System. Appl. Microbiol. 25, 423–433 (2002)
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SYSTEMATIC AND
APPLIED MICROBIOLOGY

Analysis of Genomic Diversity Among Photosynthetic Stem-nodulating Rhizobial Strains from Northeast Argentina

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Received: May 24, 2002

Summary

The genomic diversity among photosynthetic rhizobia from northeast Argentina was assessed. Forty six isolates obtained from naturally occurring stem and root nodules of *Aeschynomene rudis* plants were analyzed by three molecular typing methods with different levels of taxonomic resolution: repetitive sequence-based PCR (rep-PCR) genomic fingerprinting with BOX and REP primers, amplified 16S rDNA restriction analysis (ARDRA), and 16S-23S rDNA intergenic spacer-restriction fragment length polymorphism (IGS-RFLP) analysis.
The in vivo absorption spectra of membranes of strains were similar in the near infrared region with peaks at 870 and 800 nm revealing the presence of light harvesting complex I, bacteriochlorophyll-binding polypeptides (LHI-Bchl complex). After extraction with acetone-methanol the spectra differed in the visible part displaying peaks belonging to canthaxanthin or spirilloxanthin as the main carotenoid complement. The genotypic characterization by rep-PCR revealed a high level of genomic diversity among the isolates and almost all the photosynthetic ones have identical ARDRA patterns and fell into one cluster different from *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii.* In the combined analysis of ARDRA and rep-PCR fingerprints, 7 clusters were found including most of the isolates. Five of those contained only photosynthetic isolates; all canthaxanthin-containing strains grouped in one cluster, most of the other photosynthetic isolates were grouped in a second large cluster, while the remaining three clusters contained a few strains. The other two clusters comprising reference strains of *B. japonicum* and *B. elkanii*, respectively. The IGS-RFLP analysis produced similar clustering for almost all the strains.
The 16S rRNA gene sequence of one representative isolate was determined and the DNA sequence analysis confirmed the position of photosynthetic rhizobia in a distinct phylogenetic group within the *Bradyrhizobium* rDNA cluster.

Key words: Photosynthetic rhizobia – *Aeschynomene* – genomic fingerprinting – carotenoids – diversity

Introduction

Although rhizobia interact with most leguminous plants forming nitrogen-fixing nodules in the roots, some of them are able to nodulate stems of a few legumes, including *Aeschynomene* species (ALAZARD, 1985; LADHA et al., 1990; BOIVIN et al., 1997). A number of nodule isolates from these stem-nodulating legumes have the ability to form a photosynthetic apparatus detected through the presence of bacteriochlorophyll *a* (Bchl *a*) and carotenoids (EAGLESHAM et al., 1990; LADHA and SO, 1994; VAN BERKUM et al., 1995; MOLOUBA et al., 1999). The first and the best studied example is strain BTAi1 isolated from *Aeschynomene indica* stem nodules (EAGLESHAM and SLAZAY, 1983; EAGLESHAM et al., 1990). This strain was able to synthesize photosynthetic pigments growing heterotrophically and aerobically under regular light-dark cycles and phylogenetic analysis of that strain showed it to belong to the *Bradyrhizobium*/*Rhodopseudomonas* 16S rRNA cluster (YOUNG et al., 1991; WONG et al., 1994).

Since the first report of a photosynthetic rhizobium, other studies were carried out in different collections of *Aeschynomene* spp. isolates, mainly to examine their characteristics, diversity and also to determine their taxonomic status. A numerical taxonomy study, placed the photosynthetic rhizobia in one phenon distinct from *Bradyrhizobium* (LADHA and SO, 1994). However, phylo-

0723-2020/02/25/03-423 $ 15.00/0

genetic analysis of the same isolates based on 16S rDNA and fatty acids methyl esters data, indicated that they formed a separate subcluster in the *Bradyrhizobium* cluster (SO et al., 1994). Lately, the diversity of *Aeschynomene* spp. isolates originating from West Africa was assessed (MOLOUBA et al., 1999; WILLEMS et al., 2000) indicating that photosynthetic bradyrhizobia constituted a separate subbranch on the *Bradyrhizobium* 16S rRNA lineage, distinct from *Bradyrhizobium elkanii* and *B. japonicum*. Furthermore, the relationship between symbiotic properties and pigment synthesis was also investigated, suggesting that they were correlated. Among *Aeschynomene* species, three cross-inoculation groups were defined (ALAZARD, 1985) and the photosynthetic strains were exclusively found in groups 2 and 3. On the basis of their pigment accumulation under light-dark aerobic growth conditions, the photosynthetic rhizobia constitute different pigmentation groups that produced spirilloxanthin as the sole carotenoid or canthaxanthin as the major pigment (LORQUIN et al., 1997).

Recent information has shown that the photosynthetic bradyrhizobia belonged to at least two different genospecies among the 11 *Bradyrhizobium* genospecies recognized by DNA-DNA hybridizations, a major criterion for species delineation (WILLEMS et al., 2001 c). Alternative simple and reliable approaches to the classification of bacteria are the PCR-based methods such as repetitive sequence-based PCR (rep-PCR) (VERSALOVIC et al., 1994), amplified ribosomal DNA restriction analysis (ARDRA) (LAGUERRE et al., 1994; HEYNDRICKX et al., 1996) and 16S–23S rDNA intergenic spacer-restriction fragment length polymorphism (IGS-RFLP) analysis (LAGUERRE et al., 1996). Moreover, the combined analysis of rep-PCR genomic fingerprints and 16S rDNA restriction patterns allowed the illustration of the phylogenetic relationship among bradyrhizobia with a dynamic range from the genus to the strain level (VINUESA et al., 1998).

The genera of known stem-nodulating legumes are present in Argentina (ZULOAGA and MORRONE, 1999) but no study has been performed yet on the diversity of stem-nodulating bacteria or photosynthetic rhizobia. The purpose of this study was to characterize the rhizobia associated with *Aeschynomene* plants in northeast Argentina and to evaluate their genotypic diversity and phylogenetic relationship. With this aim rep-PCR fingerprinting, ARDRA, and IGS-RFLP analysis have been used. To confirm their phylogenetic position we determined the sequence of 16S rDNA for one representative isolate.

Materials and Methods

Strain isolation

Aeschynomene rudis plants were collected in northeast Argentina (provinces of Formosa and Chaco), and the bacteria were isolated from both stem and root nodules. The external surfaces of the nodules were sterilized and isolation of strains was carried out by classical procedures in a yeast-mannitol (YM) medium at 28 °C (SOMASEGARAN and HOBEN, 1994). All the strains were maintained on YM medium. Table 1 shows the listing of our isolates describing some of their phenotypic characteristics and origin. The reference strains used are listed in Table 2.

The isolates were tested for their ability to nodulate *Aeschynomene rudis*, *Aeschynomene indica* and *Aeschynomene americana*. The nodulation assays were carried out as described by ALAZARD (1985).

Absorption spectra

Isolates were grown in modified glutamate (MG) broth (VAN BERKUM et al., 1995), at 28 °C for 7 days in aerobiosis with light/dark cycles of 16/8 h or, alternatively under continuous dark or light growth conditions, also aerobically.

Extraction of whole cells with acetone-methanol 7:2 (vol/vol) allowed registration of the absorption characteristics of extracted pigments. Alternatively, the cells were centrifuged, washed once in 10 mM Tris-HCl buffer pH 8.0, and broken in a Branson sonifier at maximum output in 2 cycles of 15 s sonication at low temperature interspaced by 30 s in cold. The cell debris were eliminated after centrifugation at 2000 × *g* during 20 min, and the supernatant was centrifuged at 100,000 × *g* for 1 h after layering onto a 10 ml of 50% sucrose in Tris-HCl 10 mM pH 8.0. Two distinct fractions were produced; a pelleted fraction which was denominated H (heavy) and a Bchl-containing membrane fraction banding approximately at 36 to 38% sucrose (L or light membrane). Both fractions were recovered and, after appropriate dilutions, the corresponding spectra were registered in the range 900 nm to 350 nm in a Shimadzu UV 1601 spectrophotometer.

DNA preparation

Total genomic DNA of the bacteria was prepared by a cetyltrimethylammonium bromide protocol (AUSUBEL et al., 1994). Purified DNA was adjusted to a concentration of 50 ng/μl. All DNA preparations were stored at 4 °C.

Rep-PCR genomic fingerprinting.

Total DNA amplification fingerprints were performed with BOXA1R and REP (REP1R-I and REP2-I) primers, as previously described by VERSALOVIC et al. (1994) except that 5% glycerol instead of BSA, 5mM $MgCl_2$ and 50 ng of template DNA were used. Amplification was carried out in an MJ Research PTC-100 thermocycler with the following temperature program: an initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 1 min (BOX) or at 40 °C for 2 min (REP) and extension at 65 °C for 8 min, and a final extension at 65 °C for 16 min. Six μl of the PCR products were loaded onto 24 cm-long 1.5% agarose gels and run at room temperature in TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.0) at 5V/cm for 4.6 h. In each gel a 100 bp DNA ladder and lambda DNA *Hind*III markers (Promega Corp.) were run at both sides and central lane as references. Gels were stained with ethidium bromide (0.6 μg/ml), and photographed with a Polaroid type 667 film.

ARDRA

In these experiments primers fD1 and rD1 were used to amplify nearly full length 16S rDNA genes (WEISBURG et al., 1991). The reactions were carried out in a total volume of 50 μl containing 50 ng of DNA, 5% dimethyl sulfoxide, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of each primer, 1.25U of Taq polymerase (Promega Corp.) and the buffer provided with the enzyme. The following program was routinely performed: initial denaturation for 5 min at 95 °C followed by 35 cycles of denaturation (94 °C, 1 min), annealing (57 °C, 40 s) and extension (72 °C, 2 min) and a final extension at 72 °C for 8 min. PCR products were purified with QIAquik PCR purification columns (Qiagen, Germany) and aliquots (10 μl) were digested with 5U of the restriction enzymes *Dde*I, *Hha*I, *Hinf*I and *Msp*I (Promega Corp.) following the recommendations of the manufacturer.

Table 1. Summary of some phenotypic characteristics and results of fingerprinting clustering of 46 *Aeschynomene rudis* rhizobial isolates.

Strain [a]	Geographical origin [b]	Bacteriochlorophyll synthesis		Pigmentation [c]	BOX-PCR [d]	ARDRA	Combined ARDRA and rep-PCR fingerprint [d]	IGS-RFLP analysis [d]
		Under light/ dark cycles	Under continuous darkness					
C1R1	Cañada Doce	+	+	O	VI	II	IV	III
C1T1	Cañada Doce	+	+	DP	VI	II	IV	III
C1T2	Cañada Doce	+	–	LP	I	II	V	II
C1T3	Cañada Doce	+	–	LP	I	II	V	II
C1T4	Cañada Doce	+	–	LP	III	II	V	II
C2T1	Cañada Doce	+	–	LP	I	II	V	II
C2T2	Cañada Doce	+	–	LP	II	II	V	II
C3R1	Cañada Doce	+	–	LP	I	II	V	II
C3T1	Cañada Doce	+	–	LP	I	II	V	II
C4T2	Cañada Doce	+	–	LP	II	II	V	Sep
C4T3	Cañada Doce	+	+	LP	Sep	II	III	I
C5T1	Cañada Doce	+	–	LP	IV	II	V	II
C6T1	Cañada Doce	+	+	O	VI	II	IV	III
C6T2	Cañada Doce	+	+	O	VI	I	IV	III
C6T3	Cañada Doce	+	–	LP	I	II	V	II
C6T4	Cañada Doce	+	–	LP	Sep	II	V	II
C6T5	Cañada Doce	+	–	LP	III	II	V	II
C6T6	Cañada Doce	+	–	LP	I	II	V	II
C6T7	Cañada Doce	+	–	LP	I	II	V	II
C7R1	Cañada Doce	+	+	LP	Sep	II	III	I
C7T1	Cañada Doce	+	+	O	VI	II	IV	III
C8T1	Cañada Doce	+	–	LP	Sep	II	VII	II
C8T3	Cañada Doce	+	–	LP	Sep	II	VII	II
C10T3	Cañada Doce	+	+	LP	I	II	V	II
C11T2	Cañada Doce	+	–	LP	Sep	II	Sep	Sep
C12T2	Cañada Doce	+	+	O	VI	II	IV	III
V1T1	Mayor Villafañe	+	–	LP	V	II	V	II
V1T2	Mayor Villafañe	+	–	LP	III	II	V	II
V1T3	Mayor Villafañe	+	–	LP	III	II	V	II
V1T4	Mayor Villafañe	+	–	LP	III	II	V	II
V1T5	Mayor Villafañe	+	–	LP	I	II	V	II
V2T1	Mayor Villafañe	+	–	LP	III	II	V	I
V2T2	Mayor Villafañe	+	–	LP	IV	III	VI	II
V3T1	Mayor Villafañe	+	–	LP	Sep	II	VI	II
V3T2	Mayor Villafañe	+	–	LP	IV	II	V	II
G1T1	Guaycolec	–	–	W	Sep	I	Sep	Sep
G1T2	Guaycolec	–	–	W	Sep	I	Sep	Sep
G2T1	Guaycolec	+	–	LP	I	II	V	II
G3T1	Guaycolec	+	–	LP	Sep	II	VI	II
G4T2	Guaycolec	+	–	LP	I	II	V	II
G4T3	Guaycolec	+	–	LP	I	II	V	II
G5T1	Guaycolec	+	+	O	VI	II	IV	III
G6T1	Guaycolec	+	–	LP	IV	II	V	II
G6T2	Guaycolec	+	–	LP	II	II	VI	II
CV1T1	Colonia Varela	+	–	LP	II	II	V	II
CV1T2	Colonia Varela	+	–	LP	V	II	VI	II

[a] The strain designation of isolates refers to their geographical origin, the plant identification and whether they were isolated from stem (T) or root (R) nodule.
[b] Cañada Doce, Mayor Villafañe and Guaycolec are places from the province of Formosa and Colonia Varela from Chaco, Argentina.
[c] O: orange, DP: dark pink; LP: light pink; W: white.
[d] Sep: separate

The digests were run in 2% MetaPhor agarose gels (FMC Bioproducts) in TBE buffer using 10 cm long gels and at 5V/cm for 2.5 h. As size control, a 100 bp DNA ladder (Promega Corp.) was included in the central lane and both sides of each gel.

RFLP analysis of 16S–23S rDNA intergenic spacer region

To amplify the 16S-23S rDNA IGS region, primers FGPS1490 and FGPS132' were used (LAGUERRE et al., 1996). The PCR conditions were the same as those used for 16S rDNA amplification except that annealing temperature was 55 °C and 30 cycles were used. The PCR products were digested with the restriction enzymes *Dde*I, *Hae*III, *Hinf*I and *Msp*I (Promega Corp.) and analyzed as described for ARDRA.

Analysis of patterns

The digitalized images of the gels were normalized with the molecular size markers used as references and analyzed using the GelCompar *II* software (Applied Maths, Kortrijk, Belgium) as described by the manufacturer. The fingerprints obtained by BOX- and REP-PCR were separately analyzed and combined linearly as a single data set. The degree of similarity of fingerprints was determined using the Pearson product-moment correlation coefficient (r), one of the most reliable tools to compare DNA fingerprints profiles (HÄNE et al., 1993), and the dendrograms were obtained using the UPGMA (unweighted pair group method using arithmetic averages) algorithm (SNEATH and SOKAL, 1973).

The 4 restriction patterns of the 16S rDNA, as well as those of the 16S-23S rDNA IGS region, were linearly combined and compared by using the Dice similarity coefficient (S_D) to construct the similarity matrix, and the dendrograms were obtained by using the UPGMA. In order to integrate the phylogenetic information provided by rep-PCR genomic fingerprints and 16S rDNA restriction patterns, they were linearly combined and analyzed by using r and UPGMA.

Analysis of the 16S rDNA

The determination of 16S rRNA gene sequence of strain C7T1 was performed by MIDI Labs (Newark, USA). Sequences of related organisms in the α subclass of the *Proteobacteria* were aligned using the program ClustalX (THOMPSON et al., 1997). A continuous stretch of 1,388 aligned base positions including gaps, was used for further phylogenetic analysis. A phylogenetic tree was constructed by the neighbor-joining method, from distance matrix obtained using the Kimura-2 parameter model, and a bootstrap confidence analysis was performed on 1,000 replicates, using the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE from Phylogeny inference package version 3.57c (FELSENSTEIN, 1995), and the tree was displayed using TreeView (PAGE et al., 1996).

Nucleotide sequence accession number

The 16S rDNA sequence determined for strain C7T1 was submitted to the GeneBank database under accession number AF387321.

Results

Photosynthetic pigment production

We have analyzed 46 isolates mainly from stem and some from root nodules of *Aeschynomene rudis*. This leguminous plant is one out of 12 *Aeschynomene* species that were previously described for the Argentinean territory (ZULOAGA and MORRONE, 1999). All the isolates were able to nodulate their original host and *A. indica* (cross-inoculation group 3) but no *A. americana* plants (cross-inoculation group 1).

Table 2. Reference strains used and summary of fingerprinting results.

Strain	Original host plant	Source [a]	BOX-PCR [b]	ARDRA [b]	Combined ARDRA and rep-PCR fingerprint [b]	IGS-RFLP analysis [b]
Bradyrhizobium sp. BTAi1	*Aeschynomene indica*	ATCC	Sep	I	Sep	Sep
Bradyrhizobium japonicum DSM 30131^T	*Glycine max*	DSMZ	VII	I	II	Sep
Bradyrhizobium japonicum USDA 110	*Glycine max*	IMYZA	Sep	I	Sep	IV
Bradyrhizobium japonicum USDA 138	*Glycine max*	IMYZA	VII	I	II	ND
Bradyrhizobium elkanii ATCC 49852^T	*Glycine max*	ATCC	VIII	III	I	Sep
Bradyrhizobium elkanii SEMIA 5019	*Glycine max*	IMYZA	VIII	III	I	ND
Bradyrhizobium sp. CB 756	*Macrotyloma africanum*	IMYZA	Sep	I	Sep	IV
Azorhizobium caulinodans ATCC 43989^T	*Sesbania rostrata*	ATCC	Sep	Sep	Sep	Sep

[a] ATCC: American Type Culture Collection (USA); DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Germany); IMYZA: Instituto de Microbiología y Zoología Agrícola (Argentina).
[b] Sep: separate; ND: not determined

Except strains G1T1 and G1T2, all the isolates growing on YM or MG agar plates produced pink or orange colonies after incubation under light/dark regimes and some produced also pigmented colonies after growth in darkness (Table 1), suggesting the presence of photosynthetic pigments. This was later confirmed by measuring the absorption spectra of the acetone-methanol extracts of whole cells or in purified membranes. Figure 1 shows two types of spectra indicating the presence of different carotenoid pigments. Some of the isolates produced canthaxanthin as the main carotenoid pigment (type 1, orange and dark pink strains) and the rest produced spirilloxanthin (type 2, light pink strains) (Fig. 1, a and b, respectively). The in vivo absorption spectrum shows the presence of two absorption peaks centered at 870 and 800 nm characteristic of the light harvesting complex I, Bchl-binding polypeptides. (LHI-Bchl complex) (Fig. 1c). In acetone-methanol the peak shifted to 770 nm confirming the presence of mainly Bchl *a*. Upon cell breakage and sucrose gradient centrifugation, two major membrane fractions were obtained: a heavy (H) and a lighter (L) fraction. Although both contained photosynthetic pigments, the H fraction of type 1 strains was significantly enriched in canthaxanthin. The lighter fraction was enriched in spirilloxanthin and also in Bchl and the canthaxanthin was no longer detectable (Fig. 1d). No photosynthetic growth (anaerobic-light conditions) was observed for any of the strains isolated. Moreover under continuous light in aerobiosis, we observed growth and carotenoids freely accumulated in all orange and dark pink strains, while no Bchl was accumulated (Fig. 1e).

Genomic fingerprinting by rep-PCR

By means of rep-PCR an identification of isolates was carried out and the intraspecific diversity was investigated, since this procedure allows a significantly high level of taxonomic resolution. A similarity value (*r*) of approximately 0.85 was the lowest obtained when repetitively comparing the combined rep-PCR fingerprints of the same strain against each other generated in independent PCR and electrophoresis experiments. This reproducibility is consistent with those from other studies (VINUESA et al., 1998). The individual and combined analysis of BOX- and REP-PCR patterns of isolates revealed a high genomic diversity. Figure 2 shows the dendrogram resulting from the cluster analysis of BOX-PCR fingerprints. By choosing a level of similarity of 60%, 8 clusters containing about 80% of all isolates were produced. Clusters I to V consisted of only photosynthetic isolates and all canthaxanthin-containing strains grouped in cluster VI. The reference strains of *B. japonicum* used conformed cluster VII except *B. japonicum* USDA 110 strain that occupied a separate position, and *B. elkanii* strains grouped in cluster VIII. The cluster analysis of combined BOX- and REP-PCR fingerprints produced groups defined at lower level of similarity (data not shown).

ARDRA

We have carried out a computer-simulated RFLP analysis of published 16S rDNA sequences of the reference strains used. For all restriction enzymes used, the experi-

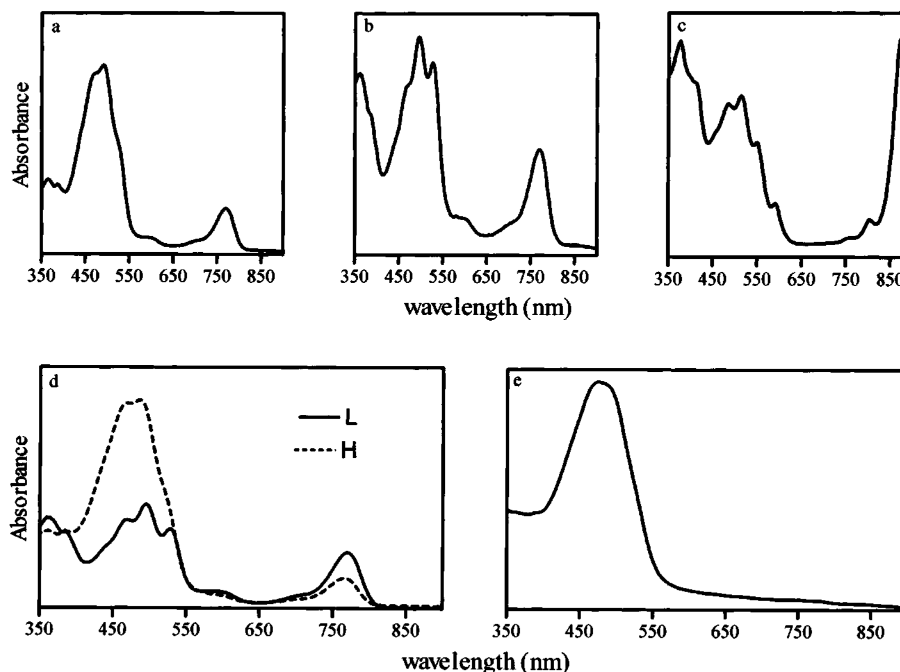

Fig. 1. In vivo and in vitro (extracted with acetone-methanol 7:2 vol/vol) absorption spectra of photosynthetic rhizobial isolates.
(a) Extracted cells of an orange G5T1 strain resulting in a so called type 1 spectrum and (b) from a light pink C4T2 strain; type 2 spectrum.
(c) Light membrane of C7T1 strain in 10 mM Tris-HCl buffer pH 8.0.
(d) Heavy (H) and light (L) membranes obtained from strain C7T1 after cell breakage and sucrose gradient centrifugation measured after extraction.
(e) Extracted cells of C7T1 strain grown under continuous illumination and in aerobiosis.

mentally observed restriction fragments matched with the theoretically expected fragments within the resolution range of agarose gels used.

All the strains yielded single amplification products of expected size (about 1,500 bp) for the 16S rRNA genes and the majority of the isolates had identical restriction profiles (data not shown). Cluster analysis of combined restriction patterns revealed three distinct bradyrhizobia clusters obtained at 90% similarity level. With the exception of strains C6T2 and V2T2, all photosynthetic isolates formed a coherent cluster (cluster II, S_D = 94%), related but distinct from the clusters that contained reference strains of *B. japonicum* (cluster I) and *B. elkanii* (cluster III), at S_D values of 88% and 82% respectively. Cluster I also contained both nonphotosynthetic isolates (G1T1 and G1T2), *Bradyrhizobium* sp. CB 756 and BTAi1 strains and one photosynthetic isolate (C6T2) (data not shown).

Combined analysis of rep-PCR and ARDRA data

The dendrogram resulting from the combination of ARDRA and rep-PCR fingerprinting data is shown in Figure 3. It is possible to observe that at 60% similarity level, 7 clusters were defined including most of our isolates. *B. elkanii* and *B. japonicum* reference strains formed clusters I and II respectively, except USDA 110 strain that occupied a separate position in the dendrogram. Cluster III grouped two strains that produced photosynthetic pigments in continuous darkness (C4T3 and C7R1) and cluster IV comprised all isolates containing canthaxanthin as the main carotenoid pigment. Cluster V comprised nearly 60% of all isolates and they were all light-pink photosynthetic strains. Cluster VI comprised five strains and cluster VII consisted of two isolates from the same plant. Only few isolates were not grouped at the level chosen for clustering; the two non-photosynthetic ones (G1T1 and G1T2) and C11T2.

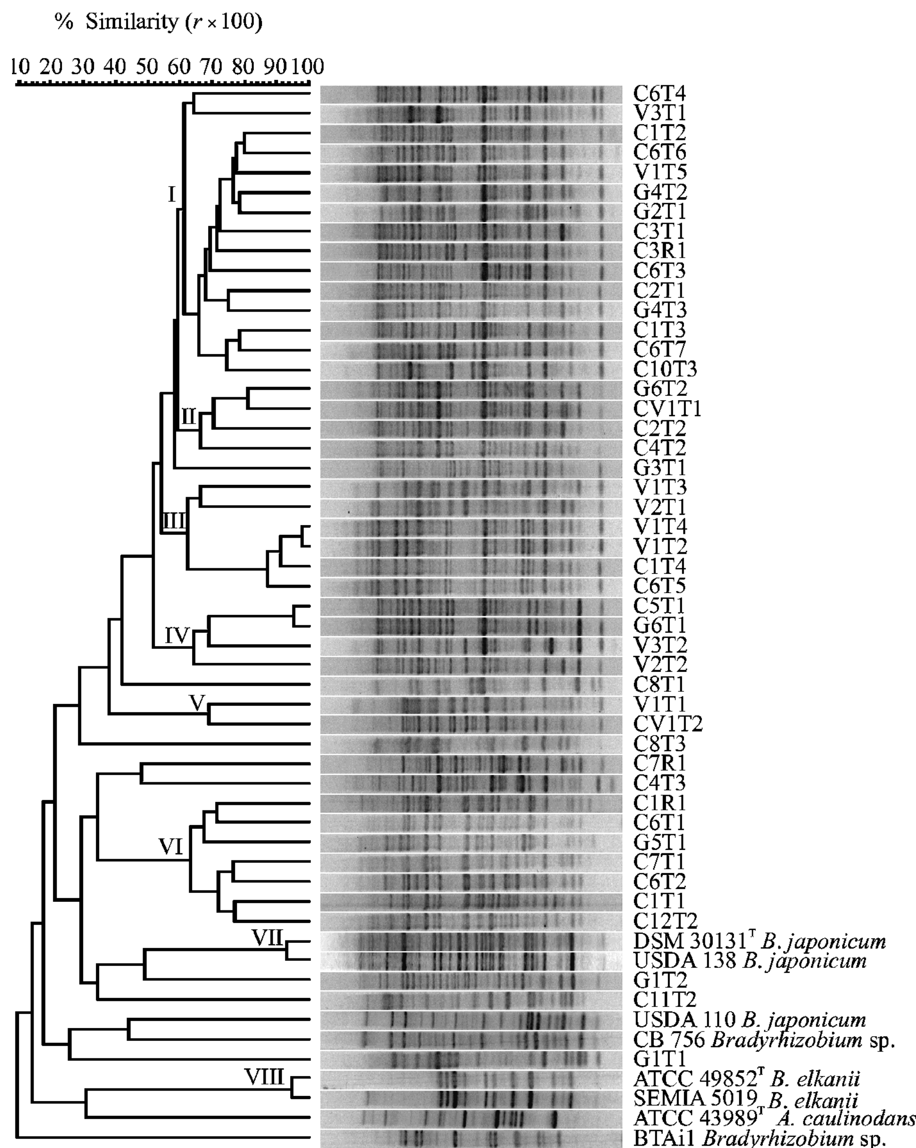

Fig. 2. Cluster analysis of BOX-PCR genomic fingerprints of isolates and reference strains using the Pearson product-moment correlation coefficient (r) and UPGMA method. The cophenetic correlation value for this dendrogram was 0.92.

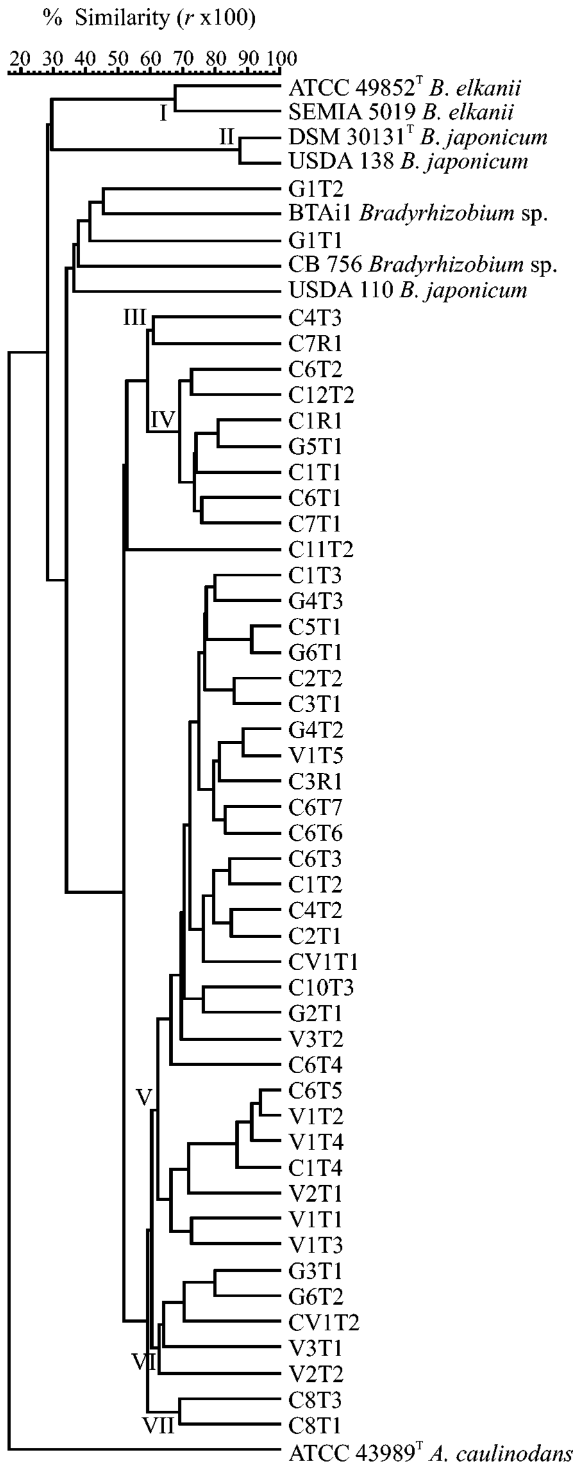

Fig. 3. Cluster analysis of linearly combined BOX and REP-PCR genomic fingerprints and 16S rDNA restriction patterns from isolates and reference strains by using the Pearson product-moment correlation coefficient (r) and UPGMA method. The clusters were delineated at above 60% similarity. The cophenetic correlation for this dendrogram was 0.93.

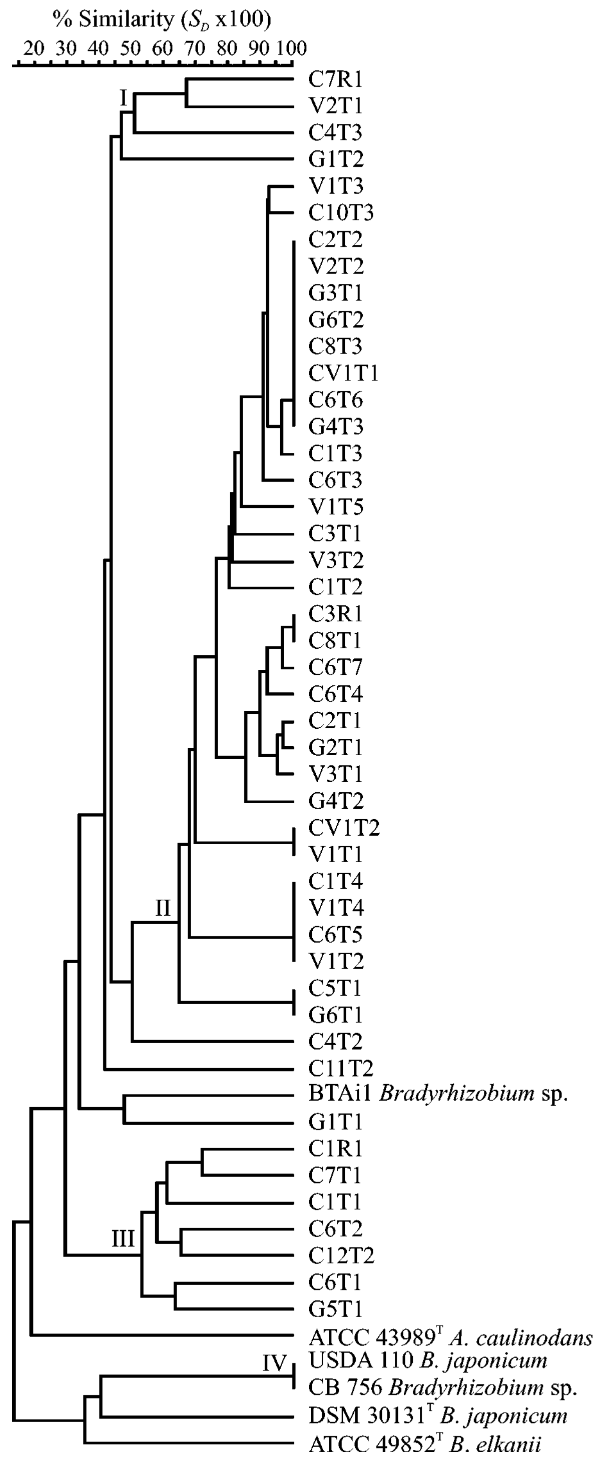

Fig. 4. Dendrogram based on cluster analysis of normalized combined 16S-23S rDNA IGS restriction patterns of isolates and reference strains using UPGMA and the Dice coefficient (S_D). Cophenetic correlation was 0.97.

16S–23S rDNA IGS-RFLP analysis

Although most isolates and reference strains produced a single amplification product ranging from about 945 bp to 1300 bp, some isolates gave repeatedly two different-length PCR products. These bands were closely spaced and indicated that several rRNA operons occur in these strains. The length of IGS amplified region of strains agrees with other reports (VINUESA et al., 1999; WILLEMS et al., 2001a). The majority of the isolates showed unique IGS-RFLP patterns, and only few strains shared the same restriction pattern type. For some strains the size of undigested IGS region did not agree with the sum of sizes of restriction fragments, indicating the existence of at least two types of IGS regions with differences in restriction sites.

The analysis of the combined restriction profiles displayed as a dendrogram in Figure 4, defined four major clusters among isolates at 50% similarity level. Cluster I comprised only three isolates, about 70% of photosynthetic strains grouped in a large cluster II (S_D = 64%), but all orange and dark pink strains grouped in a different one (cluster III). Cluster IV consisted of strains *B. japonicum* USDA 110 and *Bradyrhizobium* sp. CB 756.

16S rRNA gene sequence analysis

The 16S rRNA gene sequence determined for strain C7T1 consisted of 1,485 nucleotides. It was very similar (99% sequence similarity) to the published sequences of other photosynthetic bradyrhizobial strains and *Blastobacter denitrificans* LMG 8443. The phylogenetic tree in Figure 5 showed that these strains comprised a well-supported subcluster (bootstrap value of 100%) among related organisms from the alpha subclass of the *Proteobacteria*.

Discussion

We used genotyping methods with different levels of resolution to characterize the photosynthetic rhizobia from northeast Argentina and to explore the genomic diversity among them. Rep-PCR genomic fingerprinting was shown before to be very adequate to differentiate rhizobial strains (JUDD et al., 1993; LAGUERRE et al., 1996, 1997; RADEMAKER and DE BRUIJN, 1997; VINUESA et al., 1998), and cluster analysis of rep-PCR genomic fingerprints of closely related sub(species) or strains can provide

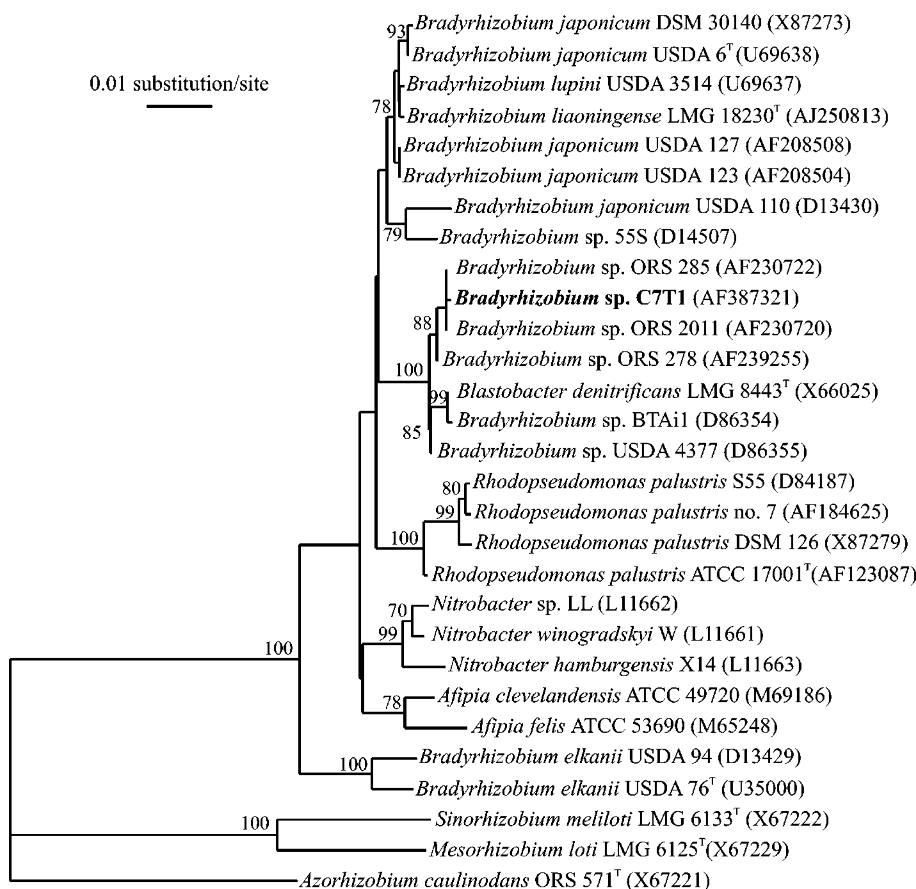

Fig. 5. Phylogenetic relationships of photosynthetic bradyrhizobia and related organisms from the alpha subclass of the *Proteobacteria* for 16S rRNA gene sequences, estimated using the neighbor-joining algorithm with the Kimura-2 parameter model. Bootstrap values of 70% or more (from 1,000 replicates) are indicated at the nodes. Sequence accession numbers are listed in parentheses.

information about their phylogenetic relationship (RADEMAKER and DE BRUIJN, 1997; RADEMAKER et al., 2000). The results showed a high genomic diversity of the photosynthetic symbionts at intraspecific level, revealed as rep-PCR, considering that all the isolates originated from the same plant species collected in different places from geographically close areas. At the taxonomic delineation level of the ARDRA technique the differences among isolates were low, because of the small 16S rDNA sequence divergence among *Bradyrhizobium* strains (WILLEMS et al., 2001a). In spite of this, in cluster analysis almost all the photosynthetic isolates grouped in a cluster related but distinct from those containing the reference strains of *B. elkanii* and *B. japonicum* respectively (not shown). This latter cluster also grouped BTAi1 strain and this is at variance with the results obtained in other report (MOLOUBA et al., 1999), but this variation may be due to the identity of the restriction enzymes used in both studies.

A. rudis belongs to cross-inoculation group 3 and about 96% of the isolated strains growing aerobically under light-dark cycles produced Bchl *a* and carotenoids. Our results agreed with those shown for the African collection of *Aeschynomene* spp. isolates (MOLOUBA et al., 1999; WILLEMS et al., 2000), and clearly indicated a correlation between nodulation characteristics and photosynthetic properties. However, VAN BERKUM et al. (1995) previously observed that approximately only 50% of soil isolates which were able to nodulate *A. indica* also synthesized Bchl and carotenoids, and only a few of those isolates accumulated photosynthetic pigments in continuous darkness. That study included only one nonphotosynthetic isolate from Argentina. Among our isolates we observed that all strains producing canthaxanthin also produced Bchl when grown under dark-aerobic conditions. It should be pointed out, however, that this property was conspicuous of that group of strains but not one of its exclusive characteristic properties. Interestingly, all the strains isolated, grown under continuous light, interrupted Bchl accumulation, but a complement of carotenoids was accumulated in all orange and dark pink isolates during cell growth (Fig. 1e). Canthaxanthin was among those carotenoid pigments, having no known photosynthetic functions. As previously suggested, probably canthaxanthin acts as a shield against deleterious radiation, and the main carotenoid pigments produced by orange and dark pink strains would not be part of the photosynthetic unit (LORQUIN et al., 1997). When cells of strain C7T1 were broken by sonication and submitted to a sucrose density gradient centrifugation, two major pigmented bands were produced (Fig. 1d). One of them contained most of the spirilloxanthin and Bchl. The other was mainly constituted by canthaxanthin and was probably associated with the cell wall. This result agrees with the possible role of canthaxanthin as radiation shield.

Integrating the information provided by ARDRA and rep-PCR fingerprints resulted in the evaluation of the overall diversity and the phylogenetic relationship existing among the isolates (Fig. 3). Thus almost all photosynthetic isolates could be included in either one of two main different groups (clusters IV and V). The main differences between them were the type of carotenoid pigments accumulated and the capacity to produce photosynthetic pigments when grown in darkness. Moreover, the strains displaying absorption spectra of type 1 (Fig. 1a) that conform a singular pigmentation group, would constitute a bradyrhizobia group different from the other isolates. The IGS-RFLP analysis is a different source of molecular evidence of their genetic relatedness. This molecular typing approach produced similar clustering of the strains analyzed, illustrating the consistence of the results. However, as it is known, the resolution of this method is lower than rep-PCR and differences among genotypes could be overestimated due to variations in the length and number of IGS regions in the strains analyzed (LAGUERRE et al., 1996). Therefore, some strains with the same IGS restriction pattern have different BOX-PCR fingerprints.

Comparative studies within different bacterial genera, showed a high correlation between rep-PCR or AFLP derived relationships and DNA hybridization groups, suggesting that these genomic fingerprinting methods can serve as an estimate of DNA relatedness (NICK et al., 1999; RADEMAKER et al., 2000; WILLEMS et al., 2001 b). We observed that all our photosynthetic isolates producing orange pigmentation grouped together irrespective of their fingerprint pattern analyses, and they could, therefore, belong to the same genospecies. However this result does not establish whether this genospecies is different from the rest of the isolates and DNA-DNA hybridization data are strictly necessary to determine the relationships among them and with other strains. Even though a DNA-DNA hybridization study was performed among representative photosynthetic *Aeschynomene* isolates and two genospecies were defined, their taxonomic status remains unclear (WILLEMS et al., 2001 c). Futhermore, the majority of *Bradyrhizobium* strains had a single rRNA operon type but some of the photosynthetic bradyrhizobial strains showed multiple 16S-23S rDNA IGS sequences (WILLEMS et al., 2001 b). In the IGS-RFLP analysis we obtained results that also indicated the presence of, at least, two types of IGS regions in some of the present strains.

The synthesis of canthaxanthin by strains C1T1, C1R1, C6T1, C6T2, C7T1, C12T2 and G5T1 constituted a distinctive characteristic of those bacteria. A comparison of the BOX-PCR with IGS-RFLP grouping demonstrate the close relationship among these strains at intraspecific level which in both analysis emerge in a single distinctive cluster (Fig. 2, 4). In the combined rep-PCR plus ARDRA analysis they also form a distinct group (Fig. 3). It would be illustrative to determine whether strains isolated from more distant regions are related to the strains described in this paper.

Based on 16S rDNA sequence similarities, all strains of photosynthetic rhizobia form a distinct phylogenetic group within the *Bradyrhizobium* cluster (Fig. 5), and as previously suggested, they would constitute different species (SO et al., 1994; FLEISHMAN and KRAMER, 1998; WILLEMS et al., 2001c). Strains isolated from widely separated geographic regions and from different *Aeschynomene* species exhibit a very high 16S rDNA sequence similarity, and although photosynthetic rhizobia

were mainly obtained from *Aeschynomene* spp., they were also found as natural endophytes within the roots of wild rice (CHAINTREUIL et al., 2000).

Using high resolution molecular typing methods based on whole genome analysis like AFLP and rep-PCR a high genetic diversity among photosynthetic rhizobia at the intraspecific to strain level was revealed (WILLEMS et al., 2000; this study). Similar results were obtained, when performing the restriction analysis of 16S-23S rDNA intergenic spacer region (DOIGNON-BOURCIER et al., 2000; this study). Moreover, from the results shown in this paper and from previous studies (LORQUIN et al., 1993; SO et al., 1994; WILLEMS et al., 2000; DOIGNON-BOURCIER et al., 2000), it was concluded that the strain BTAi1 fell within a subgroup different from the bulk of the isolates of the collections so far studied. Therefore, this strain should not be used as the unique representative strain of photosynthetic rhizobia.

Acknowledgements

We would like to acknowledge the financial support from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina), the University of Buenos Aires (UBA, Argentina), and the Volkswagen Stiftung (Germany).

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