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Restriction Fragment Length Polymorphism Analysis of 16S rDNA and Low Molecular Weight RNA Profiling of Rhizobial Isolates from Shrubby Legumes Endemic to the Canary Islands

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Summary

Thirty-six strains of slow-growing rhizobia isolated from nodules of four woody legumes endemic to the Canary islands were characterised by 16S rDNA PCR-RFLP analyses (ARDRA) and LMW RNA profiling, and compared with reference strains representing *Bradyrhizobium japonicum, B. elkanii, B. liaoningense*, and two unclassified *Bradyrhizobium* sp. *(Lupinus)* strains. Both techniques showed similar results, indicating the existence of three genotypes among the Canarian isolates. Analysis of the combined RFLP patterns obtained with four endonucleases, showed the existence of predominant genotype comprising 75% of the Canarian isolates (BTA-1 group) and the *Bradyrhizobium* sp. *(Lupinus)* strains. A second genotype was shared by nine Canarian isolates (BGA-1 group) and the *B. japonicum* and *B. liaoningense* reference strains. The BES-5 strain formed an independent group, as also did the *B. elkanii* reference strains. LMW RNA profile analysis consistently resolved the same three genotypes detected by 16S ARDRA among the Canarian isolates, and suggested that all these isolates are genotypically more related to *B. japonicum* than to *B. elkanii* or *B. liaoningense*. Cluster analysis of the combined 16S ARDRA and LMW RNA profiles resolved the BTA-1 group with the *Bradyrhizobium* sp. *(Lupinus)* strains, and the BES-5 isolate, as a well separated sub-branch of the *B. japonicum* cluster. Thus, the two types of analyses indicated that the isolates related to BTA-1 conform a group of bradyrhizobial strains that can be clearly distinguishable from representants of the tree currently described *Bradyrhizobium* species. No correlation between genotypes, host legumes, and geographic location was found.

Key words: 16S rDNA RFLP analysis – LMW RNA profiles – *Bradyrhizobium* – Canary Islands – tagasaste *(Chamaecytisus proliferus)* – genetic diversity

Introduction

The rhizobia are a group of alpha proteobacteria capable of inducing nitrogen-fixing nodules on the roots or stems of legumes. Despite the fact that the *Leguminosae* is one of the largest families of plants with more than 19,000 species, only a small number have been studied with regard to the rhizobia nodulating them. The isolation of bacteria from nodules of previously uninvestigated legumes has revealed the existence of large rhizobial diversity, leading to deep changes in the taxonomy of the *Rhizobiaceae*. Among the fast-growing rhizobia a large number of new species and genera have been described in recent years, and currently five genera are widely accepted, *Rhizobium, Azorhizobium, Sinorhizobium, Mesorhizobium* (for reviews see MARTÍNEZ-ROMEO and CABALLERO-MELLADO, 1996; YOUNG and HAUKKA, 1996; VAN BERKUM and EARDLY, 1998), and *Allorhizobium* (DE LAJUDIE et al., 1998). A polyphasic approach has been responsible for the new taxonomy in the fast-growing rhizobia, but the most relevant studies for developing the current rhizobia classification have been 16S rDNA se-

Abbreviations: LMW RNA – Low Molecular Weight RNA; ARDRA – amplified ribosomal DNA restriction analysis

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quence analyses. The taxonomy of slow-growing rhizobia, on the other hand, is less refined, due in part to the conflictive results obtained in the analyses of phenotypic and genotypic traits (So et al., 1994; VAN ROSSUM et al., 1995). The number of *Bradyrhizobium* species currently admitted has increased from one (*B. japonicum*, JORDAN, 1982) to three, with the descriptions of *B. elkani* (KUYKENDALL et al., 1991) and *B. liaoningense* (XU et al., 1995). But, as in the case of fast-growing rhizobia, the number of *Bradyrhizobium* species is expected to increase as more slow-growing strains isolated from previously unstudied legumes are characterised.

In the Canary Islands flora a large number of endemic legumes is found (HANSEN and SUNDING, 1993). Among them, some shrub legumes are of great agricultural and ecological interest. Tagasaste (*Chamaecytisus proliferus* L. fil. Link ssp. *proliferus* var. *palmensis* (Christ) Hans. and Sund. (ACEBES-GINOVÉS et al., 1991) is the most valuable native fodder shrub found in the archipelago. Its high nutritive value and palatability for grazing animals, pigs and poultry is well documented (PÉREZ DE PAZ et al., 1986; BORENS and POPPI, 1990; SNOOK, 1996). Tagasaste has been used as forage in the canaries for centuries (PÉREZ DE PAZ et al., 1986). In the last century, it was introduced in other parts of the world such as Australia and New Zealand (FRANCISCO-ORTEGA et al., 1991; SNOOK, 1996), where it has been widely cultivated, and where it has deserved preparation of an specific inoculant (ROUGHLEY, 1990). In addition to its value as fodder plant, tagasaste plantations have been used as a shelter for other crops to reduce erosion in expossed slops, for redemption of wasteland, firebreaks, or salinity control (SNOOK, 1996).

In previous works it was shown that tagasaste and other endemic legumes are nodulated in the Canaries by a group of *Bradyrhizobium* strains phenotypically and genetically diverse (LEÓN-BARRIOS et al., 1991; SANTAMARÍA et al., 1997; VINUESA et al., 1998). In this work we have used a relatively large collection of isolates (36 strains) obtained from root nodules of several genera of endemic woody legumes of the Canary Islands, collected in different islands and locations. Our aim was to study the diversity of the rhizobia isolates nodulating woody endemic legumes growing in four different Islands of the archipelago, to determine if a specific group of bradyrhizobial strains associated with these shrubs legumes are confined to these Islands (as suggested from our previous works, VINUESA, et al., 1999, 2000), and to find out the distribution and representation of different genotypes detected in the Canarian Archipelago.

Here we have used two different approaches to detect diversity: RFLP analysis of PCR-amplified 16S rDNA fragments and Low Molecular Weight RNA (LMW RNA) profiles. Amplified ribosomal DNA restriction analysis (ARDRA), has been widely and successfully used in the last years to group rhizobia at the genus or species level of taxonomic resolution (LAGUERRE et al., 1994; SESSITSCH et al., 1997; TEREFEWORK et al., 1998; MOLOUBA et al., 1999; ZHANG et al., 1999). Similar to 16S rRNA, stable LMW RNA are valuable phylogenetic markers because their ubiquitous distribution in living beings and their conserved structure and homologous biochemical functions in protein synthesis (HÖFLE, 1988). Recently, a new single dimension electrophoretic technique in polyacrylamide gels – Staircase Electrophoresis (SCE) – has permitted optimum separation of these molecules (CRUZ-SÁNCHEZ, et al., 1997), and has been successfully applied in the identification of prokaryotes (VELÁZQUEZ et al., 1998a; 198b) and eukaryotes (VELÁZQUEZ et al., 2000). This technique has already been applied to genera and species of the *Rhizobiaceae*, yielding a different profile for each bacterial species analysed (VELÁZQUEZ et al., 1998a). The LMW RNA profiles comprise the 5S rRNA, class 1 tRNA and class 2 tRNA. The 5S rRNA zone is characteristic for each genus while the tRNA profiles are characteristic for each of the species analysed. Therefore, LMW RNA profiles produce a molecular fingerprint that can be used as a valuable tool for taxonomic and diversity studies (HÖFLE, 1990; VELÁZQUEZ et al., 1998a; 1998b, 2000).

In this work we have analysed the genetic diversity found among nodule isolates from endemic woody legumes of the Canary islands. The results of 16S rDNA PCR-RFLPs and LMW RNA profiles are compared.

Materials and Methods

Bacterial strains and growth conditions

The strains used in this study are presented in Table 1. Those not previously described (indicated in Table 1) were isolated from root nodules following the procedure described by VINCENT (1970). The bacteria were grown at 28 °C in yeast mannitol (YM) medium (VINCENT, 1970).

Plant infection tests

Isolates were tested for nodulation on tagasaste and *Glycine max*. Seed surface sterilisation was carried out with 5% commercial hypochlorite as described (VINCENT, 1970). Germination of tagasaste seeds required a previous scarification with concentrated sulphuric acid.

DNA isolation

Total genomic DNA was obtained from bacterial batch cultures grown until late exponential phase using a standard cetyltrimethylammonium bromide (CTAB) protocol (WILSON, K., 1994). The DNA concentration of the preparations was estimated by visually comparing the DNA samples with samples of known concentrations of lambda-DNA in agarose gels.

PCR amplification of 16S rRNA genes

The universal primers fD1 and rD1 were used to amplify nearly full-length 16S rRNA gene (WEISBURG et al., 1991). The primers, *Taq* polymerase, dNTPs and buffers were purchased from Amersham-Pharmacia Biotech. The PCRs were performed in 50 µl reaction mixtures as described (VINUESA et al., 1998).

Restriction fragment analysis

PCR products were purified with Qiaex II gel extraction kit (Qiagen) and digested with the restriction endonuclease, *Dde*I, *Msp*I *Hha*I, and *Hin*fI (Amersham-Pharmacia Biotech), as recommended by the manufacturers. The digests were separated by electrophoresis in 2% High Resolution agarose gels (Sigma

Table 1. Strains used in this study, 16S rDNA restriction patterns and ARDRA groups.

Strain[1]	Synonym	16S rDNA pattern[2]	ARDRA genotype[3]	Original host	Geographic origin	Reference or source[4]
Bradyrhizobium sp.						
BTA-1*		aaba	I	*Chamaecytisus proliferus*	Tenerife	LEÓN-BARRIOS et al. (1991)
BTA-2		aaba	I	*Chamaecytisus proliferus*	La Palma	SANTAMARÍA et al. (1997)
BTA-3*		baaa	II	*Chamaecytisus proliferus*	La Palma	SANTAMARÍA et al. (1997)
BTA-4*		aaba	I	*Chamaecytisus proliferus*	La Palma	SANTAMARÍA et al. (1997)
BTA-6*	BC-C2	aaba	I	*Chamaecytisus proliferus*	G. Canaria	SANTAMARÍA et al. (1997)
BES-1*		aaba	I	*Chamaecytisus proliferus*	Tenerife	LEÓN-BARRIOS et al. (1991)
BES-2*		aaba	I	*Chamaecytisus proliferus*	Tenerife	SANTAMARÍA et al. (1997)
BES-3*		aaba	I	*Chamaecytisus proliferus*	Tenerife	SANTAMARÍA et al. (1997)
BES-4*		aaba	I	*Chamaecytisus proliferus*	Tenerife	SANTAMARÍA et al. (1997)
BES-5*	BC-C1, BTA-5	bbab	IV	*Chamaecytisus proliferus*	G. Canaria	SANTAMARÍA et al. (1997)
BES-7*		aaba	I	*Chamaecytisus proliferus*	Tenerife	This study
BES-8*		aaba	I	*Chamaecytisus proliferus*	Tenerife	This study
BESG-10		aaba	I	*Chamaecytisus proliferus*	La Gomera	This study
BESG-13		aaba	I	*Chamaecytisus proliferus*	La Gomera	This study
BGA-1*		baaa	II	*Teline stenopetala*	La Palma	LEÓN-BARRIOS et al. (1991)
BGA-2*		aaba	I	*Teline stenopetala*	La Palma	SANTAMARÍA et al. (1997)
BGA-3*		aaba	I	*Teline stenopetala*	La Palma	SANTAMARÍA et al. (1997)
BGA-4*		aaba	I	*Teline stenopetala*	La Palma	This study
BGA-5*		aaba	I	*Teline stenopetala*	La Palma	This study
BGA-6*		baaa	II	*Teline stenopetala*	La Palma	This study
BGA-7*		baaa	II	*Teline stenopetala*	La Palma	This study
BGA-8*		baaa	II	*Telline stenopetala*	La Palma	This study
BRE-1		baaa	II	*Teline canariensis*	Tenerife	LEÓN-BARRIOS et al. (1991)
BCO-1		aaba	I	*Adenocarpus foliolosus*	Tenerife	SANTAMARÍA et al. (1997)
BCO-2		aaba	I	*Adenocarpus foliolosus*	Tenerife	SANTAMARÍA et al. (1997)
BCO-3*		baaa	II	*Adenocarpus foliolosus*	Tenerife	SANTAMARÍA et al. (1997)
BCO-4*		baaa	II	*Adenocarpus foliolosus*	Tenerife	SANTAMARÍA et al. (1997)
BCO-5		baaa	II	*Adenocarpus foliolosus*	Tenerife	This study
BCO-6*		aaba	I	*Adenocarpus foliolosus*	Tenerife	This study
BCOG-1		aaba	I	*Adenocarpus foliolosus*	La Gomera	This study
BCOG-3		aaba	I	*Adenocarpus foliolosus*	La Gomera	This study
BRT-1*		aaba	I	*Spartocytisus supranubius*	Tenerife	SANTAMARÍA et al. (1997)
BRT-1*		aaba	I	*Spartocytisus supranubius*	Tenerife	SANTAMARÍA et al. (1997)
BRT-3		aaba	I	*Spartocytisus supranubius*	Tenerife	SANTAMARÍA et al. (1997)
BRT-4*		aaba	I	*Spartocytisus supranubius*	Tenerife	SANTAMARÍA et al. (1997)
BRT-5*		aaba	I	*Spartocytisus supranubius*	Tenerife	SANTAMARÍA et al. (1997)
Bradyrhizobium sp. *(Lupinus)*						
ISLU 16*		aaba	I	*Lupinus* sp.	Sevilla	CIFA
ISLU 65*		aaba	I	*Lupinus* sp.	Madrid	CIFA
B. japonicum						
USDA 110		baaa	II	*Glycine max*	USA	E. BEDMAR
USDA 122		baaa	II	*Glycine max*	USA	B. VAN BERKUM
LMG 6138^T*	ATCC 10324 USDA 505^T	baaa	II	*Glycine max*	Japan	LMG
B. elkanii						
LMG 6134^T*	USDA 76^T	ccbc	III	*Glycine max*	Japan	LMG
USDA 94		ccbc	III	*Glycine max*	USA	P. VAN BERKUM
*B. liaoningense**						
LMG 18230^T	USDA 3622^T	baaa	II	*Glycine max*	China	LMG
S. meliloti						
1021a		ddcd	V	*Medicago sativa*	USA	P. VAN BERKUM

[1] The first letter refers to *Bradyrhizobium* genus, the next two letters refer to the local name of the legume; a fourth letter refers to the location where them were collected. strains with asterisk were included in the LMW RNA analysis.
[2] Letters refer to the type of restriction pattern of 16S rRNA genes digested with *Dde*I, *Msp*I, *Hha*I and *Hin*fI, respectively, as assigned in Fig. 1.
[3] ARDRA genotypes represent the UPGMA groups obtained in the combined 16S rDNA RFLP analyses, which were arbitrary designed from I to V.
[4] CIFA, Centro de Investigación y Formación Agraria-Las Torres-Tomejil, Sevilla; LMG, laboratorium voor Microbiologie, Ghent.

Chemical Co., St. Louis, Mo) in Tris-borate-EDTA at 60 V for approximately 3 h. A 100-bp ladder (Amersham-Pharmacia Biotech) was used as marker.

RNA extraction and LMW RNA profile analysis

RNA was extracted according to the method described by HÖFLE (1988). LMW RNA profiles were obtained using staircase electrophoresis in polyacrylamide gel under denaturing conditions in 10 min steps, rising through a constant ramp with 50 V increases from 100 V to 2,300 V, as reported earlier (CRUZ-SÁNCHEZ et al., 1997). After electrophoresis, the gels were silver-stained according to the method described by HAAS et al. (1994). The following commercial molecules from Boehringer Mannheim (Mannheim, Germany) and Sigma (Sigma Chemical Co., St. Louis, Mo, USA) were used as reference: 5S rRNA Germany) and Sigma (Sigma Chemical Co., St. Louis, Mo, USA) were used as reference: 5S rRNA from *Escherichia coli* MRE 600 (120 and 115 nucleotides) (BIDLE and FLETCHER, 1995), Tyr-tRNA from *E. coli* (85 nucleotides), and Val-tRNA from *E. coli* (77 nucleotides) (SPRINZL et al. 1985). Samples were prepared as reported elsewhere (HÖFLE, 1988).

Data analysis

The bands present in each RFLP or LMW RNA pattern were coded in binary form, and Dice's or Jaccard's similarity coefficients were calculated, respectively, to construct a similarity matrix for the RFLP and LMW RNA analysis. A dendrogram was constructed from the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA).

Results

Cultural and infective characteristics

All isolates displayed a slow growth rate on YM medium. All the strains, independently from the original host legume genera from which they were isolated, were capable of forming effective nodules on tagasaste *(Chamaecytisus proliferus)*. Moreover, the *Bradyrhizobium* sp. *(Lupinus)* strains ISLU 16 and ISLU 65 also nodulated tagasaste producing effective nodules. The *B. japonicum* and *B. elkanii* reference strains were unable to nodulate tagasaste. On the other hand, the representative isolates BTA-1 and BGA-1 tested on *Lupinus albus* proved to be good nitrogen fixers on this legume (M. Fernández-Pascual, personal communication). None of the canarian isolates were able to nodulate *Glycine max*.

RFLP analysis of PCR-amplified 16S rRNA genes

PCR amplification of 16S rRNA genes resulted in a single fragment of about 1.5 kb for all strains tested. The purified PCR products were individually restricted with endonucleases *Dde*I, *Msp*I, *Hha*I, and *Hin*fI. All endonucleases produced polymorphic restriction patterns. The different types of RFLP patterns obtained for each enzyme are shown in Fig. 1. Table 1 shows the RFLP pattern obtained for each strain with each restriction en-

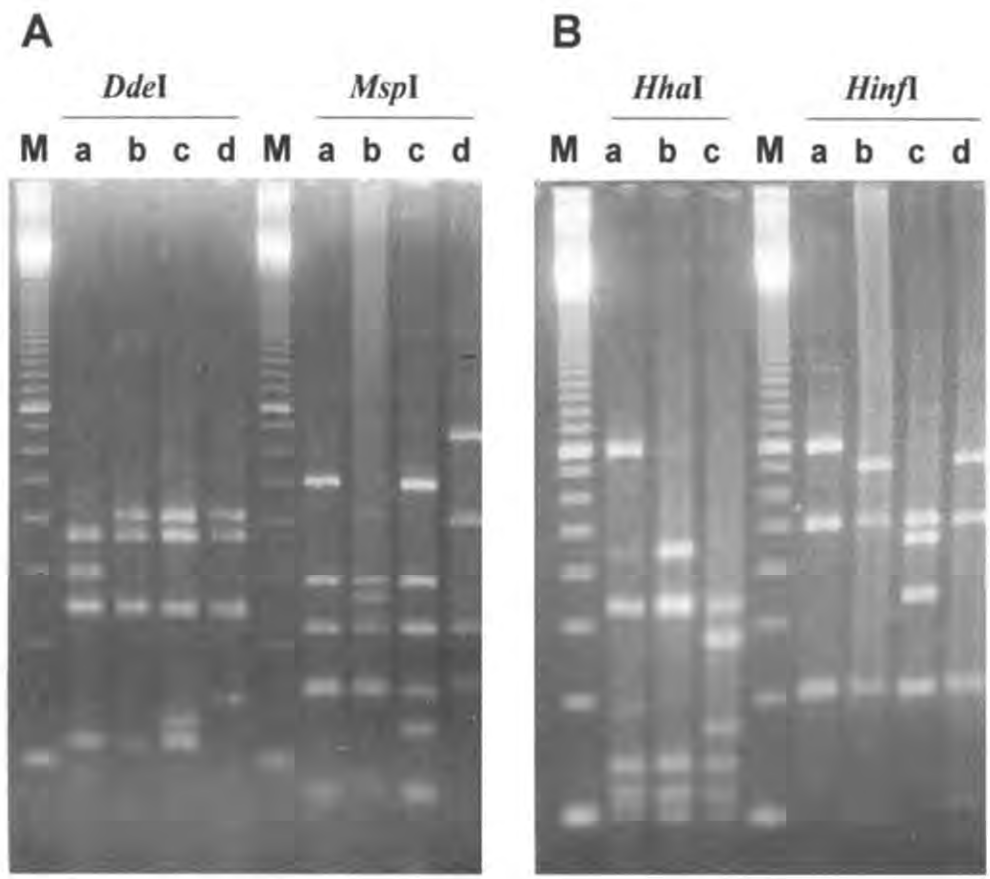

Fig. 1. Types of restriction patterns of PCR-amplified 16S rRNA genes digested with (A) *Dde*I, *Msp*I, (B) *Hha*I and *Hin*fI, obtained with strains used in this study. Lowercase letters were arbitrarily assigned to represent specific fingerprint patterns generated with each enzyme. Lanes marked with M correspond to the 100 bp ladder marker.

zyme. The combined restriction patterns of 16S rRNA genes obtained with the four restriction endonucleases were used for cluster analysis by UPGMA. Only fragments of 100 bp or larger were considered. This analysis resolved all the bradyrhizobia reference strains and isolates in four genotypes, conforming the ARDRA groups I, II, III, and IV (Table 1) at a Dice similarity (S_D) of 76%. All Canarian isolates could be resolved into three genotypes. Genotype I clustered strains characterised by an aaba pattern (Fig. 1) that grouped most of the Canarian isolates (75%) with strains ISLU 16 and ISLU 65 of *Bradyrhizobium* sp. *(Lupinus)*. The BTA-1 strain was chosen as representative strain of cluster I, which was referred to BTA-1 group. Genotype II (BGA-1 group) is formed by strains with a baaa pattern (Fig. 1), and it grouped nine of the isolates with the *B. japonicum* and *B. liaoningense* reference strains at a S_D of 100%. The Canarian isolate BES-5 showed a bbab pattern (Fig. 1) and formed an outgroup from the bradyrhizobial cluster at a Dice similarity level of 76%. The reference strains LMG 6138 (USDA 76) and USDA 94 of *B. elkanii* with a ccbc pattern (Fig. 1) were placed in a separate RFLP group (ARDRA group III, Table 1). *S. meliloti*, produced a ddcd pattern (Fig. 1) and was consequently resolved as a well separate outgroup (S_D of 55%) to the *Bradyrhizobium* spp., as expected.

LMW RNA profiles analysis

A group of twenty-six isolates and reference strains were analysed by their profiles of stable LMW RNAs. This technique resolved the Canarian isolates into three groups (groups 1, 2, and 3; Table 2) that were coincident with the 16S ARDRA genotypes I, II and IV respectively, (Table 1). The isolates included in each LMW RNA group are presented in Table 2, and the three types of LMW RNA profiles are shown in Fig. 2 (lanes 4, 5 and 6), being each pattern identical for each LMW RNA group. The two *Bradyrhizobium* sp. *(Lupinus)* strains produced a profile as that of the BTA-1 group (lane 7, Fig. 2). The three distinct LMW RNA profiles obtained for the *B. elkani*, *B. liaoningense*, and *B. japonicum* type strains are shown in Fig. 2 (lanes 1, 2 and 3, respectively). The Canarian isolates in the BTA-1 and BGA-1 groups showed a 5S rRNA zone identical to *B. japonicum*, while the BES-5 5S rRNA profile differed in the presence of an extra band (Fig. 2). The tRNA profiles of BTA-1 and BGA-1 groups, and BES-5 strain were different to each other, although that of BES-5 was more similar to those of the BTA-1 group. The tRNA profiles yielded by strains in the BGA-1 group could not be distinguished from that of *B. japonicum* type strain. However, the BTA-1 group, ISLU 16, ISLU 65 and BES-5 tRNA profiles showed patterns that could be easily distinguishable from those produced for the type strains of the three currently accepted *Bradyrhizobium* species.

The UPGMA cluster analysis of the LMW RNA profiles resulted in the dendrogram where the three *Bradyrhizobium* type strains and the isolates used in this study grouped in three main branches. One branch contained the *B. japonicum* type strain, all the Canarian isolates, and the two *Lupinus* isolates at a similarity level of 70% while the *B. liaoningense* and the *B. elkanii* type strains were resolved in other two independent branches.

Table 2. Canarian isolates groups based on LMW RNA profiles.

LMW RNA group[1]	Strains
1	BTA-1, BTA-4, BTA-6, BGA-2, BGA-3, BGA-4, BGA-5, BES-1, BES-2, BES-3, BES-4, BES-7, BES-8, BCO-6, BRT-1, BRT-2, BRT-4, BRT-5
2	BTA-3, BGA-1, BGA-6, BGA-7, BGA-8, BCO-3, BCO-4
3	BES-5

[1] Numbers were arbitrarily assigned for each type of LMW RNA profile obtained.

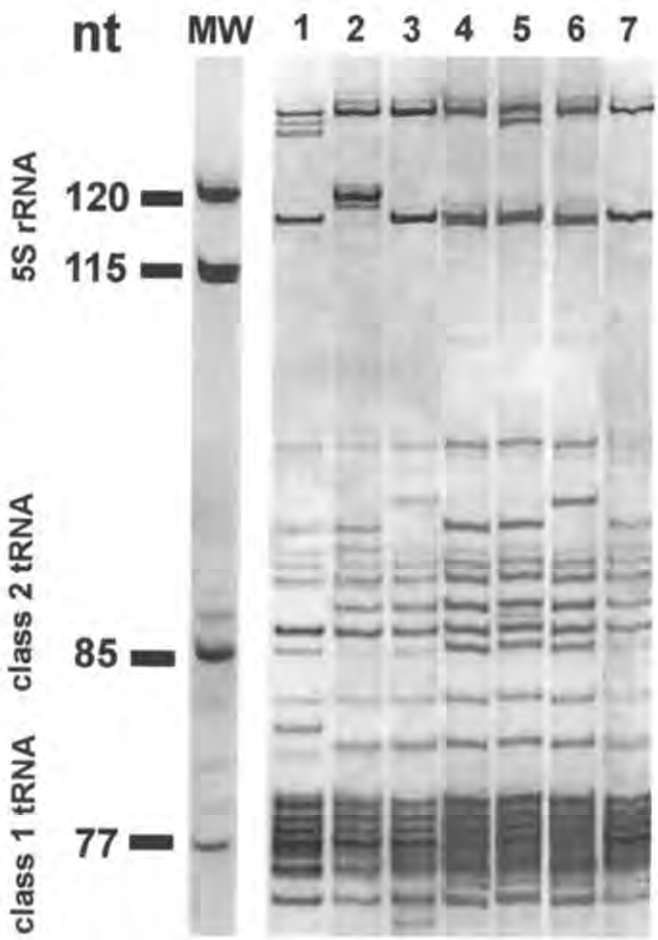

Fig. 2. LMW RNA profiles of representative strains of each group. Lane 1. *B. elkanii* LMG 6134^T; lane 2, *B. liaoningense* LMG 18230^T; lane 3, *B. japonicum* LMG 6138^T; lane 4, BTA-1; lane 5, BGA-1; lane 6, BES-5, and lane 7, *Bradyrhizobium* sp. *(Lupinus)* ISLU 65.

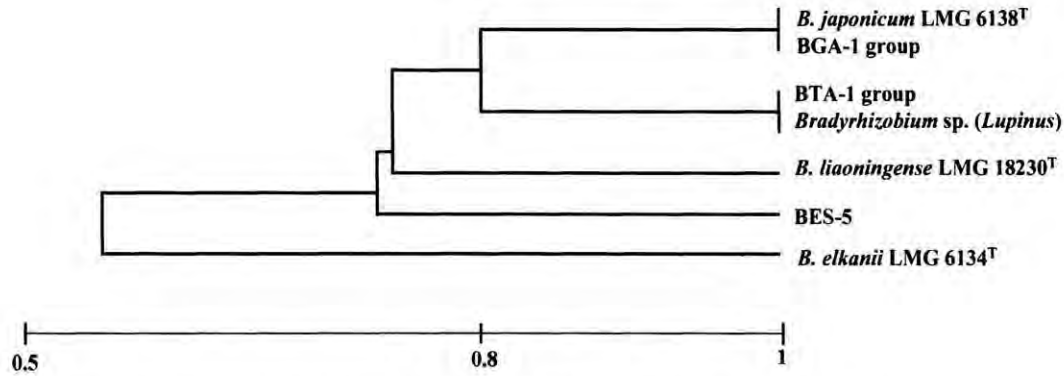

Fig. 3. Dice/UPGMA analysis of the combined 16S rDNA RFLP and LMW RNA patterns of the isolates and reference strains.

The BTA-1 group (including the ISLU 16 and ISLU 65 strains) and the BES-5 isolate formed a well separate subbranch of the *B. japonicum* cluster. According to our previous results (VELÁZQUEZ et al., 1998a) each rhizobial species displays a characteristic tRNA profile. Therefore, the LMW RNA fingerprint data presented herein suggest that the strains related to the BTA-1 might represent a new species of *Bradyrhizobium*.

Cluster analysis of the combined 16S rDNA RFLP and LMW RNA patterns

Patterns obtained from both techniques were combined in a Dice/UPGMA (Fig. 3). This analysis resulted in a dendrogram with two main branches, one contained the *B. elkanii* type strain at a S_D of 57%, and the other grouped all the rest of the bradyrhizobial strains (at a S_D of 74%) in a cluster with four subbranches. The BGA-1 group and the *B. japonicum* strains formed one branch, the BTA-1 group and the *Bradyrhizobium* sp. *(Lupinus)* strains a second one, and *B. liaoningense* and the BES-5 isolate were placed each in two independents subbranches.

Discussion

In this report we have studied the genetic diversity of the rhizobia nodulating four genera of woody legumes (into the Genisteae Tribe) endemic to the Canary Islands (*Chamaecytisus, Spartocytisus, Adenocarpus* and *Teline*). All 36 isolates belonged to *Bradyrhizobium*, as deduced from their slow growth rate in YM medium, 16S ARDRA and LMW RNA profiling. This contrasts with the results reported by GAULT et al. (1994) who found that in Australia the introduced tagasaste can be nodulated both by fast- and slow-growing rhizobia; however, the taxonomic status of these isolates was not determined in that study.

Although four genera of legume growing in four different Canary Islands were used to isolate the rhizobia, no more than three genotypes could be distinguished by 16S ARDRA, using four discriminating endonucleases. Twenty six (75) of the Canarian isolates (BTA-1 group) showed the genotype 1; nine of the isolates displayed a 16S ARDRA genotype II (BGA-1 group) identical to the *B. japonicum* and *B. liaoningense* strains; finally, the BES-5 isolate, showed a unique genotype characterised by its unique *Msp*I and *Hin*fI patterns, and consequently formed an outgroup branch of the bradyrhizobial cluster.

LMW RNA profile analysis resolved the Canarian isolates in the same three groups obtained by 16S ARDRA; and the same relationships among the BTA-1 and BGA-1 groups and *B. japonicum* and *B. elkanii* reference strains were obtained. However, discrepancies between LMW RNA profile analysis and 16S ARDRA were found for the BES-5 isolate and *B. liaoningense*. Thus, LMW RNA profile analysis placed the BES-5 isolate close to the BTA-1 group forming a subcluster from *B. japonicum* instead of a very distant ARDRA branch. On the other hand, *B. liaoningense* was resolved as an independent branch at a similarity level of 60%, while 16S rDNA RFLP analysis did not separate *B. liaoningense* from *B. japonicum*. This last result agrees with that found by DOIGNON-BOURCIER, et al. (1999) who were unable to differentiate *B. liaoningense* from *B. japonicum* type strain using five endonucleases.

Cluster analysis of the combined 16S rDNA RFLP and LMW RNA patterns yielded a dendrogram that seems to reflect better the phylogenetic relationships among the strains used in this study. In this analysis *B. elkanii* was resolved as a very distant branch of the *Bradyrhizobium* spp. cluster, in agreement with previous reports (MINAMISAWA, 1990; KUYKENDALL et al., 1992; VAN BERKUM and EARDLY, 1998). In our analysis *B. liaoningense* and the BES-5 isolate formed two separated branches within a cluster that included all the bradyrhizobial strains studied (other than *B. elkanii*). These groupings are in good agreement with partial 16S rDNA sequencing data which indicated that strain BES-5 (BC-C1) is phylogenetically related to the *B. japonicum/Rhodopseudomonas palustris* branch having a partial sequence identical to that of the photosynthetic strain. *Bradyrhizobium* sp. BTAil (VINUESA et al., 1998). Furthermore, this combined analysis is also in good agreement with the *B. liaoningense* phylogenetic relationships based on partial 16S rDNA sequence analysis (XU et al., 1995) or 16S ARDRA

(DOIGNON-BOURCIER, et al., 1999) that placed this species closer to *B. japonicum* than to *B. elkanii*.

The two technique (16S ARDRA and LMW RNA profiling) using in this work to characterise the Canarian isolates have previously demonstrated to be efficient tools to classify the rhizobia at the species and higher levels of taxonomic resolution (LAGUERRE et al., 1994; VELÁZQUEZ et al., 1998a). The isolates into the BGA-1 group showed to be close relatives of *B. japonicum* in both types of analyses. But, the differences found by both techniques for the BTA-1 group support that these isolates form a distinctive group of bradyrhizobial strains, clearly distinguishable from the three currently reported *Bradyrhizobium* species, providing preliminary evidence that they could represent a new species. Additional genotypic evidence derived from nearly full-length *rrn* operon PCR-RFLP analysis (VINUESA et al., 2000) also pointed that this group constitutes a new genomic species. A polyphasic characterisation of these strains, including total DNA-DNA hybridisation by different methods is being pursued towards a formal proposal about the taxonomic status of the BTA-1 group isolates. Establishing the taxonomic status of the BES-5 isolate also demand further investigation and the isolation of new strains with the same genotype.

The finding of two *Bradyrhizobium* sp. *(Lupinus)* strains showing an ARDRA genotype I identical to the Canarian group (BTA-1 group) was unexpected. In previous works (VINUESA et al., 1999, 2000) ARDRA of nearly full-length rDNA operon could resolve a group of ten Canarian isolates among a vast collection of over 100 *Bradyrhizobium* strains of tropical and temperate origin, including *Lupinus* isolates. This finding suggested that the Canarian isolates with a BTA-1 like genotype could represent a local population of bradyrhizobia strains confined to the Canary Islands. The fact that the two *Lupinus* isolates ISLU 16 and ISLU 65 were collected in mainland-Spain means that *Bradyrhizobium* strains with a 16S ARDRA genotype I are not restricted to the Canarian archipelago, but more investigation should be carried out to clarify the geographic distribution of this genotype. It must be highlighted, that other well characterised *Bradyrhizobium* sp. *(Lupinus)* isolates from different geographical origins show 16S ARDRA genotypes (LAGUERRE et al., 1994; NUSWANTARA et al., 1997; VINUESA et al., 2000) or 16S rRNA gene sequences (BARRERA et al., 1997) that cluster them with *B. japonicum* strains. It is worthwhile noting that *Lupinus* ssp. belongs to the tribe Genisteae as the Canarian shrub legumes studied herein. It remains to be established if these plants constitute a cross-inoculation group.

It can be concluded that a distinctive group of bradyrhizobial strains (the BTA-1 group), that might constitute a new *Bradyrhizobium* species, appears to be widely distributed in the Canarian soils. These represented 75% of the isolates included in this study and can be recovered from different genera of endemic legumes growing in different Islands, indicating they are not associate to a specific legume or particular area in the canaries. Whether or not this group of bradyrhizobia is specific to the Canary Islands requires further studies.

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References

ACEBES-GINOVÉS, J. R., DEL ARCO-AGUILAR, M., WILPRET DE LA TORRE, W.: Revisión taxonómica de *Chamaecytisus proliferus* (L. fil) Ling en Canarias. Vieraea 20, 191–202 (1991).

BARRERA, L. L., TRUJILLO, M. E., GOODFELLOW, M., GARCIA, F. J., HERNÁNDEZ-LUCAS, I., DÁVILA, G., VAN BERKUM, P., MARTÍNEZ-ROMERO, E.: Biodiversity of bradyrhizobia nodulating *Lupinus* spp. Int. J. Syst. Bacteriol. 47, 1086–1091 (1997).

BIDLE, K. D., FLETCHER, M.: Comparision of free-living and particle-associated bacterial communities in the Chesapake bay by stable low-molecular-weight RNA analysis. Appl. Environ. Microbiol. 61, 944–952 (1995).

BORENS, F., POPPI, D. P.: The nutritive value for ruminants of tagasaste *(Chamaecytisus palmensis)*, a leguminous tree. Animal feed Sci. Technol. 28, 275–292 (1990).

CRUZ-SÁNCHEZ, J. M., VELÁZQUEZ, E. MATEOS, P. F., MARTÍNEZ-MOLINA, E.: Enhancement of resolution of low molecular weight RNA profiles by staircase electrophoresis. Electroforesis 18, 1909–1911 (1997).

DE LAJUDIE, P., LAURENT-FULELE, E., WILLEMS, A., TORCK, U., COOPMAN, R., COLLINS, M. D., KERSTERS, K., DREYFUS, B. L., GILLIS, M.: *Allorhizobium undicola* gen. nov., nitrogen fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. Int. J. Syst. Bacteriol. 48, 369–382 (1998).

DOIGNON-BOURCIER, F., SY, A., WILLEMS, A., TORCK, U., DREYFUS, B., GILLUS, M., DE LAJUDIE, P.: Diversity of bradyrhizobia from 27 tropical *Leguminosae* species native of Senegal. System. Appl. Microbiol. 22, 647–661 (1999).

FRANCISCO-ORTEGA, J., JACKSON, M. T., SANTOS-GUERRA, A., FERNÁNDEZ-GALVÁN, M.: Historical aspects of the origin and distribution of tagasaste [*Chamaecytisus proliferus* (L. fil.) Link ssp. *palmensis* (Christ) G. Kunkel], a fodder tree from the Canary Islands. J. Adelaide Bot. Garden 14, 67–76 (1991).

GAULT, R. R., PILKA, A., HEBB, D. M. BROCKWELL, J.: Nodulation studies on legumes exotic to Australia: symbiotic relationships between *Chamaecytisus palmensis* (tagasaste) and *Lotus* spp. Austr. J. Exp. Agric. 34, 385–394 (1990).

HAAS, H., BUDOWLE, B. WEILER, G.: Horizontal polyacrylamide gel electrophoresis for separation of DNA fragments. Electrophoresis 15, 153–158 (1994).

HANSEN, A., SUNDING, P.: Flora of Macaronesia. Checklist of vascular plants. 4 rev. Sommerfeltia, 17, 295 pp. (1993).

HÖFLE, M. G.: Identification of bacteria by low molecular weight RNA profiles: a new chemotaxonomic approach. J. Microbiol. Methods 8, 235–248 (1988).

HÖFLE, M. G.: Transfer RNAs as genotypic fingerprints of eubacteria. Arch. Microbiol. 153, 299–304 (1990).

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, 136–139 (1982).

KUYKENDALL, L. D., SAXENA, B., DEVINE, T. E., UDELL, S. E.: Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and proposal for *Bradyrhizobium elkanii* sp. Nov. Can. J. Microbiol. 38, 501–505 (1992).

LAGUERRE, G., ALLARD, M. R., REVOY, F., AMARGER, N.: Rapid identification of rhizobia by restriction fragment length polymorphism of PCR-Amplified 16S rRNA genes. Appl. Environ. Microbiol. 60, 56–63 (1994).

LEÓN-BARRIOS, M., GUTIÉRREZ-NAVARRO, A. M., PÉREZ-GALDONA, R., CORZO, J.: Characterization of Canary islands isolates of *Bradyrhizobium* sp. *(Chamaecytisus proliferus)*. Soil Biol. Biochem. 23, 487–489 (1991).

MARTÍNEZ-ROMERO, E., CABALLERO-MELLADO, J.: *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Rev. Plant Sci. 15, 113–140 (1996).

MINAMISAWA, K.: Division of rhizobitoxine-producing and hydrogen-uptake positive strains of *Bradyrhizobium* by *nifD-KE* sequence divergence. Plant Cell Physiol. 31, 81–89 (1990).

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, 3084–3094 (1999).

PÉREZ DE PAZ, P. L., DEL ARCO-AGUILAR, M., ACEBES-GINOVÉS, J. R., WILPRET, W.: Leguminosas forrajeras de Canarias. Tenerife, Excmo. Cabildo Insular de Tenerife, ACT Serie publicaciones cientificas, Subserie Museo Insular de Ciencias Naturales 1986.

ROUGHLEY, R. J., GRIFFITH, G. W., GEMELL, L. G.: The Australian Inoculants Research and Control Service: A. I. R. C. S. procedures. p. 17. N. S. W. Agriculture and Fisheries. Gosford, New South Wales 1990.

SANTAMARÍA, M., CORZO, J., LEÓN-BARRIOS, M., GUTIÉRREZ-NAVARRO, A. M.: Characterization and differentiation of indigenous rhizobia isolated from Canarian shrub legumes of agricultural and ecological interest. Plant Soil 190, 143–152 (1997).

SESSITSCH, A., HARDANSON, G., AKKERMANS, A. D. L., DE VOS, M.: Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrain soil. Mol. Ecol. 6, 601–608 (1997).

SNOOK, L. C.: Tagasaste. A productive browse shrub for sustainable agriculture. (G. WILSON, ed.) Mansfield, QLD, Agrovision 1996.

SO, R. B., LADHA, J. K., YOUNG, J. P. W.: Photosynthetic symbionts of *Aeschynomene* spp. form a cluster with bradyrhizobia on the basis of fatty acid and rRNA analysis. Int. J. Syst. Bacteriol. 44, 392–403 (1994).

SPRINZL, M., MOLL, J., MEEISSNER, F., HATMANN, T.: Compilation of tRNA sequences. Nucleic. Acid Res. 13, 1–49 (1985).

TEREFEWORK, Z., NICK, G., SUOMALAINEN, S., PAULIN, L., LINDSTRÖM, K.: Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. Int. J. Syst. Bacteriol. 48, 349–356 (1998).

VAN BERKUM, P., EARDLY, B. D.: Molecular evolutionary systematics. pp. 1–24. In: The *Rhizobiaceae* (H. P. SPAINK, A. KONDOROSI, P. J. HOOYKAAS, eds.) Dordrecht: Kluwer Academic Publishers 1998.

VAN ROSSUM, D., SCHUURMANS, F. P., GILLIS, M., MUYOTCHA, A., VAN VERSEVELD, H. W., STOUTHAMER, A. H., BOOGERD, F. C.: Genetic and phenetic analysis of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogea* L.) roots. Appl. Environ. Microbiol. 61, 1599–1609 (1995).

VELÁZQUEZ, E., CRUZ-SÁNCHEZ, J. M., MATEOS, P. F., MARTÍNEZ-MOLINA, E.: Analysis of stable low-molecular-weight RNA profiles of members of the family *Rhizobiaceae*. Appl. Environ. Microbiol. 64, 1555–1559 (1998a).

VELÁZQUEZ, E., CERVANTES, E., IGUAL, J. M., PEIX, A., MATEOS, P. F., BENEMAR, S., MOIROUD, A., WHEELER, C. T., DAWSON, J., LABEDA, D., RODRÍGUEZ-BARRUECO, C., MARTÍNEZ-MOLINA, E.: Analysis of LMW RNA profiles of *Frankia* strains by Staircase electrophoresis. System. Appl. Microbiol. 21, 539–545 (1998b).

VELÁZQUEZ, E., CALVO, O., MATEOS, P. F., TAMME, M., MARTÍNEZ-MOLINA, E.: Staircase electrophoresis profiles of stable Low Molecular Weight RNA as yeast fingerprinting. Int. J. Syst. Bact., in press (2000).

VINCENT, J. M.: A Manual for the Practical Study of Root-Nodule Bacteria. Oxford, Blackwell Scientific Publications 1970.

VINUESA, P., RADEMAKER, J. L. W., DE BRUIJN, F. J., WERNER, D.: Genopypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-restriction fragment length polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S–23S rDNA intergenic spacers, repetitive extragenic palindromic PCR genomic fingerprinting, and partial 16S rDNA sequencing. Appl. Environ. Microbiol. 64, 2096–2104 (1998).

VINUESA, P., RADEMARKER, J. L. W., DE BRUIJN, F. J., WERNER, D.: Characterization of *Bradyrhizobium* spp. strains by RFLP analysis of amplified 16S rDNA and rDNA intergenic spacer regions. pp. 275–279. In: Highlights of nitrogen fixation research (E. MARTÍNEZ, G., HERNÁNDEZ, eds.) New York, Kluwer Academic/Plenum publishers 1999.

VINUESA, P., KURZ, E. M., THIERFELDER, H., LEÓN-BARRIOS, M., THYNN, M., SICARDI-MALLORCA, M., RADEMAKER, L. W., MARTÍNEZ-ROMERO, E., DE BRUIN, F. L., BEDMAR, E., IZAGUIRRE-MAYORAL, M. L., WERNER, D.: Genotypic diversity of *Bradyrhizobium* strains of tropical and temperate origin and the identification of new genomic species nodulating endemic woody legumes (Fabaceae: Genisteae) from the Canary Islands. p. 195. In: Nitrogen fixation: from molecules to crop productivity (F. O. PEDROSA, M. HUNGRIA, M. G. YATES, W. E. NEWTON, eds.), Kluwer Academic Publishers Dordrecht 2000.

WEISBURG, W. G., BARNS, S. M., PELLETIER, D. A., LANE, D. J.: 16S ribosomal DNA amplification for phylogenetic studies. J. Bacteriol. 173, 697–703 (1991).

WILSON, K.: Preparation of genomic DNA from bacteria, pp. 2.4.1–2.4.2. In: Current protocols in molecular biology. (F. M. AUSUBEL, R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, J. A. SMITH, K. S. STRUHL, eds.) New York, John Wiley & sons 1994.

YOUNG, J. P. W., HAUKKA, K.: Diversity and phylogeny of rhizobia. New Phytol. 133, 87–94 (1996).

XU, L. M., GE, C., CUI, Z., LI, J., FAN, H.: *Bradyrhizobium liaoningense* sp. Nov., isolated from the root nodules of soybeans. Int. J. Syst. Bacteriol. 45, 706–711 (1995).

ZHANG, X., NICK, G., KAIJALAINEN, S., TEREFEWORK, Z., PAULIN, L., TIGHE, S. W., GRAHAM, P. H., LINDSTROM, K.: Phylogeny and diversity of *Bradyrhizobium* strains isolated from the root nodules of peanut *(Arachis hypogaea)* in Sichuan, China. System. Appl. Microbiol. 22, 378–386 (1999).

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