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Taxonomic diversity and symbiotic efficiency of rhizobial strains obtained from nodules of *Medicago sativa* growing in Iran

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ABSTRACT

Forty-eight rhizobial strains isolated from root nodules of alfalfa (Medicago sativa L.) plants growing in different regions of Iran were characterized by using restriction fragment length polymorphism (RFLP) of ribosomal internal transcribed spacer (ITS) region, 16S RNA gene-RFLP and sequence analysis of symbiotic genesnodA and nifH. Taxonomic relationships of selected strains from different ITS-RFLP groups were further inferred in this study by using single and concatenated phylogenetic analysis of recA and atpD gene sequences. In all constructed trees, the strains were distributed similarly into three different rhizobial phylogenetic groups I, II and III. Group I included strains belonging to Sinorhizobium (Ensifer) meliloti and S.kummero wiae. The strains in groupsII and III were identified as E. adhaerens and Agrobacterium radiobacter, respectively. Based on the two core genes used in this study, S.kummerowiaestrains appeared to be very closely related to S. melilotireference strains. Forty-two strains induced nitrogen-fixing nodules on alfalfa plants. Remarkably, two of these strains belonged to A. radiobacter. The entire three test strains in group II (E. adhaerens) and one test strain in group III (A. radiobacter) failed to nodulate. Generally, our results confirm that S. meliloti is the most important symbionts of M. sativa growing in agricultural fields in Iran and question S. kummerowiae being a species separate from S. meliloti.

Keywords: *Medicago sativa* L, taxonomy, phylogeny, population genetics, symbiotic efficiency

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a perennial legume, which is widely grown worldwide for forage production and as green manure source for farmlands. Iran is considered being one of the origins of alfalfa along with North-East Turkey and Turkmenistan [21, 28]. Alfalfa is extensively cultivated in various regions of Iran to be used as the main protein source for livestock.

Alfalfa is known to establish a beneficial nitrogen-fixingsymbiosis with Gram-negative soil bacteria called rhizobia, mainly belonging to the genus *Sinorhizobium* (syn. *Ensifer*)[4, 6,43]. This fast-growing genus is known to include11 rhizobial species (http://www.straininfo.net/taxa/2328). Despite the importance of alfalfa in Iran, little is known about the taxonomy and phylogeny of its symbiotic nitrogen-fixing bacteria, though a few studies revealed the presence of diverse *M. sativa* nodulating *Sinorhizobium* species. According to Talebi et al.[39], bacterial isolates nodulating *M. sativa* were mainly identified as closely related to *S. meliloti* species followed by *S. medicae*, based on the analysis of *nodbox4*, 16S rRNA gene RFLP pattern and sequences of *mucR*or 16S rRNA chromosomal genes. The diversity of 48 bacterial strains isolated from root nodules of alfalfa (*M. sativa* L.) in Iran was studied by employing ITS-RFLP and 16S rRNA gene-RFLP molecular techniques [36]. With these methods the bacterial strains were clustered into six different ITS groups, however most of them (40) were clustered together with type strain *S. meliloti* USDA 1002 under ITS group I[36]. From this group, ten bacterial strains were also found to share closely similar symbiotic gene (*nodA* and *nifH*) phylogeny with the *S. meliloti* species [35].

The 16S rRNA gene has been used as a standard molecular marker to study bacterial systematics. However, many authors have reported drawbacks of this method. The 16S rRNA gene is present as multiple copies in the genome of some bacteria and thus affects its use as a genetic marker [1]. It may undergo recombination and horizontal transfer resulting in sequence mosaicism[25, 40,46]. Sometimes, it is also highly conserved among closely related species and is thus unable to classify closely related species [44]. On the contrary, housekeeping protein coding genes are more variable and conserved enough to differentiate closely related bacterial species. Thus, these genes have proved to be useful tools for the phylogenetic study and identification of rhizobia at species level [2, 3, 10, 15,26].The aim of this study was to investigate taxonomic relations of 14 selected rhizobial strains among different ITS-RFLP clusters identified by Soltani et al.[36]using phylogenic analysis of *recA* and *atpD* gene sequences. The nodulation capacity and symbiotic efficiency of all 48 rhizobial strains were evaluated under greenhouse conditions on alfalfa host plants.

MATERIALS AND METHODS

Bacterial strains representing different ITS-RFLP clusters [36]were incubated at 28^{o}C for 3-5 days in yeast extract-mannitol broth media [45]. Genomic DNAs were extracted from bacterial suspensions and purified using the PowerMicrobial® Midi DNA Isolation Kit as recommended by manufacturer (MO BIO Laboratories, Inc., 2746 Loker Avenue WestCarlsbad, CA 92010, USA). The genomic DNAs were used as templates to amplify protein coding housekeeping genes *recA* and *atpD* using primers and PCR cycling conditions listed in Table 1. The PCR reactions were done following the instructions given by the manufacturer (Finnzymes, Finland).

Table 1. Oligonucleotide primers used for the amplification and sequencing of core genes and their PCR cycling conditions

Primer	Direction	Primer sequence(5'-3')	PCR cycling	Reference
recA555R	Reverse	CGR ATC TGG TTG ATG AAG ATC	5 min 95^{O}C,32×(45 s 94^{O}C, 1 min 60^{O}C, 1 min 30 s 74^{O}C), 5 min 72^{O}C	[25]
recA63F	Forward	ATC GAG CGG TCG TTC GGC AAG GG		[25]
recA6F	Forward	CGK CTS GTA GAG GAY AAA TCG GTG GA	5 min 95^{O}C, 30× (45 s 94^{O}C, 60 s 50^{O}C, 90 s 74^{O}C), 5 min 72^{O}C	[19]
recA504	Reverse	TTG CGC AGC GCC TGG CTC AT		[19]
atpD273F	Forward	SCT GGG SCG YAT CMT GAA CGT	5 min 95^{O}C, 30× (45 s 94^{O}C, 60 s 50^{O}C, 90 s 74^{O}C), 5 min 72^{O}C	[19]
atpD771R	Reverse	GCC GAC ACT TCC GAA CCN GCC TG		[19]
atpD255F	Forward	GCT SGG CCG CAT CMT SAA CGT C	1 min 95 ^{O}C, 30× (45 s 95 ^{O}C, 60 s 70^{O}C, 90 s 74^{O}C), 8 min 74^{O}C	[46]
atpD782R	Reverse	GCC GAC ACT TCM GAA CCN GCC TG		[46]

Generally the PCR conditions, DNA sequencing, sequence analysis and tree constructions were performed as described previously [2, 3]. In short, new gene sequences were blasted into the GenBank database and a number of sequences of reference and type strains related to our strains were recovered from the database

(www.ncbi.nlm.nih.gov/BLAST). The sequences were aligned by ClustalW using Mega 6[37]. The same software was also used to construct single gene and concatenated Maximum Likelihood (ML) phylogenetic trees. For tree construction, General Time Reversible plus Gamma (GTR+G) was identified as the best fit model for all *recA* and *atpD* single gene datasets and *recA-atpD* concatenated gene sequences as calculated using Find Model (http://hfv.lanl.gov/content/sequence/NEWALIGN/align.html). Statistical supports of the trees were computed by 100 bootstrap replicates.

Nucleotide polymorphisms analyses were performed for *atpD*, *recA* and *recA-atpD* concatenated datasets. Nucleotide diversity, Tajima's D tests of neutrality, Fu and Li's D*, Fu and Li's F* test statistics were calculated using DNASP 5.10.1 [33].

All 48 strains were tested for their nitrogen fixing ability in symbiosis with alfalfa plants in the greenhouse. Seeds were surface-sterilized with acidified mercuric chloride for 5 minutes followed by rinsing 6 times with sterile water and germinated on 0.8% water agar at 28^{o}C for 3 days [45]. Five seedlings were then transferred aseptically to plastic pots containing three kilogram washed and sterilized perlite-vermiculite mixture (pH 6.7). Pots were arranged in a completely randomized design with four replicates for each treatment. The experiment also contained anun-inoculated and N-fertilized pot (5mM KNO_3) controls. After three days, seeds were inoculated with 1 ml (about 10^8cells/ml) of bacterial broth culture and irrigated with N-free Jenson´s nutrient solution according to Beck et al. [7]. Plants were harvested after eight weeks and the N_2 fixation efficiency of the inoculated plants was estimated by comparing shoot dry weights with those of un-inoculated control plants. The mean dry weight of shoots (X) was used to calculate an index of effectiveness (Ei) defined as: Ei=100(Xi-Xc/XN-Xc), where i = inoculated strain, c = un-inoculated control and N = nitrogen-fertilized treatment[17].

All test strains were deposited in CCSM-WDCM891 - Culture Collection for Soil Microorganisms in the Iranian Soil and Water Research Institute (SWRI).

Accession numbers

Nucleotide sequences generated in this study were deposited into the GenBank database and their accession numbers (JX482114, KF925266, KF986582-83, KF986585-87, KF986590-98, KF986602-09 and KT265228-29) are also shown in parenthesis in the phylogenetic trees (Figs. 1, 2).

RESULTS AND DISCUSSION

Taxonomic relationships of 14 selected bacterial strains representing different ITS clusters were studied among isolates obtained previously from nodules of *M. sativa* growing in Iran. Species identification was carried out using sequence analysis of *recA* and *atpD* protein coding housekeeping genes. For strains considered in this study, thirteen sequences were obtained for each of *recA* (443 bp) and *atpD* (437 bp) genes. Yet, we could not amplify *recA* and *atpD* fragments for strains KH133 and KH21, respectively. With a few exceptions, all test strains were classified similarly into three groups I, II and III in all *recA*, *atpD* and *recA-atpD* combined phylogenetic trees (Figs. 1, 2, 3). In the concatenated phylogenetic tree (Fig.3), the phylogenetic groups were supported with 100 bootstrap values. The test strains in group I were found belonging to *S. meliloti* and *S. kummerowiae*. The strains in group II and III were affiliated to *E. adhaerens* and *Agrobacterium radiobacter*, respectively.

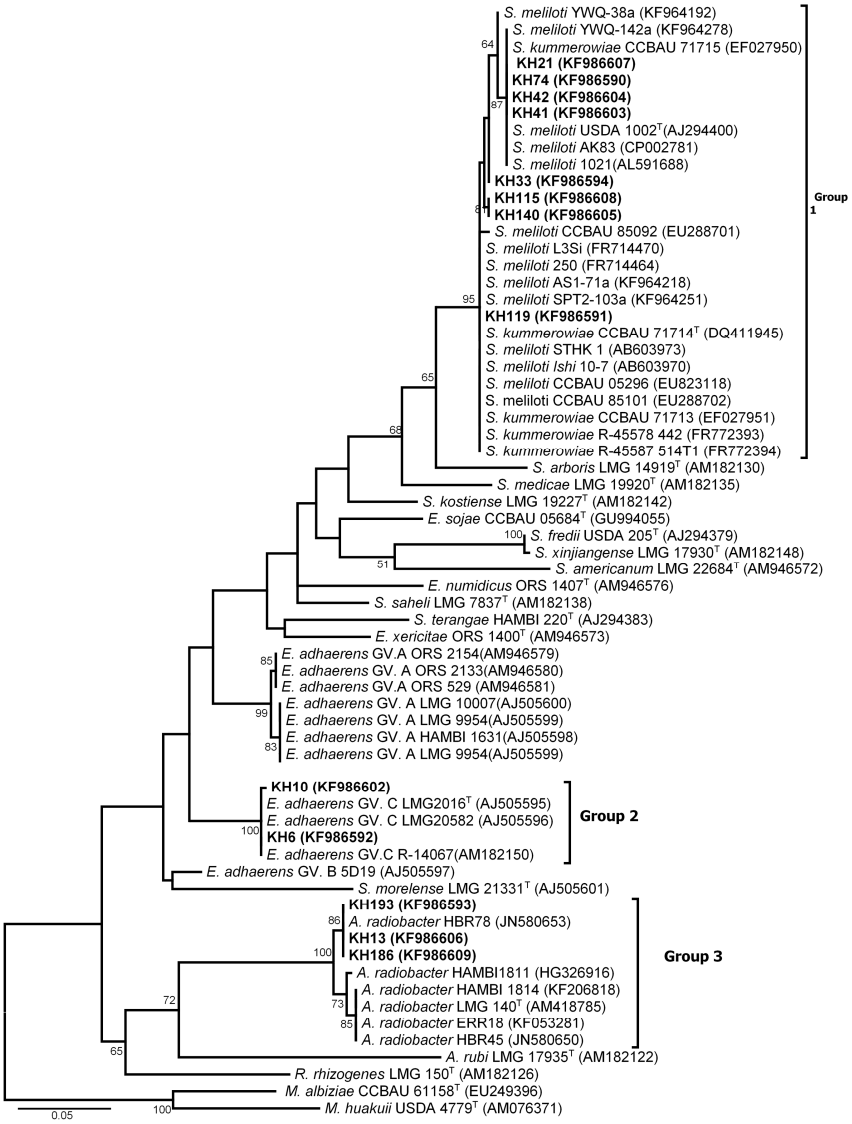

Fig.1 ML Single phylogenetic tree constructed based on *recA* gene sequence showing the taxonomic relationship of Iranian alfalfa nodulating rhizobia. Bootstrap values higher than 50% are indicated at branch points. Sequences resulted in this study are shown in bold. The scale bar 0.1% indicates number of substitutions per site. Type strains are presented by superscript "T". The tree is rooted using the sequence of the type strains *Mesorhizobium huakuii* USDA 4779 and *Mesorhizobium albiziae* CCBAU 61158

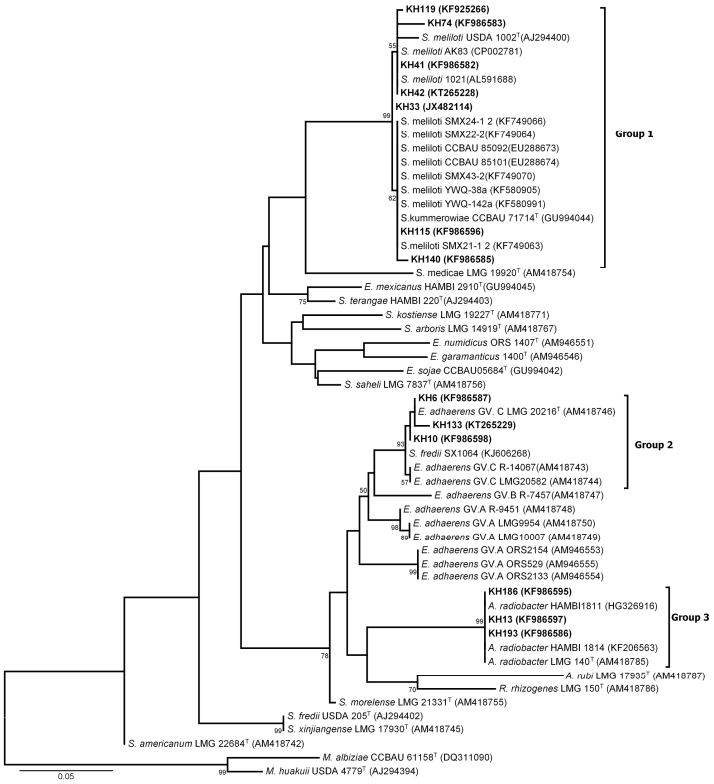

Fig.2 ML Single phylogenetic tree constructed based on atpD gene sequence showing the taxonomic relationship of Iranian alfalfa nodulating rhizobia. Bootstrap values higher than 50% are indicated at branch points. Sequences resulted in this study are shown in bold. The scale bar 0.05% indicates number of substitutions per site. Type strains are presented by superscript "T". The tree is rooted using the sequence of the type strains *Mesorhizobium huakuii* USDA 4779 and *Mesorhizobium albiziae* CCBAU 61158

According to previous studies, alfalfa nodulating rhizobia mainly belong to *S. meliloti* and *S. medicae* species [8, 11, 12,23]. Strains isolated from root nodules of the perennial legume *Kummerowiaestipulacea* growing in the Loess Plateau in North-Western China were classified as a novel species, *S.kummerowiae*. These strains could also form nodules on *M. sativa*[42]. According to the authors, this species proposal was mainly based on distinctive numerical taxonomic features of these *K. stipulacea* nodulating strains compared to other *Rhizobium* and *Sinorhizobium* species and also based on the result of DNA-DNA hybridization. Nevertheless, 16S rRNA gene sequence similarity between the type strains, *S. kummerowiae* CCBAU 71714 and *S. meliloti* USDA 1002 was reported to be 97.8%, which is above the recommended cut off value (97%) for strains belonging to different species. Similarly in our study, all *recA* and *atpD* single genes and *recA-atpD* concatenated trees showed close similarity between *S. meliloti* species and *S. kummerowiae* species in group I. Strains representing ITS clusters 1a,1b and 1c (Table 2)occupied phylogenic group I together with type strains of *S. meliloti* and *S. kummerowiae* species (Figs. 1, 2, 3).

Table 2. Geographic origin, ITS cluster, core gene group, phylogenetic affiliation and symbiotic status of strains isolated from *Medicago sativa* nodules in Iran and used in this study

Strain	Places of isolation	ITS cluster[a]	*recA* group	*atpD* group	Concatenated group	Phylogenetic affiliation based on *recA* + *atpD* sequences	Symbiotic status		
							Nodulation	Fixation	%SE[5]
KH21	Chapaghlu	**1a**	**I**	**?**	n.d	***S. meliloti***[b]	+	+	**106**
KH22	Khomajin	**1a**	n.d	n.d	n.d	n.d	+	+	**93**
KH25	Haji abad	**1a**	n.d	n.d	n.d	n.d	+	+	**90**
KH31	Dahdalilan	**1a**	n.d	n.d	n.d	n.d	+	+	**92**
KH41	**Sardaran**	**1a**	**I**	**I**	**Ia**	***S. meliloti***	+	+	**94**
KH42	**Sardarabad**	**1a**	**I**	**I**	**Ia**	***S. meliloti***	+	+	**90**
KH43	Kabudarahang	**1a**	n.d	n.d	n.d	n.d	+	+	**76**
KH44	Hatamabad	**1a**	n.d	n.d	n.d	n.d	+	+	**77**
KH49	Zangane	**1a**	n.d	n.d	n.d	n.d	+	+	**104**
KH57	Kortilabad	**1a**	n.d	n.d	n.d	n.d	+	+	**110**
KH69	Milajerd	**1a**	n.d	n.d	n.d	n.d	+	+	**101**
KH70	Pirnahan	**1a**	n.d	n.d	n.d	n.d	+	+	**84**
KH74	**Nisher**	**1a**	**I**	**I**	**Ia**	***S. meliloti***	+	+	**75**
KH95	Ghaleasijan	**1a**	n.d	n.d	n.d	n.d	+	+	**82**
KH109	Bitran	**1a**	n.d	n.d	n.d	n.d	+	+	**91**
KH121	Salam saraee	**1a**	n.d	n.d	n.d	n.d	+	+	**62**
KH126	Ghuri chai	**1a**	n.d	n.d	n.d	n.d	+	+	**114**
KH142	Samen	**1a**	n.d	n.d	n.d	n.d	+	+	**100**
KH152	Famast	**1a**	n.d	n.d	n.d	n.d	+	+	**80**
KH153	Jafarabad	**1a**	n.d	n.d	n.d	n.d	+	+	**97**
KH162	Vahman	**1a**	n.d	n.d	n.d	n.d	+	+	**84**
KH182	Kouhaven	**1a**	n.d	n.d	n.d	n.d	+	+	**85**
KH183	Shirin sou	**1a**	n.d	n.d	n.d	n.d	+	+	**96**
KH187	Targhieh	**1a**	n.d	n.d	n.d	n.d	+	+	**78**
KH198	Baba khanjar	**1a**	n.d	n.d	n.d	n.d	+	+	**86**
KH202	Razan	**1a**	n.d	n.d	n.d	n.d	+	+	**121**
KH209	Karafs	**1a**	n.d	n.d	n.d	n.d	+	+	**93**
KH212	Ghalghalabad	**1a**	n.d	n.d	n.d	n.d	+	+	**105**
KH115	**Darband**	**1b**	**I**	**I**	**Ib**	***S. meliloti/ kummerowiae***	+	+	**98**
KH140	**Malaier**	**1b**	**I**	**I**	**Ib**	***S. meliloti/ kummerowiae***	+	+	**79**
KH33	**Shara**	**1c**	**I**	**I**	**Ib**	***S. meliloti/ kummerowiae***	+	+	**97**
KH40	Sarvarabad	**1c**	n.d	n.d	n.d	n.d	+	+	**79**
KH63	Bar zoun	**1c**	n.d	n.d	n.d	n.d	+	+	**66**
KH73	Ali abad	**1c**	n.d	n.d	n.d	n.d	+	+	**92**
KH81	Siakamar	**1c**	n.d	n.d	n.d	n.d	+	+	**70**
KH82	Zaman abad	**1c**	n.d	n.d	n.d	n.d	+	+	**80**
KH105	Asadabad	**1c**	n.d	n.d	n.d	n.d	+	+	**94**
KH119	**Hasan gheshlagh**	**1c**	**I**	**I**	**Ib**	***S. meliloti/ kummerowiae***	+	+	**90**
KH150	Avar zaman	**1c**	n.d	n.d	n.d	n.d	+	+	**83**
KH173	Ghurehjenie	**1c**	n.d	n.d	n.d	n.d	+	+	**87**
KH10	**Alanje**	**2**	**II**	**II**	**II**	***E. adhaerens***	-	-	
KH6	**Chenaroli**	**3**	**II**	**II**	**II**	***E. adhaerens***	-	-	
KH133	Toushmal	**4**	**?**	II	n.d	***E. adhaerens***[2]	-	-	
KH13	**Zirebagh**	**5**	**III**	**III**	**III**	***A. radiobacter***	+	+	**63**
KH186	**Kamighale**	**5**	**III**	**III**	**III**	***A. radiobacter***	+	+	**70**
KH193	**Gonbadechai**	**5**	**III**	**III**	**III**	***A. radiobacter***	-	-	
KH16	Azandarian	**6**	n.d	n.d	n.d	n.d	-	-	
KH24	Dahlagh	**6**	n.d	n.d	n.d	n.d	-	-	
Reference									
S. meliloti HAMBI1318		**1a**	n.d	n.d	n.d		n.d	n.d	n.d
S. meliloti HAMBI2148/USD1002[T]		**1a**	**I**	**I**	**Ia**				
S.medicae HAMBI12306/LMG19920[T]		**3**	S	S	S		n.d	n.d	n.d
A. tumefaciens HAMBI1811		**5**	**III**	III	III		n.d	n.d	n.d

?, amplification was not succeeded; [a]ITS patterns based on the analysis of the intergenic spacer between 16S and 23S rDNAs (ITS), Soltani et al. [36]. [b]identifcation was based on recA or atpD gene sequence; n.d, not determined; +, nodules; - , no nodules; %SE, percent of symbiotic effectiveness; E, Ensifer; S, Sinorhzobium; A, Agrobacterium.

In this group, several test strains had 100% *recA* and/or *atpD* gene sequence similarity with each other and with *S. kummerowiae* and *S. meliloti* species. In the concatenated tree, group I split into two very close subgroups Ia and Ib that contained the type strains of *S. meliloti* and *S. kummerowiae* species, respectively. However, in the latter case other reference strains belonging to *S. meliloti* species were also tightly grouped with the type strain *S. kummerowiae* CCBAU 71714 (Fig. 3).

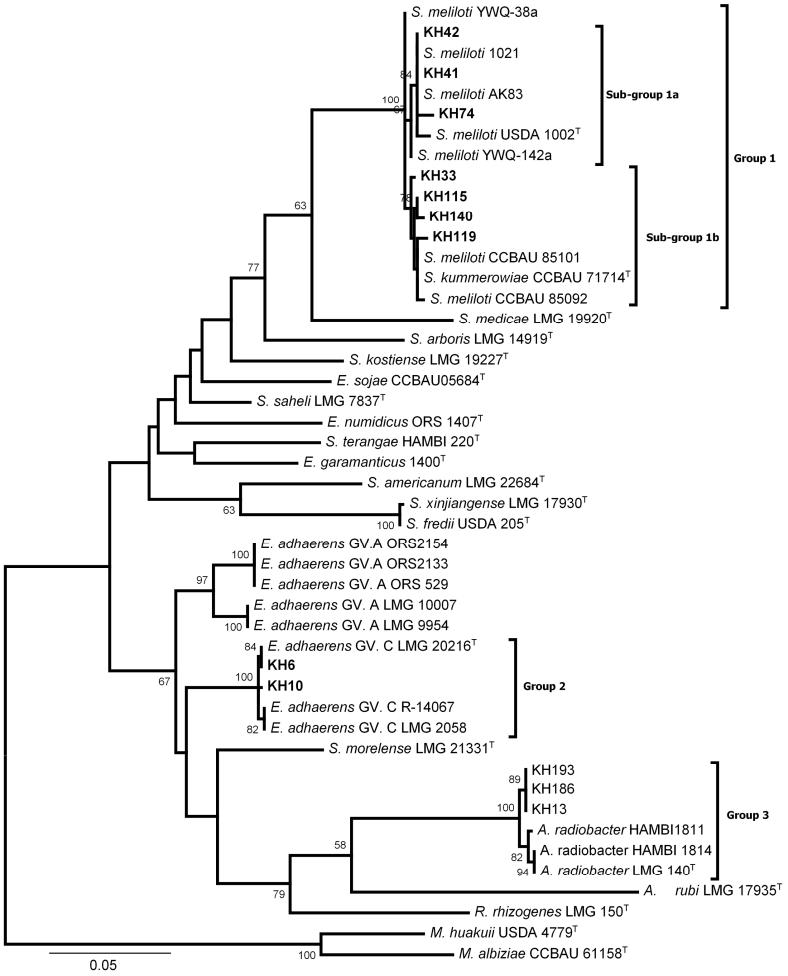

Fig. 3 ML phylogenetic tree constructed based on concatenated sequences of *recA* and *atpD* genes showing the taxonomic relationship of Iranian alfalfa nodulating rhizobia. Bootstrap values higher than 50% are indicated at branch points. Sequences resulted in this study are shown in bold. The scale bar 0.05% indicates number of substitutions per site. Type strains are presented by superscript "T". The tree is rooted using the sequence of the type strains *Mesorhizobium huakuii* USDA 4779 and *Mesorhizobium albiziae* CCBAU 61158.

Sequence similarity among strains within and between subgroups Ia and Ib was in the range of 97-100%. The type strains *S. kummerowiae* CCBAU 71714 and *S. meliloti* USDA 1002 showed 99% *recA-atpD* sequence similarity. Generally, our analysis and 16S rRNA gene sequence similarity reported by Wei et al.[42]suggest that *S. meliloti* and *S. kummerowiae* species are very similar, perhapsbelonging to one and the same species. However, further housekeeping gene phylogenetic analyses may help to know more detail taxonomic relationship between *S. kummerowiae* and *S. meliloti* species.

The genus *Sinorhizobium*(syn. *Ensifer*) comprises non-symbiotic soil bacteria known as *E. adhaerens*[13]. This species was first identified as a predator of other bacteria in the soil; however it was also found capable of forming nitrogen fixing nodules on *Phaseolus vulgaris* and *Leucaena leucocephala* after symbiotic plasmid of *Rhizobium tropici* CFN 299 was transferred into the genome of it [32]. In this study, three strains representing ITS clusters 2 and 3 including *E. adhaerans* LMG 2016^{T}were included in phylogenetic group II (100% bootstrap support) (Figs.1, 2 ,3). The sequence similarities between test strains and *E. adhaerans* reference strains were in the range 99.5-100%. Strain KH133 was identified only based on its *atpD* gene sequence similarity with *E. adhaerens* reference strains (99%). In this group *S. fredii* strain SX1064 also had 99% sequence similarity with other test and reference strains (Fig.2).

Several previous studies reported that *Agrobacterium* strains were commonly isolated from leguminous root nodules [e.g. 3,14,20,24, 27, 29, 47]. In our study, test strains representing ITS cluster 5 (Table 2) were tightly gathered in phylogenetic group III together with *A. Radiobacter* reference strains (Figs. 1, 2, 3). The test strains in this group had identical *recA* sequence similarity with *A. radiobacter* HBR78 isolated from common bean in Ethiopia [3]. The test strains also had identical *atpD* gene sequence with type strain *A. radiobacter* LMG 140 (Fig. 2).

Generally, bacterial strains belonging to different phylogenetic groups were consistent with their ITS-RFLP clusters (Table 2), implying that strains in the same ITS-RFLP cluster may represent the same taxonomic group. Thus, based on the sequence analysis of *recA* and *atpD* of core genes of selected test strains we suggest that in Iran indigenous *M. sativa* nodulating rhizobia mainly belong to *S. meliloti*and*S. kummerowiae* (40 strains).

The *recA* locus displayed higher genetic diversity per site between two strains (π = 0.108) than the *atpD* gene (π = 0.081) and *recA-atpD* dataset (π = 0.101) (Table 3). However, the combined dataset was the most polymorphic (268 segregating sites) followed by *recA* (138 segregating sites) and *atpD* (130 segregating sites). The combined dataset also exhibited a large number of haplotypes (33 from 41 total sequences) compared to single *recA* (27 haplotypes from 56 sequences) and *atpD* (haplotypes 29 from 48 sequences) genes (Table 3).

Table 3. Descriptive statistics of nucleotide polymorphisms, neutrality and population growth tests based on the *recA*, *atpD,* and concatenated *atpD- recA* dataset

Group (Genospecies)	Gene (no. of sequence)	Haplotypes	Sample size	Segregating sites	Parsimony informative sites	dN/dS	K	h/Hd	θ	π	Tajima's D	Fu and Li's D*	Fu and Li's F*
Total	*recA(65)*	30	402	140	122	0.093	40.15	30/0.932	0.081	0.110	1.253	-0.993	-0.123
	atpD (56)	33	416	130	105	0.120	32.46	33/0.953	0.087	0.082	0.519	-1.696	-1.012
	atpD-recA (47)	35	801	270	225	0.100	77.01	35/0.984	0.100	0.101	0.948	-1.103	-0.401
S. meliloti/ kummerowiae	*recA* (27)	7	402	20	7	1	4.057	7/0.695	0.013	0.010	-0.776	-2.283	-2.125
(Group1)	*atpD(19)*	7	416	54	4	0.333	6.766	7/0. 702	0.037	0.016	**-2.294***	**-3.550***	**-3.698***
	atpD-recA (15)	12	801	34	9	1	7.695	12/0.962	0.013	0.010	-1.119	-2.007	-2.027
E. adhaerens(Group2)	*recA* (5)	2	402	1	0	0	0.400	2/0.400	0.001	0.001	-0.817	-0.817	-0.772
	atpD (7)	5	416	6	4	0	2.762	5/0.0905	0.006	0.006	0.649	0.439	0.529
	atpD-recA (5)	3	801	99	7	0.279	41.00	3/0.800	0.066	0.055	-1.043	-1.043	-1.134
A. radiobacter (Group3)	*recA* (9)	3	402	8	7	0.0	4.111	3/ 0.667	0.007	0.010	1.800	1.000	1.331
	atpD (6)	1	416	**0**	**0**	**0**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**	**n.d**
	atpD-recA (6)	3	801	8	7	0/8	4.400	3/0.733	0.004	0.006	1.508	1.218	1.377

*dN/dS =nonsynonymous/synonymous changes (nucleotide diversity); K=Average number of nucleotide differences h/Hd=Number of haplotypes/haplotype (gene) diversity; Theta per site (bp) (Waterson, G. A. 1975), assuming the infinite-sites model; π =Nucleotide diversity; Tajima's D =computed based on the total number of segregating sites; Fu and Li's D and F=Population growth test statistics; *Significant values. Tajima's D: **-2.29381**, statistical significance: P < 0.01; Fu and Li's D* test statistic: **-3.55031**, statistical significance: P < 0.02; Fu and Li's F test statistic: **-3.69806**, statistical significance: P < 0.02; n.d= not determined*

Among the different phylogenetic groups, all strains related to *A.radiobacter* (group III) displayed the same haplotype for *atpD* sequence, and thus for this gene we did not calculate all other descriptive statistics and population genetic parameters. In group I, the strains belonged to *S. meliloti* and *S.kummerowiae* presented several haplotypes for each of *recA*, *atpD* and the *recA-atpD* concatenated dataset. Compared to strains in groups II and III, strains ingroup I also showed the highest level of DNA polymorphisms, in terms of number of segregating sites and nucleotide diversity for all *recA*, *atpD* and combined datasets (Table 3). Most statistical values of Tajima's D and Fu & Li's tests were negative, which may indicate population growth but in most cases these tests were non-significant. Thus, these results imply that the observed nucleotide substitutions and polymorphisms in most cases follow the normal neutral model molecular evolution (i.e., constant population size, no population subdivision) [38]. However, the neutrality and population growth tests (Tajima's D= -2.294, Fu and Li's D= -3.550and Fu and Li's F=-3.698) were significant for the *atpD* gene of *S. meliloti*and*S. kummerowiae*. This indicates that the evolution of this gene might be determined by purifying selection or suggests population expansion of *S. meliloti* and *S.kummerowiae*[18,30].

Rhizobia adapted to local soils, and having a high capacity of nodulation and nitrogen fixation improves the yield and quality of legumes [22,31].Our plant nodulation test results showed that all strains classified as *S. meliloti* and *S. kummerowiae* formed nodules on the roots of their host plant, alfalfa. The symbiotic efficiency test results (Table2) showed wide variations among test strains belonging to *S. meliloti* and *S.kummerowiae*, and which is in agreement with previous reports [9,16,23]. Nine strains showed symbiotic efficiency above 100% and strain KH202 being the most effective nitrogen fixer (SE 121%) (Table2). Though, their effectiveness need to be tested with further field

experiments, the present result can be taken as important basic information for the development of effective alfalfa nodulating rhizobial inocula which can be applied in the field for alfalfa production in Iran.
The strains identified as *E. adherens*in this study induced no nodules on alfalfa and in the previous study we could not amplify the *nifH* and *nodA* genes from them[35]. These results are also in agreement with previous studies by Casida[13]and Willems etal.[43]. Nevertheless, according to Merabet et al. [26], *E. adhaerens* strains were reported to nodulate their plant of isolation, *Lotus arabicus*.

According to preceding studies *Agrobacterium* species could not induce proper nodules on their host legumes during the re-nodulation tests [14,41, 47]. In the present study, among strains identified as *A. radiobacter* in group III, strain KH193 did not either form any nodules on their host plant, alfalfa. Conversely, strains KH13 and KH186 could form nodules on alfalfa plants, although they were relatively poorly effective (SE 63 and 70%).Previously, agrobacterial like strains were also reported to nodulate *Acacia auriculiformis*, *L. leucocephala*, *Gliricidia sepium*, *P. vulgaris* and *Sesbania sesban* plants[5]. Another study revealed nonspecific nodulation of woody legumes by *Agrobacterium* spp. and their coexistence with *S. meliloti* in the same nodules [24].Strains KH13 and KH186 identified as *A. radiobacter* showed100% *nifH* and *nodA* gene sequence similarity with *S. meliloti* 1021 or *S. meliloti* ATTCC9930 [35]. Thus, the nodulation capacity of the two *A. radiobacter* strains having identical symbiotic gene sequences with *S. meliloti* suggests that these strains might have acquired their symbiotic genes via horizontal transfer from *S.meliloti*.

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