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Molecular diversity of rhizobia isolated from root nodules of alfalfa evaluated by analysis of IGS and 16SrRNA

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ABSTRACT

Rhizobia are used extensively in agricultural and agroforestry systems for increasing the ability of legumes to fix N_2. Knowledge of the classification, genetic characterization and biodiversity of the native rhizobia population is necessary for the selection of inoculant strains. In this work the genetic diversity of 48 rhizobia isolated from root nodules of alfalfa, cultivated in different regions of Iran, was studied by restriction fragment length polymorphism (RFLP) analysis of PCR-amplified intergenic region (IGS) and 16srRNA gene. Analysis of the intergenic region between 16S and 23S rDNAs (IGS) showed a considerable diversity within these microsybionts. At the similarity of 70%, these rhizobia were clustered in to 4 groups:I, II, III and IV. Two genera,Sinorhizobium and Agrobacterium were identified among the isolates by PCR-RFLP of 16srRNA gene.

Key words: Genetic diversity**,** *Sinorhizobium*, *Agrobacterium* and alfalfa.

INTODUCTION

Symbiotic nitrogen fixation with rhizobia is the most important route for sustainable nitrogen input into agroecosystems. The legume-rhizobium symbiosis represents a significant basic model for symbiosis, evolution and differentiation in agriculture for sustainable production and other fields [13]. Alfalfa (*Medicago sativa L.*) is The most widely cultivated perennial species of *Medicago* that has great nutritive value, high digestibility. This species formes nitrogen-fixing symbiosis with the genus *Sinorhizobium* and decreases the utilization of chemical nitrogen fertilizers in agricultural and pasture systems.

Knowledge of the classification, genetic characterization and biodiversity of the native rhizobia population is necessary for the selection of inoculant strains. The diversity of alfalfa nodulating rhizobia has been investigated worldwide with various molecular techniques. PCR-RFLP analysis of 16S-23S intergenic spacer region (IGS) has been showen as a easy, profitable and potent approach to assess the genetic diversity of rhizobia belonging to different genera [4, 25, 2]. In contrast to 16S-23S rDNA intergenic spacer region, the 16S rRNA gene is considerably well conserved throughout most bacterial species [23]. Hence, analysis of this gene has a vital and key role in determining the phylogenetic relationships of bacteria and species identification [24].

Medicago sativa L. is broadly cultivated in different area of Iran and has an important role as main protein source for livestock. However, despite the importance of alfalfa cultivation in Iran little is known about the genetic diversity and phylogeny of its microsymbiont. Therefore, in this study we examine the genetic diversity and phylogeny of a collection of 48 strains from alfalfa root nodules, sampled in different fields in various regions of Iran by using IGS-RFLP and 16S rRNA as molecular markers.

MATERIALS AND METHODS

Bacterial strains

A total of 52 bacterial strains were used in this study. Fourty-eight test strains, isolated from root nodules of green and healthy alfalfa plants grown in the various fields of Iran, were chosen from microbial collection of department of soil biology, soil and water research institute of Iran. Four reference strains, comprising *S. meliloti* HAMBI1318- *S. meliloti* HAMBI2148- *S. medicae* HAMBI12306 and *A. tumefaciens* HAMBI1811,were used as controls. (Table 1)

DNA extraction

Genomic DNA from 48 field strains and four reference strains was prepared using the alkaline lysis method as described previously [3].

PCR-RFLP of 16S-23S rDNA

The primers FGPS1490[17] and FGPS132' [18] were used to amplify the IGS region.Amplification reaction was carried out in a 50µl mixture solution containing: reaction buffer (Finnzyme), 2.5 mM MgCl2, 0.2 mM of each dNTP, 1.6 U of Taq DNA polymerase (DynazymeII, Finnzyme), 10 pmol of each primer, 25 ng of template DNA. Reaction was performed by means of a PTC-200 peltier thermal cycler(MJ Research) programmed for an initial denaturation at 94^{o}c for 10 min followed by 34 cycles at 94^{o}c for 35s,52^{o}c for 1 min, 72^{o}c for 2min.A final extension step at 72^{o}c for 10 min was done.The concentration and the size of PCR product was evaluated on a 1.5% agarose gel stained with ethidium bromide.For the restriction analysis,aliquots of 5µl of the amplified 16S-23S rDNA were digested with 4.5U of each of the restriction endonucleases *HaeIII*(Promega) and *MSPI*(Fermentase). The digested DNA fragments were separated by horizontal gel electrophoresis on a 3% agarose gel pre-stained with ethidium bromide in 1X TAE buffer. The gels were run for 2h at 100v and photographed under UV illumination with kodak DC-290 camera and the kodak molecular imaging software V.40. The RFLP patterns acquired from digestion with restriction enzymes were amalgamated and utilized in clustering analysis by the unweighted pair grouping with mathematic average(UPGMA) using the Bionumerics version 6 software. The strains that had similar restriction fragment profiles were classified in to the equal IGS group.

PCR-RFLP of the 16S rRNA

The 16S rRNA gene was amplified by PCR with the primers rD1 and fD1 [22] and procedure described previously [10]. The procedure of restriction fragments analysis was same as RFLP of 16S-23S rDNA. The restriction enzymes used were *AluI* and *MspI.*

RESULTS

In our study, electrophoresis of nondigested PCR products of 16S-23S rDNA spacer revealed that most strains possess one band ranging from 1000-1300bp.The length of the IGS amplified region for strains KH16 and KH24 was 800bp. Strain KH186 produced one additional band of size 700bp(data not shown). After digestion of PCR products by restriction enzymes, 15 genotypes were distinguished among 48 test strains and 4 reference strains. In dendrogram constructed from IGS-RFLP (Fig 1), all of the 15 genotypes were grouped in to four groups at a similarity of 70%. Group I contained the majarity of the strains and two reference strains (*S.meliloti* HAMBI1318 and HAMBI2148). This group could be divided at 85% similarity in to three subgroups (I_a, I_b and I_c). Reference strain *S.medicae* HAMBI2306 and strain KH6 formed groupII. Group III consisted of three strains and *A.tumefaciens* HAMBI1811. Strains with small IGS were placed in groupIV. 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 16S rRNA gene analysis.

Table 1 Rhizobia used in this study

Strain	Host plant	Places of isolation	ITS pattern	16S rRNA pattern	Source /reference
KH21	*Medicago sativa*	Chapaghlu	2	2	Soil and Water Research Institute, Iran
KH183	*M.sativa*	Shirin sou	2	2	Soil and Water Research Institute, Iran
KH49	*M.sativa*	Zangane	2	2	Soil and Water Research Institute, Iran
KH152	*M.sativa*	Famast	2	2	Soil and Water Research Institute, Iran
KH69	*M.sativa*	Milajerd	2	2	Soil and Water Research Institute, Iran
KH162	*M.sativa*	Vahman	2	2	Soil and Water Research Institute, Iran
KH182	*M.sativa*	Kouhaven	2	2	Soil and Water Research Institute, Iran
KH198	*M.sativa*	Baba khanjar	2	2	Soil and Water Research Institute, Iran
KH95	*M.sativa*	Ghale asijan	2	2	Soil and Water Research Institute, Iran
KH44	*M.sativa*	Hatam abad	2	2	Soil and Water Research Institute, Iran
KH121	*M.sativa*	Salam saraee	2	2	Soil and Water Research Institute, Iran
KH43	*M.sativa*	Kabudar ahang	2	2	Soil and Water Research Institute, Iran
KH202	*M.sativa*	Razan	2	2	Soil and Water Research Institute, Iran
KH153	*M.sativa*	Jafar abad	2	2	Soil and Water Research Institute, Iran
KH70	*M.sativa*	Pirnahan	2	2	Soil and Water Research Institute, Iran
KH31	*M.sativa*	Dahdalilan	2	2	Soil and Water Research Institute, Iran
KH212	*M.sativa*	Ghalghal abad	2	2	Soil and Water Research Institute, Iran
KH57	*M.sativa*	Kortil abad	2	2	Soil and Water Research Institute, Iran
KH126	*M.sativa*	Ghuri chai	2	2	Soil and Water Research Institute, Iran
KH42	*M.sativa*	Sardar abad	2	2	Soil and Water Research Institute, Iran
KH25	*M.sativa*	Haji abad	2	2	Soil and Water Research Institute, Iran
KH142	*M.sativa*	Samen	2	2	Soil and Water Research Institute, Iran
KH187	*M.sativa*	Targhieh	2	2	Soil and Water Research Institute, Iran
KH209	*M.sativa*	Karafs	2	2	Soil and Water Research Institute, Iran
KH74	*M.sativa*	Nisher	2	2	Soil and Water Research Institute, Iran
KH109	*M.sativa*	Bitran	2	2	Soil and Water Research Institute, Iran
KH22	*M.sativa*	Khomajin	2	2	Soil and Water Research Institute, Iran
KH41	*M.sativa*	Sardaran	3	2	Soil and Water Research Institute, Iran
KH115	*M.sativa*	Darband	4	2	Soil and Water Research Institute, Iran
KH140	*M.sativa*	Malaier	5	2	Soil and Water Research Institute, Iran
KH82	*M.sativa*	Zaman abad	6	2	Soil and Water Research Institute, Iran
KH150	*M.sativa*	Avar zaman	6	2	Soil and Water Research Institute, Iran
KH173	*M.sativa*	Ghureh jenie	6	2	Soil and Water Research Institute, Iran
KH40	*M.sativa*	Sarvar abad	6	2	Soil and Water Research Institute, Iran
KH63	*M.sativa*	Bar zoun	6	2	Soil and Water Research Institute, Iran
KH119	*M.sativa*	Hasan gheshlagh	6	2	Soil and Water Research Institute, Iran
KH105	*M.sativa*	Asad abad	7	2	Soil and Water Research Institute, Iran
KH81	*M.sativa*	Siakamar	7	2	Soil and Water Research Institute, Iran
KH33	*M.sativa*	Shara	7	2	Soil and Water Research Institute, Iran
KH73	*M.sativa*	Ali abad	7	2	Soil and Water Research Institute, Iran
KH10	*M.sativa*	Alanje	8	1	Soil and Water Research Institute, Iran
KH6	*M.sativa*	Chenaroli	9	1	Soil and Water Research Institute, Iran
KH133	*M.sativa*	Toushmal	11	1	Soil and Water Research Institute, Iran
KH13	*M.sativa*	Zirebagh	12	3	Soil and Water Research Institute, Iran
KH193	*M.sativa*	Gonbadechai	12	3	Soil and Water Research Institute, Iran
KH186	*M.sativa*	Kamighale	14	3	Soil and Water Research Institute, Iran
KH16	*M.sativa*	Azandarian	15	4	Soil and Water Research Institute, Iran
KH24	*M.sativa*	Dahlagh	15	4	Soil and Water Research Institute, Iran
HAMBI1318			1	2	HAMBI, Finland, K.Lindstrom
HAMBI21148			1	2	HAMBI, Finland, K.Lindstrom
HAMBI12306			10	2	HAMBI, Finland, K.Lindstrom
HAMBI1811			13	3	HAMBI, Finland, K.Lindstrom

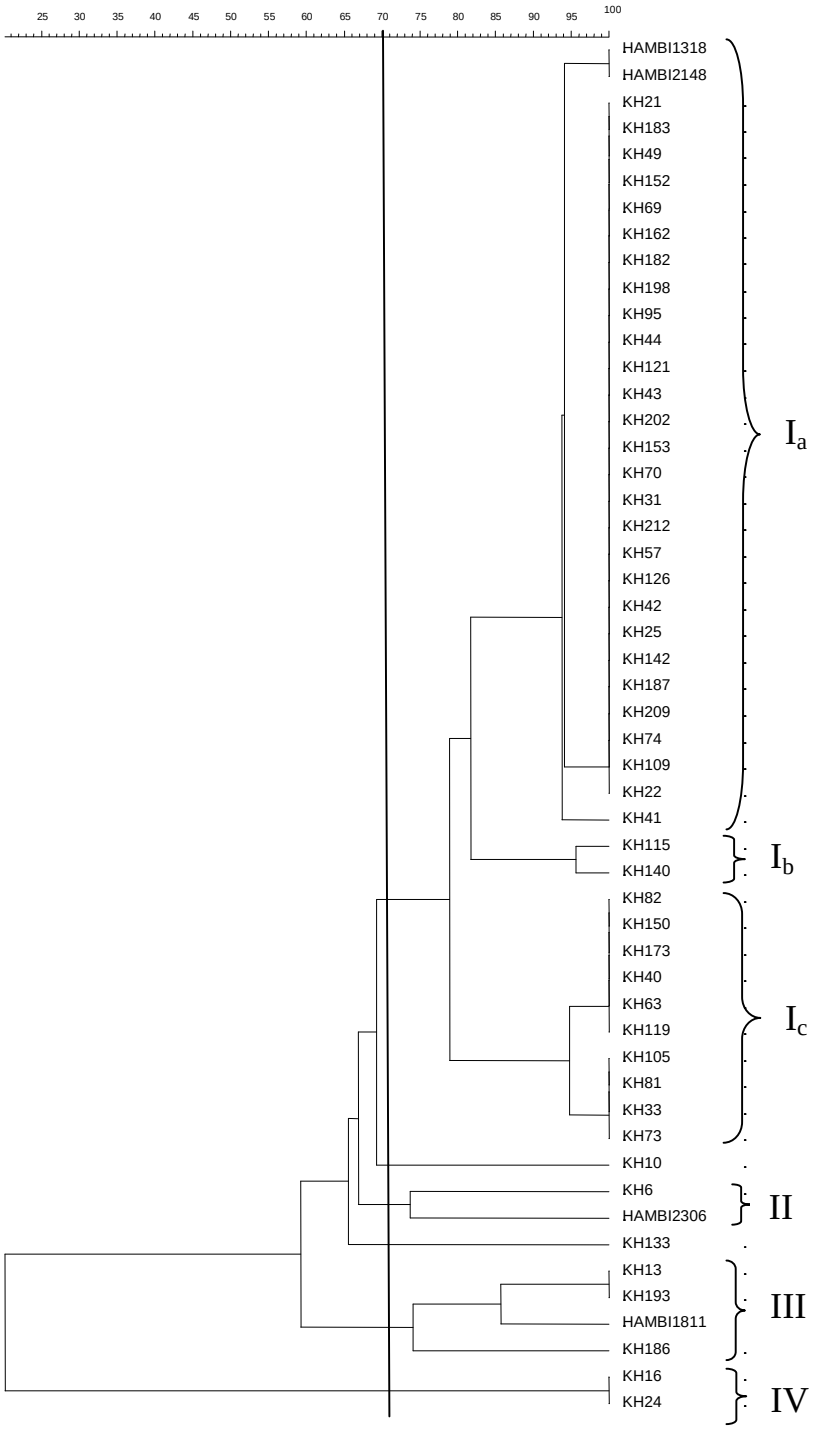

Fig. 1 Dendrogram generated from the 16S-23S IGS RFLP patterns of the alfalfa rhizobia and the reference strains grouped by UPGMA

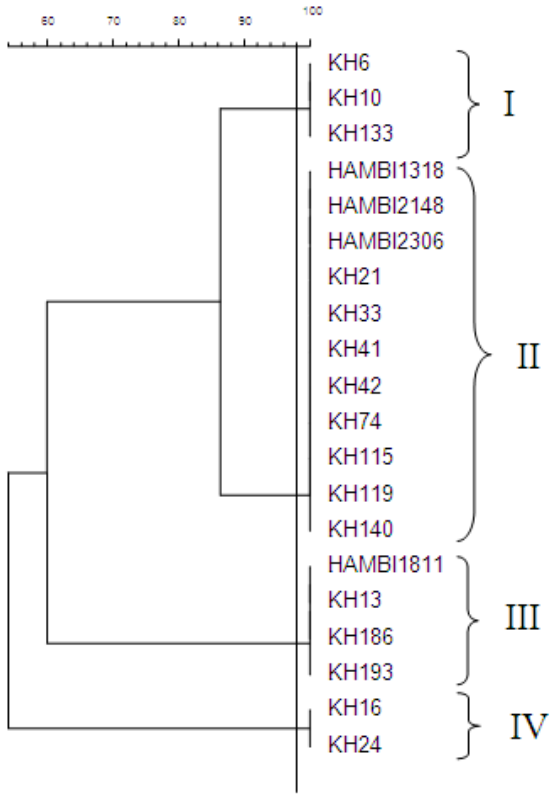

Figure 2 UPGMA dendrogram based on 16S rRNA PCR-RFLP patterns showing the genetic relationship among rhizobia isolated from alfalfa

Nearly 1500bp length of 16S rRNA from representative strains and four reference strains was amplified by PCR amplification. All the strains produced a single band. After digestion by restriction enzymes, four rDNA genotypes were distinguished among the 16 test strains and four reference strains. A dendrogram based on the combined 16S rRNA gene restriction patterns by UPGMA algorithm (Fig 2) shown that all strains were clustered in to four groups at a similarity of 98%. Group II was the largest one, including 8 test strains and the reference strains *S. meliloti* HAMBI1318 and *S. meliloti* HAMBI2148. Reference strain *A.tumefaciens* HAMBI1811 and 3 test strains formed groupIII. Groups I and IV consisted of 3 and 2 test strains repectively and none of the reference strains were clustered in to these groups.

DISCUSSION

In this research, the genetic diversity of 48 alfalfa nodulating rhizobia isolated from different parts of Iran were estimated.We used PCR-RFLP of 16S-23S spacer for diversity assessment, as PCR-based RFLP of IGS have been evidenced as rapid,effective and reliable technique for identifying genetic differences of rhizobia isolates [2, 21, 12`, 19, 9, 1]. Our results revealed that the IGS between the 16S and 23S rRNA was a suitable marker for evaluation genetic diversity. We obtained relatively high diversity either as for the lenth of IGS bands or in the patterns appeared after digestion with restriction enzymes. Several authors have reported high genetic diversity among alfalfa nodulating rhizobia [11, 5, 8, 6]. IGS-RFLP analysis showed that tested strains included 15 IGS genotypes and were clustered in to four groups at a similarity of 70%. IGS groups I_b, I_c and IV were distinct from the reference strains since none of the reference strains were grouped in to these groups. To investigate the phylogeny of the test strains we analysed the 16S rRNA gene by PCR–RFLP with two restriction enzymes *AluI* and *MspI*. Although 16S rRNA gene sequencing has become quite easy to perform, but still not every laboratory has the ability to use this technique. A good phylogenetic estimate can be gained by PCR-RFLP analysis of the 16S rRNA gene [10] a technique which does not need any special equipment. This method is also a rapid identification method [10, 7]. In this study, our results showed that PCR with RFLP analysis of 16S rRNA was a good marker for grouping new isolates. Based

upon this analysis, most of the test strains were grouped with genus *sinorhizobium*. PCR-based RFLP analysis of 16S rRNA with *AluI* and *MspI* was not able to seperate *S.meliloti* and *S.medicae* species, indicating that the set of two restriction enzymes is not enough to resolve the closely related species. Our results revealed that test strains KH13, KH186 and KH193 were clustered with *A.tumefaciens* at a similarity of 98%. Several authors have reported the isolation of *Agrobacterium* strains from the root nodules of *Phaseolus vulgaris* [15], from nodules of *Acacia tortilis* [4] from common bean nodules [16] from root nodules of *Vicia faba* [20] and from woody legumes [14]. According to the results of IGS-RFLP and 16S rRNA-RFLP test strains were identified as *Sinorhizobium* (40 strains) and *A.tumefaciens* (3 strains). Further more, 5 test strains from our collection were not identified. Other molecular technigues (genes sequencing and DNA-DNA hybridisation) are needed for further characterization of the non-identified root nodule bacteria.

CONCLUSION

According to our knowledge, little is known about the genetic diversity and phylogeny of alfalfa microsymbionts in Iran. This work is a preliminary step towards molucular identification of alfalfa nodulating rhizobia. Our result showed that *Sinorhizobium* is the dominant genus in alfalfa nodules with a reletively high genetic diversity. To better assess the phylogeny of *Medicago sativa* L. nodulating rhizobia isolated from Iran, further studies are required.

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