Relationships of bradyrhizobia from *Platypodium* and *Machaerium* (Papilionoideae: tribe Dalbergieae) on Barro Colorado Island, Panama

Matthew A. Parker and Alexandra Lunk

Author for correspondence: M. A. Parker. Tel: +1 607 777 6283. Fax: +1 607 777 6521.
e-mail: mparker@binghamton.edu

Department of Biological Sciences, State University of New York, Binghamton, NY 13902-6000, USA

Enzyme electrophoresis and rRNA sequencing indicated that root nodule bacteria from the canopy tree *Platypodium elegans* and the lianas *Machaerium milleflorum* and *Machaerium arboreum* on Barro Colorado Island, Panama, were highly diverse on a local scale. A total of 11 distinct multilocus genotypes [ETs (electrophoretic types)] was found among the 33 isolates analysed. On average, ETs differed from one another at 74% of the 11 enzyme loci assayed, and separate nodules on a single host individual were often occupied by genetically divergent ETs. Certain ETs were sampled multiple times from both *Platypodium* and *Machaerium*, suggesting a lack of specificity toward the two legume genera. Within the intervening sequence (IVS) region in the 5' end of 23S rRNA, seven ETs had a length variant similar to that of *Bradyrhizobium japonicum* USDA 110, and the other four ETs had an IVS region 26–28 bp shorter. Parsimony analysis of both partial 23S rRNA and nearly full-length 16S rRNA sequences indicated that all *Platypodium* and *Machaerium* isolates were related to *B. japonicum* rather than *Bradyrhizobium elkanii*. The 16S rRNA sequence of one isolate was >99% similar to that of *B. japonicum* USDA 110, and the closest known relatives for other isolates were Philippine bradyrhizobia from the legumes *Stylosanthes* and *Samanea*.

Keywords: *Bradyrhizobium japonicum*, 16S rRNA, 23S rRNA, tropical biodiversity

INTRODUCTION

Even though Neotropical forests are one of the main centres of legume biodiversity (Raven & Polhill, 1981), very little is known about root nodule bacteria associated with these plants. Field surveys have helped to quantify the prevalence of nodulation in different taxa (Faria *et al.*, 1984; Souza Moreira *et al.*, 1992), but relatively few studies have used nucleotide sequencing to assess relationships. 16S rRNA data reported for isolates from several genera of woody legumes in Brazil indicated that they were related to *Bradyrhizobium elkanii* (Dupuy *et al.*, 1994; Laguerre *et al.*, 1997; Lafay & Burdon, 1998). However, for the vast majority of host taxa in tropical American forests,

the diversity and phylogenetic affinities of nodule bacteria have not been investigated.

In this study, we analysed the relationships of bradyrhizobia from two genera of the papilionoid tribe Dalbergieae growing on Barro Colorado Island, Panama. Barro Colorado Island is a biological preserve in Gatun Lake in the Panama Canal covered by a semi-evergreen moist tropical forest, much of which has received little disturbance for the past several hundred years (Foster & Brokaw, 1996). *Platypodium elegans* is a canopy tree found in areas of the island covered by both old and young forest (Croat, 1978; Augspurger, 1983). The genus *Platypodium* includes only two species (Gentry, 1993), but it has a wide geographic range in the Neotropics and can be relatively common (Croat, 1978). The genus *Machaerium* has about 125 species of small trees or lianas widely distributed throughout tropical America, with one species extending to the coast of West Africa (Rudd, 1977; Bastos, 1988). We sampled root nodules

Abbreviations: ET, electrophoretic type; IVS, intervening sequence.

The GenBank accession numbers for the 16S rRNA sequences of isolates Pe1-3, Pe4 and Mm1-3 are AF159436–AF159438, respectively.

from several individuals of *P. elegans* and *M. milleflorum*, and from one individual of *M. arboreum*, because juvenile plants of these taxa are relatively common in the forest understorey, and they have distinctive vegetative morphology which makes them easy to recognize. Bacterial isolates were characterized by isozyme allele profiles using starch gel electrophoresis (Selander *et al.*, 1986) and by rRNA sequence analysis. We focused on a region in the 5' end of 23S rRNA which contains an intervening sequence (IVS) that is cleaved and removed during RNA processing (Selenska-Pobell & Evguenieva-Hackenberg, 1995; Evguenieva-Hackenberg & Selenska-Pobell, 1995). The IVS region is highly polymorphic among *Rhizobiaceae*, and we therefore felt that it could be a useful marker for analysing closely related bacterial strains. To confirm phylogenetic hypotheses derived from partial 23S rRNA sequences, we also obtained nearly full-length 16S rRNA sequences from three representative strains.

METHODS

Isolate sampling. Root nodule bacteria were collected from six individuals of *P. elegans*, five individuals of *M. milleflorum*, and one individual of *M. arboreum* (Table 1). Isolates were named by an abbreviation of the host's name followed by a number designating an individual plant. Nodules were sampled from small juvenile plants by carefully excavating around the base of the stem until roots with attached nodules were located. For half of the plant individuals sampled, only a single nodule was obtained per plant. From

the remaining plants, 2–7 nodules were collected. Isolates from separate nodules on the same host individual were designated by a dash and a number following the plant number (Table 1). All plants sampled were <1.8 km apart from one another on the northeast side of Barro Colorado Island. Pe1, Pe2, Mm1 and Mm2 grew <12 m from one another in a single light gap. Pe5, Pe6 and Marb1 also occurred within one light gap <10 m from each other. Mm3 and Mm4 grew 2 m apart, and were approximately 300 m from Pe3 and Pe4.

Nodules were washed and then stored in vials with calcium sulfate desiccant. Within 1 week, nodules were rehydrated in 0.04 M sodium phosphate buffer pH 7.0, then surface-disinfected in 3.2% sodium hypochlorite. One isolate was purified from each nodule as described previously (Spoerke *et al.*, 1996). All isolates grew slowly on yeast-mannitol agar plates (colonies were not visible before 5 d), suggesting that they were members of the genus *Bradyrhizobium*.

Enzyme electrophoresis. Bacterial isolates were grown in yeast-mannitol broth (Vincent, 1970) and enzymes were obtained from sonicated cells (Spoerke *et al.*, 1996). Isolates were characterized by starch-gel electrophoresis at the following 11 enzyme loci: alcohol dehydrogenase (ADH), alanine dehydrogenase (ALA), butyrate esterase (EST), glucose-6-phosphate dehydrogenase (G-6), β -hydroxybutyrate dehydrogenase (HBD), isocitrate dehydrogenase (IDH), malic enzyme (ME), malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), 6-phosphogluconate dehydrogenase (6-P) and shikimate dehydrogenase (SDH). HBD, IDH, MDH and 6-P were resolved on buffer 11 of Soltis *et al.* (1983) (sodium citrate pH 7.0 electrode buffer and histidine.HCl gel buffer). ADH, ALA and SDH were resolved on buffer I of Selander *et al.* (1986) (Tris/borate pH

Table 1. Bacterial strains used in this study

Host individual	Origin*	Strains	ET
<i>P. elegans</i> 1	Barbour Trail, BCI	Pe1-1, Pe1-3, Pe1-5	1
		Pe1-2	2
		Pe1-4	4
<i>P. elegans</i> 2	Barbour Trail, BCI	Pe2-1 to Pe2-5	1
		Pe2-6	7
<i>P. elegans</i> 3	Shannon Trail, BCI	Pe3-1, Pe3-2	10
<i>P. elegans</i> 4	Shannon Trail, BCI	Pe4	11
<i>P. elegans</i> 5	Fairchild Trail, BCI	Pe5	8
<i>P. elegans</i> 6	Fairchild Trail, BCI	Pe6	7
<i>M. milleflorum</i> 1	Barbour Trail, BCI	Mm1-1	7
		Mm1-2	3
		Mm1-3	6
<i>M. milleflorum</i> 2	Barbour Trail, BCI	Mm2	8
<i>M. milleflorum</i> 3	Shannon Trail, BCI	Mm3-1 to Mm3-4	7
<i>M. milleflorum</i> 4	Shannon Trail, BCI	Mm4	5
<i>M. milleflorum</i> 5	Laboratory Cove, BCI	Mm5-1, Mm5-6	8
		Mm5-2 to Mm5-4, Mm5-7	7
		Mm5-5	9
<i>M. arboreum</i> 1	Fairchild Trail, BCI	Marb1	8
<i>Glycine max</i>	USA	<i>B. japonicum</i> USDA 110	
<i>G. max</i>	USA	<i>B. elkanii</i> USDA 94	

* BCI, Barro Colorado Island, Panama.

8-0). G-6, ME and PGI were resolved on Selander *et al.* (1986) buffer F (Tris/maleate pH 8.2) and EST was resolved on Selander *et al.* (1986) buffer G (potassium phosphate pH 7.0). Each isolate was characterized by its allelic profile for the 11 enzymes, and each unique multilocus genotype was designated an electrophoretic type (ET). Pairwise genetic distances between ETs were estimated by the proportion of enzyme loci at which allelic differences occurred. ETs were then clustered by the unweighted pair group method with arithmetic averages (UPGMA; Sneath & Sokal, 1973).

DNA amplification and sequencing. DNA was purified from 18 isolates by the protocol of Wilson (1994). For reference, we also used *Bradyrhizobium japonicum* USDA 110 and *B. elkanii* USDA 94 (kindly provided by L. D. Kuykendall, USDA, Beltsville Agricultural Research Center – West, Beltsville, MD, USA). For PCR, 25 µl reaction mixtures were used containing 10 mM Tris buffer with 0.1% Triton X-100, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 µM of each primer, 0.5 µl genomic DNA and 0.5 U *Taq* polymerase. Tubes were incubated for 70 s at 94 °C and then subjected to 34 cycles of 20 s at 94 °C, 50 s at 58 °C and 60 s at 72 °C, with a final extension of 4 min at 72 °C. A 5' portion of 23S rRNA was amplified using primers 23Sup115 and 23SrIII (Sterner & Parker, 1999). These primers yield a 260 bp DNA fragment in *B. japonicum* USDA 110 (GenBank accession no. Z35330). A 5 µl aliquot of PCR product was run on a 1.9% agarose gel with a DNA size standard to analyse length variation. For seven isolates, a larger segment of DNA spanning this region was then sequenced on both strands beginning 62 bases from the 5' end of the 23S rRNA gene using primers 23Sup6n and 23Sr#2 (Sterner & Parker, 1999; these amplify 496 bp in *B. japonicum* USDA 110). For three isolates, a 1410 bp portion of 16S rRNA was amplified using primers fD1d and rPlα (Parker, 1999). This fragment was sequenced on both strands using the following internal sequencing primers [Parker, 1999; numbers in parentheses indicate primer position relative to the *Escherichia coli* *rrnB* gene (Brosius *et al.*, 1978)]: m5if (395–416), 5v2ir (420–438), 16smidf (774–795), al4r (890–911), 16cup (1069–1091) and m3ir (1178–1199). PCR-amplified DNA was sequenced using an Applied Biosystems model 310 automated sequencer with dye-terminator chemistry and the protocols recommended by the manufacturer.

Phylogenetic analyses. Sequences were first aligned using CLUSTAL W (Thompson *et al.*, 1994), and then trees were constructed by maximum-parsimony using the PAUP software (version 4.0b1, from D. L. Swofford, Smithsonian Institution, Washington, DC, USA). Relationships were also analysed by the neighbour-joining algorithm (Saitou & Nei, 1987), but results were virtually identical to the maximum-parsimony trees. To determine the degree of statistical support for branches in the phylogeny (Felsenstein, 1985), 1000 bootstrap replicates of each data set were analysed. For the 23S rRNA region, data from *Platypodium* and *Machaerium* isolates were compared to reference sequences available for *B. japonicum* USDA 110 (GenBank accession no. Z35330), *Bradyrhizobium* sp. [*Lupinus*] strain DSM 30140 (X87283), *B. elkanii* USDA 94 (AF081266) and *Bradyrhizobium* strains jwc91-2 and ApT2 from the North American legumes *Amphicarpea* and *Apios*, respectively (AF081262 and AF146821, respectively). *Rhodopseudomonas palustris* (X71839) was used as the outgroup (Sterner & Parker, 1999). For 16S rRNA, data from *Platypodium* and *Machaerium* isolates were compared to the following *Bradyrhizobium* reference sequences (strains

without formal names are identified by host legume genus in square brackets): *B. japonicum* strains USDA 6 (GenBank accession no. U69638) and USDA 110 (Z35330), strain DSM 30140 [*Lupinus*] (X87273), *B. elkanii* strains USDA 76 (U35000) and USDA 94 (D13429), strain 55S [*Samanea*] (D14507), strain 129 [*Stylosanthes*] (D14508; Oyaizu *et al.*, 1993), *Bradyrhizobium* genomic species 'A' [*Bossiaea*] (Z94811; Lafay & Burdon, 1998), *Bradyrhizobium* 'genomic species 'E' [*Podolobium*] (Z94814), *Bradyrhizobium* genomic species 'H' [*Goodia*] (Z94816), *Bradyrhizobium* genomic species 'P' [*Daviesia*] (Z94805), LMG 9514 [*Lonchocarpus*] (X70405; Dupuy *et al.*, 1994). Several related genera of the α -Proteobacteria were also included in the analysis: *Azorhizobium caulinodans* (X67221), *Paracoccus denitrificans* (X69159), *Rhodobacter sphaeroides* (D16424), *Blastobacter denitrificans* (S46917) and *Rhodopseudomonas palustris* (D25312). *A. caulinodans* was chosen as the outgroup because most 16S rRNA phylogenies place it at a basal position relative to the other taxa (Willems & Collins, 1993; Dupuy *et al.*, 1994; Young & Haukka, 1996).

Nodulation and nitrogenase activity. No seeds of *Platypodium* or *Machaerium* were available to use for inoculation studies. We thus used plants of *Vigna unguiculata* and *Macroptilium atropurpureum*, which are known to be nodulated by a broad range of tropical bradyrhizobia (Thies *et al.*, 1991; Turk & Keyser, 1992). Seven isolates representing different genotypes revealed by isozyme and rRNA sequence analyses were used to inoculate 3–4 plants of each species according to previously described procedures (Wilkinson *et al.*, 1996). Seeds were surface-disinfected with 50% ethanol and then germinated. Seedlings were planted in pots using a *Bradyrhizobium*-free mixture of sand, perlite and potting soil, and then inoculated with approximately 10⁹ cells of a particular isolate grown in yeast-mannitol broth. Plants were grown in a greenhouse for 27 d with precautions to avoid bacterial contamination across inoculation treatments (Wilkinson *et al.*, 1996). Uninoculated control plants grown simultaneously in the same room were found to be completely free of nodules. Plants were fertilized weekly with a nitrogen-free nutrient solution (Parker & Wilkens, 1990). At harvest, nodule numbers were recorded, and each plant's root system was analysed for acetylene reduction activity using a Hewlett Packard 5890 series II gas chromatograph as described by Spoerke *et al.* (1996).

RESULTS

Diversity and relationships inferred from multilocus enzyme electrophoresis (MLEE)

Among the 33 isolates sampled from *Platypodium* and *Machaerium*, variation was detected at all 11 enzyme loci examined, with a mean of 4.7 alleles per locus (range, 2–7; Table 2). A total of 11 distinct ETs was detected. Seven of these ETs were represented by only a single isolate, whilst the remaining four ETs were recovered multiple times. The two most common ETs associated with *M. milleflorum* (ET7, ET8) were also observed in three isolates from *P. elegans* (Table 1). The sole isolate from *M. arboreum* also had the ET8 multilocus allele profile. The most common ET for *P. elegans* (ET1) occurred in 8 of the 11 nodules sampled from the Barbour Trail site, but was observed at no other locations for any of the host legumes.

An average linkage cluster analysis grouped the ETs

Table 2. Allele profiles at 11 enzyme loci in 11 ETs of *Bradyrhizobium* isolates from *Platypodium* and *Machaerium*

ET	Allele at indicated enzyme locus*											No. of isolates	
	ADH	ALA	EST	G-6	HBD	IDH	ME	MDH	PGI	6-P	SDH	<i>Platypodium</i>	<i>Machaerium</i>
1	1	2	2	2	1	1	6	2	1	1	3	8	0
2	1	4	2	3	2	2	5	2	1	3	4	1	0
3	1	4	2	3	2	2	6	2	1	3	1	0	1
4	1	5	1	5	1	2	4	2	2	5	6	1	0
5	1	0	3	3	4	4	1	2	2	6	5	0	1
6	0	1	2	4	3	5	1	1	3	2	7	0	1
7	2	3	4	1	3	3	1	2	3	4	2	2	9
8	2	6	4	1	3	3	1	2	3	4	2	1	4
9	2	6	4	1	3	3	1	2	3	6	2	0	1
10	2	4	4	3	3	3	2	1	3	2	7	2	0
11	2	4	4	3	3	3	3	1	3	2	7	1	0

* Alleles are listed by relative anodal migration speed (1 = slowest; null alleles are depicted by '0'). ADH, alcohol dehydrogenase; ALA, alanine dehydrogenase; EST, butyrate esterase; G-6, glucose-6-phosphate dehydrogenase; HBD, β -hydroxybutyrate dehydrogenase; IDH, isocitrate dehydrogenase; ME, malic enzyme; MDH, malate dehydrogenase; PGI, phosphoglucose isomerase; 6-P, 6-phosphogluconate dehydrogenase; SDH, shikimate dehydrogenase.

Fig. 1. Genetic relationships among 11 ETs of *Bradyrhizobium* isolates from *Platypodium* and *Machaerium*.

into several divergent lineages (Fig. 1). Isolates from both *Platypodium* and *Machaerium* were scattered throughout all groups, and showed little tendency to cluster according to host origin. Moreover, there was wide diversity among the bacterial isolates from separate nodules on the same individual host plant. For example, the three nodules sampled from plant Mm1 were extremely different from one another (ET3, ET6 and ET7), and highly divergent ETs were also found among the nodules sampled from plants Pe1 and Pe2.

23S rRNA variation

To further characterize relationships of these divergent genotypes, primers flanking a region in the 5' portion of 23S rRNA that commonly shows length variation among taxa of *Rhizobiaceae* (Evguenieva-Hackenberg & Selenska-Pobell, 1995) were used to amplify DNA from representative isolates. Two basic length variants were detected. Isolates belonging to ET1, ET2, ET3 and ET6 had a smaller fragment that roughly corresponded to the size of DNA amplified from *B. elkanii* USDA 94 (232 bp). Strains belonging to ET4, ET5 and ET7–ET11 had a larger fragment that matched that of *B. japonicum* USDA 110 (260 bp).

A larger portion of 23S rRNA spanning this region was sequenced in seven isolates. Alignment of the IVS region indicated that isolates were polymorphic for two long insertion/deletions together with two single-nucleotide-length variants. Four isolates with the longer length variant (Pe4, Mm4, Pe2-6 and Mm3-1) were very similar to *B. japonicum* USDA 110, differing by only one single-nucleotide deletion and three or four base substitutions. Despite coming from different host legumes, the two isolates representing isozyme genotype ET7 (Pe2-6 and Mm3-1) were completely identical throughout the entire 496 bp region sequenced. Among the three isolates with the shorter length variant (Pe1-3, Mm1-2, Mm1-3), all shared an apparently identical deletion of 12 bases corresponding to positions 168–179 of the *B. japonicum* USDA 110 sequence. This condition was also seen in *B. elkanii* USDA 94 and numerous *Bradyrhizobium* isolates from the North American legumes *Amphicarpaea*, *Apios* and *Desmodium* (Sterner & Parker, 1999; Parker, 1999). However, a second gap of 15 nucleotides in *B. elkanii* USDA 94 (corresponding to positions 215–229 of the *B. japonicum* USDA 110 sequence) was not

Fig. 2. Parsimony tree for seven partial 23S rRNA sequences of *Bradyrhizobium* isolates from *Platypodium* and *Machaerium* (shown in bold). Numbers above branches are bootstrap percentages ($n=1000$ replicates). GenBank accession numbers are given after strain names.

precisely matched by any of the *Platypodium* or *Machaerium* isolates. Pe1-3 and Mm1-2 had a 13 bp gap in this area, whilst Mm1-3 had a 14 bp gap. Overall, these results indicate that the apparent 23S rRNA IVS length similarity between *B. elkanii* and certain Panama isolates may reflect parallel evolution rather than common ancestry. Indeed, if data from the two large 23S rRNA gap regions are omitted, a maximum-parsimony tree using the remaining portion of the 23S rRNA region sequenced (approx. 470 bp) grouped all *Platypodium* and *Machaerium* isolates into a clade together with *B. japonicum* USDA 110, with *B. elkanii* apparently forming a separate lineage (Fig. 2). However, the group consisting of all Panama isolates together with *B. japonicum* USDA 110 had only moderate bootstrap support (77%). Relationships of selected isolates were therefore investigated further using 16S rRNA.

16S rRNA variation

Nearly full-length sequences were obtained for isolates Pe1-3, Mm1-3 and Pe4 (representing ET1, ET6 and ET11, respectively). Mm1-3 and Pe4 showed only three nucleotide differences out of 1410 bp analysed, whilst Pe1-3 differed at 15–18 bases from the other two isolates. A search of α -Proteobacteria sequences in GenBank identified other taxa whose 16S rRNA sequences were similar to each of these isolates (Fig. 3). Pe1-3 showed >99% similarity to the 16S rRNA sequence of the non-symbiotic bacterium *Blastobacter denitrificans* (Willems & Collins, 1992). *B. japonicum* USDA 110 and *Bradyrhizobium* genomic species 'A', which Lafay & Burdon (1998) found to be widely

distributed on shrubby legumes in Australia, also showed >99% sequence similarity to Pe1-3 (Fig. 3). Photosynthetic stem-nodulating bradyrhizobia from *Aeschynomene* also show high 16S rRNA similarity to *Blastobacter denitrificans* (Wong *et al.*, 1994), but these were not included in Fig. 3 because <1100 bp of sequence data were available compared to >1400 bp for the other taxa. The most similar sequences to Mm1-3 and Pe4 proved to be two *Bradyrhizobium* isolates from the Philippines studied by Oyaizu *et al.* (1993): 55S originated from the mimosoid tree *Samanea saman*, whilst strain 129 came from the herbaceous papilionoid legume *Stylosanthes guyanensis* (tribe Aeschynomeneae). All three *Platypodium* and *Machaerium* isolates were grouped together with *B. japonicum* (USDA 110 and USDA 6) in a very high proportion of bootstrap replicates (98%). Two isolates of *B. elkanii* (USDA 76 and USDA 94) formed a lineage that was distinct from the group that included the *Platypodium* and *Machaerium* isolates. A Brazilian isolate from *Lonchocarpus costatus* (papilionoid tribe Tephrosieae; Dupuy *et al.*, 1994) and an Australian isolate from *Daviesia leptophylla* (papilionoid tribe Mirbelieae; Lafay & Burdon, 1998) also had 16S rRNA sequences that clustered with *B. elkanii* in a high proportion of bootstrap replicates, as seen in a previous analysis using the neighbour-joining method (Lafay & Burdon, 1998).

Symbiotic phenotypes

Moderate to high numbers of nodules formed on most *Vigna unguiculata* and *Macroptilium atropurpureum* plants inoculated with isolates from *Platypodium* and

Fig. 3. Phylogeny of three *Bradyrhizobium* isolates from *Platypodium* and *Machaerium* (shown in bold) based on parsimony analysis of 16S rRNA sequences. Numbers above branches are bootstrap percentages (based on 1000 replicates; for clarity, only bootstrap values >80 % are shown).

Machaerium (Fig. 4). Substantial acetylene reduction activity was evident in all combinations of plants and bacteria except those with isolate Mm1-3. This isolate formed smaller numbers of nodules on both hosts than any other isolate tested. This lower degree of nodulation compatibility was accompanied by a complete absence of measurable acetylene reduction activity for *V. unguiculata* nodules, and by relatively low activity for *M. atropurpureum* nodules (Fig. 4).

DISCUSSION

Our main finding was that root nodule bacteria associated with *Platypodium* and *Machaerium* in Panama constitute a diverse set of genotypes related to *B. japonicum* (Figs 2, 3). Bacterial genetic diversity was very high on a local scale. For each host legume species, divergent bacterial genotypes were found not only on separate plants growing <2 km apart, but also

among separate nodules on a single host individual (Table 1, Fig. 1).

This high diversity, coupled with the limited scale of sampling, means that it is not yet possible to reach conclusions about the degree of host specificity for many of the bacterial genotypes, since they were recovered only once or twice. In addition, there are numerous other potential legume host taxa sharing this environment (Croat, 1978) that have not yet been sampled. Thus, even for bacterial genotypes that seemed to be consistently associated with one host (e.g. ET1), much additional research will be required to understand host relationships. Nevertheless, certain genotypes clearly did not show strict specificity for *Platypodium* vs *Machaerium*, since they were recovered multiple times from both host taxa (Table 1, Fig. 1). Also, sequence analysis of a portion of 23S rRNA indicated very close relationships of some isolates from *Platypodium* vs *Machaerium* (Fig. 2). Moreover, an

Fig. 4. Nodulation (a) and acetylene reduction activity (b) of *V. unguiculata* (left) and *M. atropurpureum* (right) inoculated with *Bradyrhizobium* isolates from *Platypodium* and *Machaerium*. Data shown are means $\pm$ 1 SD.

inoculation experiment demonstrated that all of the isolates tested had potential host ranges that extended to other legume species as well (Fig. 4). Therefore, a lack of specificity toward *Platypodium* vs *Machaerium* would not be surprising.

The tree based on 16S rRNA sequence variation indicated that the *Platypodium* and *Machaerium* isolates apparently did not form a single phylogenetic group (Fig. 3). Instead, one Panama isolate exhibited a close relationship to the North American soybean symbiont *B. japonicum* USDA 110, whilst two other isolates clustered with bradyrhizobia from other host legumes in the Philippines. As observed in previous analyses (Barrera *et al.*, 1997; Laguerre *et al.*, 1997), the 16S rRNA phylogeny showed a strongly marked division of the genus *Bradyrhizobium* into two basic lineages allied with *B. japonicum* vs *B. elkanii*. There was strong bootstrap support for placing the Panama isolates in the *B. japonicum* group (Fig. 3). However, the structure of relationships within the *B. japonicum* group was not clearly resolved by the 16S rRNA data. Sets of apparently related sequences were marked by relatively small numbers of shared nucleotide substitutions, and bootstrap resampling analysis indicated

that alternative hypotheses about relationships could not be ruled out with high confidence. Thus, a definitive resolution of phylogenetic relationships within this group will require additional sequence information from other, more rapidly evolving loci.

Research on 23S rRNA sequence variation in *Bradyrhizobium* isolates from temperate North America has suggested that isolates related to *B. japonicum* vs *B. elkanii* show a consistent length difference in the 5' IVS region (Sterner & Parker, 1999), which therefore may be useful for typing unknown bacteria. The present study suggests caution is advisable with regard to using length variation alone as a marker for relationships, unless validated by sequencing. Several Panama isolates had a 23S rRNA length variant very similar to *B. elkanii*, but subsequent sequence analysis did not confirm a close relationship. Both *B. elkanii* and certain Panama isolates had two long deletions relative to the *B. japonicum* sequence, but the precise size and location for one of the deletions were not identical, and a parsimony tree instead grouped the Panama isolates together with *B. japonicum* (Fig. 2). Overall, the evidence currently available suggests that a short 23S rRNA IVS region is a consistent trait of isolates related to *B. elkanii* (Sterner & Parker, 1999; Parker, 1999). However, tropical bacteria related to *B. japonicum* are polymorphic for this same phenotype. Further analysis of this polymorphism, particularly its biogeographic distribution, would be valuable to better define the utility of the 23S rRNA IVS region as a diagnostic tool.

In conclusion, our results provide new information about the diversity and relationships of root nodule bacteria for the legume genera *Platypodium* and *Machaerium*, whose bacterial symbionts had not been previously characterized in detail. Much additional research on bacteria associated with these and other genera of tropical forest legumes will be necessary to build a clear systematic understanding of the nodule bacteria in this environment.

ACKNOWLEDGEMENTS

We are grateful to C. Augspurger for suggestions about plant locations on BCI, to J. Pfeil for assistance with sequencing, and to L. D. Kuykendall for providing bacterial isolates. We also thank the Smithsonian Tropical Research Institute and the Republic of Panama's Instituto de Recursos Nacionales Renovables for permission to collect on BCI, the Harpur College Dean of Arts and Sciences for travel support, and the Howard Hughes Medical Institute for other financial support.

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