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Genotyping Icelandic isolates of rhizobia based on rDNA-RFLP

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SUMMARY

Simple PCR amplification using universal rhizobial oligonucleotide primers for intergenic spacer (IGS) regions of 16S-23S ribosomal genes (rDNA) was able to discriminate species, biovars and strains of rhizobial isolates. Six isolates of *Rhizobium* and *Bradyrhizobium* species from Iceland were analysed in comparison with four isolates from Scandinavia and Siberia. The rhizobia in pure cultures were isolated from nodules of leguminous species in the genera *Trifolium*, *Lathyrus*, *Lupinus*, *Astragalus* and *Thermopsis*. The amplified DNA fragments were between 400 and 1400-bp long and all the isolates could be differentiated except one pair of samples. The discrimination of isolates corresponded reasonably well to the rhizobial classification and was independent of their geographic origin. Further analysis was performed by fractionation of the PCR amplified products with restriction endonucleases. Twelve restriction enzymes were used, one at a time. Six of these (i.e. *Eco*RI, *Hae*III, *Mbo*I, *Taq*I, *Pst*I and *Pvu*II) were able to identify all isolates except the pair that could not be differentiated by the PCR amplification. This pair was likely to be of the same genotype. The use of single enzyme digestion provided a simple means to verify rhizobial identification, whereas combinations of two or more restriction enzymes, used simultaneously, maximised resolution of the restriction fragment length polymorphism (RFLP) profiles. The genetic distance analysis based on rDNA-RFLP profiles showed two clusters: one of these included essentially *R. leguminosarum* bv. *trifolii* and the other, which was a more diverse group, included other species. The rDNA-RFLP used in this study identified rhizobial species and strains, and detected misidentification of some isolates.

Key words: rhizobia, ribosomal DNA, PCR amplification, RFLP.

YFIRLIT

Greining íslenskra ísólata af Rhizobium-*bakteríum með rDNA-RFLP aðferð*

Með notkun DNA mögnunar (PCR) með sértækum vísum fyrir IGS (intergenic spacer) svæði 16S-23S ríbósómgena var hægt að greina á milli *Rhizobium* tegunda, afbrigða og stofna. Greind voru sex sýni af íslenskum *Rhizobium* og *Bradyrhizobium* tegundum og borin saman við fjögur sýni frá Skandinavíu og Síberíu. Bakteríurnar voru einangraðar úr rótarhnýðum belgjurtategunda af ættkvíslum *Trifolium*, *Lathyrus*,

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Lupinus, Astragalus og *Thermopsis*. Við mögnun fengust bútar frá 400 til 1400 bp og greina mátti á milli allra sýnanna, nema eins pars. Aðgreining sýnanna var í nokkuð góðu samræmi við flokkun bakteríanna og var óháð landfræðilegum uppruna þeirra. Mögnuðu afurðirnar voru meltar með skerðiensímum til frekari greiningar (PCR-RFLP aðferð). Tólf skerðiensím voru notuð, eitt í senn. Sex af þessum ensímum nægðu til að greina á milli allra sýnanna, nema þeirra tveggja sem DNA mögnunin greindi ekki í sundur, en þar er líklega um sömu arfgerð að ræða. Melting með einu ensími gaf einfalda leið til að staðfesta greiningu, en notkun tveggja eða fleiri ensíma samtímis hámarkaði breytileika skerðibútastærða. Þegar breytileikinn var notaður til að finna erfðatengsl með skyldleikatré NJT (Neighbor Joining Tree) komu fram tveir hópar. Í öðrum hópnum var nær eingöngu *R. leguminosarum* bv. *trifolii*, en hinn var fjölbreyttari og taldi aðrar tegundir. Aðferðin sem notuð var í þessari rannsókn greindi á milli *Rhizobium* tegunda og stofna og leiddi í ljós að sum sýnin höfðu verið rangt greind. Í greininni er einnig fjallað um notkun þessara aðferða við greiningu á skyldleika.

INTRODUCTION

Nitrogen fixation in leguminous plants (family Fabaceae or Leguminosae) by rhizobia, gram-negative symbiotic bacteria, is important in natural ecosystems and for agriculture. This is because the rhizobia are capable of utilising dinitrogen directly with their nitrogenases, whereas the plants have to get nitrogen in a chemically reduced form, which can be costly. Both the bacteria and their host plants are involved in determining host specificity and the interaction between the host plant and the rhizobial bacteria is often highly specific. For example, *Rhizobium leguminosarum* bv. *trifolii* infects only *Trifolium*, whereas *R. leguminosarum* bv. *viceae* can infect several genera including *Vicia*, *Pisum*, *Lathyrus* and *Lens* (Heidstra and Bisseling, 1996). There are also rhizobial species with broad host range (several species of *Rhizobium* and *Bradyrhizobium* can cross-inoculate different genera like *Phaseolus* and *Glycine*, and even non-legumes) and these legumes can receive more than one rhizobial species (Doyle, 1998). Therefore in a rhizobium-based cultivation it is important to select the most effective rhizobia for each crop, for example the use of *Bradyrhizobium* with lupine in reclamation areas of low temperature in Iceland (El-Mayas, 1996) and cold tolerant *R. leguminosarum* strains for white clover fodder crop (Svenning *et al*., 1997).

During such selection and breeding programmes it is necessary that the rhizobia investigated can be identified accurately. Several molecular markers have been developed for fingerprinting purposes and for studying phylogenetic relationships based on the chromosomal genomes (reviewed in Tas, 1997). Among these, the 16S-23S rDNA (ribosomal or rRNA genes) is one of the most applicable markers especially when analysed by PCR amplification together with (RFLP) restriction fragment length polymorphism (Laguerre *et al*., 1996; Tas *et al*., 1996). Other PCR-based methods, especially RAPD (random amplified polymorphic DNA), have been used for rhizobial typing (e.g. Kishinevsky *et al*., 1996). A more powerful method, AFLP (amplified fragment length polymorphism), recently developed by Keygene (Vos *et al*., 1995), has been used successfully with other bacteria (Lin *et al*., 1996). PCR-based methods do not required prior knowledge of the sequence data, however such markers are not without drawbacks, the former being non-transferrable between laboratories or databases and the latter being expensive and laborious. The rRNA genes, on the other hand, are site-targeted and hence making the analysis informative and reproducible. They can also reveal phylogenetic (and evolutionary) relationships in rhizobia, which is a very diverse group of bacteria. At least five rhizobial genera have been recognised: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Mesorhizobium* (Young and Haukka, 1996). Sequence analysis of 16S rRNA genes has highlighted the evolutionary diversity of these symbiotic bacteria, and has ruled out any broad co-evolution with their host plants (Doyle, 1998).

Table 1. The plant species and origin of the isolates[a)].
1. tafla. Tegundir plantna, Rhizobium *tegundir og uppruni ísólatanna[a)].*

Plant species	Rhizobia	Strains: number and origin	
Trifolium spp.	*Rhizobium leguminosarum* bv. *trifolii*	2,4,5)	Isolates from various locations in Iceland
		3)	Norway
Astragalus norwegicus	*Rhizobium* sp.	6)	Jötunheimar, Norway
Astragalus sp.		7)	Ultuna, Sweden
Lupinus nootkatensis	*Rhizobium* sp.	8)	LupH1B2, Haukadalur, S-Iceland
	Bradyrhizobium sp.	9)	LupS1, Skorradalur, W-Iceland
Thermopsis lupinoides	*Rhizobium* sp.	10)	Vladivostok, eastern Siberia
Lathyrus japonicus	*R. leguminosarum* bv. *viciae*	11)	Gunnarsholt, S-Iceland

a) Strain no. 1, a clover strain from Finland was excluded from the analysis due to contamination in culture—*Stofn nr 1, sem var smárastofn frá Finnlandi, var felldur út úr greiningunni vegna mengunar í ræktun.*

The aim of the present study was therefore to develop a simple and reliable method based on the rDNA-PCR for screening of rhizobial isolates used in ecological and agricultural field experiments in Iceland.

MATERIALS AND METHODS

Rhizobial isolates

The rhizobia used in the present study were isolated from nodules of different plant species mainly from Iceland and Scandinavia (Table 1). They included species and biovars in the genera *Rhizobium* and *Bradyrhizobium*. *R. leguminosarum* bv. *trifolii* was represented by four isolates, but other species/biovars had one to two isolates each. The pure cultures were maintained in YM (yeast manitol) medium at 26–28°C in the dark. The bacterial cells in liquid culture were harvested for DNA extraction according to the standard protocol for gram-negative bacteria (Johnson, 1991) and the genomic DNA was extracted as in Anamthawat-Jónsson and Heslop-Harrison (1995). The DNA yield was in the range of 0.2–1.5 µg/µl and the fragments were over 20-kb long.

PCR amplification

Universal rhizobial primer sequences for IGS regions of the rDNA were adopted from Laguerre *et al*. (1996) where primer FGPS1490 was TGC GGC TGG ATC ACC TCC TT and primer FGPS132' was CCG GGT TTC CCC ATT CGG. The primers were synthesised by TAGCopenhagen (Denmark). The PCR reaction, 50 µl, contained 40–100 ng of template DNA, 1 µM each of the two primers, 200 µM each of the four dNTPs (Amersham, UK), 2 mM $MgCl_2$, PCR buffer accompanying the enzyme and 0.6 unit of DNA polymerase (DynaZyme^{Tm} isolated from *Thermus brockianus*). PCR reactions were carried out in a Techne thermocycler (UK) with heated lid, using 40 cycles of the following temperature regime, 94°C 1 min, 55°C 1 min and 72°C 2 min, with the first cycle starting with the 95°C 5 min and the last cycle ended with 72°C 3 min. The PCR amplified products were separated by electrophoresis in 1.5–2% agarose gel. A negative control, without template DNA, was included in every PCR run. The 100-bp size marker (Pharmacia Biotech) was used. The images were visualised on UV transilluminator and recorded using Polaroid camera.

Restriction analyses

The rDNA-PCR amplified products were digested with the following restriction enzymes: 4-base cutters *Alu*I, *Hae*II, *Hpa*II, *Mbo*I and

*Taq*I, and 6-base cutters *Bam*HI, *Cla*I, *Hind*III, *Eco*RI, *Pst*I, *Pvu*II and *Sac*I. Single enzyme digestion was made by adding 3–5 units of the enzyme directly into 8–10 µl of the PCR amplified reaction mixture, whereas double or multiple digestion was performed by adding the enzymes that required the same buffer conditions together, or else sequentially. The restricted fragments were then electrophoresed the same way as the direct PCR products described above. The rDNA-RFLP profiles were analysed visually by comparing fragment sizes. To make a dendrogram (NJT, neighbour-joining tree), the RFLP fragments were scored from each of the isolates as present (1) and absent (0) and these scores were used to calculate genetic distance according Nei and Li (1979) and Dice (1945). The NJT phenogram was constructed from this distance matrix using RAPDistance program (Armstrong *et al*., 1996), singly from individual enzyme digestions as well as from combined data of different enzymes.

RESULTS

The present study revealed the followings. (1) A simple PCR amplification in the IGS regions of the rhizobial 16S-23S rDNA was able to give amplification profiles that differentiated species, biovars and strains of rhizobial isolates from Iceland, Scandinavia and Siberia. (2) Further analysis using endonuclease restriction of the PCR products confirmed the identification and produced specific profiles for genotyping the isolates. (3) A consensus rDNA-RFLP dendrogram showing genetic distances among the isolates corresponded with the rhizobial classification. The results presented here were reproducible in our laboratory. PCR reactions with different template concentrations were also performed to ensure reliable amplification.

Simple PCR amplification using universal rhizobial rDNA-IGS primers was able to discriminate species, biovars and strains of rhizobial isolates (Figure 1a). The amplified DNA fragments were between 400 and 1400-bp long and all the isolates could be differentiated except one pair of samples, no. 6 and no. 8. The profiles based on these fragments appeared to be isolate-specific. They were reproducible experimentally and therefore should be useful for identification of unknown isolates. Interestingly this PCR amplification detected a non-variable 1-kb fragment that persisted in all isolates of *Trifolium* (*R. leguminosarum* bv. *trifolii*, no. 2–5), regardless of country of origin. This could be used as a species/biovar specific marker. The fragment was also detected in the isolate from nodules of *Astragalus* from Sweden (no. 7), but not from *Astragalus* rhizobia from Norway (no. 6). It is therefore possible that the Swedish sample was misidentified. On the other hand, the profile of the Norwegian *Astragalus* isolate (no. 6) is identical to that of isolate no. 8, rhizobia from nodules of *Lupinus* in Iceland. One of these two isolates was probably misidentified too, or there was a mix up in the laboratory as the DNA analysis was made from cultures, not directly from nodules. Further studies are required to solve the discrepancy. It is also possible that a broad-range *Rhizobium* could inoculate both *Astragalus* and *Lupinus*, and these legume genera can receive more than one rhizobial strains or species.

Further analysis of the isolates was performed by fractionation of the PCR amplified products with restriction endonucleases, creating restriction fragment length polymorphism (RFLP). Twelve restriction enzymes were used, one at a time. Six of these (i.e. *Eco*RI, *Hae*III, *Mbo*I, *Taq*I, *Pst*I and *Pvu*II) were able to differentiate all isolates except the same one pair that could not be differentiated by the PCR amplification (no. 6 and 8). An example of distinct genotype profiles generated by single enzyme digestion of the PCR products is shown in Figure 1b (*Hae*III digestion). Once again the *Trifolium* isolates are more similar to one another than they are to the other isolates. The *Hae*III restriction sites occur in all samples of *Trifolium* isolates, as the full-length PCR products were restricted resulting in smaller RFLP

fragments ranging between 200 and 800 bp. In this way the RFLP was able to increase the resolution of polymorphism, confirming the rhizobial identification. Other single enzymes were equally powerful in discriminating the genotypes. The use of single enzyme digestion therefore provided a simple means to verify rhizobial identification.

The use of more than one restriction enzymes, one after another or simultaneously, maximised resolution of the RFLP profiles (e.g. Figure 1c – *Bam*HI, *Pvu*II and *Hind*III). This approach not only confirmed the rhizobial identification by PCR alone or by PCR-RFLP using single enzymes, but it also revealed differences in certain pairs of isolates that were not apparent before. The sample no. 7 (*Astragalus* rhizobia from Sweden) is now clearly different from *R. leguminosarum* bv. *trifolii* (no. 2–5), as its 1-kb PCR species-specific band in Figure 1a has resolved differently here. The *Astragalus* rhizobia could be closely related, genetically, to the *Trifolium* rhizobia, rather than the sample being misidentified. The multiple enzymatic approach in general is not necessary for identification of isolates, especially where rapid screening is required. But in certain situations, such as the pair of isolates no. 6 and 8, the approach revealed differences for the first time. The 500-bp fragment is present in no. 8 but not in no. 6 (Figure 1c). These two isolates are therefore not identical. However, such relatively small difference is probably not significant enough to conclude that they are different isolates. Therefore, the conclusion is that the same rhizobial strain inoculated roots of Norwegian *Astragalus* (no. 6) and Icelandic *Lupinus* (no. 8), or one of these isolates was misidentified or there was a mix up of pure cultures. According to the nodule morphology, this lupine *Rhizobium* appeared very different from lupine rhizobia in general, and this must be further investigated.

The rDNA-RFLP not only confirmed the species and strain identification, but also revealed genetic relationship among the rhizobial isolates (Figure 2a – a consensus dendrogram based on eight enzymes; Figure 2b – based on

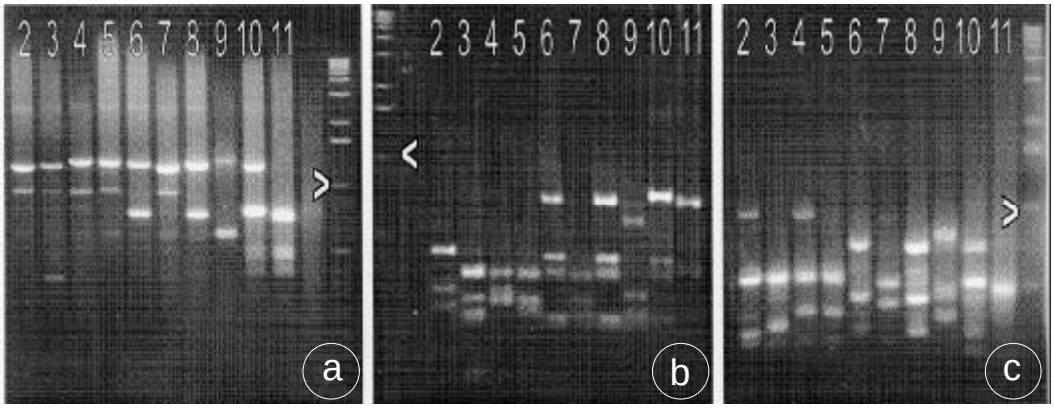

Figure 1. Gel electrophoresis in 2% agarose of (a) PCR amplification in the IGS regions of the rhizobial 16S-23S rDNA, (b) *Hae*III digestion of the PCR amplified products and (c) triple enzyme digestion of the PCR products using *Bam*HI, *Pvu*II and *Hind*III. Lanes 2 to 11 correspond to the rhizobial isolates numbered as in Table 1. Size marker is with 100-bp intervals and the arrow indicates 1 kb-fragment. The unnumbered lane in gel (a) shows the standard negative PCR reaction control without template DNA.
1. mynd. Rafdráttur í 2% agarósa. (a) DNA mögnun á IGS svæði 16S-23S ríbósómgena í rhizobíu, (b) Hae*III melting á afurðum DNA mögnunar, (c) melting á DNA mögnun með þremur ensímum,* Bam*HI,* Pvu*II og* Hind*III. Í rásum 2–11 eru rhizobíu sýni. Númerin svara til 1. töflu. Stærðarviðmiðun hleypur á um 100 bp og örin bendir á 1 kb bút. Ómerkta rásin á geli (a) inniheldur viðmiðun.*

one enzyme *Hpa*II). The genetic distance analysis showed two clusters: one of these included essentially *R. leguminosarum* bv. *trifolii* and the other, which was a more diverse group, included other species. Within the *Trifolium* biovar, the isolates are genetically similar and this seems to be independent of their geographic origin. The Swedish and Norwegian *Astragalus* rhizobia (no. 7 and no. 6) were placed in different clusters (Figure 2), indicating that they are at least not the same rhizobial species. The *Bradyrhizobium* from lupine (no. 9) is distinct. Overall, the genetic distances correspond relatively well to the rhizobial classification and identification.

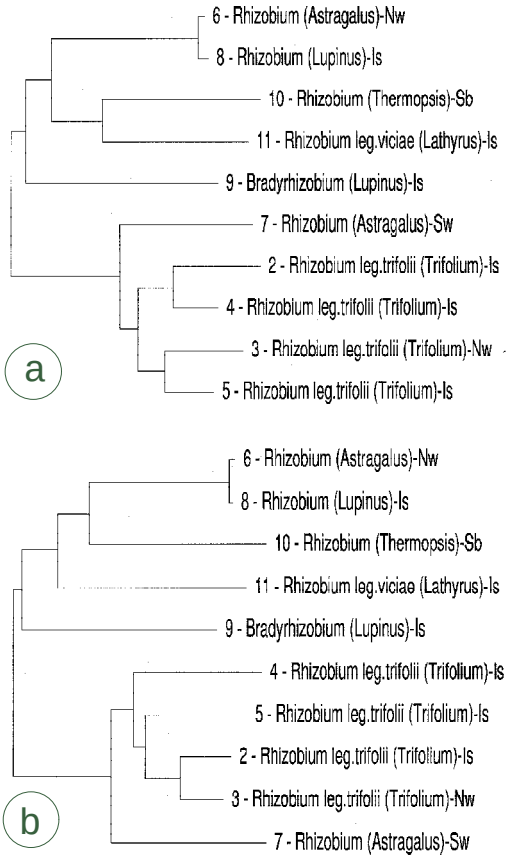

Figure 2. Neighbour joining tree of rDNA-RFLPs of ten rhizobial isolates (as in Table 1) based on (a) 105 polymorphic bands generated by restriction of the PCR amplified products with eight endonucleases, *Alu*I, *Hae*II, *Hpa*II, *Mbo*I, *Taq*I, *Bam*HI, *Hind*III and *Pvu*II, and (b) 11 bands generated by single *Hpa*II digestion.
2. mynd. Skyldleikatré af tíu rhizobíu sýnum sem byggir á breytileika skerðibútastærða rDNA svæða. Tréið er byggt á (a) 105 skerðibútum sem urðu til við meltingu á DNA mögnunum með átta ensímum, Alu*I,* Hae*II,* Hpa*II,* Taq*I,* Mbo*I,* Bam*HI,* Hind*III og* Pvu*II og (b) 11 böndum sem urðu til við meltingu á DNA mögnunum með* Hpa*II.*

DISCUSSION

Genotyping ten pure culture isolates of rhizobia belonging to four species of *Rhizobium* and one species of *Bradyrhizobium*, from Iceland, Scandinavia and Siberia, was performed using PCR amplification of the IGS region of 16S-23S ribosomal genes (rDNA-PCR) and restriction fragment length polymorphism (RFLP) of the PCR products. The simple PCR amplification itself appears to be sufficient to differentiate all the isolates except a pair of probably the same isolate. The PCR-RFLP, a procedure for higher resolution, confirms the simple PCR identification and produces genotype-specific profiles that can be used to identify unknown samples. The method does not require a prior knowledge of sequence data or an elaborate fingerprint database, hence is suitable as a rapid screening and preliminary analysis of unknown material, which is the situation often encountered in agricultural and field experiments.

This simple and rapid method for typing of rhizobial species and strains is very useful as a tool for managing biological studies of the legume-rhizobium establishment in the field, especially in the situation like ours where laboratory facilities and staffs are extremely limited. Although the study was made on bacterial pure cultures and full scale DNA purification, there is little doubt that the method can be modified to suit applications such as identification in mixed cultures, complex samples or crude extracts from nodules. A Southern hybridisation experiment showed undetectable homology between rhizobial (prokaryotic) and plant (eukaryotic) rDNA, and so a significant

competition in a PCR reaction would be unlikely. Hybridisation or PCR methods do not necessary require DNA extraction but can be performed directly on crushed nodules or sonicated samples (Nick and Lindström, 1994). The PCR is also sensitive and the amount of template DNA required is in a nano-scale and hence making the typing possible with small sample sizes. Furthermore, a species-specific primer, which can be obtained for example by cloning and sequencing of the *R. leguminosarum* bv. *trifolii* PCR fragment detected in our study (Figure 1a), can be used to positively identify a species and type the isolates within species without cross-detection with other rhizobia or other bacteria. A few such rDNA sequences have already been made including both broad range rhizobial probes and species- or strain-specific probes (reviewed in Ludwig *et al*., 1998).

The polymorphism in the IGS regions of the rDNA detected in the present study seems to be larger than that from other studies, for example Laguerre *et al*. (1996) where the same primers were used but the PCR amplification could not differentiate the rhizobia without further RFLP analysis. Although the samples examined here are very few, the results may still indicate high level of genetic variation and differentiation among rhizobia, which in turns may be the characteristics of sub-arctic species. ECM mycorrhizal fungi in the arctic-alpine regions, for example, are found to be very diverse in species number and composition, possibly due to extreme environments and unique history of glaciation and species migration to these regions (Gardes and Dahlberg, 1996).

A consensus tree based on the PCR-RFLPs generated in the present study is in a reasonable agreement with the recognised classification of rhizobia. It shows the rhizobia from *Trifolium* to be clearly divergent from all the others, whereas the genetic relationships among the other rhizobia seem to be intermixed even after eliminating samples that may have been misidentified (Figure 2). This is not surprising because the rhizobia analysed here have been obtained from nodules of legumes in the genera *Trifolium*, *Lathyrus*, *Lupinus*, *Astragalus* and *Thermopsis*, all from the largest subfamily Papilionoideae (Leguminosae). In this subfamily, the nodules are highly diverse and consisting of virtually all types, e.g. the branched and indeterminate *astragaloid*, the round and determinate *desmodioid* and the unbranched, elongate and indeterminate *caesalpinoid* nodules (Young and Haukka, 1996; Doyle, 1998). Some rhizobial species have broad host range, others are species- or biovar-specific, and some legumes can receive more than one rhizobial species. *R. leguminosarum* bv. *trifolii* in particular is known to be highly specific and infect only *Trifolium* (Heidstra and Bisseling, 1996) and hence is likely to be genetically isolated and consequently becomes divergent. It has been shown often that estimates of rhizobial phylogeny can only be obtained from the sequence data, whereas the PCR-rDNA is a simple and powerful method to detect and characterise diversity even with species (Laguerre *et al*., 1997; Khbaya *et al*., 1998; Moreira *et al*., 1998). However, analysis of PCR-RFLP of 23S rRNA genes in *R. galegae* was shown to correlate with sequence data analysis, confirming its validity in phylogeny study (Terefework *et al*., 1998). This is supported by the study of *Bradyrhizobium* strains in Canary Islands (Vinuesa *et al*., 1998) which is based on PCR-RFLPs of the IGS regions of the 16S-23S rDNA the same way as in our study that is presented here. The analysis of 16S-23S rDNA-RFLP should therefore be useful to study phylogenetic relationships among these isolates further and more specifically.

ABBREVIATIONS

PCR (polymerase chain reaction), rDNA (ribosomal DNA, rRNA genes), IGS (intergenic spacer), RFLP (restriction fragment length polymorphism), RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), YM (yeast manitol), bv. (biovar).

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