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Genetic Diversity of *Sinorhizobium meliloti* Isolated from Root Nodules of Alfalfa (*Medicago sativa*) Growing in Hamadan Soils (Iran) Using Plasmid Profile and PCR/RFLP

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ABSTRACT

Genetic diversity of 196 isolates of *Sinorhizobium* from nodules of *Medicago sativa* (cv. Hamadani) grown in different soil samples was investigated. Plasmid profile and the PCR/RFLP of 16S-23S rRNA were employed for this study. The results of the plasmid profile distinguished 27 groups among 196 isolates. PCR/RFLP of 16S-23S rRNA from 150 isolates of *Sinorhizobium* showed that they could be divided into three distinct groups. The two major groups were identified as *S. meliloti* and *S. medicae* and a third minor group appeared different from the other two. Shannon's index was different in each land type in Hamadan province. Results also indicated that there was a positive correlation between some soil characteristics and Shannon's index.

Key Words: Diversity; *Sinorhizobium*; Plasmid profiles; PCR/RFLP; 16SrRNA

INTRODUCTION

Alfalfa (*Medicago sativa* L.) as a forage legume is one of the main sources of protein for livestock (Lesins & Lesins, 1979). More than 6,000 km^2 of arable land in Iran are under alfalfa cultivation. Alfalfa plants form symbiotic relation with nitrogen fixing bacteria of the *Sinorhizobium* genera (Paffetti *et al*., 1998; Bradic *et al.*, 2003). It is very important to study the native population of rhizobial bacteria in Iran, where it is supposed to be the origin of the host plant for *Sinorhizobium* genus (Karimi, 1990). On the other hand it has been documented that the evolution of symbiotic rhizobacteria may be influenced by environmental factors such as biological barriers of gene exchange, physiographical units, soil types and host plant genotypes (Krasovsky & Stotzky, 1987; Souza *et al*., 1994; Mendes & Bottomley, 1998; Lakzian & Bromfield, 2004).

The aim of this study was to investigate the native population diversity of indigenous *Sinorhizobium* bacteria isolated from Hamadan soils by using Plasmid profile and PCR/RFLP of 16S-23S rRNA. The relationship between the genetic diversity and some soil properties were also evaluated.

MATERIALS AND METHODS

Bacterial isolates. One hundred ninety six isolates of *Sinorhizobium* bacteria from nodules of alfalfa (*Medicago sativa* L cv. Hamadani) plants were grown in soil samples collected from different physiographical units in Hamadan province, Iran. Fig. 1 shows the soil sampling sites. All isolates of *Sinorhizobium* bacteria were purified and preserved on YEMA media for later use (Somasegaran & Hoben, 1994).

Plasmid profile procedure. The plasmid profile analysis was carried out by using Ekhardt (1987) gel and later modified by Hynes *et al*. (1984).

Genomic DNA extraction. Total genomic DNAs of 150 *Sinorhizobium* isolates were extracted using Phenol-chloroform extraction and ethanol precipitation (Somasegaran & Hoben, 1994). The IGS of 16S-23S rRNA genes was amplified by PCR technique using two specific primers FGPS1490 (5´TGC GGC TGG ATC ACC TCC TT3´) and FGPS132 (5´CCG GGT TTC CCC ATT CGG3´) (Peng *et al.,* 2002). The PCR reaction was carried out in 50 µl (200 µM of dNTPs, 0.1 µM of each primes, 2.5 U of *Taq* DNA polymerase, 2.5 mm $MgCl_2$). The thermal program for PCR reactions carried out at 95^{o}C for 5 min; 35 cycling times at 94^{o}C for 1 min, 55^{o}C for 45 sec. and 72^{o}C for 1 min and a final cycle was 72^{o}C for 5 min.

Digestion condition. Aliquots of PCR products were digested with the restriction endonucleases *HaeIII* and *HinfI* according to the manufacturer's instruction. The restricted DNA was analyzed by electrophoresis in 4% agarose gel. The gels were stained with Ethidium bromide and photographed under 312 nm ultraviolet light.

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Diversity index. The Shannon and Weaver's Index (H) (Shannon & Weaver, 1949) and Similarity Index (S) were calculated to determine the diversity and similarity groups within each physiographic unit, respectively. The Shannon and Weaver's Index and Similarity Index were calculated by the following equation:

$$H = -\sum_{i=1}^{k} P_i \ln P_i \quad \text{and} \quad S = \frac{2C_{ij}}{C_i + C_J}$$

Where k is the number of operational taxonomic units (OTU) defined by similar plasmid profile or PCR/RFLP), Pi: the relative abundance of isolates of each OTU (i = 1, 2, …k) (Coutinho *et al.*, 1999) and the Similarity Index C_{ij} is the number of common OTU between two separated physiographical units, C_i: number of OTU in the i physiographical unit and C_j: number of OTU in the j physiographical unit.

RESULTS AND DISCUSSION

Plasmid profile. The results of the plasmid analysis showed that all isolates contain one to four plasmids. The molecular weight of plasmids varied from 50 to 200 kb (Fig. 2). All isolates of *Sinorhizobium* were grouped based on the number and size of their plasmid profiles and hence 27 distinct plasmid groups were identified. Rhizobial plasmid size varied among rhizobial bacteria isolates (Lakzian, 1998; Zou *et al*., 1998; Jebara *et al*., 2001). Thirteen different plasmid sizes were identified among 196 isolates of *Sinorhizobium*. Our results were in agreement with those obtained by Hartman *et al*. (1998) and Jebara *et al*. (2001). It seems that nodulation gene was carried on the heaviest plasmid (200 kb), which was common among all isolates.

It has been well documented that the *nod* and *nif* genes in fast growing rhizobia is located on the large plasmid similar to the plasmid in this study (Finan *et al*., 1986; Barloy *et al*., 2000; Melanie *et al*., 2001). The plasmid with 130 kb had the most frequency among isolates (Fig. 3). Our results showed that H and S were different in each physiographical unit (Table I). By using a regression model, no significant difference was found between H (dependent variable) and soil properties (independent variable) individually. Stepwise regression model was used to study the combination effects of soil properties (independent variable) on H (dependent variable). Nine different models were tested and a model with four variables was finally selected (Table II). The results revealed that organic carbon, nitrate and ammonium and clay content had positive effect on H, which clay and organic content had a larger effect on diversity of plasmids compared with other examined soil factors. Compared to clay and organic carbon, nitrate and ammonium had less effect on H (Table II). Data from different studies have shown that genetic diversity affected by environmental conditions such as biological barrier of gene exchange, geographical isolation, soil types and genotype of the host plant (Krasovsky & Stotzky, 1987;

Fig. 1. Sampling sites in Hamadan province (20172 km^2), sea level 3574 m, west Iran

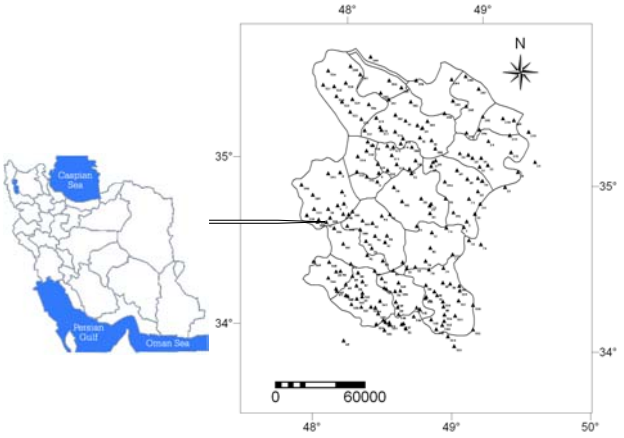


Fig. 2. Plasmid profile (partial results) of 15 *Sinorhizobium* isolates. MVI is the marker strain

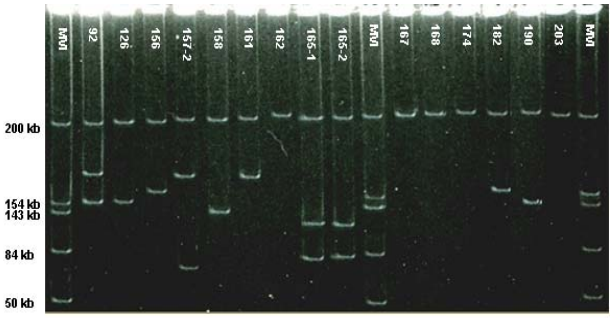
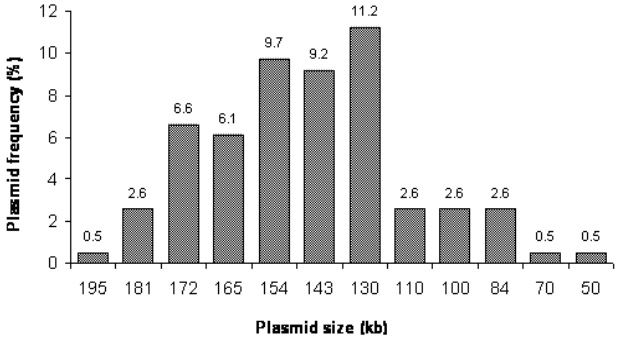


Fig. 3. Plasmid frequency of different plasmid identified among 196 *Sinorhizobium* isolates



Souza *et al*., 1994; Mendes & Bottomley, 1998; Coutinho, 1999; Lakzian & Bromfield, 2004). Our results also indicated that genetic variations among the studied isolates are different in each physiographical unit based on differentiation of soils.

PCR/RFLP. The amplified PCR products of IGS of the 16S-23S rRNA gene from 150 *Sinorhizobium* isolates varied from 900 to 2,000 bp. This variation with four different IGS sizes (200, 1,800, 1,200, 1,931 bp) were identified among all isolates. These differences are in part due to variation in number and type of tRNA genes found in

Table I. Changes of H and S in physiographical units based on PCR/RFLP 16S-23S rRNA

Physiographical Unit code	H	Number of strain
17	0.41	21
18	0.57	3
28	0.3	2
30	0.25	33
43	0.27	6
44	0.24	4
45	0.43	20
47	0.41	7
55	0.39	30
83	0	5
85	0.47	19

Physiographical Unit code	30	85	17	45	47	43	44	55	28	83	18
30	1	0.67	0.57	0.67	0.80	0.50	0.77	0.57	0.50	0.67	0.87
85		1	0.44	0.50	0.57	0.33	0.66	0.67	0.33	0.40	0.67
17			1	0.44	0.50	0.23	0.57	0.47	0.29	0.33	0.57
45				1	0.57	0.33	0.67	0.67	0.23	0.40	0.67
47					1	0.40	0.80	0.50	0.40	0.50	0.80
43						1	0.50	0.29	0.50	0.67	0.50
44							1	0.57	0.50	0.67	0.65
55								1	0.29	0.37	0.57
28									1	0.67	0.50
83										1	0.67
18											1

Fig. 4. Relation of 150 isolates of *Sinorhizobium* isolated from Hamadan soil samples based on PCR/RFLP of IGS of 16S-23S rRNA gene

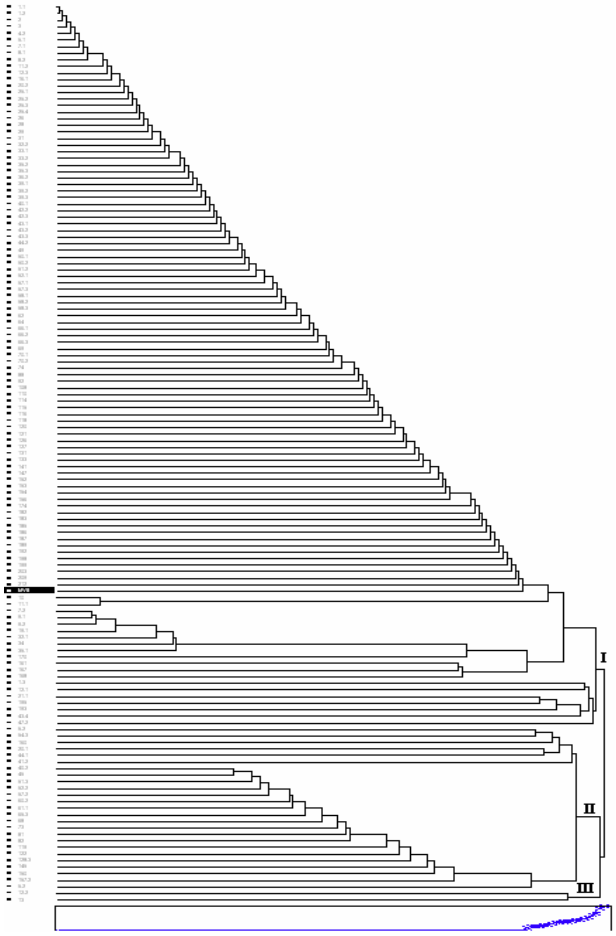


Table II. Stepwise model regression among plasmid groups and soil properties

Model No.	Independent variables	Maloo Cof.	R^2	Adj Rsqr	MS
1	1	5.60	0.36	0.29	0.0190
2	2	1.54	0.67	0.59	0.0111
3	3	2.03	0.74	0.63	0.0097
4	4	2.33	0.83	0.72	0.0075
5	5	3.99	0.84	0.70	0.0081
6	6	5.55	0.87	0.67	0.0086
7	7	6.71	0.91	0.71	0.0077
8	8	8.35	0.93	0.66	0.0091
9	9	10.00	0.95	0.50	0.0136

Model	4	$R^2 = 0.83$		
Variation	Coefficients	Std. Error	t	P
Intercept	1.200	0.200	5.549	0.001
Organic matter	0.398	0.188	2.107	0.080
NO_3^-	0.012	0.006	1.916	0.103
NH_4^+	0.117	0.067	1.745	0.131
Clay	0.037	0.006	-5.386	0.001
Analysis of Variance				
	DF	SS	MS	F
Regression	4	0.223	0.056	7.36
Residua	6	0.045	0.007	
Total	10	0.267	0.026	

this spacer (Peng *et al*., 2002) Ninety percent of isolates had one IGS with 200 bp and some isolates (1.5%) had two different IGS sizes. Digestion of amplified PCR products of IGS of 16S-23S rRNA with *HinfI* endonuclease enzyme produced 3 to 5 different fragments and all isolates were grouped into five different profiles. *HaeIII* endonucleases enzyme produced 1 to 6 different restriction fragments and all isolates of *Sinorhizobium* were grouped into ten different profiles based on the restriction patterns. However, a high level of size homogeneity in the rhizobial IGS was reported by Paffetti (1996) and Lakzian (1998). The results of PCR/RFLP of two enzymes were combined and a dendo-

gram was illustrated (Fig. 4) based on the UPGMA algorithm by analyzing the similarities among the restriction fragments. One hundred and fifty isolates of *Sinorhizobium* were clustered into three distinct groups based on IGS/RFLP patterns (Fig. 4). Group I contained 123 isolates of *Sinorhizobium* and was identified as S. *meliloti.* Group II contained 25 isolates and was identified as *S. medicae*. Group III differed from I and II included only two isolates each with two different IGS sizes. The H and S were calculated to determine the diversity and similarity of groups within each physiographic unit based on PCR/RFLP, as done for plasmid profiles in this study. The highest correlation (R^2) was found between soil silt content and H.

In conclusion, genetic diversity of *Sinorhizobium* bacteria isolated from Hamadan province was different in each physiographical unit based on Plasmid profile and PCR/RFLP of IGS from 16S-23S rRNA. Stepwise regression of data showed that soil properties affect plasmid diversity. H was higher for plasmids compared with the data from PCR/RFLP. Plasmids play important roles for bacteria grown under specific environmental conditions. Therefore, plasmid transfer may occur in soils, which may increase plasmid profile diversity.

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