International Journal of Systematic Bacteriology (1999), 49, 1359–1368 Printed in Great Britain

Sinorhizobium arboris sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya

Giselle Nick,[1] Philippe de Lajudie,[2,5]† Bertrand D. Eardly,[3] Sini Suomalainen,[4] Lars Paulin,[4] Xiaoping Zhang,[1,6] Monique Gillis[5] and Kristina Lindström[1]

Author for correspondence: Giselle Nick. Tel: +358 9 708 59281. Fax: +358 9 708 59322. e-mail: giselle.nick-maenpaa@helsinki.fi

1 Department of Applied Chemistry and Microbiology, PO Box 56, Viikki Biocentre, FIN-00014 University of Helsinki, Finland

2 Laboratoire de Microbiologie des Sols, ORSTOM BP 1386, Dakar, Senegal

3 Pennsylvania State University, Berks Campus, POB 7009, Reading, PA 19610-6009, USA

4 Institute of Biotechnology, POB 56, Biocentre 1, FIN-00014 University of Helsinki, Finland

5 Laboratorium voor Microbiologie, Universiteit Gent, K. L. Ledeganckstraat, 35, B-9000 Gent, Belgium

6 Department of Applied Microbiology, Sichuan Agricultural University, Yaan 625014, People's Republic of China

SDS-PAGE of total bacterial proteins was applied to the classification of 25 Sudanese and five Kenyan strains isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis*. Twenty strains were also studied by multilocus enzyme electrophoresis (MLEE) and the whole 16S rRNA gene was sequenced from two strains representing the two major clusters. These results, together with the previously reported numerical taxonomy analysis, pulsed-field gel electrophoresis studies, DNA–DNA dot-blot hybridization, genomic fingerprinting using repetitive sequence-based PCR, DNA base composition analysis, DNA–DNA reassociation analysis, partial sequencing of the 16S rRNA gene and RFLP analysis of the amplified 16S rRNA gene, showed that all 30 strains belong to the genus *Sinorhizobium*. Two of the strains grouped with *Sinorhizobium saheli* and seven with *Sinorhizobium terangae*, while the rest did not cluster with any of the established species. The majority of the strains formed two phenotypically and genotypically distinct groups and we therefore propose that these strains should be classified as two new species, *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov.

Keywords: MLEE, SDS-PAGE, 16S rDNA sequencing, *Sinorhizobium arboris*, *Sinorhizobium kostiense*

Fonds Documentaire IRD
Cote : B*23997 Ex : 1

INTRODUCTION

The bacteria that are able to form nitrogen-fixing nodules on leguminous plants and trees are currently divided into five genera, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium*. *Azorhizobium caulinodans*, a stem-nodulating species, is the only species of the genus *Azorhizobium* (Dreyfus *et al.*, 1988). The bradyrhizobia, with species *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992), *Bradyrhizobium japonicum* (Jordan, 1984) and *Bradyrhizobium liaoningense* (Xu *et al.*, 1995), are root-nodulating but slow-growing bacteria. From the fast-growing rhizobia, the genus *Sinorhizobium*, with the species *Sinorhizobium fredii* (Scholla & Elkan, 1984), *Sinorhizobium meliloti* (Jordan, 1984), *Sinorhizobium saheli* and *Sinorhizobium terangae*, was emended by de Lajudie *et al.* (1994) and recently Rome *et al.* (1996) described the newest member of this genus, *Sinorhizobium medicae*. The genus *Mesorhizobium* (Jarvis *et al.*, 1997) consists of the species *Mesorhizobium loti* (Jarvis *et al.*, 1982),

†**Present address:** LSTM-ORSTOM/CIRAD-Forêt, Baillarget, BP 5035, 34032 Montpellier, France.

Abbreviations: ACO, aconitase; ADK, adenylate kinase; ET, electrophoretic type; G6P, glucose-6-phosphate dehydrogenase; HBD, hydroxybutyrate dehydrogenase; HEX, hexokinase; IDH, isocitrate dehydrogenase; IPO, indophenol oxidase; LAP, leucine aminopeptidase; MDH, NAD-malate dehydrogenase; MLEE, multilocus enzyme electrophoresis; PEP, leucylalanine peptidase; 6-PG, 6-phosphogluconate dehydrogenase; PGI, phosphoglucose isomerase; PGM, phosphoglucomutase; rep-PCR, repetitive-sequence-based PCR.

The EMBL accession numbers for the 16S rRNA sequences of *S. arboris* HAMBI 1552[T] and *S. kostiense* HAMBI 1489[T] are Z78204 and Z78203.

00999 © 1999 IUMS

Table 1. Strains used in this study

The strain numbers of Sudanese and Kenyan strains are according to Zhang *et al.* (1991), otherwise the original strain number or strain number as received is listed. ATCC, American Type Culture Collection, Manassas, VA, USA; CCBAU, Culture Collection of Beijing Agricultural University, Beijing, China; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Aútonoma de México, Cuernavaca, Mexico; CIAT, *Rhizobium* Collection, Centro International de Agricultura Tropical, Cali, Colombia; HAMBI, Culture Collection of the Division of Microbiology, University of Helsinki, Helsinki, Finland; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, Ghent, Belgium; NZP, Culture Collection of the Department for Scientific and Industrial Research, Biochemistry Division, Palmerston North, New Zealand; ORS, ORSTOM Collection, Institut Français de Recherche Scientifique pour le Développement en Coopération, Dakar, Senegal; USDA, US Department of Agriculture, Beltsville, MD, USA. NK, Not known.

Strain	Strain number		Cluster*	Host plant	Geographical origin†	Reference/source
	HAMBI	LMG				
Sinorhizobium arboris						
EO2	1706	15624	11	*Acacia senegal*	Sudan, EO	Zhang *et al.* (1991)
EO3	1624	–	11	*Acacia senegal*	Sudan, EO	Zhang *et al.* (1991)
GB9	1700	15626	11	*Acacia senegal*	Sudan, Kh	Zhang *et al.* (1991)
GB10	1704	15629	11	*Acacia senegal*	Sudan, Kh	Zhang *et al.* (1991)
HAMBI 1396	–	14917	15	*Prosopis chilensis*	Kenya	Zhang *et al.* (1991)
TTR 3	1680	15621	11	*Prosopis chilensis*	Sudan, Te	Zhang *et al.* (1991)
TTR 22	1707	–	11	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
TTR 33	1685	15637	r 18	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
TTR 38^T	1552^T	14919^T	11	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
Sinorhizobium kostiense						
TTR 1	1679	–	1	*Prosopis chilensis*	Sudan, Te	Zhang *et al.* (1991)
TTR 2	1476	–	8	*Prosopis chilensis*	Sudan, Te	Zhang *et al.* (1991)
TTR 9	1482	14924	8	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
TTR 11	1484	15609	8	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
TTR 12	1485	15610	8	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
TTR 15^T	1489^T	15613^T	8	*Acacia senegal*	Sudan, Ko	Zhang *et al.* (1991)
TTR 18	1492	14926	8	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
TTR 19	1493	14927	r 1–9	*Prosopis chilensis*	Sudan, Kh	Zhang *et al.* (1991)
TTR 27	1498	15615	r 8	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
TTR 31	1501	–	9	*Acacia senegal*	Sudan, Ko	Zhang *et al.* (1991)
TTR 34	1502	14911	8	*Acacia senegal*	Sudan, Te	Zhang *et al.* (1991)
TTR 36	1504	14929	r 1–8	*Prosopis chilensis*	Sudan, Kh	Zhang *et al.* (1991)
TTR 37	1505	14912	8	*Prosopis chilensis*	Sudan, Ko	Zhang *et al.* (1991)
***Sinorhizobium* sp.**						
HAMBI 1395	–	15632	12	*Prosopis chilensis*	Kenya	Zhang *et al.* (1991)
TTR 6	1480	14923	9	*Acacia senegal*	Sudan, Ko	Zhang *et al.* (1991)
TTR 7	1681	15634	r 13	*Acacia senegal*	Sudan, Ko	Zhang *et al.* (1991)
TTR 28	1500	–	1	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
TTR 30	1499	15618	9	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
TTR 40	1506	14909	r 1–7	*Acacia senegal*	Sudan, EO	Zhang *et al.* (1991)
Sinorhizobium fredii						
USDA 191	1337	8317	6	Soil	China	Scholla & Elkan (1984)
USDA 205^T	2075^T	6217^T	–	*Glycine max*	China	Scholla & Elkan (1984)
Sinorhizobium medicae						
CC 169	1838	16582	–	*Medicago rugosa*	Australia	Eardly *et al.* (1990)
M 75	1808	16579	–	*Medicago radiata*	Syria	Eardly *et al.* (1990)
M 102	1809	16580	–	*Medicago truncatula*	Syria	Eardly *et al.* (1990)
M 158	1837	16581	–	*Medicago noeana*	Syria	Eardly *et al.* (1990)
Sinorhizobium meliloti						
CC 1002	–	–	–	NK	NK	B. D. Eardly
CC 2013	–	–	–	*Medicago sativa*	Australia	Eardly *et al.* (1990)
NZP 4009	–	6130	–	*Medicago sativa*	Australia	LMG
NZP 4027^T (= ATCC 9930^T)	–	6133^T	–	*Medicago sativa*	USA	LMG

Table 1 *(cont.)*

Strain	Strain number		Cluster*	Host plant	Geographical origin†	Reference/source
	HAMBI	LMG				
Sinorhizobium saheli						
ORS 600	217	11864	–	*Sesbania cannabina*	Senegal	de Lajudie *et al.* (1994)
ORS 609ᵀ	215ᵀ	7837ᵀ	–	*Sesbania pachycarpa*	Senegal	de Lajudie *et al.* (1994)
TTR 21	1495	14914	14	*Acacia senegal*	Sudan, Kh	Zhang *et al.* (1991)
TTR 23	1496	14928	14	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
Sinorhizobium terangae						
ORS 51	199	6464	–	*Sesbania rostrata*	Senegal	de Lajudie *et al.* (1994)
ORS 1009ᵀ	220ᵀ	7834ᵀ	–	*Acacia laeta*	Senegal	de Lajudie *et al.* (1994)
ORS 1013	–	7844	–	*Acacia senegal*	Senegal	de Lajudie *et al.* (1994)
EO6	248	15631	12	*Acacia senegal*	Sudan, EO	Zhang *et al.* (1991)
EO7	1550	14918	12	*Acacia senegal*	Sudan, EO	Zhang *et al.* (1991)
HAMBI 1392	–	15635	14	*Prosopis chilensis*	Kenya	Zhang *et al.* (1991)
TSO1	1703	15627	11	*Acacia senegal*	Sudan, Te	Zhang *et al.* (1991)
TTR 41	1551	14931	14	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
TTR 39	1698	15630	11	*Acacia senegal*	Sudan, EF	Zhang *et al.* (1991)
Rhizobium etli						
CFN 42ᵀ	1727ᵀ	11937ᵀ	–	*Phaseolus vulgaris*	Mexico	Segovia *et al.* (1993)
Rhizobium galegae						
HAMBI 540ᵀ	–	6214ᵀ	–	*Galega orientalis*	Finland	Lindström (1989)
HAMBI 1147	–	6215	–	*Galega orientalis*	Finland	Lindström (1989)
Rhizobium leguminosarum						
ATCC 10004ᵀ	–	8817ᵀ	–	*Pisum sativum*	NK	LMG
ATCC 14482	–	8819	–	NK	NK	LMG
ATCC 14480	–	8820	–	*Trifolium pratens*	NK	LMG
CNPAF 512	–	9505	–	NK	NK	LMG
Rhizobium tropici						
CFN 299	–	9517	–	*Phaseolus vulgaris*	Brazil	Martinez-Romero *et al.* (1991)
CIAT 899ᵀ	1163ᵀ	9503ᵀ	–	*Phaseolus vulgaris*	Colombia	Martinez-Romero *et al.* (1991)
Mesorhizobium ciceri						
UPM-Ca7ᵀ	1750ᵀ	17150ᵀ	–	*Cicer arietinum*	Spain	Nour *et al.* (1994)
522	–	17149	–	*Cicer arietinum*	Russia	Nour *et al.* (1994)
Mesorhizobium huakuii						
CCBAU 2609ᵀ	1674ᵀ	14107ᵀ	–	*Astragalus sinicus*	China	Chen *et al.* (1991)
Mesorhizobium loti						
NZP 2213ᵀ	1129ᵀ	6125ᵀ	5	*Lotus corniculatus*	New Zealand	Jarvis *et al.* (1982)
NZP 2230	–	6126	–	*Lotus maroccanus*	Morocco	de Lajudie *et al.* (1994)
Mesorhizobium mediterraneum						
Ca-36ᵀ	–	17148	–	*Cicer arietinum*	Spain	Nour *et al.* (1995)
Mesorhizobium plurifarium						
BR 3804	–	9970	–	*Chamaecrista ensiformis*	Brazil	de Lajudie *et al.* (1998)
INPA 78B	204	10056	–	*Leucaena diversifolia*	Brazil	de Lajudie *et al.* (1998)
ORS 1001	193	7836	–	*Acacia senegal*	Senegal	de Lajudie *et al.* (1998)
ORS 1030	1995	11890	–	*Acacia senegal*	Senegal	de Lajudie *et al.* (1998)
Mesorhizobium tianshanense						
A-1BSᵀ	1870ᵀ	15767ᵀ	–	*Glycyrrhiza pallidiflora*	China	Chen *et al.* (1995)
6	1956	15769	–	*Glycyrrhiza uralensis*	China	Chen *et al.* (1995)
017A	–	15768	–	*Sophora alopecuroides*	China	Chen *et al.* (1995)

* Clusters according to Zhang *et al.* (1991); r, related to.

† The five locations in the Sudan from which strains were isolated are abbreviated as Khartoum (Kh), Tendelti (Te), El Obeid (EO), Kosti (Ko) and El Fau (EF).

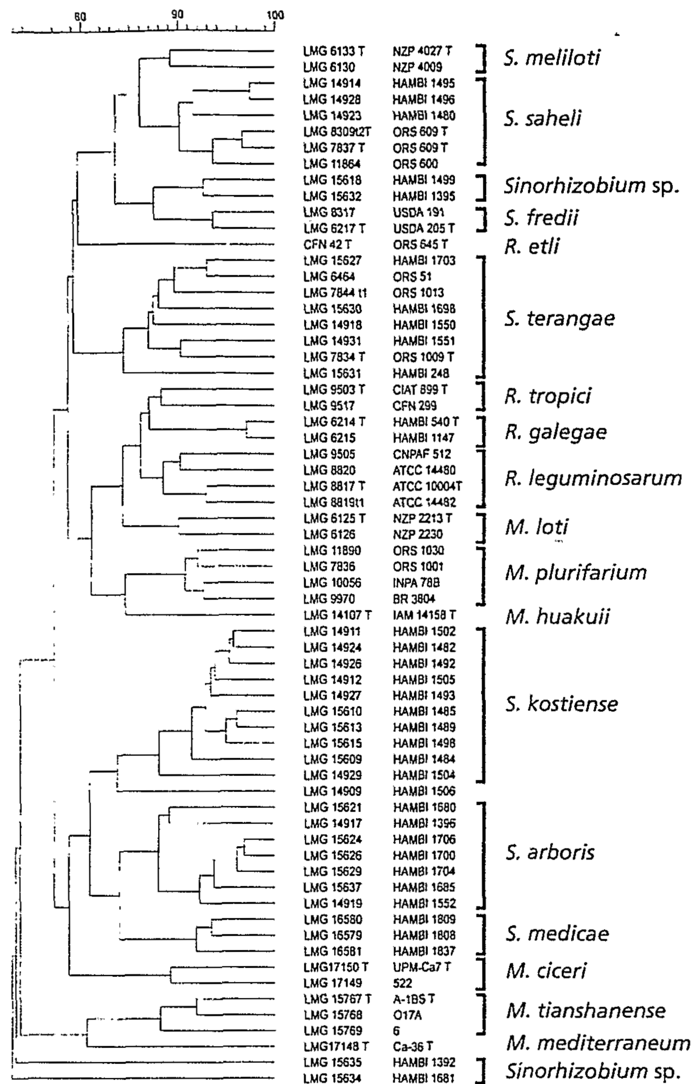

Fig. 1. UPGMA dendrogram of the SDS-PAGE analysis showing the relationships between electrophoretic protein patterns of Sudanese, Kenyan and reference strains of established rhizobial species.

Mesorhizobium ciceri (Nour *et al.*, 1994), *Mesorhizobium huakuii* (Chen *et al.*, 1991), *Mesorhizobium mediterraneum* (Nour *et al.*, 1995), *Mesorhizobium plurifarium* (de Lajudie *et al.*, 1998) and *Mesorhizobium tianshanense* (Chen *et al.*, 1995). The genus *Rhizobium* is phylogenetically heterogeneous with two different groups: *Rhizobium etli* (Segovia *et al.*, 1993), *Rhizobium gallicum* (Amarger *et al.*, 1997). *Rhizobium hainanense* (Chen *et al.*, 1997), *Rhizobium leguminosarum* (Jordan, 1984), *Rhizobium mongolense* (van Berkum *et al.*, 1998) and *Rhizobium tropici* (Martinez-Romero *et al.*, 1991) form one rRNA branch and *Rhizobium galegae* (Lindström, 1989), *Rhizobium huautlense* (Wang *et al.*, 1998) and *Rhizobium giardinii* (Amarger *et al.*, 1997) are in a separate branch together with *Agrobacterium vitis* (Ophel & Kerr, 1990) and *Agrobacterium* biovar 1 (Kersters & De Ley, 1984).

De Lajudie *et al.* (1994) have shown that tropical, fast-growing rhizobia isolated in West Africa are very diverse but are clearly different from previously established species. Sudanese and Kenyan tree nodule isolates showed wide diversity by numerical taxonomy (Zhang *et al.*, 1991) and this was later confirmed by several genotypic analyses (Haukka & Lindström, 1994; Haukka *et al.*, 1996; Nick, 1998; Nick *et al.*, 1999). The previous studies led us to complete the polyphasic taxonomy of the Sudanese and Kenyan strains by using SDS-PAGE of total bacterial proteins, multilocus enzyme electrophoresis (MLEE) and total 16S rRNA sequence analysis. On the basis of previous and current results, we propose two new species, *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov.

METHODS

Bacterial strains and growth conditions. Strains used in this study are listed in Table 1. Sudanese and Kenyan strains were isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis* (Zhang *et al.*, 1991) and the other strains were representatives of *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species. All strains were maintained in yeast extract-mannitol (YEM) broth (Lindström *et al.*, 1985) containing 20% (v/v) glycerol at −70 °C and freeze-dried. For transmission electron microscopy observations, the type strains of *S. arboris* and *S. kostiense* were grown in non-agitated YEM broth, which contained only 1 g mannitol in 1000 ml medium.

PAGE of total bacterial proteins. SDS-PAGE was performed by using the procedure of Laemmli (1970) with slight modifications, as described previously (Kiredjian *et al.*, 1986; de Lajudie *et al.*, 1994). The normalized densitometric traces of the protein electrophoretic patterns were grouped by numerical analysis, using the GelCompar 2.2 software package (Applied Maths) (Vauterin & Vauterin, 1992). The similarity between all pairs of traces was expressed by the Pearson product–moment correlation coefficient (r) converted for convenience to a percentage value (Pot *et al.*, 1989, 1994).

MLEE. Nineteen Sudanese strain and one Kenyan strain were used in MLEE analysis and compared with *S. saheli*, *S. terangae*, two *S. medicae* and four *S. meliloti* strains. The techniques used for starch-gel electrophoresis and selective staining of enzymes have been described previously (Eardly *et al.*, 1990; Eardly, 1994; Selander *et al.*, 1986). The electrophoretic buffer systems and the thirteen enzymes assayed were as follows: Tris/citrate (pH 6·7) for 6-phosphogluconate dehydrogenase (6PG), hydroxybutyrate dehydrogenase (HBD), aconitase (ACO) and NAD-malate dehydrogenase (MDH); Tris/citrate (pH 8·0) for phosphoglucose isomerase (PGI), isocitrate dehydrogenase (IDH), glucose-6-phosphate dehydrogenase (G6P), leucyl-alanine peptidase (PEP) and leucine aminopeptidase (LAP); and borate (pH 8·2) for indophenol oxidase (IPO), hexokinase (HEX), adenylate kinase (ADK) and phosphoglucomutase (PGM). Distinctive mobility variants (electromorphs) of each enzyme, numbered in order of decreasing anodal mobility,

Table 2. Allele profiles at 13 enzyme loci for 18 ETs of *Sinorhizobium*

Strain	Allele at indicated enzyme locus												
	MDH	6PG	HBD	ACO	PGI	IDH	G6P	PEP	LAP	IPO	HEX	ADK	PGM
S. arboris													
HAMBI 1396	3	3	1	1	5	1	3	4	1	2	4	5	3
HAMBI 1552^T, 1680	3	3	3	2	4	1	3	3	3	2	2	5	3
HAMBI 1624	3	3	2	2	5	1	3	3	3	2	2	5	3
HAMBI 1685	3	3	4	2	5	1	3	3	4	2	2	5	3
HAMBI 1707	3	3	4	2	4	1	3	3	4	2	2	5	3
HAMBI 1700, 1704, 1706	3	3	4	2	4	1	3	3	5	2	2	5	3
S. kostiense													
HAMBI 1476, 1679	2	1	4	2	4	1	3	5	3	2	1	2	2
HAMBI 1501	2	1	4	1	4	1	3	5	3	1	1	1	2
HAMBI 1482, 1489^T, 1493, 1498, 1504	2	2	4	3	6	1	3	5	3	2	2	1	3
***Sinorhizobium* sp.**													
HAMBI 1499	4	4	6	2	5	2	1	3	3	4	3	5	0
HAMBI 1500, 1506	1	6	6	2	3	1	4	5	2	2	5	3	1
S. medicae													
M 102	3	4	3	2	7	1	2	1	4	1	3	6	6
M 158	3	5	3	2	7	3	2	1	3	1	3	6	6
S. meliloti													
ATCC 9930^T, CC 1002	3	6	4	1	2	3	3	3	4	1	5	5	5
CC 169	3	7	3	2	7	1	2	1	3	1	3	6	6
CC 2013	3	4	3	1	3	1	2	2	2	1	5	5	7
S. saheli													
ORS 609^T	0	8	5	3	1	1	4	5	3	4	1	5	4
S. terangae													
ORS 1009^T	4	7	5	4	5	2	3	4	5	3	3	4	0

were equated with alleles at the corresponding structural gene locus. Allele profiles, or electrophoretic types (ETs), were equated with multilocus genotypes. The genetic distance between pairs of ETs was estimated as the proportion of loci at which dissimilar alleles (mismatches) occurred and clustering was performed by the unweighted pair group method (Sneath & Sokal, 1973).

Sequencing of 16S rRNA genes. The 16S rRNA gene sequences of the type strains *S. arboris* HAMBI 1552^T and *S. kostiense* HAMBI 1489^T were determined. A large fragment of the 16S rRNA of about 1522 bp was amplified by using primers pA (5′-biotin-AGAGTTTGATCCTGGCTCAG 3′) and pH* (5′ AAGGAGGTGATCCAGCCGCA 3′) (Edwards *et al.*, 1989). The PCR was carried out in a 100 µl reaction volume containing polymerase reaction buffer [10 mM Tris/HCl (pH 8·8 at 25 °C), 1·5 mM $MgCl_2$, 50 mM KCl, 0·1 % Triton X-100], 200 µM dNTPs (Pharmacia), 1 µM of both primers, 2 U DynaZyme II DNA polymerase (Finnzymes) and about 30 ng pure total DNA as template. Amplification was done in an MJ Research MiniCycler with the following temperature profile: initial denaturation at 95 °C for 3 min, 30 cycles at 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min, final extension at 72 °C for 3 min. The PCR product was sequenced directly by using the solid-phase method as described previously (Hultman *et al.*, 1991) with an automated ALF DNA sequencer (Pharmacia) (Ridell *et al.*, 1995). The determined sequences, together with reference sequences obtained from the EMBL database, were aligned by using the PILEUP program in the Wisconsin package of the Genetics Computer Group (GCG). All the sequences used were almost full length and were derived from the type strain wherever possible. A phylogenetic tree was created by the CLUSTAL W 1.7 program (Thompson *et al.*, 1994) using the neighbour-joining method based on a distance matrix with the distance correction calculated by Kimura's two-parameter method. The tree was drawn using the TREEVIEW program (Page, 1996). Identities were calculated using the GAP program in the GCG package.

Morphological and physiological tests. Cell dimensions and morphology were determined under light microscopy. The type of flagellation was determined for the type strains of *S. arboris* and *S. kostiense* by transmission electron microscopy in the electron microscopy laboratory at the Institute of Biotechnology, University of Helsinki.

RESULTS

SDS-PAGE of total bacterial proteins

Seven representative strains of *S. arboris* and ten representative strains of *S. kostiense* constituted two main electrophoretic groups, three strains (HAMBI 1480, 1495 and 1496) clustered with *S. saheli* and five strains (HAMBI 248, 1550, 1551, 1698 and 1703) with *S. terangae*. Two strains (HAMBI 1395 and 1499) were related to *S. fredii* while two others (HAMBI 1392 and 1681) had a separate position (Fig. 1).

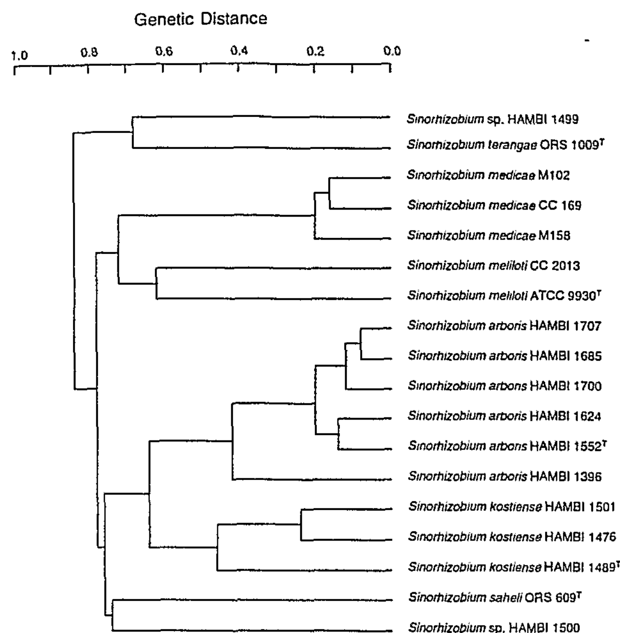

Fig. 2. Genetic relationships among 18 ETs of *Sinorhizobium* estimated on the basis of electrophoretically detectable allelic variation at 13 loci. A representative isolate is listed for each ET.

MLEE analysis

Nineteen Sudanese strains and one Kenyan strain were used in MLEE analysis. The *S. arboris* strains revealed six and *S. kostiense* three distinctive multilocus genotypes (ETs). Four of the *S. arboris* and one of the *S. kostiense* ETs were represented by single strains (Table 2). Cluster analysis of the ETs grouped nine *S. arboris* and eight *S. kostiense* strains together, and the genetic distances ranged from 0·08 to 0·19 and from 0·23 to 0·46, respectively (Fig. 2). Strains HAMBI 1500 and 1506 gave identical ETs but were clearly different from other strains and species examined, at a genetic distance of 0·78. The ET of strain HAMBI 1499 was not identical or close to *S. saheli* and was thus in agreement with the result of repetitive-sequence-based (rep)-PCR. *S. fredii* strains were not included in the MLEE analysis as 16S rDNA PCR-RFLP analysis (Nick, 1998) and protein analysis (data not shown) has shown that the *S. fredii* strains do not form a homogeneous group as the other *Sinorhizobium* species do and thus need to be studied more thoroughly.

16S rRNA gene sequencing

The 16S rRNA sequences determined were compared with the 16S rRNA sequences of other members of the α-2-subclass of the *Proteobacteria* available from the EMBL database. Strains HAMBI 1489^T and 1552^T were found to be closely related but distinct members of the *Sinorhizobium* lineage (Fig. 3). The levels of sequence identity obtained by using the GAP program are shown in Table 3.

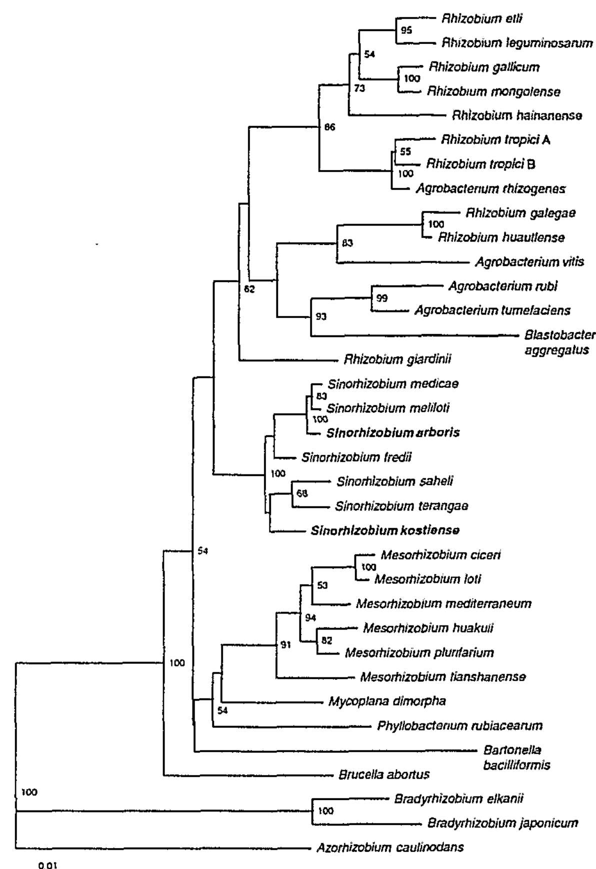

Fig. 3. Phylogenetic tree of rhizobia and some related bacteria in the α-subclass of the *Proteobacteria*. The tree was constructed by the neighbour-joining method from 16S rRNA sequences. Bootstrap probability values greater than 50% are indicated at the branch-points. Bar, 0·01 substitution per site. The sequences are derived from the type strains wherever possible. The GenBank/EMBL accession numbers used are (from top to bottom): U28916, U29386, U86343, U89816, U71078, X67233, X67234, X67224, X67226, AF025852, X67225, X67228, X67223, X73041, U86344, L39882, X67222, Z78204, X67231, X68390, X68388, Z78203, U07934, X67229, L38825, D12797, Y14158, U71079, D12786, D12790, Z11683, X13695, U35000, X66024 and X67221.

DISCUSSION

The relationships between rhizobia isolated from the root nodules of *Acacia senegal* and *Prosopis chilensis* trees in Sudan and Kenya were studied by using a polyphasic approach. These rhizobia were first found to be very diverse by numerical taxonomy (Zhang *et al.*, 1991) and were further studied by molecular biological methods including pulsed-field gel electrophoresis (Haukka & Lindström, 1994), 16S rDNA analyses (Haukka *et al.*, 1996; Nick, 1998), rep-PCR genomic fingerprinting, dot-blot DNA–DNA hybridization, DNA base composition and DNA–DNA reassociation analysis (Nick *et al.*, 1999) and cellular fatty acid analysis (S. W. Tighe & B. D. W. Jarvis, unpublished results). Two of the strains have been found to belong to the new species *M. plurifarium* (de Lajudie *et al.*, 1998).

Table 3. 16S rRNA sequence identity levels between sinorhizobia

The values in the lower left part of the table are percentage identities and those in the upper right part of the table are numbers of nucleotide differences observed. The strains used in the comparison are indicated in the left-hand column.

Strain	No. of nucleotide differences/Percentage identity						
	1	2	3	4	5	6	7
1. *S. arboris* HAMBI 1552^T	–	17	21	8	8	26	35
2. *S. fredii* USDA 205^T	98·7	–	15	16	14	12	23
3. *S. kostiense* HAMBI 1489^T	98·5	99·0	–	27	25	21	21
4. *S. medicae* A-321^T	99·4	98·9	98·1	–	3	27	35
5. *S. meliloti* NZP 4027^T	99·4	99·0	98·3	99·7	–	24	35
6. *S. saheli* ORS 609^T	98·2	99·2	98·5	98·0	98·3	–	16
7. *S. terangae* ORS 1009^T	97·6	98·4	98·5	97·5	97·6	98·9	–

The 16S rRNA and cellular fatty acid analyses showed that almost all the Sudanese and Kenyan isolates belong to the genus *Sinorhizobium* (Haukka *et al.*, 1996; Nick, 1998; S. W. Tighe & B. D. W. Jarvis, unpublished results). Numerical taxonomy had grouped the majority of these strains in two main clusters, which were phenotypically separate from established species (Zhang *et al.*, 1991), and this finding was confirmed by several genomic analyses (Haukka & Lindström, 1994; Haukka *et al.*, 1996; Nick, 1998; Nick *et al.*, 1999). To complete the classification of the sinorhizobial isolates, 30 strains were further compared with established rhizobial species by SDS-PAGE of total proteins, 20 strains were studied by MLEE and the full length sequences of the 16S rRNA genes from two strains representing the two main clusters were determined. The results were in good agreement with previous studies and led to the proposal of two new species.

Among the Sudanese and Kenyan strains, we found two strains that belong to *S. saheli* and six strains that belong to *S. terangae*. Strains HAMBI 1495 and 1496 both fell into cluster 14 in the numerical taxonomy analysis (Table 1). They grouped with the Senegalese *S. saheli* strains by dot-blot hybridization, rep-PCR and protein analysis. By 16S rDNA analyses they were slightly different from the type strain but very close to it (Haukka *et al.*, 1996; Nick, 1998; Nick *et al.*, 1999). In DNA–DNA reassociation analysis, strain HAMBI 1496 was a typical *S. saheli* strain (Nick *et al.*, 1999). Three strains (HAMBI 248, 1550 and 1703) gave similar results to the type strain of *S. terangae* in dot-blot hybridization, rep-PCR (Nick *et al.*, 1999), 16S rDNA sequence (Haukka *et al.*, 1996) and PCR-RFLP analyses (Nick, 1998) and protein analysis. Strain HAMBI 1550 was also used in DNA–DNA reassociation analysis and showed very strong homology to *S. terangae* (Nick *et al.*, 1999). Strain HAMBI 1703 behaved differently only in numerical taxonomy, where it was in cluster 11, whereas the other two originated from cluster 12 (Zhang *et al.*, 1991). Strains HAMBI 1392, 1551 and 1698 clustered with the *S. terangae* type strain in dot-blot hybridization (Nick *et al.*, 1999) and also, in the case of the two latter strains, in protein analysis. They were very closely related to the type strain by 16S rDNA analyses and rep-PCR, which probably means that these strains represent the borderlines of the *S. terangae* species, since the DNA-binding level of strain HAMBI 1551 in the *S. terangae* cluster was not high, although significant (Haukka *et al.*, 1996; Nick, 1998; Nick *et al.*, 1999). In numerical taxonomy, strain HAMBI 1698 belonged to cluster 11 and strains HAMBI 1392 and 1551 to cluster 14 (Zhang *et al.*, 1991). Strain HAMBI 1395 clearly clustered with *S. terangae* in dot-blot hybridization and the short sequenced fragment of the 16S rRNA gene placed it in the *S. terangae* continuum (Haukka *et al.*, 1996; Nick *et al.*, 1999). Numerical taxonomy placed it in cluster 12, together with strains HAMBI 248 and 1550 (Zhang *et al.*, 1991). It had a separate position in SDS-PAGE, and therefore we could not place it with *S. terangae* until the DNA–DNA reassociation results were available. The rest of the sinorhizobial isolates did not group with any of the known species.

In numerical taxonomy, the two main clusters, 8 and 11, contained 15 and 18 strains, respectively. In addition, three strains were related to cluster 8. In further analysis by dot-blot hybridization, rep-PCR (Nick *et al.*, 1999) and 16S rDNA PCR-RFLP (Nick, 1998), all the cluster 8 strains except one, which was not studied further, formed a homogeneous group. This group also contained the three strains related to cluster 8 and two strains from cluster 1 and one strain from cluster 9. We found that phenotypic cluster 11 was more heterogeneous and only eight strains from it formed a distinct group clearly different from the established species. Two other strains, one from cluster 15 and the other related to cluster 18, also belonged to this second main cluster. The representative strains from both of these clusters also formed separate groups by protein analysis (Fig. 1), MLEE (Fig. 2) and

DNA–DNA reassociation analysis (Nick *et al.*, 1999). A short sequence (230 bp) of the 16S rRNA gene grouped the cluster 11 strains with *S. medicae* (Haukka *et al.*, 1996), but the full-length sequence showed that clusters 8 and 11 represent two new *Sinorhizobium* species, *S. kostiense* and *S. arboris*, respectively (Fig. 3). In the dendrogram based on the neighbour-joining method, *S. arboris* forms a branch with *S. medicae* and *S. meliloti*, with the two latter species more closely related, whereas *S. kostiense* is by itself. Also, pairwise comparison of sequences by the GAP program showed that the sequence identity was highest between *S. meliloti* and *S. medicae* (99·7%).

Eight of the Sudanese and Kenyan isolates did not cluster unambiguously with any of the *Sinorhizobium* species or clusters 8 or 11 (Table 1). Numerical taxonomy placed five of them in cluster 9 (Zhang *et al.*, 1991), but on further analysis they did not form a separate group. Strains HAMBI 1500 and 1506 were included in the MLEE analysis, since they grouped with *S. kostiense* by 16S rDNA PCR-RFLP (Nick, 1998) and with *S. arboris* by rep-PCR (Nick *et al.*, 1999). These strains where not members of any clusters by dot-blot hybridization (Nick *et al.*, 1999). Strain 1506 also had a separate position by SDS-PAGE. MLEE analysis showed that these strains are closely related only to each other. Strain HAMBI 1499 is an interesting isolate; 16S rDNA PCR-RFLP grouped it with *S. saheli*, otherwise it remained unclustered. Haukka *et al.* (1996) found that it has two 16S sequences among its rRNA operons, which were similar to the sequences of strains HAMBI 1393 and 1394. These eight strains clearly belong to *Sinorhizobium* but additional similar strains have to be studied before they can be assigned to a species.

Among the Sudanese and Kenyan isolates, the two main clusters (8 and 11) have been shown to form two distinct groups different from established species. We therefore propose that these strains should be assigned to two new species, *Sinorhizobium kostiense* sp. nov. and *Sinorhizobium arboris* sp. nov., respectively.

Description of *Sinorhizobium arboris* sp. nov.

Sinorhizobium arboris (ar'bo.ris. L. fem. n. *arbor* tree: L. fem. gen. n. *arboris* of the tree).

Short, aerobic, Gram-negative, non-spore-forming rods that are 0·5–0·7 μm wide by 1·5–2·0 μm long. Motile by means of one or two polar or subpolar flagella. The maximum growth temperature on YEM medium is 41–43 °C. Colonies of most strains on YEM are circular, cream-coloured, semi-translucent and mucilaginous and often spread over an entire plate within 2–4 d. A wide range of carbohydrates and amino acids are utilized as sole carbon sources for growth. Physiological and biochemical features typical for this species include growth on L-(+)-isoleucine and tolerance of 3% (w/v) NaCl, heavy metals (but not aluminium) and a wide range of antibiotics. Produces melanin and grows at pH 8·5. However, this species cannot be identified by biochemical and physiological characters alone. Strains can nodulate *Acacia senegal* and *Prosopis chilensis*. At the molecular level, this species can be differentiated by SDS-PAGE whole-cell protein pattern, MLEE, rep-PCR genomic fingerprinting (Nick *et al.*, 1999), RFLP analysis of the amplified 16S rDNA (Nick, 1998), total DNA–DNA hybridization (Nick *et al.*, 1999) and sequencing the of the whole 16S rRNA gene. *S. arboris* can be assigned to the genus *Sinorhizobium* by cellular fatty acid analysis (S. W. Tighe & B. D. W. Jarvis, unpublished results). The G+C content of the DNA is 60·6–61·8 mol% (Nick *et al.*, 1999). Strain HAMBI 1552^T (= LMG 14919^T) is the type strain. All the *Sinorhizobium arboris* strains identified in this paper have been deposited in the Culture Collections of the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland, and the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium.

Description of *Sinorhizobium kostiense* sp. nov.

Sinorhizobium kostiense (kos.ti.en'se. L. neut. adj. *kostiense* pertaining to Kosti, the region in Sudan from which the majority of the organisms were isolated).

Short, aerobic, Gram-negative, non-spore-forming rods that are 0·5–0·7 μm wide by 1·5–2·0 μm long. Motile by means of one or two polar or subpolar flagella. The maximum growth temperature of the majority of the strains on YEM medium is 38–40 °C. Colonies of most strains on YEM are circular, cream-coloured, semi-translucent and mucilaginous and often spread over an entire plate within 2–4 d. A narrow range of carbohydrates and amino acids are utilized as sole carbon sources for growth. Physiological and biochemical features typical for this species include tolerance of only 1% (w/v) NaCl and sensitivity to heavy metals (except copper and lead) and to a wide range of antibiotics. Produces melanin but does not grow at pH 5·5 or 8·5. However, this species cannot be identified by biochemical and physiological characters alone. Strains can nodulate *Acacia senegal* and *Prosopis chilensis*. At the molecular level, this species can be differentiated by SDS-PAGE whole-cell protein pattern, MLEE, rep-PCR genomic fingerprinting (Nick *et al.*, 1999), RFLP analysis of amplified 16S rDNA (Nick, 1998), total DNA–DNA hybridization (Nick *et al.*, 1999) and sequencing of the whole 16S rRNA gene. *S. kostiense* can be assigned to the genus *Sinorhizobium* by cellular fatty acid analysis (S. W. Tighe & B. D. W. Jarvis, unpublished results). The G+C content of the DNA is 57·9–61·6 mol% (Nick *et al.*, 1999). Strain HAMBI 1489^T (= LMG 15613^T) is the type strain. All the *Sinorhizobium kostiense* strains identified in this paper have been deposited in the Culture Collections of the Department of Applied Chemistry and Microbiology, University of Helsinki, Helsinki, Finland, and the Laboratorium voor Microbiologie, University of Ghent, Ghent, Belgium.

ACKNOWLEDGEMENTS

We thank Sirpa Tiikkainen, Kari Lounatmaa and Eeva-Liisa Paunonen for helping us with the transmission electron microscopy. This work was supported by a grant from the University of Helsinki and by the Commission of the European Communities (STD3 Programme contract no. TS3-CT93-0232). M. G. is indebted to the Fund of Scientific Research – Flanders (Belgium), for research and personal grants.

REFERENCES

Amarger, N., Macheret, V. & Laguerre, G. (1997). *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. *Int J Syst Bacteriol* 47, 996–1006.

van Berkum, P., Beyene, D., Bao, G., Campbell, T. A. & Eardly, B. D. (1998). *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. *Int J Syst Bacteriol* 48, 13–22.

Chen, W. X., Li, G. S., Qi, Y. L., Wang, E. T., Yuan, H. L. & Li, J. L. (1991). *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. *Int J Syst Bacteriol* 41, 275–280.

Chen, W. X., Wang, E. T., Wang, S., Li, Y., Chen, X. & Li, Y. (1995). Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an arid saline environment in Xinjiang, People's Republic of China. *Int J Syst Bacteriol* 45, 153–159.

Chen, W.-X., Tan, Z.-Y., Gao, J.-L., Li, Y. & Wang, E.-T. (1997). *Rhizobium hainanense* sp. nov., isolated from tropical legumes. *Int J Syst Bacteriol* 47, 870–873.

Dreyfus, B., Garcia, J. L. & Gillis, M. (1988). Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem-nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. *Int J Syst Bacteriol* 38, 89–98.

Eardly, B. D. (1994). Multilocus enzyme electrophoresis methods for the analysis of bacterial population genetic structure. In *Methods of Soil Analysis, Part 2. Microbiological and Biochemical Properties*, pp. 557–573. Edited by R. W. Weaver, J. S. Angle & P. J. Bottomley. Madison, WI: Soil Science Society of America.

Eardly, B. D., Materon, L. A., Smith, N. H., Johnson, D. A., Rumbaugh, M. D. & Selander, R. K. (1990). Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. *Appl Environ Microbiol* 56, 187–194.

Edwards, U., Rogall, T., Blöcker, H., Emde, M. & Böttger, E. C. (1989). Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. *Nucleic Acids Res* 17, 7843–7853.

Haukka, K. & Lindström, K. (1994). Pulsed-field gel electrophoresis for genotypic comparison of *Rhizobium* bacteria that nodulate leguminous trees. *FEMS Microbiol Lett* 119, 215–220.

Haukka, K., Lindström, K. & Young, J. P. W. (1996). Diversity of partial 16S rRNA sequences among and within strains of African rhizobia isolated from *Acacia* and *Prosopis*. *Syst Appl Microbiol* 19, 352–359.

Hultman, T., Bergh, S., Moks, T. & Uhlén, M. (1991). Bidirectional solid-phase sequencing of in vitro-amplified plasmid DNA. *BioTechniques* 10, 84–93.

Jarvis, B. D. W., Pankhurst, C. E. & Patel, J. J. (1982). *Rhizobium loti*, a new species of legume root nodule bacteria. *Int J Syst Bacteriol* 32, 378–380.

Jarvis, B. D. W., van Berkum, P., Chen, W. X., Nour, S. M., Fernandez, M. P., Cleyet-Marel, J. C. & Gillis, M. (1997). Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* 47, 895–898.

Jordan, D. C. (1984). Family III. *Rhizobiaceae* Conn 1938. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–256. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Kersters, K. & De Ley, J. (1984). Genus III. *Agrobacterium* Conn 1942. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 244–254. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Kiredjian, M., Holmes, B., Kersters, K., Guilvout, I. & De Ley, J. (1986). *Alcaligenes piechaudii*, a new species from human clinical specimens and the environment. *Int J Syst Bacteriol* 36, 282–287.

Kuykendall, L. D., Saxena, B., Devine, T. E. & Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* 38, 501–505.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.

de Lajudie, P., Willems, A., Pot, B. & 7 other authors (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.

de Lajudie, P., Willems, A., Nick, G. & 9 other authors (1998). Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. *Int J Syst Bacteriol* 48, 369–382.

Lindström, K. (1989). *Rhizobium galegae*, a new species of legume root nodule bacteria. *Int J Syst Bacteriol* 39, 365–367.

Lindström, K., Sarsa, M., Polkunen, J. & Kansanen, P. (1985). Symbiotic nitrogen fixation of Rhizobium (Galega) in acid soils and its survival in soil under acid and cold stress. *Plant Soil* 87, 293–302.

Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A., Graham, P. & Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* 41, 417–426.

Nick, G. (1998). *Polyphasic taxonomy of rhizobia isolated from tropical tree legumes*. PhD thesis. Dissertationes Biocentri Viikki Universitatis Helsingiensis. Department of Applied Chemistry and Microbiology, University of Helsinki.

Nick, G., Jussila, M., Hoste, B., Niemi, M., Kaijalainen, S., de Lajudie, P., Gillis, M., de Bruijn, F. J. & Lindström, K. (1999). Rhizobia isolated from the root nodules of tropical leguminous trees characterized using DNA-DNA dot-blot hybridisation and rep-PCR. *Syst Appl Microbiol* (in press).

Nour, S. M., Fernandez, M. P., Normand, P. & Cleyet-Marel, J.-C. (1994). *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *Int J Syst Bacteriol* 44, 511–522.

Nour, S. M., Cleyet-Marel, J.-C., Normand, P. & Fernandez, M. P. (1995). Genomic heterogeneity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov. *Int J Syst Bacteriol* 45, 640–648.

Ophel, K. & Kerr, A. (1990). *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *Int J Syst Bacteriol* 40, 236–241.

Page, R. D. M. (1996). TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.

Pot, B., Gillis, M., Hoste, B., Van De Velde, A., Bekaert, F., Kersters, K. & De Ley, J. (1989). Intra- and intergeneric relationships of the genus *Oceanospirillum*. *Int J Syst Bacteriol* **39**, 23–34.

Pot, B., Vandamme, P. & Kersters, K. (1994). Analysis of electrophoretic whole organism protein fingerprints. In *Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & T. O'Donnell. Chichester: Wiley.

Ridell, J., Siitonen, A., Paulin, L., Lindroos, O., Korkeala, H. & Albert, M. J. (1995). Characterization of *Hafnia alvei* by biochemical tests, random amplified polymorphic DNA PCR, and partial sequencing of 16S rRNA gene. *J Clin Microbiol* **33**, 2372–2376.

Rome, S., Fernandez, M. P., Brunel, B., Normand, P. & Cleyet-Marel, J.-C. (1996). *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. *Int J Syst Bacteriol* **46**, 972–980.

Scholla, M. H. & Elkan, G. H. (1984). *Rhizobium fredii* sp. nov., a fast-growing species that effectively nodulates soybeans. *Int J Syst Bacteriol* **34**, 484–486.

Segovia, L., Young, J. P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 374–377.

Selander, R. K., Caugant, D. A., Ochman, H., Musser, J. M., Gilmour, M. N. & Whittam, T. S. (1986). Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. *Appl Environ Microbiol* **51**, 873–884.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy*. San Francisco: W. H. Freeman.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.

Vauterin, L. & Vauterin, V. (1992). Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. *Eur Microbiol* **1**, 37–41.

Wang, E. T., van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X. & Martínez-Romero, E. (1998). *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbacea* that has a close phylogenetic relationship with *Rhizobium galegae*. *Int J Syst Bacteriol* **48**, 687–699.

Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* **45**, 706–711.

Zhang, X., Harper, R., Karsisto, M. & Lindström, K. (1991). Diversity of *Rhizobium* bacteria isolated from the root nodules of leguminous trees. *Int J Syst Bacteriol* **41**, 104–113.