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MULTILOCUS SEQUENCE ANALYSIS (MLSA) OF *BRADYRHIZOBIUM* STRAINS: REVEALING HIGH DIVERSITY OF TROPICAL DIAZOTROPHIC SYMBIOTIC BACTERIA

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ABSTRACT

Symbiotic association of several genera of bacteria collectively called as rhizobia and plants belonging to the family Leguminosae (=Fabaceae) results in the process of biological nitrogen fixation, playing a key role in global N cycling, and also bringing relevant contributions to the agriculture. *Bradyrhizobium* is considered as the ancestral of all nitrogen-fixing rhizobial species, probably originated in the tropics. The genus encompasses a variety of diverse bacteria, but the diversity captured in the analysis of the 16S rRNA is often low. In this study, we analyzed twelve *Bradyrhizobium* strains selected from previous studies performed by our group for showing high genetic diversity in relation to the described species. In addition to the 16S rRNA, five housekeeping genes (*recA*, *atpD*, *glnII*, *gyrB* and *rpoB*) were analyzed in the MLSA (multilocus sequence analysis) approach. Analysis of each gene and of the concatenated housekeeping genes captured a considerably higher level of genetic diversity, with indication of putative new species. The results highlight the high genetic variability associated with *Bradyrhizobium* microsymbionts of a variety of legumes. In addition, the MLSA approach has proved to represent a rapid and reliable method to be employed in phylogenetic and taxonomic studies, speeding the identification of the still poorly known diversity of nitrogen-fixing rhizobia in the tropics.

Key words: Biological nitrogen fixation; *Bradyrhizobium*; multilocus sequence analysis; phylogeny; taxonomy

INTRODUCTION

Species of the large family Leguminosae (Fabaceae in the USA) occupy a broad-range of terrestrial biomes, with several of them being capable of establishing symbioses with bacteria collectively called rhizobia, starting the process of fixing atmospheric nitrogen (N_2). Diazotrophic bacteria were first isolated from root nodules and characterized in 1888 by Martinus Willem Beijerinck (2) as *Bacillus radicicola*, but then reclassified into the genus *Rhizobium*, with the taxonomy based

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on nodulation with certain host plants, establishing the "cross-inoculation group" concept (11). Taxonomical classification remained unaltered until the early 1980s, when numerical taxonomy considering morphological, physiological and genetic patterns led to the positioning of some strains into a new genus, *Bradyrhizobium*, with a unique defined species, *B. japonicum* (18). *Bradyrhizobium* includes slow growers that produce alkaline reaction in culture medium with mannitol as carbon source, while *Rhizobium* contains fast growing acid producers (18, 19).

A few years later, high diversity among *Bradyrhizobium* strains led to the description of a new species named *Bradyrhizobium elkanii* (23); however, the development of several molecular techniques helped to identify high genetic diversity among *Bradyrhizobium* strains isolated from a wide range of leguminous plants (e.g., 13, 28, 29, 46). Nowadays, there are nine defined *Bradyrhizobium* species (7), but certainly many more should be described in the next years.

Sequencing of the 16S rRNA has become the method of choice for tracing bacterial phylogenies (12, 50, 52), but in several genera including *Bradyrhizobium* variability in the 16S rRNA is often low and may not reflect the diversity detected by other morpho-physiological and genetic properties (e.g., 13, 28, 42, 46, 51). To detect higher diversity, other ribosomal genes or regions have been chosen, as the 23S rRNA and the 16S-23S rRNA intergenic transcribed spacer (ITS), since they evolve at a faster rate than the 16S rRNA, thus adding valuable information to the analysis (e.g., 29, 41, 42, 46, 51). However, proximity of ribosomal genes might not properly indicate the correct phylogeny in the case of horizontal gene transfer (43).

The multilocus sequence analysis (MLSA) method has been increasingly used in phylogeny and taxonomy studies. The method consists of the analysis of several conserved housekeeping genes dispersed in at least 100 kb of the genome (4, 6, 15, 25). Successful definitions of phylogenetic groups of rhizobia have also been achieved with the use of the MLSA (29, 31, 36).

Tropical rhizobia represent a key component for the sustainability of tropical soils, and the few results obtained so far clearly indicate that diversity is largely underestimated within the *Bradyrhizobium* genus (13, 28, 29). Therefore in this study the MLSA approach was applied to achieve a better phylogenetic resolution of twelve strains showing high genetic diversity of the 16S rRNA gene in relation to the described *Bradyrhizobium* species. The objective is to delineate strategies which may help to more promptly define diversity of tropical *Bradyrhizobium*.

MATERIALS AND METHODS

Strains

Twelve *Bradyrhizobium* strains were used in this study and are listed in Table 1. The strains were chosen from previous studies from our group (3, 13, 28, 29, 37), based on the high level of genetic diversity observed in comparison to the described species of *Bradyrhizobium*. The strains have been isolated from members of two subfamilies and five tribes of the family Leguminosae, and originated from four countries located in different continents: Australia, Brazil, Malaysia and Zimbabwe (Table 1). The strains were purified on yeast extract-mannitol agar (YMA) medium (45) containing Congo red (0.00125%) and stocks were prepared on YMA and kept at -80^{o}C (under 30% of glycerol) for long-term storage and at 4^{o}C as source cultures. The strains are deposited at the ''Culture Collection of Diazotrophic and PGPR Bacteria" of Embrapa Soja (http://www.bmrc.lncc.br) and at the Brazilian official culture collection "SEMIA" of rhizobial strains (IBP World Catalogue of *Rhizobium* Collections nº443 in the WFCC World Data Center on Microorganisms).

Table 1. Information about the *Bradyrhizobium* strains from the Embrapa Soybean culture collection used in this study

SEMIA number	Other designations[a]	Origin of the strain	Country of origin	Host species[b]	Subfamily[b]	Tribe[b]	Previous studies[c]
656	SEMIA original, CNPSo 988	FEPAGRO	Brazil	*Neonotonia wightii* (Wight & Arn.) Lackey	Papilionoideae	Phaseoleae	a, b, d
662	CB 188, CNPSo 990	CSIRO	Australia	*Vigna unguiculata* (L.) Walp	Papilionoideae	Phaseoleae	a, b, d
696	CB 627, CNPSo 993	CSIRO	Australia	*Desmodium uncinatum* (Jacq.) DC	Papilionoideae	Demodieae	a, b, d, e
6002	CB 756, TAL 309, RCR 3824, CNPSo 1092	CSIRO	Zimbabwe	*Vigna unguiculata* (L.) Walp	Papilionoideae	Phaseoleae	a, b, d
6028	TAL 569, SPRL 472, MAR 472, CNPSo 1094	NIFTAL	Zimbabwe	*Desmodium uncinatum* (Jacq.)DC	Papilionoideae	Demodieae	a, b, d
6053	TAL 827, UMKL 28, CNPSo 1095	NIFTAL	Malaysia	*Clitoria ternatea* L.	Papilionoideae	Phaseoleae	a, b, d, e
6144	SMS 400, USDA 3187, MAR 11, CNPSo 1109	IAC	Zimbabwe	*Arachis hypogaea* L.	Papilionoideae	Aeschynomeneae	a, b, d
6145	BR 2001, CNPSo 1110	Embrapa Agrobiologia	Brazil	*Crotalaria juncea* L.	Papilionoideae	Crotalarieae	a, b, d
6148	SMS 303, CNPSo 1112	IAC	Brazil	*Neonotonia wightii* (Wight & Arn.) Lackey	Papilionoideae	Phaseoleae	a, b, d
6154	BR 446, CNPSo 1117	Embrapa Agrobiologia	Brazil	*Stylosanthes* spp.	Papilionoideae	Aeschynomeneae	a, c, e
6160	BR 5610, CNPSo 1123	Embrapa Agrobiologia	Brazil	*Albizia lebbeck* (L.) Benth.	Mimosoideae	Ingeae	a, b, d
6395	BR 4301, CNPSo 1161	Embrapa Agrobiologia	Brazil	*Calliandra houstoniana* (Mill.) Standl.	Mimosoideae	Ingeae	c

[a] Culture collections: BR (Brazil, Embrapa Agrobiologia, Seropédica, Brazil); CB (Commonwealth Scientific and Industrial [a] Research Organization – CSIRO, Canberra, Australia); CNPSo (Centro Nacional de Pesquisa de Soja, Brazil); MAR (Marondera, Grasslands *Rhizobium* Collection, Soil Productivity Research Laboratory, Marondera, Zimbabwe; also called SPRL); SEMIA (Seção de Microbiologia Agrícola, FEPAGRO, Porto Alegre, Brazil); SMS (Seção de Microbiologia do Solo, IAC, Campinas, Brazil); TAL (NifTAL, Nitrogen Fixation by Tropical Agricultural Legumes Project, University of Hawaii, Paia, USA); USDA (United States Department of Agriculture, Beltsville, USA);[b] Taxonomy based on ILDIS (www.ildis.org);[c] Previous studies with the strains: (a) Germano *et al*. (2006); (b) Menna *et al*. (2006); (c) Binde *et al*. (2009); (d) Menna *et al*. (2009); (e) Roma Neto *et al*. (2010).

DNA extraction and sequencing analysis of the 16S rRNA

The DNA samples were extracted from bacterial batch cultures grown in YM broth until late exponential phase (10^9 cells mL^{-1}) as described before (20). The DNAs were submitted to the amplification with primers for the 16S rRNA, *recA*, *atpD, glnII, gyrB* and *rpoB* genes, as listed in Table 2. The PCR products were purified with the PureLink™ PCR Purification Kit (Invitrogen), and the reactions for the sequencing analysis contained purified PCR products of each bacterium culture (80 ng per reaction), 3 µL of dye (DYEnamic ET terminator reagent premix for the MEGA BACE) and 3 pmol of each primer (Table 2). The conditions of PCR amplification were as described by Menna *et al*. (28). The sequencing was performed on a MEGA BACE 1000 (Amersham Biosciences) capillary sequencer according to the manufacturer's instructions.

Table 2. Primers and DNA amplification conditions used in this study

Primer	Sequence (5`- 3`)[a]	Target gene (position) †	PCR cycling	Reference
TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	*recA* (8-32)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	Stepkowski *et al*. (2005)
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	*recA* (620-594)		
TSatpDf	TCTGGTCCGYGGCCAGGAAG	*atpD* (189-208)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	Stepkowski *et al*. (2005)
TSatpDr	CGACACTTCCGARCCSGCCTG	*atpD* (804-784)		
TSglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	*glnII* (13-38	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	Stepkowski *et al*. (2005)
TSglnIIr	SGAGCCGTTCCAGTCGGTGTCG	*glnII* (681-660)		
gyrB343F	TTCGACCAGAAYTCCTAYAAGG	*gyrB* (343-364)	5 min 95°C, 5X (2 min 94°C, 2 min 58°C, 1 min 72°C) 28 X (30s 94°C, 1 min 58°C, 1 min 72°C and 5 min 72°C.	Martens *et al*. (2008)
gyrB1043R	AGCTTGTCCTTSGTCTGCG	*gyrB* (1061-1043)		
rpoB83F	CCTSATCGAGGTTCACAGAAGGC	*rpoB* (83-103)	5 min 95°C, 3X (2 min 94°C, 2 min 58°C, 1 min 72°C) 30 X (30s 94°C, 1 min 58°C, 1 min 72°C and 5 min 72°C.	Martens *et al*. (2008)
rpoB1061R	AGCGTGTTGCGGATATAGGCG	*rpoB* (1081-1061)		
fD1	AGAGTTTGATCCTGGCTCAG	16S rRNA (9-29)	2 min 95°C, 30 X (15s 94°C, 45s 93°C, 45s 55°C, 2 min 72°C and 5 min 72°C.	Weisburg *et al*. (1991)
rD1	CTTAAGGAGGTGATCCAGCC	16S rRNA (1474-1494)		

[a] Mixtures of bases used at certain positions are given as: K, T or G; S, G or C; Y, C or T; R, A or G; M, A or C
† Position of the primer in the corresponding sequence of *Bradyrhizobium japonicum* USDA 110

Cluster analyses

The 16S rRNA, *recA*, *atpD, glnII, gyrB* and *rpoB* sequences generated were analyzed with the programs Phred (8, 9), Phrap (http://www.phrap.org) and Consed (16). The consensus sequences obtained and confirmed in the 5´ and 3´ directions were submitted to the GenBank database and received the accession numbers listed in Table 3. Some genes have been previously sequenced by our group, but were re-sequenced in this study; as the identities were confirmed in 100% of the nucleotide bases, the original accession numbers were maintained (Table 3). Sequences for other reference/type strains were retrieved from the GenBank database and are also listed in Table 3. *Caulobacter crescentus* strain CB 15 (genome, AE005673), was used as an outgroup.

The MLSA analysis was performed considering only the complete aligned sequences (size among parenthesis) obtained for the *Bradyrhizobium* strains and for the type/reference strains retrieved from GenBank: 16S rDNA (1,347 bp), *recA* (293 bp), *atpD* (395 bp), *glnII* (442 bp), *gyrB* (434 bp) and *rpoB* (395 bp).

All sequences obtained in this study or retrieved from GenBank were analyzed individually and concatenated using the MEGA (Molecular Evolutionary Genetics Analysis) software version 4.0 with the default parameters, K2P distance model (21), and the Neighbor-Joining algorithm (38). Statistical support for tree nodes was evaluated by bootstrap (10) analyses with 1000 samplings (17).

Table 3. GenBank/EMBL/DDBJ accession numbers for the sequences of the *Bradyrhizobium* strains used in this study and of the reference/type strains

Strain	6S rRNA	*recA*	*atpD*	*glnII*	*gyrB*	*rpoB*
			Bradyrhizobium strains			
SEMIA 656	AY904732[a]	FJ391146[c]	FJ390946[c]	FJ391026[c]	HQ634882[e]	HQ634901[e]
SEMIA 662	AY904734[a]	HQ634894[e]	HQ634871[e]	HQ634877[e]	HQ634883[e]	HQ634902[e]
SEMIA 696	AY904736[a]	HQ634895[e]		GQ160506[d]	HQ634884[e]	HQ634903[e]
SEMIA 6002	AY904743[a]	HQ634896[e]	HQ634872[e]	HQ634878[e]	HQ634885[e]	HQ634904[e]
SEMIA 6028	AY904744[a]	FJ391159[c]	FJ390959[c]	FJ391039[c]	HQ634886[e]	HQ634905[e]
SEMIA 6053	AY904745[a]	FJ391160[c]	FJ390960[c]	FJ391040[c]	HQ634887[e]	HQ634906[e]
SEMIA 6144	AY904750[a]	HQ634897[e]	HQ634873[e]	HQ634879[e]	HQ634888[e]	HQ634907[e]
SEMIA 6145	AY904751[a]	HQ634898[e]	HQ634874[e]	HQ634880[e]	HQ634889[e]	HQ634908[e]
SEMIA 6148	AY904753[a]	FJ391168[c]	FJ390968[c]	FJ391048[c]	HQ634890[e]	HQ634909[e]
SEMIA 6154	FJ025100[b]	HQ634899[e]	HQ634875[e]	GQ160500[d]	HQ634891[e]	HQ634910[e]
SEMIA 6160	AY904762[a]	FJ391171[c]	FJ390971[c]	FJ391051[c]	HQ634892[e]	HQ634911[e]
SEMIA 6395	FJ025101[b]	HQ634900[e]	HQ634876[e]	HQ634881[e]	HQ634893[e]	HQ634912[e]
			Reference/type strains			
B. betae LMG 21987^T	AY372184[f]	AB353734[f]	FM253129[f]	AB353733[f]	FM253217[f]	FM253260[f]
B. jicamae PAC 68^T	AY624134[f]	HM047133[f]	FJ428211[f]	FJ428204[f]	HQ873309[f]	HQ587647[f]
B. pachyrhizi PAC 48^T	AY624135[f]	HM047130[f]	FJ428208[f]	FJ428201[f]	HQ873310[f]	HQ587648[f]
B. canariense LMG 22265^T	AJ558025[f]	FM253177[f]	AY386739[f]	AY386765[f]	FM253220[f]	FM253263[f]
B. yuanmingense LMG 21827^T	AF193818[f]	AM168343[f]	AY386760[f]	AY386780[f]	FM253226[f]	FM253269[f]
B. liaoningense LMG 18230^T	AF208513[f]	AY591564[f]	AY386752[f]	AY386775[f]	FM253223[f]	FM253266[f]
B. elkanii USDA 76^T	U35000[f]	AY591568[f]	AY386758[f]	AY599117[f]	AM418800[f]	AM295348[f]
B. japonicum USDA 6^T	X66024[f]	AM182158[f]	AM168320[f]	AF169582[f]	AM418801[f]	AM295349[f]
B. iriomotense EK05^T	AB300992[f]	AB300996[f]	AB300994[f]	AB300995[f]	AB300997[f]	HQ587646[f]
Rhizobium tropici CIAT 899^T	EU488752[f]	EU488815[f]	AM418789[f]	EU488791[f]	AM418836[f]	AM295354[f]
Rhizobium etli CFN 42^T	EU488751[f]	EU488824[f]	$^+$NC_007761[f]	EU488776[f]	$^+$NC_007761[f]	$^+$NC_007761[f]
Rhizobium rhizogenes ATCC 11325^T	AY945955[f]	AM182126[f]	AM418786[f]	FJ816281[f]	AM418833[f]	AM295353[f]
Mesorhizobium loti LMG 6125^T	X67229[f]	AM182156[f]	AM946552[f]	AF169581[f]	EU273810[f]	$^+$A000012.4[f]
Mesorhizobium huakuii USDA 4779^T	D13431[f]	AJ294370[f]	AJ294394[f]	AF169588[f]	AM076344[f]	FJ393283[f]

[a] From the study by Menna *et al.* (2006); [b] From the study by Binde *et al.* (2009); [c] From the study by Menna *et al.* (2009); [d] From the study by Roma Neto *et al.* (2010); [e] From this study; [f] From the GenBank (www.ncbi.nlm.nih.gov); [+] Genome.

RESULTS

Diversity in the 16S rRNA

The phylogenetic tree built with the 16S rRNA sequences split the *Bradyrhizobium* strains in two large groups, with final bootstrap supports for each group of 70 and 94%, respectively (Fig. 1). The first group (G-I) comprised eight SEMIA strains, all grouped with *B. elkanii* and also with *B. pachyrhizi* and *B. jicamae*. The second group (G-II) included SEMIAs 6395, 656, 6002 and 6144 showing closer relation with *B. iriomotense*, in addition to reference/type strains of *B. betae*, *B. canariense*, *B. yuanmingense*, *B. liaoningense* and *B. japonicum*. Fig. 1 also highlights that the clustering analysis based on the 16S rRNA gene did not define clear positions for the SEMIA strains.

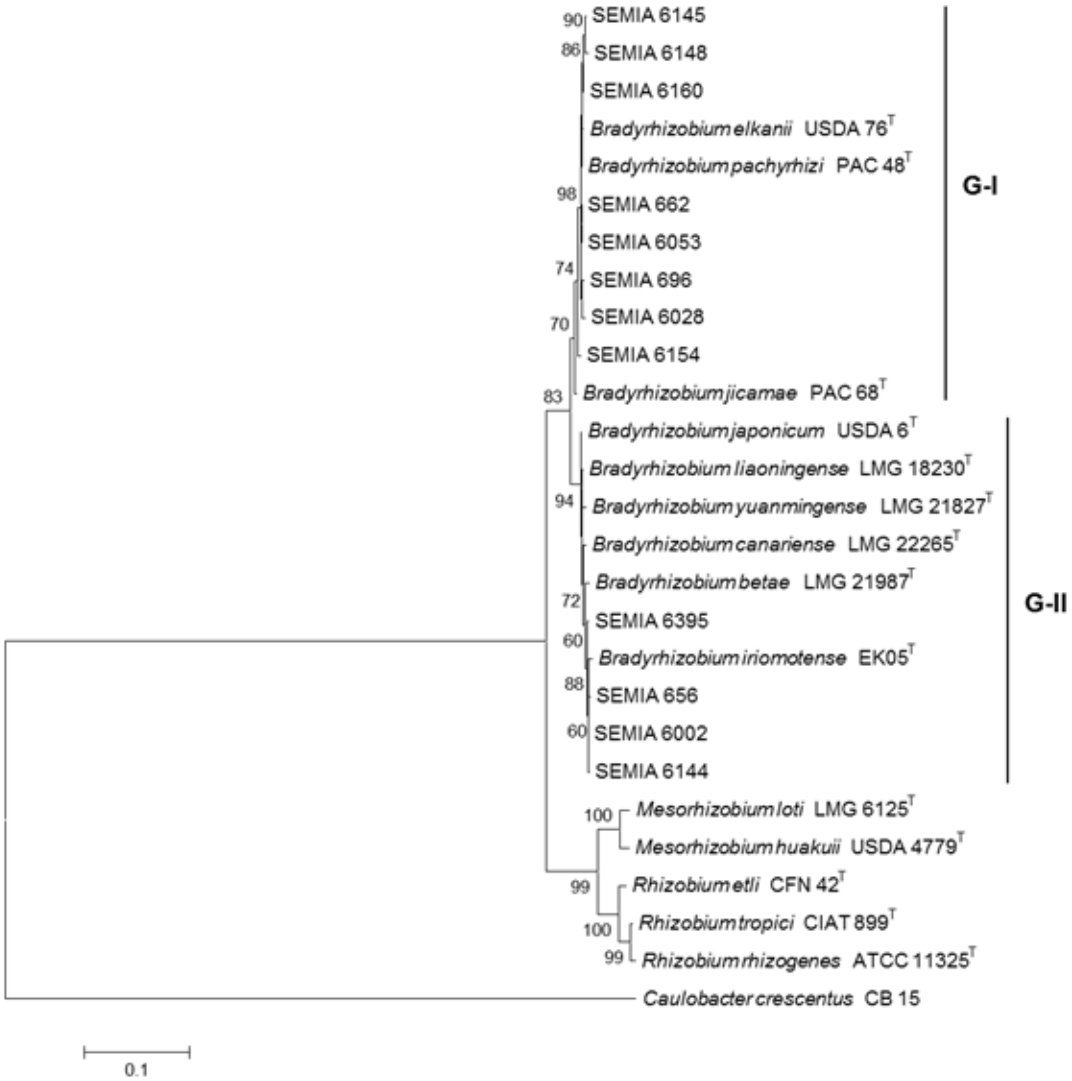


Figure 1. Phylogenetic relationships of *Bradyrhizobium* strains from this study and of reference/type rhizobial strains based on the 16S rRNA. Phylogeny was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

Diversity in the *atpD*, *glnII*, *gyrB*, *recA* and *rpoB* genes

The additional housekeeping genes selected to refine the phylogeny analysis in this study are highly conserved among bacteria of the order Rhizobiales, are dispersed in the genome of *B. japonicum* strain USDA 110 and encode important proteins. For each housekeeping gene, phylogenetic trees were constructed and resulted in distinct groups (Fig. 2).

When compared to the 16S rRNA, higher variability was detected in the analysis of the *recA* gene, and strains also fit into two groups (Fig. 2A). All four strains previously positioned in G-II of the 16S rRNA were closer to *B. iriomotense*, but with the appearance of at least one subgroup including SEMIA 6002 and SEMIA 6144 strains. This first group of strains had a high bootstrap support, of 99%. Subgroups were even more evident with the strains previously positioned in G-I of the 16S rRNA, with the delineation of four subgroups based on the *recA* gene: one including strains SEMIAs 6160, 662 and *B. pachyrhizi*, the second with SEMIA 696 and *B. elkanii* and two new subgroups including exclusively SEMIA strains, one with SEMIAs 6148 and 6154 and the other with SEMIAs 6028, 6053 and 6145. These subgroups had bootstrap supports ranging from 81 to 99% (Fig. 2A).

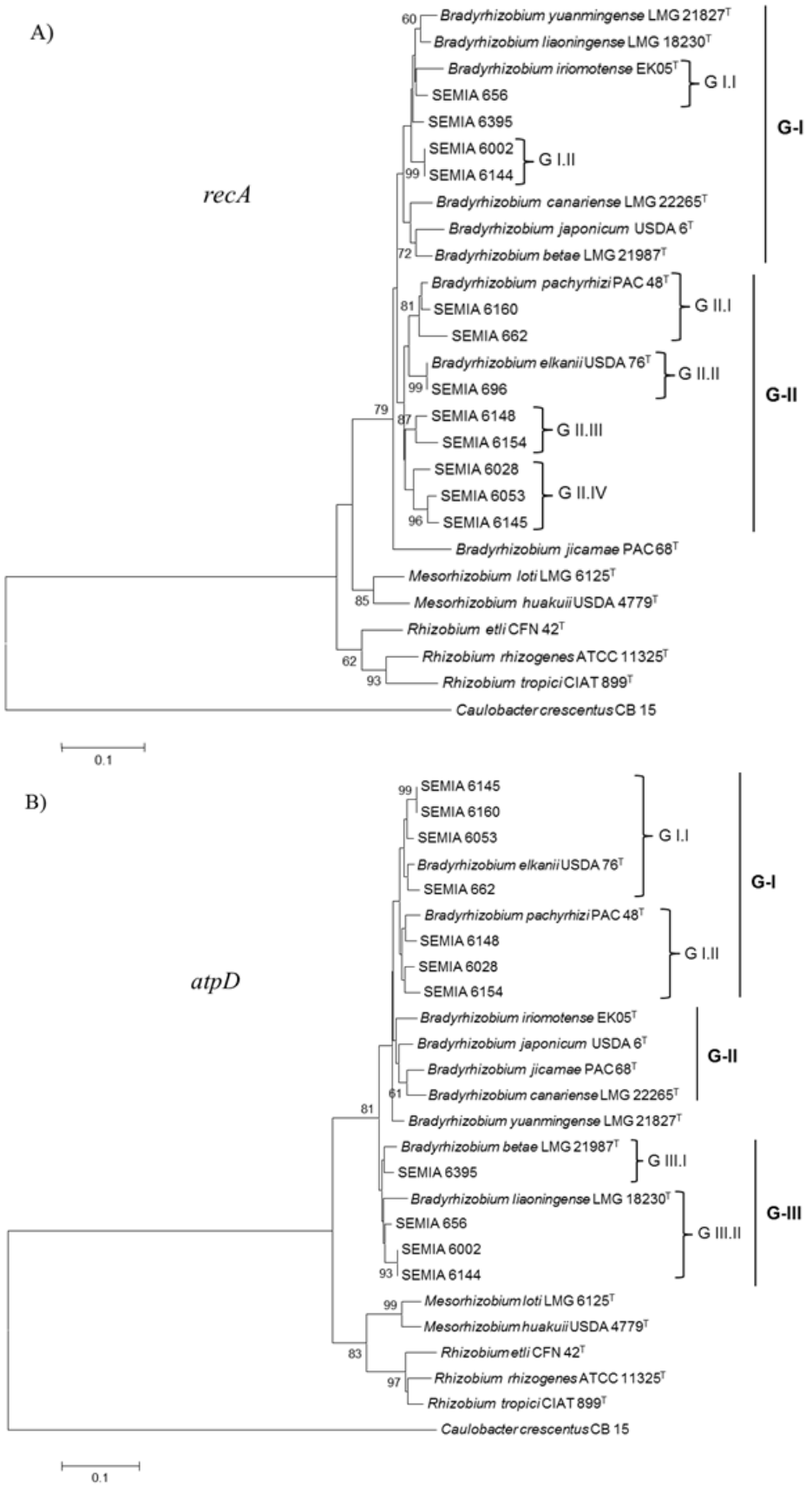
Greater variability was also observed with *atpD* gene (Fig. 2B), when compared to the 16S rRNA. The tree built with the *atpD* gene resulted in the definition of three main groups (G-I, G-II and G-III), with a final bootstrap support of 81%. Although the DNA of strain SEMIA 696 amplified with the *atpD* primers, sequencing of the fragment failed, therefore the strain was not included in the analysis. In G-I, strains were split in two subgroups, the first clustering four strains with higher resemblance with *B. elkanii*, and the second with three strains grouping with *B. pachyrhizi* (Fig. 2B). The other four SEMIA strains from this study were positioned in G-III of the *atpD* tree, with SEMIA 6395 showing higher similarity with *B. betae*, while the other strains were grouped with *B. liaoningense* (Fig. 2B).

The tree built with the *glnII* gene also resulted in two groups. In G-I four SEMIA strains were clustered with *B. pachyrhizi*, SEMIA 696 was clustered with *B. elkanii*, followed by the inclusion of SEMIA 662 and *B. jicamae* (Fig. 2C). In G-II of the *glnII*, it is worth mentioning the clustering of strains SEMIAs 6002 and 6144 with a bootstrap support of 97% (Fig. 2C), also confirmed with high support in the previous trees of *recA* (Fig. 2A) and *atpD* (Fig. 2B), but not well defined in the 16S rRNA (Fig. 1).

In the trees built with the *gyrB* and *rpoB* genes the strains were also split in two groups, with the formation of a third group in the *gyrB* tree, which clustered only the type strains of *B. betae* and *B. canariense*. In general, a better definition of the strains positioned in G-I of the 16S rRNA (Fig. 1) was not achieved with the analysis of the *gyrB* (Fig. 2D) and *rpoB* (Fig. 2E) genes. The other four SEMIAs analyzed were positioned in G-II in both trees. In G-II of *gyrB*, the SEMIA 6395 clustered with *B. iriomotense*, while SEMIAs 656, 6002 and 6144 were closer to *B. yuanmingense*. In the *rpoB* tree, these four SEMIAs were clustered in two well defined subgroups (656-6395 and 6002-6144).

It is also interesting that, excepting for the *atpD* gene, *B. pachyrhizi*, *B. jicamae* and *B. elkanii* were positioned in the same group in the analysis of the 16S rRNA, *recA*, *glnII*, *gyrB* and *rpoB* (Fig. 1 and Figs. 2A to 2E). On the other hand, definition of the other *Bradyrhizobium* species considered in this study was not completely clear when considering those genes (Fig. 1 and Figs. 2A to 2E).

From the analysis of the 16S rRNA (Fig. 1) and of five housekeeping genes (Fig. 2), the division of *Bradyrhizobium* in two main groups was clear, with the appearance of a third group not completely well defined only in the trees built with the *atpD* and *gyrB* genes. Each SEMIA strain used in our study was always positioned in the same great group in all six trees.


A)
recA
Bradyrhizobium yuanmingense LMG 21827T
Bradyrhizobium liaoningense LMG 18230T
Bradyrhizobium iriomotense EK05T
SEMIA 656
SEMIA 6395
SEMIA 6002
SEMIA 6144
Bradyrhizobium canariense LMG 22265T
Bradyrhizobium japonicum USDA 6T
Bradyrhizobium betae LMG 21987T
Bradyrhizobium pachyrhizi PAC 48T
SEMIA 6160
SEMIA 662
Bradyrhizobium elkanii USDA 76T
SEMIA 696
SEMIA 6148
SEMIA 6154
SEMIA 6028
SEMIA 6053
SEMIA 6145
Bradyrhizobium jicamae PAC 68T
Mesorhizobium loti LMG 6125T
Mesorhizobium huakuii USDA 4779T
Rhizobium etli CFN 42T
Rhizobium rhizogenes ATCC 11325T
Rhizobium tropici CIAT 899T
Caulobacter crescentus CB 15
G I.I
G I.II
G II.I
G II.II
G II.III
G II.IV
G-I
G-II
60
99
72
81
99
79
87
96
85
62
93
0.1
B)
atpD
SEMIA 6145
SEMIA 6160
SEMIA 6053
Bradyrhizobium elkanii USDA 76T
SEMIA 662
Bradyrhizobium pachyrhizi PAC 48T
SEMIA 6148
SEMIA 6028
SEMIA 6154
Bradyrhizobium iriomotense EK05T
Bradyrhizobium japonicum USDA 6T
Bradyrhizobium jicamae PAC 68T
Bradyrhizobium canariense LMG 22265T
Bradyrhizobium yuanmingense LMG 21827T
Bradyrhizobium betae LMG 21987T
SEMIA 6395
Bradyrhizobium liaoningense LMG 18230T
SEMIA 656
SEMIA 6002
SEMIA 6144
Mesorhizobium loti LMG 6125T
Mesorhizobium huakuii USDA 4779T
Rhizobium etli CFN 42T
Rhizobium rhizogenes ATCC 11325T
Rhizobium tropici CIAT 899T
Caulobacter crescentus CB 15
G I.I
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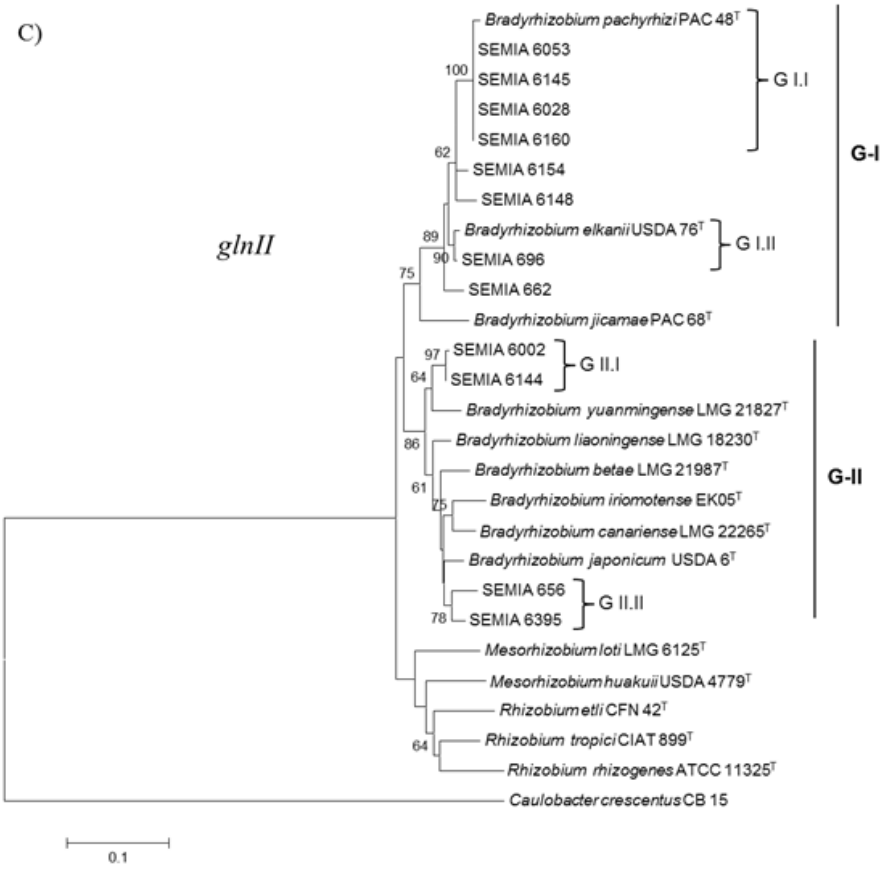
C)
glnII
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SEMIA 6053
SEMIA 6145
SEMIA 6028
SEMIA 6160
SEMIA 6154
SEMIA 6148
Bradyrhizobium elkanii USDA 76T
SEMIA 696
SEMIA 662
Bradyrhizobium jicamae PAC 68T
SEMIA 6002
SEMIA 6144
Bradyrhizobium yuanmingense LMG 21827T
Bradyrhizobium liaoningense LMG 18230T
Bradyrhizobium betae LMG 21987T
Bradyrhizobium iriomotense EK05T
Bradyrhizobium canariense LMG 22265T
Bradyrhizobium japonicum USDA 6T
SEMIA 656
SEMIA 6395
Mesorhizobium loti LMG 6125T
Mesorhizobium huakuii USDA 4779T
Rhizobium etli CFN 42T
Rhizobium tropici CIAT 899T
Rhizobium rhizogenes ATCC 11325T
Caulobacter crescentus CB 15
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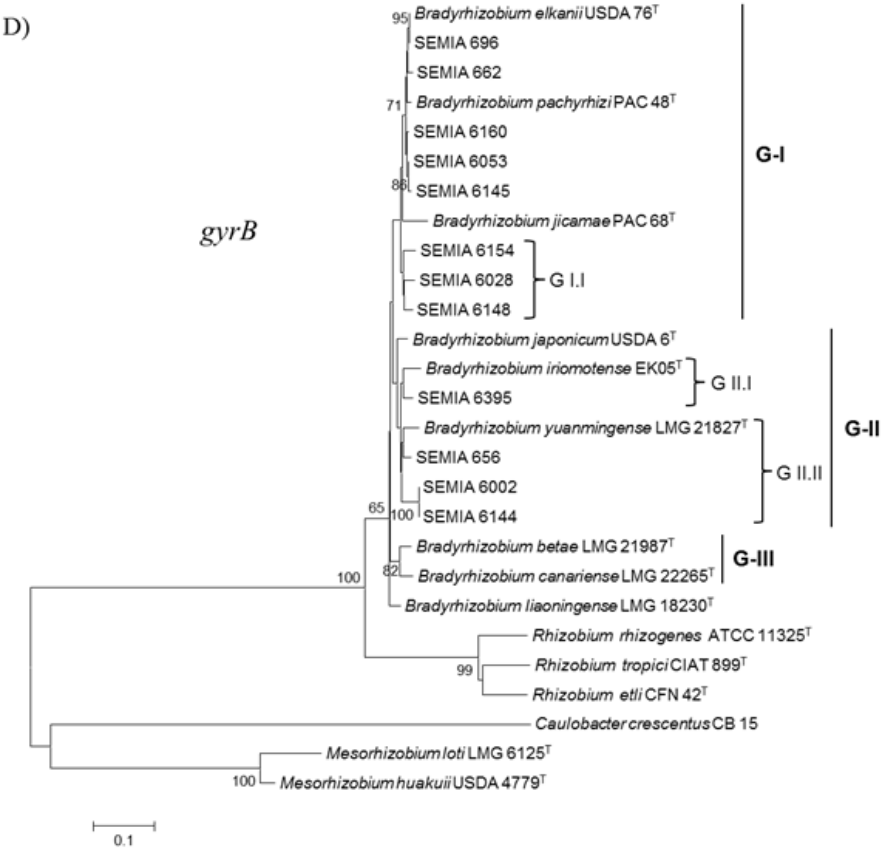
D)
gyrB
Bradyrhizobium elkanii USDA 76T
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Bradyrhizobium pachyrhizi PAC 48T
SEMIA 6160
SEMIA 6053
SEMIA 6145
Bradyrhizobium jicamae PAC 68T
SEMIA 6154
SEMIA 6028
SEMIA 6148
Bradyrhizobium japonicum USDA 6T
Bradyrhizobium iriomotense EK05T
SEMIA 6395
Bradyrhizobium yuanmingense LMG 21827T
SEMIA 656
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Bradyrhizobium betae LMG 21987T
Bradyrhizobium canariense LMG 22265T
Bradyrhizobium liaoningense LMG 18230T
Rhizobium rhizogenes ATCC 11325T
Rhizobium tropici CIAT 899T
Rhizobium etli CFN 42T
Caulobacter crescentus CB 15
Mesorhizobium loti LMG 6125T
Mesorhizobium huakuii USDA 4779T
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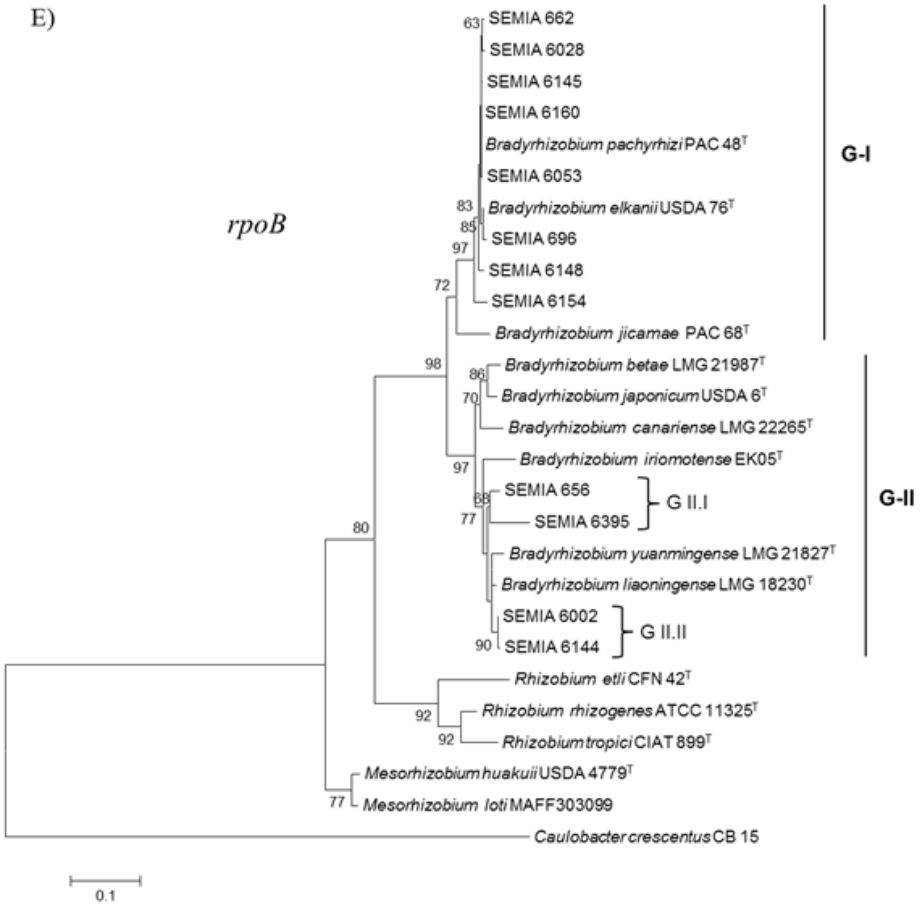

Figure 2. Phylogenetic relationships of *Bradyrhizobium* strains from this study and of reference/type rhizobial strains based on the (A) *recA*, (B) *atpD*, (C) *glnII*, (D) *gyrB* and (E) *rpoB* genes. Method and parameters of analysis were as described for Figure 1.

Concatenated analysis of *recA*, *atpD*, *glnII*, *gyrB* and *rpoB*

All five sequences of the housekeeping genes were concatenated to gain a better understanding of the strains; SEMIA 696 was not included. A concatenated sequence with 1,959 bp was obtained and 2,028 sites were analysed, resulting in 1,496 conserved, 463 variable and 304 parsimony-informative sites (Table 4).

The tree built with the concatenated genes resulted in two great groups, with a bootstrap support of 100% (Fig. 3). G-I assembled SEMIAs 656, 6395, 6002 and 6144 together with type/reference strains of *B. yuanmingense*, *B. liaoningense*, *B. iriomotense*, *B. japonicum*, *B. betae* and *B. canariense*. Within G-I, subclusters that were not well defined in the 16S rRNA (Fig. 1) tree were now shown (Fig. 3), including the pairs of strains SEMIAs 656-6395 and SEMIAs 6002-6144, with bootstrap supports of 95 and 100%, respectively. The results highlight that these strains deserve further studies, as they may represent new species. The concatenated tree has also detected higher diversity of the strains occupying G-II, with the SEMIAs 6160, 6028, 6053 and 6145 showing similarity with the type strain of *B. pachyrhizi*. The pair of strains 6148-6154 could also represent a new species. Finally, it is important to mention that in all trees *Bradyrhizobium* was clearly apart from the other rhizobial genera.

Another important observation is that clustering of strains by means of both 16S rRNA and housekeeping genes showed no relation with the host plant. Strains clustered in G-I of the MLSA were isolated from subfamilies Papilionoideae

(SEMIAs 656, 6002 and 6144) and Mimosoideae (SEMIA 6395), and G-II also included strains from both subfamilies. Other examples refer to isolates from *Vigna*, clustered in both G-I (SEMIA 6002) and G-II (SEMIA 662), while isolates from tribe Ingeae were clustered in both G-I (SEMIA 6395) and G-II (SEMIA 6160) of the MLSA (Fig. 3).

Table 4. Sequence information obtained in this study. Twelve strains were analysed, together with nine type and reference strains, as described in Methods.

Locus	Strains analysed (*n*)	Nucleotides (%)				Frequency T/C/A/G (%)
		Conserved	Variable	Parsimony-informative	Total*	
16S rRNA	21	1,266 (91,5)	86 (6,2)	52 (3,7)	1,347/1,383	20.4/24.0/24.7/30.9
atpD	20	318 (77,6)	77 (18,8)	57 (13,9)	395/410	15.7/33.3/19.6/31.3
glnII	21	334 (72,8)	108 (23,5)	77 (16,8)	442/459	16.2/32.7/19.9/31.2
gyrB	21	338 (74,8)	96 (21,2)	56 (12,4)	434/452	21.7/29.9/16.5/32.0
recA	21	217 (72,6)	76 (25,4)	52 (17,4)	293/299	15.8/32.3/17.3/34.7
rpoB	21	287 (70,8)	108 (26,7)	63 (15,5)	395/405	17.4/32.8/18.7/31.1
Concatenated genes	20	1,496 (73,7)	463 (22,8)	304(15)	1,959/2,028	17.5/32.2/18.4/31.9

* Mean number of nucleotides amplified/number of sites analysed, including gaps.

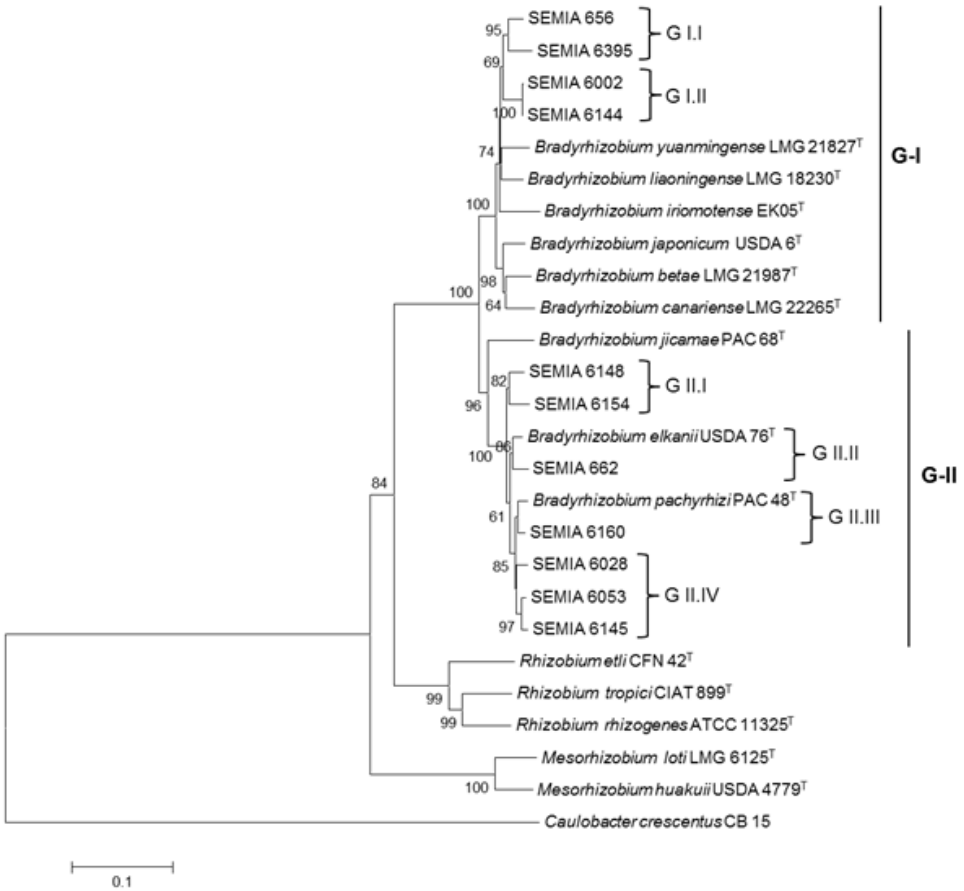

Figure 3. Evolutionary tree inferred using the Neighbor-Joining method for 22 strains based on concatenated genes *(recA, atpD, glnII, gyrB, rpoB)*. The percentage of replicate trees in which the associated strains clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

DISCUSSION

Bradyrhizobium is an intriguing genus of bacteria encompassing a number of interesting features. First, the genus has been considered as the ancestor of all rhizobia (24, 32, 35, 49), and it has been isolated from a variety of legumes distributed worldwide. However, the great majority of the reports on diversity and genetics of diazotrophic symbiotic bacteria has been performed with fast-growing rhizobia, thus studies with *Bradyrhizobium* may reveal new insights into the evolution of rhizobia. The second important feature is that *Bradyrhizobium* has probably originated in the tropical region (24, 32), and indeed bradyrhizobia seem to represent the majority of the isolates from leguminous trees in Brazilian tropical forests (30). As it has been pointed out since the pioneer studies of ribosomal genes, apparently there are many more varieties of rhizobia in tropical and subtropical than in temperate regions (33, 46); therefore, studies on the genetic diversity of *Bradyrhizobium* may expose a high level of genetic diversity. Finally, the third important feature relies on the reports of high rates of nitrogen fixation related to bacteria belonging to the genus *Bradyrhizobium* (14), and excellent examples comprise the symbioses with cowpea (*Vigna* spp.) and soybean (*Glycine max*). Indeed, the SEMIA strains used in our study are highly effective in fixing nitrogen with their host plants.

Previous studies from our group employing analyses of the 16S rRNA, 23S rRNA, ITS and housekeeping genes have indicated an unexpected genetic diversity of *Bradyrhizobium* (13, 28, 29). In our study the use of five housekeeping genes in the MLSA approach highlighted a far higher diversity in comparison to the single analysis with the 16S rRNA, clearly indicating putative new species.

DNA-DNA hybridization is still required to define new species (12), but arguments against its obligatory use have been raised, including: high cost and intensive work (5, 44), existence of more accurate approaches (22), doubts about its adequacy (1). MLSA has then been proposed as a more accessible tool for assessing phylogeny and taxonomy of prokaryotes (4, 6, 15, 25). Also in this context, the use of at least four housekeeping genes for the phylogenetic analysis and taxonomic classification of bradyrhizobia has been proposed (29, 34, 39, 40, 48), and was confirmed as a successful approach in our study.

It is also interesting that host specificity was not related to genetic clustering, confirming previous reports from our group (3, 13, 28, 29, 37), and indicating that other genes must be searched aiming at getting a better understanding of the evolution of the symbioses, probably nodulation and nitrogen fixation genes.

Biological nitrogen fixation plays a key role in the environment and agriculture sustainability. The results from our study highlight the high genetic variability associated with *Bradyrhizobium* microsymbionts of a variety of legumes. Our results confirm that the MLSA approach can represent an important, effective, fast and low-cost strategy to reveal the still poorly known diversity of *Bradyrhizobium* and certainly other nitrogen-fixing rhizobial species (26, 29, 36, 40, 47). In our study MLSA clearly contributed to a better phylogeny definition, as well as to the identification of new subgroups indicative of new species.

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