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Original article

Evaluation of AFLP for the grouping of *Bradyrhizobium* strains

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Abstract – In recent years we have characterized 250 *Bradyrhizobium* strains, mainly from Senegal, using several taxonomic techniques *i.e.* numerical taxonomy of phenotypic features, Biolog system, SDS-PAGE of total cellular proteins, 16S rDNA RFLP and sequence analyses, 16S-23S rDNA intergenic gene spacer RFLP and sequence analyses, AFLP, DNA:DNA hybridizations. Here we evaluate the taxonomic resolving power of these techniques by comparing the results obtained on various subsets of the same strains. We conclude that AFLP is a useful method for an initial grouping of *Bradyrhizobium* strains and provides infraspecific information. However, from a limited comparison, it appears that the less labor-intensive 16S-23S rDNA intergenic gene spacer analysis gives similar results and may provide additional information on deeper groupings.

AFLP / *Bradyrhizobium* / polyphasic taxonomy / ribosomal operon analysis

Résumé – Évaluation de l'AFLP pour le groupage des souches de *Bradyrhizobium*. Au cours des dernières années, nous avons caractérisé 250 souches de *Bradyrhizobium*, principalement du Sénégal, par différentes techniques, *i.e.*, analyse numérique de caractères phénotypiques, analyse Biolog, SDS-PAGE des protéines cellulaires totales, RFLP et séquençage du gène codant pour l'ARN ribosomique 16S ou de l'espace intergénique entre les gènes ribosomiques 16S et 23S, AFLP et hybridations ADN:ADN. Nous évaluons ici le niveau de résolution taxonomique de ces différentes techniques par comparaison des résultats obtenus sur différentes sélections des mêmes souches. L'AFLP s'avère être une technique efficace pour le groupement

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initial des souches de *Bradyrhizobium* qui renseigne au niveau infraspécifique. Cependant l'analyse de l'intergène 16S-23S, moins lourd à mettre en œuvre, apparaît plus performant en permettant un groupement plus fin.

AFLP / *Bradyrhizobium* / taxonomie polyphasique / analyse de l'opéron ribosomique

1. INTRODUCTION

The genus *Bradyrhizobium* was created to accommodate the slow-growing rhizobia, bacteria capable of nitrogen fixation and nodule formation on certain leguminous plants [10]. Currently it contains three named species, *Bradyrhizobium japonicum* (type species), *Bradyrhizobium elkanii* and *Bradyrhizobium liaoningense*. However, many other *Bradyrhizobium* sp. strains with unresolved taxonomic status have been described worldwide from different legumes in recent years. Several authors have pointed out the lack of a suitable tool to assess relationships among bradyrhizobia [11, 17, 20].

In particular in Senegal, our research group reported on the diversity of *Bradyrhizobium* sp. strains from various *Aeschynomene* species [1, 12, 13] and *Faidherbia albida*, [4], and from 27 small legumes [2]. For initial grouping of the bradyrhizobial isolates, we first assessed various techniques such as numerical taxonomy of phenotypic features, the Biolog system, SDS-PAGE of total cellular proteins, 16S rDNA sequence and RFLP analysis. Each of these techniques had its drawbacks, limiting their use for the study of bradyrhizobia. Some were rather laborious, but the main problem was often that techniques were not sufficiently discriminative (16S-rDNA RFLP) or reproducible (Biolog, SDS-PAGE protein analysis). In a further attempt to identify a fast initial grouping method for *Bradyrhizobium*, we tested AFLP and 16S-23S rDNA spacer RFLP and sequencing. Results were then evaluated using extensive DNA:DNA hybridizations. These experiments proved that AFLP clusters as well as 16S-23S rDNA intergenic gene spacer groupings are correlated with genomic species [19]. Here we synthesize all the results obtained by this polyphasic approach, and define a valuable strategy for further study of *Bradyrhizobium* taxonomy.

2. MATERIALS AND METHODS

2.1. Bacterial strains

We performed AFLP analysis on a total of ca. 250 *Bradyrhizobium* strains, including 79 nodule isolates from 9 *Aeschynomene* species (*A. indica, A. sensitiva, A. afraspera, A. elaphroxylon, A. americana, A. uniflora, A. nilotica, A. schimperi, A. tambacoundensis*), 81 nodule isolates from *Faidherbia albida* [18] and 64 nodule isolates from 27 native leguminous plants species in

Senegal (West-Africa) belonging to the genera *Abrus, Alysicarpus, Bryaspis, Chamaecrista, Cassia, Crotalaria, Desmodium, Eriosema, Indigofera, Moghania, Rhynchosia, Sesbania, Tephrosia, Zornia* ([3]). For comparison, 14 reference strains from the three described *Bradyrhizobium* species and 10 representatives from previous studies [14, 17] were also included.

2.2. Culture medium

Strains were grown on yeast mannitol agar (YMA; pH, 6.8; composition in g per liter: mannitol, 10; sodium glutamate, 0.5; K_2HPO_4, 0.5; $MgSO_47H_2O$, 0.2; NaCl, 0.05; $CaCl_2$, 0.04; $FeCl_3$, 0.004; yeast extract, 1; agar, 20).

2.3. AFLP analysis

Cells were grown on plates (2 per strain) and DNA was extracted by the method of Pitcher *et al.* [16]. High resolution DNA fingerprints, or AFLP patterns, were prepared by selective amplification of restriction fragments obtained with the enzymes *Apa*I and *Taq*I. Briefly, the AFLP technique consists of the following steps: (1) 1 μg of total DNA is digested with two restriction enzymes, a tetracutter and a hexacutter; (2) short double-stranded, restriction-halfsite specific adapter molecules are ligated to the restriction fragments; (3) a selective PCR amplification of those fragments flanked by both a hexacutter and a tetracutter site is carried out by using oligonucleotide primers complementary to the adapters, but with one or more extra bases at their 3′ end; one of these primers is ^{32}P-labeled; (4) the products thus amplified are separated by acrylamide gel electrophoresis and the resulting banding pattern is revealed through autoradiography. Films are then scanned into a computer for further numerical analyses.

The procedures and conditions for preparation of template DNA, PCR reactions, electrophoresis, visualization of amplified fragments, data processing and analysis using the GelCompar software version 4.2 (Applied Maths, Kortrijk), using the Dice coefficient and the UPGMA clustering algorithm, were as described by Huys *et al.* [8]. The only modification to the procedure of Huys *et al.* [8] was that the PCR primers used for the amplification of restriction fragments in the present study carried different selective bases at the 3′ end. Several primer combinations with different selective bases were tested (data not shown) and the primers B07 (5'-GACTGCGTACAGGCCC**G**-3′) and T12 (5′-GATGAGTCCTGACCGA**A**-3′) were selected because they produced evenly distributed patterns of about 40 to 50 bands with the bradyrhizobia. For the numerical analysis, of a total of 1500 datapoints per AFLP pattern, positions 55 to 1432 were used in the calculations. *Bordetella holmesii* strain LMG 15945 was used as the reference strain because AFLP analysis of this strain generated a banding pattern displaying evenly distributed and well-separated bands over the entire length of the gel.

3. RESULTS AND DISCUSSION

We performed AFLP on 250 *Bradyrhizobium* strains and evaluate here the taxonomic resolving power of the technique by comparing the results with those obtained from other taxonomic techniques applied in recent years to various subsets of the same strains, *i.e.* numerical taxonomy of phenotypic features, Biolog system, SDS-PAGE of total cellular proteins, 16S rDNA RFLP and sequence analyses, 16S-23S rDNA spacer RFLP and sequence analyses, DNA:DNA hybridizations.

3.1. AFLP

3.1.1. Optimization of the AFLP technique for Bradyrhizobium *strains*

In initial tests several restriction enzymes (*Taq*I, *Mse*I, *Hind*III, *Apa*I and *Eco*RI) were compared. A hexacutter and a tetracutter were selected which produced a large number of fragments (30 to 50) of many different lengths so that, after electrophoresis, an evenly distributed banding pattern would be obtained. For *Bradyrhizobium*, having a high mol G+C% in their genome (61-65 mol% (T_m)), the combination of *Taq*I (T/CGA) and *Apa*I (GGGCC/C) proved the most useful. In the same way, four combinations of primers with different selective bases were tested, and primers 5′-GACTGCGTACAGGCCC**G**-3′ and 5′-GATGAGTCCTGACCGA**A**-3′ were retained (characters in bold indicate the selective bases). The reproducibility of the technique was good with 89.1±2.5% similarity obtained between 62 repeated runs of *Bordetella holmesii* LMG 15945, the reference strain used for normalization. In seven instances the same original strain was included twice under a different LMG number and such patterns grouped at 91 ± 7% (large standard deviation is caused by two cases grouping at 80%).

3.1.2. Numerical analysis

Considerable profile heterogeneity between strains was revealed among the 250 *Bradyrhizobium* strains studied [3,18]. After cluster analysis of the *Bradyrhizobium* AFLP patterns, 48 clusters (Fig. 1) were delineated at a level of 50% or more similarity (Pearson product moment correlation coefficient, expressed as a percentage). This cut-off level was chosen because similar or slightly lower levels have been used in studies of other bacterial groups (*Aeromonas*, 43% [8], *Acinetobacter*, 45% [9], *Vibrio*, 50% [15]).

Four AFLP clusters represented the known species: *Bradyrhizobium japonicum* strains were recovered in two clusters (12 and 15) grouping at 19.2% similarity, which correspond to the DNA homology groups Ia and I, respectively, of Hollis *et al.* [7]; *Bradyrhizobium elkanii* strains made up AFLP cluster

Figure 1. (see next pages). Dendrogram showing the grouping of AFLP patterns of *Bradyrhizobium* strains. Similarities were expressed as Pearson product moment correlation coefficients r, converted to percentage values, and clustering was performed by UPGMA analysis. Clusters are delineated at 50% similarity. In addition to strain numbers, the host plant is given and for strains belonging to named species, the species name is also listed.

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32 together with a nodule isolate from *Aeschynomene indica*; *Bradyrhizobium liaoningense* strains formed AFLP cluster 16.

Considerable diversity of *Bradyrhizobium* sp. strains isolated in Senegal from various host plants was demonstrated by the fact that they made up 44 separate clusters, distinct from the four clusters representing the known species. In addition, 50 strains occupied separate positions in the dendrogram. The majority of the AFLP clusters consisted of strains from Senegal originating either from *Faidherbia albida* (9 clusters) or from *Aeschynomene* species (12 clusters) or from small legumes (12 clusters). Comparison of the AFLP results with groupings obtained by other methods is only partially possible because not all strains have been studied with all methods.

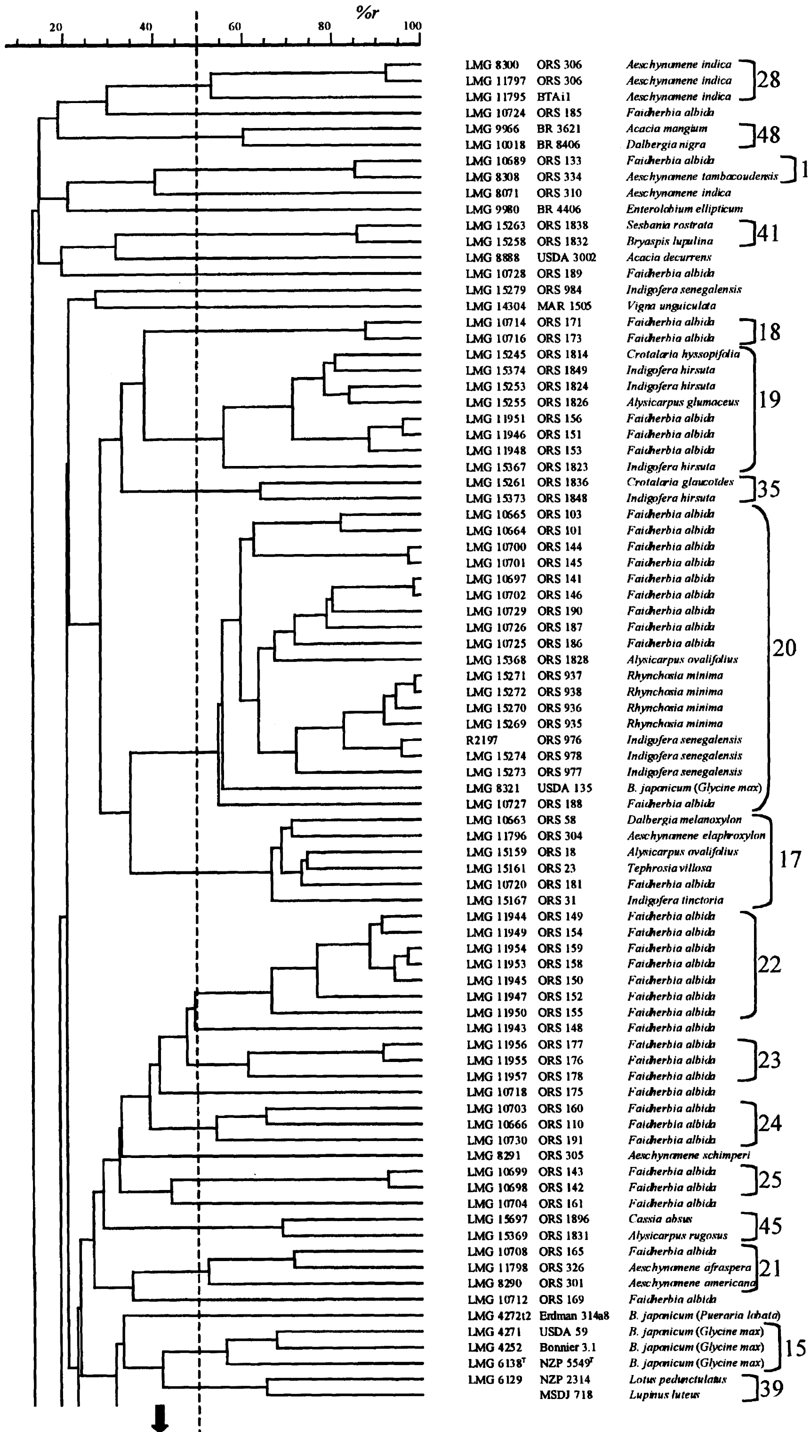
3.2. AFLP/SDS-PAGE comparison

Eighty-four *Faidherbia albida* and 64 small legume isolates were studied using SDS-PAGE protein electrophoresis together with 33 reference strains [2,4].

From the comparison of the AFLP cluster numbers with PAGE groups, AFLP appears to have a higher resolution than protein electrophoresis. Although a few AFLP clusters correspond to a single PAGE group each, several AFLP clusters contain strains of various PAGE groups. These discrepancies between the two sets of data may be due to the low reproducibility and fuzzy nature of protein profiles of bradyrhizobia which have been reported previously [2,4].

3.3. AFLP/Biolog comparison

Ninety *F. albida* isolates and 4 *Bradyrhizobium* reference strains were phenotypically characterized using the Biolog system with a modified protocol [4] and observations largely similar to those for comparison with SDS-PAGE can be made when comparing with the AFLP grouping. Each of the five main phenotypic groups [4] comprises several AFLP clusters. However, there are several AFLP clusters that contain strains belonging to different Biolog groups. Dupuy *et al.* [4] observed that the Biolog system provided few differentiating features for the five groups, because for most substrates the strains had different reactions, resulting in many "d" scores (10 to 90% positive). They called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21
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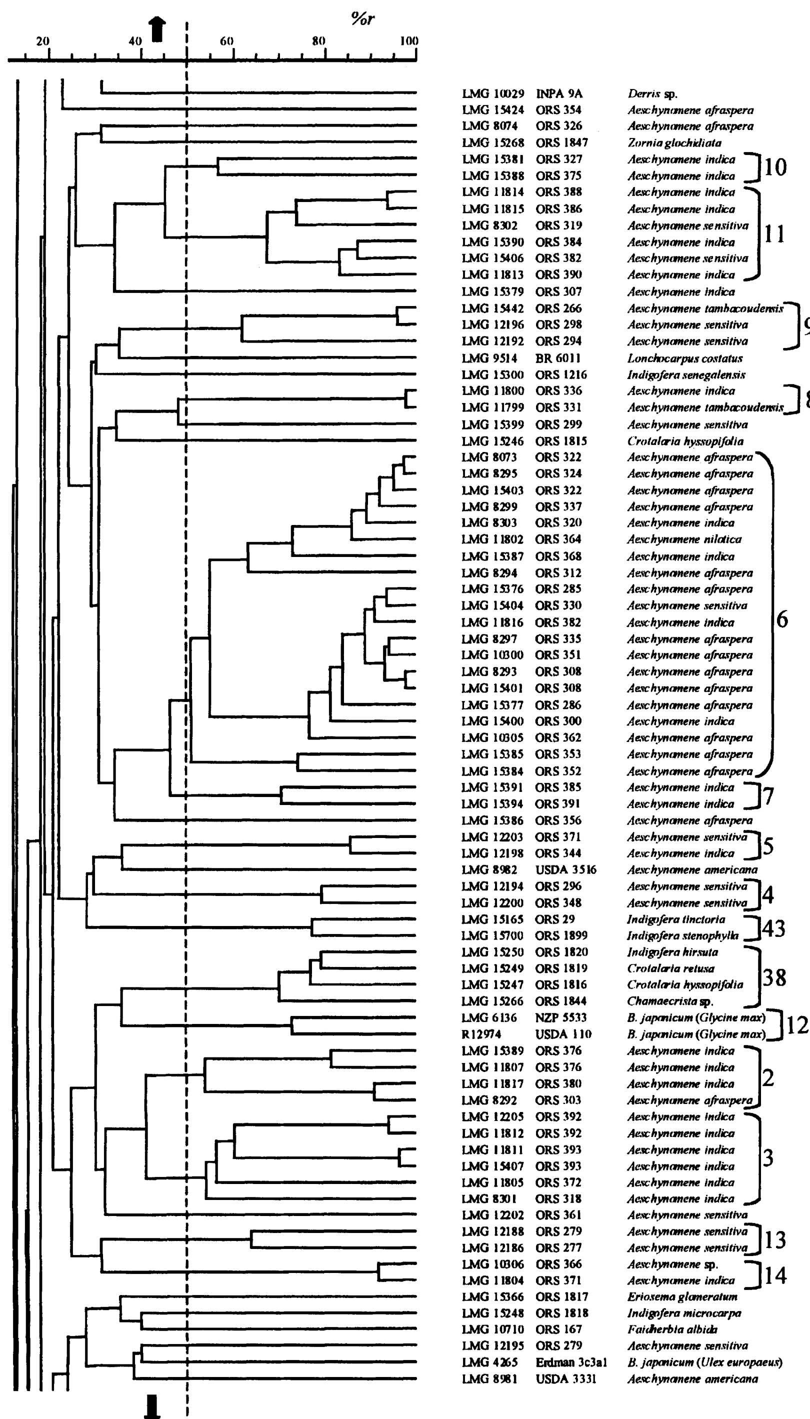
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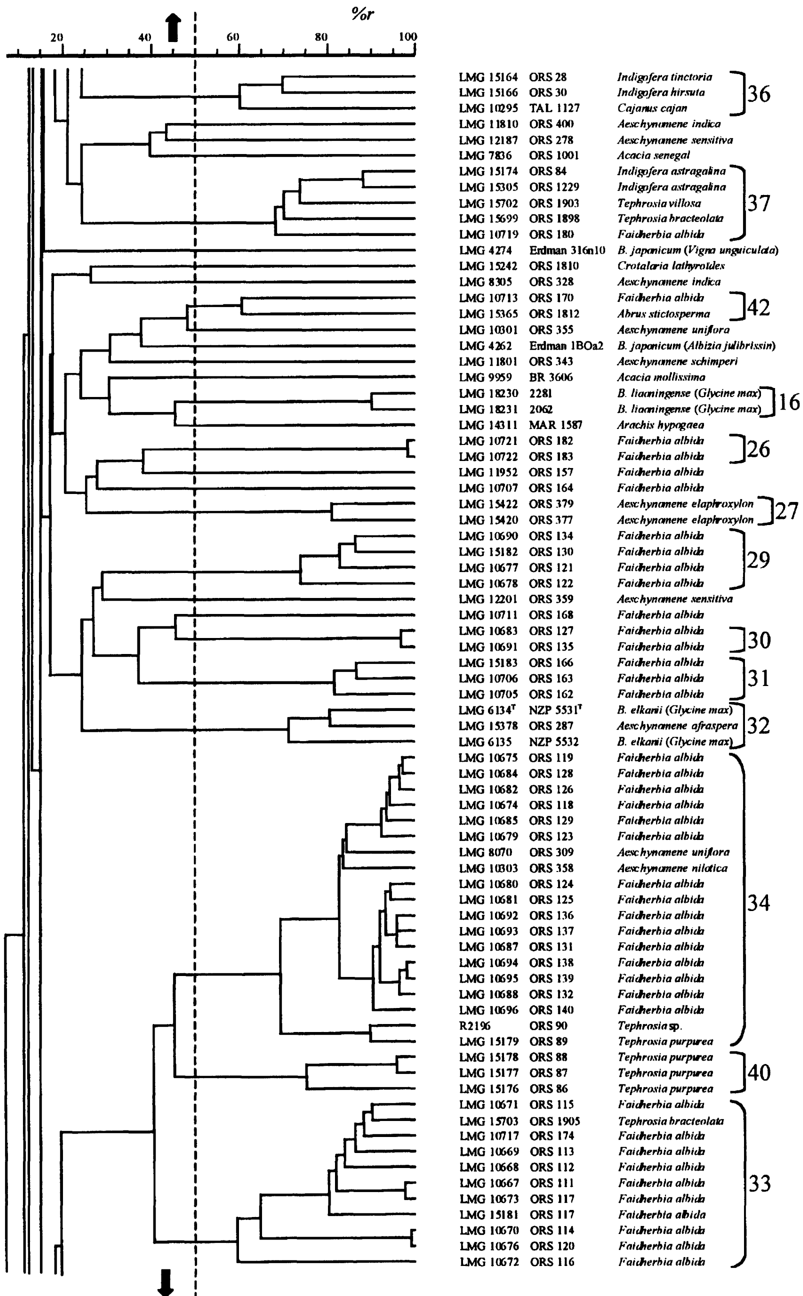
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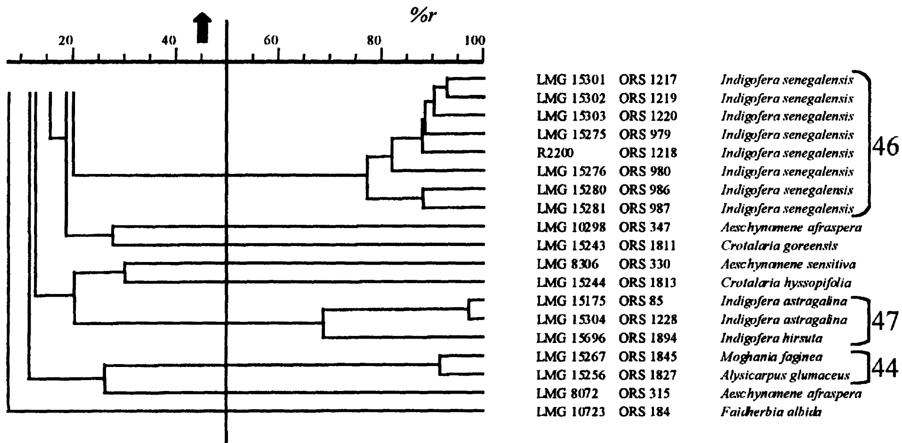

for genotypic data to assess the significance of the phenotypic groups. Our comparison with AFLP data demonstrates that, in general, the Biolog groups poorly reflect the genotypic groups.

3.4. AFLP/16S-ARDRA comparison

We amplified the gene coding for 16S rRNA (Fig. 2) of 36 *Aeschynomene*, 59 small legume and 9 *F. albida* isolates. The PCR products were compared to those of 11 reference strains by restriction analysis (16S-ARDRA) using five restriction enzymes [2, 13]. Cluster analysis of results delineates seven 16S-ARDRA groups each of them corresponding to several AFLP clusters, 27 in all [2, 18]. Groupings by both techniques are consistent in that each ARDRA group contained one or more different AFLP groups. As expected, our data show that AFLP, as a technique that samples the total genome, was more discriminative than 16S ARDRA, which is based on one conserved gene.

3.5. IGS PCR RFLP and sequencing/AFLP comparison

We amplified DNA corresponding to the 16S-23S rRNA intergenic gene spacer (Fig. 2) of 104 strains (nodule isolates from small legumes and reference strains). The PCR products were compared by Restriction Fragment Length Polymorphism using 8 restriction enzymes [3]. They produced 70 types of combined restriction profiles forming 16 groups. By AFLP, the strains formed 27 groups. In most cases agreement between results from the two techniques is very good and each AFLP cluster contains strains with the same or very similar rDNA IGS types and belonging to one IGS PCR-RFLP cluster.

Sequencing of the same spacer region was performed on a selection of strains from 9 AFLP clusters. Similarities ranged from 63 to 100%, and relatively little variation (94 to 100% sequence similarity) was observed within most of the

Figure 2. Scheme of part of the rRNA operon of *Bradyrhizobium.*

different AFLP clusters [19]. Although only a limited comparison is possible, correspondence between AFLP groupings and the grouping of spacer sequences was very good. However, the spacer sequence data provided additional detail on the deeper groupings of the AFLP clusters included [19].

3.6. AFLP/DNA:DNA hybridizations comparison

DNA:DNA hybridizations using a microplate method described by Ezaki *et al.* [5] were performed on 43 strains representative of nine of the AFLP clusters [19]. This method provides results comparable to those of the renaturation rate hybridization method [6]. By using the criterion of at least 60% hybridization within a genospecies, seven *Bradyrhizobium* genospecies were revealed, three of which correspond to the named species *B. japonicum*, *B. elkanii* and *B. liaoningense* [19]. Strains belonging to the same AFLP group belong to the same genospecies, but a genospecies may include several AFLP groups, with AFLP similarity levels between them sometimes as low as 20%. Therefore, under our experimental conditions, AFLP similarities within *Bradyrhizobium* define clusters at an infraspecific level. This is consistent with the general observation, made also in other bacterial groups, that AFLP analysis is a very fine typing technique that provides information at infraspecific to strain level.

4. CONCLUDING REMARKS

Previous studies of the diversity of *Bradyrhizobium* demonstrated that SDS-PAGE protein analysis is not very suitable for the study of bradyrhizobia because of the low reproducibility of the groupings [2,4]. In addition, numerical taxonomy and 16S ARDRA proved not very discriminative in this group [2,18]. In an effort to identify a reliable grouping technique for *Bradyrhizobium* strains, we carried out a large-scale AFLP analysis among these organisms. Our data demonstrated the reproducibility of this technique and resulted in the definition of 48 AFLP clusters [3,18]. Because the AFLP technique involves several steps and is quite laborious, we also looked at alternative techniques. IGS-RFLP analysis [3] and sequencing of the 16S-23S spacer region [19] were found to provide very similar groupings. In addition, the spacer sequence data provided information with regard to the relationships between different AFLP clusters [19]. DNA-DNA hybridizations carried out for a limited number of AFLP clusters, confirm the AFLP groupings [19].

At this stage, study of the 16S-23S rDNA spacer (PCR-RFLP, sequencing) and AFLP analysis proved to be very useful methods for an initial grouping of *Bradyrhizobium* strains, consistent with each other and with DNA hybridization values. However, AFLP analysis provides infraspecific information, whereas spacer analysis (PCR-RFLP and sequencing) on a subset of strains gave essentially similar groups to those found by AFLP [3, 19], but groupings deeper in the spacer sequence dendrogram were better resolved [19].

The genus *Bradyrhizobium* currently contains three species (*B. japonicum*, *B. elkanii* and *B. liaoningense*) and a large number of unnamed groups and strains that are referred to as *Bradyrhizobium* sp. Our results indicate that AFLP analysis and 16S-23S rDNA spacer analysis are useful tools for grouping unnamed strains. To establish whether such groups represent separate (geno)species, DNA-DNA hybridizations remain necessary. However, here the results from the initial grouping techniques, in particular the spacer sequence analysis, can reduce the number of hybridizations required. This polyphasic approach has been applied to a limited subset of *Bradyrhizobium* AFLP clusters and has so far identified at least 7 distinct genospecies (including the three named species) among these strains [19]. Additional phenotypic data will be required to permit formal description of the new groups.

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REFERENCES

[1] Alazard D., La nodulation caulinaire dans le genre *Aeschynomene*, Ph.D. thesis, University Claude Bernard-Lyon I, France, 1991, 176 p.

[2] Doignon-Bourcier F., Sy A., Willems A., Torck U., Dreyfus B., Gillis M., de Lajudie P., Diversity of bradyrhizobia from 27 tropical *Leguminosae* species native of Senegal, System. Appl. Microbiol. 22 (1999) 647–661.

[3] Doignon-Bourcier F., Willems A., Coopman R., Laguerre G., Gillis M., de Lajudie P., Genotypic characterization of *Bradyrhizobium* strains nodulating small Senegalese legumes by 16S-23S rRNA intergenic gene spacers and amplified fragment length polymorphism fingerprint analyses. Appl. Environ. Microbiol. 66 (2000) 3987–3997.

[4] Dupuy N., Willems A., Pot B., Dewettinck D., Vandenbruaene I., Maestrojuan G., Dreyfus B.L., Kersters K., Collins M.D., Gillis M., Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree *Acacia albida*, Int. J. Syst. Bacteriol. 44 (1994) 461–473.

[5] Ezaki T., Hashimoto Y., Yabuuchi E., Fluorometric deoxyribonucleic acid - deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains, Int. J. Syst. Bacteriol. 39 (1989) 224–229.

[6] Goris J., Suzuki K.-I., De Vos P., Nakase T., Kersters K., Evaluation of a microplate DNA-DNA hybridization method compared with the initial renaturation method, Can. J. Microbiol. 44 (1998) 1148–1153.

[7] Hollis A.B., Kloos W.E., Elkan B.E., DNA:DNA hybridization studies of *Rhizobium japonicum* and related *Rhizobiaceae*, J. Gen. Microbiol. 123 (1981) 215–222.

[8] Huys G., Kersters I., Coopman R., Janssen P., Kersters K., Genotypic diversity among *Aeromonas* isolates recovered from drinking water production plants as revealed by AFLP^{TM} analysis, System. Appl. Microbiol. 19 (1996) 428–435.

[9] Janssen P., Coopman R., Huys G., Swings J., Bleeker M., Vos P., Zabeau M., Kersters K., Evaluation of the DNA fingerprinting method AFLP(tm) as a new tool in bacterial taxonomy, Microbiol. 142 (1995) 1881–1893.

[10] Jordan D.C., Transfer of *Rhizobium japonicum* Buchanan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing root nodule bacteria from leguminous plants, Int. J. Syst. Bacteriol. 32 (1982) 136–139.

[11] Ladha J.K., So. R.B., Numerical taxonomy of photosynthetic rhizobia nodulating *Aeschynomene* species, Int. J. Syst. Bacteriol. 44 (1994) 62–73.

[12] Lorquin J., Molouba F., Dupuy N., N'diaye S., Alazard D., Gillis M., Dreyfus B.L., Diversity of photosynthetic *Bradyrhizobium* strains from stem nodules of *Aeschynomene* species, in: New Horizons in Nitrogen Fixation Palacios R., Mora J., Newton W. E. (Eds.), Dordrecht, Boston, Kluwer Academic Publishers, 1993, pp. 683–689.

[13] Molouba F., Lorquin J., Willems A., Hoste B., Giraud E., Dreyfus B., Gillis M., de Lajudie P., Masson-Boivin C., Photosynthetic bradyrhizobia from *Aeschynomene* spp. are specific to stem-nodulated species and form a separate 16S ribosomal DNA restriction fragment length polymorphism group, Appl. Environ. Microbiol. 65 (1999) 3084–3094.

[14] Moreira F.M.S., Gillis M., Pot B., Kersters K., Franco A.A., Characterization of rhizobia isolated from different divergence groups of tropical *Leguminosae* by comparative polyacrylamide gel electrophoresis, System. Appl. Microbiol. 16 (1993) 135–146.

[15] Pedersen K., Verdonck L., Austin B., Austin D. A., Blanch A. R., Grimont P.A.D., Jofre J., Koblavi S., Larsen J. L., Tiainen T., Vigneulle M., Swings

J., Taxonomic evidence that *Vibrio carchariae* Grimes *et al.* 1985 is a junior synonym of *Vibrio harveyi* (Johnson and Shunk 1936) Baumann *et al.* 1981, Int. J. Syst. Bacteriol. 48 (1998) 749–758.

[16] Pitcher D.G., Saunders N.A., Owen R.J., Rapid extraction of bacterial genomic DNA with guanidinium thiocyanate, Lett. Appl. Microbiol. 8 (1989) 151–156.

[17] Van Rossum D., Schuurmans F.P., Gillis M., Muyotcha A., Van Verseveld H.W., Stouthamer A.H., Boogerd F.C., Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots, Appl. Environ. Microbiol. 61 (1995) 1599-1609.

[18] Willems A., Doignon-Bourcier F., Coopman R., Hoste B., de Lajudie P., Gillis M., AFLP fingerprint analysis of *Bradyrhizobium* strains isolated from *Faidherbia albida* and *Aeschynomene* species, System. Appl. Microbiol. 23 (2000) 137–147.

[19] Willems A., Coopman R., Gillis M., Comparison of sequence analysis of 16S - 23S rDNA spacer regions, AFLP analysis, and DNA-DNA hybridizations in *Bradyrhizobium*, Int. J. Syst. Evol. Microbiol. 51 (2001) 623–632.

[20] Young J. P. W., Downer H. L., Eardley B. D., Phylogeny of the phototrophic rhizobium strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment, J. Bacteriol. 173 (1991) 2271–2277.