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Molecular Diversity of Rhizobia Occurring on Native Shrubby Legumes in Southeastern Australia

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The structure of rhizobial communities nodulating native shrubby legumes in open eucalypt forest of southeastern Australia was investigated by a molecular approach. Twenty-one genomic species were characterized by small-subunit ribosomal DNA PCR-restriction fragment length polymorphism and phylogenetic analyses, among 745 rhizobial strains isolated from nodules sampled on 32 different legume host species at 12 sites. Among these rhizobial genomic species, 16 belonged to the *Bradyrhizobium* subgroup, 2 to the *Rhizobium leguminosarum* subgroup, and 3 to the *Mesorhizobium* subgroup. Only one genomic species corresponded to a known species (*Rhizobium tropici*). The distribution of the various genomic species was highly unbalanced among the 745 isolates, legume hosts, and sites. *Bradyrhizobium* species were by far the most abundant, and *Rhizobium tropici* dominated among the *Rhizobium* and *Mesorhizobium* isolates in the generally acid soils where nodules were collected. Although a statistically significant association occurred between the eight most common genomic species and the 32 hosts, there was sufficient overlap in distributions that no clear specificity between rhizobial genomic species and legume taxa was observed. However, for three legume species, some preference for particular genomic species was suggested. Similarly, no geographical partitioning was found.

The family Fabaceae is one of the most successful families of angiosperms. It is the third largest, with approximately 650 genera and 20,000 species (16), and is most remarkable for its wide evolutionary diversification (56) and cosmopolitan distribution (58). Many of its members are of considerable agricultural or ecological importance, generally reflecting their ability to develop symbiotic associations with nitrogen-fixing soil bacteria, a feature which is widespread within the family (1). The bacteria inducing nitrogen-fixing nodules on leguminous plants correspond to five formally recognized genera (34, 67); *Rhizobium* (23), *Sinorhizobium* (9), *Bradyrhizobium* (36), *Azorhizobium* (17), and *Mesorhizobium* (34). These genera all belong to the alpha subdivision of the proteobacteria but represent separate lineages, relatively distant from one another and each more closely related to nonnodulating taxa.

In Australia, the Fabaceae constitute a significant part of the vascular flora, representing about 10% of the estimated 18,000 native plant species (14). Several tribes (e.g., Mirbeliae and Bossiaeae) and a number of genera (e.g., *Daviesia*, *Bossiaea*, and *Pultenaea*) of the family are endemic. Native legumes are widely distributed throughout the continent, occurring in all vegetation types except salt marshes and marine aquatic communities (14). They are often a dominant part of ecosystems in which they occur, whether this is measured in terms of structural position, numbers, or overall biomass. This dominance may reflect the advantage that legumes gain in soils of low fertility (a characteristic feature of the majority of Australian ecosystems) from symbiotic nitrogen-fixing associations with rhizobia. In such situations, plant-microbial associations that help circumvent nutrient deficiencies are likely to be of considerable significance in determining the species and structural diversity of individual ecosystems.

Paradoxically, relatively few studies have aimed to uncover the nature of these bacterial symbionts in their native environments. A synthesis of the work conducted in Australia over the past 40 years shows that the comprehension of native rhizobia in the country is mainly based on nodulation experiments and growth characteristics (14). These two criteria are now held to be insufficient (28) and can be misleading, e.g., slow-growing *Mesorhizobium ciceri* (51). The aim of the present study has been to improve our knowledge of Australian native rhizobial diversity and to analyze the influence of the nature of the associated host legume as well as the geographic origin on the structure of the native rhizobial communities. We tried to ensure that sampling was restricted to rhizobial communities that were not invaded by strains exotic to Australia (e.g., previously used as crop inoculants) by collecting rhizobia at sites located in national parks or away from cropping systems, and we focused on shrubby legumes composing the undercover in woodland and forest ecosystems of southeastern Australia. A molecular systematics approach combining small-subunit (SSU) ribosomal DNA (rDNA) PCR-restriction fragment length polymorphism (RFLP) analysis and sequencing was adopted to facilitate rapid identification of a large number of strains.

MATERIALS AND METHODS

Nodule collection and isolation of bacterial strains. Plants of 32 legume species (Table 1) were excavated at various field sites in southeastern Australia (Fig. 1) during spring and early summer for all but the Island Bend site and for *Gompholobium huegelii* at Lobs Hole, which were both sampled at the end of summer. At each site, up to 10 individuals of a minimum of two legume species were sampled. From these, segments of roots with attached nodules were excised and transported in plastic bags to the laboratory, where bacterial strains were isolated the following day. In the process, the nodules were separated from the root, washed in distilled water, and then surface sterilized following the technique of Cannon et al. (8) with a nodule-sterilizing apparatus (25). The nodules were crushed, and the exudate was streaked onto yeast-mannitol agar medium (64). Pure cultures were obtained with one or more further subculturing steps.

DNA preparation. Bacterial DNA was prepared following the method described by Sritharan and Barker (61). Bacteria were grown on yeast-mannitol agar medium and colonies were collected, suspended in 100 μl of 10 mM Tris (pH 8.0)–1 mM EDTA–1% Triton X-100 solution, and boiled for 5 min. After a single chloroform extraction, 5 μl of the supernatant was used in the amplification reaction.

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TABLE 1. Number of rhizobial strains according to their legume host and origin site

Host[a]					No. of strains at site[b]											
Subfamily	Tribe	Group	Genus	Species	BBNP	BM	BR	GR	IB	LFPR	LH	MF	MR	TF	TR	TSR
Mimosoideae	Acaciaeae		*Acacia*	*obliquinervia*					12							
Papilionideae	Bossiaeae	*Bossiaea*	*Bossiaea*	*buxifolia*			24									
				ensata	12									15		
				foliosa					26		15	10				
			Goodia	*lotifolia*						70						
			Platylobium	*formosum*							10				11	
	Brongniartieae		*Hovea*	*linearis*							9					
				purpurea							15					
	Indigofereae		*Indigofera*	*australis*			18									
	Mirbelieae	*Daviesia*	*Daviesia*	*buxifolia*	11											
				latifolia							9					
				leptophylla			6	2					20			
				mimosoides		13						5				
				ulicifolia					5	36					9	13
		Gompholobium	*Gompholobium*	*huegelii*							14		10			
		Oxylobium	*Mirbelia*	*oxylobioides*							7					
				rubiifolia										24		
			Oxylobium	*ellipticum*			24									29
			Podolobium	*alpestre*								2				
				ilicifolium	7					8					5	
		Pultenaea	*Aotus*	*ericoides*	3											
			Dillwynia	*brunioides*										6		
				glaberrima	9											
				ramosissima										66		
				retorta		35										
				sericea	13											
			Phyllota	*phylicoides*										4		
			Pultenaea	*capitellata*	10											
				daphnoides	15											
				procumbens		27	6									
				scabra	18											
	Phaseoleae	*Kennediinae*	*Hardenbergia*	*violacea*		18		9								

[a] Legume classification is according to Crisp and Weston (13).
[b] BBNP, Ben Boyd National Park; BM, Black Mountain; BR, Boboyan Road; GR, Gunning Road; IB, Island Bend; LFPR, Lowden Forest Park Road; LH, Lobs Hole; MF, Mount Franklin; MR, Mundoonen Range; TF, Tianjiara Falls; TR, Turpentine Road; TSR, Two Sticks Road.

SSU rRNA gene amplification. Primers corresponding to positions 8 to 28 and 1492 to 1509 (39) in the *Escherichia coli* SSU rRNA sequence (7) were used for amplification of the SSU rRNA genes by PCR. PCRs were carried out in a 100-μl volume containing 5 μl of template DNA solution, 50 pmol of each of the two primers, 200 μM deoxynucleoside triphosphate (Boehringer Mannheim), and 2.5 U of Amplitaq DNA polymerase (Perkin-Elmer) in Amplitaq DNA polymerase reaction buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$). Amplifications were performed with a Hybaid Omnigene thermocycler with the following temperature profile: an initial cycle consisting of a denaturation step at 95°C for 5 min, an annealing step at 52°C for 120 s, and an extension step at 72°C for 90 s; 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 60 s, and extension at 72°C for 60 s; and a final extension step at 72°C for 5 min.

SSU rDNA PCR-RFLPs. Aliquots of PCR products (10 μl) were digested with restriction endonucleases as described by Laguerre et al. (38). Nine restriction enzymes, *Alu*I, *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Nde*II, *Rsa*I, and *Taq*I (New England Biolabs), were first used to screen a limited number of bacterial strains. These had no greater discriminating power than a combination of only four enzymes (*Hha*I, *Hinf*I, *Msp*I, and *Rsa*I) as observed by Laguerre et al. (38). Restricted fragments were separated by electrophoresis on 3% NuSieve 3:1 agarose gels at 80 V for 5 h and visualized by ethidium bromide staining.

Nodulation tests. The nodulation ability of the representative strains of each PCR-RFLP genotype was verified by inoculation onto the young plants of the original host grown from seeds which had been collected at their corresponding site at the end of summer. The seeds were dried over silica gel and surface sterilized in concentrated H_2SO_4 for 20 min. After the acid was drained, the seeds were washed thoroughly with 10 changes of sterile water and placed in moistened steam-sterilized sand to germinate at 22°C for 7 days. The seedlings were then planted in a steam-sterilized sand-vermiculite mix in 12-cm-diameter pots and left to establish in the greenhouse at 25°C. After 2 days, they were inoculated at the base of the stem with 1 ml of the appropriate bacterial inoculum (heavy suspension of the log-phase culture on yeast-mannitol agar in 10 ml of N-free Jensen solution, pH 6.8). The surface was covered with polyurethane beads to prevent evaporation and contamination. The plants were grown in the greenhouse at 25°C and watered with a sterile N-free nutrient solution twice each week. After 10 weeks of growth in the greenhouse, nodules were observed on the seedling roots for all genomic species. Control uninoculated plants were unnodulated.

PCR product sequencing. Representative examples of isolates possessing each of the distinct PCR-RFLP genotypes detected, with a minimum of two for the most frequent genotypes, were used in a subsequent sequence comparison. SSU rDNA PCR products were purified with a Wizzard PCR Preps DNA purification system (Promega) as specified by the manufacturer. The sequencing reaction was performed with the ABI PRISM dye terminator cycle-sequencing ready-reaction kit with Amplitaq DNA polymerase FS as specified by the manufacturer, on an FTS-1 thermal cycler (Corbett). Sequencing products were analyzed with an ABI automatic sequencer model 377. Sense and antisense synthetic primers complementary to conserved eubacterial domains corresponding to positions 100 to 120, 243 to 263, 343 to 357, 518 to 536, 685 to 704, 787 to 803, 907 to 926, 1100 to 1115, 1224 to 1241, and 1385 to 1401 in the *E. coli* SSU rRNA sequence (7) were used to sequence both strands of the SSU rRNA gene.

Sequence analysis. The SSU rRNA gene sequences were aligned manually by comparison with a database of alpha proteobacteria SSU rRNA sequences aligned on the basis of their phylogenetic relationships by using the program VSM 4.0 for SSU rRNA sequence database management (11). All of the sites were included in the phylogenetic analysis, except in the case of *Bradyrhizobium* species analysis, for which a short stretch of the sequences (positions 997 to 1041 in the *E. coli* SSU rRNA sequence [7]) was excluded. Phylogenetic analyses were performed by the neighbor-joining method (59) with the program NEIGHBOR in PHYLIP version 3.5c (21). Distances were computed with DNADIST under the Jin and Nei distance (35). One thousand bootstrap replications were performed with SEQBOOT. The graphic manipulation of the tree was realized with NJplot (55).

Nucleotide sequence accession numbers. The SSU rRNA gene sequences corresponding to the rhizobial genomic species identified have been deposited in the EMBL nucleotide database under accession no. Z94803 to Z94823. The accession numbers of the nucleotide sequences of the SSU rRNA genes of the *Rhizobiaceae* and related alpha proteobacteria used for comparison are as follows: *Afipia clevelandensis*, M69186; *Afipia felis*, M65248; *Agrobacterium rhizogenes* LMG152, X67224; *Agrobacterium tumefaciens* Ch-Ag-4, D14505; *Agromonas oligotrophica*, D78366; *Blastobacter denitrificans*, X66025; *Bradyrhizobium*

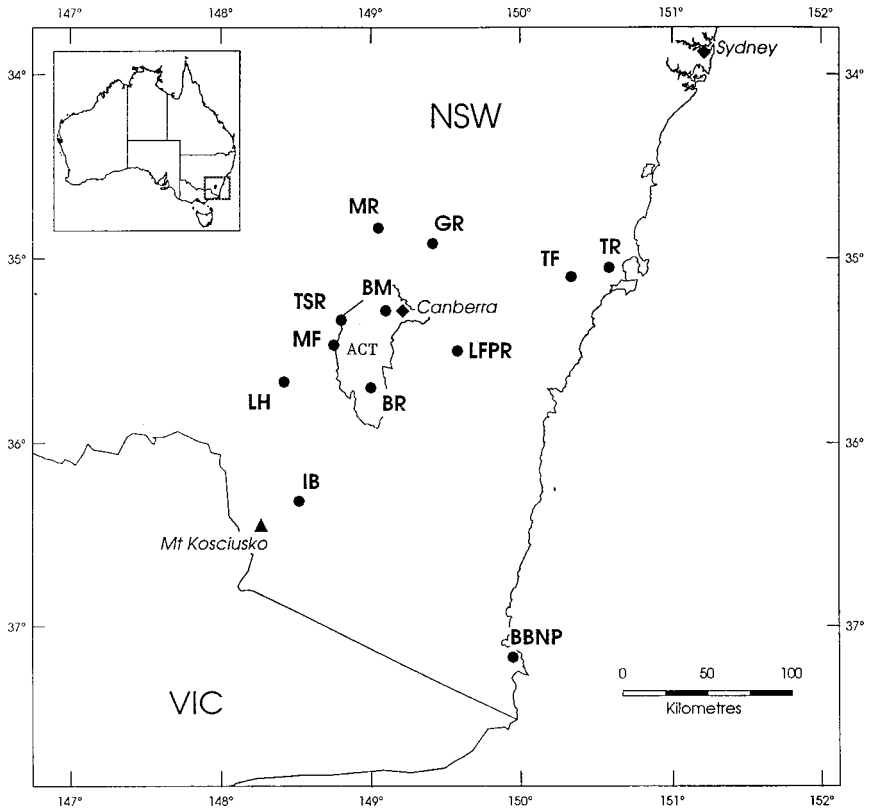

FIG. 1. Geographical location of the 12 sites where nodules were collected: Ben Boyd National Park (BBNP), Black Mountain (BM), Boboyan Road (BR), Gunning Road (GR), Island Bend (IB), Lowden Forest Park Road (LFPR), Lobs Hole (LH), Mount Franklin (MF), Mundoonen Range (MR), Tianjiara Falls (TF), Turpentine Road (TR), and Two Sticks Road (TSR). Abbreviations: ACT, Australian Capital Territory; NSW, New South Wales; VIC, Victoria.

elkanii, U35000; *Bradyrhizobium japonicum* USDA 6^T, U69638, and USDA 110, D13430; *Bradyrhizobium* spp. 129, D14508; 55S, D14507; LMG 9514, X70401; LMG 9520, X70403; LMG 9580, X70404; LMG 9966, X70403; LMG 10698, X70405; *Brucella melitensis*, L26166; *Mesorhizobium ciceri*, U07934; *Mesorhizobium huakuii*, D12797; *Mesorhizobium loti* A, X67229, and B, X67230; *Mesorhizobium mediterraneum*, L38825; *Mesorhizobium* spp. LMG7836, XZ68389, and LMG7854, X68391; *Mesorhizobium tianshanense*, U71079; *Mycoplana dimorpha*, D12786; *Ochrobactrum anthropi*, D12794; "*Photorhizobium*" *thompsonianum*, L23405; *Phyllobacterium myrsinacearum*, D12789; *Phyllobacterium rubiacearum*, D12790; *Rhizobium etli*, U28939; *Rhizobium gallicum*, U86343; *Rhizobium hainanense*, U71078; *Rhizobium leguminosarum*, X67227; *Rhizobium tropici*, D12798; *Rhizobium* sp. LMG 9509, X67232; *Rhodopseudomonas palustris*, D25312; and *Zoogloea ramigera*, X74915.

Statistical analyses. Association between rhizobium genomic species and hosts, or between rhizobium genomic species and sites, was tested by using the log-likelihood ratio statistic (15). This test has been shown to perform well for large sample sizes, such as those in our study (37), even when there are low expected numbers for some combinations. The nature of the associations was then examined by correspondence analysis (29).

RESULTS

SSU rDNA PCR-RFLP study. A total of 745 strains, representing all the strains isolated from all the hosts, were included in the RFLP study. Gel electrophoresis of the PCR products revealed that the amplification reaction produced a single DNA molecule slightly less than 1.5 kb long for all strains. Four restriction endonucleases, *Hha*I, *Hinf*I, *Msp*I, and *Rsa*I, were used to characterize the whole collection. From 4 to 10 distinct restriction patterns were detected with each of these enzymes. The combination of the four patterns identified 21 SSU rDNA types that we arbitrarily named A to U.

SSU rDNA sequence analyses. SSU rRNA gene sequences of the 21 rRNA genomic species were aligned by comparison with a database containing about 500 aligned SSU rRNA sequences of alpha proteobacteria. Phylogenetic analyses including representatives of all rhizobial genera and related alpha proteobacteria revealed that all 21 SSU rRNA genomic species detected belonged to the *Rhizobium-Agrobacterium* group as defined in the Ribosomal Database Project (45). Sixteen of the SSU rRNA genomic species clustered within the *Bradyrhizobium* subgroup, and three (genomic species S, T, and U) grouped within the *Mesorhizobium* subgroup, while the remaining two (genomic species Q and R) grouped within the *R. leguminosarum* subgroup.

Genomic species were further studied according to the subgroup to which they were related. In each case, outgroups were chosen as the most closely related species in accordance with the Ribosomal Database Project general phylogeny of procaryotes. Within the *R. leguminosarum* subgroup, genomic species Q and R clustered with *R. tropici* (Fig. 2A), Q being much more closely related to *R. tropici* (with a difference of one base between their SSU rDNA sequences) than to genomic species R (with a difference of 40 bases). Genomic species S and species T and U formed two individualized lineages which were each clearly affiliated with one of the two major groups within the *Mesorhizobium* subgroup (Fig. 2B). Genomic species S was closely related to the *M. loti-M. ciceri* cluster, its SSU rDNA differing from *M. loti* A and B sequences by four and three bases, respectively, and differing from *M. ciceri* SSU rRNA by six bases. The lineage formed by genomic species T and U clustered with *M. huakuii*, from which they differed by four and two bases, respectively, at the SSU rDNA level. Their SSU rDNA sequences differed from each other by only two bases.

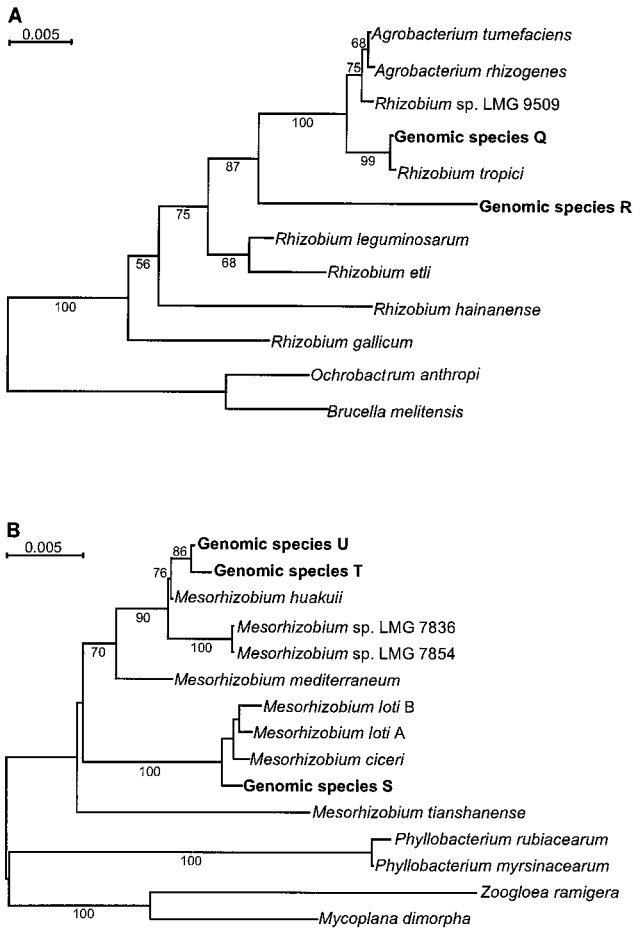

FIG. 2. Phylogenetic relationships among genomic species belonging to the genera *Rhizobium* and *Mesorhizobium* characterized by SSU rDNA PCR-RFLPs. The phylogenetic trees were constructed by the neighbor-joining method. The numbers correspond to the percentage of bootstrap support for internal branches, based on 1,000 replications. The scale bar corresponds to 0.005 substitution per site. (A) Phylogenetic positions of genomic species Q and R within the *R. leguminosarum* subgroup. (B) Phylogenetic positions of genomic species S, T, and U within the *Mesorhizobium* subgroup.

In both analyses, internal branches linking the genomic species to known rhizobial species were supported above the 70% level by 1,000 bootstrap replications and are thus expected to represent true clades according to Hillis and Bull (32).

The phylogenetic positions of 16 genomic species within the *Bradyrhizobium* subgroup were investigated by comparing their SSU rDNA sequences to those of representatives of the various genera within this group. Numerous sequences are available in the DNA sequence databases for *B. japonicum*; the choice of representative SSU rRNA sequences for this species was made according to the results of Barrera et al. (6). The resulting phylogenetic tree (Fig. 3) exhibited a poor level of resolution for some of the internal branches, due to the small divergence between the various sequences. Consequently, the phylogenetic position of the rhizobial genomic species identified in the PCR-RFLP study, in particular for genomic species E, G, and H, as well as the branching order of the various groupings, could not be resolved. With the exception of L and P, none of the genomic species identified in this study clustered with any of the known species of this group at a significant level by 1,000 bootstrap replications. Eleven of them (A, B, C, D, F, I, J, K, M, N, and O) formed a group of closely related species, relatively distant from any known species. The sequences of any two of these genomic species exhibited a very high degree of similarity: the minimum difference was one base (genomic species A and M); the maximum was 18 bases (genomic species B and F), which represents less than 4% of the sequence. Genomic species L and P clustered with the *B. elkanii* cluster, which is the only one supported at a significant level by 1,000 bootstrap replications. SSU rDNAs of all the species included in this cluster present a characteristic sequence from positions 997 to 1041, according to *E. coli* SSU rRNA sequence numbering (7), which was also found in the genomic species L and P sequences. This part of the SSU rDNA was not included in the construction of the phylogeny presented in Fig. 3 (see Discussion). The other three genomic species, E, G, and H, did not show a significant phylogenetic affinity for any particular branch and thus are likely to constitute separate lineages within the *Bradyrhizobium* subgroup.

Analysis of rhizobial diversity. The distribution of the 745 strains among the various rhizobial genomic species was highly unbalanced (Table 2). Most of them (94.3%) were *Bradyrhizobium* species, with one genomic species, A, representing more than half of the total number of strains (57.6%). Among the 21 genomic species, only 8 constituted 97.05% of the entire collection. The remaining 13 genomic species each represented less than 1% of the strains and, in most cases, were only isolated once. Various combinations of rhizobial genomic species occurring on the same host plant were observed, most of them logically involving genomic species A strains. *Rhizobium* and *Mesorhizobium* genomic species were found on 14% of the

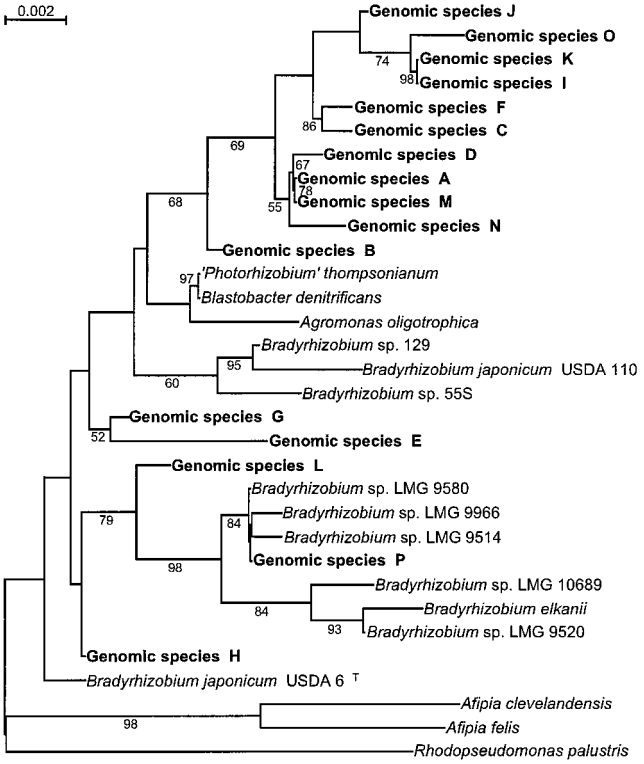

FIG. 3. Phylogenetic relationships among genomic species belonging to the genus *Bradyrhizobium* characterized by SSU rDNA PCR-RFLPs. The phylogenetic tree was constructed by the neighbor-joining method. The numbers correspond to the percentage of bootstrap support for internal branches, based on 1,000 replications. The scale bar corresponds to 0.002 substitution per site.

TABLE 2. Distribution of 745 rhizobial isolates among 21 genomic species identified by RFLP analysis of PCR-amplified SSU rRNA genes

Genomic species		No. of isolates	% of total isolates
Bradyrhizobium	A	429	57.58
	P	82	11.01
	B	77	10.34
	F	35	4.70
	H	24	3.22
	I	20	2.68
	D	18	2.42
	O	6	0.81
	E	3	0.40
	J	2	0.27
	C	1	0.13
	G	1	0.13
	K	1	0.13
	L	1	0.13
	M	1	0.13
	N	1	0.13
Rhizobium	Q	38	5.10
	R	1	0.13
Mesorhizobium	S	2	0.27
	T	1	0.13
	U	1	0.13

plants hosting a minimum of two nodules and always co-occurred with various *Bradyrhizobium* species.

Frequencies of the various rhizobial genomic species were calculated for each host summed across all sites (Table 3) and for each site regardless of their host origin (Table 4). All genomic species isolated more than once were found on several hosts and at more than one site, including species J, which was isolated only twice. For all but one host and at all sites, one genomic species dominated the rhizobial community. At 10 of 12 sites and for most hosts (21 of 32) this was genomic species A. Genomic species P dominated among the rhizobia found on four of the nine host species from Ben Boyd National Park site (*Aotus ericoides*, *Dillwynia glaberrima*, *Dillwynia sericea*, and *Pultenaea daphnoides*), and B was the dominant genomic species isolated on the roots of four of the five hosts collected exclusively at Lobs Hole site (*Daviesia latifolia*, *Gompholobium huegelii*, *Hovea linearis*, *Hovea purpurea*, and *Mirbelia oxylobioides*). Genomic species co-occurring either on plants of the same host or at the same site were each present at much lower frequencies than the dominant rhizobial species and were generally widely distributed among a number of hosts and occurred at several sites. Only in the case of three minor genomic species (D, F, and H) was one particular host predominantly nodulated (Table 3).

The 13 minor rhizobial genomic species were excluded from the association analyses because of the paucity of data. There was a highly significant association between the remaining eight rhizobial genomic species and both the 32 hosts (maximum-likelihood chi-square = 790.79 with 217 df; $P < 0.001$) and the 12 sampling sites (maximum-likelihood chi-square = 973.98 with 77 df; $P < 0.001$). This association was clearly visible on the projection of rhizobial genomic species and either legume hosts or sampling sites along the two first axes generated by correspondence analyses (Fig. 4A and B). Hosts for which several rhizobial genomic species were relatively abundant had an intermediate position between the two or three major rhizobial species, such as *Bossiaea foliosa*, *Platylobium formosum*, and *G. huegelii* between A and B; *Daviesia leptophylla* and *Daviesia buxifolia* between A and D; and *Pultenaea capitellata* between A, and F and P (Fig. 4A).

The number and frequency of rhizobial genomic species varied among host species belonging to the same genus (Table 3). Although genomic species A was prevalent for all species of *Bossiaea* and *Pultenaea*, the relative distribution of the rhizobia between A and all other genomic species varied significantly from one taxon to another within either genus (chi-square = 41.4 with 18 df [$P < 0.005$] and chi-square = 32.3 with 15 df [$P < 0.001$], respectively). In contrast, in the case of the two *Hovea* species, this was not significant (chi-square = 4.1 with 3 df; $P = 0.25$ [B is the prevalent rhizobial genomic species in this case]). The genera *Daviesia*, *Dillwynia*, and *Mirbelia* exhibited species differences in both the predominant rhizobial genomic species and the distribution of rhizobial genomic species. No clear specificity could be observed at a higher taxonomic rank between the two subfamilies represented among the sampled hosts. The strains isolated from the only member of the subfamily Mimosoideae represented in the present sample, *Acacia obliquinervia*, belonged to two of the most abundant rhizobial genomic species, A and F, also isolated from various Papilionoideae species. For this host, however, F represented more than half of the strains (58.3%) and A represented only 16.7%.

DISCUSSION

Rhizobial diversity. Early reports indicated that symbionts of native legumes in Australia were typical slow-growing bacteria with the characteristics of *Bradyrhizobium* species (31, 40, 47, 50). Later studies, however, have suggested a higher level of diversity. Ninety-eight percent of the strains isolated from acacias at Fowlers Gap, near Broken Hill in arid northwestern New South Wales (NSW), were fast growers (3), and slow- and fast-growing rhizobia were shown to occur in temperate Australia (4, 41, 63). Nevertheless, a high predominance of *Bradyrhizobium* species has generally been observed in temperate southeastern Australia (3, 4, 41, 63) as well as in other parts of Australia, e.g., Queensland (57) and Western Australia (40). Barnet and Catt (3) isolated atypical very slow growing rhizobia with high host specificity in the alpine area of Kosciusko National Park. Very slow growing rhizobia were also isolated at Bridge Hill near Bulahdelah (4). In contrast, Barnet et al. (5) showed that fast-growing rhizobia isolated from *Acacia* spp. in NSW were diverse and belonged to various *Rhizobium* species, suggesting that some represented a new genus more closely related to *Bradyrhizobium* than to *Rhizobium*. Unfortunately, the various previous descriptions of rhizobia occurring on Australian native plants were based solely on growth features and the cross-inoculation concept and thus do not provide precise information on the real nature and structure of the rhizobial communities in Australian ecosystems.

From these various studies, and as the range of sites studied in the past was limited, we expected to encounter high diversity among indigenous rhizobial strains in Australia. Among the 745 strains that we typed, 21 rhizobial genomic species were identified. This might seem low; however, whereas the results of SSU rDNA analyses clearly show differences between strains, SSU rDNA is not appropriate for the formal delineation of species (44, 65). Stackebrandt and Goebel (62) showed that two procaryotes are unlikely to have more than 60 to 70% DNA similarity, and hence to be related at the species level, when their SSU rDNA sequences have less than 97% homology but that above 97% SSU rDNA homology, the DNA sim-

TABLE 3. Frequencies[a] of 21 rhizobial genomic species among legume host species from which nodules were collected

Host[b]	*Bradyrhizobium*																*Rhizobium*		*Mesorhizobium*			No.[c]			
	A	P	B	F	H	I	D	O	E	J	M	C	G	K	L	N	Q	S	R	T	U	GS	N	S	P
Ao	0.167			0.583		0.083												0.083		0.083		5	12	1	1
Ae	0.333	0.667																				2	3	1	3
Bb	0.708	0.042	0.167					0.083														4	24	1	11
Be	0.630	0.259	0.037	0.037									0.037									5	27	2	8
Bf	0.451	0.020	0.216	0.098		0.078				0.020							0.098	0.020				8	51	3	16
Db	0.545	0.273					0.182															3	11	1	5
Dla			0.889			0.111																2	9	1	5
Dle	0.500	0.107					0.321										0.071					4	28	3	14
Dm	0.833				0.111					0.056												3	18	2	9
Du	0.587	0.032	0.016	0.048	0.032	0.063	0.016	0.016									0.190					9	63	4	20
Dwb	1.000																					1	6	1	2
Dwg	0.444	0.556																				2	9	1	4
Dwra	0.864	0.121															0.015					3	66	1	5
Dwre	0.971		0.029																			2	35	1	10
Dws	0.231	0.769																				2	13	1	10
Gh	0.250	0.208	0.375					0.042									0.125					5	24	2	9
Gl	0.200	0.143		0.229	0.271	0.057		0.014						0.014			0.057		0.014			9	70	1	10
Hv	0.630		0.037			0.037									0.037	0.037	0.222					6	27	2	10
Hl	0.111		0.667			0.222																3	9	1	5
Hp	0.133		0.800		0.067																	3	15	1	6
Ia	0.944																0.056					2	18	1	8
Mo		0.143	0.857																			2	7	1	4
Mr	0.875		0.042						0.042		0.042											4	24	1	6
Oe	0.755	0.075	0.057			0.019		0.019	0.019			0.019					0.019				0.019	9	53	2	9
Php	0.250						0.750															2	4	1	2
Pf	0.571		0.429																			2	21	2	10
Poa	1.000																					1	2	1	1
Poi	0.400	0.200	0.100				0.150		0.050								0.100					6	20	3	14
Pc	0.600	0.200		0.200																		3	10	1	5
Pd	0.467	0.467		0.067																		3	15	1	5
Pp	0.848		0.061			0.061											0.030					4	33	2	13
Ps	0.611	0.389																				2	18	1	6

[a] The highest frequency is underlined for each host.
[b] Ao, *A. obliquinervia*; Ae, *A. ericoides*; Bb, *B. buxifolia*; Be, *Bossiaea ensata*; Bf, *B. foliosa*; Db, *D. buxifolia*; Dla, *D. latifolia*; Dle, *D. leptophylla*; Dm, *Daviesia mimosoides*; Du, *D. ulicifolia*; Dwb, *Dillwynia brunioides*; Dwg, *D. glaberrima*; Dwra, *Dillwynia ramosissima*; Dwre, *Dillwynia retorta*; Dws, *D. sericea*; Gh, *G. huegelii*; Gl, *G. lotifolia*; Hv, *Hardenbergia violacea*; Hl, *H. linearis*; Hp, *H. purpurea*; Ia, *Indigofera australis*; Mo, *M. oxylobioides*; Mr, *Mirbelia rubiifolia*; Oe, *Oxylobium ellipticum*; Php, *P. phylicoides*; Pf, *P. formosum*; Poa, *Podolobium alpestre*; Poi, *Podolobium ilicifolium*; Pc, *P. capitellata*; Pd, *Pultenaea daphnoides*; Pp, *Pultenaea procumbens*; Ps, *Pultenaea scabra*.
[c] GS, number of rhizobial genomic species; N, number of nodules collected; S, number of sites where the genomic species occurred; P, number of plants sampled.

ilarity can vary greatly, from 10 to 100%. Thus, a very high SSU rDNA similarity, as high as 99.8%, can be observed for different species (22). In contrast, heterogeneity between SSU rDNA sequences has been documented within the seven rRNA operons of *E. coli* (12). It will thus be necessary to perform DNA-DNA hybridization and thermal denaturation analyses, which constitute the criteria commonly used to define bacterial species (65), to evaluate the full extent of taxonomic diversity among our strains. Nevertheless, with one exception, none of these genomic species corresponded to previously described rhizobia—a pattern that has generally been observed in other studies of wild rhizobial communities (10, 18, 49, 53, 68) and

TABLE 4. Frequencies[a] of 21 rhizobial genomic species among sites where nodules were sampled

Site[b]	*Bradyrhizobium*																*Rhizobium*		*Mesorhizobium*			No.[c]			
	A	P	B	F	H	I	D	O	E	J	C	G	K	L	M	N	Q	S	R	T	U	GS	N	H	P
BBNP	0.469	0.439	0.010	0.031			0.051										0.043					5	98	9	46
BM	0.914		0.022		0.011									0.011			0.013					5	93	4	31
BR	0.782	0.038	0.115			0.026		0.026									0.455					6	78	5	31
GR	0.364					0.091										0.091						4	11	2	7
IB	0.395			0.279		0.163				0.023							0.070	0.047		0.023		7	43	3	8
LFPR	0.289	0.140		0.167	0.184	0.053	0.009	0.018	0.009			0.009					0.114		0.009			11	114	3	25
LH	0.165	0.013	0.709		0.013	0.038		0.013									0.051					7	79	7	34
MF	0.750	0.063			0.063					0.063							0.063					5	17	3	10
MR	0.433	0.233	0.033				0.300															4	30	2	12
TF	0.852	0.070	0.009	0.009			0.026		0.009			0.009			0.009		0.009					9	115	5	19
TR	0.720		0.280																			2	25	3	14
TSR	0.674	0.070				0.023		0.023	0.023		0.023						0.140				0.023	8	42	2	9

[a] The highest frequency is underlined for each site.
[b] BBNP, Ben Boyd National Park; BM, Black Mountain; BR, Boboyan Road; GR, Gunning Road; IB, Island Bend; LFPR, Lowden Forest Park Road; LH, Lobs Hole; MF, Mount Franklin; MR, Mundoonen Range; TF, Tianjiara Falls; TR, Turpentine Road; TSR, Two Sticks Road.
[c] GS, number of rhizobial genomic species; N, number of nodules collected; H, number of hosts on which species occurred; P, number of plants sampled.

which suggests a very high level of diversity within the rhizobium taxonomy.

Geographical localization. Barnet and Catt (3) found marked geographical localization of the various rhizobial types according to their rates of growth: fast growers in arid northwestern NSW, typical *Bradyrhizobium* at the two distant coastal heath areas (Myall Lakes National Park and Wanda Beach) and a rain forest site in Blue Mountains National Park, and slow and very slow growers in the alpine area of Kosciusko National Park. In contrast, other studies of rhizobial diversity in other parts of the world failed to identify a geographical specificity of particular rhizobial types (52, 68). We did not observe such a geographical partitioning of the various genomic species, and we found no difference in the geographic distribution of *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium* species (we have to assume that fast growers identified by Barnet and Catt belong to *Rhizobium* and slow growers belong to *Bradyrhizobium*). On the contrary, most genomic species were found at several sites, even in the case of the species isolated on just a few occasions (e.g., genomic species E, J, and O). In general, one prevalent genomic species was recovered from a particular site, with a number of additional species present at much lower frequencies. Our sampling, however, did not cover the same climatic range as the Barnet and Catt study. A study conducted a few years ago at Mount Cootha in Queensland investigated the diversity of soil bacterium communities by using a molecular approach, in which partial SSU rDNA sequences (about 250 bases long) were generated from DNA directly extracted from the soil (42). A phylogenetic analysis including members of the alpha proteobacteria division revealed that some of the clones belonged to the *Rhizobium-Agrobacterium* group. None was strictly identical to any SSU rRNA sequence already available in nucleic acid databases at that time. When compared to our sequences, two clones, MC6 and MC23 (accession no. X65573 and X65578 in the GenBank/EMBL/DDJB DNA sequence database), showed perfect identity with the corresponding parts of the sequences of genomic species G and L, a difference of one base with species H, and a difference of two bases with species P and E. In our phylogenetic analysis (Fig. 3), P and L clustered with the *B. elkanii* clade. E, G, and H did not show any affinity to any particular previously characterized lineage within the *Bradyrhizobium-Rhodopseudomonas* subgroup and thus are expected to constitute a new lineage (genus?) within this group. These results can only be indicative, since a different phylogeny can sometimes be obtained when the complete SSU rRNA gene is considered, as in the case of *Rhizobium galegae* (51, 66) or *Rhizobium etli* (46). However, it appears that there are strong similarities between rhizobial communities at the sites that we sampled and that at the distant and climatically different site in Queensland. Species that we identified in the temperate zone thus appear to be widespread geographically under very different climate conditions.

All 12 sites presented some degree of diversity of vegetation and soil characteristics, but all had acid or near-neutral soil, conditions which favor *Bradyrhizobium* species over *Rhizobium*, *Mesorhizobium*, or *Sinorhizobium* species (27, 50). Bradyrhizobia can survive at low pH, which is not the case for most strains of the other rhizobial genera (27). The predominance of *Bradyrhizobium* species among our isolates is thus not surprising, as is the presence of slow-growing strains in similar sites (Kosciusko National Park, Wanda Beach, Myall Lakes National Park, and Blue Mountains National Park) studied by Barnet and Catt (3). Among the *Rhizobium* and *Mesorhizobium* species that we isolated, the most abundant (*R. tropici*) was also one of the more acid tolerant (27). It is thus likely that there is a relation between the level of soil acidity and the nature of the

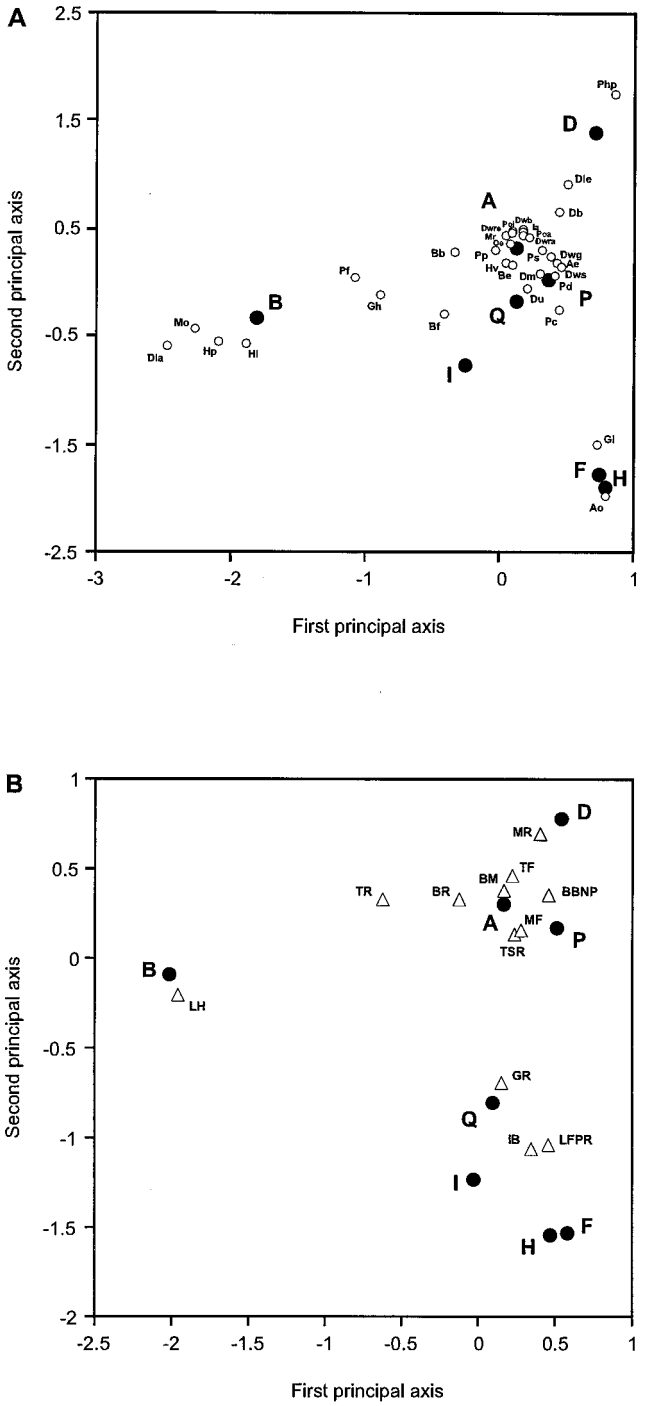

FIG. 4. Position of the eight more-abundant rhizobial genomic species and legume hosts or sites along the first and second principal axes. (A) Association between genomic species (solid circles) and legume hosts (open circles). Abbreviations: Ao, *A. obliquinervia*; Ae, *A. ericoides*; Bb, *Bossiaea buxifolia*; Be, *Bossiaea ensata*; Bf, *B. foliosa*; Db, *D. buxifolia*; Dla, *D. latifolia*; Dle, *D. leptophylla*; Dm, *Daviesia mimosoides*; Du, *D. ulicifolia*; Dwb, *Dillwynia brunioides*; Dwg, *D. glaberrima*; Dwra, *Dillwynia ramosissima*; Dwre, *Dillwynia retorta*; Dws, *D. sericea*; Gh, *G. huegelii*; Gl, *G. lotifolia*; Hv, *Hardenbergia violacea*; Hl, *H. linearis*; Hp, *H. purpurea*; Ia, *Indigofera australis*; Mo, *M. oxylobioides*; Mr, *Mirbelia rubiifolia*; Oe, *Oxylobium ellipticum*; Php, *P. phylicoides*; Pf, *P. formosum*; Poa, *Podolobium alpestre*; Poi, *Podolobium ilicifolium*; Pc, *P. capitellata*; Pd, *P. daphnoides*; Pp, *Pultenaea procumbens*; Ps, *Pultenaea scabra*. (B) Association between genomic species (solid circles) and legume hosts (open triangles). Abbreviations: BBNP, Ben Boyd National Park; BM, Black Mountain; BR, Boboyan Road; GR, Gunning Road; IB, Island Bend; LFPR, Lowden Forest Park Road; LH, Lobs Hole; MF, Mount Franklin; MR, Mundoonen Range; TF, Tianjiara Falls; TR, Turpentine Road; TSR, Two Sticks Road.

rhizobial species present at our sampling sites. This had also been observed in Africa for *Rhizobium* species nodulating *Phaseolus vulgaris*. An apparently similar degree of diversity was found at two sites with different soil pH levels. However, *R. tropici* predominated at the acid soil site, and *R. etli* predominated at the site with a near-neutral soil (2, 26). The apparent geographical specificity described by Barnet and Catt (3) certainly reflects the lack of resolution of the rhizobium identification methodology applied but also reflects pH differences between the sites.

Host specificity. We did not observe any clear host specificity at either the host species or genus level between any particular rhizobial species and its leguminous host, as was previously reported by several authors (24, 33, 48, 68). Likewise, no clear specificity could be seen at a higher taxonomic rank or within a particular plant. In particular, *Rhizobium* and *Mesorhizobium* genomic species were never found to nodulate exclusively either a particular host or a particular plant. This co-occurrence of slow- and fast-growing rhizobia on the same host genus or species appears to happen quite commonly (24, 43, 49, 54). Even when several legume species occurred at a site, in most cases, all of the species were predominantly nodulated with the commonest rhizobial species found at that site. Clearly, in very many cases, this involved rhizobial genomic species A. However, without further detailed testing, this association cannot simply be ascribed to a generally better "fit" of genomic species A to all legumes as, even at sites dominated by other genomic species (e.g., Lobs Hole with genomic species B and Gunning Road with genomic species Q), genomic species A was also present.

In contrast, in the cases of *A. obliquinervia*, *Goodia lotifolia*, and *Phyllota phylicoides*, we did observe some suggestion of preference for particular rhizobial species. Different rhizobial genomic species were recovered from their root nodules, but the dominant species isolated (which was different for each of the three species) also differed from the prevalent species isolated from nodules on other legume hosts occurring at the same sites. Thus, at Island Bend, where genomic species A was predominant on *B. foliosa* and *Daviesia ulicifolia*, almost 60% of isolates recovered from *A. obliquinervia* were of genomic species F. Similarly, for *G. lotifolia* at Lowden Forest Park Road the dominant species was H, and for *P. phylicoides* at Tianjiara Falls it was D, although genomic species A was commonest on all co-occurring species (two and four species, respectively). Furthermore, the correspondence analyses (Fig. 4A and B) indicated stronger links in the host-rhizobium comparisons than in the site-rhizobium comparisons for the genomic species prevalent on each of these three legume species. The differences observed for *A. obliquinervia*, *G. lotifolia*, and *P. phylicoides* are consistent with the suggestion that one rhizobial species is being selected by these legume hosts regardless of the apparently most abundant rhizobial species at those sites. These three host species could only be sampled at one site each, and our results need confirming by further sampling at additional sites. However, it is of particular interest to note that *A. obliquinervia* is the only member of the subfamily Mimosoideae from which nodules were isolated for the present study, all others belonging to the Papilionoideae. This might indicate a specificity difference between the Fabaceae subfamilies. Further investigation is needed to evaluate this observation.

Considering the broad range of specificities either of rhizobial species towards their hosts or of the legume species towards their symbionts, molecular identification appears to be a prerequisite to any study of rhizobial population structure. Indeed, it is fundamental to differentiate between members of the same species and members of a group of species, to be able to provide some insight on the relationship between the two partners, and to infer the factors determining the legume-rhizobium symbiotic association. Interestingly, species of the *B. elkanii* cluster can be differentiated from other *Bradyrhizobium* species by a short part of their SSU rRNA gene sequence, corresponding to a highly variable part of the molecule (30). Although it was fully conserved between sequences of species within the *B. elkanii* subgroup, it was highly divergent from that of other species in the *Bradyrhizobium* subgroup, to the extent that no homology could be safely identified. The other species, in turn, were characterized by a unique sequence. In contrast, the *B. elkanii* subgroup "signature" sequence showed a reasonably good level of similarity to SSU rDNA sequence from species of the *Mesorhizobium* subgroup, which represents a comparatively distant lineage. The occurrence of recombination in SSU rRNA genes has been documented in *Aeromonas* (60), as well as among *Rhizobium* and *Agrobacterium* species (19, 20); it is thus possible that SSU rDNA of the ancestor of the *B. elkanii* cluster evolved by recombination between distant lineages, possibly representing the same lifestyle. Further analyses of rRNA genes might bring insight into the mode of evolution of the different rhizobial lineages and the emergence and spreading of nodulation ability.

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