APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2004, p. 5980–5987 Vol. 70, No. 10
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.10.5980–5987.2004

Unexpectedly Diverse *Mesorhizobium* Strains and *Rhizobium leguminosarum* Nodulate Native Legume Genera of New Zealand, while Introduced Legume Weeds Are Nodulated by *Bradyrhizobium* Species

Bevan S. Weir,[1,2]* Susan J. Turner,[2] Warwick B. Silvester,[3] Duck-Chul Park,[1] and John M. Young[1]

Landcare Research[1] and School of Biological Sciences, University of Auckland,[2] Auckland, and Department of Biological Sciences, University of Waikato, Hamilton,[3] New Zealand

Received 29 January 2004/Accepted 11 June 2004

The New Zealand native legume flora are represented by four genera, *Sophora*, *Carmichaelia*, *Clianthus,* and *Montigena*. The adventive flora of New Zealand contains several legume species introduced in the 19th century and now established as serious invasive weeds. Until now, nothing has been reported on the identification of the associated rhizobia of native or introduced legumes in New Zealand. The success of the introduced species may be due, at least in part, to the nature of their rhizobial symbioses. This study set out to address this issue by identifying rhizobial strains isolated from species of the four native legume genera and from the introduced weeds: *Acacia* spp. (wattles), *Cytisus scoparius* (broom), and *Ulex europaeus* (gorse). The identities of the isolates and their relationship to known rhizobia were established by comparative analysis of 16S ribosomal DNA, *atpD*, *glnII*, and *recA* gene sequences. Maximum-likelihood analysis of the resultant data partitioned the bacteria into three genera. Most isolates from native legumes aligned with the genus *Mesorhizobium*, either as members of named species or as putative novel species. The widespread distribution of strains from individual native legume genera across *Mesorhizobium* spp. contrasts with previous reports implying that bacterial species are specific to limited numbers of legume genera. In addition, four isolates were identified as *Rhizobium leguminosarum*. In contrast, all sequences from isolates from introduced weeds aligned with *Bradyrhizobium* species but formed clusters distinct from existing named species. These results show that native legume genera and these introduced legume genera do not have the same rhizobial populations.

Rhizobia are soil-inhabiting bacteria that form symbiotic relationships with plant legume species in root nodules. The bacteria fix nitrogen from the atmosphere to form ammonia, which is assimilated by the plant. This relationship has been exploited by agriculture to enhance legume crop growth without the addition of nitrogen-containing fertilizers. For this reason, the majority of research in this field has focused on the herbaceous crop legumes of agricultural significance. In contrast, few studies have been made of rhizobial associations among noncrop legumes, despite the fact that they may be important in ecological interactions.

Worldwide, there are an estimated 17,000 to 19,000 legume species (19). However, symbiotic bacterial species have only been identified for a small proportion of these. To date, 45 symbiotic nodulating bacterial species have been identified in 10 genera: *Azorhizobium*, *Blastobacter*, *Bradyrhizobium*, *Burkholderia*, *Devosia*, *Mesorhizobium*, *Methylobacterium*, *Ralstonia*, *Rhizobium*, and *Sinorhizobium* (28). Most of the species are in the genera *Rhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium* and are related to one another in the order *Rhizobiales*, with *Rhizobium* and *Sinorhizobium* in the family *Rhizobiaceae*, and *Mesorhizobium* in the *Phyllobacteriaceae* (28).

The symbiotic relationships of nitrogen-fixing members of the Rhizobiaceae have been intensively studied (for reviews, see references 5, 6, 15, and 16). Some of these rhizobial species are reported to form host specific associations with particular legumes. For example, *Rhizobium leguminosarum* biovar *trifolii* is considered specific to clover (*Trifolium* spp.) (12), and *Rhizobium galegae* is reported only on goat's rue (*Galega* spp.) (16). Many other *Rhizobium* spp. and *Mesorhizobium* spp. appear to be more or less "promiscuous," nodulating more than one plant genus, and most nodulate two or more plant genera (5, 16). *Sinorhizobium fredii* strain NGR234 forms rhizobial associations with 232 species in 112 genera (12, 26, 27). Generally, rhizobia of herbaceous host species are reported to be more promiscuous than those of woody legumes (24). Despite more than a century of research, the host ranges for rhizobial species are known for fewer than 200 plant species, most being crop, forage, or grain legumes (16, 17).

New Zealand has a number of native (naturally occurring) woody legumes comprising 34 species in the genera *Carmichaelia*, *Clianthus*, *Montigena*, and *Sophora* (9, 10). There are also over 100 naturalized woody legume species that were introduced into New Zealand since colonization by Europeans in the 19th century. These include the woody legumes *Cytisus* spp. (brooms), gorse (*Ulex europaeus*) from Europe, and vari-

* Corresponding author. Mailing address: Landcare Research, Private Bag 92170, Auckland, New Zealand. Phone: 64 9 574 4200. Fax: 64 9 574 4101. E-mail: weirb@LandcareResearch.co.nz.

TABLE 1. Genomic grouping of rhizobia isolated from native and introduced plants in New Zealand

Legumes	ICMP no.	Host legume	Genomic group	GenBank accession no.[a]			
				16S rDNA	*atpD*	*glnII*	*recA*
Native	11727	*Carmichaelia australis*	E	AY491060	ND	ND	ND
	12687	*Carmichaelia australis*	E	AY491061	ND	ND	ND
	13190	*Carmichaelia australis*	D	AY491071	AY493456	AY494808*	AY494822
	15054	*Carmichaelia australis*	A	AY491068	ND	ND	ND
	11708	*Carmichaelia nana*	D	AY491073	AY493454	AY494792	AY494818
	11722	*Carmichaelia nana*	D	AY491072	AY493458	AY494810	AY494814
	14319	*Carmichaelia odorata*	D	AY491074	AY493459	AY494812	AY494817
	12635	*Carmichaelia petriei*	D	AY491075	AY493457	AY494811	AY494815
	12649	*Carmichaelia petriei*	A	AY491064	ND	ND	ND
	11541	*Clianthus puniceus*	D	AY491070	AY493455	AY494809	AY494821
	11542	*Clianthus puniceus*	E	AY491059	ND	ND	ND
	11721	*Clianthus puniceus*	C	AY491077	ND	ND	ND
	11726	*Clianthus puniceus*	C	AY491078	ND	ND	ND
	12685	*Montigena novae-zelandiae*	B	AY491069	AY493452	AY494793	AY494823
	12690	*Montigena novae-zelandiae*	D	AY491076	ND	ND	ND
	14642	*Sophora chathamica*	E	AY491062	AY493451	AY494795	AY494813
	11736	*Sophora microphylla*	A	AY491063	ND	ND	ND
	12637	*Sophora microphylla*	A	AY491066	ND	ND	ND
	14330	*Sophora microphylla*	A	AY491067	AY493461	AY494806	AY494820
	11719	*Sophora tetraptera*	A	AY491065	X	AY494805	AY494819
Introduced	12835	*Acacia dealbata*	G	AY491090	AY493444	AY494799	AY494826
	14754	*Acacia longifolia*	G	AY491094	AY493448	AY494801	AY494832
	14755	*Acacia longifolia*	G	AY491089	AY493449	AY494802	AY494828
	14752	*Albizia julibrissin*	J	AY491081	AY493443	AY494798	AY494830
	14753	*Albizia julibrissin*	F	AY491082	AY493447	AY494797	AY494831
	12624	*Cytisus scoparius*	J	AY491079	ND	ND	ND
	14291	*Cytisus scoparius*	J	AY491084	AY493442	AY494796	AY494829
	14309	*Cytisus scoparius*	J	AY491087	ND	ND	ND
	14310	*Cytisus scoparius*	J	AY491088	ND	ND	ND
	14328	*Cytisus scoparius*	J	AY491086	ND	ND	ND
	12674	*Ulex europaeus*	I	AY491080	ND	ND	ND
	14292	*Ulex europaeus*	J	AY491085	ND	ND	ND
	14304	*Ulex europaeus*	H	AY491091	ND	ND	ND
	14306	*Ulex europaeus*	J	AY491083	ND	ND	ND
	14320	*Ulex europaeus*	I	AY491092	ND	ND	ND
	14533	*Ulex europaeus*	G	AY491093	AY493445	AY494800	AY494827

[a] It was not always possible to obtain both sequences for *glnII*. * indicates relative position of the missing section. ND, not determined. X, PCR amplification failed.

ous *Acacia* and *Albizia* spp. (wattles) from Australia (21), introduced as ornamental or hedge plants. In their native habitats, these shrubs are in equilibrium with their natural flora, but in New Zealand, they have become serious invasive noxious weeds. The New Zealand native legumes, together with broom and gorse, are members of the subfamily *Faboideae* (*Papilionoideae*), distinct from *Acacia* and *Albizia* in the *Mimosoideae*.

The ability of legume plants to become established in soils of low fertility and to compete successfully with other plants can be attributed in part to the symbiotic associations that give them the capacity to fix atmospheric nitrogen. The plant-rhizobial association usually forms immediately following germination if the nodulating rhizobia are present naturally in the soil. Because New Zealand became geographically isolated about 80 million years ago (29), it is postulated that the native legume genera coevolved with nitrogen-fixing bacterial symbionts in isolation from the regions of major legume evolution. However, the source of rhizobial symbionts of introduced legumes is unknown. Either these rhizobia were introduced at the same time as the plants, or the plants were able to use indigenous rhizobia associated with native legumes. Alternatively, they made use of a diazotrophic bacterial population preexisting in New Zealand soils.

The primary objective of this research was to identify the rhizobial symbionts of both native and introduced New Zealand legumes and to determine whether these are putatively indigenous or cosmopolitan strains. To this end, rhizobial isolates have been obtained from native and introduced legumes and sets of gene sequences from them were compared in order to establish the identity and relationships of the bacteria to their legume hosts.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains (Table 1) were either obtained from the International Collection of Microorganisms from Plants (ICMP, Landcare Research, Auckland, New Zealand; http://www.landcareresearch.co.nz/research/biodiversity/fungiprog/icmp.asp) or directly isolated from wild plants (usually as young seedlings). Nodules of native legumes were obtained from pristine sites on conservation lands, distant from agricultural plantings, throughout the country. Introduced legume samples were obtained from arable lands or from conservation lands.

Bacterial isolation. Root nodules were dissected from roots, rinsed thoroughly in water and Tween 80 (0.001%), surface sterilized by immersion in a 5% solution of commercial sodium hypochlorite (3% active hypochlorite) for 30 min,

TABLE 2. Primers used in this study

Primer	Sequence	Target gene	Reference
16S-1F	AGCGGCGGACGGGTGAGTAATG		11
16S-485F	CAGCAGCCGCGGTAA		11
16S-1100R	GGGTTGCGCTCGTTG	16S rDNA	11
16S-1509R	AAGGAGGGGATCCAGCCGCA		11
16S-PB36	AGRGTTTGATCMTGGCTCAG		4
atpD-273F	SCTGGGSCGYATCMTGAACGT		7
atpD-771R	GCCGACACTTCCGAACCNGCCTG	ATP synthase beta-subunit	7
atpD-294F	ATCGGCGAGCCGGTCGACGA		7
GSII-1	AACGCAGATCAAGGAATTCG		34
GSII-2	ATGCCCGAGCCGTTCCAGTC		34
GSII-3	AGRTYTTCGGCAAGGGYTC	Glutamine synthetase II	34
GSII-4	GCGAACGATCTGGTAGGGGT		34
recA-6F	CGKCTSGTAGAGGAYAAATCGGTGGA		7
recA-555R	CGRATCTGGTTGATGAAGATCACCAT		7
recA-63F	ATCGAGCGGTCGTTCGGCAAGGG	DNA recombinase A	7
recA-504R	TTGCGCAGCGCCTGGCTCAT		7
recA-BF	CGTACTGTCGAAGGTTCTTCCATGGA		This study

and rinsed in sterile water. Nodules were individually comminuted, and the suspension was streaked onto surface-dried yeast mannitol agar plates (YMA; 10 g of active dried baker's yeast, 10 g of mannitol [Sigma], 2.5 g of peptone [Difco], 15 g of agar, 1 liter of deionized water). Plates were incubated at 28°C for 3 to 10 days. Individual colonies were selected, restreaked onto YMA plates, and subcultured onto slopes of YMA supplemented with 3 g of calcium carbonate and 1.5 g of calcium gluconate per liter for short-term storage at 8°C. The strains used and reported in this study were deposited in the ICMP.

DNA extraction, PCR amplification, and sequencing. DNA was isolated from bacterial cultures with a standard phenol-chloroform extraction method (2). Primers for PCR amplification, with their sources and sequences, are shown in Table 2. All amplification conditions were performed as specified by their authors. Either 16S-1F or 16S-PB36 was used as the forward primer for the 16S ribosomal DNA (rDNA) sequence. No *recA* PCR products for *Bradyrhizobium* spp. were obtained with the published *recA* primers (7).

After aligning the sequences, it was found that the forward primer was placed in a variable region of the gene. A new forward primer, recA-BF, was designed based on the *recA* sequence from the related bacterium *Rhodopseudomonas palustris* (GenBank accession no. D84467). With this primer, *recA* PCR products were obtained for all New Zealand *Bradyrhizobium* strains and the type strain of *Bradyrhizobium liaoningense*. However, no PCR product was obtained from *Bradyrhizobium elkanii* or *Bradyrhizobium japonicum*. PCR amplifications were performed with an Applied Biosystems 9700 thermal cycler. PCR products were column purified with a Roche High Pure PCR product purification kit. Purified products were cycle sequenced with the appropriate primers with BigDye terminator ready reaction mix (ABI) (version 3.0 or 3.1), and sequences were obtained in both directions with an ABI 310 Prism genetic analyzer. Sequences were assembled and edited with Sequencher 3.11 (Gene Codes Corp.).

Phylogenetic analysis. Nucleotide alignments were constructed with ClustalX 1.83 (19) and edited manually with GeneDoc 2.6.02 (33). The four primers for *glnII* amplify two overlapping sections. However, when one of the two sequences for some *glnII* genes was not amplified, the standard method of replacing missing data with the symbol "?" was used. The alignments were checked for chimeras with the Bellerophon server (http://foo.maths.uq.edu.au/~huber/bellerophon.pl) (13). None were found. GenBank sequences from the type strains of representative species from *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* were also included for comparison (Table 3). The outgroup for each alignment of 16S rDNA, *atpD*, and *recA* was the appropriate sequence from *Caulobacter crescentus* strain CB15, obtained from the complete genome (GenBank accession no. NC_002696) in which homologues for sequences were found. There is no outgroup for *glnII* because there is too little homology between the glutamine synthase II gene of the rhizobia and other taxa that could act as an appropriate outgroup.

Preliminary analyses were performed with the ClustalX and PAUP* neighbor-joining methods, with 1,000 bootstrap replicates. Maximum likelihood was compared with neighbor joining and was chosen as the preferred method of analysis because, although more computationally demanding, the assumptions of this model are more rigorous (4). The appropriate maximum-likelihood parameters were selected with the application MODELTEST, version 3.5 (25). This computer program tests nucleotide alignments against 56 models of DNA evolution with maximum likelihood. The resultant negative log likelihood (−lnL) scores and associated parameters were subjected to a hierarchical likelihood ratio test to determine which model best fitted the sequence data. In the 16S rDNA analysis, the TrN+I+Γ model of DNA evolution was selected. In the *atpD*, *glnII,* and *recA* analyses, the GTR+I+Γ model of DNA evolution was selected. The model parameters (base frequencies, proportion of invariable sites, gamma distribution shape parameter, and substitution rate matrix) were then specified in PAUP* 4.0b10 (32) to build phylograms with tree-bisection-reconnection heuristics.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences reported in this study are AY491059 to AY491094 (16S rDNA), AY493442 to AY493461 (*atpD*), AY494791 to AY494812 (*glnII*), and AY494813 to AY494833 (*recA*).

RESULTS

The 16S rDNA sequences were obtained from all isolates. Sequences were obtained for the *atpD*, *glnII*, and *recA* genes from at least one isolate from each of the 14 legume species considered. Sequences of *glnII* could only be partially amplified for four strains: *Mesorhizobium plurifarium* and strain ICMP 14753 (48% sequence coverage obtained) and *Mesorhizobium amorphae* and strain ICMP 13190 (67% sequence coverage obtained). Gaps were treated as missing data as described in Materials and Methods.

Maximum-likelihood and neighbor-joining trees for each gene had similar overall tree topologies (neighbor joining trees, with bootstrap values, are available as supplementary data at http://www.rhizobia.co.nz/papers.html). Groups were selected on the basis of the minimum standard changes between named species in the 16S rDNA phylogram (Fig. 1), and all groups were well supported in both maximum-likelihood and neighbor-joining analyses except for the monotypic groups H and F, which had less than 50% bootstrap support in the neighbor-joining tree. The sequences from rhizobia isolated from New Zealand legumes are distributed in 10 genomic groups (A to J). Sequences from native legumes, *Carmichaelia*,

TABLE 3. Type strains of *Mesorhizobium* spp., *Rhizobium* spp., *Sinorhizobium* spp., and *Caulobacter crescentus*, showing the GenBank records of sequences used in analyses

ICMP no.[a]	Species	GenBank accession no.[b]			
		16S rDNA	*atpD*	*glnII*[c]	*recA*
15022[T]	*M. amorphae*	*AF041442*	AY493453	AY494807*	AY494816
14587[T]	*M. chacoense*	*AJ278249*	AY493460	AY494791	AY494825
13641[T]	*M. ciceri*	*U07934*	*AJ294395*	*AF169580*	*AJ294367*
11069[T]	*M. huakuii*	*D12797*	*AJ294394*	*AF169588*	*AJ294370*
4682[T]	*M. loti*	*X67229*	*AJ294393*	*AF169581*	*AJ294371*
13644[T]	*M. mediterraneum*	*L38825*	*AJ294391*	*AF169578*	*AJ294369*
13640[T]	*M. plurifarium*	*Y14158*	X	*AY494794	AY494824
13645[T]	*M. tianshanense*	*AF041447*	*AJ294392*	*AF169579*	*AJ294368*
13642[T]	*R. etli*	*U28916*	*AJ294404*	*AF169585*	*AJ294375*
13689[T]	*R. leguminosarum*	*U29386*	*AJ294405*	*AF169586*	*AJ294376*
13646[T]	*R. tropici*	*U89832*	*AJ294397*	*AF169584*	*AJ294373*
11139[T]	*S. fredii*	*D01272*	*AJ294402*	*AF169571*	*AJ294379*
12623[T]	*S. meliloti*	*X67222*	*AJ294400*	*AF169593*	*AJ294382*
13648[T]	*S. saheli*	*X68390*	*AJ294399*	*AF169589*	*AJ294380*
13638[T]	*B. elkanii*	*U35000*	AY493446	AY494804	X
2864[T]	*B. japonicum*	*U69638*	*AJ294388*	*AF169582*	X
13639[T]	*B. liaoningense*	*AB029402*	AY493450	AY494803	AY494833
—	*B. yuanmingense*	*AF193818*	—	—	—
—	*C. crescentus*	*AJ227757*	*AE006004*	—	*AE005786*

[a] Type strain indicated by [T]; —, strain not present in the ICMP.
[b] Italicized sequences were obtained from GenBank. It was not always possible to obtain both sequences for *glnII*. *, relative position of the missing section; X, PCR amplification failed; —, sequence data not available.

Clianthus, *Montigena*, and *Sophora* spp., were distributed in groups A to D, together with the reference sequences representing *Mesorhizobium* spp. Other sequences from native legumes also formed a clade (group E) with *Rhizobium leguminosarum*. All rhizobia isolated from introduced legumes, *Acacia*, *Albizia*, *Cytisus*, and *Ulex* spp., were in the *Bradyrhizobium* clade (groups F to J).

Group A comprised strains 14330 (*Sophora* sp.), 11719 (*Sophora* sp.), 11736 (*Sophora* sp.), 12637 (*Sophora* sp.), 12649 (*Carmichaelia* sp.), and 15054 (*Carmichaelia* sp.). These strains were most closely grouped to *Mesorhizobium ciceri* and *Mesorhizobium loti*.

Group B comprised a single strain 12685 (*Montigena* sp.) as an outgroup to *Mesorhizobium ciceri* and *Mesorhizobium loti*.

Group C comprised 11726 and 11721, both from *Clianthus*. These strains were most closely grouped to *Mesorhizobium amorphae*.

Group D comprised strains 11708 (*Carmichaelia* sp.), 14319 (*Carmichaelia* sp.), 12690 (*Montigena* sp.), 12635 (*Carmichaelia* sp.), 13190 (*Carmichaelia* sp.), 11722 (*Carmichaelia* sp.), and 11541 (*Clianthus* sp.). These strains were most closely grouped to *Mesorhizobium huakuii*.

Group E comprised 14642 (*Sophora* sp.), 12687 (*Carmichaelia* sp.), 11542 (*Clianthus* sp.), and 11727 (*Carmichaelia* sp.). These strains were members of the *Rhizobium leguminosarum* clade.

Group F comprised the single isolate 14753 (*Albizia* sp.).

Group G comprised 14754 (*Acacia* sp.), 14755 (*Acacia* sp.), 12835 (*Acacia* sp.) and 14533 (*Ulex* sp.), an outlier group to *Bradyrhizobium liaoningense* and *Bradyrhizobium yuanmingense*.

Group H comprised the single isolate 14304 (*Ulex* sp.).

Group I comprised isolates 14320 (*Ulex* sp.) and 12674 (*Ulex* sp.).

Group J comprised isolates 14309 (*Cytisus* sp.), 14310 (*Cytisus* sp.), 14291 (*Cytisus* sp.), 14328 (*Cytisus* sp.), 14292 (*Ulex* sp.), 14306 (*Ulex* sp.), 14752 (*Albizia* sp.), and 12624 (*Cytisus* sp.).

The trees with the other partial gene sequences (*atpD*, *glnII*, and *recA*) all concurred with the 16S gene tree with respect to placement of the strains into the three genera, *Rhizobium*, *Mesorhizobium*, and *Bradyrhizobium* (Fig. 2, 3, and 4). However, the branching order of the individual sequences in groups A to J represented by 16S rDNA differed from that represented by the 16S rDNA tree.

A comparison of individual base variations in 16S rDNAs between sequences within the different groups showed that there were no base differences in group A (100% similarity), 4 bases (99.7% similarity) for group D in the *Mesorhizobium* spp., 4 bases (99.7% similarity) within group E (*R. leguminosarum*), and 27 bases (98.0% similarity) for all strains within the *Bradyrhizobium* clade.

DISCUSSION

Rhizobial isolates of the three most common and geographically widespread species of *Carmichaelia* and *Sophora* and from the monotypic genera *Clianthus* and *Montigena* (Table 1) were used to infer the phylogenetic relationships of the rhizobia of the native legume genera in New Zealand. These were compared with the rhizobia of important introduced legumes, *Acacia*, *Cytisus*, and *Ulex* spp., which are noxious weeds in New Zealand. Reference sequences from *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* type strains were included. Phylogenetic inference, as an approach to clarifying bacterial relationships, is usually based on the comparative analysis of 16S rDNA sequences and has been used in past investigations of rhizobia (5, 6, 15, 16, 17, 37). In this study,

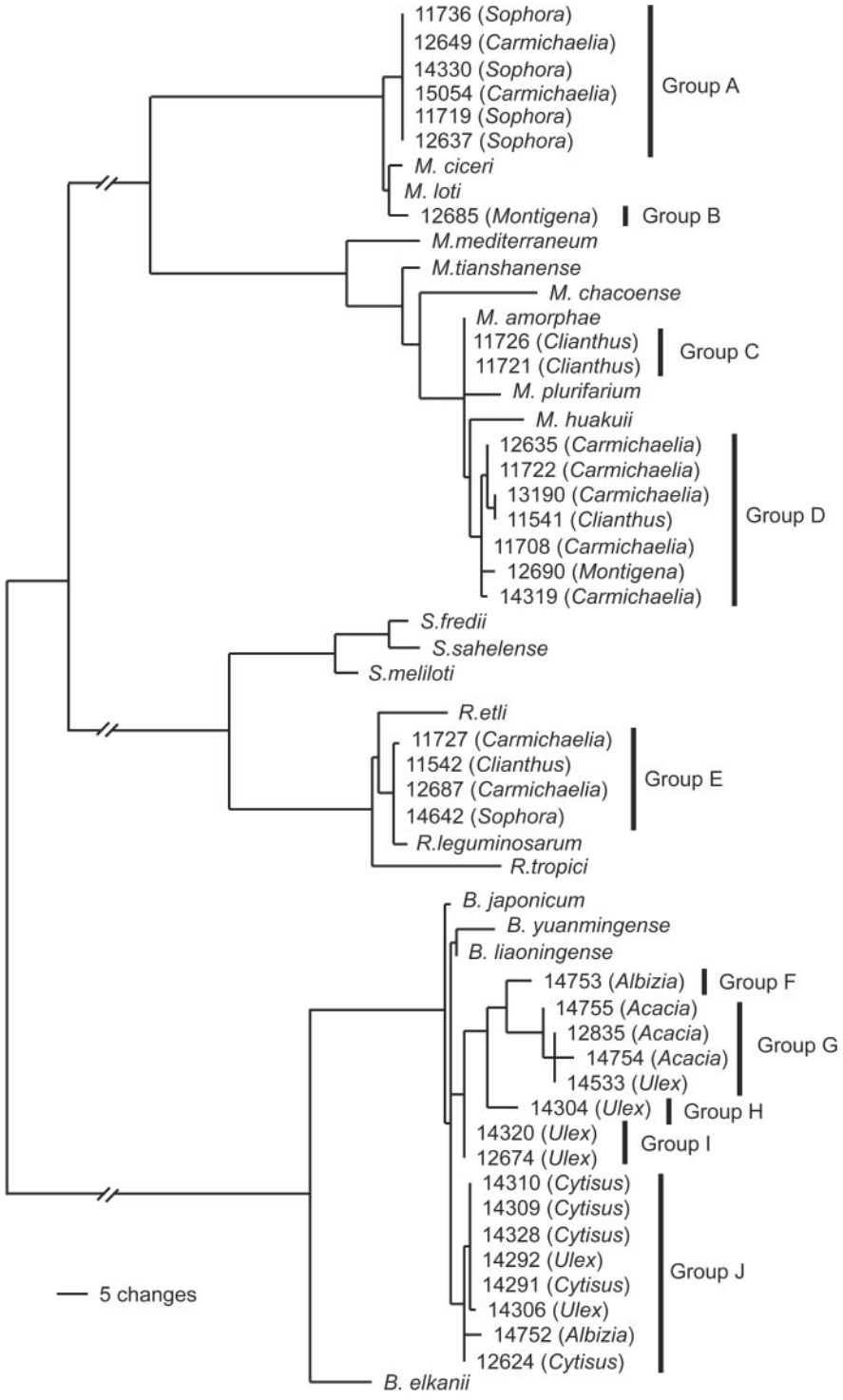

FIG. 1. Maximum-likelihood tree based on 16S rDNA gene sequence data (1,321 bp), showing the relationships of rhizobial isolates from New Zealand legume flora to type strains of rhizobia. The isolate names and the accession number in the ICMP culture collection are shown. The genus of the legume from which the bacteria were isolated is shown in parentheses. Genomic grouping is shown by the vertical bars. The value of −lnL for this tree is 4,565.84.

three additional genes were used to derive more reliable phylogenetic inferences (1, 7, 11).

Tree topologies for *Mesorhizobium*, *Rhizobium*, and *Bradyrhizobium*. Partial sequences of the three housekeeping genes (*atpD*, *glnII*, and *recA*) were also used to generate phylograms, which were then compared. The topologies of all four trees are congruent in indicating that New Zealand's native legumes are nodulated by members of the genera *Mesorhizobium* and *Rhi-*

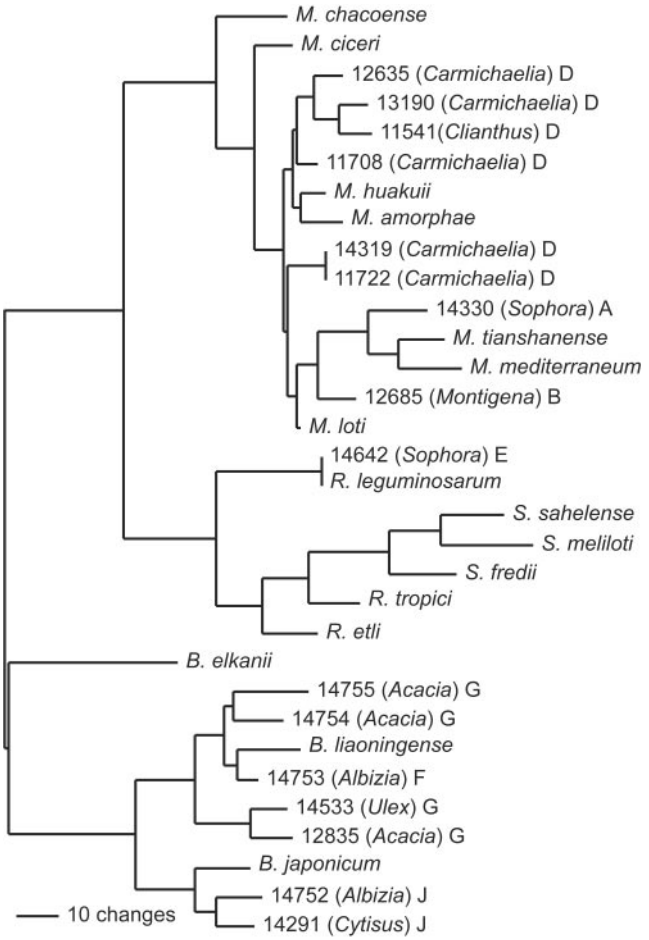

FIG. 2. Maximum-likelihood tree based on *atpD* gene sequence data (464 bp), showing the relationships of rhizobial isolates from New Zealand legume flora to type strains of rhizobia. The isolate names and the accession number in the ICMP culture collection are shown. The genus of the legume from which the bacteria were isolated is shown in parentheses. The letter following the parentheses indicates the genomic grouping as defined by the 16S rDNA data. The value of −lnL for this tree is 3,633.07.

zobium. Based on the analysis of 16S rDNA, individual rhizobial strains were assigned to 10 groups (A to J). Sequences representing rhizobial strains from a single plant genus are distributed between groups. With the exception of group C, which includes two strains from *Clianthus* spp., and group D, which is dominated by strains from *Carmichaelia* spp., the groups generally do not represent bacterial strains from particular host legumes. Homogeneous groups such as group C are probably a reflection of the small sample size of this legume genus. The presence of an outlying *Clianthus* strain in group D suggests that larger representations of strains will result in groups that are more heterogeneous. All other groups are heterogeneous with respect to the host sources of strains. For instance, group A comprises four strains from *Sophora* and two from *Carmichaelia* plants.

Groups A to D are in a clade represented entirely by known *Mesorhizobium* spp. The clade formed by strains in group E, from *Sophora*, *Carmichaelia*, and *Clianthus* plants, includes the sequence representing *Rhizobium leguminosarum*. Group G in

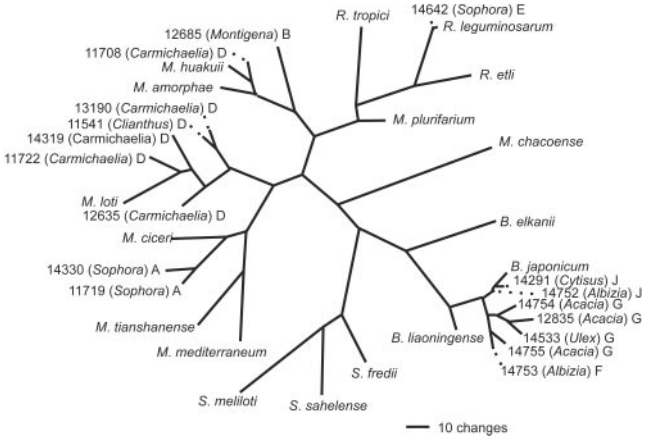

FIG. 3. Maximum-likelihood tree based on *glnII* gene sequence data (828 bp), showing the relationships of rhizobial isolates from New Zealand legume flora to type strains of rhizobia. The isolate names and the accession number in the ICMP culture collection are shown. The genus of the legume from which the bacteria were isolated is shown in parentheses. The letter following the parentheses indicates the genomic grouping as defined by the 16S rDNA data. The value of −lnL for this tree is 6,404.27.

Bradyrhizobium contains all sequences from *Acacia* spp.. The topologies of the different sequences place the strains isolated from New Zealand native legumes in the genus *Mesorhizobium* and in *Rhizobium leguminosarum* and those from all the introduced legumes in the genus *Bradyrhizobium*. However, consideration of the individual gene trees shows that they are not mutually congruent at the species level.

In some cases, sequences are as similar to one another as to the neighboring known species and therefore they may be members of these species. For instance, the sequences in group C may represent strains of *Mesorhizobium amorphae*. The placement of many strains into clusters that are distinct from existing named species indicates possible novel species. However, the absence of criteria relating sequences directly to taxonomic differences means that further data must be obtained by other methods before these strains can be properly classified (35).

These data confirm a preliminary study which showed that isolates from *Carmichaelia* plants were members of the genus *Mesorhizobium* (N. McCallum and C. W. Ronson, personal communication).

Establishment of symbioses. Studies of *Lotus corniculata* have shown that this legume species was not nodulated in pristine soils because no effective bacteria were present (8). Nodulation and fixation were initiated when *Lotus* plants were inoculated with an effective rhizobial strain (22). Since then, it has been shown that the effective rhizobial symbiont of *Lotus corniculata*, *Mesorhizobium loti*, carries nodulating and nitrogen-fixing genes on a large transmissible element, a symbiosis island, of 500 kb (31). This symbiosis island can be transmitted to and incorporated by a range of *Mesorhizobium* strains already present in the soil, converting them into effective strains (30). Symbiosis islands may therefore also be involved with transfer and fixation in the native New Zealand *Mesorhizobium* spp. If so, then the observation that sequences representative of isolates from *Carmichaelia* and *Sophora* plants are distrib-

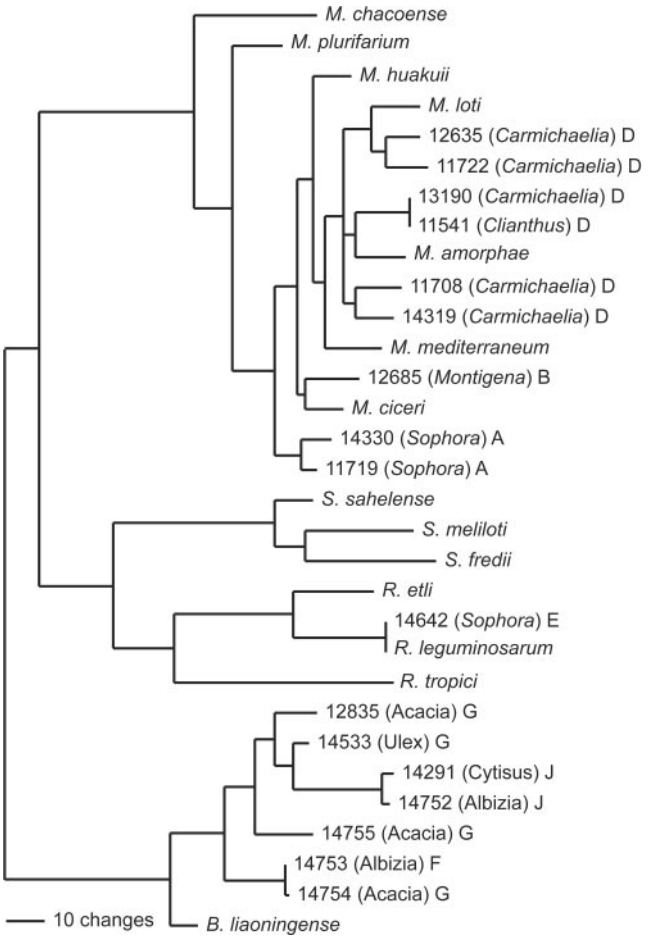

FIG. 4. Maximum-likelihood tree based on *recA* gene sequence data (828 bp), showing the relationships of rhizobial isolates from New Zealand legume flora to type strains of rhizobia. The isolate names and the accession number in the ICMP culture collection are shown. The genus of the legume from which the bacteria were isolated is shown in parentheses. The letter following the parentheses indicates the genomic grouping as defined by the 16S rDNA data. The value of −lnL for this tree is 4,114.36.

uted across the *Mesorhizobium* clade indicates either that a single symbiosis island with a broad host range is responsible for nodulation and fixation of several native legume genera or that symbiosis islands specific for each native legume genus are distributed across the genus. By their nature, symbiosis islands are incorporated into the bacterial genome of recipient strains. It seems clear that these genes may be transferred to many, if not all, *Mesorhizobium* species. The distribution of sequences in the genus *Mesorhizobium*, apparently representing several species, raises a fundamental question concerning the specificity of the association of the effective nodulating strains. Further studies will establish the extent and genetic basis of the host specificity of these strains.

It appears that many, if not all, known *Mesorhizobium* spp. reported in other countries (5) are present in New Zealand and have the capacity to nodulate the native legumes. Presumably these species were present prior to the separation of New Zealand from the regions of major legume evolution 80 million years ago (29).

***Rhizobium leguminosarum*.** An exception to the general association of native legume strains with *Mesorhizobium* spp. was four isolates from *Sophora chathamica*, *Carmichaelia australis*, and *Clianthus puniceus* that had very high sequence identity to *Rhizobium leguminosarum*. This demonstrates the ability of this species to nodulate native legumes. These strains of *R. leguminosarum* may have acquired Sym plasmids that enable wider nodulating host ranges than are currently recognized, including woody legumes. Alternatively, they may have acquired specific nodulating plasmids, one or more of which enable the nodulation of *Carmichaelia*, *Clianthus*, and *Sophora* plants. As yet it is not known if the strains from the native legumes represent one or more of the known *R. leguminosarum* biovars or if they are novel and specific. These *Rhizobium* strains may be endemic in New Zealand, or they may be strains introduced as commercial inoculum to enhance crop legume development and have acquired either the necessary symbiosis genes alone or the entire symbiosis island from a *Mesorhizobium* sp. The recorded host range of *R. leguminosarum* includes *Lathyrus* spp., *Lens* spp., *Phaseolus* spp., *Pisum* spp., *Trifolium* spp., and *Vicia* spp., allocated to three biovars, named according to the host plants with which they are associated (16). These isolations represent extensions of the known host range of *R. leguminosarum*.

A primary question of this research was to determine if the legume weeds (broom, gorse, and wattle) introduced into New Zealand were being nodulated by rhizobia that were cosmopolitan and already present in New Zealand, were introduced with them during colonization, or were able to take advantage of a native New Zealand rhizobial flora. This study indicates that most rhizobia isolated from New Zealand native legumes are members of the genus *Mesorhizobium*, and all isolates obtained from the introduced legumes studied are members of the genus *Bradyrhizobium*. Therefore it is clear that the two groups of legume plants from different origins are nodulated by unrelated rhizobial populations. The nodulating bradyrhizobia may have been transmitted in the course of dispersal of the plants (36). For instance, *Bradyrhizobium* spp. could be introduced either with adventive legumes, in soil imported with other plants, or with seed (23). Alternatively, these bacteria may occur naturally in New Zealand soils without being involved in symbiotic associations but have been available to nodulate the introduced legumes. The heterogeneity of the *Bradyrhizobium* sequences, which is substantially greater than the recorded difference between *B. liaoningense* and *B. yuanmingense*, may be an indication of a long presence and evolution in New Zealand rather than of a small recent founder population. Similar heterogeneity has been recorded for *Bradyrhizobium* spp. elsewhere (14, 18).

ACKNOWLEDGMENTS

This study was supported by a grant from the Marsden Fund of the Royal Society of New Zealand under contract 97-LAN-LFS-002 and by a grant from the Non-Specific Output Fund of Landcare Research.

P. J. Bellingham, P. B. Heenan, and the staff of the Department of Conservation assisted in identifying native legume sampling sites. T. Armstrong, H. M. Harman, and R. Howitt offered constructive comments on the manuscript.

REFERENCES

1. **Anzai, Y., H. Kim, J.-Y. Park, H. Wakabayashi, and H. Oyaizu.** 2000. Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. Int. J. Syst. Evol. Microbiol. **50:**1563–1589.
2. **Ausubel, F. M., R. Brent, A. Moore, J. G. Seidman, J. A. Smith, and K. Struh.** 1987. Current protocols in molecular biology. Greene Publishing Associates and Wiley-Interscience, Sunderland, Mass.
3. **Baxevanis, A. D., and B. F. Oulette.** 2001. Bioinformatics: a practical guide to the analysis of genes and proteins, 2nd ed. John Wiley and Sons, Inc., New York, N.Y.
4. **Bell, P. J. L., A. Sunna, M., D Gibbs, N. C. Curach, H. Nevalainen, and P. L. Bergquist.** 2002. Prospecting for novel lipase genes using PCR. Microbiology **148:**2283–2291.
5. **Chen, W. X., E. T. Wang, and L. D. Kuykendall.** Bergey's manual of systematic bacteriology, 2nd ed., vol. 2, in press. Springer-Verlag, New York, N.Y.
6. **De Lajudie, P., A. Willems, B. Pot, et al.** 1994. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. nov., *Sinorhizobium saheli* sp. nov. and *Sinorhizobium teranga* sp. nov. Int. J. Syst. Bacteriol. **44:**715–733.
7. **Gaunt, M. W., S. L. Turner, L. Rigottier-Gois, S. A. Lloyd-Macgilp, and J. P. Young.** 2001. Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. Int. J. Syst. Evol. Microbiol. **51:**2037–2048.
8. **Greenwood, R. M., and C. E. Pankhurst.** 1977. The *Rhizobium* component of the nitrogen-fixing symbiosis. Proc. N. Z. Grassland Assoc. **38:**147–150.
9. **Heenan, P. B.** 1998. Phylogenetic analysis of the *Carmichaelia complex, Clianthus*, and *Swainsona* (Fabaceae), from Australia and New Zealand. N. Z. J. Bot. **36:**21–40.
10. **Heenan, P. B., P. J., de Lange, and A. D. Wilton.** 2001. *Sophora* (Fabaceae) in New Zealand: taxonomy, distribution, and biogeography N. Z. J. Bot. **39:**17–34.
11. **Hilario, E., T. R. Buckley, and J. M. Young.** 2004. Improved resolution of the phylogenetic relationships among *Pseudomonas* by the combined analysis of *atpD*, *carA*, *recA* and 16S rDNA. Antonie van Leeuwenhoek **86:**51–64.
12. **Hirsch, A. M., M. R. Lum, and J. Downie.** 2001. What makes the rhizobia-legume symbiosis so special. Plant Physiol. **127:**1484–1492.
13. **Hugenholtz, P., and T. Huber,** 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. Int. J. Syst. Evol. Microbiol. **53:**289–293.
14. **Jarabo-Lorenzo, A., R. Perez-Galdona, J. Donate-Correa, et al.** 2003. Genetic diversity of bradyrhizobial population from diverse geographic origins that nodulate *Lupinus* spp. and *Ornithopus* spp. Syst. Appl. Microbiol. **26:** 611–623
15. **Jarvis, B. D. W., P. van Berkum, W. X. Chen, Nour, S. M., M. Fernandez, J.-C. Cleyet-Marel, and M. Gillis.** 1997. Transfer of *Rhizobium loti*, Rhizobium huakuii, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshansense* to *Mesorhizobium* gen. nov. Int. J. Syst. Bacteriol. **47:**895–898.
16. **Kuykendall, L. D., J. M. Young, E. Martínez-Romero, A. Kerr, and H. Sawada.** Bergey's manual of systematic bacteriology, 2nd ed., vol. 2. Springer-Verlag, New York, N. Y., in press.
17. **Kuykendall, L. D., F. M. Hashem, and E. T. Wang.** Bergey's manual of systematic bacteriology, 2nd ed., vol. 2, in press. Springer-Verlag, New York, N.Y.
18. **Lafay, B., and J. J. Burdon.** 1998. Molecular diversity of rhizobia occurring on native shrubby legumes in southeastern Australia. Appl. Environ. Microbiol. **64:**3989–3997.
19. **Martínez-Romero, E., and J. Caballero-Mellado.** 1996. *Rhizobium* phylogenies and bacterial genetic diversity. Crit. Revs. Plant Sci. **15:**113–140.
20. **Nicholas, K. B., H. B. Nicholas, Jr., and D. W. Deerfield II.** 1997. GeneDoc: analysis and visualization of genetic variation. EMBNEW News **4:**14.
21. **Parsons, M. J., P. Douglas, and J. McMillain.** 1998. Current names for wild plants in New Zealand. Manaaki Whenua Press, Lincoln, New Zealand.
22. **Patrick, H. N., and W. L. Lowther.** 1992. Response of *Lotus corniculatus* to inoculation and pelleting on a range of Otago tussock grassland environments. Proc. N. Z. Grassland Assoc. **54:**105–109.
23. **Pérez-Romírez, N. O., M. A. Rogel, E. Wang, J. Z. Castellanos, and E. Martinez-Romero.** 1998. Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*. FEMS Microbiol. Ecol. **26:**289–296.
24. **Perret, X., C. Staehelin, and W. J. Broughton.** 2000. Molecular basis of symbiotic promiscuity. Microbiol. Mol. Biol. Rev. **64:**180–201.
25. **Posada, D., and K. A. Crandall.** 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:**817–818.
26. **Pueppke, S. G., and W. J. Broughton.** 1999. *Rhizobium* sp. NGR234 and *R. fredii* USDA257 share exceptionally broad, nested host-ranges. Mol. Plant-Microbe Interact. **12:**293–318.
27. **Saldana, G., V. Martinez-Alcantara, J. M. Vinardell, R. Bellogin, J. E. Ruiz-Sainz, and P. A. Balatti.** 2003. Genetic diversity of fast-growing rhizobia that nodulate soybean (*Glycine max* L. Merr). Arch. Microbiol. **180:**45–52.
28. **Sawada, H., L. D. Kuykendall, and J. M. Young.** 2003. Changing concepts in the systematics of bacterial nitrogen-fixing legume symbionts. J. Gen. Appl. Microbiol. **49:**155–179.
29. **Stevens, G., M. McGlone, and B. McCulloch.** 1988. Prehistoric New Zealand. Heinemann Reed, Auckland, New Zealand.
30. **Sullivan, J. T., H. N. Patrick, W. L. Lowther, D. B. Scott, and C. W. Ronson.** 1995. Nodulating strains of *Rhizobium loti* arose through chromosomal symbiotic gene transfer in the environment. Proc. Natl. Acad. Sci. USA **92:**8985–8989.
31. **Sullivan, J. T., and C. W. Ronson.** 1998. Evolution of rhizobia by acquisition of a 500-kb symbiosis island that integrates into a phe-tRNA gene. Proc. Natl. Acad. Sci. USA **95:**5145–5149.
32. **Swofford, D. L.** 2003. PAUP* phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, Mass.
33. **Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins.** 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876–4882.
34. **Turner, S. L., and J. P. Young.** 2000. The glutamine synthetases of rhizobia: phylogenetics and evolutionary implications. Mol. Biol. Evol. **17:**309–319.
35. **Vandamme, P., B. Pot, M. Gillis, P. De Vos, K. Kersters, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol. Rev. **60:**407–438.
36. **Wang, E. T., F. L. Kan, Z. Y. Tan, I. Toledo, W. X. Chen, and E. Martínez-Romero.** 2003. Diverse *Mesorhizobium plurifarium* populations native to Mexican soils. Arch. Microbiol. **180:**444–454.
37. **Young, J. M., L. D. Kuykendall, E. Martínez-Romero, A. Kerr, and H. Sawada,** 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie *et al*. 1998 as new combinations: *Rhizobium radiobacter*, *R. rhizogenes, R. rubi, R. undicola*, and *R. vitis*. Int. J. Syst. Evol. Microbiol. **51:**89–103.