Bradyrhizobium viridifuturi sp. nov., encompassing nitrogen-fixing symbionts of legumes used for green manure and environmental services

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Symbiotic nitrogen-fixing bacteria, commonly called rhizobia, are agronomically important because they can provide significant amounts of nitrogen to plants and help in recovery of impoverished soils and improvement of degraded environments. In recent years, with advances in molecular techniques, several studies have shown that these bacteria have high levels of genetic diversity, resulting in taxonomic reclassifications and descriptions of new species. However, despite the advances achieved, highly conserved 16S ribosomal genes (16S rRNA) do not elucidate differences between species of several genera, including the genus *Bradyrhizobium*. Other methodologies, such as multilocus sequence analysis (MLSA), have been used in such cases, with good results. In this study, three strains (SEMIAs 690^T, 6387 and 6428) of the genus *Bradyrhizobium*, isolated from nitrogen-fixing nodules of *Centrosema* and *Acacia* species, without clear taxonomic positions, were studied. These strains differed from genetically closely related species according to the results of MLSA of four housekeeping genes (*dnaK*, *glnII*, *gyrB* and *recA*) and nucleotide identities of the concatenated genes with those of related species ranged from 87.8 % to 95.7 %, being highest with *Bradyrhizobium elkanii*. DNA–DNA hybridization (less than 32 % DNA relatedness) and average nucleotide identity values of the whole genomes (less than 90.5 %) indicated that these strains represented a novel species, and phenotypic traits were determined. Our data supported the description of the SEMIA strains as *Bradyrhizobium viridifuturi* sp. nov., and SEMIA 690^T (=CNPSo 991^T=C 100a^T=BR 1804^T=LMG 28866^T), isolated from *Centrosema pubescens*, was chosen as type strain.

Abbreviations: ANI, average nucleotide identity; DDH, DNA–DNA hybridization; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the *dnaK* gene sequences of *B. viridifuturi* SEMIA 690^T, SEMIA 6387 and SEMIA 6428 are KR149128–KR149130; those for the *glnII* sequences of SEMIA 690^T, SEMIA 6387 and SEMIA 6428 are KR149131–KR149133; those for the *gyrB* sequences of SEMIA 690^T, SEMIA 6387 and SEMIA 6428 are KR149134–KR149136; those for the *recA* sequences of SEMIA 690^T, SEMIA 6387 and SEMIA 6428 are KR149140–KR149142; those for the *nifH* sequences of SEMIA 690^T, SEMIA 6387 and SEMIA 6428 are KR149137–KR149139.

Three supplementary tables and six supplementary figures are available with the online Supplementary Material.

Biological nitrogen fixation has been recognized for over 130 years as a key process for environmental sustainability, but lately it has become the subject of increased interest, with an emphasis on the symbioses with legumes, as a means of increasing nutrient and energy balances in agriculture, in environmentally beneficial reforestation efforts and in the mitigation of greenhouse-gas emissions (e.g. Hungria *et al.*, 2005, 2013; Ormeño-Orrillo *et al.*, 2013). However, although our knowledge of the rhizobia–legume symbioses is rapidly increasing, driven largely by 'omics' studies, we are still far from fully understanding this biological process, which has evolved over millions of years. A good example is our still poor knowledge of

the phylogeny and taxonomy of the genus *Bradyrhizobium*, considered to be the ancestor of all rhizobia (Norris, 1965; Lloret & Martínez-Romero, 2005; Germano *et al.*, 2006; Menna et al., 2006; Binde et al., 2009; Menna & Hungria, 2011; Delamuta *et al.*, 2013). One limitation in defining species within the genus *Bradyrhizobium* is that 16S rRNA genes are highly conserved. On the other hand, the multilocus sequence analysis (MLSA) technique, applied to concatenated housekeeping genes, has greatly clarified phylogenetic relationships and elucidated novel species within the genus (e.g. Menna *et al.*, 2009; Delamuta *et al.*, 2013; Durán *et al.*, 2014a, b; Parker & Rousteau, 2014). New insights into the evolution of the symbiosis with *Bradyrhizobium* have also been obtained by the analysis of nodulation and nitrogen-fixation genes (Menna & Hungria, 2011; Parker & Rousteau, 2014; Zhang *et al.*, 2014).

Leguminous species used as green manure, for reforestation and for remediation of degraded areas are key for sustainability, considering that the global loss of fertile soil has been estimated at 24 billion tonnes per year, adversely affecting 1.5 billion people (United Nations, 2015). The three strains (SEMIA 690^T, SEMIA 6387 and SEMIA 6428) used in this study were isolated from different sites in Brazil from legumes used for those three purposes, *Centrosema pubescens*, *Acacia auriculiformis* and *Acacia saligna*, respectively. SEMIA 690^T was isolated at the Instituto de Pesquisas e Experimentação Agropecuária do Centro-Sul (IPEACS), Rio de Janeiro, Brazil, and SEMIA 6387 and SEMIA 6428 by Dr Sergio M. Faria at the Embrapa Agrobiologia, Seropédica, Rio de Janeiro, Brazil. The strains are recognized as the most effective for fixing nitrogen with the legumes from which they were isolated; they have been authorized for inclusion in commercial inoculants for their respective host legumes by the Ministry of Agriculture in Brazil since 1994 (MAPA, 2011).

The strains of members of the genus *Bradyrhizobium* used in this study have been deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054), located at Londrina (State of Paraná, Brazil) and at the Center for Genomic Sciences Culture Collection (Cuernavaca, Mexico). They are also deposited at the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO; WDCM # 443, Porto Alegre, Rio Grande do Sul), Embrapa Agrobiologia (WDCM # 364, Seropédica, Rio de Janeiro) Culture Collections. Unless otherwise indicated, strains were grown on yeast extract–mannitol agar (YMA) medium at 28 °C (Vincent, 1970). Stock cultures were maintained on YMA at 4 °C, while long-term preservation was performed in liquid YM medium containing 30 % glycerol (v/v) at −80 °C and −150 °C, or by lyophilization.

Fingerprinting analysis of the strains under study was performed by BOX-PCR (Kaschuk *et al.*, 2006) and the profiles were compared with those of five type strains of the *Bradyrhizobium elkanii* superclade. The BioNumerics program (Applied Mathematics, Kortrijk, Belgium, v.7.1) was used to generate the clusters, with the UPGMA (Sneath & Sokal, 1973) algorithm and the Jaccard coefficient (Jaccard, 1912) with 3 % tolerance. The three strains had similarities higher than 72 % among themselves and less than 66 % with closely related species (Fig. S1, available in the online Supplementary Material).

Phylogenetic trees were reconstructed with 16S rRNA gene sequences obtained from the GenBank database (accession numbers are given on the trees and in Supplementary Table S1) of the strains from this study and 29 species of the genus *Bradyrhizobium*. *Xanthobacter autotrophicus* Py2 was used as outgroup. The MEGA 6.0 program (Tamura *et al.*, 2013) was used to generate the alignments and phylogenies, using maximum-likelihood (ML) (Felsenstein, 1981) and neighbour-joining (NJ) (Saitou & Nei, 1987) algorithms and Tamura–Nei distance (Tamura & Nei, 1993). Statistical support for the trees was assessed by bootstrapping (Felsenstein, 1985) with 1000 replicates. NJ and ML reconstructions gave similar results; therefore, only the ML phylogram is presented. The SEMIA strains were positioned in the *B. elkanii* clade, and their closest neighbours were *Bradyrhizobium neotropicale*, *Bradyrhizobium jicamae* and *Bradyrhizobium erythrophlei* (Fig. 1).

The MLSA approach using housekeeping concatenated genes has been successfully used to define many groups of the genus *Bradyrhizobium* (Menna *et al.*, 2009; Rivas *et al.*, 2009; Chang *et al.*, 2011; Delamuta *et al.*, 2012, 2013; Durán *et al.*, 2014a, b). MLSA phylograms were reconstructed as described for the 16S rRNA gene with four housekeeping genes (*dnaK*, *glnII*, *gyrB* and *recA*, accession numbers in Supplementary Table S1) and it clearly separated the three strains from this study into a single and consistent group with 100 % bootstrap support, isolated from all described species of the genus *Bradyrhizobium* (Fig. 2). Their closest neighbours were *B. elkanii* and *Bradyrhizobium pachyrhizi*. Each individual phylogenetic tree reconstructed with each of the four housekeeping genes supported the distinctiveness between the cluster of SEMIA strains and the other species of the genus *Bradyrhizobium*. In all four trees, the strains were clustered with high bootstrap support and the most closely related species were *B. pachyrhizi, Bradyrhizobium ferriligni* and *B. elkanii* (Figs S2–S5). The three reconstructed with three genes (*glnII*+*gyrB*+*recA*) also supported the proposal of the novel species (Fig. S6).

Nucleotide identities of every analysed gene and of concatenated sequences are shown in Table S2. The SEMIA strains were compared both with each other and with the other species of the genus *Bradyrhizobium*. Considering the 16S rRNA gene, values within SEMIA strains ranged from 99.5 % to 100 % and from 99.3 % to 99.5 % for the four concatenated housekeeping genes. Considering the SEMIA strains and the other species of the genus *Bradyrhizobium*, values ranged from 96.2 % to 100 % for the 16S rRNA gene and from 87.8 % to 95.7 % for the four concatenated genes. These values are lower than the 97.0 % suggested as a cut-off level for definition of species of the genus *Bradyrhizobium*

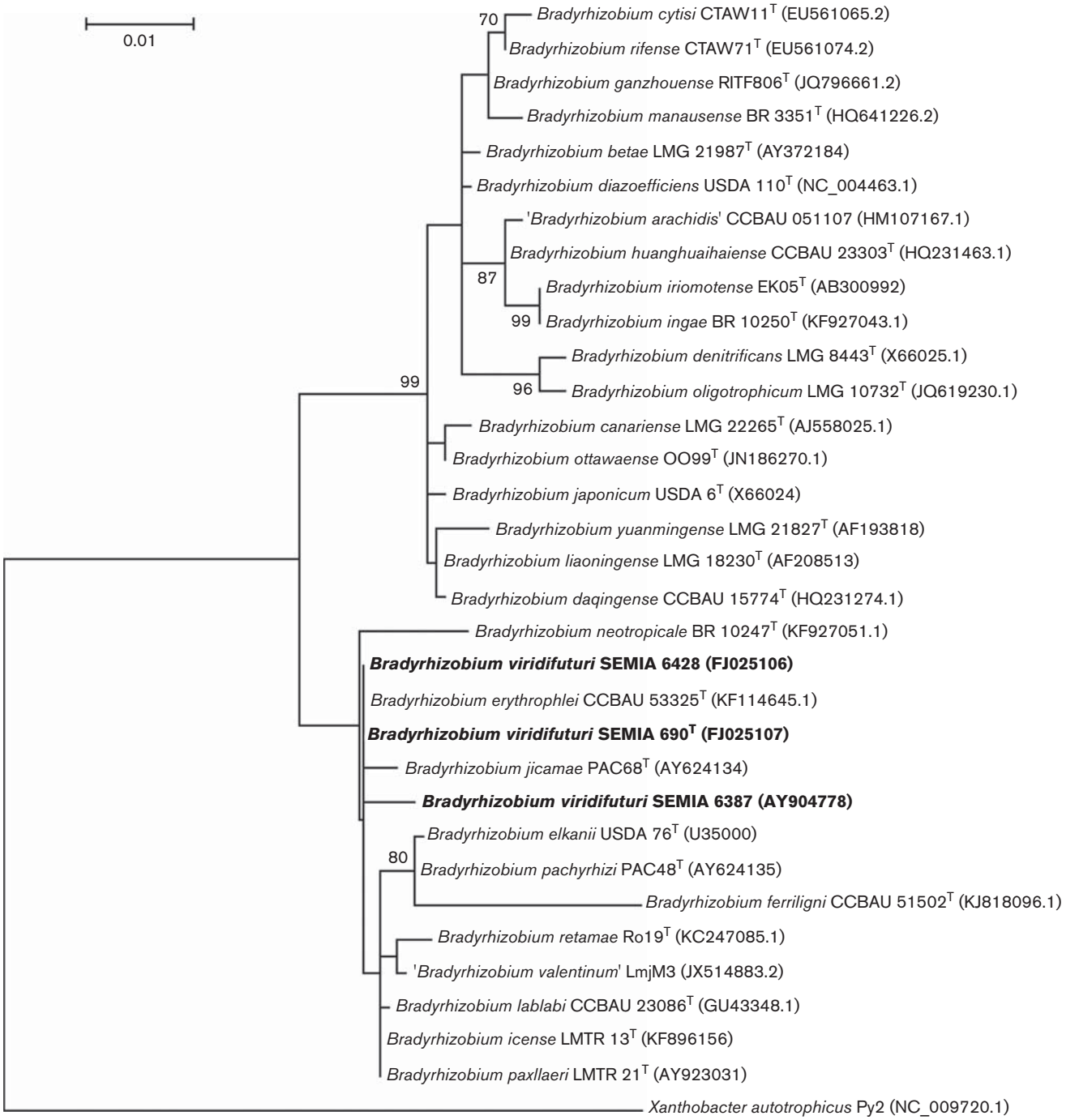

Fig. 1. Maximum-likelihood tree based on 16S rRNA sequences of SEMIA strains (indicated by bold type) and type/reference strains. Bootstrap support values 70 % or greater are shown at tree nodes. GenBank accession numbers are provided in parentheses. Bar, one substitution per 100 nucleotide positions.

by Durán *et al.* (2014a), supporting the hypothesis that the SEMIA strains represent a novel species.

Average nucleotide identity (ANI) of genome sequences has been increasingly used as an alternative to DNA–DNA hybridization (DDH) to estimate genome relatedness, including in the genus *Bradyrhizobium* (Delamuta *et al.*, 2013; Durán *et al.*, 2014a, b). Richter & Rosselló-Móra (2009) suggested that ANI values of 95–96 % would correspond to 70 % DDH, and Kim *et al.* (2014) confirmed this range studying more than 6000 genomes. A genome draft was obtained for SEMIA 690^T (SAMN03890369) and for the

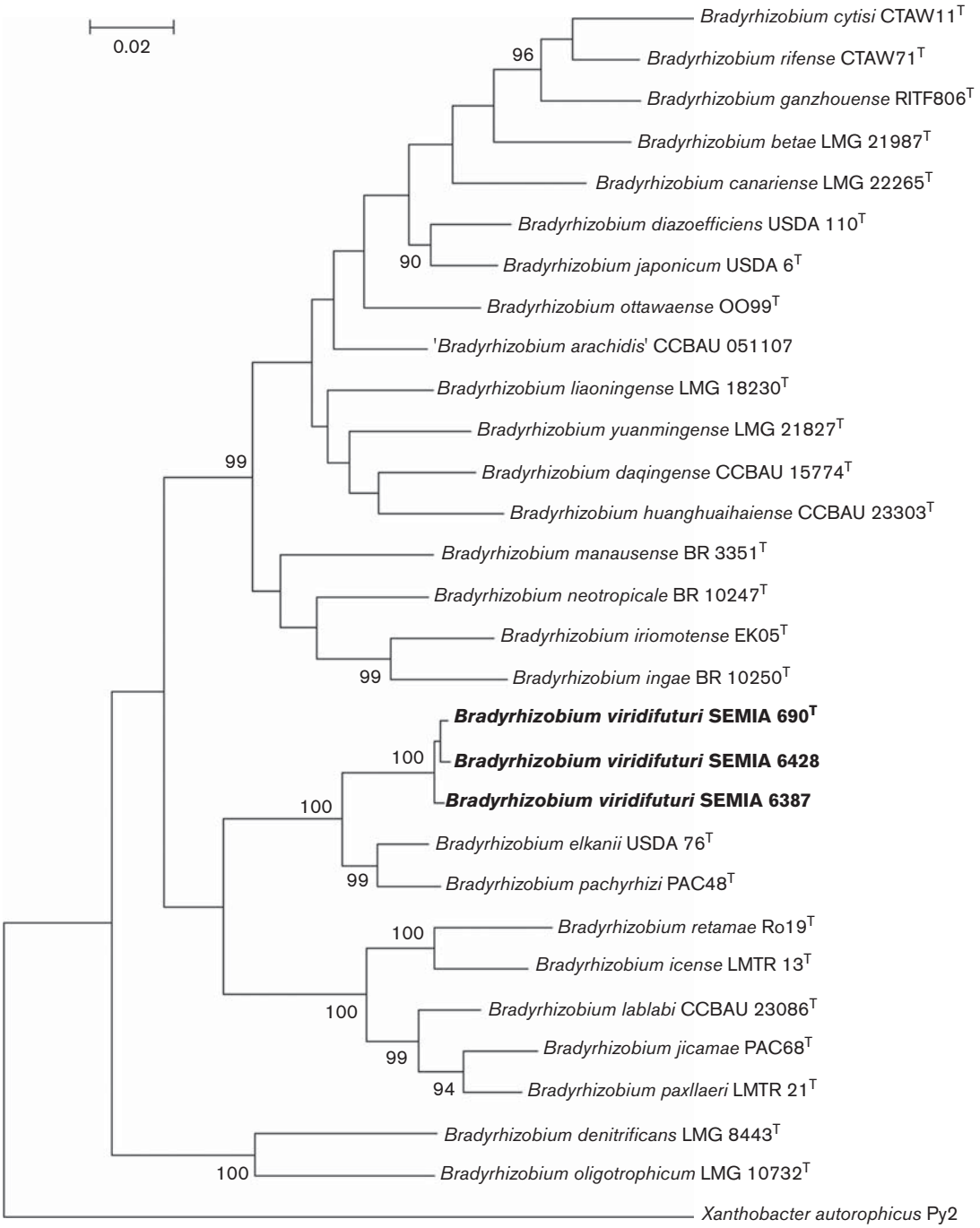

Fig. 2. Maximum-likelihood phylogenetic tree based on a concatenated alignment of *dnaK*, *glnII*, *gyrB* and *recA* sequences of SEMIA strains (indicated by bold type) and type/reference strains. Bootstrap support values of 70 % or greater are shown at tree nodes. GenBank accession numbers are provided in parentheses. Bar, two substitutions per 100 nucleotide positions.

closely related *B. pachyrhizi* PAC 48^T (SAMN03782120). Other seven genomes available used as comparison were of the close species *B. elkanii* (GenBank accession number NZ_ARAG00000000), *Bradyrhizobium paxllaeri*, *Bradyrhizobium icense* (Durán *et al.*, 2014a), '*Bradyrhizobium valentinum*', *Bradyrhizobium retamae*, *Bradyrhizobium lablabi* and *B. jicamae* (Durán *et al.*, 2014b). ANI values were calculated using JSpecies (Richter & Rosselló-Móra, 2009) and Mummer for sequence alignment. The values for comparisons of SEMIA 690^T with *B. jicamae*, *B. paxllaeri*, *B. retamae*, *B. lablabi*, *B. icense* and '*B. valentinum*' were all below 85.5 %. With the most closely related species being *B. elkanii*

and *B. pachyrhizi*, the ANI values were lower than 90.5 %, all below the species circumscription threshold (Table 1).

DDH was conducted by a filter hybridization methodology (Martínez-Romero *et al.*, 1991). The genome of SEMIA 690^T strain was used as the basis for hybridization and was compared with the most closely related species *B. elkanii* (USDA 76^T) and *B. pachyrhizi* (PAC 48^T). The DNA relatedness values obtained between SEMIA 690^T and those type strains were 30.3±3.6 % and 25.5±2.6 %, respectively, supporting the hypothesis that the SEMIA strains represent a novel species.

The DNA G+C content of the SEMIA 690^T genome was also determined based on the draft genome obtained

Table 1. Percentages of average nucleotide identity (ANI) of whole genome sequences between *B. viridifuturi* and related species

Strain used as reference	SEMIA 690^T
B. pachyrhizi PAC 48^T	90.4
B. elkanii USDA 76^T	90.5
B. jicamae PAC 68^T	85.3
B. paxllaeri LMTR 21^T	85.4
B. lablabi CCBAU 23086^T	85.3
B. retamae Ro19^T	85.0
B. icense LMTR 13^T	85.1
'*B. valentinum*' LmjM3	85.1

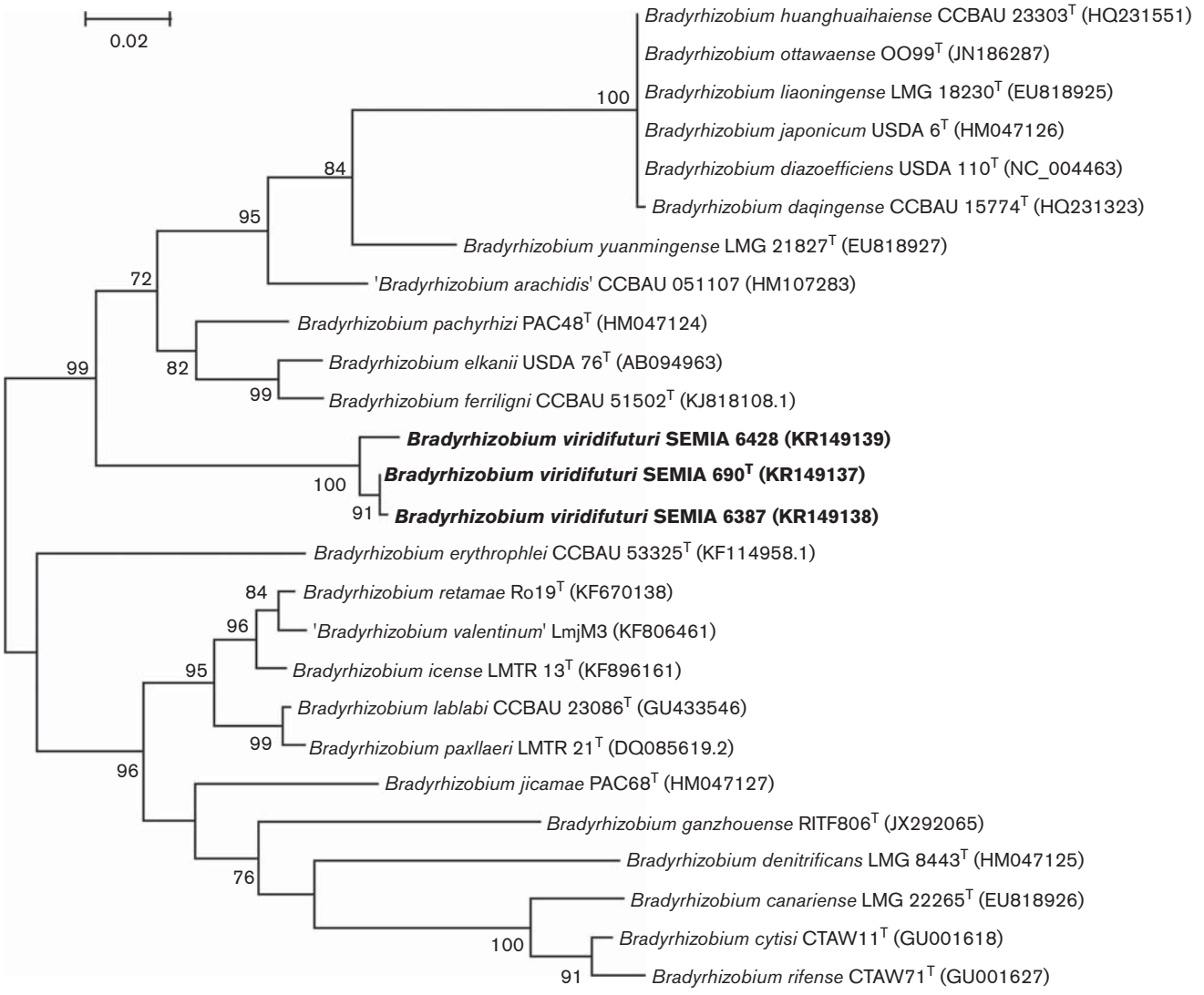

Fig. 3. Maximum-likelihood phylogenetic tree based on *nifH* sequences of SEMIA strains (indicated by bold type) and type/-reference strains. Bootstrap support values of 70 % or greater are shown at tree nodes. GenBank accession numbers are provided in parentheses. Bar, two substitutions per 100 nucleotide positions.

Table 2. Distinctive phenotypic features of SEMIA strains and phylogenetically related species of the genus *Bradyrhizobium*.

Strains: 1, SEMIA 690^T; 2, SEMIA 6387; 3, SEMIA 6428; 4, *B. elkanii* USDA 76^T; 5, *B. pachyrhizi* PAC48^T; 6, *B. jicamae* PAC68^T; 7, *B. lablabi* CCBAU 23086^T, 8, *B. retamae* Ro19^T. Data represent the means of two biological replicates. +, Growth; −, no growth; w, weakly positive.

	1	2	3	4	5	6	7	8
Growth								
pH 4.5	+	+	+	−	w	−	−	−
pH 8.0	w	+	+	+	+	+	+	+
Urea 2%	+	+	w	+	+	−	+	−
Tolerance (μg per disc)								
Erythromycin (15)	+	+	+	+	+	w	+	+
Cefuroxime (15)	+	+	+	+	+	−	+	−
Neomycin (30)	+	+	+	−	−	−	−	w
Tetracycline (30)	+	+	+	+	+	+	−	−
Streptomycin (10)	+	+	+	+	+	−	−	−
Carbohydrates								
L-Arabinose	+	+	+	+	+	+	w	+
D-Xylose	+	+	+	+	+	+	w	w
D-Adonitol	w	w	w	w	w	−	w	−
D-Galactose	w	w	+	+	+	+	+	w
D-Mannose	w	+	+	+	+	+	w	−
L-Sorbose	w	w	−	−	−	−	−	−
L-Rhamnose	w	w	w	w	w	+	+	w
Dulcitol	−	−	−	w	−	−	−	−
D-Mannitol	w	w	w	w	w	−	w	−
D-Sorbitol	w	w	w	w	w	−	−	−
Esculin	+	+	w	w	−	−	+	w
Glycogen	−	−	−	−	−	−	+	−
Xylitol	w	w	w	w	−	−	−	−
D-Lyxose	w	+	+	+	+	+	+	+
D-Fucose	+	+	+	+	+	+	+	w
D-Arabitol	w	w	w	w	w	−	w	−
L-Arabitol	+	w	w	w	w	−	−	−

in this study (SAMN03890369). The contigs were concatenated and the proportion of G+C bases was calculated with BioEdit (Hall, 1999). The SEMIA 690^T genome had a G+C content of 63.46 mol%, within the range for species of the genus *Bradyrhizobium* (Xu *et al.*, 1995).

The fatty acid profile of strain SEMIA 690^T was determined using the MIDI Sherlock Microbial Identification System with the TSBA6 database after growth on YMA (Vincent, 1970) for 7 days; details are given in Supplementary Table S3. The analyses revealed summed feature 8 ($C_{18:1}\omega6c$/$C_{18:1}\omega7c$) together with $C_{16:0}$ to be major fatty acids in SEMIA 690^T (Table S3), a typical characteristic of members of the genus *Bradyrhizobium* (Tighe *et al.*, 2000).

The SEMIA strains are recognized for their high capacity for fixing nitrogen with their host legumes and stability in their symbiotic properties, which is the reason why they are authorized for the production of commercial inoculants in Brazil: SEMIA 690^T for *Centrosema pubescens* (Subfamily Papilionoideae, Tribe Phaseoleae); SEMIA 6387 for *Acacia auriculiformis* (Subfamily Mimosoideae, Tribe Acacieae); SEMIA 6428 for *Acacia saligna* (Subfamily Mimosoideae, Tribe Acacieae) (MAPA, 2011). To obtain information about the evolution of nitrogen-fixation genes we obtained the sequences of *nifH* genes and reconstructed a phylogenetic tree (Fig. 3). SEMIA strains clustered in a separate group from other species of the genus *Bradyrhizobium* with 100 % bootstrap support.

Several phenotypic characteristics were evaluated in order to compare the SEMIA strains with those of closely related type strains of species of the genus *Bradyrhizobium*. Unless indicated, all tests were performed at 28 °C. Characteristics evaluated were colony morphology, acid/alkaline reaction in YMA medium containing bromothymol blue and tolerance to 1 % NaCl on YM medium, all performed as described previously (Hungria *et al.*, 2001). Growth at different pHs (pH 4.5 and pH 8.0), different temperatures (28, 37 and 40 °C) and in Luria–Bertani (LB) medium were also evaluated as described previously (Hungria *et al.*, 2001). Enzymic degradation of urea was determined in YMA medium supplemented with 2 % urea and phenol red indicator. For the evaluation of use of carbon sources we used the API 50CH kit (BioMérieux) with YM-minus-mannitol as the basal medium, and the tests were performed as described by the manufacturer's instructions. Tolerance to antibiotics was assessed by the disk diffusion method on YMA plates with the following antibiotics: cefuroxime, bacitracin, chloramphenicol, neomycin, nalidixic acid, tetracycline, streptomycin and erythromycin. All tests were performed in duplicate, each with three replicates. Table 2 shows the most relevant data. In general the phenotypic results are in agreement with those commonly found in species of the clade of *B. elkanii*, but differences were detected, being specific to the novel species, e.g. the ability to grow well in medium with an acid pH and tolerance of antibiotics. The properties that characterized the SEMIA strains are given in the species description.

Results of the polyphasic analysis, including phenotypic, genotypic and phylogenetic tests indicate that the SEMIA strains represent a novel species, within the genus *Bradyrhizobium*. We propose the name *Bradyrhizobium viridifuturi* sp. nov. for this novel taxon.

Description of *Bradyrhizobium viridifuturi* sp. nov.

Bradyrhizobium viridifuturi (vi.ri.di.fu.tu′ri. L. adj. viridis green; L. neut. n. futurum future; N.L. gen. n. viridifuturi of a green future, referring to the future use of strains of this species for a green economy).

Cells are Gram-stain-negative, aerobic, non-spore-forming rods. Colonies on YMA medium are circular, translucent, display low production of mucus and are 0.5–1.5 mm in diameter within 7 days of incubation at 28 °C. Strains alkalinize YMA medium containing bromothymol blue in 7 days, and optimum growth occurs at pH 6.8 and 28 °C. Strains do not grow in LB medium, in the presence of 1 % NaCl or at 37 or 40 °C, but grow at pH 4.5. Test for urease activity is positive. Tolerant to bacitracin, cefuroxime, chloramphenicol, erythromycin, nalidixic acid, neomycin, tetracycline and streptomycin. With respect to carbon sources in API tests, they are positive for D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, aesculin, starch, D-fucose, L-fucose and L-arabitol, weakly positive for glycerol, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-sorbose L-rhamnose, D-mannitol, D-sorbitol, xylitol, D-lyxose and D-arabitol and negative for erythritol, methyl-β-D-xylopyranoside, dulcitol, inositol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, glycogen, gentiobiose, turanose, tagatose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate.

The type strain is SEMIA 690^T (CNPSo 991^T=C 100a^T=BR 1804^T=LMG 28866^T) isolated from *Centrosema pubescens* in Brazil. The DNA G+C content of strain SEMIA 690^T is 63.46 mol%.

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