Photosynthetic Symbionts of *Aeschynomene* spp. Form a Cluster with Bradyrhizobia on the Basis of Fatty Acid and rRNA Analyses

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The relationship between photosynthetic rhizobia that nodulate 10 *Aeschynomene* species (*Aeschynomene afraaspera*, *Aeschynomene denticulata*, *Aeschynomene evenia*, *Aeschynomene indica*, *Aeschynomene nilotica*, *Aeschynomene pratensis*, *Aeschynomene rudis*, *Aeschynomene scabra*, *Aeschynomene schimperii*, and *Aeschynomene sensitiva*) and reference strains of the genera *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* was investigated by analyzing cellular fatty acid methyl esters (FAME) and 16S rRNA sequences. The members of each genus produced very distinct FAME patterns, and the photosynthetic rhizobia formed a subcluster in the *Bradyrhizobium* cluster. The absence of the cyc C_{19:0} type of fatty acid in all of the photosynthetic rhizobium strains isolated from 10 *Aeschynomene* species distinguished these microorganisms from other known rhizobia, including strain BTAi 1, a photosynthetic symbiont of *A. indica*. We sequenced a 264-base segment of the 16S rRNA genes of selected strains after amplification by the PCR and compared the results with previously published sequences for species of rhizobia and related photosynthetic bacteria. Photosynthetic strains IRBG 2 (from *A. afraaspera*), IRBG 230 (from *A. nilotica*), and ORS 322 (from *A. afraaspera*) had identical sequences but were distinct from strain BTAi (from *A. indica*) and from strain IRBG 231 (from *A. denticulata*), which is similar to the type strain (DNA homology group Ia) of *Bradyrhizobium japonicum*. Nonphotosynthetic strain IRBG 274 (from *A. afraaspera*) was closely related to *Bradyrhizobium elkanii* (DNA homology group II). All of the photosynthetic rhizobia clearly fell into the *Bradyrhizobium* cluster. Although the results of the FAME and 16S rRNA analyses were in excellent agreement, our placement of the photosynthetic rhizobia is in apparent conflict with phenotypic data, as determined by numerical taxonomy (Ladha and So, Int. J. Syst. Bacteriol., in press) which placed the photosynthetic rhizobia in a coherent cluster that is as far from the genus *Bradyrhizobium* as the genera *Rhizobium* and *Azorhizobium* are. While the FAME and 16S rRNA data probably provide a more reliable indication of phylogeny, the degree of phenotypic divergence observed raises questions concerning the polyphasic approach to bacterial systematics.

The root- and stem-nodulating bacterial symbionts of legumes are currently classified in three genera (the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*), and the physiologically diverse relatives of these organisms belonging to the alpha subgroup of the *Proteobacteria* include photosynthetic anaerobes such as *Rhodobacter* and *Rhodospseudomonas* spp. (39, 40). Recently, the members of a group of symbiotic rhizobia isolated from stem nodules of *Aeschynomene* species have been shown to produce bacteriochlorophyll and to be capable of photosynthesis (6, 15). Solely on the basis of the presence of bacteriochlorophyll, Eaglesham et al. (6) suggested that strain BTAi 1 (isolated from *Aeschynomene indica*) might represent a new genus and proposed the name "*Photorhizobium thompsonianum*" (6). Subsequently, a study of part of the 16S rRNA sequence showed that strain BTAi 1 fell within the range of variation found in the genus *Bradyrhizobium*, as did the nonsymbiotic photosynthetic organism *Rhodospseudomonas palustris* (40).

Ladha and So (16) characterized 52 isolates obtained from nine *Aeschynomene* species that produce stem nodules and compared these isolates with 80 *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* strains. On the basis of the results of numerical taxonomy of 150 phenotypic traits, at least eight

phenotypes were distinguished. All of the photosynthetic rhizobia fell into a group that was well separated from other rhizobia. Although these results supported proposal that a new genus should be created for photosynthetic rhizobia, Ladha and So (16) stressed the need for additional tests before a final decision is made. The international subcommittee responsible for the taxonomy of rhizobia has recently recommended that proposals for new genera or species should be based on both phenotypic and phylogenetic traits (7). The study described in this paper was a continuation of our work on the characterization of rhizobia isolated from aquatic legumes belonging to the genera *Sesbania* and *Aeschynomene*. Representative strains of groups distinguished by phenotype-based numerical taxonomy were further characterized by fatty acid methyl ester (FAME) analysis and 16S rRNA sequencing. Comparisons of 16S rRNA sequences are now very widely used to determine phylogenetic relationships of bacteria (35, 36), and the significance of fatty acids in bacterial taxonomy has also been recognized (12). MacKenzie et al. (20) and Kuykendall et al. (13) have used fatty acid composition to determine similarities among strains of rhizobia. The objective of this work was to determine whether FAME and 16S rRNA sequence data support the conclusions of a previous numerical taxonomy study (16).

MATERIALS AND METHODS

Table 1 lists the sources and origins of 100 rhizobial strains used in this study. The strains were routinely maintained at the Soil Microbiology Section of The International Rice Research

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TABLE 1. Origins and hosts of bacterial strains used

Genus	Cluster	Subcluster	Strain	Host	Nodule or strain origin	Source ^a	Reference no.
<i>Bradyrhizobium</i>	I	IA	IRBG 231	<i>Aeschynomene denticulata</i>	Laguna, Philippines	IRRI	1
	I	IA	IRBG 284	<i>Aeschynomene pratensis</i>	Iloilo, Philippines	IRRI	2
	I	IA	IRBG 287	<i>Aeschynomene pratensis</i>	Laguna, Philippines	IRRI	3
	I	IA	IRBG 114	<i>Aeschynomene sensitiva</i>	Laguna, Philippines	IRRI	4
	I	IA	IRBG 115	<i>Aeschynomene sensitiva</i>	Laguna, Philippines	IRRI	5
	I	IA	IRBG 122	<i>Aeschynomene sensitiva</i>	Iloilo, Philippines	IRRI	6
	I	IA	IRBG 2	<i>Aeschynomene afraspera</i>	Laguna, Philippines	IRRI	7
	I	IA	IRBG 79	<i>Aeschynomene afraspera</i>	Laguna, Philippines	IRRI	8
	I	IA	IRBG 81	<i>Aeschynomene afraspera</i>	Laguna, Philippines	IRRI	9
	I	IA	IRBG 124	<i>Aeschynomene pratensis</i>	Laguna, Philippines	IRRI	10
	I	IA	IRBG 126	<i>Aeschynomene pratensis</i>	Iloilo, Philippines	IRRI	11
	I	IA	IRBG 119	<i>Aeschynomene sensitiva</i>	Laguna, Philippines	IRRI	12
	I	IA	IRBG 92	<i>Aeschynomene indica</i>	Laguna, Philippines	IRRI	13
	I	IA	IRBG 91	<i>Aeschynomene indica</i>	Laguna, Philippines	IRRI	14
	I	IA	IRBG 348	<i>Aeschynomene indica</i>	Laguna, Philippines	IRRI	15
	I	IA	IRBG 125	<i>Aeschynomene pratensis</i>	Laguna, Philippines	IRRI	16
	I	IA	IRBG 127	<i>Aeschynomene pratensis</i>	Iloilo, Philippines	IRRI	17
	I	IA	IRBG 297	<i>Aeschynomene sensitiva</i>	Iloilo, Philippines	IRRI	18
	I	IA	IRBG 123	<i>Aeschynomene pratensis</i>	Iloilo, Philippines	IRRI	19
	I	IA	IRBG 250	<i>Aeschynomene schimperii</i>	Laguna, Philippines	IRRI	20
	I	IA	IRBG 234	<i>Aeschynomene evenia</i>	Laguna, Philippines	IRRI	21
	I	IA	IRBG 233	<i>Aeschynomene evenia</i>	Laguna, Philippines	IRRI	22
	I	IA	IRBG 229	<i>Aeschynomene evenia</i>	Laguna, Philippines	IRRI	23
	I	IA	IRBG 238	<i>Aeschynomene rudis</i>	Laguna, Philippines	IRRI	24
	I	IA	IRBG 83	<i>Aeschynomene afraspera</i>	Laguna, Philippines	IRRI	25
	I	IA	ORS 322	<i>Aeschynomene afraspera</i>	Senegal	ORSTOM	26
	I	IA	IRBG 228	<i>Aeschynomene pratensis</i>	Laguna, Philippines	IRRI	27
	I	IA	IRBG 210	<i>Aeschynomene indica</i>	Iloilo, Philippines	IRRI	28
	I	IA	IRBG 224	<i>Aeschynomene indica</i>	Iloilo, Philippines	IRRI	29
	I	IA	IRBG 67	<i>Aeschynomene scabra</i>	Laguna, Philippines	IRRI	30
	I	IA	IRBG 230	<i>Aeschynomene nilotica</i>	Laguna, Philippines	IRRI	31
	I	IA	IRBG 118	<i>Aeschynomene sensitiva</i>	Laguna, Philippines	IRRI	32
	I	IA	IRBG 296	<i>Aeschynomene sensitiva</i>	Iloilo, Philippines	IRRI	33
	I	IA	IRBG 294	<i>Aeschynomene sensitiva</i>	Iloilo, Philippines	IRRI	34
	I	IB	A1017	<i>Glycine max</i>	Japan	IB	35
	I	IB	IRBG 271	<i>Aeschynomene fluminensis</i>	Laguna, Philippines	IRRI	36
	I	IB	IRBG 272	<i>Aeschynomene fluminensis</i>	Laguna, Philippines	IRRI	37
	I	IB	USDA 110	<i>Glycine max</i>	United States?	USDA	38
	I	IB	IRBG 301	<i>Aeschynomene fluminensis</i>	Laguna, Philippines	IRRI	39
	I	IB	NIAES 3316	<i>Glycine max</i>	Japan	IB	40
	I	IB	IRBG 270	<i>Aeschynomene fluminensis</i>	Laguna, Philippines	IRRI	41
	I	IB	TAL 835	<i>Macrotyloma africanum</i>	Zimbabwe	NiFTAL	42
	I	IB	LB 5	<i>Acacia mangium</i>	Philippines	BIOTECH	43
	I	IB	LB 6	<i>Acacia mangium</i>	Philippines	BIOTECH	44
	I	IB	TAL 1521	<i>Acacia mangium</i>	Hawaii	NiFTAL	45
	I	IB	TAL 289	<i>Indigofera endecaphylla</i>	Hawaii	NiFTAL	46
	I	IB	TAL 760	<i>Indigofera hirsuta</i>	Mexico	NiFTAL	47
	I	IB	TAL 761	<i>Indigofera hirsuta</i>	Mexico	NiFTAL	48
	I	IB	TAL 290	<i>Indigofera suffruticosa</i>	Hawaii	NiFTAL	49
	I	IB	TAL 350	<i>Indigofera suffruticosa</i>	Hawaii	NiFTAL	50
	I	IB	TAL 169	<i>Vigna unguiculata</i>	Wisconsin	NiFTAL	51
	I	IB	TAL 1038	<i>Indigofera</i> sp.	Kenya	NiFTAL	52
	I	IB	IRBG 309	<i>Crotalaria juncea</i>	India	IRRI	53
	I	IB	TAL 209	<i>Vigna radiata</i>	Thailand	NiFTAL	54
	I	IB	BTAi 1	<i>Aeschynomene indica</i>	United States	BTI	55
	I	IC	IRBG 251	<i>Aeschynomene afraspera</i>	Thailand	IRRI	56
	I	IC	IRBG 274	<i>Aeschynomene afraspera</i>	Thailand	IRRI	57
	I	IC	USDA 31	<i>Glycine max</i>	United States?	USDA	58
	I	IC	USDA 76 ^T	<i>Glycine max</i>	United States?	USDA	59
	I	IC	IRBG 342	<i>Glycine max</i>	Laguna, Philippines	IRRI	60
	I	IC	IRBG 343	<i>Glycine max</i>	Laguna, Philippines	IRRI	61
	I	IC	J501	<i>Glycine max</i>	Japan	IB	62
	I	IC	JIR 33	<i>Glycine max</i>	Japan	IB	63
	I	IC	TAL 1037	<i>Indigofera brevicalyx</i>	Kenya	NiFTAL	64
<i>Rhizobium</i>	II	IIA	IRBG 350	<i>Neptunia oleracea</i>	Maahas, Philippines	IRRI	65
	II	IIA	RG 504 ^T	<i>Galega officinalis</i>	New Zealand	NZP	66
	II	IIB	TAL 1436	<i>Acacia farnesiana</i>	Senegal	NiFTAL	67
	II	IIB	IC 3100	<i>Cajanus cajan</i>	India	ICRISAT	68

Continued on following page

TABLE 1—Continued

Genus	Cluster	Subcluster	Strain	Host	Nodule or strain origin	Source	Reference no.
<i>Azorhizobium</i>	II	IIB	TAL 20	<i>Cajanus cajan</i>	Hawaii	NifTAL	69
	II	IIB	TAL 1113	<i>Sesbania</i> sp.	India	NifTAL	70
	II	IIB	TAL 1115	<i>Sesbania</i> sp.	India	NifTAL	71
	II	IIB	NZP 4017	<i>Medicago sativa</i>	New Zealand	NZP	72
	II	IIB	TAL 380	<i>Medicago sativa</i>	New South Wales, Australia	NifTAL	73
	II	IIB	TAL 1372	<i>Medicago sativa</i>	Brazil	NifTAL	74
	II	IIB	USDA 205 ^T	<i>Glycine sojae</i>	People's Republic of China	USDA	75
	II	IIB	TAL 638	<i>Lens culinaris</i>	Hawaii	NifTAL	76
	II	IIB	TAL 1383	<i>Phaseolus vulgaris</i>	Guatemala	NifTAL	77
	II	IIB	TAL 382	<i>Trifolium semipilosum</i>	Australia	NifTAL	78
	II	IIB	RL 8002	<i>Phaseolus vulgaris</i>	United Kingdom	JII	79
	II	IIB	TAL 182	<i>Phaseolus vulgaris</i>	Hawaii	NifTAL	80
	II	IIB	TAL 674	<i>Sesbania rostrata</i>	Hawaii	NifTAL	81
	II	IIB	TAL 1123	<i>Sesbania macrocarpa</i>	Missouri	NifTAL	82
	II	IIB	TAL 1797	<i>Phaseolus vulgaris</i>	United States	NifTAL	83
	II	IIB	TAL 634	<i>Lathyrus hirsutus</i>	Missouri	NifTAL	84
	II	IIB	TAL 1298	<i>Galega orientalis</i>	Texas	NifTAL	85
	II	IIB	TAL 1768	<i>Gliciridia sepium</i>	Mexico	NifTAL	86
	III		IRBG 32	<i>Sesbania rostrata</i>	Cotabato, Philippines	IRRI	87
	III		IRBG 320	<i>Sesbania rostrata</i>	Maahas, Philippines	IRRI	88
	III		IRBG 313	<i>Sesbania rostrata</i>	India	IRRI	89
	III		IRBG 317	<i>Sesbania rostrata</i>	India	IRRI	90
	III		IRBG 314	<i>Sesbania rostrata</i>	India	IRRI	91
	III		IRBG 316	<i>Sesbania rostrata</i>	India	IRRI	92
	III		IRBG 315	<i>Sesbania rostrata</i>	India	IRRI	93
	III		IRBG 319	<i>Sesbania rostrata</i>	Maahas, Philippines	IRRI	94
	III		IRBG 318	<i>Sesbania rostrata</i>	Maahas, Philippines	IRRI	95
	III		IRBG 44	<i>Sesbania rostrata</i>	Tiaong, Philippines	IRRI	96
	III		ORS 571 ^T	<i>Sesbania rostrata</i>	Senegal	ORSTOM	97
	III		IRBG 9	<i>Sesbania rostrata</i>	Maahas, Philippines	IRRI	98
	III		IRBG 21	<i>Sesbania rostrata</i>	Maligaya, Philippines	IRRI	99
	III		IRBG 45	<i>Sesbania rostrata</i>	Tiaong, Philippines	IRRI	100

^a BIOTECH, National Institutes of Applied Microbiology and Biotechnology, University of the Philippines at Los Baños, Los Baños, Philippines; BTI, Boyce Thompson Research Institute, New York, N.Y.; IB, Ibaraki University Department of Agricultural Chemistry, Ibaraki, Japan; ICRISAT, International Centre for Research in Semi-Arid Tropics, Patancheru, India; IRBG, The International Rice Research Institute Bacterial Germplasm, IRRI, Manila, Philippines; JII, John Innes Institute, Norwich, United Kingdom; NifTAL, Nitrogen Fixation in Tropical Agricultural Legumes, University of Hawaii, Paia, Hawaii; NZP, Culture Collection of the Department for Scientific and Industrial Research, Palmerston North, New Zealand; ORSTOM, Institut Français de Recherche Scientifique Pour le Développement en Coopération, Paris, France; USDA, U.S. Department of Agriculture, Beltsville, Md. T = type strain.

Institute (IRRI) as part of the Biofertilizer Germplasm Collection (30). Most of the strains were characterized in a previous study in which numerous cultural and phenotypic characteristics were determined (16).

Cultural conditions. Mannitol-yeast extract medium was the routine medium used for isolation, purification, and maintenance of rhizobia (16). The photosynthetic rhizobia grew better in a glucose-based medium than in the mannitol-based mannitol-yeast extract medium in which organisms were initially isolated from *Aeschynomene* nodules. Although growth medium has been shown to affect FAME profiles in certain cases (24), preliminary studies showed that the FAME profiles of the photosynthetic bacteria were not significantly different when the organisms were grown in glucose- and mannitol-

based media (Table 2). Hence, glucose-yeast extract medium was used to grow photosynthetic rhizobia and mannitol-yeast extract medium was used to grow nonphotosynthetic rhizobia throughout this study.

FAME analyses. Freeze-dried bacterial cells (approximately 20 mg) were methanolized with 2 M HCl in 5% methanol as previously described (31). The FAME fraction was obtained by extracting the preparation three times with petroleum benzene and then washing the preparation with an equal volume of distilled water. After the solvent phase was separated, it was dried under a stream of nitrogen and redissolved in petroleum benzene. The extract was divided into two portions; the first portion was used to determine the total FAME content, and the second portion was spotted onto a thin-layer chromatog-

TABLE 2. Effects of carbon sources on the FAME compositions of photosynthetic rhizobia

Carbon source	Fatty acid composition (% of total peak area) ^a									
	C _{10:0}	C _{12:0}	C _{14:1}	C _{16:0}	C _{16:1}	C _{17:0}	C _{18:0}	C _{18:1}	C _{18:1} ^b	cyc C ₁₉ ^c
Glucose	0.8 ± 0.46	0.1 ± 0.15	0.2 ± 0.16	18.7 ± 1.46	0.6 ± 0.54	0.1 ± 0.07	0.7 ± 0.92	3.2 ± 2.22	72.9 ± 6.23	3.9 ± 1.21
Mannitol	0.8 ± 1.48	0.3 ± 0.21	0.2 ± 0.39	19.1 ± 7.55	0.5 ± 0.49	0.1 ± 0.08	1.1 ± 1.45	2.0 ± 1.92	74.2 ± 8.25	2.3 ± 1.03

^a The values are means ± standard deviations for two or three determinations. The strains used were strains IRBG 114, IRBG 115, IRBG 122, IRBG 125, IRBG 127, BTAi 1, and ORS 322. The nutrient compositions of the culture media were the same except for the carbon sources (see Materials and Methods).

^b *cis*-9-Octadecenoate (*cis*-vaccenic acid).

^c cyc C_{19:0} was found only in strain BTAi 1.

raphy plate (precoated Silica Gel 60; Merck) to separate the polar and nonpolar FAMES. The plates were developed in an elution tank by using hexane-diethyl ether (90:10, vol/vol) as the mobile phase. The extracts were visualized with UV light and then were eluted with diethyl ether, evaporated to dryness, and redissolved in 100 μ l of diethyl ether.

The FAMES were analyzed with a Hitachi model 263-30 gas chromatograph equipped with a flame ionization detector. The following two types of columns were used: 5% DEGS-PS in Supelcoport 100/200 and 3% SP 2100 DOH in Supelcoport 100/200. The injection and detector temperatures were 250°C. Isothermal and gradient temperature profiles were used for each analysis. The oven temperatures used for isothermal conditions were 190°C for nonpolar column types and 210°C for polar column types. For temperature gradients, the oven temperature was programmed to increase from 150 to 250°C at a ramp rate of 15°C $\cdot$ min⁻¹. Nitrogen was used as the carrier gas at flow rates of 40 and 20 ml⁻¹ min⁻¹ for isothermal conditions and temperature gradients, respectively. FAMES were identified by comparing their retention times with the retention times of known authentic standards and computing the equivalent chain lengths. The identities of some FAMES were confirmed by gas chromatography-mass spectrometry, using a Hewlett-Packard model 5985 gas chromatography-mass spectrometer, an ionizing energy of 70 eV, and a type DB-1 fused silica column, which was subjected to the following temperature program: 50°C for 1 min, increased at a rate of 30°C $\cdot$ min⁻¹ until the temperature was 270°C, and 270°C for 15 min. The gas chromatography-mass spectrometry analyses were carried out by using helium as the carrier gas.

16S rRNA PCR amplification and sequencing. The sequences of portions of the 16S rRNA genes of photosynthetic rhizobial strains IRBG 2, IRBG 230, IRBG 231, and ORS 322 and nonphotosynthetic strains IRBG 274 and IRBG 309 were determined after PCR amplification. The sequences corresponding to nucleotides 44 to 377 in the *Escherichia coli* 16S rRNA sequence (2) were amplified with forward primer Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and reverse primer Y2 (5'-CCCCTGCTGCCTCCCGTAGGAGT-3'), essentially as described by Young et al. (40). DNA amplicons were 312 bp long and were purified by using Qiagen anion-exchange columns (Diagen GmbH, Dusseldorf, Germany). Purified DNA products were sequenced by using the amplifying primers and a T7 sequencing kit (Pharmacia LKB), which yielded sequence information for 264 bases between the primers. Up to 10 bases at the beginning and up to 20 bases at the end were unreadable for certain strains, but an examination of previously published sequences showed that these regions normally provide little useful information.

Data analyses. The peak areas of individual FAMES were expressed as relative peak percentages, which were determined by dividing the area of each FAME by the total peak area of all FAMES. The means, minimums, maximums, and standard deviations were derived from the relative peak percentage values. The mean FAME values used for each strain were derived from replicate values from two or three analyses and were used in the multivariate statistical analyses. Levels of similarity were calculated by using Euclidean distance coefficients derived from the standardized FAME values. Hierarchical clustering was performed by using the mean linkage method (28).

Sequence data for 16S rRNAs were aligned by using the Wisconsin programs (4) and were compared with previously published sequence data for *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium* spp. and photosynthetic bacteria (9, 34, 40). The computer programs used to analyze 16S rRNA data were

NTSYS-pc (Applied Biostatistics, Inc.) and SIMGEND (similarity for genetic data using the Jukes-Cantor method [11]), and a dendrogram was generated by using the NJOIN program (neighbor-joining method of Saitou and Nei [27]). Only positions represented by a known nucleotide in each of the sequences in the alignment were considered in the analysis.

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rRNA sequences of some of the organisms included in this study are as follows: IRBG 2, U04715; IRBG 230, U04719; IRBG 231, U04720; IRBG 274, U04718; IRBG 309, U04717; ORS 322, U04716; BTAi 1, M55492; and *B. elkanii* USDA 76^T, M55490.

RESULTS

FAMES and carbon sources. The use of glucose or mannitol as the carbon source in the culture medium did not affect the FAME profiles of the photosynthetic rhizobia (Table 2). The relative levels of cyc C_{19:0}, a FAME unique to strain BTAi 1 (Fig. 1), also were not affected.

FAME analyses. On the basis of cellular FAME profiles, the 100 rhizobial strains which we studied fell very clearly into three clusters corresponding to the three currently recognized genera, *Bradyrhizobium* (cluster I), *Rhizobium* (cluster II), and *Azorhizobium* (cluster III) (Fig. 2 and Table 1). The photosynthetic rhizobia fell into cluster I together with the bradyrhizobia. Subcluster IA contained only photosynthetic rhizobia and included all of the photosynthetic rhizobia except BTAi 1, which fell into subcluster IB. Subcluster IB also contained the nonphotosynthetic rhizobial strains isolated from *Aeschynomene fluminensis* (IRBG 270, IRBG 271, IRBG 272, and IRBG 301) and strains isolated from a number of other hosts, including *Acacia mangium* (LB5, LB6, and TAL 1521), *Crotalaria juncea* (IRBG 309), *Glycine max* (USDA 110, A1017, and NIAES 3316), *Indigofera endecaphylla* (TAL 289), *Indigofera hirsuta* (TAL 760 and TAL 761), *Indigofera suffruticosa* (TAL 290 and TAL 350), *Indigofera* sp. (TAL 1038), *Macrotyloma africanum* (TAL 835), *Vigna radiata* (TAL 209), and *Vigna unguiculata* (TAL 169). Strain BTAi1 could be differentiated from the other photosynthetic rhizobia by the presence of cyc C_{19:0} and by differences in the amounts of C_{18:0} and C_{18:1} FAMES. A third subcluster, subcluster IC, contained the two nonphotosynthetic rhizobial strains isolated from *Aeschynomene afraspera* (IRBG 251 and IRBG 274), a strain isolated from *Indigofera brevicalyx* (TAL 1037), and six strains symbiotic on *Glycine max* (IRBG 342, IRBG 343, J501, JIR 33, USDA 31, and USDA 76^T [T = type strain]). The latter strains included the type strain of *Bradyrhizobium elkanii*, strain USDA 76.

On the basis of FAME profiles, of all of the fast-growing rhizobia formed cluster II. Two strains, which were isolated from *Galega officinalis* and *Neptunia oleracea*, formed a very distinct subcluster (subcluster IIA), while 20 strains isolated from several legumes (*Acacia farnesiana*, *Cajanus cajan*, *Galega orientalis*, *Gliciridia sepium*, *Glycine sojae*, *Lens culinaris*, *Lathyrus hirsutus*, *Medicago sativa*, *Phaseolus vulgaris*, *Sesbania macrocarpa*, *Sesbania rostrata*, *Sesbania* sp., and *Trifolium semipilosum*) made up subcluster IB.

All of the stem- and root-nodulating azorhizobia (eight strains from The Philippines, five strains from India, and one strain from Senegal) formed another homogeneous cluster, cluster III. The azorhizobia could be distinguished from the stem- and root-nodulating photosynthetic rhizobia by differences in the amounts of some types of FAMES, namely, cyc C_{19:0}, C_{16:0}, C_{18:0}, and 3-OH C_{14:0} (Table 3).

16S rRNA sequence analysis. Figure 3 shows the sequences

FIG. 1. Chromatograms of FAME profiles for photosynthetic rhizobium strain IRBG 2 (from *Aeschynomene afraspera*) (A) and strain BTAi 1 (from *Aeschynomene indica*) (B).

of the 16S rRNA gene fragments for several photosynthetic rhizobium strains isolated from *Aeschynomene* spp. (IRBG 2, IRBG 230, IRBG 231, and ORS 322), nonphotosynthetic *Aeschynomene* rhizobium strain IRBG 274, and *Bradyrhizobium* sp. strain IRBG 309 (isolated from *Crotalaria* sp.), along with previously published sequences for comparison. Three of the photosynthetic rhizobia (IRBG 2, IRBG 230, and ORS 322) had identical sequences, but these organisms differed at two or three nucleotide sites from two other photosynthetic rhizobia, IRBG 231 and BTAi 1, and by up to six nucleotides from the most dissimilar bradyrhizobia (Table 4). On the other hand, all of the photosynthetic rhizobia differed at more than 20 nucleotide sites from *Rhizobium* and *Azorhizobium* spp. The phylogenetic tree in Fig. 4 was obtained by using the neighbor-joining method (27) of the Jukes-Cantor distance matrix for partial 16S rRNA sequences (Table 4). This tree is in good agreement with a previously described phylogeny for the alpha subgroup of the *Proteobacteria* (35). All of the photosynthetic bacteria were found on the *Bradyrhizobium-Rhodospseudomonas palustris* branch.

DISCUSSION

The FAME analysis in this study provided very clear discrimination of the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*. MacKenzie et al. (20) obtained clear separation of the genera *Rhizobium* and *Bradyrhizobium* on the basis of FAME profiles, but were not able to distinguish additional groups at a lower level. Kuykendall et al. (13), using capillary column chromatography, were able to subdivide soybean *Bradyrhizobium* strains and found that their FAME groups correlated with the DNA homology groups of Hollis et al. (8). These authors argued that MacKenzie et al. (20) may have been unable to separate strains at the subgeneric level because they used packed-column chromatography. However, in this study we used packed-column chromatography but distinguished clear subgeneric groups. In our study we included a large number of strains and used a numerical statistical approach.

On the basis of FAME profiles, the *Aeschynomene* rhizobia exhibited much closer affinity with the *Bradyrhizobium* group (cluster I) than with the *Rhizobium* group (cluster II). The absence of cyc C_{19:0} in all of the photosynthetic rhizobium strains (except BTAi 1) (Fig. 1) separated our strains from the nonphotosynthetic rhizobium strains belonging to all three currently recognized genera (Fig. 2). On the other hand, the 3-hydroxy type of FAME, which has been reported to be a characteristic of gram-negative bacteria (12), was found in *Rhizobium* and *Azorhizobium* spp. (this study) but not in photosynthetic rhizobium strains or *Bradyrhizobium* strains (Table 3).

In the *Rhizobium* cluster, the relationships suggested by the results of the FAME comparisons are very similar to the relationships established by other methods. Two examples should serve to illustrate this. The FAME profile of *Rhizobium fredii* USDA 205^T is very similar to the FAME profiles of *Rhizobium meliloti* NZP 4017, TAL 380, and TAL 1372. This finding is consistent with previously published DNA-DNA hybridization data (33), DNA-rRNA hybridization data (10), serological test data (28), and 16S rRNA sequence data (9) and further supports the hypothesis that *Rhizobium fredii* belongs to the genus *Rhizobium* (9) and does not require a new genus, *Sinorhizobium*, as has been proposed by Chen et al. (3). Second, *Rhizobium galegae* RG 504, together with an isolate obtained from *Neptunia* sp., had a FAME profile that placed it well outside subcluster IIB, which included representatives of the species *Rhizobium leguminosarum*, *Rhizobium meliloti*, and

FIG. 2. Dendrogram showing the relationship between the FAME profiles of photosynthetic *Aeschynomene* rhizobia and the FAME profiles of *Bradyrhizobium* strains (cluster I), *Rhizobium* strains (cluster II), and *Azorhizobium* strains (cluster III), determined by using mean average linkage data. Subcluster IA comprises all of the photosynthetic rhizobia, while subclusters IB and IC contain all of the nonphotosynthetic rhizobia except strain BTAi 1, which is in subcluster IB (reference no. 55 [Table 1]).

Rhizobium fredii. This is entirely consistent with the 16S rRNA sequence data (34, 37), which indicated that *Rhizobium galegae* is a rather distantly related member of the genus, and with data from previous studies of *Rhizobium galegae* in which DNA-DNA hybridization, phage typing, cross-inoculation, and phenotypic traits were examined (17-19).

The FAME profiles of the strains in cluster III, which included 14 strains of azorhizobia from three countries, were homogeneous. Our data further confirmed the distinctness of the genus *Azorhizobium* (5). However, strain TAL 674, which was described as an isolate obtained from *S. rostrata*, fell into the *Rhizobium* cluster. Furthermore, we failed to induce stem

TABLE 3. FAME profiles of photosynthetic rhizobia and the genera *Rhizobium*, *Bradyrhizobium*, and *Azorhizobium*

Cluster	Sub-cluster	Parameter ^a	Fatty acid composition (% of total peak area) ^b																				
			C _{10:0}	C _{12:0}	C _{13:0}	C _{13:1}	C _{14:0}	C _{14:1}	C _{15:0}	C _{15:1}	C _{16:0}	C _{16:1}	C _{17:0}	C _{17:1}	C _{18:0}	C _{18:1}	C _{18:1} *	C _{19:0}	C _{19:1}	3-OH C _{14:0}	X1	X2	X3
I	IA	Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	14.57	0.05	0.00	0.00	0.00	0.80	70.59	0.00	0.00	0.00	0.00	0.00	0.00
		Maximum	0.33	0.68	0.00	0.00	0.93	0.00	0.00	0.21	21.52	1.34	0.47	0.85	0.85	7.41	82.44	0.00	0.00	0.00	0.00	0.00	0.00
		Mean	0.02	0.23	0.01	0.00	0.14	0.00	0.05	0.02	17.18	0.59	0.15	0.12	0.58	4.14	76.45	0.00	0.00	0.00	0.00	0.00	0.00
		SD	0.11	0.37	0.01	0.00	0.25	0.00	0.04	0.07	3.11	0.38	0.24	0.34	0.72	2.46	4.60	0.00	0.00	0.00	0.00	0.00	0.00
	IB	Minimum	0.00	0.00	0.00	0.00	0.06	0.00	0.00	0.00	3.11	0.06	0.00	0.00	1.02	0.00	4.60	0.00	0.12	0.00	0.00	0.00	0.00
		Maximum	0.11	0.93	0.00	0.00	0.30	0.07	0.15	0.35	20.54	2.40	0.69	0.97	9.05	2.46	80.79	0.07	7.80	0.00	0.00	0.00	1.50
		Mean	0.00	0.16	0.00	0.00	0.18	0.02	0.01	0.05	16.76	0.78	0.24	0.17	2.33	0.36	76.60	0.01	3.10	0.00	0.00	0.00	0.45
		SD	0.05	0.31	0.00	0.00	0.12	0.02	0.05	0.11	2.62	0.75	0.29	0.32	2.89	0.45	4.09	0.02	2.40	0.00	0.00	0.00	0.67
	IC	Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	17.80	0.20	0.00	0.00	0.26	0.00	46.00	0.00	0.20	0.00	0.00	0.00	0.00
		Maximum	0.89	1.62	0.95	0.34	1.07	0.00	0.44	0.96	25.20	4.60	2.08	0.37	2.60	12.99	74.94	0.10	23.29	0.00	0.00	0.05	7.94
		Mean	0.30	0.15	0.36	0.01	0.27	0.00	0.09	0.22	20.54	0.81	0.72	0.10	1.35	3.44	59.62	0.02	11.06	0.00	0.16	0.00	1.79
		SD	0.28	0.46	0.69	0.11	0.37	0.00	0.14	0.33	2.27	1.33	1.54	0.13	0.87	4.42	8.46	0.03	7.86	0.00	0.64	0.01	2.43
II	IIA	Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	2.27	1.20	0.00	0.00	0.26	0.00	8.46	0.00	0.20	0.00	0.00	0.00	
		Maximum	0.89	1.62	0.95	0.34	1.07	0.00	0.44	0.96	25.20	4.60	2.08	0.37	14.20	12.99	74.94	0.10	28.30	8.82	0.64	0.05	7.94
		Mean	0.17	0.00	0.31	0.06	0.27	0.00	0.00	0.00	16.75	3.47	1.02	0.03	12.38	4.38	26.98	0.01	26.25	5.29	0.00	0.00	3.14
		SD	0.19	0.00	0.36	0.06	0.21	0.00	0.00	0.00	3.08	2.49	0.01	0.05	1.26	3.12	4.45	0.00	4.63	4.25	0.00	0.00	3.46
	IIB	Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	10.72	0.26	0.00	0.18	1.14	0.52	31.74	0.00	11.05	3.55	0.00	0.80	2.05
		Maximum	0.00	0.96	0.00	0.00	1.00	0.00	0.08	0.00	16.05	4.41	0.32	3.07	6.36	4.65	54.17	0.86	23.61	9.00	0.55	6.64	11.45
		Mean	0.00	0.05	0.00	0.00	0.24	0.00	0.00	0.00	11.31	2.78	0.01	2.69	1.47	1.44	47.04	0.39	19.01	6.28	0.07	2.14	5.64
		SD	0.00	0.10	0.00	0.00	0.19	0.00	0.00	0.00	0.47	1.34	0.03	0.43	0.11	0.31	2.64	0.34	3.10	2.62	0.14	0.34	1.63
	III	Minimum	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	3.10	0.23	0.22	0.00	3.43	0.14	37.90	0.00	12.43	0.00	0.00	0.00	2.68
		Maximum	0.00	0.16	0.34	0.00	0.32	0.00	0.00	0.18	5.62	0.66	0.63	0.26	11.00	4.99	60.49	0.40	34.32	0.95	1.58	2.41	11.57
		Mean	0.00	0.05	0.08	0.00	0.09	0.00	0.00	0.02	4.46	0.41	0.41	0.08	7.68	2.35	51.39	0.11	24.00	0.52	0.52	0.37	7.63
		SD	0.00	0.07	0.13	0.00	0.14	0.00	0.00	0.07	0.91	0.12	0.12	0.10	2.15	1.24	6.66	0.15	6.72	0.42	0.69	0.80	2.62

^a The means are means from two or three determinations for each strain.^b The number before the colon indicates the number of carbon atoms in the chain, and the number after the colon indicates the number of double bonds. C_{10:0}, decanoate; C_{12:0}, dodecanoate; C_{13:0}, tridecanoate; C_{13:1}, *cis*-9-tridecanoate; C_{14:0}, tetradecanoate; C_{14:1}, *cis*-9-tetradecanoate; C_{15:0}, pentadecanoate; C_{15:1}, *cis*-9-pentadecanoate; C_{16:0}, hexadecanoate; C_{16:1}, *cis*-9-hexadecanoate; C_{17:0}, heptadecanoate; cyc C_{17:0}, *cis*-9,10-methylenehexadecanoate; C_{18:0}, octadecanoate; C_{18:1}, *trans*-9-octadecanoate; C_{18:1}*, *cis*-9-octadecanoate (*cis*-vaccinic acid); C_{19:0}, nonadecanoate; cyc C_{19:0}, *cis*-9,10-methyleneoctadecanoate; 3-OH C_{14:0}, 3-hydroxytetradecanoate; X1, X2, and X3, unknown peaks.

or root nodules on *S. rostrata* with TAL 674 (data not shown), confirming our previous hypothesis that the culture of TAL 674 that we used is not an *Azorhizobium* culture and probably is a contaminant or is mislabeled (16).

The 16S rRNA data placed all of the photosynthetic rhizobia in the *Bradyrhizobium* group (Fig. 4), but none of the newly studied strains had a sequence identical to the sequence of BTAi 1, which was the only photosynthetic rhizobium studied previously (40). Three strains of photosynthetic rhizobia (IRBG2, IRBG 230, and ORS 322) had identical sequences, even though they were isolated from different host species or at distant geographical locations. The *Bradyrhizobium* cluster is very tight; even when *Rhodopseudomonas palustris* and the photosynthetic rhizobia are included, the most dissimilar members of the cluster are separated by just 12 nucleotide substitutions in the 16S rRNA segment which we studied. For this reason, it is not possible to be very precise about relationships within the cluster, but the branches of the 16S rRNA dendrogram (Fig. 4) correlate remarkably well with groups defined by other criteria and in particular with the groups of soybean bradyrhizobia defined by DNA homology data (8). Five branches of bradyrhizobia can be discerned in Fig. 4. The first branch is the most distantly related and contains *B. elkanii*

USDA 31 and USDA 76^T (i.e., homology group II of the soybean bradyrhizobia [14]) together with IRBG 274, a non-photosynthetic symbiont of *Aeschynomene afraspera*. The second branch contains ATCC 1032, which is the type strain of *Bradyrhizobium japonicum*, and USDA 59, both of which are included in homology group I of soybean symbionts. This branch also contains the nonsymbiotic phototroph *Rhodopseudomonas palustris* (two strains that have the same sequence) and *Crotalaria* symbiont IRBG 309. The third branch contains soybean symbionts belonging to homology group Ia (USDA 110 and RCR 3407), as well as a *Lotus* bradyrhizobium (NZP 2257) and BTAi 1, which is of course both symbiotic and photosynthetic. The last two branches also contain photosynthetic rhizobia; one branch contains IRBG 231 by itself, and the other branch contains three strains that have identical sequences (IRBG 2, IRBG 230, and ORS 322).

The phylogenetic conclusions described above were based on comparisons of a 264-base segment of the 16S rRNA gene, which is a relatively short sequence for this purpose, although it includes some of the most informative parts of the gene. Nevertheless, conclusions based on data from this segment agree very well with other data and have generally been confirmed by data for more extensive 16S rRNA sequences

FIG. 3. Aligned sequences of parts of the 16S rRNAs of strains IRBG 2, IRBG 230, IRBG 231, IRBG 274, IRBG 309, and ORS 322. The corresponding sequences of BTAi 1 and the type strains of *B. japonicum* (4a) and *B. elkanii* are included for comparison. The blank spaces indicate positions where the sequences are identical to the consensus sequence (shown at the top), the dots indicate positions where there are alignment gaps, and N indicates an undetermined base. The nucleotides at positions 221 to 264 are identical in all of the sequences.

	10	20	30	40	50	60
	a g g c t	T A A C A C A T G C A A G T C G A g C G g g C a t a g c a a t a t G l c A G c G G C A G A C G G G T G A G T A				
IRBG 2	N N N N T		G	GG	ATAGCAATAT	TC C
IRBG 230	N N N N T		G	GG	ATAGCAATAT	TC C
ORS 322	N N N N T		G	GG	ATAGCAATAT	TC C
IRBG 231	A G G C T		G	GG	GTAGCAATAC	TC C
IRBG 274	N N N N N		G	GG	ATAGCAATAT	TC C
IRBG 309	N N N N N		G	GG	GTNNNNNTAC	TC C
BTai 1	A G G C T		G	GG	GTAGCAATAC	TC C
<i>Azorhizobium caulinodans</i> T	A G G C T		A	GG	CC . TTC . . GG	TC T
<i>Bradyrhizobium elkanii</i> T	A G G C T		G	GG	ATAGCAATAT	TC C
<i>B. japonicum</i>	A G G C T		G	GG	GTAGCAATAC	TC C
<i>Rhizobium leguminosarum</i>	A G G C T		G	. C	CC . GCAA . GG	G . C
<i>R. fredii</i> T	A G G C T		G	. C	CC . GCAA . GG	G . C
<i>R. meliloti</i> T	A G G C T		G	. C	CC . GCAA . GG	G . C
<i>Agrobacterium tumefaciens</i> T	A G G C T		A	. C	CC . GCAA . GG	G . T

	70	80	90	100	110	120
	A C G C G T G G G A A c g T a C C t T t t g g T t C G G A A c A a C a c a G G G A A A c T t g t g c T A A T A C C G g A					
IRBG 2		CG A T TTGG T		C A ACA		TGTGC G
IRBG 230		CG A T TTGG T		C A ACA		TGTGC G
ORS 322		CG A T TTGG T		C A ACA		TGTGC G
IRBG 231		CG A T TTGG T		C A ACA		TGTGC G
IRBG 274		CG A T TTGG T		C A TGA		TCAGC G
IRBG 309		CG A T TTGG T		C A ACA		TGTGC G
BTai 1		CG A T TTGG T		C A ACA		TGTGC G
<i>Azorhizobium caulinodans</i> T		CG G C TCAG T		T A CCA		TGGGC G
<i>Bradyrhizobium elkanii</i> T		CG A T TTGG T		C A TGA		TCAGC G
<i>B. japonicum</i>		CG A T TTGG T		C A ACA		TGTGC G
<i>Rhizobium leguminosarum</i>		TC A C TGAC A		T A GCA		TGTGC T
<i>R. fredii</i> T		TC A C TTTC A		T A GCA		TGTGC T
<i>R. meliloti</i> T		TC A C TTTC A		T A GCA		TGTGC T
<i>Agrobacterium tumefaciens</i> T		TC A G GCCC G		T G TCC		GGAAT C

	130	140	150	160	170	180
	T a a G c C C t t a c G G g G A A A G A T T t A T C G c c g a A a G A T c g G C C C G C G T c t G A T T A G C T A G T T					
IRBG 2	AA C TTAC G		CCGA A	CG		CT
IRBG 230	AA C TTAC G		CCGA A	CG		CT
ORS 322	AA C TTAC G		CCGA A	CG		CT
IRBG 231	AA C TTAC G		CCGA A	CG		CT
IRBG 274	AA C TTAC G		CCGA A	CG		CT
IRBG 309	AA C TTAC G		CCGA A	CG		CT
BTai 1	AA C TTAC G		CCGA A	CG		CT
<i>Azorhizobium caulinodans</i> T	AC T GAAA A		G	CTGA G	CG	CT
<i>Bradyrhizobium elkanii</i> T	AA C TTAC G		T	CCGA A	CG	CT
<i>B. japonicum</i>	AA C TTAC G		T	CCGA A	CG	CT
<i>Rhizobium leguminosarum</i>	GT T TTCG A		T	GTCA G	GA	TG
<i>R. fredii</i> T	GA C TTCG G		T	GGAA G	GA	TG
<i>R. meliloti</i> T	GA C TTCG G		T	GGAA G	GA	TG
<i>Agrobacterium tumefaciens</i> T	AC C TACG G		T	GGGT T	GA	TG

	190	200	210	220
	G G T g g G T A A t G G C c c A C C A A G G C G A C G A T C a g T A G C T G G			
IRBG 2	GG	T CC		AG
IRBG 230	GG	T CC		AG
ORS 322	GG	T CC		AG
IRBG 231	GG	T CC		AG
IRBG 274	GA	T TC		AG
IRBG 309	GA	T TC		AG
BTai 1	AG	T CT		AG
<i>Azorhizobium caulinodans</i> T	GA	T TC		AG
<i>Bradyrhizobium elkanii</i> T	GA	T TC		AG
<i>B. japonicum</i>	GA	T TC		AG
<i>Rhizobium leguminosarum</i>	GG	A CT		CA
<i>R. fredii</i> T	GG	A CT		CA
<i>R. meliloti</i> T	GG	A CT		CA
<i>Agrobacterium tumefaciens</i> T	GG	A CT		CA

230 239 250 260
T C T G A G A G G A T G A T C A G C C A C A T T G G G A C T G A G A C A C G G C C C A A

TABLE 4. Nucleotide sequence distance matrix

		No. of nucleotide differences or Jukes-Cantor evolutionary distance ^a																				
sm		IRBG 2	IRBG 230	ORS 322	IRBG 231	BTAl 1	IRBG 274	IRBG 309	<i>B. elkanii</i> USDA 31	<i>B. elkanii</i> USDA 76 ^T	<i>B. japonicum</i> USDA 59	<i>B. japonicum</i> USDA 110	<i>B. japonicum</i> NZP 2257	<i>B. japonicum</i> USDA 3407	<i>Azorhizobium caulinodans</i> ^T	<i>Rhizobium leguminosarum</i>	<i>Rhizobium meliloti</i>	<i>Rhizobium fredii</i> ^T	<i>Rhodospirillum rubrum</i>	<i>Agrobacterium tumefaciens</i> ^T	<i>Erythrobacter longus</i>	
	31 ^c	0.000	0	0	2	4	5	4	5	5	4	5	6	5	25	33	29	29	38	39	33	5
	76 ^c	0.000	0.000	0	2	4	6	4	5	5	4	5	6	4	25	33	29	29	37	40	33	5
	DA 59 ^c	0.008	0.008	0.008	2	3	6	4	5	5	4	5	6	4	25	33	29	29	38	38	33	5
	DA 110 ^{c,d}	0.008	0.008	0.008	2	8	2	7	7	7	2	3	4	4	25	33	32	29	38	38	32	3
	P 2257 ^c	0.012	0.012	0.008	0.004	2	8	4	7	8	4	2	3	3	26	34	30	30	38	39	34	2
	DA 3407 ^c	0.025	0.025	0.020	0.033	0.037	8	5	0	0	5	11	12	10	23	38	34	34	41	40	35	7
	<i>linodans</i> ^{Tc}	0.016	0.016	0.025	0.008	0.012	0.025	5	5	5	0	6	7	7	23	35	31	31	40	40	34	2
	<i>inosarum</i> ^c	0.033	0.033	0.033	0.031	0.037	0.000	0.033	0	0	5	10	10	9	22	38	33	33	40	40	34	6
	<i>brum</i> ^c	0.002	0.025	0.025	0.033	0.037	0.000	0.025	0.000	0.025	5	10	11	8	22	37	33	33	40	40	35	6
	<i>nefaciens</i> ^{Tc}	0.016	0.016	0.016	0.008	0.012	0.025	0.000	0.025	0.046	0.020	5	5	6	23	35	31	31	35	40	33	1
	<i>guis</i> ^c	0.020	0.020	0.020	0.012	0.008	0.046	0.020	0.046	0.050	0.041	0.029	3	0	28	33	29	29	37	41	33	4
	<i>palustris</i> ^{Tc}	0.025	0.025	0.025	0.016	0.012	0.050	0.025	0.041	0.050	0.025	0.012	0.020	4	28	34	30	28	41	38	34	5
	<i>eroides</i> ^c	0.020	0.020	0.020	0.020	0.016	0.041	0.029	0.050	0.041	0.029	0.000	0.020		27	33	30	29	34	38	31	5
		0.108	0.108	0.108	0.108	0.113	0.099	0.099	0.099	0.099	0.099	0.122	0.127	0.122	0.151	33	37	37	40	39	40	24
		0.156	0.156	0.150	0.156	0.161	0.177	0.166	0.177	0.177	0.166	0.156	0.156	0.151	0.151	0.029	6	7	46	27	35	36
		0.131	0.137	0.137	0.137	0.142	0.156	0.147	0.156	0.156	0.147	0.137	0.137	0.132	0.166	0.029	0	0	46	23	35	30
		0.131	0.137	0.137	0.137	0.142	0.156	0.147	0.156	0.156	0.147	0.137	0.137	0.132	0.166	0.029	0.000	0.224	46	23	35	30
		0.182	0.182	0.182	0.182	0.187	0.197	0.192	0.197	0.197	0.197	0.187	0.202	0.187	0.171	0.219	0.224	0.085	38	46	38	38
		0.187	0.187	0.187	0.187	0.192	0.192	0.192	0.192	0.192	0.197	0.187	0.187	0.187	0.182	0.104	0.085	0.085	0.192	0.192	0.187	34
		0.177	0.177	0.177	0.177	0.182	0.182	0.187	0.182	0.182	0.187	0.187	0.182	0.177	0.197	0.182	0.187	0.187	0.208	0.192	0.187	0.187
		0.025	0.025	0.025	0.016	0.012	0.033	0.008	0.033	0.033	0.008	0.020	0.025	0.029	0.097	0.177	0.156	0.156	0.192	0.187	0.187	0.187
		0.127	0.127	0.127	0.127	0.132	0.142	0.137	0.142	0.142	0.137	0.132	0.137	0.132	0.161	0.197	0.182	0.182	0.240	0.229	0.252	0.1

the upper right are the numbers of nucleotide differences, and the values on the lower left are Jukes-Cantor evolutionary distances. T = type strain.

A sequence was determined in this study.

A sequence was obtained from reference 40.

A sequence was identical to the 16S rRNA sequence of the type strain.

A sequence was obtained from reference 9.

FIG. 4. Phylogenetic tree derived from 16S rRNA gene fragment data. The tree was constructed by using the neighbor-joining method and the distance matrix data in Table 4. Bar = 2% sequence divergence.

when such data are available. In particular, the close relationship between *Rhodopseudomonas palustris* and *B. japonicum* was clear when longer sequences were obtained for two of the photosynthetic rhizobia considered in this study (BTai 1 and IRBG 230) and for a strain isolated from *Aeschynomene aspera* (MKAa2), and these data confirmed the close relationship with the genus *Bradyrhizobium* (36a). There is not sufficient variation in the 264-base segment to permit reliable conclusions concerning the root or exact branching order within this group of organisms, but in fact the members of the group are so closely related that even almost full-length 16S rRNA sequences are not conclusive (unpublished data from bootstrap analyses of previously published data). In any case, our major conclusion is very robust and is that all of the photosynthetic rhizobia examined in this study are very closely related to the genus *Bradyrhizobium* and distantly related to the genera *Rhizobium* and *Azorhizobium*; this finding is clearly inconsistent with the clustering data derived from numerical taxonomy (16).

Overall, the agreement between the results of the FAME analysis and the 16S rRNA sequence analysis was excellent. Not only were the genera *Bradyrhizobium*, *Rhizobium*, and *Azorhizobium* readily separated, but some of the relationships within these genera were also supported by both lines of evidence. Within the genus *Bradyrhizobium*, *B. elkanii* and IRBG 274 form a very distinct subgroup, and most of the photosynthetic bradyrhizobia also form a coherent subgroup; the exception is BTai 1, which is more closely related to *B. japonicum* and other nonphotosynthetic bradyrhizobia. The results of both techniques also indicate that *Rhizobium meliloti* and *Rhizobium fredii* are very similar and that *Rhizobium leguminosarum* is much more closely related to these taxa than *Rhizobium galegae* is. It is widely accepted that rRNA sequences provide some of the most reliable evidence available for determining phylogenetic relationships (35), but it is always valuable to have independent support. The close agreement between the conclusions from the 16S rRNA sequence and FAME profile analyses shows that fatty acids not only are a useful tool for determining the taxonomic positions of bacteria (12), including members of the family *Rhizobiaceae* (20, 38),

but also provide a good guide to phylogenetic relationships (1, 21–23).

Although the FAME and 16S rRNA data described above are in good agreement in indicating that the photosynthetic rhizobia belong in the genus *Bradyrhizobium*, perhaps as one or more new species, there is an apparent conflict between this conclusion and results obtained from numerical taxonomy. In a separate study of many of the same strains, in which a large number of phenotypic characteristics were used, we found that the photosynthetic rhizobia belong to a phenon that is separate from the genera *Bradyrhizobium*, *Azorhizobium*, and *Rhizobium* (16). A dendrogram derived by mean linkage cluster analysis showed that the phenon containing the photosynthetic rhizobia is linked to the genera *Azorhizobium* and *Rhizobium*. However, this does not necessarily mean that the photosynthetic rhizobia are more closely related to the genera *Azorhizobium* and *Rhizobium* than to the genus *Bradyrhizobium*. The cluster analysis of phenotypic data was not explicitly designed to reveal evolutionary relationships, although in the absence of other information taxonomists often have used such analyses to define taxonomic groups. The problems encountered with the use of phenotypic data for this purpose include the lack of reliability of properties that are variable or unstable in culture, the fact that properties may evolve in parallel or convergently in different lineages in response to similar selection pressures, the absence of any objective method for weighting different characters, and the different results produced by alternative clustering algorithms. Phenotypic clustering neither equates directly with phylogenetic grouping (29) nor takes into account the origin of the resemblance or the rate of change of resemblance (ancestor-descendant relationship). This is not the first time that a disparity between phylogenetic and phenotypic data has been found in studies of the rhizobia. As determined by phenetic analysis, the fast-growing symbionts of soybeans were sufficiently distinct from the previously described *Rhizobium* species that a new genus, *Sinorhizobium*, seemed necessary and appropriate (3), but in terms of rRNA data and certain other characteristics (including the FAME profiles reported in this paper), the same isolates are so similar to *Rhizobium meliloti* that they can scarcely be distinguished (9).

The current practice in bacterial systematics is to take a "polyphasic" approach, that is, to put together evidence of a variety of different kinds into a composite assessment of relationships. However, it has been recognized that evidence from sequence comparisons is particularly powerful and is likely to provide the best guide to evolutionary relationships in cases where different lines of evidence point to different conclusions (7, 25, 32). On balance, therefore, it seems likely that the photosynthetic rhizobia do belong in the *Bradyrhizobium* cluster, forming a distinct group within it (with the exception of BTai 1). This group probably deserves species status, but it would be valuable to have DNA-DNA hybridization data before a formal proposal is made.

We are still left with some interesting general questions posed by the apparent conflict between sequence and FAME data on the one hand and numerical taxonomy on the other. Should this example, and the comparable case of the genus *Sinorhizobium*, persuade us that numerical taxonomy based on phenotypic characteristics does not provide reliable evidence concerning evolutionary relationships? Or should it instead remind us that sequence data do not by themselves tell us everything (or even anything) that is interesting about the biology of organisms? Both views are probably true.

ACKNOWLEDGMENTS

We thank Maggie Knox for training R.B.S. in PCR and DNA sequencing techniques, Helen Downer for help with the University of Wisconsin Genetics Computer Group, software, and the John Innes Institute (United Kingdom) for supplies and facilities. Reference rhizobia were provided by the NifTAL Project at Paia, Hawaii, and Bernard Dreyfus provided *Azorhizobium caulinodans* ORS 571.

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