***Allorhizobium undicola* gen. nov., sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal**

Philippe de Lajudie,^{1,2} Etike Laurent-Fulele,¹ Anne Willems,^{2,3}
Urbain Torck,² Renata Coopman,² Matthew D. Collins,³ Karel Kersters,²
Bernard Dreyfus^{1†} and Monique Gillis²

Author for correspondence: Monique Gillis. Tel: +32 9 264 5117. Fax: +32 9 264 5092/5346.
e-mail: Moniek.Gillis@rug.ac.be

¹ Laboratoire de
Microbiologie des Sols,
ORSTOM BP 1386, Dakar,
Senegal, West Africa

² Laboratorium voor
Microbiologie, Universiteit
Gent, K.-L.
Ledeganckstraat, 35, B-
9000 Ghent, Belgium

³ Microbiology Department,
Reading Laboratory,
Institute of Food Research,
Earley Gate, Whiteknights
Road, Reading RG6 6BZ,
UK

A group of nodule isolates from *Neptunia natans*, an indigenous stem-nodulated tropical legume found in waterlogged areas of Senegal, was studied. Polyphasic taxonomy was performed, including SDS-PAGE of total proteins, auxanography using API galleries, host-plant specificity, PCR-RFLP of the internal transcribed spacer region between the 16S and the 23S rRNA coding genes, 16S rRNA gene sequencing and DNA–DNA hybridization. It was demonstrated that this group is phenotypically and phylogenetically separate from the known species of *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium*, *Bradyrhizobium* and *Azorhizobium*. Its closest phylogenetic neighbour, as deduced by 16S rRNA gene sequencing, is *Agrobacterium vitis* (96.2% sequence homology). The name *Allorhizobium undicola* gen. nov., sp. nov., is proposed for this group of bacteria, which are capable of efficient nitrogen-fixing symbiosis with *Neptunia natans*, and the type strain is ORS 992^T (= LMG 11875^T).

Keywords: *Allorhizobium undicola*, *Neptunia natans*, tropical rhizobia, polyphasic taxonomy, nitrogen fixation

INTRODUCTION

Neptunia natans L.f. (Druce), previously *Neptunia oleracea* Lour., McVaugh 1987 (Subba Rao *et al.*, 1995), is an annual aquatic legume that is indigenous to waterlogged areas of Senegal. *N. natans* produces floating stems and roots containing white, spongy, internodal tissue and nodes with bright-red nodules and adventitious roots (Allen & Allen, 1981; Schaeede, 1940). The mode of root infection of *N. natans* (Subba Rao *et al.*, 1995) is similar in many respects to that of other tropical legumes, such as *Aeschynomene americana* (Napoli *et al.*, 1975), *Neptunia plena* (James *et al.*, 1992) and *Sesbania rostrata* (Ndoye *et al.*, 1994).

Bacteria enter natural wounds caused by splitting of the epidermis and emergence of young lateral roots. Bacteria spread first intercellularly, then through intercellular infection threads towards the meristematic cells of the nodule (Schaeede, 1940). The vascular bundles of the nodules are connected to the vasculature of the adventitious roots and not to that of the stem, indicating that they are root nodules rather than true stem nodules (Schaeede, 1940; James *et al.*, 1992; Subba Rao *et al.*, 1995). *N. natans* is being evaluated as green manure for rice cultivation in India and is consumed in South-East Asia (Subba Rao *et al.*, 1995). *N. natans* nodule bacterial isolates have been reported to induce small, white ineffective nodules on *Medicago sativa* and *Ornithopus* spp. (Subba Rao *et al.*, 1995) but not on roots of *Cicer arietinum*, *Lupinus albus*, *Lupinus angustifolius*, *Vicia faba*, *Trifolium subterraneum*, *Glycine max* and *Macroptilium atropurpureum*. *N. natans* was recently reported to be nodulated by *Mesorhizobium plurifarium* strains isolated from *Acacia* (de Lajudie *et al.*, 1998). *N. natans* nodule isolates have been reported to be fast growers (Dreyfus *et al.*, 1984) but have not yet been taxonomically characterized.

† Present address: LSTM ORSTOM/CIRAD-Forêt, Baillarguet, BP 5035, 34032 Montpellier Cedex 1, France.

Abbreviations: ITS, internal transcribed spacer; YMA, yeast mannitol agar; YEB, yeast extract peptone medium; TY, tryptone yeast extract medium.

The EMBL accession number for the 16S rRNA gene sequence of strain LMG 11875 reported in this paper is Y17047.

Phylogenetically, rhizobia belong in the alpha-2 subclass of the *Proteobacteria* (Stackebrandt *et al.*, 1988; Sawada *et al.*, 1993; Willems & Collins, 1993; Yanagi & Yamasato, 1993; Young, 1991), and several genera have been recognized, i.e. *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Mesorhizobium* (for a review see Young & Haukka, 1996). Polyphasic taxonomy has revealed that members of the genus *Rhizobium* are phylogenetically intertwined with members of the genus *Agrobacterium*, to which they are more closely related than to *Azorhizobium* and *Bradyrhizobium* (Sawada *et al.*, 1993; Willems & Collins, 1993; Yanagi & Yamasato, 1993). In *Agrobacterium*, the original species (*Agrobacterium tumefaciens*, *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*) were created on the basis of their phytopathogenic properties, which are mainly governed by plasmid-borne genes and do not correlate with the taxa found by polyphasic taxonomy (Kerstens & De Ley, 1984). For nomenclatural reasons, *Agrobacterium tumefaciens* must be retained as the type species of *Agrobacterium*. Consequently, no definite renaming of the species was proposed by Kersters & De Ley (1984), although a temporary division into four groups was suggested, reflecting the polyphasic results. *Agrobacterium* bv. 1 (containing the type strains of *Agrobacterium tumefaciens* and *Agrobacterium radiobacter*) constitutes the first group, *Agrobacterium* bv. 2 including the type strain of *Agrobacterium rhizogenes* constitutes a second group and a third taxon corresponds to the species *Agrobacterium vitis*; *Agrobacterium rubi* was considered to have a separate position and represents the fourth group. Later, comparison of the sequences of the 16S rRNA genes (Sawada *et al.*, 1993; Willems & Collins, 1993; Yanagi & Yamasato, 1993) revealed four phylogenetic sublineages on the *Agrobacterium*-*Rhizobium* branch: (i) a first sublineage contains *Agrobacterium* bv. 1, *Agrobacterium rubi* and *Agrobacterium vitis*; *Rhizobium galegae* and the recently proposed new species *Rhizobium giardinii* (Amarger *et al.*, 1997) also belong to this sublineage but have somewhat separate positions; (ii) a second phylogenetic sublineage contains *Rhizobium leguminosarum* (type species of *Rhizobium*), *Rhizobium tropici*, *Rhizobium etli*, *Agrobacterium* bv. 2 and a recently proposed new species, *Rhizobium gallicum* (Amarger *et al.*, 1997); (iii) a third sublineage was considered sufficiently different to deserve a separate genus status, for which the name *Sinorhizobium* had priority. *Sinorhizobium* contains *Sinorhizobium meliloti*, *Sinorhizobium fredii*, *Sinorhizobium xinjiangense*, *Sinorhizobium terangaie*, *Sinorhizobium saheli* (de Lajudie *et al.*, 1994; Trüper & de Clari, 1997) and *Sinorhizobium medicae* (Rome *et al.*, 1996); (iv) the fourth sublineage consists of species recently transferred in the new genus *Mesorhizobium* (Jarvis *et al.*, 1997), namely *Mesorhizobium loti*, *Mesorhizobium huakuii*, *Mesorhizobium ciceri*, *Mesorhizobium tianshanense*, *Mesorhizobium mediterraneum* (Jarvis *et al.*, 1997) and *Mesorhizobium plurifarum* (de Lajudie *et al.*, 1998).

Following the proposition of Sawada *et al.* (1993) to name *Agrobacterium* bv. 1 strains and *Agrobacterium* bv. 2 strains *Agrobacterium radiobacter* and *Agrobacterium rhizogenes* respectively, Bouzar (1994) requested a Judicial Opinion to decide whether *Agrobacterium radiobacter* or *Agrobacterium tumefaciens* should be the type species of *Agrobacterium*. Because this decision is still pending, we use here the nomenclature proposed by Kersters & De Ley (1984). Here we study a new group of fast-growing rhizobia isolated from *N. natans* nodules collected in Dakar and in the Sine Saloum region of Senegal, where this plant grows naturally. We performed whole-cell protein analysis by SDS-PAGE, PCR-RFLP of the internal transcribed spacer (ITS) region between 16S and 23S rRNA genes, 16S rRNA gene sequencing, DNA-DNA hybridizations, and auxanographic tests using API 50 galleries. Based on the findings of this polyphasic study, we conclude that this group of rhizobia belongs to the *Agrobacterium* rRNA sublineage, with *Agrobacterium vitis* as its closest phylogenetic neighbour, and deserves a separate genus and species status, for which the name *Allorhizobium undicola*, gen. nov., sp. nov., is proposed.

METHODS

Bacterial strains. *Rhizobium* strains were isolated as previously described (de Lajudie *et al.*, 1994) from naturally occurring nodules on adventitious roots of *N. natans*. All strains used are listed in Table 1. They were checked for purity by repeated streaking and by microscopical examination. The identity of the nodulating strains was verified by plant infection tests on the original host plants. We included type or representative strains of the different *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Agrobacterium* species. *Mycoplana*, *Ochrobactrum* and *Phyllobacterium* representatives were included in the auxanographic tests.

Growth and culture conditions. All *Rhizobium* and *Bradyrhizobium* strains were maintained on yeast mannitol agar (YMA), containing (g l⁻¹): mannitol, 10; sodium glutamate, 0.5; K₂HPO₄, 0.5; MgSO₄·7H₂O, 0.2; NaCl, 0.05; CaCl₂, 0.04; FeCl₃, 0.004; yeast extract (Difco), 1; agar, 20; pH 6.8. *Azorhizobium* and *Agrobacterium* strains were maintained on yeast extract peptone medium (YEB) containing in g l⁻¹ of 0.01 M phosphate buffer, pH 7.2: peptone (Oxoid), 5; yeast extract (Oxoid), 1; beef extract (Oxoid), 5; sucrose, 5 and MgSO₄·7H₂O, 0.592. All strains were stored at -80 °C on the same medium plus 15% (v/v) glycerol. For protein and DNA preparations we used tryptone yeast extract medium (TY) containing (g l⁻¹, pH 6.8-7): tryptone (Oxoid), 5; yeast extract (Oxoid), 0.75; KH₂PO₄, 0.454; Na₂HPO₄·12H₂O, 2.388; CaCl₂, 1; agar, 20. For protein preparation, TY with LabM agar was used. *Mycoplana*, *Ochrobactrum* and *Phyllobacterium* strains were maintained on nutrient agar containing (g l⁻¹): beef extract (Oxoid), 1; yeast extract (Oxoid), 2; peptone (Oxoid), 5; NaCl, 5; pH 7.4; agar, 20.

Morphological tests. Cell dimensions and morphology were determined on living cells by phase-contrast microscopy.

Plant infection tests. The seeds were scarified and surface sterilized with concentrated sulfuric acid. The duration of treatment (min) in H₂SO₄ for the different plant species was

Table 1. Strains used

ATCC, American Type Culture Collection, Rockville, MD, USA; BR and FL, strains from the CNPDS/EMBRAPA, Centro Nacional de Pesquisa em Biologia do Solo, Seropédica 23851, Rio de Janeiro, Brazil/Emprasa Brasileira de Pesquisa Agropecuária; CFN, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Cuernavaca, Mexico; CIAT, *Rhizobium* Collection, Centro Internacional de Agricultura Tropical, Cali, Columbia; HAMBI, Culture Collection of the Department of Microbiology, University of Helsinki, Helsinki, Finland; IAM, Institute for Applied Microbiology, University of Tokyo, Tokyo, Japan; LMG, Collection of Bacteria of the Laboratorium voor Microbiologie, K.-L. Ledeganckstraat, 35, B-9000 Ghent, Belgium; NCPPB, National Collection of Plant-pathogenic bacteria, Harpenden Laboratory, Hertfordshire, UK; 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, BP 1386, Dakar, Senegal; Pan., Panagopoulos, C., Crete, Greece; USDA, US Department of Agriculture, Beltsville, MD, USA; UPM, Universidad Politécnica Madrid, Spain.

Strain*	LMG no.	Other strain designation	Host plant or origin	Geographical origin	Reference or source
<i>Allorhizobium undicola</i>					
ORS 991	11874		<i>Neptunia natans</i>	Senegal (North Kaolack)	This work
ORS 992 ^T	11875 ^T		<i>Neptunia natans</i>	Senegal (Kaolack)	This work
ORS 995	11876		<i>Neptunia natans</i>	Senegal (South Kaolack)	This work
ORS 996	11877		<i>Neptunia natans</i>	Senegal (South Kaolack)	This work
ORS 997	11878		<i>Neptunia natans</i>	Senegal (North Kaolack)	This work
ORS 998	11879		<i>Neptunia natans</i>	Senegal (Dakar–Bel Air)	This work
<i>Mesorhizobium plurifarum</i>					
ORS 1001	7836		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
ORS 1014t1	7849t1		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
ORS 1002	7854		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
ORS 13	7921		<i>Acacia</i> sp.	Senegal	de Lajudie <i>et al.</i> (1998)
ORS 1018	11881		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
ORS 1037	11895		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
ORS 1040	11898		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1998)
HAMBI 1487	14925		<i>Acacia senegal</i>	Soudan	de Lajudie <i>et al.</i> (1998)
<i>Mesorhizobium loti</i>					
3F3C1	4269		<i>Wisteria frutescens</i>		Jarvis <i>et al.</i> (1986)
NZP 2230	6126		<i>Lotus maroccanus</i>	Morocco	Jarvis <i>et al.</i> (1986)
NZP 2213 ^T	6125 ^T	ORS 664 ^T	<i>Lotus tenuis</i>	New Zealand	Jarvis <i>et al.</i> (1986)
NZP 2037	6123	ORS 652	<i>Lotus divaricatus</i>	New Zealand	Jarvis <i>et al.</i> (1986)
NZP 2014	6124		<i>Lotus corniculatus</i>		Jarvis <i>et al.</i> (1986)
<i>Mesorhizobium ciceri</i>					
UPM-Ca7 ^T	17150 ^T	ORS 2738 ^T	<i>Cicer arietinum</i> L.	Spain	Nour <i>et al.</i> (1994)
522	17149		<i>Cicer arietinum</i> L.	Russia	Nour <i>et al.</i> (1994)
<i>Mesorhizobium mediterraneum</i>					
Ca-36 ^T	17148 ^T	ORS 2739 ^T	<i>Cicer arietinum</i> L.	Spain	Nour <i>et al.</i> (1995)
UPM-Ca142	14990		<i>Cicer arietinum</i> L.	Spain	Nour <i>et al.</i> (1995)
<i>Mesorhizobium</i> sp. (<i>Cicer</i>) genospecies 4					
IC-60	14995		<i>Cicer arietinum</i> L.	India	Nour <i>et al.</i> (1995)

[Continued overleaf]

Table 1 (cont.)

Strain*	LMG no.	Other strain designation	Host plant or origin	Geographical origin	Reference or source
<i>Mesorhizobium huakuii</i> IAM 14158 ^T	14107 ^T	ORS 1752 ^T	<i>Astragalus sinicus</i>	Nanjing, China	Chen <i>et al.</i> (1991)
<i>Mesorhizobium tianshanense</i> A-1BS ^T	15767 ^T	ORS 2640 ^T	<i>Glycyrrhiza pallidiflora</i>	Xinjiang, China	Chen <i>et al.</i> (1995)
<i>Sinorhizobium fredii</i> USDA 205 ^T USDA 191	6217 ^T 8317	ORS 669 ^T	<i>Glycine max</i> Soil	Honan, China Shanghai, China, 1978	Jarvis <i>et al.</i> (1986) Jarvis <i>et al.</i> (1986)
USDA 208	6219		<i>Glycine max</i>	Honan, China	LMG
<i>Sinorhizobium meliloti</i> NZP 4009 NZP 4027 ^T 102F34 L5-30 RCR 2011 3DOa30	6130 6133 ^T 4266	ORS 665 ^T ORS 620 ORS 621 ORS 634	<i>Medicago sativa</i> <i>Medicago sativa</i>	Australia Virginia, USA	LMG LMG ORS ORS ORS LMG
<i>Sinorhizobium medicae</i> HAMBI 1808 (m75) HAMBI 1809 (m102) HAMBI 1837 (m158)	16579 16580 16581	ORS 504	<i>Medicago sativa</i> <i>Medicago sativa</i>		Eardly <i>et al.</i> (1990) Eardly <i>et al.</i> (1990) Eardly <i>et al.</i> (1990)
<i>Sinorhizobium terengae</i> ORS 15	7833		<i>Sesbania</i> sp.	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 51	7843		<i>Sesbania rostrata</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 604	11865		<i>Sesbania aculeata</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 1007	7847		<i>Acacia laeta</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 1009 ^T	7834 ^T		<i>Acacia laeta</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 1073	11926		<i>Acacia senegal</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Sinorhizobium saheli</i> ORS 609 ^T	7837 ^T		<i>Sesbania cannabina</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 609t2	8309t2		<i>Sesbania cannabina</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 611	7842		<i>Sesbania grandiflora</i>	Senegal	de Lajudie <i>et al.</i> (1994)
ORS 611	8310		<i>Sesbania grandiflora</i>	Senegal	de Lajudie <i>et al.</i> (1994)
<i>Sinorhizobium</i> sp. BR 816 NGR 234		ORS 2645 ORS 644	<i>Lablab purpureus</i>		ORS Trinick (1980)
<i>Rhizobium leguminosarum</i> CNPAF 146 NZP 561 ATCC 14482	9504 6122 8819t1		<i>Trifolium repens</i>	Australia	LMG B. Jarvis LMG
<i>Rhizobium tropici</i> group a CNPAF 119	9502		<i>Phaseolus vulgaris</i> L.	Brazil	LMG
CFN 299	9517	ORS 651	<i>Phaseolus vulgaris</i> L.	Brazil	Martínez-Romero <i>et al.</i> (1991)

Table 1 (cont.)

Strain*	LMG no.	Other strain designation	Host plant or origin	Geographical origin	Reference or source
<i>Rhizobium tropici</i> group b					
CIAT 899 ^T	9503 ^T	ORS 1163 ^T	<i>Phaseolus vulgaris</i> L.	Columbia	Martínez-Romero <i>et al.</i> (1991)
C-05	9518		<i>Phaseolus vulgaris</i> L.		Martínez-Romero <i>et al.</i> (1991)
<i>Rhizobium etli</i>					
CFN 42 ^T		ORS 645 ^T	<i>Phaseolus vulgaris</i> L.	Mexico	Segovia <i>et al.</i> (1993)
<i>Rhizobium galegae</i>					
HAMBI 540 ^T	6214 ^T	ORS 668 ^T	<i>Galega orientalis</i>	Finland	LMG
HAMBI 1147	6215		<i>Galega orientalis</i>	Russia	LMG
HAMBI 1428/2	15143		<i>Galega orientalis</i>	Russia	LMG
<i>Agrobacterium</i> bv. 2					
ATCC 11325 ^T	150 ^T				
<i>Agrobacterium</i> bv. 1					
ATCC 19358 ^T	140 ^T	ORS 1351			LMG
M2/1	147		Ditch water	Belgium	Kerstens <i>et al.</i> (1973)
B6 ^T	187 ^T		<i>Lycopersicon lycopersicon</i>	USA	Kerstens <i>et al.</i> (1973)
ICPB TT111	196		Crown gall	USA	Kerstens <i>et al.</i> (1973)
B2a	268		<i>Lycopersicon lycopersicon</i>		Kerstens <i>et al.</i> (1973)
IICHR 28	303		<i>Chrysanthemum frutescens</i>	Germany, 1927	Kerstens <i>et al.</i> (1973)
CDC A6597	383		Vagina	South Carolina, USA	LMG
<i>Agrobacterium rubi</i>					
ICPB TR2	159	ORS 1353	<i>Rubus</i> sp.	USA, 1942	Kerstens <i>et al.</i> (1973)
ATCC 13335 ^T	156 ^T		<i>Rubus ursinus</i>	USA, 1942	LMG
<i>Agrobacterium vitis</i>					
Pan. AG61	257	ORS 2643	<i>Vitis vinifera</i>	Crete, Greece	LMG
Pan. AG63	258		<i>Vitis vinifera</i>	Crete, Greece	LMG
NCPPB 1771	233		<i>Vitis vinifera</i>	Iran	Kerstens <i>et al.</i> (1973)
<i>Azorhizobium caulinodans</i>					
ORS 571 ^T	6465 ^T		<i>Sesbania rostrata</i>	Senegal	Dreyfus <i>et al.</i> (1988)
FY12	11352		<i>Sesbania rostrata</i>	Senegal	Rinaudo <i>et al.</i> (1991)

* Original strain number, or as received.

as follows: *Acacia senegal*, 14; *Acacia seyal*, 30; *Acacia tortilis* subsp. *raddiana*, 150; *Sesbania rostrata*, 30–60; *Sesbania pubescens*, 60; *Sesbania grandiflora*, 60; *Neptunia natans*, 30; *Medicago sativa*, 25; *Macroptilium atropurpureum* (Siratro), 3. For *Vigna unguiculata* (niebe), seeds were left for 3 min in 96% alcohol followed by 5 min in 1% HgCl₂. After acid or HgCl₂ treatment, the seeds were washed with water until all traces of acid or HgCl₂ were removed.

The seeds were incubated in sterile Petri dishes on 1% water agar for 24–48 h to allow germination and then transferred to tubes containing Jensen seedling slant agar (Vincent, 1970) for root nodulation trials (8–10 plants were routinely tested with each strain). Root nodules appeared around 10–20 d after inoculation, and 3 weeks later they were fully developed. Nitrogen-fixing potential was estimated by visual observation of plant vigour and foliage colour of 30- to 45-

d-old plants and also by measuring the fresh and dry weights of aerial parts; infected plants were compared with control uninoculated plants.

PAGE of total bacterial proteins. PAGE was performed using small modifications of the procedure of Laemmli (1970), as described previously (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 (Vauterin & Vauterin, 1992). Similarity between 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).

PCR-RFLP of the ITS of 16S–23S rRNA genes. Strains were grown at 28 °C for 36–48 h on YMA, according to the method of Vincent (1970). Total DNA was purified with Chelex 100 (Sigma). Cells resuspended in a 5% suspension of Chelex 100 were boiled for 15 min. After centrifugation, the supernatant was used directly for PCR amplification. For strains for which the above procedure did not result in optimum DNA amplification, total DNA was purified using the phenol-chloroform method as described by Boucher *et al.* (1987). Primers FGPL 132'–38 and FGPS 1490–72, as described by Normand *et al.* (1996), were used for PCR amplification. These primers are derived from conserved regions of the 23S and 16S rRNA genes, respectively, and can be used to amplify the ITS of all prokaryotic DNAs tested so far. The oligonucleotides were purchased from Pharmacia. PCR amplification was carried out in a 100 µl reaction volume containing template DNA (50 µg), reaction buffer (Appligene), 20 mM of each dNTP (Pharmacia), 0.1 mM of each of the primers and 1 U *Taq* polymerase (Appligene). Amplifications were carried out in a GeneAmp PCR System 2400 (Perkin Elmer) using the following programme: initial denaturation for 5 min at 94 °C, 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C) and extension (1 min at 72 °C) and a final extension (5 min at 72 °C). PCR-amplified DNAs were visualized by electrophoresis of 4 µl of the amplified mixture on 1.4% (w/v) horizontal agarose gel (type 11; Sigma) in TBE buffer (83 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 4 V cm⁻¹ for 1 h. The gels were stained in an aqueous solution of 1 mg ethidium bromide l⁻¹ and photographed with Polaroid Type 667 positive film using a 260 nm UV source.

Aliquots of 6 µl of PCR products were digested in a 10 µl final volume with restriction endonucleases as specified by the manufacturer but with an excess of enzyme (5 U per reaction). The following enzymes were used: *Alu* I, *Dde* I, *Hinf* I, *Pal* I (Pharmacia), *Cfo* I (Boehringer Mannheim), *Msp* I (Gibco-BRL), *Rsa* I (Amersham or Gibco-BRL). Restricted DNA was analysed by horizontal electrophoresis in 3% (w/v) agarose gel (Nusieve 3:1; FMC). Electrophoresis was run at 2.3 V cm⁻¹ for 3 h. Gels were stained and photographed as described previously.

Clustering was obtained using the GelCompar 2.2 software package (Vauterin & Vauterin, 1992).

DNA base composition. Cells were grown for 2–3 d in Roux flasks on TY medium. High-molecular-mass DNA was prepared using the method of Marmur (1961). The G+C content was determined by thermal denaturation (De Ley, 1970) and calculated by using the equation of Marmur & Doty (1962), as modified by De Ley (1991). DNA from *Escherichia coli* LMG 2093 was used as a reference.

DNA–DNA hybridization. DNA–DNA hybridizations were performed with the initial renaturation rate method (De Ley, 1970). Renaturations using approximately 50 mg DNA ml⁻¹ were carried out at 79.8 °C, which is the optimum renaturation temperature, in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7).

Analysis of the 16S rRNA genes. The nearly complete 16S rRNA gene of strain LMG 11875, a representative of the new group, was determined. Lyophilized cells were resuspended in 500 ml TES buffer (0.05 M Tris/HCl, 0.005 M EDTA, 0.05 M NaCl, pH 8.0) and DNA was extracted by the method of Lawson *et al.* (1989). A large fragment of the 16S rRNA gene (corresponding to positions 28–1521 of the *Escherichia coli* 16S rRNA gene) was amplified by PCR. The PCR products were purified using a Prep-A-Gene kit (Bio-Rad) and sequenced using a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an automatic DNA sequencer (model 373A; Applied Biosystems). The new sequence was aligned, together with reference sequences obtained from the EMBL database, using the program PILEUP of the Genetics Computer Group package (Devereux *et al.*, 1984). Altogether a continuous stretch of 1348 base positions (including gaps) was used for further analysis. This corresponded to positions 84–1480 of the *Escherichia coli* 16S rRNA gene. Distances, modified according to the Kimura-2 model, were calculated using the DNADIST program of the Phylogeny Inference Package (Felsenstein, 1982), and the program NEIGHBOR of the same package was used to produce an unrooted phylogenetic tree. The stability of the groupings was verified by bootstrap analysis (500 replications) using the programs DNABOOT, DNADIST, NEIGHBOR and CONSENSE (Felsenstein, 1982). Uncorrected distances were calculated using the DISTANCES program of the GCG package and used to calculate similarity values.

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, and the results of auxanographic tests were scored as described previously (de Lajudie *et al.*, 1994).

3-Ketolactose test. The 3-ketolactose test was performed using the original method of Bernaerts & De Ley (1963) as modified by Bouzar *et al.* (1995).

RESULTS

Six isolates were purified from root nodules collected either on *N. natans* plants growing naturally in waterlogged areas around the town of Kaolack in the Sine Saloum region of Senegal or on *N. natans* plants seeded in our experimental field at Dakar-Bel Air in Senegal.

Host specificity

The six *Neptunia* isolates induced nodules on their original host, resulting in a very efficient nitrogen-fixing symbiosis. They were also found to be effective on *Acacia* species (*Acacia tortilis* subsp. *raddiana*, *Acacia senegal*, *Acacia seyal*), *Faidherbia albida*, and some strains were effective on *Lotus arabicus*, but ineffective on *Medicago sativa*, *Sesbania* species (*Sesbania rostrata*, *Sesbania pubescens*, *Sesbania gran-*

Table 2. Host specificity of *N. natans* isolates

All strains nodulated *N. natans*, *Acacia seyal*, *Faidherbia albida* and *Acacia tortilis* subsp. *raddiana*. No strain nodulated *Sesbania rostrata*, *Sesbania pubescens* or *Sesbania grandiflora*.

Strain	<i>Acacia senegal</i>	<i>Lotus arabicus</i>	<i>Medicago sativa</i>	<i>Vigna unguiculata</i>	<i>Macroptilium atropurpureum</i>
ORS 991	+	±	±	NT	NT
ORS 992 ^T	NT	—	—	—	—
ORS 995	NT	±	—	NT	NT
ORS 996	NT	+	—	NT	NT
ORS 997	+	+	—	—	—
ORS 998	+	—	—	NT	NT

+, Nodulation; —, no nodulation; ±, 10–30% plants were nodulated; NT, not tested.

Fig. 1. Dendrogram showing the relationships between the electrophoretic protein patterns from nodule isolates of *N. natans* and reference strains of *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Agrobacterium* species. The mean correlation coefficient (r) was represented as a dendrogram and calculated by the unweighted pair group method with averages. Positions 10–320 of the 400 point traces were used for calculation of similarities between individual pairs of traces. T indicates type strain. The scale represents the r values converted to percentages.

Fig. 2. Dendrogram showing the relationships between the PCR-RFLP profiles of the ITS of *N. natans* nodule isolates and representatives of different *Mesorhizobium*, *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium* and *Agrobacterium* species. The mean correlation coefficient (r) was represented as a dendrogram and calculated by the unweighted pair group method with averages. T indicates type strain. The scale represents the r value converted to percentages.

Fig. 3. Dendrogram showing the phylogenetic relationships of strain LMG 11875 and representatives of the alpha subclass of the *Proteobacteria*. The tree was calculated from a distance matrix (modified according to the Kimura-2 model) using the neighbour-joining method. Bootstrap values, expressed as a percentage of 500 replications, are given at the branching points. Numbers in parentheses are the accession numbers of the sequences used. The bar represents one expected substitution per 100 nucleotide positions.

diflora), *Vigna unguiculata* and *Macroptilium atropurpureum* (Table 2).

SDS-PAGE of total bacterial proteins

We purified whole-cell proteins from the *N. natans* isolates, performed SDS-PAGE in standardized conditions (de Lajudie *et al.*, 1994) and compared the normalized patterns (Vauterin & Vauterin, 1992) with those in our database, which contains profiles of strains of different species of *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium* and *Azorhizobium*. *Sinorhizobium terengae*, *Sinorhizobium saheli*, *Sinorhizobium fredii*, *Sinorhizobium meliloti*, *Sinorhizobium medicae*, *Rhizobium leguminosarum*, *Rhizobium tropici*, *Rhizobium galegae*, *Azorhizobium caulinodans*, *Mesorhizobium plurifarium*, *Mesorhizobium ciceri*, *Meso-*

Fig. 4. Dendrogram obtained from an unweighted pair group method with averages cluster analysis of Canberra metric similarity coefficients based on 147 auxanographic characteristics. Reference strains included in this study were essentially the same as those we used in a previous report (de Lajudie *et al.*, 1994). Numbers of strains used were as follows: *Agrobacterium* bv. 1, 9; *Agrobacterium* bv. 2, 3; *Agrobacterium rubi*, 1; *Agrobacterium vitis*, 3; *Rhizobium galegae*, 2; *Sinorhizobium meliloti*, 3; *Sinorhizobium fredii*, 2; *Sinorhizobium terengae*, 20; *Sinorhizobium saheli*, 4; *Rhizobium leguminosarum*, 1; *Rhizobium tropici* a, 3; *Rhizobium tropici* b, 3; *Mesorhizobium plurifarium*, 26; *Azorhizobium caulinodans*, 1; *Phyllobacterium*, 5; *Ochrobactrum anthropi*, 10; *Mycoplasma*, 2.

rhizobium sp. (*Cicer*) formed separate clusters. This is illustrated in Fig. 1, which presents a limited dendrogram with a few representatives of the different species. Only one strain each of *Mesorhizobium huakuii*, *Mesorhizobium mediterraneum*, *Agrobacterium* bv. 2, *Agrobacterium vitis* and *Agrobacterium rubi* were included, and these each had separate positions. Strains of *Agrobacterium* bv. 1 exhibited diverse protein patterns and could be grouped into two different clusters, as observed previously (de Lajudie *et al.*, 1998). The strains of *Mesorhizobium loti* did not group together and were found to fall into three clusters. The six *N. natans* isolates were related with a

correlation coefficient of 92% and formed a rather homogeneous gel electrophoretic cluster, distinct from all described species and clusters contained in our database. The highest correlation coefficient between the cluster of *Neptunia* strains and the other rhizobial species and groups was 70%.

PCR-RFLP of the ITS

We performed PCR-RFLP analysis of the ITS region between the 16S and 23S rRNA genes of the *Neptunia* strains and some representative strains of rhizobial species of *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* and *Agrobacterium*. The size of the amplified ITS fragment varied from 1000 to 1450 bp depending on strains and was 1380–1390 bp for the *Neptunia* isolates. The results of RFLP analysis are shown in Fig. 2. All strains belonging to the same species, except *Mesorhizobium loti*, grouped together. At a correlation coefficient of 30%, three main branches could be distinguished. The first branch consisted of the *Rhizobium*, *Sinorhizobium* and *Agrobacterium* species, together with two *Sinorhizobium* sp. strains, BR 816 and NGR 234. The second branch consisted of the *Mesorhizobium* species (*Mesorhizobium loti*, *Mesorhizobium huakuii*, *Mesorhizobium ciceri*, *Mesorhizobium tianshanense*, *Mesorhizobium mediterraneum*, *Mesorhizobium plurifarium*). All isolates from *N. natans* could be grouped in a third branch as a separate homogeneous group (internal correlation coefficient of 73%) distinct from every other described species.

G + C content of DNA and DNA–DNA hybridizations

The G + C content of ORS 992^T is 60.1 mol%. We found a high degree of DNA–DNA binding (89%) between two strains (ORS 992^T and ORS 997) of the new *N. natans* group.

Analysis of the 16S rRNA genes

The determined sequence of the 16S rRNA gene of strain LMG 11875 consisted of 1433 bases. A search in the EMBL database revealed the new sequence to be most similar to the 16S rRNA gene sequence of *Agrobacterium vitis*, thereby placing the new isolates in the *Rhizobium*–*Agrobacterium* group of the alpha subclass of the *Proteobacteria*. A dendrogram showing the phylogenetic relationships of strain LMG 11875 and representatives of the alpha subclass of the *Proteobacteria* is shown in Fig. 3.

Numerical analysis of auxanographic results

The six *Neptunia* isolates were tested for assimilation of 147 organic compounds as sole carbon source using the API 50 system, and the results were compared with those of representative strains of *Sinorhizobium fredii*, *Sinorhizobium meliloti*, *Sinorhizobium teranga*, *Sinorhizobium saheli*, *Mesorhizobium loti*, *Mesorhizobium huakuii*, *Mesorhizobium plurifarium*, *Rhizobium leguminosarum*, *Rhizobium tropici*, *Rhizobium galegae*,

Agrobacterium rhizogenes, *Agrobacterium tumefaciens*, *Agrobacterium vitis*, *Agrobacterium rubi*, *Azorhizobium caulinodans*, *Ochrobactrum*, *Phyllobacterium* and *Mycoplana* available in our database (de Lajudie *et al.*, 1994). The dendrogram obtained by numerical analysis of these results (Fig. 4) showed that the *Neptunia* strains formed a very homogeneous group distinct from every other species. They were related to *Agrobacterium vitis* at a correlation coefficient of 0.695. Table 3 shows the results of the *Neptunia* strains and their nearest phylogenetic relatives, namely *Agrobacterium vitis*, *Agrobacterium* bv. 1, *Agrobacterium rubi* and *Rhizobium galegae*.

3-Ketolactose test

The six strains from *Neptunia* were negative, as were the four control strains of *Agrobacterium* bv. 2 tested (LMG 150^T, LMG 155, LMG 161, LMG 341). Control *Agrobacterium* bv. 1 strains LMG 64, LMG 146, LMG 196, LMG 201, LMG 296t2 were positive; *Agrobacterium* bv. 1 strain LMG 26 and *Agrobacterium* sp. strain LMG 294 were negative.

DISCUSSION

We isolated six new strains from naturally occurring nodules of *Neptunia natans* plants in Senegal. These strains grew rapidly on YMA, produced exopolysaccharides and exhibited a particular spectrum of carbon source utilization. We employed a polyphasic approach to the taxonomic characterization of this new group of tropical rhizobia, using techniques with wide discriminative powers (16S rRNA gene sequencing and auxanography) and others at the species and infra-species levels (DNA–DNA hybridization, SDS-PAGE, PCR-RFLP of the ITS). Two screening methods, SDS-PAGE protein profile analysis and numerical analysis of auxanographic data, indicated that these isolates constitute a homogeneous phenon, distinct from a wide range of rhizobia, agrobacteria and other related bacteria (Figs 1 and 4). These results suggested that this group constituted a separate species, which we further characterized using genotypic techniques with diverse taxonomic discriminative powers.

PCR-RFLP analysis of the ITS of 16S–23S genes demonstrated that the new *Neptunia* isolates constitute a homogeneous genotypic group (Fig. 2), and this was further supported by the high level of DNA–DNA binding (89%) found between two representative strains. Our RFLP analysis of the 16S–23S ITS confirmed that the new group is also genotypically distinct from representatives of all known species of the *Rhizobium*–*Agrobacterium* group (Fig. 2). To determine precisely the phylogenetic position of the *Neptunia natans* isolates, the 16S rRNA gene sequence of a representative strain (LMG 11875) was determined. Phylogenetic analysis revealed that this strain is related to the *Agrobacterium* lineage that contains

Table 3. Results of carbon assimilation tests performed with *Allorhizobium undicola* and reference strains of *Rhizobium galegae*, *Agrobacterium* bv. 1, *Agrobacterium vitis* and *Agrobacterium rubi*

n = Number of strains studied; results recorded for strains *Agrobacterium vitis* LMG 257 and LMG 258, *Allorhizobium undicola* ORS 991, ORS 992^T, ORS 995, ORS 996, ORS 997 and ORS 998, *Rhizobium galegae* LMG 6214^T and LMG 6215, *Agrobacterium* bv. 1 LMG 140, LMG 147, LMG 187^T, LMG 196, LMG 268, LMG 303, LMG 383 and *Agrobacterium rubi* LMG 156^T. +, All strains are positive; —, all strains are negative; the values are the percentage of positive strains. The main discriminative results between *Allorhizobium undicola* and *Agrobacterium vitis* are given in bold face. The reaction of the type strain is given in parentheses. All strains grew in API 50 on glycerol, ribose, L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, D-cellobiose, D-maltose, lactose, rhamnose, D-turanose, D-lyxose, inositol, mannitol, D-arabitol, fumarate, DL-lactate, D-malate, L-(α)-alanine, L-proline and L-histidine and did not grow on erythritol, aesculin, inulin, starch, glycogen, isobutyrate, *n*-valerate, isovalerate, *n*-caproate, heptanoate, caprylate, pelargonate, caprate, maleate, oxalate, adipate, pimelate, suberate, azelate, sebacate, glycolate, laevulinate, citraconate, itaconate, mesaconate, phenylacetate, benzoate, *o*-hydroxybenzoate, D-mandelate, L-mandelate, phthalate, isophthalate, terephthalate, glycine, DL-norvaline, DL-2-aminobutyrate, L-methionine, L-phenylalanine, L-tyrosine, D-tryptophan, L-tryptophan, DL-kynurenine, creatine, urea, acetamide, ethylamine, butylamine, amylamine, benzylamine, diaminobutane, spermine, histamine and tryptamine.

Substrate	<i>Ag. vitis</i> (<i>n</i> = 2)	<i>Al. undicola</i> (<i>n</i> = 6)	<i>R. galegae</i> (<i>n</i> = 2)	<i>Ag. bv. 1</i> (<i>n</i> = 7)	<i>Ag. rubi</i> (<i>n</i> = 1)
Dulcitol, methyl α -D-glucoside, D-melezitose, D-tagatose, L-arabitol, 5-ketogluconate, propionate, aconitate, L-lysine, L-citrulline, sarcosine, ethanolamine	—	—	—	+	+
Methyl α -D-xyloside, xylitol	—	—	+	+	+
D-Tartrate, mesotartrate, <i>m</i> -hydroxybenzoate	—	—	+	—	—
Arbutin	+	+	—	+	+
L-Ornithine	—	+	—	+	+
DL-Glycerate, adonitol, <i>N</i> -acetylglucosamine, D-melibiose, gluconate, D-raffinose, L-fucose	+	—	+	+	+
L-Tartrate, citrate	+	—	—	—	—
Glutarate	+	—	—	50	—
Butyrate	+	—	50	—	—
Malonate	—	—	—	—	+
L-Sorbose	—	—	—	75	+
D-Arabinose, 2-ketogluconate	50	—	+	+	+
Methyl α -D-mannoside	50	—	—	—	—
Amygdalin	50	—	—	+	+
L-Xylose	50	—	—	50	—
β -Gentiobiose	50	+	+	+	+
Sorbitol, acetate	+	85 (+)	+	+	+
Salicin	+	75 (+)	—	+	+
Trehalose, D-fucose	+	30 (—)	+	+	+
Succinate	+	60 (+)	+	+	+
Sucrose, DL-3-hydroxybutyrate, L-malate	+	80 (+)	+	+	+
Pyruvate	+	+	+	75	+
2-Ketoglutarate	—	30 (—)	—	50	+
<i>p</i> -Hydroxybenzoate	+	80 (+)	+	75	+
L-Leucine	—	—	50	25	—
L-Isoleucine, L-valine	—	—	—	25	—
D-(α)-Alanine, L-norleucine	—	—	—	50	—
L-Cysteine	—	—	—	75	—
L-Serine, L-threonine	50	+	—	+	+
Trigonelline	+	30 (—)	—	25	—
L-Aspartate	+	30 (—)	—	+	+
L-Glutamate	50	+	—	+	+
L-Arginine	—	60 (—)	—	+	+
Betaine	50	30 (+)	+	+	+
β -Alanine	—	60 (+)	—	50	+
DL-3-Aminobutyrate	—	30 (+)	—	—	—
DL-4-Aminobutyrate	+	80 (+)	—	75	+
DL-5-Aminovalerate	—	30 (+)	—	25	+
2-Aminobenzoate, 3-aminobenzoate, 4-aminobenzoate	—	15 (—)	—	—	—
Glucosamine	+	60 (—)	+	75	+

Table 4. Discriminatory utilization of carbohydrates as sole carbon source in *Allorhizobium* and other related genera and phylogenetic groups

Results from this work, de Lajudie *et al.* (1994, 1998), Nour *et al.* (1994, 1995) and Chen *et al.* (1991, 1995). +, All strains are positive; —, all strains are negative; d, some strains are positive.

Carbohydrate	<i>Allorhizobium</i>	<i>Agrobacterium</i> bv. 1	<i>Rhizobium</i> <i>vitis</i>	<i>Rhizobium</i> <i>galegae</i>	<i>Rhizobium</i> *	<i>Sinorhizobium</i> †	<i>Mesorhizobium</i> ‡	<i>Azorhizobium</i>
Adonitol	—	+	+	+	+	+	+	—
D-Arabinose	—	+	d	+	+	d	d	—
L-Fucose	—	+	+	+	+	d	d	—
N-Acetylgluco-amine	—	+	+	+	d	+	+	—
D-Melibiose	—	+	+	+	+	+	d	—
D-Raffinose	—	+	+	+	+	+	d	—
Trehalose	d	+	+	+	+	+	+	—
Methyl xyloside	—	+	—	+	+	d	—	—
Sucrose	d	+	+	+	+	+	d	—
Xylitol	—	+	—	+	+	d	d	—
L-Arabitol	—	+	—	—	+	d	d	—
Gluconate	—	+	+	+	+	d	d	+
Succinate	d	+	+	+	d	+	+	+
DL-glycerate	—	+	+	+	d	d	d	+

* Results for *Rhizobium tropici*, *Rhizobium leguminosarum* and *Agrobacterium* bv. 2.

† Results for *Sinorhizobium fredii*, *Sinorhizobium teranga*, *Sinorhizobium saheli* and *Sinorhizobium meliloti*.

‡ Results for *Mesorhizobium loti*, *Mesorhizobium huakuii*, *Mesorhizobium ciceri*, *Mesorhizobium mediterraneum*, *Mesorhizobium tianshanense* and *Mesorhizobium plurifarum*.

Agrobacterium tumefaciens bv. 1, *Agrobacterium rubi*, *Agrobacterium* strains isolated from *Ficus*, *Agrobacterium vitis* and *Rhizobium galegae* (Fig. 3). Sequence similarity values of members of this lineage with strain LMG 11875 ranged from 94.5 to 95.2%, with the highest value found between this strain and *Agrobacterium vitis* strain LMG 8750 (96.3% sequence similarity, corresponding to at least 55 base differences). From the level of these similarity values, it can be presumed that there is no significant DNA–DNA binding between these groups of organisms; in consequence, and following the recommendation of Stackebrandt & Goebel (1994), no additional DNA–DNA hybridizations were performed.

The results of all the techniques used converge to the conclusion that the new isolates from *N. natans* nodules form a homogeneous group that can be phenotypically and genotypically distinguished from other described species of rhizobia and agrobacteria. It is clear that this taxon represents at least a new species. The genus allocation of this group is less clear-cut and several possibilities are apparent:

(i) The new group could be described as a new *Rhizobium* species, because it lives in a nitrogen-fixing symbiosis with leguminous plants and is phylogenetically related to *Rhizobium galegae* (Fig. 3, sequence similarity 95.1%). However, it is clear that both the new group and *Rhizobium galegae* are phylogenetically distinct from the lineage that contains the type species of the genus *Rhizobium*, *Rhizobium leguminosarum*, and therefore represents the true genus *Rhizobium* (Fig. 3, sequence similarity of the *Rhizobium leguminosarum* lineage with strain LMG 11875 ranges from 92.9 to 93.5%). Furthermore, several

other genera [*Agrobacterium* (94.5–96.3% sequence similarity, *Sinorhizobium* (94.2% sequence similarity with *Sinorhizobium fredii*) and *Mesorhizobium* (93.1% sequence similarity with *Mesorhizobium loti*)] are also closely related to the new group. The proposal of a new *Rhizobium* species for the *Neptunia* isolates is thus excluded on phylogenetic grounds.

(ii) The new group could be described as a new *Agrobacterium* species, because phylogenetically it belongs to the bv. 1 *Agrobacterium* lineage, which contains the type strain of the type species of the genus *Agrobacterium*. However, it is generally recognized that the species delineation in this genus is unclear and needs revision. Although the new group is clearly distinct from all other *Agrobacterium* species in the bv. 1 lineage (Fig. 3), the peripheral position of *Agrobacterium vitis* in this lineage and the presence of *Rhizobium galegae*, together with the low bootstrap values (Fig. 3), indicate that this lineage may represent several genera, and it therefore seems unwise to create a new *Agrobacterium* species in this group. In addition to these phylogenetic considerations, the inclusion of a non-tumorigenic species in the genus *Agrobacterium* would undoubtedly raise opposition from phytopathologists and lead to considerable practical problems.

(iii) The new group could be described as a new genus of nitrogen-fixing legume symbionts. It is most closely related to *Rhizobium galegae* and *Agrobacterium vitis*, but the 16S rRNA gene sequence similarity levels (approx. 95.5–96%) and the low bootstrap values (Fig. 3) suggest that none of these relationships is particularly significant at present. In view of the data

presented above, we propose to create a new genus, *Allorhizobium*, with one new species, *Allorhizobium undicola*, to describe the new *N. natans* isolates.

A number of discriminative features between *Allorhizobium* and its phylogenetic relatives, *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium*, *Rhizobium galegae* and *Agrobacterium* species can be found in Tables 3 and 4. In particular, at least 12 features can be used to discriminate between *Allorhizobium undicola* and its closest phylogenetic neighbour, *Agrobacterium vitis*: growth on adonitol, *N*-acetylglucosamine, D-melibiose, D-raffinose, L-fucose, gluconate, butyrate, glutarate, DL-glycerate, L-tartrate, citrate and L-ornithine (Table 3).

In recent years, polyphasic research into the genus *Rhizobium* has resulted in its gradual subdivision, with the proposal of *Bradyrhizobium* (Jordan, 1982), *Sinorhizobium* (Chen *et al.*, 1988) and, more recently, *Mesorhizobium* (Jarvis *et al.*, 1997). The proposal of *Allorhizobium* is a further step in this process. From the phylogenetic data (Fig. 3), it is evident that the taxonomic position of *Rhizobium galegae* and *Agrobacterium vitis* should be revised because these species are distinct from the phylogenetic groups containing the type strain of their genus. However, our study mainly concerned the new *Neptunia* isolates and therefore we refrain from making any formal proposals for these other taxa just yet. The taxonomic revision of *Rhizobium galegae* and *Agrobacterium vitis* is a complex issue, linked with the revision of the genus *Agrobacterium*, and can only be attempted after extensive study of the literature data and international consultation.

Description of *Allorhizobium* gen. nov.

Allorhizobium gen. nov. (Al.lo.rhi.zo'bi.um. Gr. adj. *allos* other; M.L. neut. n. *Rhizobium* a bacterial generic name; M.L. neut. n. *Allorhizobium* the other *Rhizobium*, to refer to the fact that it is phylogenetically separate from other rhizobia).

Aerobic, Gram-negative, non-spore-forming rods that are 0.5–0.7 µm wide by 2–4 µm long. Strains grow fast and form colonies of 0.5–3 mm diameter within 1–2 d on yeast mannitol mineral salts agar. Pronounced turbidity develops after 1–2 d in agitated broth media. Chemo-organotrophic, utilizing a wide range of carbohydrates, organic acids and amino acids as sole carbon sources for growth (Table 3). Discriminative features between *Allorhizobium* and other related genera and phylogenetic groups are shown in Table 4. 3-Ketolactose is not produced from lactose. Growth on carbohydrate media is usually accompanied by extracellular polysaccharide production. The organisms are typically able to invade the root hairs of some temperate-zone (*Medicago sativa*) and some tropical zone (*Neptunia natans*, *Acacia senegal*, *Acacia seyal*, *Acacia tortilis* subsp. *raddiana*, *Lotus arabicus*, *Faidherbia albida*) leguminous plants (family *Leguminosae*) and induce the production of root nodules,

wherein the bacteria occur as intracellular symbionts. All strains exhibit host specificity. No strain was found to nodulate *Sesbania rostrata*, *Sesbania pubescens*, *Sesbania grandiflora*, *Vigna unguiculata* or *Macroptilium atropurpureum*. The G+C content of the DNA is 60.1 mol% (by T_m). The type species is *Allorhizobium undicola*. At the molecular level the genus can be recognized by SDS-PAGE whole-cell protein analysis, ITS PCR-RFLP and 16S rRNA gene sequencing.

Description of *Allorhizobium undicola* sp. nov.

Allorhizobium undicola (un.di'co.la. L. n. *unda* water; L. suff. *cola* dweller; L. n. *undicola* water-dweller, referring to the isolation of these strains from nodules of the aquatic plant *Neptunia natans*).

Strains have all the characteristics of the genus *Allorhizobium*. They grow fast and form colonies of 0.5–3 mm diameter within 1–2 d on YMA. Colonies are round, creamy, convex to drop-like, beige-coloured; margin and surface have a smooth aspect. Aerobic, Gram-negative, non-spore-forming rods that are 0.5–0.7 µm wide by 2–4 µm long. Motile in liquid medium. A wide range of carbohydrates, organic acids and amino acids are utilized as sole carbon sources for growth (Table 3). Discriminating features from related species are given in Table 3. Strains are 3-ketolactose-negative. Strains can induce nitrogen-fixing nodules on their original host *N. natans* and can also nodulate *Medicago sativa*, *Acacia senegal*, *Acacia seyal*, *Acacia tortilis* subsp. *raddiana*, *Lotus arabicus* and *Faidherbia albida*, but nodules do not always fix nitrogen. No strain was found to nodulate *Sesbania rostrata*, *Sesbania pubescens*, *Sesbania grandiflora*, *Vigna unguiculata* or *Macroptilium atropurpureum*.

They can be differentiated by SDS-PAGE of their total cellular proteins, and at the molecular level by PCR-RFLP profiles of the ITS and the sequence of their 16S rRNA gene.

The well-studied strain ORS 992^T (=LMG 11875), isolated from *N. natans* in Senegal, is designated as the type strain and its features are given in Tables 2 and 3. The G+C content of ORS 992^T is 60.1 mol%. All *Allorhizobium undicola* strains have been deposited in the Culture Collection of the Laboratorium voor Microbiologie, University of Gent, in the Culture Collection of the Laboratory of Soil Microbiology, ORSTOM, Dakar, Senegal, and in the Culture Collection of LSTM, CIRAD-ORSTOM, Baillarguet, France.

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