Genetic diversity and phylogeny of rhizobia isolated from agroforestry legume species in southern Ethiopia

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The genetic diversity within 195 rhizobial strains isolated from root nodules of 18 agroforestry species (15 woody and three herbaceous legumes) growing in diverse ecoclimatic zones in southern Ethiopia was investigated by using PCR–RFLP of the ribosomal operon [16S rRNA gene, 23S rRNA gene and the internal transcribed spacer (ITS) region between the 16S rRNA and 23S rRNA genes] and 16S rRNA gene partial sequence (800 and 1350 bp) analyses. All of the isolates and the 28 reference strains could be differentiated by using these methods. The size of the ITS varied among test strains (500–1300 bp), and 58 strains contained double copies. UPGMA dendrograms generated from cluster analyses of the 16S and 23S rRNA gene PCR–RFLP data were in good agreement, and the combined distance matrices delineated 87 genotypes, indicating considerable genetic diversity among the isolates. Furthermore, partial sequence analysis of 67 representative strains revealed 46 16S rRNA gene sequence types, among which 12 were 100% similar to those of previously described species and 34 were novel sequences with 94–99% similarity to those of recognized species. The phylogenetic analyses suggested that strains indigenous to Ethiopia belonged to the genera *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium* and *Sinorhizobium*. Many of the rhizobia isolated from previously uninvestigated indigenous woody legumes had novel 16S rRNA gene sequences and were phylogenetically diverse. This study clearly shows that the characterization of symbionts of unexplored legumes growing in previously unexplored biogeographical areas will reveal additional diversity.

INTRODUCTION

Rhizobia are soil bacteria that are capable of forming nitrogen-fixing symbiosis with leguminous plants. The family Leguminosae comprises about 650 genera and 18 000 species, and includes short-lived annual herbs and woody perennials that are distributed over a wide range of ecological conditions (Doyle, 1994). Many of its members are of

considerable agricultural and ecological importance, generally reflecting the beneficial symbiotic association with rhizobia. In sub-Saharan Africa, woody legumes are an integral part of traditional agroforestry systems and are considered to be low-cost alternatives to fertilizers for soil fertility improvement and land reclamation (Giller, 2001).

Currently, there are 44 recognized species of nodule-forming bacteria on legumes, within 12 genera, 10 of which belong to the class ‘Alphaproteobacteria’ (*Allorhizobium*, *Azorhizobium*, *Blastobacter*, *Bradyrhizobium*, *Devosia*, *Ensifer*, *Mesorhizobium*, *Methylobacterium*, *Rhizobium* and *Sinorhizobium*), and two to the class ‘Betaproteobacteria’ (*Burkholderia* and *Ralstonia*) (Sawada *et al.*, 2003). In the last few years, many studies investigating rhizobia isolated from tree legumes in East Africa (notably Kenya and Sudan) have revealed considerable phenotypic and genetic diversity among strains, and several distinct groups have been identified and novel species described (Zhang *et al.*, 1991;

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Abbreviation: ITS, internal transcribed spacer.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains included in this study are given in Table 1.

UPGMA dendrograms based on combined distance matrices from PCR–RFLP of 16S and 23S rRNA genes and RFLP analysis of PCR-amplified 23S rRNA genes, and data on the grouping of the Ethiopian rhizobial isolates and reference strains with various molecular biological methods are available as supplementary material in IJSEM Online.

Odee *et al.*, 1997, 2002; Nick *et al.*, 1999; McInroy *et al.*, 1999). However, despite its designation as a centre of diversity and origin of some of the major legume crops, such as pea, lentils, clover and chickpea (Raven & Polhill, 1981), no information is available on rhizobia from Ethiopia, except for a recent article by Beyene *et al.* (2004), in which a unique natural rhizobial population that nodulates *Phaseolus vulgaris* was reported. Exploration of new biogeographical regions and the investigation of legumes that have not been checked for nodulation not only helps to uncover unknown rhizobia, but also supports research efforts aimed at selecting effective combinations of rhizobium-legume genotype to exploit the enormous potential of increased nitrogen fixation. In view of this, we previously surveyed the nodulation status of a variety of indigenous and exotic woody legumes in 14 ecologically diverse zones in southern Ethiopia, and isolated a large number of root-nodulating bacteria (Wolde-meskel *et al.*, 2004a). The strains were phenotypically diverse and comprised several metabolically and genomically distinct groups that were not related to reference rhizobial species (Wolde-meskel *et al.*, 2004a, b, c).

In this study, the strains were characterized by using PCR-RFLP of the 16S rRNA gene, the internal transcribed spacer (ITS) and the 23S rRNA gene, and partial 16S rRNA gene sequencing, and their genetic diversity and phylogenetic relationships were determined. To infer the phylogenetic relatedness of the unknown strains, the 16S rRNA gene sequences of currently recognized nitrogen-fixing root/stem nodule bacterial species were retrieved from the GenBank/EMBL database and included in the analysis.

METHODS

Bacterial strains. The 223 strains (195 novel isolates and 28 reference strains) used are listed in Tables 1 and 2 and Supplementary Tables S1 and S2 in IJSEM Online. The Ethiopian strains were isolated as described previously (Wolde-meskel *et al.*, 2004a) from naturally occurring root nodules of 15 woody species (indigenous and introduced) and three herbaceous legumes, growing either in the field or under greenhouse conditions in soil samples from diverse agroecological zones in southern Ethiopia. The indigenous woody legumes included *Acacia abyssinica*, *Acacia senegal*, *Acacia seyal*, *Acacia tortilis*, *Albizia gummifera*, *Erythrina brucei*, *Faidherbia albida*, *Milletia ferruginea* and *Sesbania sesban*, whereas the introduced species were *Acacia saligna*, *Cajanus cajan*, *Calliandra calothyrsus*, *Gliricidia sepium*, *Leucaena diversifolia* and *Leucaena leucocephala*. The herbaceous species were *Phaseolus vulgaris*, *Vicia faba* and *Vigna unguiculata*.

Growth of bacteria and DNA isolation. Bacteria were grown on yeast extract mannitol broth at 28 °C for 3–8 days depending on the strain (Somasegaran & Hoben, 1994). Total genomic DNA was isolated from 1.5 ml cultures according to Boom *et al.* (1990) with slight modifications (Terefeework *et al.*, 2001), where diatomaceous earth or Celite analytical filter aid (BDH) was used as a DNA-binding solid support. The concentrations were estimated by comparing the DNA samples with known concentrations of λ DNA using agarose gel electrophoresis.

PCR-RFLP of the ITS, and 16S and 23S rRNA genes. PCR of the 16S rRNA gene was carried out with primers fD1 and rD1

(Weisburg *et al.*, 1991), as described by Zhang *et al.* (1999b), whereas the 23S rRNA gene was amplified with primers 3 (5'-CCGTGAGGGAAAGGTGAAAAGTACC-3') and 4 (5'-CCCCTTATGATGCTTTTCAGC-3'), as described previously (Terefeework *et al.*, 1998). For amplification of the ITS, we used primers FGPS1490-72 and FGPL132', as described by Normand *et al.* (1992). DNA amplification was performed by using a PTC-200 Peltier thermal cycler (MJ Research); the cycling profile used was according to Zhang *et al.* (1999b). The size of the amplification products was verified by electrophoresis in 1% agarose gels. Aliquots of 8–12 μ l of the amplified 16S and 23S rRNA genes were digested with 1.5 U of each of the restriction endonucleases *AluI*, *HaeIII*, *MspI* and *MboI*, at 37 °C overnight. The ITS region was restricted with the first three enzymes. The digested rRNA genes were separated in 3% agarose gels.

Partial 16S rRNA gene sequencing. Based on the PCR-RFLP results, 67 test strains were chosen for partial sequencing of the 16S rRNA gene, which was performed directly from PCR products (800 bp for 57 strains and 1350 bp for the other 10). The 16S rRNA genes were amplified as described by Zhang *et al.* (1999a), except that primers pA (5'-AGAGTTTGATCCTGGCTCAG-3') and pF' (5'-ACGAGCTGACGACAGCCATG-3') were used for the first 57 strains, and primers 1F (5'-GAGTTTGATCCTGGCTCAG-3'), 15F (5'-ACGGGAGGCAGCAGT-3') and 16F (5'-AACTCAAA-TGAATTFGACGGG-3') were used for the other 10 strains. The amplified fragments were sequenced from both strands by using the solid-phase method, with an automatic laser fluorescence DNA sequencer (Pharmacia). The quality of the sequences was verified by sequencing both strands. These sequences were added to the GenBank/EMBL/DBJ database (Table 1).

Data analysis. Analysis of the restriction fragments and construction of dendrograms were performed as described by Terefeework *et al.* (1998). During separation of the fragments by agarose gel electrophoresis, the smaller fragments (100 bp or less) appeared diffuse and therefore were not used in the RFLP analysis. The CLUSTAL_X (version 1.83) program (Thompson *et al.*, 1997) was used to align the sequences, and phylogenetic trees were constructed using the neighbour-joining method in MEGA program version 2.1 (Kumar *et al.*, 2001). The trees were displayed using TreeView, as described by Zhang *et al.* (1999a). The 16S rRNA gene sequences of the type strains of the various genera used in this study were retrieved from the GenBank/EMBL database and used for cladistic analysis. The stability of the groupings was estimated by bootstrap analysis on 100 trees in the same package.

RESULTS AND DISCUSSION

Characterization by 16S rRNA and 23S rRNA PCR-RFLP

PCR of the 16S rRNA and 23S rRNA gene loci from each of the strains produced a single band of 1.5 and 2.3 kb fragments, respectively, which correspond to the expected size reported previously (Weisburg *et al.*, 1991; Terefeework *et al.*, 1998). The grouping of representative strains by using these methods, as well as the Biolog and AFLP data reported previously (Wolde-meskel *et al.*, 2004b, c), are presented in Tables 1 and 2 (data for all strains are given in Supplementary Tables S1 and S2 in IJSEM Online). Fingerprints of the strains generated by PCR-RFLP of the ribosomal genes were used to construct dendrograms by using UPGMA analysis in the Bionumerics software

Table 1. Representative Ethiopian rhizobial isolates and their grouping results with various molecular biological methods

–, 16S rRNA gene sequences not determined or aligned; Comb., combined distance matrices from PCR–RFLP of 16S and 23S rRNA genes; U, strains unclustered on the dendrogram. GenBank/EMBL/DBJ database accession numbers given are from this study. Biolog and AFLP groupings are from Wolde-meskel *et al.* (2004b, c).

Strain*	Host plant (provenance)	Geographical origin†	Accession no.	PCR–RFLP genotype				Biolog	AFLP	Closest partial 16S rRNA gene sequence	Similarity (%)
				16S	23S	Comb.	ITS				
AC22d (10)	<i>Acacia seyal</i> , <i>Acacia tortilis</i>	NZ, RFC	AY776185	1	7	9	8, 9, 10, 11, 23	1C–1E, 3	6B, 7	<i>Sinorhizobium fredii</i>	100
AC18a (4)	<i>Acacia seyal</i> , <i>Acacia tortilis</i>	DZ, RFC	AY776180	2	7	10	8, 11, 12	U, 3	7	<i>Sinorhizobium fredii</i>	100
AC24d1 (2)	<i>Acacia tortilis</i> (Mega)	DZ	–	3	16	7	19	5	5	<i>Sinorhizobium</i> sp.‡	–
AC27c (4)	<i>Acacia tortilis</i> (Meki)	DZ	–	4	7	11	24	1C, 1D	7	<i>Sinorhizobium</i> sp.‡	–
AC27e (3)	<i>Acacia tortilis</i> (Meki)	DZ	AY776187	5	7	11	24	1C, 1D, 4	7	<i>Sinorhizobium fredii</i>	100
AC01b (14)	<i>Acacia seyal</i> (four provenances)	DZ, NZ, RFC	AY776181	6	4, 5, 8	1, 2, 3	2, 22, 82	1B–4, U	6A, 7	<i>Sinorhizobium saheli</i>	99
AC20b	<i>Acacia tortilis</i> (Arba-minch)	AK	AY776182	7	16	8	18	1A	5	<i>Sinorhizobium fredii</i>	100
AC10d (3)	<i>Acacia seyal</i> (two provenances)	ACA, DZ, NZ	AY776178	8	2, 17	5, 6	17, 21, 46	1A, 1B, U	5	<i>Sinorhizobium fredii</i>	100
AC10a1 (3)	<i>Acacia seyal</i> (two provenances)	ACA, NZ	AY776177	9	6	4	98, 99	1A, 2B, U	4	<i>Sinorhizobium saheli</i>	99
AC38b2 (3)	<i>Acacia abyssinica</i>	AK, DZ	AY776190	10	27, 30	19, 21	16, 126	1A, 1D	6B, 7	<i>Sinorhizobium saheli</i>	99
AC38d1 (2)	<i>Acacia abyssinica</i>	AK	–	11	28	22	20	1B	5	<i>Sinorhizobium</i> sp.‡	–
AC47c	<i>Sesbania sesban</i>	AM	AY776196	12	12	28	125	U	7	<i>Sinorhizobium fredii</i>	100
AC52c (3)	<i>Sesbania sesban</i>	ACA	AY776201	15	10	27	81	12	3	<i>Sinorhizobium meliloti</i>	99
AC52a1 (3)	<i>Sesbania sesban</i>	ACA	–	18	9	26	81	12	3	<i>Sinorhizobium</i> sp.‡	–
AC50e (2)	<i>Sesbania sesban</i>	DZ	AY776199	19	43	25	73	2A	8	<i>Sinorhizobium meliloti</i>	100
AC28a (2)	<i>Acacia tortilis</i> (Meki)	RFC	AY776188	20	29	23	4	2B	12	<i>Sinorhizobium medicae</i>	99
AC25d	<i>Acacia tortilis</i> (Mega)	RFC	–	21	1	12	7	2B	12	<i>Sinorhizobium</i> sp.‡	–
AC07b1 (5)	<i>Acacia seyal</i> (three provenances)	RFC	–	22	3, 5	14, 15	1, 6, 25	2A, 2B, 12	3, 12	<i>Sinorhizobium</i> sp.‡	–
AC17d (2)	<i>Acacia seyal</i> , <i>Acacia tortilis</i>	RFC	–	23	1	13	5	2B	12	<i>Sinorhizobium</i> sp.‡	–
AC47a (3)	<i>Sesbania sesban</i>	AM	AY776195	24	36	29	113	13	2	<i>Ensifer adhaerens</i>	99
AC82b (4)	<i>Albizia gummifera</i> , <i>Vicia faba</i>	ACA	AY776222	25	60	34	33	U, 1, 6	5	<i>Rhizobium leguminosarum</i>	100
AC100b	<i>Acacia senegal</i>	LK	AY776212	26	21	40	101	9	U	<i>Rhizobium leguminosarum</i>	99
AC100a	<i>Acacia senegal</i>	LK	AY776243	27	53	32	100	1	6	<i>Rhizobium leguminosarum</i>	100
AC56b	<i>Vigna unguiculata</i>	AK	AY776202	32	54	33	86	U	1B	<i>Rhizobium etli</i>	100
AC04d	<i>Acacia seyal</i> (Abergele)	RFC	AY776198	33	58	31	88	U	2	<i>Rhizobium etli</i>	99
AC87k1 (2)	<i>Milletia ferruginea</i>	ACA	AY776226	34	59	30	31	14	25	<i>Rhizobium etli</i>	100
AC90e1 (5)	<i>G. sepium</i>	ACA	AY776234	35	19	44	43	5	4	<i>Rhizobium mongolense</i>	99
AC91e (2)	<i>Calliandra calothyrsus</i>	ACA	AY776235	36	18, 19	45	28, 42	1, 3	8, 18	<i>Rhizobium mongolense</i>	99
AC89a (6)	<i>L. leucocephala</i>	ACA	–	37	19	46	28, 42	1, 3, 4	3, 8	<i>Rhizobium</i> sp.‡	–
AC80a (5)	<i>L. diversifolia</i>	ACA	–	38	24	42	30	1	8	<i>Rhizobium</i> sp.‡	–
AC86c1 (2)	<i>Cajanus cajan</i>	ACA	AY776225	39	61	43	35	9	9	<i>Rhizobium</i> sp.	98
AC93e (5)	<i>G. sepium</i>	RFC	AY776238	40	23	41	29	4	8	<i>Rhizobium mongolense</i>	99
AC86a	<i>Cajanus cajan</i>	ACA	AY776223	41	20	47	34	10	5	<i>Rhizobium</i> sp.	98

Table 1. cont.

Strain*	Host plant (provenance)	Geographical origin†	Accession no.	PCR-RFLP genotype				Biolog	AFLP	Closest partial 16S rRNA gene sequence	Similarity (%)
				16S	23S	Comb.	ITS				
AC26e	<i>Acacia tortilis</i> (Meki)	AK	AY776186	44	55	50	111	U	1B	<i>Rhizobium</i> sp.	97
AC87k3	<i>Millettia ferruginea</i>	ACA	AY776227	45	62	51	27	U	9	<i>Rhizobium</i> sp.	97
AC88c	<i>Acacia abyssinica</i>	ACA	AY776232	46	79	87	117	U	U	<i>Mesorhizobium chacoense</i>	98
AC88a	<i>Acacia abyssinica</i>	ACA	AY776231	47	46	52	97	11	19	<i>Rhizobium giardinii</i>	99
AC79c1	<i>Erythrina brucei</i>	ACA	AY776219	51	52	57	90	8	2	<i>Agrobacterium radiobacter</i>	100
AC42c	<i>Acacia abyssinica</i>	RFC	AY776194	52	39	65	115	7	8	<i>Agrobacterium tumefaciens</i>	99
AC97c1 (2)	<i>Acacia saligna</i>	WG	AY776241	54	38	63	47	13	3	<i>Agrobacterium vitis</i>	99
AC77b	<i>Acacia senegal</i>	RFC	AY776218	56	51	56	36	U	U	' <i>Agrobacterium albertimagni</i> '	99
AC11a (3)	<i>Acacia seyal</i> (two provenances)	ACA, DZ	AY776179	57	63	54	26, 112	8	U	' <i>Agrobacterium albertimagni</i> '	99
AC51e (9)	<i>Sesbania sesban</i> , <i>Vigna unguiculata</i>	NZ	AY776200	60	37, 40, 41	58, 60	40, 41, 116	6A, 6B	1A, 1B	<i>Rhizobium huautilense</i>	99
AC73d (6)	<i>Phaseolus vulgaris</i>	DZ	AY776210	61	50	61	37	6A, 6B	1A, 1B	<i>Rhizobium huautilense</i>	100
AC100e (2)	<i>Acacia senegal</i>	LK	AY776213	67	72	78	50, 77	2	15	<i>Mesorhizobium plurifarium</i>	99
AC39a (6)	<i>Acacia abyssinica</i> , <i>Acacia tortilis</i>	CF, RFC	AY776191	68	64–68	70–74	52, 54, 119–121	U, 9, 14	4, 10	<i>Mesorhizobium plurifarium</i>	99
AC99a (4)	<i>Sesbania sesban</i>	WG	—	69	72–74	76, 77	48–50, 118	2, 12	12, 24	<i>Mesorhizobium</i> sp.‡	—
AC99d	<i>Sesbania sesban</i>	WG	AY776211	70	72	75	107	2	24	<i>Mesorhizobium plurifarium</i>	99
AC98a	<i>Acacia abyssinica</i>	WG	AY776242	71	75, 76	79, 80	51, 74–76	U	15	<i>Mesorhizobium plurifarium</i>	99
AC21c2 (2)	<i>Acacia tortilis</i> (Arba-minch)	NZ	AY776184	72	77, 78	85, 86	53, 69	9	8, 16	<i>Mesorhizobium</i> sp.	99
AC72a	<i>Phaseolus vulgaris</i>	CF	AY776209	73	103	114	45	U	4	<i>Methylobacterium</i> sp.	94
AC101b (3)	<i>Acacia saligna</i>	LK	AY776214	74	88	92	78	15	3, 10	<i>Bradyrhizobium liaoningense</i>	100
AC29c	<i>F. albida</i> (Arba-minch)	RFC	AY776189	76	93	107	65	16	4	<i>Bradyrhizobium liaoningense</i>	100
AC79a (3)	<i>Erythrina brucei</i>	ACA	AY776220	77	82	88	57	15	3	<i>Bradyrhizobium liaoningense</i>	100
AC104a (3)	<i>Acacia saligna</i>	BL	AY776215	78	86	90	105	18	13	<i>Bradyrhizobium</i> sp.	99
AC104c1 (2)	<i>Acacia saligna</i>	BL	AY776216	79	87	91	106	U	7	<i>Bradyrhizobium</i> sp.	99
AC62a	<i>Vigna unguiculata</i>	DL	AY776203	81	94	99	67	16	1B	<i>Bradyrhizobium japonicum</i>	99
AC64a (4)	<i>Vigna unguiculata</i>	NZ	AY776204	82	92, 94	100, 102	61, 66, 68	16	4, 5	<i>Bradyrhizobium yuanmingense</i>	99
AC87b1 (2)	<i>Millettia ferruginea</i>	ACA	AY776228	83	99	112	70	16	5	<i>Bradyrhizobium elkanii</i>	100
AC86b2 (2)	<i>Cajanus cajan</i>	ACA	AY776224	84	81	106	55	15	1	<i>Bradyrhizobium japonicum</i>	99
AC79c2	<i>Erythrina brucei</i>	ACA	—	85	80	105	57	15	1	<i>Bradyrhizobium</i> sp.‡	—
AC82d	<i>Albizia gummifera</i>	ACA	AY776221	86	81	104	55	15	1	<i>Bradyrhizobium</i> sp.	99
AC87h (3)	<i>Millettia ferruginea</i>	ACA	—	87	81	103	64	15	1	<i>Bradyrhizobium</i> sp.‡	—
AC92d (2)	<i>Albizia gummifera</i>	ACA	AY776237	88	83, 89	95, 96	60, 62	15	3	<i>Bradyrhizobium japonicum</i>	100
AC70c	<i>Phaseolus vulgaris</i>	AK	AY776208	89	95	101	58	16	1B	<i>Bradyrhizobium liaoningense</i>	100

Table 1. cont.

Strain*	Host plant (provenance)	Geographical origin†	Accession no.	PCR-RFLP genotype			Biolog	AFLP	Closest partial 16S rRNA gene sequence	Similarity (%)
				16S	23S	Comb.				
AC97a (3)	<i>Acacia saligna</i>	WG	AY776240	90	85, 91	97, 98	U, 15	U, 16	<i>Bradyrhizobium japonicum</i>	100
AC94a	<i>F. albida</i>	RFC	AY776239	91	98	111	16	20	<i>Bradyrhizobium elkanii</i>	100
AC87n (3)	<i>Milletia ferruginea</i>	ACA	AY776230	92	97	108	U, 15	2	<i>Bradyrhizobium</i> sp.	100
AC92b	<i>Albizia gummifera</i>	ACA	—	93	90	93	17	21	<i>Bradyrhizobium</i> sp.‡	—
AC92c	<i>Albizia gummifera</i>	ACA	AY776236	94	84	94	15	U	<i>Bradyrhizobium liaoningense</i>	100
AC107e (2)	<i>Milletia ferruginea</i>	DL	AY776217	95	100	109	16	20	<i>Bradyrhizobium elkanii</i>	100

*Data from Wolde-meskel *et al.* (2004a); AC, Awassa College culture collection; numbers in parentheses indicate the number of strains with the same 16S rRNA gene PCR-RFLP patterns.

†Locations in southern Ethiopia (Wolde-meskel *et al.*, 2004a): ACA, Awassa College of Agriculture campus; AK, Akaki; AM, Arba-minch; BL, Bule; CF, Chofa; DL, Dilla; DZ, Debrezeit; LK, Leku; NZ, Nazret; RFC, Research and Farm Centre of the ACA; WG, Wondogenet.

‡16S rRNA genotypes (partial sequence not determined).

(Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online). The cophenetic correlation coefficient indicates the consistency of a cluster or the whole dendrogram and calculates the correlation between the dendrogram-derived similarities and the matrix similarities. In this study, cluster analysis based on the Dice coefficient yielded a significantly high mean cophenetic correlation coefficient (generally > 92 %), suggesting that the level of distortion between the similarity matrix and cluster analysis was low. In 58 strains, the amplified fragments of the ITS existed as multiple copies of variable size (Supplementary Table S1 in IJSEM Online). This rendered the ITS region unsuitable for use for overall comparison of all the strains.

Characterization based on PCR-RFLP of the 16S rRNA gene distinguished 96 different combinations of patterns, representing 96 distinct 16S rRNA genotypes among all the strains, including the reference species (Tables 1 and 2). The clustering of the strains in the dendrogram (Fig. 1) was in agreement with previously reported RFLP groupings and phylogenetic trees based on whole 16S rRNA gene sequences for rhizobia (Young & Haukka, 1996; Terefeework *et al.*, 1998; Nick *et al.*, 1999; Gao *et al.*, 2001). The 71 16S rRNA genotypes identified among the test strains corresponded to five major groups (Fig. 1); 78 strains were variously assigned to the *Sinorhizobium* branch, 49 to *Rhizobium*, 8 to *Agrobacterium*, 20 to *Mesorhizobium* and 40 to the *Bradyrhizobium* lineage.

Application of the 23S rRNA gene PCR-RFLP delineated 103 different 23S rRNA genotypes among all the strains studied (78 in the test strains and 25 in the reference strains) (Tables 1 and 2, and Supplementary Fig. S1 in IJSEM Online). The 23S rRNA gene, because of its large size and greater phylogenetic information content, gives better resolution than the 16S rRNA gene (Terefeework *et al.*, 1998; Zhang *et al.*, 1999a; Gao *et al.*, 2001). Discrimination of the test strains into a large number of 23S rRNA genotypes in this study supported previous findings. However, the genotypes defined by RFLP of the 16S and 23S rRNA genes were in good agreement in most cases. Exceptions were found among isolates of 12 16S rRNA genotypes (6, 8, 10, 22, 36, 60, 68, 69, 71, 72, 82 and 88) that were divided into two or more 23S rRNA genotypes, and among strains of 16S rRNA genotypes 1, 2, 4 and 5 that belonged to a single 23S rRNA genotype, genotype 7 (Table 1 and Supplementary Table S1 in IJSEM Online).

The combined distance matrices of the 16S and 23S rRNA gene PCR-RFLP patterns revealed the diverse nature of our collection more than either gene alone, and thus identified 114 genotypes among the strains (87 and 27 genotypes among test and reference strains, respectively). The dendrogram constructed (Supplementary Fig. S2 in IJSEM Online) showed more extensive and deeper branching than either of the 16S or 23S rRNA gene PCR-RFLP dendrograms. Whereas all but 13 isolates (representing 16S rRNA genotypes 25, 26, 27, 46, 51, 56, 57 and 73) were classified into the same taxonomic groups as with the 16S rRNA gene

Table 2. Reference strains and their grouping results with various molecular biological methods

Abbreviations: CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico; CIAT, CIAT Rhizobium Collection, Centro Internacional de Agricultura Tropical, Columbia; Comb., combined distance matrices from PCR-RFLP of 16S and 23S rRNA genes; HAMBI, culture collection of the Division of Microbiology, University of Helsinki, Finland; LMG, Laboratorium voor Microbiologie, University of Ghent, Belgium; ORS, IRD/ORSTOM collection, Institut de Recherche pour le Développement, Dakar, Senegal; USDA, US Department of Agriculture, Beltsville, MD, USA; U, strains unclustered on the dendrogram. Sequence accession numbers were retrieved from GenBank/EMBL. Biolog and AFLP groupings are from Wolde-meskel *et al.* (2004b, c).

Strain	Accession no.	PCR-RFLP genotype				Biolog	AFLP
		16S	23S	Comb.	ITS		
<i>Sinorhizobium kostiense</i> HAMBI 1489 ^T (=LMG 15613 ^T)	Z78203	13	13	16	91	U	9
<i>Sinorhizobium saheli</i> HAMBI 215 ^T (=ORS 609 ^T)	X68390	13	11	17	127	1A	9
<i>Sinorhizobium teranga</i> HAMBI 220 ^T (=ORS 1009 ^T)	X68388	14	14	18	104	2A	11
<i>Sinorhizobium arboris</i> HAMBI 1552 ^T (=LMG 14919 ^T)	Z78204	16	15	20	15	2A	9
<i>Sinorhizobium medicae</i> HAMBI 1838 (=LMG 16582)	L39882	17	44	24	13	2A	9
<i>Sinorhizobium meliloti</i> HAMBI 2148 ^T (=LMG 6133 ^T)	X67222	17	44	24	14	2A	9
<i>Rhizobium leguminosarum</i> HAMBI 12 ^T (=LMG 14904 ^T)	U29386	28	56	35	110	15	1A
<i>Rhizobium etli</i> HAMBI 1727 ^T (=CFN 42 ^T)	U28916	29	57	36	93	U	11
<i>Rhizobium hainanense</i> HAMBI 1930 ^T	U71078	30	49	39	92	10	9
<i>Rhizobium tropici</i> HAMBI 1163 ^T (=CIAT 899 ^T)	X67234	30	47	37	94	10	U
<i>Agrobacterium rhizogenes</i> HAMBI 1816 ^T (=LMG 150 ^T)	X67224	31	48	38	87	10	U
<i>Rhizobium mongolense</i> HAMBI 2349 ^T (=USDA 1844 ^T)	U89816	42	22	48	44	U	15
<i>Rhizobium gallicum</i> HAMBI 2326 ^T	U86343	43	26	49	95	15	15
<i>Rhizobium giardinii</i> HAMBI 2323 ^T	U86344	48	45	53	103	U	U
<i>Agrobacterium tumefaciens</i> HAMBI 1811 ^T (=LMG 187 ^T)	X67223	49	31	66	102	11	13
<i>Agrobacterium radiobacter</i> HAMBI 1814 ^T (=LMG 140 ^T)	AJ389904	50	31	67	102	11	13
<i>Agrobacterium rubi</i> HAMBI 1812 ^T (=LMG 156 ^T)	X67228	53	32	68	79	7	8
<i>Agrobacterium vitis</i> HAMBI 1817 ^T (=LMG 8750 ^T)	X67225	55	34	64	84	U	13
<i>Rhizobium galegae</i> HAMBI 540 ^T (=LMG 6214 ^T)	X67226	58	25	55	80	U	U
<i>Rhizobium huaulense</i> HAMBI 2409 ^T	AF025852	59	35	59	38	6B	16
<i>Allorhizobium undicola</i> LMG 11875 ^T	Y17047	62	33	69	83	8	9
<i>Mesorhizobium ciceri</i> HAMBI 1750 ^T (=LMG 14989 ^T)	U07934	63	69	81	123	U	14
<i>Mesorhizobium loti</i> HAMBI 1129 ^T (=LMG 6125 ^T)	X67229	64	69	82	122	U	14
<i>Mesorhizobium mediterraneum</i> HAMBI 2096 ^T	L38825	65	70	83	89	U	U
<i>Mesorhizobium huakuii</i> HAMBI 1674 ^T (=LMG 14107 ^T)	D12797	66	71	84	124	7	16
<i>Bradyrhizobium japonicum</i> HAMBI 2314 ^T (=USDA 6 ^T)	X66024	75	96	89	108	U	U
<i>Bradyrhizobium elkanii</i> LMG 6134 ^T	U35000	80	101	110	109	U	15
<i>Azorhizobium caulinodans</i> HAMBI 216 ^T (=ORS 571 ^T)	X67221	96	102	113	85	U	U

PCR-RFLP method, cluster analysis of the combined 16S and 23S rRNA PCR-RFLP data resulted in further discrimination of the strains, and hence identified more genotypes within the same taxon. Thus, the number of different genotypes assigned to the *Rhizobium* branch increased from 20 (16S rRNA genotypes) to 24 (combined 16S and 23S rRNA genotypes), and from 20 to 24 in the *Sinorhizobium* branch, 6 to 13 in the *Mesorhizobium* branch and 20 to 23 in the *Bradyrhizobium* branch. The more-heterogeneous nature of the isolates that the combined RFLP pattern revealed in the *Mesorhizobium* branch was remarkable (Supplementary Fig. S2 in IJSEM Online), and supports previous reports on strains belonging to the genus *Mesorhizobium* (de Lajudie *et al.*, 1998; Zhang *et al.*, 1999a; Wang *et al.*, 2003).

Interestingly, apart from a few isolates, all the test strains in the different taxonomic branches formed a number of tightly clustered, separate subgroups in all the parameters studied (Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online) and were related to the reference strains at most with 88 % similarity, hence reflecting their distinct genotypic nature. This was supported by the tight and separate clusters that the reference species consistently formed in all the dendrograms constructed (Fig. 1 and Supplementary Figs S1 and S2 in IJSEM Online). In addition, in previous studies, a large number of test strains (80 %) in our collection had metabolic and genomic (AFLP) profiles that were not related to reference species (Wolde-meskel *et al.*, 2004b, c). This contrasts with the findings of Odee *et al.* (2002) and Bala *et al.* (2002, 2003) where, in a study of a large number

Fig. 1. Dendrogram based on the RFLP analysis of PCR-amplified 16S rRNA genes of test and reference strains. The distinct genotypes are numbered in order of their appearance. Numbers in parentheses indicate the number of strains that had the same restriction pattern (genotypes). The data were clustered by using UPGMA in the Bionumerics program.

of tree rhizobia isolated from Kenya and southern parts of Africa, many strains had identical 16S rRNA genotypes to the reference rhizobial strains included in the study.

Phylogenetic analysis

To elucidate the taxonomic positions of the isolates, we partially sequenced the 16S rRNA genes of 67 strains representing the various 16S rRNA PCR-RFLP genotypes. The aligned sequences of the Ethiopian strains, and those of recognized species of rhizobia, agrobacteria and other related symbiotic and non-symbiotic species, were used in the phylogenetic analysis. The results are presented in Fig. 2, and were consistent with previous reports (Young *et al.*, 2001; Toledo *et al.*, 2003; Sawada *et al.*, 2003). Overall, 46 different 16S rRNA gene sequence types representing six genera were found (Fig. 2); 13 were clustered within the *Rhizobium* branch, 12 within the *Bradyrhizobium* branch, four within the *Agrobacterium* branch, eight each within *Sinorhizobium* and *Mesorhizobium* and one within the *Methylobacterium* branch. Twelve of the 46 rRNA genotypes had 100 % partial sequence similarity with one or more species of the first four genera, whereas the other 34 were novel and were related (with 94–99 % similarity) to members of one of these six genera (Table 1 and Supplementary Table S1 in IJSEM Online).

Rhizobium and Agrobacterium branches. Nine of the 13 *Rhizobium* genotypes (in groups I, III, V, VII and IX; Fig. 2) were typical of previously described species and showed high sequence similarity (99–100 %) to *Rhizobium etli*, *Rhizobium leguminosarum*, *Rhizobium mongolense*, *Rhizobium giardinii* and *Rhizobium huautlense*. However, four 16S rRNA gene sequences in groups II, IV, VI and VIII (representing strains AC86a, AC86c1, AC26e and AC87k3, respectively) were distinct, with 16S rRNA gene similarities with the most similar published sequences of as low as 97 %, a 'threshold' for species delineation (Stackebrandt & Goebel, 1994), hence possibly representing novel species. Interestingly, several *Rhizobium* genotypes in this study showed high similarity to non-described *Rhizobium* strains isolated from China.

(i) The two genotypes in group V, which were related to *Rhizobium mongolense* (99 %) and represented 23 strains isolated from three host species (Supplementary Table S1 in IJSEM Online), instead showed 100 % partial sequence similarity to strain X59, an isolate from *Astragalus adsurgens* (Gao *et al.*, 2001). In a polyphasic study, Gao *et al.* (2001) reported that strain X59 had a unique genotype that was closely related to a strain isolated from another *Astragalus* species, *Astragalus membranaceus* (Wang & Chen, 1996).

(ii) Strains SDW024 and SDW058, which were isolated from *Astragalus adsurgens* (Gao *et al.*, 2001), and USDA 1920, a strain isolated from *Medicago ruthenica* in China (van Berkum *et al.*, 1998), had the most similar published sequences found in the database (97–98 %), matching strains AC86a (group II), AC87k3 (VIII) and AC86c1(IV), respectively, in our study.

There is no known documented history of the introduction of rhizobial inoculants from China into Ethiopia, or vice versa, nor, in fact, from any other part of the world. However, because of the highly conserved nature of the 16S rRNA gene sequence, the existence of alike genotypes or similar sequences in widely separated geographical regions under varying environmental conditions is to be expected (Martínez-Romero & Caballero-Mellado, 1996; Moreira *et al.*, 1998). Although allopatry could not be implicated and recombination within and between ribosomal genes has been shown (Young & Haukka, 1996), it is also possible that divergent evolution in rhizobia took place independently in several locations. A thorough study of the core and accessory genome of these and other Ethiopian isolates might reveal very interesting insights into rhizobial evolution.

The partial sequence analysis of AC26e, a strain isolated from *Acacia tortilis*, showed a mosaic 16S rRNA gene, which was related (97 %) to *Sinorhizobium* sp. 9702-M4, a strain reported to synthesize an extracellular polymer that facilitates the transport of hydrophobic pollutants as well as toxic metals, lead and cadmium, in soil (Janeca *et al.*, 2002). However, it formed a separate phylogenetic branch within the genus *Rhizobium* and showed distinct metabolic reactions in a previous Biolog study (Wolde-meskel *et al.*, 2004b).

Among the eight strains isolated from root nodules of *Erythrina brucei* and *Acacia* species, four *Agrobacterium* genotypes were identified (Fig. 2). These included *Agrobacterium radiobacter* (100 % partial sequence similarity), *Agrobacterium tumefaciens* (99 %), *Agrobacterium vitis* (99 %) and '*Agrobacterium albertimagni*' (99 %). In previous studies, a large number of strains with 16S rRNA gene sequences that were very similar to that of *Agrobacterium tumefaciens* were isolated from herbaceous (*Phaseolus vulgaris* and *Vigna unguiculata*) and tree legume species grown in African soils (Anyango *et al.*, 1995; Khbaya *et al.*, 1998; de Lajudie *et al.*, 1999; Odee *et al.*, 2002; Bala & Giller, 2001; Bala *et al.*, 2003). A cross-inoculation study conducted to evaluate symbiotic effectiveness (data not presented) showed that the strains were not capable of eliciting nodulation on homologous or other host species, in agreement

Fig. 2. Phylogenetic tree of selected Ethiopian rhizobial strains and related bacteria within the class 'Alphaproteobacteria'. The tree was constructed using the neighbour-joining method from partial 16S rRNA gene sequences. Bootstrap probability values greater than 50 % are indicated at the branch points. Bar, 0.1 substitution per site. Roman numerals indicate groups of different test strains in the respective genus, whereas numbers in parentheses represent the various 16S rRNA gene sequence types in the group. ●, Closely related but non-symbiotic bacteria.

with other studies. The agrobacterial isolates that were reported to be capable of infection after isolation from nodules (Bala & Giller, 2001) thus seem to represent an exception. However, all of our strains except one had novel sequences, with 99 % partial sequence similarity to each of their described counterparts, and were classified in different AFLP and Biolog groups in previous studies (Wolde-meskel *et al.*, 2004b, c). In addition, this is the first report of strains with high similarity to '*Agrobacterium albertimagni*', an arsenite-oxidizing bacterium from aquatic macrophytes in a hot creek (Salmassi *et al.*, 2002), being isolated from root nodules of plants in African soil.

Sinorhizobium lineage. All representative isolates in this group were phylogenetically affiliated to one of five species: *Sinorhizobium fredii*, *Sinorhizobium meliloti*, *Sinorhizobium medicae*, *Sinorhizobium saheli* and *Ensifer adhaerens* (Fig. 2). Although only partial sequences were used, all isolates except those identified as belonging to the species *Sinorhizobium fredii* had novel 16S rRNA gene sequences, with 99 % sequence similarity to the respective reference species (Table 1). It is interesting to note that, with the exception of 11 strains isolated from *Sesbania sesban*, all of the other 61 strains associated with the *Sinorhizobium* branch were isolated from *Acacia* species (Table 1 and Supplementary Table S1 in IJSEM Online). The type strain of *Sinorhizobium terengae* was isolated from *Sesbania* and *Acacia* species in Senegalese soils (de Lajudie *et al.*, 1994), whereas *Sinorhizobium arboris* and *Sinorhizobium kostiensense* were isolated from *Acacia senegal* and *Prosopis chilensis*, in Kenyan and Sudanese soils, respectively (Nick *et al.*, 1999). Despite extensive sampling that covered a wide range of ecoclimatic and altitudinal zones in Ethiopia (Wolde-meskel *et al.*, 2004a), and the use of the same and/or related trap host species of similar rhizobial affinities, no strains closely related to these genotypes were found. However, two and three different genotypes were represented in *Sinorhizobium meliloti* and *Sinorhizobium saheli*, respectively (Fig. 2).

Three strains (AC47a, AC47b and AC47d) that were closely related to *Ensifer adhaerens* (99 % partial sequence similarity) were isolated from a location (Arba-minch) that is characterized by a high soil pH (8.6) and temperature (Wolde-meskel *et al.*, 2004a). Interestingly, these strains were 100 % similar to an undescribed high-temperature and halotolerant *Sinorhizobium* species isolated from seaside areas in Taiwan (Chen *et al.*, 2000). This may reflect the role of the habitats of the strains in shaping the rhizobial genotypes, and possibly the *in situ* population structure of rhizobia (Wang *et al.*, 1999, 2003; Wang & Martínez-Romero, 2000). These strains offer the possibility of enhancing nitrogen fixation in salty-alkaline conditions where the efficiency of rhizobium-legume symbiosis is hampered.

Mesorhizobium branch. Phylogenetic analysis of nine strains, representing 20 isolates in this group (Fig. 2 and

Supplementary Table S1 in IJSEM Online), identified two species, *Mesorhizobium chacoense* (one strain) and *Mesorhizobium plurifarium* (eight strains), with 98 and 99 % partial sequence similarities, respectively. However, with the exception of two strains (AC100e and AC98a), which had 100 % sequence similarity, all of the strains in the latter species exhibited nucleotide differences (substitutions, insertions or gaps) at up to 13 positions (depending on the strain) (data not shown). Hence, these represented seven different genotypes supported by a high bootstrap value (Fig. 2). All the strains linked to this species were shown to be metabolically and genomically diverse in previous studies (Supplementary Table S1 in IJSEM Online). The heterogeneous nature of these strains is in agreement with a previous report on strains of *Mesorhizobium plurifarium* isolated from *L. leucocephala* and *Sesbania herbacea* in Mexican soils (Wang *et al.*, 2003). The relatively low sequence similarity of strain AC88c to *Mesorhizobium chacoense*, a *Prosopis alba* symbiont from Argentina (Velázquez *et al.*, 2001) not previously reported from an African soil, suggests that this strain might represent a novel species.

Bradyrhizobium lineage. Compared with the large number of fast-growing rhizobial species, until recently only four species of *Bradyrhizobium* had been described (*Bradyrhizobium japonicum*, *Bradyrhizobium elkanii*, *Bradyrhizobium yuanmingense* and *Bradyrhizobium liaoningense*) (Sawada *et al.*, 2003). However, by using several taxonomic techniques, such as numerical taxonomy, Biolog, SDS-PAGE, AFLP fingerprinting, and ITS and 16S rRNA gene sequencing, a large number of bradyrhizobia from various parts of the world have been studied, and several groups of strains with different phenotypic features and genomic profiles have been identified (Zhang *et al.*, 1999b; van Berkum & Fuhrmann, 2000; Lafay & Burdon, 2001; Willems *et al.*, 2001, 2003). In previous studies, we reported large metabolic and genomic diversity in 40 slow-growing strains isolated from eight different host species (Wolde-meskel *et al.*, 2004b, c). Phylogenetic analysis of 21 representative strains in this study delineated eight distinct groups, in which 12 different partial 16S rRNA gene sequence types (genotypes) were represented (Fig. 2). All strains in groups I and VIII (except one strain in each) showed 100 % partial sequence similarity to the type strains of *Bradyrhizobium liaoningense* and *Bradyrhizobium elkanii*, respectively (Table 1). Exceptions were strains AC86b2 (group I) and AC87b1 (group VIII), which had nucleotide substitutions at positions 29 (A→C) and 61 (G→C), respectively. All strains in the other groups (II–VII) had novel sequences, but were closely related (99 %) to *Bradyrhizobium japonicum* or *Bradyrhizobium yuanmingense*. Interestingly, the extra-slow-growing strains (Wolde-meskel *et al.*, 2004a) in group II and the three others in group VII, which showed distinct metabolic and genomic profiles in previous studies (Table 1), also formed separate phylogenetic groups in this study (Fig. 2). The 16S rRNA gene

of bradyrhizobia has been reported to show little variation (Barrera *et al.*, 1997) and can be identical as, for example, *Bradyrhizobium liaoningense* and *Bradyrhizobium japonicum* (van Berkum & Fuhrmann, 2000). Hence, RFLP or sequence analysis of this molecule may provide little discrimination. In view of this, and the high metabolic and genomic diversity of the test strains, which did not relate to reference species in earlier studies (Wolde-meskel *et al.*, 2004b, c), our collection may represent a number of yet unrecognized taxa in the genus *Bradyrhizobium*, and further taxonomic analysis would be justified.

Methylobacterium strain. Phylogenetic analysis of an almost full-length (1387 bp) 16S rRNA gene sequence of AC72a, a strain from a root nodule of *Phaseolus vulgaris*, revealed that it belonged to the *Methylobacterium* lineage of the class 'Alphaproteobacteria' (Fig. 2). It showed 90 % sequence similarity with *Methylobacterium nodulans*, the only symbiotic *Methylobacterium* species identified to date, from root nodules of *Crotalaria* sp. in Senegal (Sy *et al.*, 2001). Other published closest phylogenetic neighbours in the genus were *Methylobacterium organophilum* (93 %) and *Methylobacterium* sp. strain F48 (94 %), suggesting that strain AC72a might represent another novel symbiotic species in the genus. The strain also formed a separate cluster using Biolog (Wolde-meskel *et al.*, 2004b), 23S rRNA gene and combined 16S rRNA and 23S rRNA gene PCR-RFLP studies (Table 1 and Supplementary Figs S1 and S2 in IJSEM Online). Characteristically, AC72a was a fast-growing strain (>3 mm colony size in 2–3 days) on Yeast Mannitol Agar at 28 °C, which is in agreement with previous reports for strains belonging to *Methylobacterium nodulans* (Samba *et al.*, 1999; Sy *et al.*, 2001). However, poor growth occurred in liquid culture medium (yeast mannitol broth), even when a longer incubation time (up to 9 days) was used, after which characteristic pink-pigmented clumps in the solution were produced. In contrast to *Methylobacterium nodulans*, AC72a formed effective nodules on *Vigna unguiculata* and intermittently elicited nodules on *Sesbania sesban* and *F. albida* seedlings (data not shown). Although further phenotypic characterization of strain AC72a, and isolation and analysis of various nodulation genes are under way to establish whether the strain uses the same molecular mechanisms as rhizobia, our results emphasize that much greater diversity can be expected following the characterization of symbionts of unexplored legumes and by focusing on previously unexplored biogeographical areas.

Symbionts of previously unexplored woody legume species. *Acacia abyssinica*, *Albizia gummifera*, *Erythrina brucei* and *Milletia ferruginea* have been little studied, but are known to be locally important, indigenous woody legumes that have been integrated into traditional agroforestry systems in the highlands of Ethiopia as well as in East Africa (Hunde & Thulin, 1989; Al Amin, 1990; Mbuya *et al.*, 1994). This is the first reported phylogenetic analysis of isolates from these trees, and the results show

associated rhizobia belonging to diverse groups. For example, strains of *Acacia abyssinica* were phylogenetically related to seven different rhizobial species in four genera (including *Agrobacterium*). It is interesting to note that a number of the strains associated with the trees were novel, with sequence similarities of 98–99 % with the recognized species. In view of this and previous reports (Wolde-meskel *et al.*, 2004b, c), which showed that there was a wide range of metabolic and genomic diversity that was not related to reference species, it is likely that several groups of these strains represent potentially novel species. The diversity shown in this study supports the view that long-term association between rhizobia and indigenous host species would allow gradual differentiation and diversity in the natural rhizobial population resident in the host's native soils (Andronov *et al.*, 2003; Wang *et al.*, 2003). Furthermore, the observed genetic diversity in indigenous rhizobial populations provides an opportunity to improve nitrogen fixation in agroforestry systems through the selection of efficient rhizobium–legume combinations.

Comparative grouping of strains by using the various methods. The 16S rRNA gene PCR-RFLP pattern has been used to detect potential novel taxa of new isolates (Laguerre *et al.*, 1994; Heyndrickx *et al.*, 1996). It is also known that a longer stretch of the 16S rRNA gene (e.g. 800 instead of 300 bp) contains a conserved region that is sufficient to show the variation within groups of root-nodule bacteria, hence its frequent use to infer the phylogenetic affiliation of novel isolates (Terefework *et al.*, 1998; Odee *et al.*, 2002; Bala *et al.*, 2003). In this study, by using 16S rRNA gene PCR-RFLP, several subgroups were identified that had relatively low similarity (at most 85 %) to recognized species within the various genera (Fig. 1). This was also supported by the 23S rRNA gene and the combined PCR-RFLP pattern analyses results (Supplementary Figs S1 and S2 in IJSEM Online). However, strains representing distinct PCR-RFLP subgroups [for example, AC01b (16S rRNA PCR-RFLP genotype 6) and AC47c (genotype 12) in the *Sinorhizobium* branch; Fig. 1] showed 99–100 % partial sequence similarity to the recognized species (*Sinorhizobium saheli* and *Sinorhizobium fredii*, respectively; Table 1). While the disparity of these methods in resolving the test rhizobial strains into species remains, the possibility that undetected differences might exist in the second 800 bp stretch of the 16S rRNA gene cannot be excluded. Previously, various segments of the 16S rRNA gene sequence have been reported to provide differing phylogenetic signals (Eardly *et al.*, 1996).

van Berkum *et al.* (2003) reported discordant phylogenies within the *rrn* loci of rhizobia, after comparative analyses of groupings based on 16S and 23S rRNA gene sequences, resulting in different phylogenetic tree topologies for *Rhizobium* and *Sinorhizobium* species. Indeed, our 23S rRNA gene PCR-RFLP data are in line with their findings, and the reference *Sinorhizobium* species used in this study were nested among *Rhizobium* strains (Supplementary

Fig. S1 in IJSEM Online). It is also interesting to note the differences among grouping results obtained with the various approaches/methodologies used: Biolog and AFLP fingerprinting, ITS, 16S and 23S rRNA gene PCR-RFLP, and 16S rRNA gene partial sequence analyses (Table 1 and Supplementary Table S1 in IJSEM Online). For example, strains representing 16S rRNA PCR-RFLP genotype 6 were grouped into each of three different groups of the 23S rRNA and combined PCR-RFLP genotypes, whereas the same strains were grouped into four and two different Biolog and AFLP groups, respectively (Table 1). A similar situation has been reported in other studies (Wang *et al.*, 2003), further demonstrating the necessity for polyphasic approaches in bacterial taxonomy.

In conclusion, by using metabolic and several modern molecular biological methodologies, we identified, within 195 rhizobial strains, several groups of rhizobia and a *Methylobacterium* strain that were not related to the recognized taxa. Phylogenetically, 34 out of 46 identified genotypes (74%) represented novel partial 16S rRNA gene sequence types, which related to six known genera. This wide phylogenetic diversity of the strains that were isolated from a relatively small number of leguminous species has further strengthened the views of other workers (Odee *et al.*, 2002) that this sub-Saharan region might be an important centre of rhizobial biodiversity. Ethiopia, in particular, as the origin of several legumes and a centre of diversity for other plants, is a promising prospect for unearthing previously unidentified rhizobia that are more diverse and for elucidating the molecular evolution of rhizobium-legume symbiosis.

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