International Journal of Systematic and Evolutionary Microbiology (2000), **50**, 159–170 Printed in Great Britain

Polyphasic characterization of rhizobia that nodulate *Phaseolus vulgaris* in West Africa (Senegal and Gambia)

Adama Diouf,[1] Philippe de Lajudie,[2] Marc Neyra,[1] Karel Kersters,[3] Monique Gillis,[3] Esperanza Martinez-Romero[4] and Mamadou Gueye[1]

Author for correspondence: Mamadou Gueye. Tel: +221 849 33 21. Fax: +221 832 16 75. e-mail: mamadou.gueye@orstom.sn

[1] MIRCEN/Centre ISRA-IRD, BP 1386, Dakar, Senegal, West Africa

[2] Laboratoire des Symbioses Tropicales et Méditerranéennes, IRD, Campus de Baillarguet, BP 5035, 34032 Montpellier Cédex 1, France

[3] Laboratorium voor Microbiologie, Universiteit Gent, K.-L. Ledeganckstraat 35, B-9000 Ghent, Belgium

[4] Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, AP 565-A Cuernavaca, Morelos, Mexico

Fifty-eight new isolates were obtained from root nodules of common bean (*Phaseolus vulgaris*) cultivated in soils originating from different agroecological areas in Senegal and Gambia (West Africa). A polyphasic approach including both phenotypic and genotypic techniques was used to study the diversity of the 58 *Rhizobium* isolates and to determine their taxonomic relationships with reference strains. All the techniques performed, analysis of multilocus enzyme electrophoretic patterns, SDS-PAGE profiles of total cell proteins, PCR-RFLP analysis of the genes encoding 16S rRNA and of the 16S–23S RNA intergenic spacer region (ITS-PCR-RFLP), auxanographic tests using API galleries and nodulation tests lead to the consensus conclusion that the new rhizobial isolates formed two main distinct groups, I and II, belonging to *Rhizobium tropici* type B and *Rhizobium etli*, respectively. By MLEE *R. etli* and group II strains showed several related electrophoretic types, evidencing some extent of internal heterogeneity among them. This heterogeneity was confirmed by other techniques (ITS-PCR-RFLP, SDS-PAGE and host-plant-specificity) with the same nine distinct strains of group II showing some differences from the core of group II (54 strains).

Keywords: *Rhizobium*, root nodules, common bean (*Phaseolus vulgaris*)

INTRODUCTION

During recent years the classification of rhizobia that nodulate common bean (*Phaseolus vulgaris*) plants has been progressively revised as more rhizobial diversity is gradually discovered in different parts of the world (Martínez-Romero *et al*., 1991; Segovia *et al*., 1993; Amarger *et al*., 1997). These rhizobia were initially assigned to *Rhizobium leguminosarum* bv. phaseoli on the basis of their host specificity, separate from *R. leguminosarum* bv. viciae and *R. leguminosarum* bv. trifolii, symbionts of peas (*Pisum* spp., *Vicia* spp.) and clovers (*Trifolium* spp.) respectively (Jordan, 1984). This subdivision was also supported by their different symbiotic plasmids, encoding the nodulation specificities (Martínez *et al*., 1985, 1988). *R. leguminosarum* bv. phaseoli was long recognized to be taxonomically heterogeneous as evidenced by protein profile analysis (Roberts *et al*., 1980), multilocus enzyme electrophoresis (MLEE) (Piñero *et al*., 1988; Eardly *et al*., 1995), DNA relatedness analysis (Laguerre *et al*., 1993; van Berkum *et al*., 1996) and 16S rRNA genes (rDNA) sequencing (Segovia *et al*., 1993; Laguerre *et al*., 1993; Hernandez-Lucas *et al*., 1995). *R. leguminosarum* bv. phaseoli from Mexico and South America was first divided into *R. leguminosarum* bv. phaseoli type I and type II (Martínez *et al*., 1988). *Rhizobium tropici* types A and B were first proposed for type II strains carrying a single *nifH* gene copy. *R. tropici* types A and B are distinguished by their DNA–DNA hybridization values, a number of phenotypic characteristics and the presence of a specific megaplasmid (Martínez-Romero *et al*., 1991; Geniaux *et al*., 1995). *Rhizobium etli* was then proposed for *R. leguminosarum* bv. phaseoli type I strains which contain multiple copies of the nitrogenase reductase gene (*nifH*) on their symbiotic plasmids (Martínez *et al*., 1985; Segovia *et al*., 1993). More recently, two additional groups of *Rhizobium* strains nodulating bean plants were characterized in European soils and proposed as two new species,

Abbreviations: ET, electrophoretic type; ITS, internal transcribed spacer; MLEE, multilocus enzyme electrophoresis.

Rhizobium gallicum and *Rhizobium giardinii* (Amarger *et al*., 1997). *Phaseolus vulgaris* is commonly reported to originate from America whereas its symbiotic rhizobial partners are thought to be diverse and widely spread around the world. Native rhizobia nodulating common bean in African soils have been reported to be taxonomically related to *R. tropici* in East and South Africa (Anyango *et al*., 1995; Dagutat & Steyn, 1995) and to *R. tropici* and *R. etli* in Central Africa (Tjahjoleksono, 1993).

In this paper, we studied the diversity and taxonomic relationships among 58 rhizobial strains originating from different agroecological areas in Senegal and Gambia. Using techniques with various discriminative powers such as physiological and auxanographic tests, host specificity, MLEE, analysis of total cell protein profiles by SDS-PAGE, RFLP analysis of PCR-amplified ITS (internal transcribed spacer) 16S–23S and 16S rDNA we showed that, in West Africa, *Phaseolus vulgaris* is naturally nodulated by *Rhizobium* strains belonging to *R. tropici* type B and *R. etli*.

METHODS

Bacterial strains. Rhizobial strains (Table 1) were isolated from root nodules of common bean cultivated during 15–20 d either in field conditions or in pots containing soils sampled at 5–20 cm depth in different places in Senegal and Gambia.

These rhizobial strains were compared to several reference strains representing the different *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Agrobacterium* species or groups.

Growth and culture conditions. New isolates and reference *Rhizobium*, *Sinorhizobium* and *Mesorhizobium* strains were maintained on yeast mannitol agar (YMA), containing (g l^{-1}): mannitol, 10; sodium glutamate, 0·5; K_2HPO_4, 0·5; $MgSO_4.7H_2O$, 0·2; NaCl, 0·05; $CaCl_2$, 0·04; $FeCl_3$, 0·004; yeast extract, 1; pH 6·8; agar, 20. *Agrobacterium* strains were maintained on yeast extract peptone-glucose medium, which contained (g l^{-1} of 0·01 M phosphate buffer, pH 7·2): peptone, 5; yeast extract, 1; beef extract, 5; sucrose, 5 and $MgSO_4.7H_2O$, 0·592.

Morphological and physiological tests. Cell dimensions and morphology were determined by phase-contrast microscopy. Growth of strains was also studied on Luria broth (LB) medium containing (g l^{-1}): bacto-tryptone, 50; yeast extract, 5; sodium chloride, 5 and on peptone yeast extract (PY) medium containing (g l^{-1}): peptone, 5; yeast extract, 3; supplemented with 10 ml of sterile solution of $CaCl_2$ 0·7 M. The maximum growth temperature on YM medium was determined for all strains.

PCR/RFLP of 16S–23S rDNA ITS region. Bacterial genomic DNA was extracted and purified as described by Boucher *et al*. (1987). Primers FGPS 1490, corresponding to positions 1521–1541 of *Escherichia coli* (Navarro *et al*., 1992), and FGPL 132′, corresponding to positions 114–132 of *E. coli* (Ponsonnet & Nesme, 1994), were used for PCR amplification. PCR was carried out in a 100 µl reaction volume by mixing 2 µl DNA extract with the polymerase reaction buffer (10 mM Tris/HCl, pH 8·3; 50 mM KCl, 0·0.1% gelatin, 2 mM $MgCl_2$); *Taq* polymerase (Bioprobe), 0·8 µl (5 U/reaction); 2 µl each of the 10 µM dNTPs (dATP, dCTP, dGTP, dTTP), 1 µl each primer (0·05 µM). PCR amplification was performed in a Gene Amp PCR System 2400 thermal cycler adjusted to the following temperature profile: initial denaturation at 94 °C for 5 min; 35 amplification cycles (denaturation at 94 °C for 1 min, hybridization of primers at 55 °C for 1 min and extension at 72 °C for 1·5 min); final extension at 72 °C for 5 min. Amplification was checked by horizontal agarose (1%, w/v) gel electrophoresis using 10 µl of the PCR product.

Aliquots (8 µl) of PCR products were digested with the following restriction endonucleases (5 U/reaction): *Alu*I, *Dde*I, *Hha*I, *Pal*I (Pharmacia), *Hinf*I, *Msp*I (Gibco-BRL), *Rsa*I and *Taq*I (Boehringer Mannheim). Restricted DNA was analysed by horizontal agarose (NuSieve 3:1, FMC 3%, w/v) gel electrophoresis. Electrophoresis was carried out at 60 V for 4 h and gels were stained as described above and photographed under UV illumination with Polaroid 665 positive/negative film.

MLEE. Isolates and reference strains were grown overnight on an orbital shaker incubator at 30 °C in 30 ml PY medium. Cells were harvested by centrifugation at 6000 ***g*** for 10 min at 4 °C. After suspension in 0·2 ml $MgSO_4$ 10 mM, the bacteria were lysed by adding 40 µl of a lysozyme solution (10 mg ml^{-1} $MgSO_4$ 10 mM). The lysate maintained on ice was then placed for 15 min at room temperature and stored at −70 °C for 10 min before use.

Techniques of starch gel electrophoresis and selective staining of enzymes were performed as described by Selander *et al*. (1986). The electrophoretic buffer system, Tris-citrate (pH 8·0) was used for the following enzymes assayed: malate dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, glutamate dehydrogenase, xanthine dehydrogenase, phosphoglucomutase, esterases, aconitase, indophenol oxidase (super oxide dismutase), hexokinase and alanine dehydrogenase.

Distinctive mobility variants (electromorphs) of each enzyme, numbered in order of decreasing anodal mobility, were equated with alleles at the corresponding gene locus, and electromorph profiles for the 12 enzymes (electrophoretic types, ETs) were considered to be multilocus genotypes (Table 2).

The genetic diversity (h) for an enzyme locus was calculated from allele frequencies for ETs as $h = 1-\Sigma x_i^2\ (n/(n-1))$ where x_i is the frequency of the ith allele and n is the number of ETs. The mean genetic diversity per locus (H) is the arithmetic mean of h values for the 12 loci (Selander *et al*., 1986). The genetic distance between each pair of ETs was estimated; clustering at a matrix of pairwise genetic distances was performed by the unweighted pair group method with averages of Nei & Li (1979).

PAGE of total bacterial proteins (SDS-PAGE). All strains were grown at 28 °C for 48 h in Roux flasks on tryptone yeast extract (TY) medium containing (g l^{-1}): tryptone extract, 5; yeast extract, 0·75; KH_2PO_4, 0·454; $Na_2HPO_4.12H_2O$, 2·388; $CaCl_2.6H_2O$, 1; agar (Lab M), 20; pH 6·8–7. Whole-cell protein extracts were prepared from 80 mg cells and SDS-PAGE was performed using slight modifications of the Laemmli (1970) procedure, as described previously (Kiredjian *et al*., 1986). The normalized densitometric traces of the protein electrophoretic patterns were grouped by numerical analysis, using the GelCompar software package (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).

Table 1. *Rhizobium* strains ISRA isolated from *Phaseolus vulgaris* and reference strains used in this study

Abbreviations: CIAT, *Rhizobium* Collection, Centro International de Agricultura Tropical, Cali, Columbia; CIFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, AP 565-A Cuernavaca, Morelos, Mexico; ISRA, Institut Sénégalais de la Recherche Agricole, Centre ISRA-ORSTOM, BP 1386, Dakar, Senegal; BCCM/LMG™, Bacteria Collection, Laboratorium voor Microbiologie, K.-L. Ledeganckstraat 35, B9000 Gent, Belgium; ORSTOM, Institut Français de Recherche Scientifique pour le Développement en Coopération, BP 1386, Dakar, Senegal; USDA, US Department of Agriculture, Beltsville, MD, USA; Sn, Senegal.

Strain	Host plant or origin	Geographical origin	Source or reference
New isolates*			
Group I			
ISRA 350	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 352	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 354	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 554	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
Group II			
Subgroup II.1			
ISRA 30	*Phaseolus vulgaris*	Niayes, Sn	This work
ISRA 319	*Phaseolus vulgaris*	Niayes, Sn	This work
ISRA 351	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 353	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 355	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 356	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 357	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 361	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 362	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 363	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 364	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 365	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 553	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 555	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 556	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 557	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 558	*Phaseolus vulgaris*	Sylvopastorale, Sn	This work
ISRA 559	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 561	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 562	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 563	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 564	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 566	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 567	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 568	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 569	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 570	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 571	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 572	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 573	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 574	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 575	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 576	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 577	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 578	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 579	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 580	*Phaseolus vulgaris*	Gambia	This work

Table 1 (*cont.*)

Strain	Host plant or origin	Geographical origin	Source or reference
ISRA 581	*Phaseolus vulgaris*	Gambia	This work
ISRA 582	*Phaseolus vulgaris*	Gambia	This work
ISRA 583	*Phaseolus vulgaris*	Gambia	This work
ISRA 584	*Phaseolus vulgaris*	Gambia	This work
ISRA 585	*Phaseolus vulgaris*	Gambia	This work
ISRA 586	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 587	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
ISRA 588	*Phaseolus vulgaris*	Bassin arachidier, Sn	This work
Subgroup II.2			
ISRA 21	*Phaseolus vulgaris*	Casamance, Sn	This work
ISRA 77	*Phaseolus vulgaris*	Casamance, Sn	This work
ISRA 78	*Phaseolus vulgaris*	Casamance, Sn	This work
ISRA 560	*Phaseolus vulgaris*	Fleuve, Sn	This work
ISRA 565	*Phaseolus vulgaris*	Fleuve, Sn	This work
Subgroup II.3			
ISRA 27	*Phaseolus vulgaris*	Niayes, Sn	This work
ISRA 59	*Phaseolus vulgaris*	Niayes, Sn	This work
ISRA 61	*Phaseolus vulgaris*	Niayes, Sn	This work
ISRA 69	*Phaseolus vulgaris*	Niayes, Sn	This work
Reference strains			
Rhizobium tropici			
Type A			
CFN 299^T	*Phaseolus vulgaris*	Brazil	Martinez-Romero *et al.* (1991)
LMG 9502	*Phaseolus vulgaris*		LMG
Type B			
CIAT 899^T	*Phaseolus vulgaris*	Colombia	Martinez-Romero *et al.* (1991)
LMG 9519	*Phaseolus vulgaris*		LMG
LMG 9518	*Phaseolus vulgaris*		LMG
Rhizobium etli			
CFN 42^T	*Phaseolus vulgaris*	Mexico	Segovia *et al.* (1993)
BRA 5	*Phaseolus vulgaris*	Brazil	CIFN
F6	*Phaseolus vulgaris*	Mexico	CIFN
F8	*Phaseolus vulgaris*	Mexico	CIFN
F16	*Phaseolus vulgaris*	Mexico	CIFN
Viking 1	*Phaseolus vulgaris*		Segovia *et al.* (1993)
USDA 9041	*Phaseolus vulgaris*		
USDA 2667	*Phaseolus vulgaris*		
Rhizobium leguminosarum			
biovar phaseoli			
LMG 8820	*Phaseolus vulgaris*		LMG
biovar viciae			
USDA 2370	*Pisum sativum*		CIFN
biovar trifolii			
LMG 8819	*Trifolium pratense*		LMG
Rhizobium leguminosarum			
LMG 9505			LMG
LMG 6122	*Trifolium repens*	Australia	LMG
LMG 9504			LMG
316C10A			

Table 1 (*cont.*)

Strain	Host plant or origin	Geographical origin	Source or reference
Rhizobium galegae			
LMG 6214^T	*Galega orientalis*	Finland	LMG
LMG 6215	*Galega orientalis*	USSR	LMG
Rhizobium sp.			
ORS 240	*Pterocarpus lucens*	Senegal	ORSTOM
ORS 1181	*Acacia senegal*	Senegal	ORSTOM
ORS 248	*Pterocarpus lucens*	Senegal	ORSTOM
Mesorhizobium plurifarium			
ORS 1001	*Acacia senegal*	Senegal	de Lajudie *et al*. (1998)
ORS 1004	*Acacia senegal*	Senegal	de Lajudie *et al*. (1998)
ORS 1002	*Acacia senegal*	Senegal	de Lajudie *et al*. (1998)
Mesorhizobium loti			
LMG 6125^T	*Lotus corniculatus*	New Zealand	LMG
Sinorhizobium saheli			
ORS 609^T	*Sesbania canabina*	Senegal	de Lajudie *et al*. (1994)
ORS 600	*Sesbania pachycarpa*	Senegal	de Lajudie *et al*. (1994)
Sinorhizobium terangae			
ORS 51	*Sesbania rostrata*	Senegal	de Lajudie *et al*. (1994)
ORS 15	*Sesbania* sp.	Senegal	de Lajudie *et al*. (1994)
ORS 1009^T	*Acacia laeta*	Senegal	de Lajudie *et al*. (1994)
ORS 1016	*Acacia laeta*	Senegal	de Lajudie *et al*. (1994)
LMG 6463	*Sesbania rostrata*	Senegal	de Lajudie *et al*. (1994)
Sinorhizobium meliloti			
LMG 6133^T	*Medicago sativa*	Virginia, USA	LMG
LMG 6130	*Medicago sativa*	Australia	LMG
Agrobacterium biovar 2			
LMG 150^T			
Agrobacterium rubi			
LMG 156^T	*Rubus ursinus*	USA, 1942	
Agrobacterium biovar 1			
LMG 196	crown gall	USA	Kersters *et al*. (1973)
Agrobacterium vitis			
LMG 257	*Vitis vinifera*	Crete, Greece	

* Grouping according to ITS-PCR-RFLP.

PCR/RFLP of 16S rRNA genes. A bacterial colony was homogenized vigorously into 100 µl of 0·1% Tween 20 solution. The cell suspension was heated at 95–100 °C for 10 min and was directly used for PCR assay. DNA was amplified by mixing 3 µl of the suspension with the 16S PCR buffer (10 mM Tris/HCl, pH 8·3; 50 mM KCl; 0·01% gelatin), 2 µl each of the 10 mM dNTPs (dATP, dCTP, dGTP, dTTP), 1·5 µl 100 mM $MgCl_2$, 1 µl each primer fD1 and rD1 (Weisburg *et al*., 1991) which correspond to positions 8–27 and 1524–1540 respectively, of the *E. coli* 16S rRNA gene and 80·2 µl sterilized water. The mixture was sealed with a thin layer of paraffin oil and heated for 3 min at 95 °C. A 2·5 µl aliquot of diluted *Taq* polymerase in Extender and deionized water (1:1:3, by vol.) was added to the mixture. The PCR amplification was carried out in a 100 µl reaction volume and performed with a thermal reactor HYBAID. The PCR temperature profile was as follows: initial denaturation at 94 °C for 3 min; 35 amplification cycles (denaturation 94 °C for 1 min, primer annealing 55 °C for 1 min and extension 72 °C for 2 min); final extension at 72 °C for 3 min.

An excess of restriction enzymes (5 U/reaction) was used to digest a 10 µl aliquot of PCR product. Restricted DNAs with *Hha*I, *Hinf*I, *Msp*I and *Sau*3AI were analysed by horizontal agarose (3%, w/v) gel electrophoresis carried out at 80 V for 3·5 h. Gels were stained in an aqueous solution of ethidium bromide (1 mg ml^{-1}) and photographed under UV illumination using a Polaroid 55 positive/negative film.

Auxanographic tests. API galleries (API 50CH, API 50AO and API 50AA; bioMérieux) were used to test the assimilation of 147 organic compounds as sole carbon sources (Kersters *et al*., 1984). Inocula were obtained from 36 h YMA slant cultures. After incubation of the galleries for 1, 2, 4 and 7 d at 30 °C, growth of the strains was checked and scored as described previously (Kersters *et al*., 1984). The levels of interstrain similarity (S) were calculated by using a similarity distance coefficient derived from the Canberra metric coefficient (dCanb) (Sneath & Sokal, 1973) as follows: $S = 100 \times (1 - \mathrm{dCanb})$.

Plant nodulation tests. *Phaseolus vulgaris*, *Vigna unguiculata* and *Glycine max* seeds were surface sterilized successively

Table 2. Allele profiles at 12 enzyme loci forming 13 ETs among reference strains of *R. etli* and *R. tropici* and *Rhizobium* strains isolated from *Phaseolus vulgaris* in West Africa

ET	Strain	Alleles at the following enzyme loci*											
		IDH	MDH	G6P	XDH	HEX	GD2	ACO	ALA	PGM	IPO	EST1	EST2
1	ISRA 27, ISRA 59	7	5	4	6	7	7	7	4	7	5	4	5
2	ISRA 61	7	5	5	6	7	7	7·2	4	7	5	4	5
3	ISRA 69	7	5	4	6	7	7	7·2	4	7	5	3·5	5
4	Groups II.1 & II.2 strains	5	5	5	5	5	5	5	3	5	5	3	5
5	Group I strains	5	7	3	6·5	5	5	5	3	5	7	6	8
6	*R. etli* BRA 5	5	5	5	6	5	5	5	3	5	5	3	5
7	*R. etli* CFN 42^T	5	5	5	5	5	5	5	3	5	5	4·5	5
8	*R. tropici* type A CFN 299^T	5	6	3	6	5	5	5	3	5	3	6	6·5
9	*R. tropici* type B CIAT 899^T	5	7	3	6·5	5	5	5	3	5	5	6	8
10	*R. etli* F 6	5	5	4	6	5	5	5	3	5	5	4	5
11	*R. etli* F 8	7	5	5	6	7	7	7	4	7	5	4	5
12	*R. etli* F 16	7	5	4	5·5	7	7	7	4	7	5	3	5
13	*R. etli* Viking 1	5	5	**3**	6	5	5	5	3	5	5	5·5	5·5

*IDH, Isocitrate dehydrogenase; MDH, malate dehydrogenase; G6P, glucose-6-phosphate dehydrogenase; XDH, xanthine dehydrogenase; HEX, hexokinase; GD2, glutamate dehydrogenase; ACO, aconitase; ALA, alanine dehydrogenase; PGM, phosphoglucomutase; IPO, indophenol oxidase; and EST, esterases.

Table 3. Groupings of ISRA *Rhizobium* strains nodulating *Phaseolus vulgaris* using PCR-RFLP analysis of the 16S–23S RNA intergenic spacer

$^-$, DNA not digested with the corresponding enzyme.

Group or subgroup of strains	Restriction pattern of amplified ITS gene digested with:							
	*Alu*I	*Dde*I	*Hha*I	*Hinf*I	*Msp*I	*Pal*I	*Rsa*I	*Taq*I
Group I	A1	D1	Hh1	Hi$^-$	M1	P1	R1	T1
Subgroup II.1	A2	D2	Hh2	Hi$^-$	M2	P2	R$^-$	T2
Subgroup II.2	A2	D2	Hh2	Hi3	M2	P2	R$^-$	T2
Subgroup II.3	A4	D4	Hh4	Hi$^-$	M4	P4	R$^-$	T4

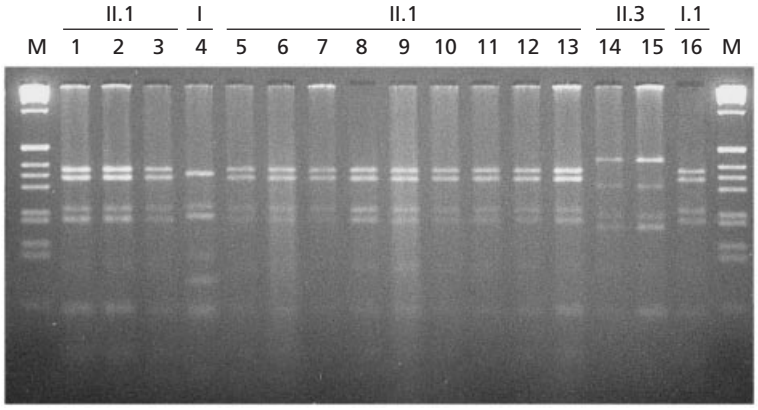

Fig. 1. Restriction patterns of PCR-amplified fragment of 16S–23S rDNA digested with *Taq*I. Lanes: M, molecular mass marker; (subgroup II.1) 1, ISRA 351; 2, ISRA 355; 3, ISRA 553; 5, ISRA 556; 6, ISRA 558; 7, ISRA 562; 8, ISRA 563; 9, ISRA 567; 10, ISRA 570; 11, ISRA 576; 12, ISRA 586; 13, ISRA 77; 16, ISRA 319; Group I; 4, ISRA 554; (subgroup II.3) 14, ISRA 27; 15, ISRA 59.

with 95 % (v/v) ethanol for 3 min and 0·1 % $HgCl_2$ for 3 min followed by several washes in sterile distilled water. *Acacia senegal*, *Leucaena leucocephala*, *Acacia tortilis* subsp. *raddiana*, *Faidherbia albida*, *Sesbania rostrata* and *Acacia seyal* seeds were scarified and surface-sterilized with concentrated sulfuric acid for 14, 20, 25, 30, 45 and 60 min, respectively. After treatment, the seeds were washed several times with water to eliminate any trace of acid and $HgCl_2$. The seeds were first allowed to germinate in sterile Petri dishes containing 0·8 % water agar for 24–48 h, then

Table 4. Number of alleles and genetic diversity (*h*) at the loci encoding the 12 enzymes in ETs observed in the *Rhizobium* strains ISRA isolated from *Phaseolus vulgaris* and the reference strains

$h = 1-\Sigma x_i^2(n/(n-1))$ where x_i is the frequency of the ith allele and n is the number of ETs.

Enzyme locus*	Thirteen ETs†		Four ETs‡	
	No. alleles	*h*	No. alleles	*h*
IDH	2	0·513	2	0·499
MDH	3	0·011	1	0·000
G6P	3	0·717	2	0·667
XDH	3	0·616	2	0·499
HEX	2	0·513	2	0·499
GD2	2	0·513	2	0·499
ACO	3	0·590	3	0·832
ALA	2	0·513	2	0·499
PGM	2	0·513	2	0·499
IPO	3	0·295	1	0·000
EST1	5	0·846	3	0·832
EST2	4	0·527	1	0·000
Mean	**2·83**	**0·547**	**1·91**	**0·444**

* For abbreviations of enzyme loci see the footnote to Table 2.

† The 13 ETs of ISRA and reference strains.

‡ The four ETs of ISRA *R. etli* strains.

transferred to tubes containing Jensen slant agar (Vincent, 1970) for root nodulation tests (6–8 plants were routinely tested with each strain). All 58 new isolates were tested for their ability to nodulate these plants.

RESULTS AND DISCUSSION

Collection of strains

Fifty-eight rhizobial isolates (Table 1) were purified from root nodules of common bean and included in the Senegalese Institute of Agricultural Research (ISRA)/Microbiological Resources Center (MIRCEN) culture collection. These isolates originate from different geographical and ecological areas in Senegal and Gambia, including fields in the Niayes zone where common bean is mainly cultivated in Senegal, and were obtained either by direct isolation from naturally occurring nodules or by trapping *Rhizobium* on sterile young plants inoculated with collected soil samples.

Morphological and physiological characteristics

All isolates were fast growers and, based on their morphological and physiological characteristics, we defined two groups (I and II) among them. In YM liquid medium, strains ISRA 350, 352, 354 and 554 (forming the so-called group I) grew at temperatures up to 44 °C whereas the maximum growth temperature of the 54 other isolates (forming the so-called group II) was 40 °C. On PY medium, the texture of colonies of group I strains was creamy whereas colonies of group II had a gummy texture. Strains of group I were able to grow on LB medium whereas strains of group II could not grow on this medium. These morphological and physiological characteristics of group I strains such as the ability to grow on LB medium and colony morphology on PY medium as well as their maximal growth temperature matched characteristics reported for *R. tropici* (Martínez-Romero *et al.*, 1991). On the other hand, group II strains shared characteristics of *R. etli* (Segovia *et al.*, 1993).

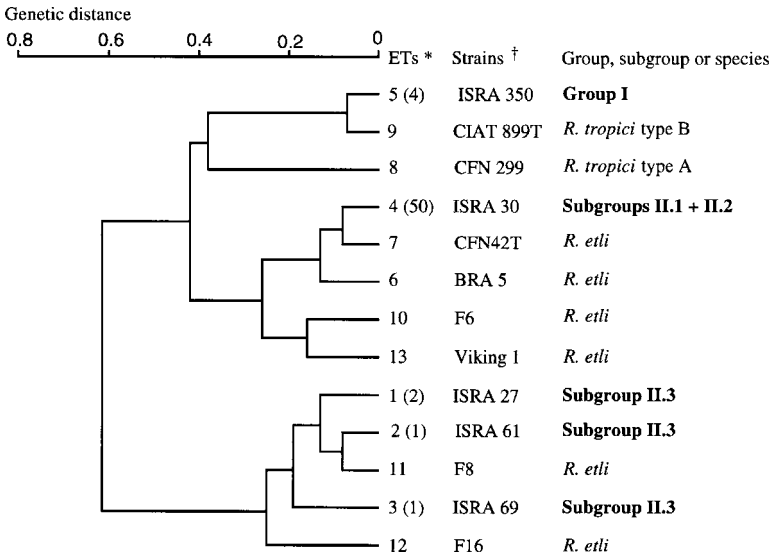

Fig. 2. Similarity among 13 ETs of ISRA *Rhizobium* strains isolated from common bean (*Phaseolus vulgaris*) and reference strains based on electrophoretically detectable allele variation at 12 enzyme loci. *Values in parentheses correspond to the number of strains in each ET. †Only one strain was represented for each ET.

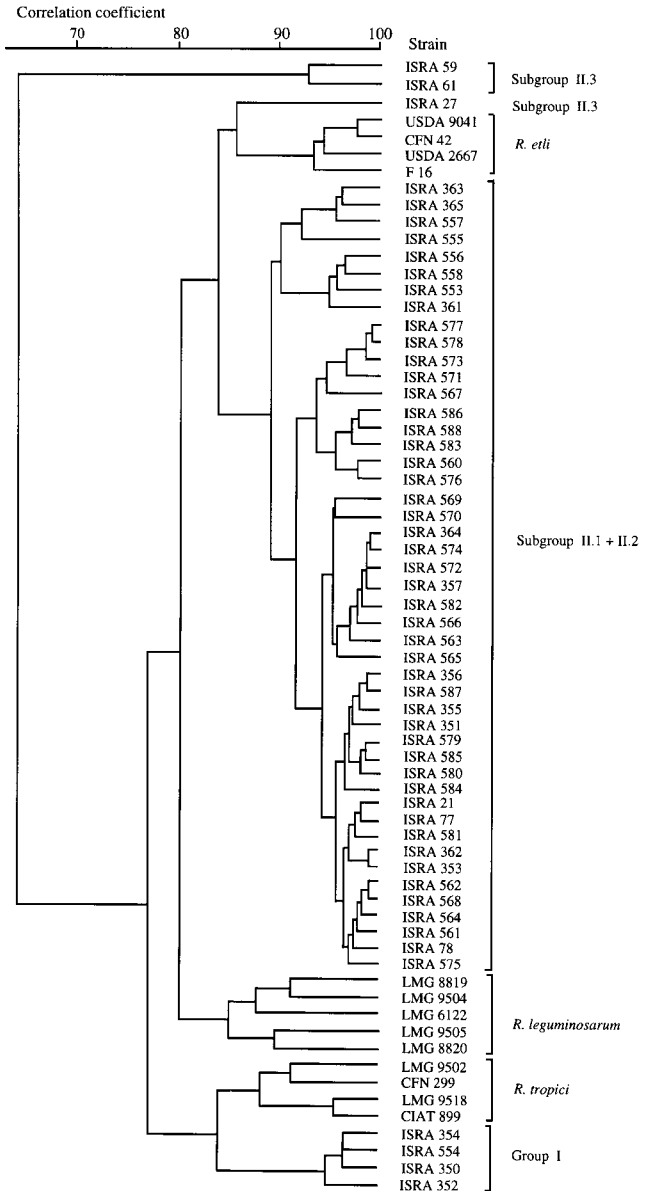

Fig. 3. Dendrogram showing the similarity among the electrophoretic protein patterns (SDS-PAGE) of ISRA and reference strains based on the mean correlation coefficient values which were grouped by the unweighted pair group method with averages.

PCR/RFLP of 16S–23S ITS region

Analysis of the PCR product of the 16S–23S ITS DNA region was reported as a useful method to evidence diversity among bacterial populations even at the intraspecific level (Barry *et al.*, 1991; Jensen *et al.*, 1993; Laguerre *et al.*, 1996). The PCR amplified 16S–23S rDNA intergenic spacer regions of the 58 new isolates were digested with eight restriction enzymes, leading to polymorphic patterns (Table 3, Fig. 1). Four different groups of strains were identified. One corresponded to the four group I strains which shared the same restriction pattern type. Group II strains formed a separate type with three subgroups, one major (subgroup II.1, 45 strains, see Table 1), and two minor (subgroups II.2 and II.3). The only difference between subgroups II.1 and II.2 was that DNA of the former could not be digested by *Hinf*I. More important were the differences exhibited by the four strains of subgroup II.3 (see Table 1) showing a separate profile. *Rsa*I restricted only ITS amplificates of strains from group I.

MLEE

The genetic relationships between the 58 isolates and reference strains of *Rhizobium tropici* and *Rhizobium etli* were further examined by performing MLEE, a technique previously employed in taxonomic studies of rhizobia that nodulate bean plants (Piñero *et al.*, 1988; Martínez-Romero *et al.*, 1991; Segovia *et al.*, 1991; Souza *et al.*, 1994). The twelve enzyme loci analysed were polymorphic with a number of alleles ranging from two to five electromorphs. The mean number of alleles was 2·83 (Table 4). There were 13 distinctive multilocus genotypes or ETs as indicated in Table 2. The electrophoretic mobilities at the twelve enzyme loci showed five ETs among the 58 isolates. The genetic diversity at the enzyme locus for the four ETs of the 54 ISRA strains of group II was 0·44 (Table 4). On the basis of the analysis of the genetic distance, the rhizobial strains nodulating bean in Senegal and Gambia are clustered into two groups. The four strains of group I (ISRA 350, ISRA 352, ISRA 354 and ISRA 554) corresponded to ET 5 and grouped with *R. tropici* type B. Group II strains exhibited four ETs, different from, but all similar to, those of *R. etli*. The majority of group II strains (the 50 strains of subgroup II.1 and II.2) have a single ET 4, highly similar to that of the type strain of *R. etli* CFN 42^T. The four remaining strains of group II (belonging to subgroup II.3) corresponded to ET 1 (ISRA 27 and ISRA 59), ET 2 (ISRA 61) and ET 3 (ISRA 69), all similar to other *R. etli* reference strains (Table 2, Fig. 2). Both group II and *R. etli* reference strains showed ET heterogeneity among them but their ETs all clustered together.

Nour *et al.* (1994) reported good agreement of results obtained by RFLP analysis of the rDNA 16S–23S intergenic spacer and MLEE for rhizobia nodulating chickpea (*Cicer arietinum*). Here both methods confirmed on the whole the distinction of the two main groups I and II among the new isolates, similar to *R. etli* and *R. tropici* type B, but with different subgroupings: three ETs were found among subgroup II.3 of ITS-PCR-RFLP, while two ITS-PCR-RFLP subgroups were distinguished among the single ET 4.

SDS-PAGE of total bacterial proteins

The SDS-PAGE whole-cell protein patterns of 54 of the rhizobial isolates from Senegal were scanned and numerically analysed, together with those of reference strains available in our database and representing *Rhizobium* species nodulating bean plants, *R. tropici*,

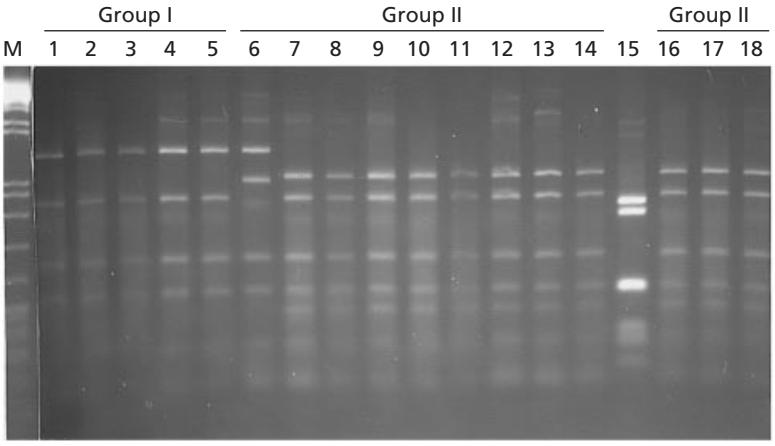

Fig. 4. Restriction patterns of PCR-amplified fragment of 16S rRNA genes digested with *Msp*I. Lanes: M, molecular mass marker; 1, ISRA 350; 2, ISRA 352; 4, ISRA 354; 5, ISRA 554; reference strains 3, *R. tropici* type B CIAT 899; 6, *R. tropici* type A CFN 299; 7, ISRA 27; 8, ISRA 59; 9, ISRA 61; 10, ISRA 69; 12, ISRA 30; 13, ISRA 77; 14, ISRA 353; 16, ISRA 566; 17, ISRA 577; 18, ISRA 584; reference strain 11, *R. etli* CFN 42; 15, *R. leguminosarum* USDA 2370. Group I, restriction pattern types of *R. tropici* type B; group II, restriction pattern types of *R. etli*.

R. etli and *R. leguminosarum*. Bean isolates from Senegal and Gambia essentially grouped in two separate protein gel electrophoretic clusters. The results are presented as a similarity dendrogram in Fig. 3. The four isolates of group I clustered together at a mean correlation coefficient (r) of 94%. The majority of group II strains formed a second cluster at a mean correlation coefficient of 89%. Group I and group II were respectively related to reference strains of *R. tropici* and *R. etli* at a moderate mean coefficient correlation of 83·5%. Strain ISRA 27 (belonging to subgroup II.3) grouped separately from the core of other isolates of group II, closer to *R. etli*. Two other strains of subgroup II.3, ISRA 59 and ISRA 61, grouped together, outside the big groups I and II. The fourth strain of subgroup II.3, ISRA 69, was not included in this study.

PCR-RFLP of 16S rRNA genes

PCR-RFLP analysis of 16S rRNA coding gene has been reported to be useful in *Rhizobium* taxonomy since results obtained are in good agreement with those from sequence analysis of the 16S rRNA coding gene and DNA–DNA hybridization (Laguerre *et al*., 1993). Four restriction endonucleases *Hha*I, *Hinf*I, *Msp*I and *Sau*3AI were used to digest 16S rRNA gene PCR amplificates of a selection of 29 Senegalese isolates, including all strains of group I, subgroup II.2 and II.3 (Table 1), and 16 representative strains of subgroup II.1 (ISRA 30, ISRA 353, ISRA 355, ISRA 580, ISRA 576, ISRA 584, ISRA 555, ISRA 577, ISRA 562, ISRA 566, ISRA 567, ISRA 570, ISRA 571, ISRA 578, ISRA 581, ISRA 584). The restriction patterns generated by *Hha*I, *Hinf*I and *Sau*3AI were similar for all isolates, but two distinct restriction patterns were detected with *Msp*I among our isolates (Fig. 4): the first was exhibited by group I strains and *R. tropici* CIAT 899^T; the second pattern was exhibited by group II strains and *R. etli* CFN 42^T. *R. tropici* strains, genomically described by sequence of their 16S rRNA gene, DNA–DNA hybridization and rDNA organization, were separated into two subgroups, namely type A and type B (Martínez-Romero *et al*., 1991; Geniaux *et al*., 1993). Restriction patterns of *R. tropici* type A and of *R. tropici* type B were different, and the pattern of group I rhizobial strains was related to that of the latter.

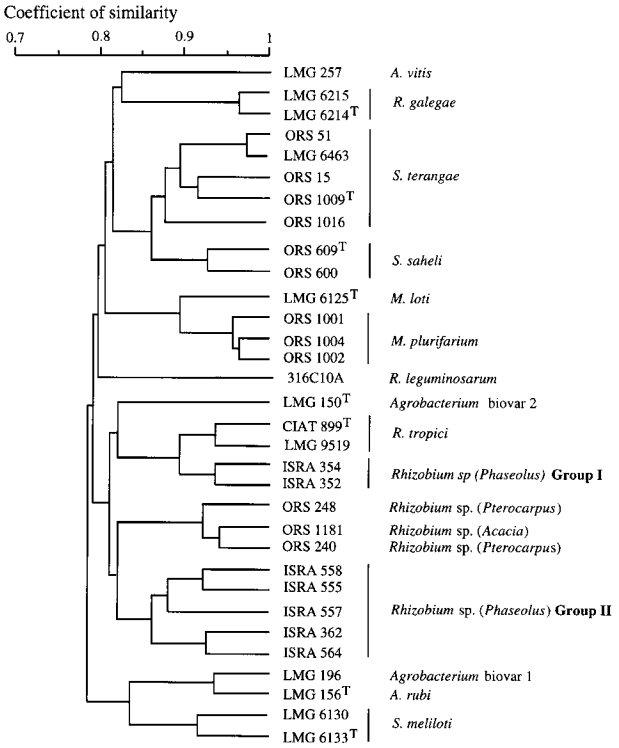

Fig. 5. Dendrogram showing the differences of auxanographic characteristics among ISRA strains and reference strains, obtained from an unweighted pair group method with averages cluster analysis of Canberra metric similarity coefficients based on 147 auxanographic characteristics.

Analysis of auxanographic results

Only the first acquired isolates were tested for assimilation of 147 organic compounds as sole carbon sources, using the API 50 system. Group I was represented by strains ISRA 352, ISRA 354 and group

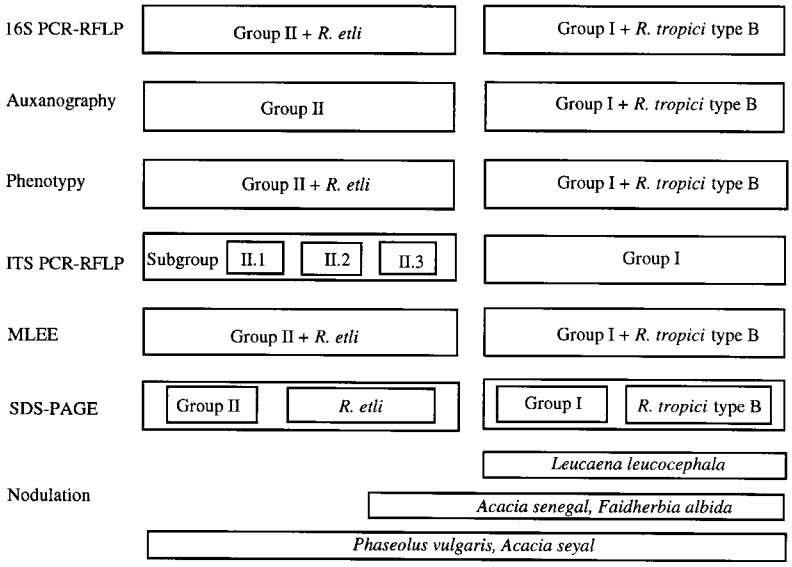

Fig. 6. Diagram summarizing results of polyphasic characterization of bean-nodulating rhizobia in Senegal and Gambia.

II was represented by ISRA 555, ISRA 557, ISRA 558, ISRA 564 and ISRA 362. The reproducibility of the test was good. The mean interstrain similarity values for strains tested in duplicate were between 88 and 92%. Results for representative strains of several *Rhizobium* species and related groups (including representatives of the *Agrobacterium* biovars, but not *R. etli*) were available in the database of our research group and were included in the numerical analysis. The results are shown as a limited dendrogram in Fig. 5. Group I formed a common cluster with *R. tropici* (similarity coefficient of 89%). At a similarity coefficient of 86%, cluster II could be distinguished as a separate cluster.

Host specificity

Bean nodulating rhizobia have been reported to have a broad host range (Hernandez-Lucas *et al.*, 1995; Geniaux *et al.*, 1993; Amarger *et al.*, 1997). The 58 new nodule isolates could all nodulate their original host plant, *Phaseolus vulgaris*, and *Acacia seyal* whereas none of them nodulated *Glycine max* and *Sesbania rostrata*. Some differences in host ranges were found among the isolates. Only strains of group I were able to effectively nodulate *Leucaena leucocephala*, similarly to *R. tropici* (Martínez-Romero *et al.*, 1991). The four strains of group I and the four strains of subgroup II.1 (ISRA 27, ISRA 59, ISRA 61 and ISRA 69) nodulated *Faidherbia albida* and *Acacia senegal*.

By all the criteria tested and results summarized in Fig. 6, group I African isolates were *R. tropici* type B strains while group II corresponded to *R. etli*.

Population diversity studies of bean nodulating rhizobia from areas where this plant is native and extensively grown have shown a large genetic diversity. This has been observed both for *R. tropici* from South American soils (Martínez-Romero *et al.*, 1991) and for *R. etli* in Mesoamerica (Piñero *et al.*, 1988; Segovia *et al.*, 1991; Eardly *et al.*, 1995). For both species a large number of ETs have been obtained with *H* values (diversity index) of 0·363 for *R. tropici* type B strains and larger than 0·5 for *R. etli*. In comparison we report here only one ET for the *R. tropici* strains in contrast to the 18 ETs for the type B *R. tropici* strains analysed previously (Martínez-Romero *et al.*, 1991). Similarly, only four ETs were obtained for our *R. etli* strains while a maximum of 70 ETs was reported in *R. etli* isolated from a limited geographical area in Mexico (Caballero-Mellado & Martínez-Romero, 1999). Also indicative of the restricted genetic diversity of the bean rhizobia population in Africa is the large dominance of a single genotype: 50 strains have a single ET. The limited genetic diversity encountered in Senegal and Gambia may be related to the fact that bean is an introduced crop in Africa. It is remarkable that in West, East and South Africa, bean is naturally nodulated by the same species of rhizobia as those nodulating bean at its site of origin. It has been supposed that bean symbiotic rhizobia are cosmopolitan. An alternative hypothesis was suggested by Sessitsch *et al.* (1997) to explain the presence of *R. etli* in Austrian soil. They proposed that the rhizobia was co-introduced with bean seeds. The presence of *R. etli* on *Phaseolus vulgaris* seeds was demonstrated, and the rhizobia remain viable on the seeds for years (Pérez-Ramírez *et al.*, 1998). We may suppose that *R. tropici* could be also transported on bean seeds harvested from South America where *R. tropici* is native. The existence of *Rhizobium* on seeds may explain the presence of *R. etli* in Africa and the limited genetic diversity encountered may be related to the founder principle, with only a few clones (founders) migrating and surviving at the introduced place.

Africa has been proposed to be the site of origin of the *Leguminosae* family (Raven & Polhill, 1981), and as such a large diversity of legume species and their symbionts exist. If the symbionts of *Phaseolus vulgaris*

were originally from Africa, we should recover the largest diversity there, but that does not seem to be the case. It seems more probable that bean symbionts co-evolved with their host at the site where they diverged and prospered.

ACKNOWLEDGEMENTS

We thank bioMérieux, Montalieu-Vercieu, France, for supplying API galleries. This work was partially supported by UNESCO grant SC/RP/206.575.6. We are grateful to J. Bakhoum, ORSTOM, Senegal, and M. A. Rogel, CIFN/UNAM, Mexico, for technical assistance. M. G. is indebted to the Fund for Scientific Research-Flanders (Belgium), for research and personnel 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.

Anyango, B., Wilson, K. J., Beynon, J. L. & Giller, K. E. (1995). Diversity of *Rhizobium* nodulating *Phaseolus vulgaris* L. in two Kenyan soils with contrasting pHs. *Appl Environ Microbiol* **61**, 4016–4021.

Barry, T., Colleran, G., Glennon, M., Ducican, L. K. & Gannon, F. (1991). The 16S/23S ribosomal spacer region as a target for DNA probes identity eubacteria. *PCR Methods Applic* **1**, 51–56.

van Berkum, P., Beyene, B. & Eardly, B. D. (1996). Phylogenetic relationships among *Rhizobium* species nodulating the common bean (*Phaseolus vulgaris* L.). *Int J Syst Bacteriol* **46**, 240–244.

Boucher, C. A., Van Gijsegem, F., Barberis, P., Arlat, A. M. & Zischek, C. (1987). *Pseudomonas solanacearum* genes controlling both pathogenicity on tomato and hypersensitivity on tobacco are clustered. *J Bacteriol* **169**, 5626–5632.

Caballero-Mellado, J. & Martínez-Romero, E. (1999). Soil fertilization limits the genetic diversity of *Rhizobium* in bean nodules. *Symbiosis* **26**, 111–121.

Dagutat, H. & Steyn, P. L. (1995). Taxonomy and distribution of rhizobia indigenous to South African soils. In *Nitrogen Fixation: Fundamentals and Applications*, pp. 683–686. Edited by I. A. Tikhonovich, N. A. Provorov, V. I. Romanov & W. E. Newton. Boston: Kluwer.

Eardly, B. D., Wang, F., Whittam, T. S. & Selander, R. K. (1995). Species limits in *Rhizobium* populations that nodulate the common bean (*Phaseolus vulgaris*). *Appl Environ Microbiol* **61**, 507–512.

Geniaux, E., Laguerre, G. & Amarger, N. (1993). Comparison of geographically distant populations of *Rhizobium* isolated from root nodules of *Phaseolus vulgaris*. *Mol Ecol* **2**, 295–302.

Geniaux, E., Flores, M., Palacios, R. & Martínez, E. (1995). Presence of megaplasmids in *Rhizobium tropici* and further evidence of differences between the two *Rhizobium tropici* subtypes. *Int J Syst Bacteriol* **45**, 392–394.

Hernandez-Lucas, I., Segovia, L., Martínez-Romero, E. & Pueppke, S. G. (1995). Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. *Appl Environ Microbiol* **61**, 2775–2779.

Jensen, M. A., Webster, J. A. & Straus, N. (1993). Rapid identification of bacteria on the basis of polymerase chain reaction-amplification ribosomal DNA spacer polymorphisms. *Appl Environ Microbiol* **59**, 945–952.

Jordan, D. C. (1984). Family III *Rhizobiaceae* Conn 1938. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 234–245. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.

Kersters, K., De Ley, J., Sneath, P. H. A. & Sackin, M. (1973). Numerical taxonomic analysis of *Agrobacterium*. *J Gen Microbiol* **78**, 227–239.

Kersters, K., Hinz, K. H., Hertle, A., Segers, P., Lievens, A., Siegmann, O. & De Ley, J. (1984). *Bordetella avium* sp. nov., isolated from the respiratory tracts of turkeys and other birds. *Int J Syst Bacteriol* **34**, 56–70.

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

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

Laguerre, G., Fernandez, M. P., Edel, V., Normand, P. & Amarger, N. (1993). Genomic heterogeneity among French *Rhizobium* strains isolated from *Phaseolus vulgaris* L. *Int J Syst Bacteriol* **43**, 761–767.

Laguerre, G., Mavingui, P., Allard, M. R., Charnay, M. P., Louvrier, P., Mazurier, S. I., Rigottier-Gois, L. & Amarger, N. (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl Environ Microbiol* **62**, 2029–2036.

de Lajudie, P., Willems, A., Pot, B. & 7 other authors (1994). Polyphasic taxonomy of rhizobia: Emendation of the genus *Sinorhizobium* and description of *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.

Martínez, E., Pardo, M. A., Palacios, R. & Cevallos, M. A. (1985). Reiteration of nitrogen fixation gene sequences and specificity of *Rhizobium* in nodulation and nitrogen fixation in *Phaseolus vulgaris*. *J Gen Microbiol* **131**, 1779–1786.

Martínez, E., Flores, M., Brom, S., Romero, D., Davila, G. & Palacios, R. (1988). *Rhizobium phaseoli*, a molecular genetic view. *Plant Soil* **108**, 179–184.

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.

Navarro, E., Simonet, P., Normand, P. & Bardin, R. (1992). Characterisation of natural populations of *Nitrobacter* spp. using PCR/RFLP analysis of the ribosomal intergenic spacer. *Arch Microbiol* **157**, 107–115.

Nei, M. & Li, W. H. (1979). Mathematical model for studying genetic variations in terms of restriction endonucleases. *Proc Natl Acad Sci USA* **76**, 5269–5273.

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.

Pérez-Ramírez, N. O., Rogel, M. A., Wang, E., Castellanos, J. Z. & Martínez-Romero, E. (1998). Seeds of *Phaseolus vulgaris* bean carry *Rhizobium etli*. *FEMS Microbiol Ecol* **26**, 289–296.

Piñero, D., Martínez, E. & Selander, R. K. (1988). Genetic diversity and relationships among isolates of *Rhizobium leguminosarum* biovar phaseoli. *Appl Environ Microbiol* **54**, 2825–2832.

Ponsonnet, C. & Nesme, X. (1994). Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. *Arch Microbiol* **161**, 300–309.

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 proteins fingerprints. In *Modern Microbial Methods. Chemical Methods in Prokaryotic Systematics*, pp. 493–521. Edited by M. Goodfellow & A. G. O'Donnell. Chichester: Wiley.

Raven, P. H. & Polhill, R. M. (1981). Biogeography of the *Leguminosae*. In *Advances in Legume Systematics VI*, pp. 27–34. Edited by R. M. Polhill & P. H. Raven. Kew: Royal Botanic Gardens.

Roberts, G. P., Leps, W. T., Selver, L. E. & Brill, W. J. (1980). Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. *Appl Environ Microbiol* **39**, 414–422.

Segovia, L., Piñero, D., Palacios, R. & Martínez-Romero, E. (1991). Genetic structure of a soil population of nonsymbiotic *Rhizobium leguminosarum*. *Appl Environ Microbiol* **57**, 426–433.

Segovia, L., Young, J. P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strain 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.

Sessitsch, A., Hardarson, G., Akkermans, A. D. & de Vos, W. M. (1997). Characterization of *Rhizobium etli* and other *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. in an Austrian soil. *Mol Ecol* **6**, 601–608.

Sneath, P. H. A. & Sokal, R. R. (1973). *Numerical Taxonomy. The Principles and Practice of Numerical Classification*. San Francisco: Freeman.

Souza, V., Eguiarte Lavila, G., Cappello, R., Gallardo, C., Montoya, J. & Piñero, D. (1994). Genetic structure of *Rhizobium etli* biovar phaseoli associated with wild and cultivated bean plants (*Phaseolus vulgaris* and *Phaseolus coccineus*) in Morelos, Mexico. *Appl Environ Microbiol* **60**, 1260–1268.

Tjahjoleksono, A. (1993). *Caractérisation et diversité des souches de Rhizobium nodulant le haricot* (*Phaseolus vulgaris*) *cultivé en trois sites tropicaux*. PhD thesis, Université de Lyon.

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

Vincent, J. M. (1970). *A Manual for Practical Study of Root Nodule Bacteria. IBP Handbook No. 15*. Oxford: Blackwell.

Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697–703.