

RESEARCH TECHNIQUES FOR ESTIMATING PHENOTYPIC AND GENOTYPIC DIVERSITY OF ROOT- AND STEM-NODULE BACTERIA

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Abstract

Root- and stem-nodule bacteria symbiotically nodulate and fix nitrogen with certain leguminous plants. Members in 14 genera have been reported as nitrogen-fixing symbionts. Phenotypic and genotypic diversity of these symbiotic bacteria have been revealed, providing valuable information, through the use of methodologies for phylogenetic analyses. Numerical analysis based on phenotypic characteristics and techniques based on DNA analysis, including 16S rDNA sequence analysis, RAPD, rep-PCR, AFLP, RFLP and PCR-RFLP, have been widely used for this purpose. A brief overview of these techniques is required to evaluate their suitability, discriminating power and limitation.

Keywords: Diversity, Root- and stem-nodule bacteria, numerical analysis, RAPD, rep-PCR, AFLP, RFLP, PCR-RFLP.

Introduction

Biological nitrogen fixation represents the major source of nitrogen input in agricultural soils. The major N₂-fixing systems are the symbiotic systems which can play a significant role in improving the fertility and productivity of low-N soils (Zahran, 1999). The specific groups of bacteria have the ability to infect in roots (or stems) of leguminous plants, causing the formation of a new organ called “nodule” and establishing a nitrogen-fixing symbiosis. Within the nodules these symbiotic bacteria fix

atmospheric nitrogen into ammonia, providing the nitrogen requirements of cultivated legumes (Hartman and Amarger, 1991). Nitrogen fixation, that is a reduction of nitrogen gas into ammonia, is catalyzed by nitrogenase. Nitrogenase is a complex enzyme consisting of two components. Component I or dinitrogenase or FeMo protein is an $\alpha_2\beta_2$ heterotetramer of about 220 KDa which is the catalytic site for N₂ reduction. Component II or dinitrogenase reductase or Fe protein is an homodimer of about 60 KDa which

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is reduced *in vivo* by low-potential electron donors. Electrons are then transferred one at a time to the FeMo protein in a process that involves MgATP hydrolysis. This cycle repeats until enough electrons have been provided for complete substrate reduction (Kaminski *et al.*, 1998). Consequently, these bacteria are of enormous agricultural and economic value (de Philip *et al.*, 1992). Formerly these bacteria, collectively called “rhizobia”, belonged in the family Rhizobiaceae which consists of two genera, *Rhizobium* and *Bradyrhizobium*. The classification of these genera is based on growth rate, host specificity and production of acid or alkaline (Jordan, 1984; Somasegaran and Hoben, 1994). Classification of root- and stem-nodule bacteria is becoming increasingly complex and is revised periodically because of new findings that propose new genera and new species. DNA related values, 16S rDNA homology values and some phenotypic characteristics provide more and deeper information for the classification of these bacteria. Some species in genera *Rhizobium* and *Bradyrhizobium* were later moved into new genera based on phylogenetic analyses. At the present time, rhizobia have been classified, mainly by comparison of the sequences of the 16S rRNA genes, into 6 genera (*Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Rhizobium*, *Sinorhizobium*) of the α -2 subclass of Proteobacteria (Wang and Martinez-Romero, 2000; Ngom, *et al.*, 2004). Recently, members of α -Proteobacteria including *Methylobacterium nodulans* isolated from the herbal legume, *Crotalaria* (Sy *et al.*, 2001), *Blastobacter denitrificans* isolated from *Aeschynomene indica* (van Berkum and Eardly, 2002), *Devosia neptuniae* isolated from the aquatic legume, *Neptunia natans* (Rivas *et al.*, 2002), *Ochrobactrum* isolated from *Acacia mangium* (Ngom *et al.*, 2004) and *Phyllobacterium trifolii* isolated from *Trifolium pratense* (Valverde *et al.*, 2005) have been reported as nitrogen-fixing symbionts of legumes. Furthermore, three members of the β -Proteobacteria, *Burkholderia* (Moulin *et al.*, 2001; Chen *et al.*, 2005; Barrett and Parker, 2006; Chen *et al.*, 2006), *Cupriavidus*

taiwanensis (formerly, *Ralstonia taiwanensis*) isolated from *Mimosa* (Chen *et al.*, 2001) and *Herbaspirillum lusitanum* isolated from *Phaseolus vulgaris* (Valverde *et al.*, 2003) have also been reported as symbiotic nitrogen-fixing members. The classification of these root- and stem-nodule bacteria are shown in Table 1.

Diversity in root- and stem-nodule bacteria has been revealed by many studies and almost all of the data reported previously indicate that there is a high level of genetic diversity in these bacteria. An assessment of the genetic diversity and genetic relationships among strains could provide valuable information about bacterial genotypes that are well adapted to a certain environment (Niemann *et al.*, 1997). Numerical analysis based on phenotypic characteristics and techniques based on DNA analysis have been used. In particular, the techniques based on DNA analysis that are frequently used are i) sequence analysis of 16S rDNA; ii) random amplified polymorphic DNA (RAPD); iii) analysis of repetitive sequences including Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC) and BOX sequence; iv) amplified fragment length polymorphism PCR (AFLP); v) restriction fragment length polymorphism (RFLP) and PCR-RFLP. These techniques are powerful tools for revealing the genetic diversity and phylogeny of bacteria.

Numerical Analysis

Numerical analysis has been used widely to study and compare rhizobia. Many previous studies have used a large range of biochemical and metabolic tests to differentiate between rhizobial species. The phenotypic characteristics used frequently in the numerical analysis are i) utilization of carbon sources such as L-rhamnose, D-arabinose, L-(+)-arabinose, L-fucose, D-(+)-raffinose, D-xylose, D-mannose, L-sorbose, fructose, D-galactose, D-cellobiose, inulin, D-(+)-melezitose, D-turanose, D-lyxose, D-trehalose, maltose, lactose, sucrose, glucose, D-(-)-ribose, D-meli-biose, D-(-)-tagatose, mannitol, sorbitol, dulcitol, inositol, meso-

Table 1. The classification of root- and stem-nodule bacteria

Class	Family	Genus	Species
Alpha-proteobacteria	Rhizobiaceae	<i>Rhizobium</i>	<i>R. cellulosilyticum</i> ; <i>R. daejeonense</i> ; <i>R. etli</i> ; <i>R. galegae</i> ; <i>R. gallicum</i> ; <i>R. genosp.</i> ; <i>R. giardinii</i> ; <i>R. hainanense</i> ; <i>R. huautlense</i> ; <i>R. indigoferae</i> ; <i>R. leguminosarum</i> ; <i>R. loessense</i> ; <i>R. lupini</i> ; <i>R. lusitanum</i> ; <i>R. mongolense</i> ; <i>R. phaseoli</i> ; <i>R. sullae</i> ; <i>R. tianshanense</i> ; <i>R. tropici</i> ; <i>R. yanglingense</i> ; <i>Rhizobium</i> sp.
		<i>Sinorhizobium</i>	<i>S. arboris</i> ; <i>S. fredii</i> ; <i>S. kostiense</i> ; <i>S. kummerowiae</i> ; <i>S. medicae</i> ; <i>S. meliloti</i> ; <i>S. morelense</i> ; <i>S. saheli</i> ; <i>S. teranga</i> ; <i>S. xinjiangense</i> ; <i>Sinorhizobium</i> sp.
		<i>Allorhizobium</i>	<i>A. undicola</i>
	Bradyrhizobiaceae	<i>Bradyrhizobium</i>	<i>B. canariense</i> ; <i>B. denitrificans</i> ; <i>B. elkani</i> ; <i>B. genosp.</i> ; <i>B. japonicum</i> ; <i>B. liaoningense</i> ; <i>B. yuanmingense</i> ; <i>Bradyrhizobium</i> sp.
Alpha-proteobacteria	Phyllobacteriaceae	<i>Mesorhizobium</i>	<i>M. albisiae</i> ; <i>M. amorphae</i> ; <i>M. chacoense</i> ; <i>M. ciceri</i> ; <i>M. huakuii</i> ; <i>M. loti</i> ; <i>M. mediterraneum</i> ; <i>M. plurifarium</i> ; <i>M. septentrionale</i> ; <i>M. temperatum</i> ; <i>M. tianshanense</i> ; <i>Mesorhizobium</i> sp.
		<i>Phyllobacterium</i>	<i>P. trifolii</i>
	Hyphomicrobiaceae	<i>Azorhizobium</i>	<i>A. caulinodans</i> ; <i>A. sesbaniae</i> ; <i>Azorhizobium</i> sp.
		<i>Devosia</i>	<i>D. neptuniae</i>
	Methylobacteriaceae	<i>Methylobacterium</i>	<i>M. nodulans</i>
	Brucellaceae	<i>Ochrobactrum</i>	<i>O. cytisi</i> ; <i>O. lupini</i>
Beta-proteobacteria	Burkholderiaceae	<i>Cupriavidus</i>	<i>C. taiwanensis</i>
		<i>Burkholderia</i>	<i>B. nodosa</i> , <i>B. caribensis</i> ; <i>B. mimosarum</i> ; <i>B. phymatum</i> ; <i>B. tuberum</i>
	Oxalobacteriaceae	<i>Herbaspirillum</i>	<i>H. lusitanum</i>

erythritol, glycerol, isopropylalcohol, adonitol, ethylene glycol, esculin, salicin, creatinine, L-glutamine, casein hydrolysate, sodium lactate, ammonium oxalate, sodium citrate, sodium D-gluconate, vanillic acid, calcium malonate, sodium succinate, sodium D-malate, sodium pyruvate, sodium hippurate, and D-arabitol; ii) utilization of nitrogen sources such as DL-arginine hydrochloride, L-methionine,

6-furfurylaminopurine, thymine, L-threonine, L-serine, DL-threonine, L-tryptophan, L-lysine, glycine, D-serine, DL-methionine, DL-phenylalanine, L-cysteine, DL-proline, L-arginine, D-methionine, L-cystine, L-tyrosine, L-leucine, L-proline, L-isoleucine, L-histidine, L-valine, DL- α -alanine, DL-valine, aspartic acid, D-histidine, L-glutamic acid and cytosine; iii) requirement of vitamins; iv) tolerance to dyes;

v) tolerance to antibiotics; vi) growth at different pH; vii) growth in NaCl; viii) reaction in litmus milk; ix) growth in peptone broth; x) reduction of methyl blue; xi) nitrate reduction; xii) growth in different temperature; xiii) production of enzyme; xiv) acid and alkaline production; xv) colony morphology; xvi) the presence of peritrichous or polar flagella, and xvii) growth rate (Chen *et al.*, 1995; Xu *et al.*, 1995; Chen *et al.*, 1997). According to Xu *et al.* (1995), extra-slowly growing (ESG) soybean rhizobia were compared with reference strains belonging to the genera *Bradyrhizobium*, *Rhizobium*, and *Agrobacterium* by performing a numerical analysis of 191 phenotypic features. *Bradyrhizobium* and *Rhizobium* strains were placed in two distinct phenotypic groups. All of the ESG strains examined clustered closely in the genus *Bradyrhizobium* but were separated from *Bradyrhizobium japonicum* at the species level. On the basis of numerical analysis and techniques based on DNA analysis including G+C content, DNA-DNA hybridization, 16S rDNA sequence analysis, and N/C ratio analysis, the ESG soybean rhizobia were proposed as *Bradyrhizobium liaoningense*. Chen *et al.* (1995) performed a numerical analysis of 148 phenotypic characteristics of root nodule bacteria isolated from an arid saline desert soil. The results obtained from numerical analysis and DNA homology values supported that moderately and slow-growing strains which produced acid are members of a new species *Rhizobium tianshanense*. Dendrogram obtained from numerical analysis showed the relationship among *R. tianshanense* and related species. Chen *et al.* (1997) proposed *Rhizobium hainanense* on the basis of 16S rRNA gene sequencing, DNA-DNA hybridization and phenotypic characterization. However, the use of traditional methods have been limited mainly by highly similar phenotypic characteristics that usually occur among closely related strains.

Sequence Analysis of 16S rDNA

The most dramatic progress in the construction of microbial phylogeny is based on sequencing

analysis of the ribosomal genes. The 16S or small subunit ribosomal RNA gene is useful for estimating evolutionary relationships among bacteria because it is slowly evolving and the gene product is both universally essential and functionally conserved (van Berkum and Eardly, 1998). Direct sequencing of genes coding for 16S rRNA (16S rDNA) has been used to establish genetic relationships and to characterize strains at the species or higher level (Laguerre *et al.*, 1996). The full-length sequence analysis of 16S rDNA is one of the most important methods to estimate the phylogeny of rhizobia (Young and Haukka, 1996), while the 900 bp partial 16S rDNA sequencing correlated well with full-length 16S rDNA sequencing (Terefework *et al.*, 1998) and has been used for rapid screening of the phylogenetic relationships among a large number of rhizobia. Sequences of 16S rDNA are known to be highly conserved among eubacteria (Woese, 1987) and analysis of genetic variations in this region is not appropriate to differentiate strains within species (Laguerre *et al.*, 1996). However, it is very useful for identification of species. Pairs of universal primers, forward and reverse primers, were design for amplification of 16S rDNA regions in most eubacteria. Pairs of universal primers were used to amplify 16S rDNA (Lane, 1991; van Berkum and Fuhrmann, 2000) to ascertain the non-symbiotic isolates belonging to the genus *Bradyrhizobium* (Pongsilp *et al.*, 2002). Novel nitrogen-fixing symbionts in genera *Methylobacterium*, *Blastobacter*, *Burkholderia*, *Ralstonia*, *Ochrobactrum*, *Devosia*, *Phyllobacterium* and *Herbaspirillum* have been discovered by 16S rDNA sequence analysis (Chen *et al.*, 2001; Sy *et al.*, 2001; Rivas *et al.*, 2002; van Berkum and Eardly, 2002; Valverde *et al.*, 2003; Ngom *et al.*, 2004; Chen *et al.*, 2005; Valverde *et al.*, 2005; Barrett and Parker, 2006; Chen *et al.*, 2006). These findings suggest that the gene responsible for symbiosis with legumes is transmissible horizontally and functions in a relatively wide range of bacterial taxa (Fuentes *et al.*, 2002; Rivas *et al.*, 2002). Phylogenetic analysis of the 16S rDNA has been constructed in many previous

studies. According to Ngom *et al.* (2004), the clusters in the phylogenetic tree, which was constructed based on nearly the full length of 16S rDNA, correlated well with the taxonomy of strains: i) a first cluster contains *Bradyrhizobium* and *Blastobacter* in Bradyrhizobiaceae; ii) a second cluster contains *Ochrobactrum* in Brucellaceae; iii) a third cluster consists of two genera *Phyllobacterium* and *Mesorhizobium* in Phyllobacteriaceae; iv) a fourth cluster consists of genera *Sinorhizobium*, *Allorhizobium* and *Rhizobium* in Rhizobiaceae. Besides 16S rDNA, sequence analysis of 23S or large subunit ribosomal RNA gene has been also studied. However, the 23S rRNA gene has not been extensively used to estimate the genetic relationships among the Rhizobiaceae, but there are several dramatic differences which may be helpful for classification and identification purposes (van Berkum and Eardly, 1998). Terefework *et al.* (1998) reported that the 23S dendrogram showed deeper branching than the 16S dendrogram and more genotypes were resolved, although in some cases the sequence divergence is not particularly high.

Random Amplified Polymorphic DNA (RAPD)

This technique is a type of polymerase chain reaction (PCR). A single primer called "arbitrary primer" (8-12 nucleotides) binds and amplifies the segments of DNA randomly throughout the genome. DNA fragments of different molecular sizes are generated from template DNA. Separation of the products by agarose gel electrophoresis often reveals polymorphism among isolates (Strange, 2003). The gels are photographed for quantitative comparisons of the lanes according to the presence of products or their absence across lanes. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction. The resultant binary matrix is used to produce a dendrogram (van Berkum and Eardly, 1998). The approach relies upon genetic variation among genomes for the relative locations of the targets of the primers, which results in amplification products varying

in molecular size. The development of RAPD marker provided a powerful tool for investigating genetic polymorphisms in many different organisms and recently this method has been used for *Rhizobium* identification and *Bradyrhizobium* genetic analyses (Kosier *et al.*, 1993; Kay *et al.*, 1994). Lunge *et al.* (1994); Nishi *et al.* (1996); Nuntagij *et al.* (1997) reported the usefulness of RAPD analyses in the characterization of *Bradyrhizobium* strains. Paffetti *et al.* (1996) and de Oliveira *et al.* (2000) investigated the genetic diversity of *Rhizobium* populations by RAPD. Paffetti *et al.* (1996) demonstrated the considerable level of genetic diversity among *Rhizobium meliloti* strains which were phenotypically indistinguishable. de Oliveira *et al.* (2000) showed the great genetic heterogeneity among *Rhizobium tropici* and *Rhizobium leguminosarum* bv. *phaseoli* strains. Besides being simpler and cheaper, this method is as effective as the more labor intensive RFLP for establishing genetic relationships and identifying *Rhizobium* strains (Laguerre *et al.*, 1996; Selenska-Pobell *et al.*, 1996; de Oliveira *et al.*, 2000). The results of Niemann *et al.* (1997) showed that RAPD PCR discriminated slightly better among *Rhizobium meliloti* strains than ERIC PCR. This might be explained by minor changes in RAPD primer binding sites which are under no constraints to sequence conservation among closely related strains. A significant limitation is the identity of each product across the different genome templates. Products of identical molecular size need not represent the identical region in each genome but could be the same size by mere coincidence. Therefore, dendrograms constructed need not reflect accurately the genetic relationships among genomes (van Berkum and Eardly, 1998) and they do not permit the investigation of the diversity of symbiotic plasmids among chromosomally closely related strains (Laguerre *et al.*, 1996). The technique is likely to be useful for determining the variation within species (van Berkum and Eardly, 1998). Another limitation is that the performance of RAPD is also sensitive to many factors such as selection of primers,

magnesium concentration in the PCR buffers and the thermocycler used for PCR (Lin *et al.*, 1996).

Analysis of Repetitive Sequences Including Repetitive Extragenic Palindromic (REP), Enterobacterial Repetitive Intergenic Consensus (ERIC) and BOX Sequence

Rep-PCR DNA fingerprints can also be generated by primers derived from conserved repeat sequences present in bacterial genome (Versalovic *et al.*, 1991). These include pairs of primers for amplification of repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC) sequences (Versalovic *et al.*, 1991; Laguerre *et al.*, 1996) and a single primer for BOX sequences (Stern *et al.*, 1984; Hulton *et al.*, 1991; Martin *et al.*, 1992). This PCR-based method relies upon the same approach as RAPD, but different primers are used. The comparison of DNA sequences of the conserved inverted repeats in both REP- and ERIC-type elements has allowed the derivation of REP and ERIC consensus sequences (Hulton *et al.*, 1991). de Bruijn (1992) demonstrated the usefulness of DNA fingerprinting by PCR using REP and ERIC primers for the identification and classification of members of several *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* species. The patterns of the resulting PCR products were found to be highly specific for each strain, suggesting that the REP and ERIC-PCR method is useful for the identification and classification of bacterial strains since they allow the fingerprinting of individual genera, species and strains and help to determine phylogenetic relationships. The methods have been used to type several rhizobial strains (Laguerre *et al.*, 1996) and offer an alternative or additional approach for the measurement of genetic diversity within rhizobial species (van Berkum and Eardly, 1998). Many previous studies demonstrated that rep-PCR methods are suitable for distinguishing strains at the species level and below (Versalovic *et al.*, 1991; de Bruijn, 1992; Martin *et al.*, 1992;

Nick and Lindström, 1994; Versalovic *et al.*, 1994; Vos *et al.*, 1995; Janssen *et al.*, 1996; Huys *et al.*, 1996; Nick *et al.*, 1999). A high level of genetic diversity among soybean rhizobia was detected by an ERIC-rep-PCR analysis (Chen *et al.*, 2000). Gao *et al.* (2001) investigated the genetic diversity among 95 isolates from *Astragalus adsurgens* and found that all of the isolates and 24 reference strains could be differentiated by REP-, ERIC- and BOX-PCR fingerprinting analysis. Vinuesa *et al.* (1998) reported the use of rep-PCR fingerprinting with BOX, ERIC and REP primers to study genotypic diversity among *Bradyrhizobium* strains and exploited the taxonomic resolution of rep-PCR by combining BOX, ERIC and REP-PCR genomic fingerprints, maximizing strain discrimination and the phylogenetic coherency of the obtained cluster. Although the PCR-based methods are thought to be limited for the investigation of diversity of symbiotic genes among chromosomally closely related strains, Pongsilp *et al.* (2002) indicated that the symbiotic strains and the non-symbiotic strains of *Bradyrhizobium japonicum* and *Bradyrhizobium elkanii* could be separated into two distinct clusters based on rep-PCR fingerprinting analyses, using the BOXA1R primer. Like RAPD analysis, this PCR-based method offers a convenient alternative to conventional RFLP analyses with the same range of levels of resolution and the same possibility of typing either the whole genome or specific DNA regions. As they are much less time-consuming, avoiding fastidious DNA extraction and hybridization, they are more suitable for large scale identification and classification of bacterial collections and for study of large populations at the intraspecies level (Laguerre *et al.*, 1996).

Amplified Fragment Length Polymorphism PCR (AFLP)

AFLP is a PCR-based DNA fingerprinting technique. AFLP overcomes many of the problems of RFLP and RAPD. In AFLP analysis, there are three major steps in the procedure: i) restriction endonuclease digestion of genomic DNA and

the ligation of double-stranded adapters. These consist of a core sequence and an enzyme-specific sequence and serve as primer sites for amplification of the restriction products (Strange, 2003); ii) amplification of the restriction fragments by PCR using primer pairs containing common sequences of the adapter and one to three arbitrary nucleotides; iii) analysis of the amplified fragments using gel electrophoresis. The combination of different restriction enzymes and the choice of selective nucleotides in the primers for PCR make AFLP a useful new system for molecular typing of microorganisms (Lin *et al.*, 1996). Like rep-PCR methods, AFLP has been reported to be suitable for distinguishing strains at the species level and below (Versalovic *et al.*, 1991; de Bruijn, 1992; Martin *et al.*, 1992; Nick and Lindström, 1994; Versalovic *et al.*, 1994; Vos *et al.*, 1995; Huys *et al.*, 1996; Janssen *et al.*, 1996; Nick *et al.*, 1999). Janssen *et al.* (1996) demonstrated the superior discriminative power of AFLP towards the differentiation of highly related bacterial strains that belong to the same species or even biovar (i.e. to characterize strains at the infrasubspecific level). In case of root- and stem-nodule bacteria, Gao *et al.* (2001) used molecular biological methods to investigate the genetic diversity among 95 isolates from *Astragalus adsurgens*. All of the isolates and 24 reference strains could be differentiated by AFLP, REP-, ERIC- and BOX-PCR fingerprinting analysis and some of the AFLP groups also covered several rep-PCR groups. The diversity of bradyrhizobia from *Faidherbia albida* and various *Aeschynomene* species was estimated using AFLP analysis. The AFLP technique was shown to provide an insight into the extent of genotypic diversity of *Bradyrhizobium* isolates. As a grouping method for new isolates it was superior to restriction analysis of amplified 16S rDNA (ARDRA), analysis of BIOLOG metabolic profiles and SDS-PAGE analysis of proteins because of its higher taxonomic resolution (Willems *et al.*, 2000). AFLP data produced grouping in line with analysis of the sequences of the 16S-23S rDNA intergenic spacer data. However, the limitation

of AFLP was previously reported. The AFLP procedure is rather laborious (Willems *et al.*, 2000) and it could not reflect more remote relationships (DNA homology level of 40 - 60%) between species (Willems *et al.*, 2001).

Restriction Fragment Length Polymorphism (RFLP) and PCR-RFLP

RFLP approaches in determining genetic relationships are based on restriction site analysis and hybridization. Total DNA digested are examined for fingerprint patterns with specific probes after standard southern hybridization (van Berkum and Eardly, 1998). RFLP has been used to examine the genetic diversity of *Rhizobium* (Demezas *et al.*, 1991; Paffetti *et al.*, 1996). RFLP analysis has demonstrated the diversity of sym (symbiotic) plasmid types within *Rhizobium leguminosarum* populations (Demezas *et al.*, 1991). Paffetti *et al.* (1996) investigated the genetic diversity of *Rhizobium meliloti* populations and found that RFLP analysis of nod/nol operon were consistent with RAPD results. PCR-RFLP is used in determining genetic relationships based upon the PCR and restriction site analysis. Specific regions of the genome are amplified and fingerprint patterns are obtained after restriction digestion of the amplification products. The banding pattern across different enzyme digests are used to estimate the genetic diversity (van Berkum and Eardly, 1998). PCR-RFLP of the 16S rDNA has been used in the analysis of legume symbionts (Laguerre *et al.*, 1994). The 16S rDNA has turned out to be a very good tool for the assessment of organismal phylogenies down to the genus level (Terefework *et al.*, 1998). PCR-RFLP analysis of 16S rDNA has been used to determine the phylogenic position of root- and stem-nodule bacteria including *Rhizobium* (Terefework *et al.*, 1998; Wang *et al.*, 1999; Diouf *et al.*, 2000), *Mesorhizobium* (Wang *et al.*, 1999). Besides PCR-RFLP of the 16S rRNA gene, some other genes have been used. These include 23S rRNA gene (Terefework *et al.*, 1998), 16S-23S rRNA intergenic spacer region (ITS-PCR-RFLP) (Laguerre *et al.*, 1996; Diouf *et al.*,

2000; Laguerre *et al.*, 2003), and symbiotic genes (Laguerre *et al.*, 1996; Laguerre *et al.*, 2003).

PCR-RFLP with nine restriction enzymes was applied to the 16S and 23S rRNA gene of rhizobial strains to determine the phylogenetic position of *Rhizobium galegae*. The results showed that clustering of the strains in the dendrogram constructed from RFLP analysis of 16S rRNA was in agreement with the tree based on whole 16S sequences and the 23S dendrogram showed deeper branching than the 16S dendrogram and more genotypes were resolved, although in some cases the sequence divergence is not particularly high (Terefework *et al.*, 1998). Intrabiovar variation within symbiotic gene regions was detected by PCR-RFLP analysis of *nifDK* and *nodD* regions (Laguerre *et al.*, 1996). The PCR-RFLP method provides a rapid tool for the identification of root nodule isolates and the detection of new taxa (Laguerre *et al.*, 1994). Isolates grouped by PCR-RFLP of the 16S rRNA genes were also separated into groups by variation in multilocus enzyme electrophoresis (MLEE) profiles and DNA-DNA hybridization (Wang *et al.*, 1999).

Conclusions

Research techniques described above are instances of the methods which have been extensively used in estimating phenotypic and genotypic diversity of root- and stem-nodule bacteria. These techniques provide a different level of perspective to interpret the phenotypic and genotypic variations amongst legume symbionts. Most studies employed the combination of several methodologies to characterize and to examine genetic relationships of these specific groups of bacteria. The ranges of discriminating power and respective levels of resolution and limitations have been evaluated. The application of these techniques relies upon many factors such as desired discriminative power, sensitivity of methods, precision of results, specificity of the regions, amount of DNA used, fragment of interests, sequence knowledge, complexity of the populations, procedures, instruments, specialized software

packages, time and labor consumption. Numerical analysis has been used as background knowledge of strains and phenotypic groups could correspond to the genera in the some studies. However, the species can not be distinguishable from a classification based entirely on numerical analysis. Among the techniques based on DNA analysis, sequence analysis of 16S rDNA provides the least discriminating power. It is useful to identify species rather than determine the genetic variation within species, whereas other techniques including RAPD, rep-PCR, AFLP, RFLP and PCR-RFLP, are suitable for distinguishing strains at the species level and intra-species level. Although these research techniques have been proved to be powerful tools for estimating the diversity of root- and stem-nodule bacteria, there is a challenge to develop or apply the novel techniques needed for the specific conditions. For instance, primers that can recognize only the organisms of interest are required in complex substrates such as rhizosphere or nodules. Moreover, cultivation-independent techniques, that can investigate the genetic diversity without prior cultivation, should be possible to reveal a diversity among root- and stem-nodule bacterial populations which are not detected by other molecular approaches.

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