

## Genetic diversity and phylogeny of alfalfa nodulating rhizobia assessed by *nifH* and *nodA* genes

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### Abstract

The alfalfa (*Medicago sativa* L.) is broadly cultivated in various regions of Iran, but information on the genetic structure and phylogeny of the rhizobia nodulating this legume crop is scarce. The genetic diversity and phylogeny of 16 strains, isolated from root nodules of alfalfa was assessed by *nifH* and *nodA* genes sequencing. Results showed that, *nifH* and *nodA* fragments for Six strains could not be amplified and There were no nodules obtained from the plants inoculated with these strains as well. These findings suggest that they may be non-symbiotic and opportunistic bacteria that occupy root nodules. Based on the sequences of *nodA* and *nifH* genes, ten strains belonged to the *S. meliloti*.

**Keywords:** alfalfa, *Sinorhizobium meliloti*, *nifH* and *nodA* genes.

### Introduction

The gram negative soil bacteria, *sinorhizobium meliloti* and *sinorhizobium medicae* are capable of forming root nodules on *Medicago sativa* L. (alfalfa), in which they convert nitrogen to ammonia that is used by host (Bailey et al., 2006). The host, alfalfa is a perennial forage legume that has great nutritive value, high digestibility and a high biomass yield (Campilo et al., 2003). This legume is one of the main sources of protein for livestock (Lesins and Lesins, 1979).

Rhizobia require several classes of specific genes for formation an effective symbiosis. These include *nod* genes, which encode the production of Nod factors, which stimulate the plants to produce symbiotic nodules, and *nif* genes, which produce the nitrogen-fixing nitrogenase enzyme. The *nod* genes are unique to rhizobia, and the *nodA*, *nodB* and *nodC*, which are found in all rhizobia, are all functionally similar among rhizobial species. These genes are involved in the very early stages of symbiosis and all three genes are necessary for nodulation (Triplett and Sadowsky, 1992). As a result of conservation and ubiquity, several nodulation genes such as; *nodA*, *nodD*, *nodC*, *nodZ* have been used for phylogenetic studies of rhizobia (Dresler et al., 2007).

By contrast, *nif* genes are found in many bacteria besides rhizobia (Haukka et al., 1998). In the case of rhizobia, the most studied nitrogen fixation genes are *nifH* and *nifD*. The *nifH* coding for two identical subunits of component II dinitrogenase reductase, is the most studied nitrogen fixation gene (Chen et al. 2003; Dobert et al. 1994; Haukka et al. 1998; Laguerre et al. 2001; Rivaset al. 2002; Trujillo et al. 2005). In rhizobia *nifH* can exist in multiple copies. However, sequencing of multiple copies has shown them to be practically identical. The *nifD* codes for the alpha subunits of component I in nitrogenase reductase (Parker et al. 2002; Ueda et al. 1995c). The *nif* genes are found within highly conserved operons and several authors have used these genes as

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molecular markers for phylogeny study of rhizobia (Haukka et al., 1998; Laggure et al., 2001; Parker et al., 2002; Rincoń-Rosales et al., 2008).

In Iran *Medicago sativa* L. is widely cultivated in different areas as an important protein source for farm animals. In spite of the fact that alfalfa has key roles as a perennial forage legume in the country, no information exist about the phylogeny of its microsymbiont. Hence, the aim of this work was to investigate the phylogeny of the strains isolated from root nodules of *Medicago sativa* L. by sequence analysis of the symbiotic genes, *nifH* and *nodA*.

## Material and methods

### Bacterial strains

In our previous study, the genetic diversity of a collection of 48 strains from alfalfa root nodules, assessed by using IGS-RFLP (Soltani et al., 2012). According to the result of the IGS-RFLP, strains KH21, KH42, KH74, KH41, KH115, KH140, KH119, KH33, KH10, KH6, KH133, KH13, KH193, KH186, KH16 and KH24 were chosen as representative for the genetic diversity and phylogeny study by symbiotic genes, *nifH* and *nodA* (Table 1).

### DNA extraction

Genomic DNA from tested strains was prepared using the alkaline lysis method as described previously (Baele et al., 2000).

### DNA amplification of the symbiotic genes

Symbiotic genes, *nifH* and *nodA*, were amplified for tested strains using primers and the PCR procedures listed in Table 2. The PCR products and their concentration were checked by electrophoresis of 5µl product on a 1% agarose gel containing ethidium bromide using a pGEM DNA marker as reference .

### Gene sequencing and phylogenetic data analysis

The PCR products were sequenced using the normal Sanger method. All the genes were sequenced using the respective pair of primers that were used for amplification. The quality of the sequences was assessed and edited using BioEdit program (Hall, 2004). *nifH* and *nodA* gene sequences of the tested strains were compared to the Genbank database by using the nucleotide blast program. For further study *nifH* and *nodA* gene sequences of a number of reference and type strains related to our strains were achieved from the database ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)) as well. Nucleotide Sequence alignments for each gene were made using ClustalW as implemented in MEGA version 5 (Tamura et al., 2011) and manually corrected when necessary. After trimmed to the same length, protein-encoding gene sequences were concatenated. For each symbiotic gene, both Neighbour Joining (NJ) and Maximum Likelihood (ML) phylogenetic trees were constructed using MEGA version 5. The NJ analyses were performed with default parameters and the Kimura 2 model. For ML analyses, the best fit model estimated by the Akaike information criteria, using MEGA version 5 for each protein-encoding and concatenated genes was T92+I (Tamura 3-parameter plus Has Invariant sites). For ML, statistical support of the trees was calculated by bootstrap analyses using 100 replications and for NJ, 1000 replications. Nucleotide sequences generated in this study were deposited in GenBank.

### Nodulation test

Nodulation test was performed according to Vincent (1970).

## Result and discussion

In this work, Sequences for the *nifH* and *nodA* were acquired for most of the tested strains. We could not amplify the *nifH* and *nodA* fragments for KH16, KH24 KH6, KH10, KH133 and KH193, using different primers and PCR profile for each gene. Altogether, 20 new sequences, containing 10 *nifH*, 10 *nodA*, gene sequences, were deposited in GenBank. For each of symbiotic gene, separate phylogenetic trees constructed using NJ and ML methods, resulted in analogous groupings. Therefore, for each gene only phylogenetic tree constructed using ML method is shown. Phylogenetic trees constructed from nucleotide sequences of *nifH* and *nodA* (Fig1, 2 respectively), grouped bacterial strains with *S. meliloti*.

In the *nifH* tree topology (Fig 1), all of the tested strains were clustered with *S. meliloti* reference strains with high (98%) bootstrap value. In this group, KH186, KH119, KH13, KH21 had identical *nifH* sequence with *S. meliloti* reference strains 1021, CCBAU 65135 and CCBAU83493. The *nifH* sequence of KH33 and KH115 was equal to reference strain *S. meliloti* JO1781. Test strains KH41, KH42 and KH74 made a subgroup with relatively

low bootstrap support of 58%. The sequence similarity between KH42 and strains KH186 and KH33 was 97.3% and 97% respectively. KH33 and KH186 had 99.5% sequence homogeneity.

Based on sequence of *nodA* (Fig 2), all of the test strains belonged to the *S. meliloti*. Bacterial strains used in this study had identical sequence *nifH* sequence with each other and *S. meliloti* reference strains.

Plant infection test result showed that, ten strains formed nodules on the roots of host plant, alfalfa. No nodules were observed on the roots of alfalfa inoculated with bacterial strains KH16, KH24, KH6, KH10, KH133 and KH193 (Table1).

As representatives of the nodulation and nitrogen fixation genes we chose *nodA* and *nifH*, respectively, because a considerable number of comparative sequences already are known and these genes have a number of other advantages. For *nodA*, only one copy has ever been detected, and although reiteration of *nifH* has been reported for several rhizobia, sequencing of the multiple copies has shown that they are identical or nearly so (Badenoch-Jones et al., 1989; Norel et al., 1987; Quinto et al., 1985). In this research, the phylogeny study of *nifH* and *nodA* genes showed that all strains belonged to *S. meliloti*. The exclusive presence of *S. meliloti* strains suggests that this species is the dominant and more specific rhizobium for alfalfa. This result has been reported by several authors (Carelli et al., 2000; Bromfield et al., 2010; Biondi et al., 2003; Langer et al., 2008). The phylogenetic trees based on the *nodA* and *nifH* genes of the alfalfa rhizobia showed that all tested strains were classified with *S. meliloti*. Correlations between *nod* and *nif* genes have also been showed in several studies (Urtz and Elkan, 1996; Laguerre et al., 2001; Lei et al., 2008). It would not be surprising if *nod* and *nif* genes showed similar evolutionary patterns, as these genes are often linked on the same plasmid (Dresler et al., 2007; Laguerre et al., 2001).

We could not amplify the *nifH* and *nodA* fragments for tested strains, KH16, KH24 KH6, KH10, KH133 and KH193, and There were no nodules obtained from the plants inoculated with these strains as well. These findings suggest that they may be non-symbiotic and opportunistic bacteria that occupy root nodules. Other molecular markers, such as housekeeping genes sequencing, are needed for further identification of these strains. The presence of non-symbiotic bacteria in the root nodules has been reported by some authors (Lei et al., 2008; Bromfield et al., 2010).

Table 1. Bacterial strains used in this study and nodulation test result

Strain	Host plant	Places of isolation	Nodulation test	Source /reference
KH21	<i>Medicago sativa</i>	Chapaghlu	+	Soil and Water Research Institute, Iran
KH42	<i>M.sativa</i>	Sardar abad	+	Soil and Water Research Institute, Iran
KH74	<i>M.sativa</i>	Nisher	+	Soil and Water Research Institute, Iran
KH41	<i>M.sativa</i>	Sardaran	+	Soil and Water Research Institute, Iran
KH115	<i>M.sativa</i>	Darband	+	Soil and Water Research Institute, Iran
KH140	<i>M.sativa</i>	Malaier	+	Soil and Water Research Institute, Iran
KH119	<i>M.sativa</i>	Hasan gheslagh	+	Soil and Water Research Institute, Iran
KH33	<i>M.sativa</i>	Shara	+	Soil and Water Research Institute, Iran
KH10	<i>M.sativa</i>	Alanje	-	Soil and Water Research Institute, Iran
KH6	<i>M.sativa</i>	Chenaroli	-	Soil and Water Research Institute, Iran
KH133	<i>M.sativa</i>	Toushmal	-	Soil and Water Research Institute, Iran
KH13	<i>M.sativa</i>	Zirebagh	+	Soil and Water Research Institute, Iran
KH193	<i>M.sativa</i>	Gonbadechai	-	Soil and Water Research Institute, Iran
KH186	<i>M.sativa</i>	Kamighale	+	Soil and Water Research Institute, Iran
KH16	<i>M.sativa</i>	Azandarian	-	Soil and Water Research Institute, Iran
KH24	<i>M.sativa</i>	Dahlagh	-	Soil and Water Research Institute, Iran

+ Nodules and - No nodules

Table 2. Oligonucleotide primers used in this study

Primer	Direction	Sequence (5'-3')	PCR cycling conditions	Reference
nifHF	Forward	TACGGNAARGGSGGNATCGGCAA	3 min 95 °C, 35x (1 min 94°C, 1 min 57°C, 2 min 72 °C), 3 min 72 °C	Laguerre et al., (2001)
nifHI	Reverse	AGCATGTCYTCSAGYTCNTCCA		Laguerre et al., (2001)
nifH-1F	Forward	GTC TCC TAT GAC GTG CTC GG		Rivas et al., (2002)
nifH-1R	Reverse	GCT TCC ATG GTG ATC GGG GT	9 min 95 °C, 35x (1 min 95°C, 2 min 60°C, 2 min 72 °C), 7 min 72 °C	Rivas et al., (2002)
nodA-1	Forward	TGCRGTGGAARNTRNCTGGGAAA	2 min 93 °C, 35x (45 S 93°C, 1 min 49 °C, 2 min 72 °C), 5 min 72 °C	Haukka et al., (1998)
nodA-2	Reverse	GGNCCGTCRTCRAAWGTCARGTA		Haukka et al., (1998)
nodA-3	Forward	TCA TAG CTC YGR ACC GTTCCG	2 min 97 °C, 25x (40 S 92°C, 1 min 55 °C, 1.5 min 72 °C), 5 min 72 °C	Zhang et al., (2000)
nodA-4	Reverse	ATC ATC KYN CCG GNN GGC CA		Zhang et al., (2000)

Symbols used: N = A, C, G or T; R = A or G; S = C or G; W = A or T, Y = C or T.

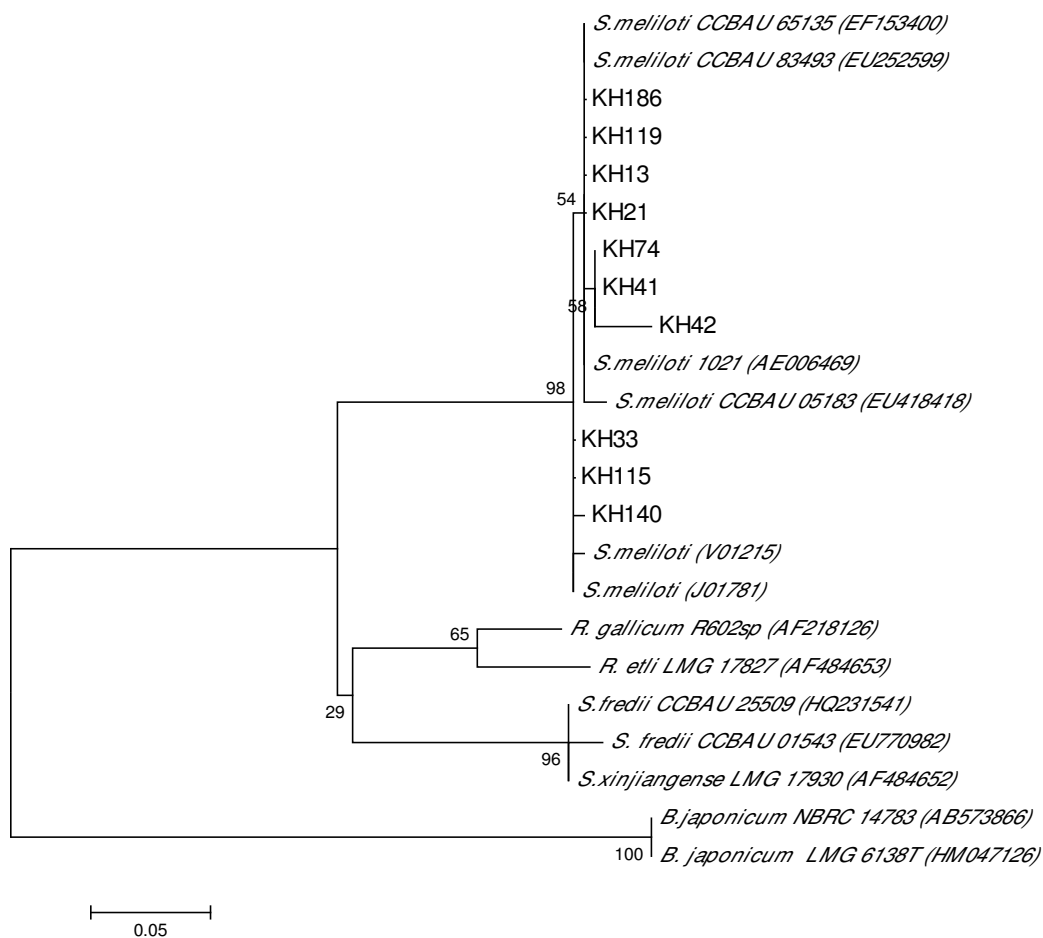


Figure 1. Phylogenetic tree estimated using the ML method with partial sequences of the symbiotic gene, *nifH*. The tree was created using MEGA version 5.0. Numbers at nodes reveal levels of bootstrap support (using 100 replicates). Scale bar 0.05% indicates number of substitutions per site. Type strains are presented by superscript T. The accession numbers for the sequences are indicated within parentheses. Only bootstrap values  $\geq 50\%$  are shown. The tree is rooted using the sequences of the *B. japonicum*.

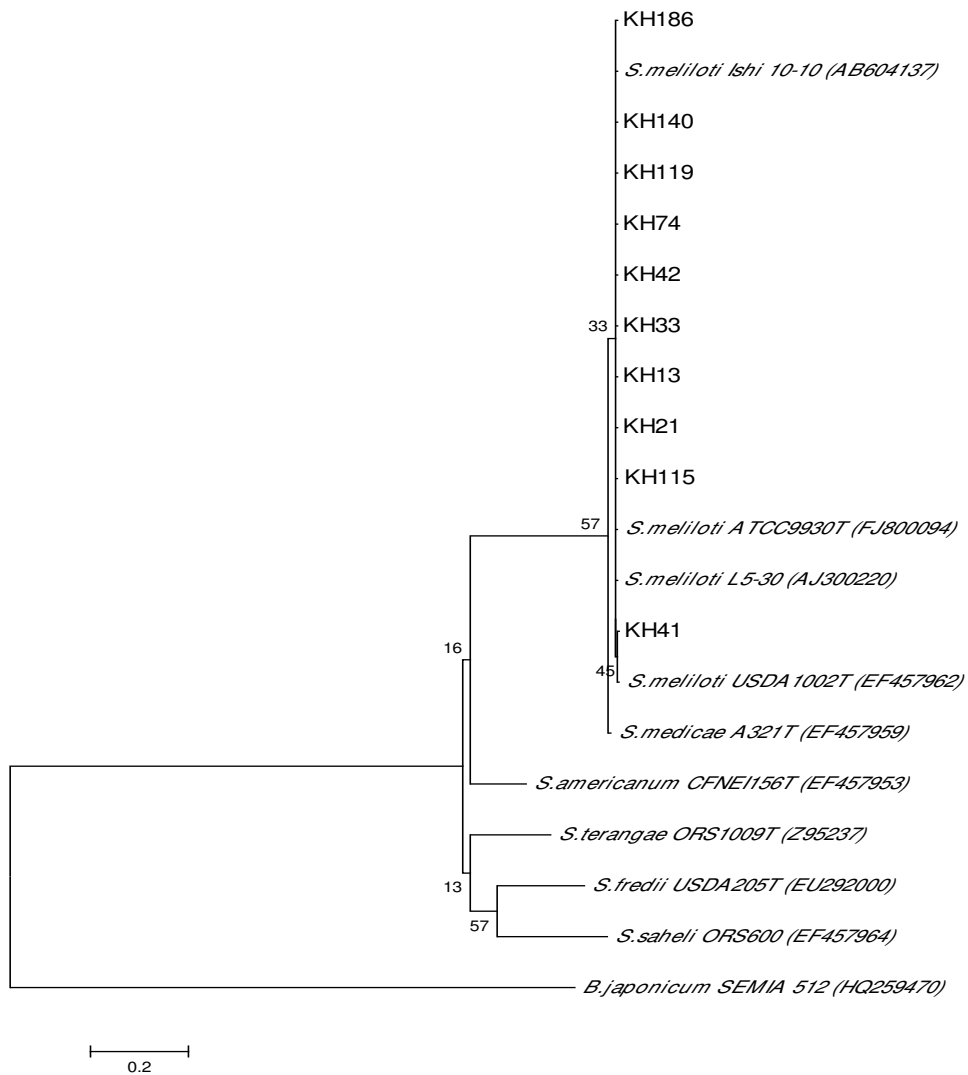


Figure 2. Phylogenetic tree estimated using the ML method with partial sequences of the symbiotic gene, *nodA*. The tree was created using MEGA version 5.0. Numbers at nodes reveal levels of bootstrap support (using 100 replicates). Scale bar 0.05% indicates number of substitutions per site. Type strains are presented by superscript T. The accession numbers for the sequences are indicated within parentheses. Only bootstrap values  $\geq 50\%$  are shown. The tree is rooted using the sequence of the *B. japonicum*.

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