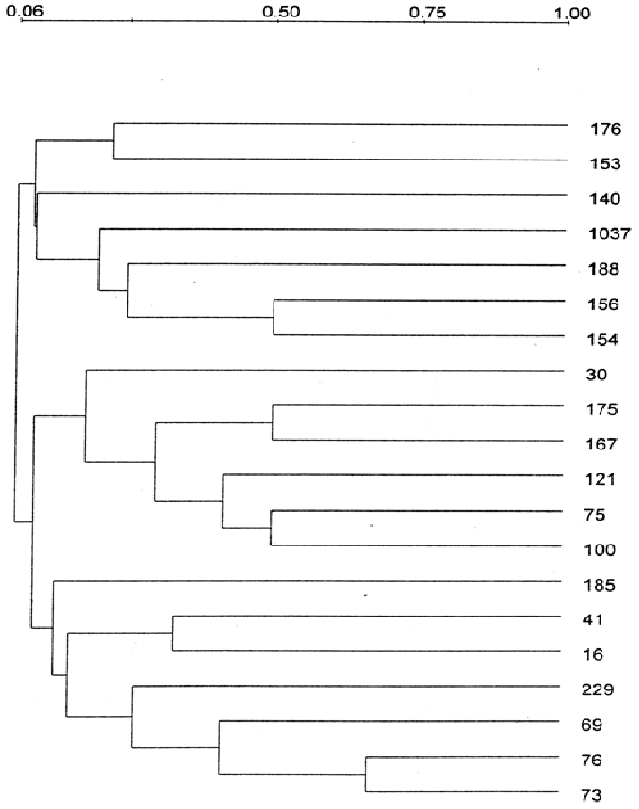

Figure 2. Dendogram of *Sinorhizobium meliloti* isolates derived from rep-PCR fingerprints.

number of amplified fragments ranged from 5-17 and from 4–12 per isolate, with rep-PCR and RAPD-PCR, respectively depending on the primer used in the amplification reaction. The dendrogram obtained by numerical analysis (diversity data base for DNA v2.1.1) of gel data (Figure 2) shows that all tested isolates could be divided into two major clusters (similarity level of 1.00). Only six *S. meliloti* field isolates were grouped within the first major cluster included the isolate No. 156 which was capable to grow on temperature of 50°C, together with

reference strain USDA 1037 which is distinguished and completely different from the isolates indigenous to Saudi Arabian soils. The second major cluster comprised most of *S. meliloti* field isolates (13 isolates) included the isolate No. 185 which was capable to grow on 12.5% NaCl.

The diversity of USDA 1037 reference strains and nineteen field isolates was also estimated by RAPD-PCR fingerprinting. The amplification reaction with five arbitrarily chosen primers (P 1, P 16, P 5, P 2, P4) resulted in specific and different fingerprints for all isolates tested. The fragment sizes ranged from 736-23130 bp. The dendrogram derived from combined RAPD profiles showed that all isolates could be divided into two major clusters which diverged at the similarity level of 1.00 (Figure 3). The majority of the isolates (12 isolates) were found to belong to the second cluster. The first cluster consisted of seven isolates, together with ref-erence strain USDA 1037.

Analysis of both rep-PCR and RAPD-PCR indicated that both methods are suitable for the fingerprinting of *S. meliloti* isolates indigenous to Saudi Arabian soils. Both methods divided all isolates into two major clusters. Results also showed that 86% of the isolates in the first cluster of RAPD-PCR were among the second cluster of rep-PCR. This clearly proved that both methods could be effectively differentiated between closely related strains

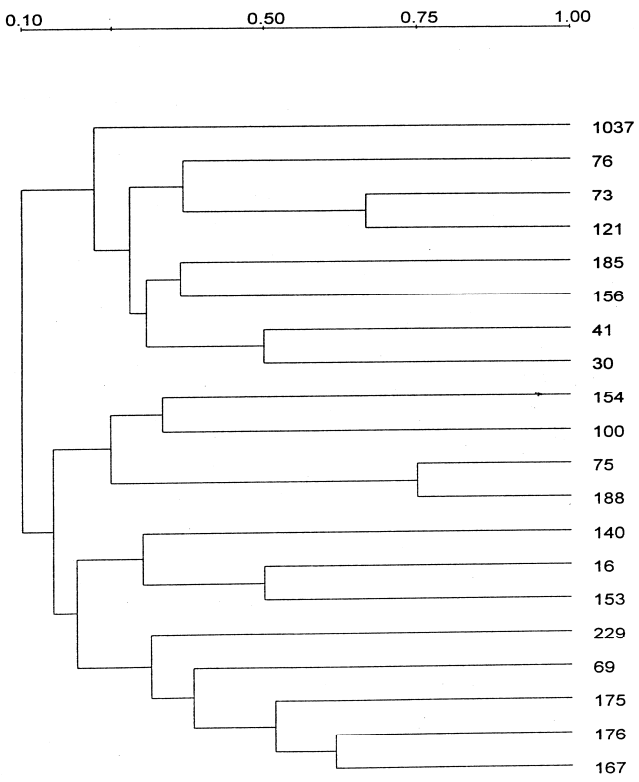

Figure 3. Dendogram of *Sinorhizobium meliloti* isolates derived from RAPD-PCR fingerprints.

DISCUSSION

Salt affected soils occupy vast areas of Saudi Arabia, in addition to spots of deteriorated soils scattered along the east and west areas. Saline conditions may limit the symbiosis by affecting survival and proliferation of *Rhizobium spp* in the soil and rhizosphere. Selection of effective highly tolerant strains of *S. meliloti* will be of great impacts in such conditions.

The large native population of *S. meliloti* presents in Saudi Arabian soils appeared to be sensitive to ecological factor of salt stress. These results are in line with those of Jebara et al. (2001) and Al-Asmary (2007) who stated that, the majority of sinorhizobia in Tunisian and Saudi Arabian soils, respectively were sensitive to high salinity.

Temperature is one of the most important factors affecting the survival of rhizobia in soil. High temperature is an essential ecological factor especially in arid and semiarid area as in Saudi Arabia. Rhizobia capable to survive and grow under these conditions are very important for inoculants production. The large native population of *S. meliloti* presents in Saudi Arabian soils (about 95%) appeared to be sensitive to ecological factor of high temperature more than 35°C. Only one isolate of *S. meliloti* (isolate No. 156) was tolerant to high temperature of 50°C. Selection of these effective highly tolerant strains of *S. meliloti* to inoculate alfalfa in Saudi Arabian soils will be of great impacts in such high temperature conditions.

The results on symbiotic effectiveness confirmed observations of the previous studies (Jebara et al., 2001; Mihaela et al., 2003; Al-Asmary, 2007) that isolates of *S.meliloti* vary in their N_2 fixation ability. In the present study, the majority of the field isolates were low in N_2 fixation effectiveness. It is, therefore, apparent that inoculation of alfalfa plants with selected highly effective and competitive rhizobial strains is needed. Differences in N_2 fixation capacity within the tested isolates might be attributed to ecological factors in the ecosystem from which the strains were isolated or may be related to the loss of important genetic information related to symbiotic performance of the isolates due to long exposure to the environmental stress in the soil. Such variability was reported previously in field population of *R. leguminosarum* bv *trifolii* (Gibson et al., 1975; Valdivia et al., 1988), *S. meliloti* (Jebara et al., 2001; Mihaela et al., 2003; Al-Asmary, 2007).

The plasmid content of 19 isolates from a local collection of sinorhizobia nodulating *M. sativa* shows high plasmid diversity. The dominant type contained 2 bands. However, the plasmid profile grouping did not appear to be correlated with the phenotypic traits of sinorhizobia analyzed, but more with physical and chemical properties of the soils from which they were derived.

Bromfield et al. (1987) and Broughton et al. (1987) examined the diversity of plasmid content among field

populations of *S.meliloti* and found a wide variety of distinct plasmid profile. Hartmann and Amarger (1991) showed that DNA restriction patterns and IS fingerprints were strongly correlated with the plasmid profiles of the strains, confirming the validity of plasmid grouping in assessing diversity of natural populations of *S.meliloti*.

Other studies have also suggested that symbiotic plasmid and chromosomal genotypes are correlated Young and Wexler (1988) and that plasmid sequences might be more variable than chromosomal sequences (Paffetti et al., 1996). However, Laguerre et al. (1993) observed that the same type of chromosomal background may harbour different plasmid types. This may be explained by lateral transfer of symbiotic and (or) cryptic plasmid as suggested by Velazquez et al. (1995). Therefore, plasmid analysis appeared to be a highly discriminating method for *Rhizobium* isolates. However, Jebara et al. (2001) found that plasmid content seems not to be correlated to the other molecular criteria used.

In this work, distinction of isolates at the strain level was determined using the RAPD PCR and rep-PCR methods (Sikora et al., 2002; Mihaela et al., 2003; Al-Asmary 2007). All strains isolated from different field sites in Saudi Arabia had specific RAPD and rep-PCR patterns significantly different from the reference strains USDA 1037. The results presented here showed that both methods grouped strains almost identically, which demonstrated that both PCR fingerprinting methods were equally suited for the characterisation of *S. meliloti* field populations. These results are in agreement with the results obtained by other authors (Niemann et al., 1997; Mihaela et al., 2003; Al-Asmary, 2007). The most different two isolates from all isolates used in this study were indigenous strain 156 and 185. Strain differentiation on the basis of their RAPD and rep-PCR fingerprints resulted in similar groupings. RAPD and rep-PCR data revealed genetic diversity among alfalfa isolates and indicate the presence of indigenous field population of *S. meliloti* in different field sites in Saudi Arabia.

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