GENOTYPIC CHARACTERIZATION OF BRADYRHIZOBIA FROM SMALL LEGUMES BY rDNA PCR-RFLP AND AFLP FINGERPRINT ANALYSES

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ABSTRACT

At the present time, the precise taxonomical status of many strains classified as *Bradyrhizobium* still remains unclear. There is a need to develop a reliable grouping method, specifying the genetic relationships between these strains. Phenotypic methods as auxanography or protein profiling did not prove valuable for classification of bradyrhizobia and were not in good agreement with phylogenetic data. The purpose of this work is to develop a strategy to analyse the diversity of bradyrhizobia and to identify genomic groups among our collection of strains isolated from 9 small legume species in Senegal. *B. japonicum*, *B. elkanii* and representatives of previously described groups by Moreira et al. (1993) and Dupuy et al. (1994) were included as references. Bacterial diversity was assessed by two different techniques, (1) PCR-RFLP analysis of the IGS region between 16S and 23S rDNA (IGS) and (2) the AFLP technique. Groupings of strains obtained by the two methodologies will be presented and compared. 16S rDNA PCR-RFLP analysis of strains representative of IGS clusters from small legumes was also performed.

ABBREVIATIONS: 16S rRNA- 16S ribosomal ribonucleic acid, IGS- Intergenic spacer between 16S and 23S rRNA genes, PCR-RFLP- Polymerase chain reaction-restriction fragment length polymorphism, AFLP- Amplified fragment length poly-

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morphism, ARDRA- Amplified ribosomal DNA restriction analysis, UPGMA- Unweighted-pair group method using average linkages.

1. INTRODUCTION

The precise taxonomical status of many *Bradyrhizobium* strains isolated from different legumes species is not well-defined (Lorquin et al., 1993; Van Rossum et al., 1995).

Several authors have reported the lack of consistency between taxonomical results obtained by different techniques (Young et al., 1991; Ladha and So, 1994; Dupuy et al., 1994). Phenotypic methods such as auxanography or protein profiling did not prove valuable for classification of bradyrhizobia and were not in good agreement with phylogenetic data.

The aim of this work is to develop a strategy to analyze the diversity of bradyrhizobia among our collection of strains isolated from 9 different small legumes species in Senegal. We used two different techniques, (1) PCR-RFLP analysis of the IGS region between 16S and 23S rRNA genes and (2) AFLP (Amplification Fragment Length Polymorphism) analysis.

2. MATERIALS AND METHODS

All information on strains used is given in Table 1.

PCR-RFLP of the IGS region. Primers FGPS1490 (Navarro et al., 1992) and FGPS132' (Ponsonnet and Nesme, 1994) and the protocol described by Laguerre et al. (1994) were used. Pattern analysis was performed using the Gel Compar software (Vauterin and Vauterin, 1992), (version 4.0, Applied maths, Kortrijk, Belgium) and a dendrogram was constructed using the UPGMA method. The AFLP technique (Zabeau and Vos, 1993) is based on the selective amplification of genomic restriction fragments by PCR. The experimental protocol used was modified from Vos et al. (1995) as described by Huys et al. (1996).

The 16S ARDRA method used was that described by Heyndrickx et al. (1996), except that we used the forward primer described by (Weisburg et al., 1991).

3. RESULTS

3.1. PCR-RFLP Analysis of IGS

PCR performed on all strains studied produced a single band of about 1000 bp for strains from small legumes, 1300 bp for photosynthetic strains from *Aeschynomene* species and 900 bp for *B. japonicum* strain LMG 8321 and *Bradyrhizobium* sp. (*Acacia*) strain LMG 8888 (Fig. 1). This is in agreement with the recent report of Laguerre et al. (1996).

One to 6 DNA fragments were generated by each restriction enzyme for the 57 strains studied. Patterns obtained for each strain with the 8 enzymes were combined resulting in 39 different combinations referred to as IGS rDNA types.

Table 1. *Bradyrhizobium* isolates used

Strains (LMG no.)	Host-plant	Geographic source
New isolates from Senegal		
15165	*Indigofera tinctoria*	West Senegal
15167	*Indigofera tinctoria*	North Senegal
15175	*Indigofera astragalina*	Central Senegal
15176	*Tephrosia purpurea*	North Senegal
15177	*Tephrosia purpurea*	North Senegal
15178	*Tephrosia purpurea*	North Senegal
15179	*Tephrosia purpurea*	North Senegal
15245	*Crotalaria hyssopifolia*	South Senegal (Casamance)
15249	*Crotalaria retusa*	South Senegal (Casamance)
15250	*Indigofera hirsuta*	South Senegal (Casamance)
15253	*Indigofera hirsuta*	Senegal
15255	*Alysicarpus glumaceus*	South Senegal (Casamance)
15258	*Bryaspis lupulina*	Senegal
15261	*Crotalaria glaucoïdes*	South Senegal (Casamance)
15263	*Sesbania rostrata*	Central Senegal
15267	*Moghania faginea*	South Senegal (Casamance)
15269	*Rhynchosia minima*	North Senegal
15275	*Indigofera senegalensis*	North Senegal
15276	*Indigofera senegalensis*	North Senegal
15279	*Indigofera senegalensis*	North Senegal
15304	*Indigofera astragalina*	Central Senegal
15365	*Abrus stictosperma*	South Senegal
15696	*Indigofera hirsuta*	South Senegal
15699	*Tephrosia bracteolata*	South Senegal
15700	*Indigofera stenophylla*	South Senegal
15702	*Tephrosia villosa*	South Senegal
Representative strains from the study of Dupuy *et al.*[a]		
Bradyrhizobium sp. (Faidherbia) strains		
10664	*Faidherbia albida*	West Senegal (Dakar)
10665	*Faidherbia albida*	West Senegal (Dakar)
10666	*Faidherbia albida*	North-West Senegal
10668	*Faidherbia albida*	North-West Senegal
10673	*Faidherbia albida*	North-West Senegal
10677	*Faidherbia albida*	North-West Senegal
10686	*Faidherbia albida*	North-West Senegal
10689	*Faidherbia albida*	North-West Senegal
10705	*Faidherbia albida*	South Senegal (Casamance)
10706	*Faidherbia albida*	South Senegal (Casamance)
10709	*Faidherbia albida*	South Senegal (Casamance)
10713	*Faidherbia albida*	South Senegal (Casamance)
10723	*Faidherbia albida*	North Senegal
10727	*Faidherbia albida*	North Senegal
Bradyrhizobium sp. (Aeschynomene) strains		
8069	*Aeschynomene elaphroxylon*	North Senegal
8295	*Aeschynomene afraspera*	North Senegal
8300	*Aeschynomene indica*	North Senegal
10298	*Aeschynomene afraspera*	Senegal
11795 BTAil	*Aeschynomene idica*	United States

(*Continued*)

Table 1. (*Continued*)

Strains (LMG no.)	Host-plant	Geographic source
	New isolates from Senegal	
12186	*Aeschynomene sensitiva*	South Senegal (Casamance)
12187	*Aeschynomene sensitiva*	South Senegal (Casamance)
15384	*Aeschynomene afraspera*	South Senegal (Casamance)
15385	*Aeschynomene afraspera*	South Senegal (Casamance)
	Reference strains	
Bradyrhizobium japonicum strains		
USDA 110	*Glycine max*	United States
4252	*Glycine max*	nd*
4262	*Albizia julibrissin*	nd
4265	*Ulex europeaus*	nd
4271	*Glycine max*	U.S.A
4272	*Pueraria lobata*	nd
6136	*Glycine max*	United States
6138^T (type strain)	*Glycine max*	Japan
8321	*Glycine max*	United States
Bradyrhizobium elkanii strains		
USDA 61		
6134^T (type strain)	*Glycine max*	United States
6135	*Glycine max*	United States
	Representative strains from the study of Moreira *et al.*[a]	
Bradyrhizobium sp. (Acacia) strains		
8888	*Acacia decurens*	Brazil
9959	*Acacia molissima*	Brazil
9966	*Acacia mangium*	Brazil
	Bradyrhizobium sp. (*Lupinus*) strains.	
MSDJ 718	*Lupinus luteus*	France

*LMG, Gulture Collection, Laboratorium voor Mikrobiologie, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium; MSDJ, Institut National de la Recherche Agronomique (INRA), Microbiologie des Sols, Dijon, France; USDA, U.S Department of Agriculture, Beltsville, Md, United States.

[a]See references; *nd, not determined.

At a correlation coefficient of about 70%, the different IGS rDNA types formed 10 clusters, four of which consisted of the majority of the strains studied. Isolates from small legumes had representatives in all the groups, except one group which only consisted of strains from *F. albida* and two groups, which only consisted of strains from *Aeschynomene*. PCR-RFLP groupings were related to the geographic origin of the strains.

3.2. Optimization of the AFLP Technique for *Bradyrhizobium* Strains

Several enzyme combinations were tested to detemine the most suitable one, that is the one producing a large number of fragments of many different lengths resulting in a well spread out banding pattern. For *Bradyrhizobium*, the combination of *Taq*I (T/CGA), and *Apa*I (GGGCC/C) proved the most useful.

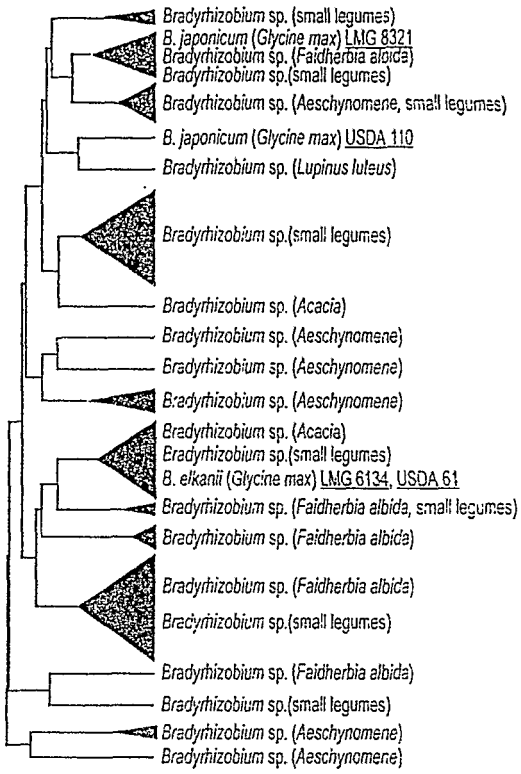

Figure 1. Dendrogram (UPGMA) of similarities between *IGS restriction patterns*. Clusters of strains were delineated above 70% similarity.

3.3. Numerical Analysis of *Bradyrhizobium* AFLP Patterns

The similarity of the AFLP patterns was calculated using the dice coefficient and the patterns were then grouped by UPGMA cluster analysis. Our analysis revealed considerable heterogeneity among the strains studied. The UPGMA dendrogram consisted of 19 groups of profiles with an internal similarity of a least 50% (Fig. 2).

Of these clusters, three contained only *Faidherbia albida* strains, 8 contained only small legume isolates, and 2 contained only *Aeschynomene* isolates. Two clusters consisted of strains from various host plants. The strains from *Acacia* had separate positions.

Reference strains grouped mostly in separate clusters. The *Bradyrhizobium japonicum* strains were recovered in two clusters, while *Bradyrhizobium elkanii* strains grouped in one cluster. Three *Bradyrhizobium japonicum* strains (LMG 4262, LMG 4265 and LMG 4272) had separate positions and one strain (LMG 8321) grouped together with *Faidherbia albida* and *Rynchosia minima* isolates.

3.4. Analysis of 16S rDNA Restriction Patterns

The analysis was performed on 11 strains representing the different IGS clusters. All the strains yielded a single band of about 1500 bp. The UPGMA dendrogram revealed heterogeneity among the strains studied and consisted of 3 groups with a similarity coefficient of about 82% (Fig. 3).

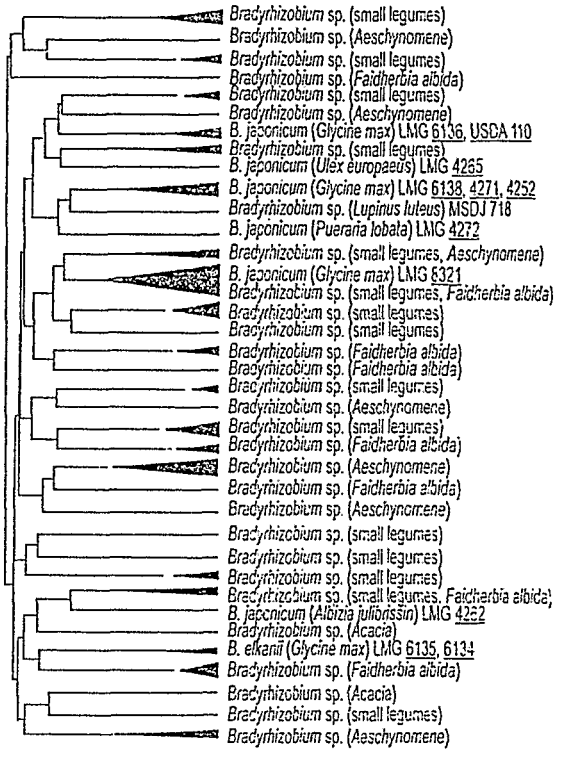

Figure 2. Dendrogram (UPGMA) of similarities between AFLP *patterns*. Clusters of strains were delineated above 50% similarity.

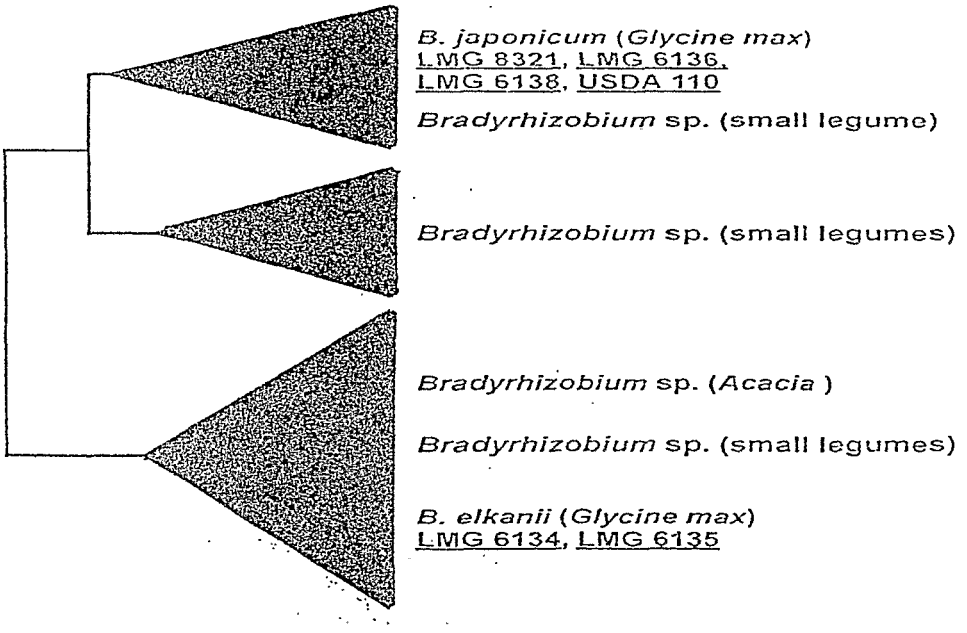

Figure 3. Dendrogram (UPGMA) of similarities between 16S restriction patterns. Clusters of strains were delineated above 80% similarity.

References strains of *Bradyrhizobium japonicum* grouped together with one strain from small legumes. The reference strains of *Bradyrhizobium elkanii* grouped together with a number of strains from small legumes. One cluster contained only small legume isolates.

4. DISCUSSION

From IGS PCR-RFLP and AFLP groupings, it is apparent that *Bradyrhizobium* isolates from small legumes exhibit a large diversity.

The photosynthetic strains from *Aeschynomene* are closely related (similarity coefficient of 75%) and form two separate clusters. This result is in agreement with previous studies showing that photosynthetic strains form a single phylogenetic group within *Bradyrhizobium* (Wong et al., 1994; So et al., 1994).

In the AFLP analysis, the reference strains grouped in several clusters. One cluster consisted only of three *Bradyrhizobium japonicum* strains, including the type strain which belongs to DNA homology group I of Hollis et al. (1981). One cluster consisted of 2 *Bradyrhizobium japonicum* strains, USDA 110, and LMG 6136. Surprisingly these strains belong to DNA homology group Ia and I, respectively (Hollis et al., 1981) and therefore these experiments should be repeated and the identity of the strains checked.

The fact that three *Bradyrhizobium japonicum* strains (LMG 4262, LMG 4265 and LMG 4272) occupied separate positions and one *Bradyrhizobium japonicum* strain (LMG 8321 = USDA 135) grouped together with 10 isolates from *Faidherbia albida* and one strain from *Rynchosia minima* seems to indicate that this species is indeed diverse.

16S ARDRA grouping confirms that isolates from small legumes belong to the *Bradyhizobium* phylogenetic branch where part of the small legume isolates form a separate cluster, distinct from other species.

In most cases, each AFLP group contains strains with the same or very similar IGS rDNA types* (Table 2). At present, the AFLP technique is the most discriminatory method to characterize bradyrhizobia. This result is not unexpected since this method studies the whole bacterial genome and has been reported to be a powerful taxonomic technique (Janssen et al., 1996).

The IGS PCR-RFLP seems to possess an intermediate discriminative power between the 16S ARDRA and AFLP technique. DNA : DNA hybridizations between representatives strains of selected genomic groups are planned.

New isolates from senegalese small legumes and reference strains of *Bradyrhizobium liaoningense* will be included in the 16S ARDRA, IGS PCR-RFLP, and AFLP databases.

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* A type is a specific combination of restriction patterns obtained with the eight enzymes.

Table 2. Comparison between clusters obtained by ARDRA16S, IGS PCR-RFLP, and AFLP analyses

ARDRA 16S groups	IGS PCR-RFLP groups	AFLP groups	IGS PCR-RFLP types
1	II	9	9–10–11–12–13
	SEP	11	14
2	III	10	16–17
		28	18
	IV	2	2–5
		25	4
		27	1
		43	3
		SEP	7
3	I	29	27
	VI	7	29
		36	32
		40	31
		SEP	30
	VII	39	36–35
	IX	5	20
		30	19
		31	19
		32	20–21
		33	22–23
		42	19
	SEP	SEP	25
nd*	V	14	41–42
nd	VIII	35	28
nd	X	41	37–38

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