Bradyrhizobium stylosanthis sp. nov., comprising nitrogen-fixing symbionts isolated from nodules of the tropical forage legume *Stylosanthes* spp.

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The introduction of legumes and nitrogen-fixing bacteria in tropical areas under pasture is a key factor for improvement of soil fertility. However, there are still very few studies concerning the symbionts of tropical forage legumes. We performed a polyphasic study with three strains representing the genus *Bradyrhizobium* (BR 446^T, BR 510 and BR 511) isolated from the tropical perennial forage legume of the genus *Stylosanthes*. On the basis of 16S rRNA gene sequences, the three strains showed highest similarity with *B. huanghuaihaiense*, and in the analysis of the intergenic transcribed spacer (ITS) they showed less than 93.4 % similarity to all described species of the genus *Bradyrhizobium*. Multilocus sequence analysis (MLSA) with three, four or five (*dnaK*, *glnII, gyrB, recA* and *rpoB*) housekeeping genes confirmed that the BR strains belong to a distinct clade, with <96.5 % nucleotide identity with other members of the genus *Bradyrhizobium.* Average nucleotide identity (ANI) of genome sequences between strain BR 446^T and *B. huanghuaihaiense* was below the threshold for species circumscription (90.7 %). DNA-DNA hybridization resulted in ΔT_m values over 6.7 °C with the most closely related species. Similarities among the BR strains and differences from other species were confirmed by rep-PCR analysis. Interestingly, the BR strains were grouped in the analysis of *nifH* and *nodC* genes, but showed higher similarity with *B. iriomotense* and *B. manausense* than with *B. huanghuaihaiense*, indicating a different evolutionary history for nitrogen-fixation genes. Morpho-physiological, genotypic and genomic data supported that these BR strains represent a novel species for which the name *Bradyrhizobium stylosanthis* sp. nov. is

Abbreviations: DDH, DNA-DNA hybridization; ITS, intergenic transcribed spacer; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are as follows: 16S of *B. stylosanthis* BR 446^T (KU724142), BR 510 (KU724143), BR 511 (KU724144); *dnaK* of *B. stylosanthis* BR 446^T (KU724145), BR 510 (KU724146), BR 511 (KU724147); *glnII* of *B. stylosanthis* BR 446^T (KU724148), BR 510 (KU724149), BR 511 (KU724150); *gyrB* of *B. stylosanthis* BR 446^T (KU724151), BR 510 (KU724152), BR 511 (KU724153); *ITS* of *B. stylosanthis* BR 446^T (KU724154), BR 510 (KU724155), BR 511 (KU724156); *nifH* of *B. stylosanthis* BR 446^T (KU724157), BR 510 (KU724158), BR 511 (KU724159); *nodC* of *B. stylosanthis* BR 446^T (KU724160), BR 510 (KU724161), BR 511 (KU724162); *recA* of *B. stylosanthis* BR 446^T (KU724163), BR 510 (KU724164), BR 511 (KU724165); *rpoB* of *B. stylosanthis* BR 446^T (KU724166), BR 510 (KU724167), BR 511 (KU724168); *rpoB* of *B. viridifuturi* SEMIA 690^T (KU724169).

The BioSample accession number for the genome sequence of BR 446^T is SAMN04549886.

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

suggested. The type strain is BR 446^T (=CNPSo 2823^T=HAMBI 3668^T=H-8^T), isolated from *Stylosanthes guianensis*.

The importance of legumes for human and animal nutrition is broadly known, and forage species are receiving increased attention, due to their contribution towards more sustainable livestock practices. The benefits of using legumes rely mainly on their capacity of establishing symbiotic partnerships with nitrogen-fixing bacteria – rhizobia – resulting in economic profits for the farmers by the replacement of chemical N fertilizers, and environmental contributions by decreasing the emission of greenhouse gases and pollution of water sources by nitrate (Hungria *et al.*, 2005, 2015; Ormeño-Orrillo *et al.*, 2013).

The genus *Bradyrhizobium* has been considered the ancestor of all rhizobia, with a probable origin in tropical acid soils (Lloret & Martínez-Romero, 2005; Norris, 1965). The genus includes root-nodulating bacteria, stem-nodulating bacteria with photosynthetic and nitrogen-fixing capacity, as well as non-symbiotic species, which occupy a variety of ecosystems (Eaglesham *et al.*, 1990; Islam *et al.*, 2008; Ormeño-Orrillo *et al.*, 2013; Rivas *et al.*, 2004). In the past few years, large biodiversity among strains of the genus *Bradyrhizobium* has been reported; however, the genus still encompasses few described species (Delamuta *et al.*, 2012, 2015; Germano *et al.*, 2006; Helene *et al.*, 2015; Hungria *et al.*, 2015; Menna *et al.*, 2009; Parker, 2015; Roma Neto *et al.*, 2010).

In the tropics, pastures for livestock occupy far larger areas than all agricultural crops together. However, most pastures are composed exclusively of naturally occurring or introduced grasses and are subjected to a continuous deterioration in soil fertility and a consequent plant–soil system degradation. Therefore, the inclusion of legumes inoculated with elite nitrogen-fixing strains could have great impact on sustainability. In this study, we investigated strains of the genus *Bradyrhizobium* that were isolated from species of the *Stylosanthes*, an important genus of perennial tropical forage legumes; the Brazilian Cerrados represent one main center of diversity of this legume.

Strains of the genus *Bradyrhizobium* used in this study are listed in Table 1. Strain BR 446^T was isolated from *Stylosanthes guianensis* in Brazil in the early 1970s at the Instituto de Pesquisas Agropecuárias do Centro-Sul (IPEACS), State of Rio de Janeiro, Brazil, and it has also been used in commercial inoculants for this legume since 1994. Strains BR 510 and BR 511 were isolated at the International Center for Tropical Agriculture (CIAT) from root nodules of *Stylosanthes capitata* collected in Manaus, Brazil. All strains are deposited at the 'Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja' (WFCC Collection no. 1213, WDCM Collection no. 1054), Londrina, State of Paraná, Brazil, and at the 'Biological Resources Center – CRB – Johanna Döbereiner' of Embrapa Agrobiologia, in Seropédica, State of Rio de Janeiro, Brazil. 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, and long-term storage took place by cryopreservation in liquid YM with 30 % (v/v) glycerol at −80 °C and −150 °C and by lyophilization.

The 16S rRNA gene and the partial sequences of the *dnaK*, *glnII*, *gyrB*, *recA*, *rpoB*, *nodC* and *nifH* genes and of the 16S–23S rRNA intergenic transcribed spacer (ITS) were amplified using primers and conditions described by Delamuta *et al.* (2013) and Menna & Hungria (2011), and were sequenced in an ABI3500xL sequence analyzer (Applied Biosystems). Other sequences representing members of the genus *Bradyrhizobium* used in this study were retrieved from the GenBank database. Accession numbers are shown in the phylograms or in Table S1 (available in the online Supplementary Material). Maximum-likelihood phylogenies were obtained with MEGA 6 (Tamura *et al.*, 2013) using the Tamura–Nei model (Tamura & Nei, 1993), and a multiple sequence alignment was constructed with MUSCLE (Edgar, 2004). Tree node support was evaluated with bootstrap analysis (Felsenstein, 1985) using 1000 pseudoreplicates. For the ITS, a neighbour-joining phylogram was reconstructed, based on a matrix of uncorrected distances (Willems *et al.*, 2001).

In the 16S rRNA gene phylogram, strains BR 446^T, BR 510 and BR 511 were grouped in a well-supported clade with 87 % bootstrap support (Fig. 1). The 16S rRNA gene sequences of the BR strains were 100 % identical to each other and showed 94.7 to 99.8 % similarity with other described species of the genus *Bradyrhizobium* (Table 2). Highest similarity was observed with *Bradyrhizobium huanghuaihaiense*, followed by *Bradyrhizobium arachidis* (Table 2). Despite the 16S rRNA gene being broadly used in phylogenetic analyses, in some bacterial genera, such as *Bradyrhizobium*, the sequences are considered highly conserved, limiting species delineation (Delamuta *et al.*, 2015; Helene *et al.*, 2015; Menna *et al.*, 2009). However, it is noteworthy that this new group with the BR strains could be observed in the analysis of the 16 rRNA gene (Fig. 1).

The higher variability of the ITS region in comparison with the 16S rRNA gene among species of the genus *Bradyrhizobium* allows a better delineation of species (Germano *et al.*, 2006; Menna *et al.*, 2009). In the ITS phylogram, the BR strains were clustered in a clade distinct from other bradyrhizobia, with a bootstrap support of 100 %, and showed higher similarity with *Bradyrhizobium japonicum* (Fig. S1). The level of similarity among the BR strains was 99 %, and

Table 1. Strains used in this study

Species/strain name	Other strain nomenclature	Original host species	Geographical origin	Reference
Bradyrhizobium stylosanthis BR 446^T	CNPSo 2823^T, H-8^T, HAMBI 3668^T	*Stylosanthes guianensis*	Brazil	Vargas *et al.* (1974)
Bradyrhizobium stylosanthis BR 510	CNPSo 2825, CIAT 2400	*Stylosanthes capitata*	Brazil	This study
Bradyrhizobium stylosanthis BR 511	CNPSo 2826, CIAT 2403	*Stylosanthes capitata*	Brazil	This study
Bradyrhizobium huanghuaihaiense CCBAU 23303^T	CNPSo 2790^T, LMG 26136^T, CGMCC 1.10948^T, HAMBI 3180^T,	*Glycine max*	China	Zhang *et al.* (2012)
Bradyrhizobium arachidis CCBAU 051107^T	CNPSo 2791^T, LMG 26795^T, CGMCC 1.12100^T, HAMBI 3281^T	*Arachis hypogaea*	China	Wang *et al.* (2013)

less than 93.4 % in relation to other species of the genus *Bradyrhizobium* (Table 2). These results support that the BR strains may represent a new clade, as strains sharing less than 95.5 % similarity in the ITS region would correspond to a DNA-DNA hybridization (DDH) value below 60 % (Willems *et al.*, 2003).

A multilocus sequence analysis (MLSA) approach was also employed in our study, as there is an increasing number of reports showing the suitability of this method for the taxonomic classification of bradyrhizobia (Delamuta *et al.*, 2012, 2013, 2015; Helene *et al.*, 2015; Menna *et al.*, 2009; Parker, 2015). In addition to providing a greater number of informative nucleotide sites, the effects of gene recombination and horizontal transfer are mitigated by the analysis of at least three concatenated protein-encoding gene sequences in the MLSA analysis (Gevers *et al.*, 2005; Schleifer, 2009). The MLSA phylogram built considering five housekeeping genes (*dnaK+glnII+recA+rpoB+gyrB*; Fig. 2) grouped the BR strains in a separate cluster with high bootstrap support (100 %), with *B. huanghuaihaiense* representing the closest taxon. We also present phylograms considering three (*dnaK+glnII+recA*; Fig. S2) and four (*dnaK+glnII+recA+rpoB*; Fig. S3) housekeeping genes, encompassing larger number of species, and the results confirm a distinct clade for the BR strains. The nucleotide identity (NI) evaluated for the five concatenated genes ranged from 99 to 100 % among the BR strains and from 88 % to 96.5 % with other bradyrhizobial species (Table 2). It is worth mentioning that, recently, a value of 97 % for NI of five concatenated genes was proposed as a cut-off level for definition of species of the genus *Bradyrhizobium* (Durán *et al.*, 2014), giving more support to the proposal of the BR strains as representatives of a novel species.

The fingerprinting of the BR strains was evaluated by the rep-PCR methodology with BOX-A1R primer, as described by Kaschuk *et al.* (2006). The dendrogram was built with the Bionumerics 7.5 software using the UPGMA algorithm and the Jaccard coefficient, and revealed a similarity of 87.9 % among the BR strains, which in turn were distinct from other species of the genus *Bradyrhizobium* (Fig. S4).

DDH is commonly used for the definition of bacterial species. The values are expressed as percentage relatedness, or as the difference in denaturation temperature of DNA strands (ΔT_m), in which strains of the same species share values above 70 % relatedness and 5 °C or less difference in ΔT_m values (Rosselló-Móra & Amann, 2001; Tindall *et al.*, 2010). The DNA–DNA relatedness (DDH) was determined on the basis of the thermal denaturation temperatures of hybrid and homologous genomic DNA, as described by Gonzalez & Saiz-Jimenez (2005), except that the DNA concentration was adjusted to 2 μg per reaction and that formamide was added (5 % final concentration) for fluorescent measurement. The experiments were performed in 96-well optical plates in three replicates and included samples without DNA as negative controls. Thermal conditions consisted of a denaturation step of 99 °C for 10 min, followed by an annealing period of 8 h at 72 °C (optimum temperature for renaturation) (De Ley, 1975; Gonzalez & Saiz-Jimenez, 2005; Radl *et al.*, 2014). This was followed by progressive 10 min steps, each at 1.8 °C below the previous one, until reaching 25 °C, when the temperature was held for 30 min before refrigeration at 4 °C. The fluorescence was measured using a denaturation ramp settled at the step and hold mode. Heating rate was 0.2 °C s^{-1} with a fluorescence decreasing measurement at each 0.2 °C step, during a 12 s hold, and between 25 and 99.9 °C (Moreira *et al.*, 2011). The BR strains displayed ΔT_m values with less than 5 °C difference among them, indicating that they belong to the same species, but BR 446^T displayed values of 6.7 °C and 13.7 °C with *B. huanghuaihaiense* and *B. arachidis*, respectively, confirming that the BR strains represent a novel species (Table S2).

Recently, efforts have been made to correlate the DDH values with the indexes obtained by the analysis of whole genomes. The most promising proposal to estimate genome relatedness in prokaryotic taxonomy is the ANI (average nucleotide identity), and values of 95–96 % would correspond to 70 % DDH (Richter & Rosselló-Móra, 2009). The draft genome sequence of BR 446^T (BioSample accession SAMN04549886, obtained in this study) and the genome

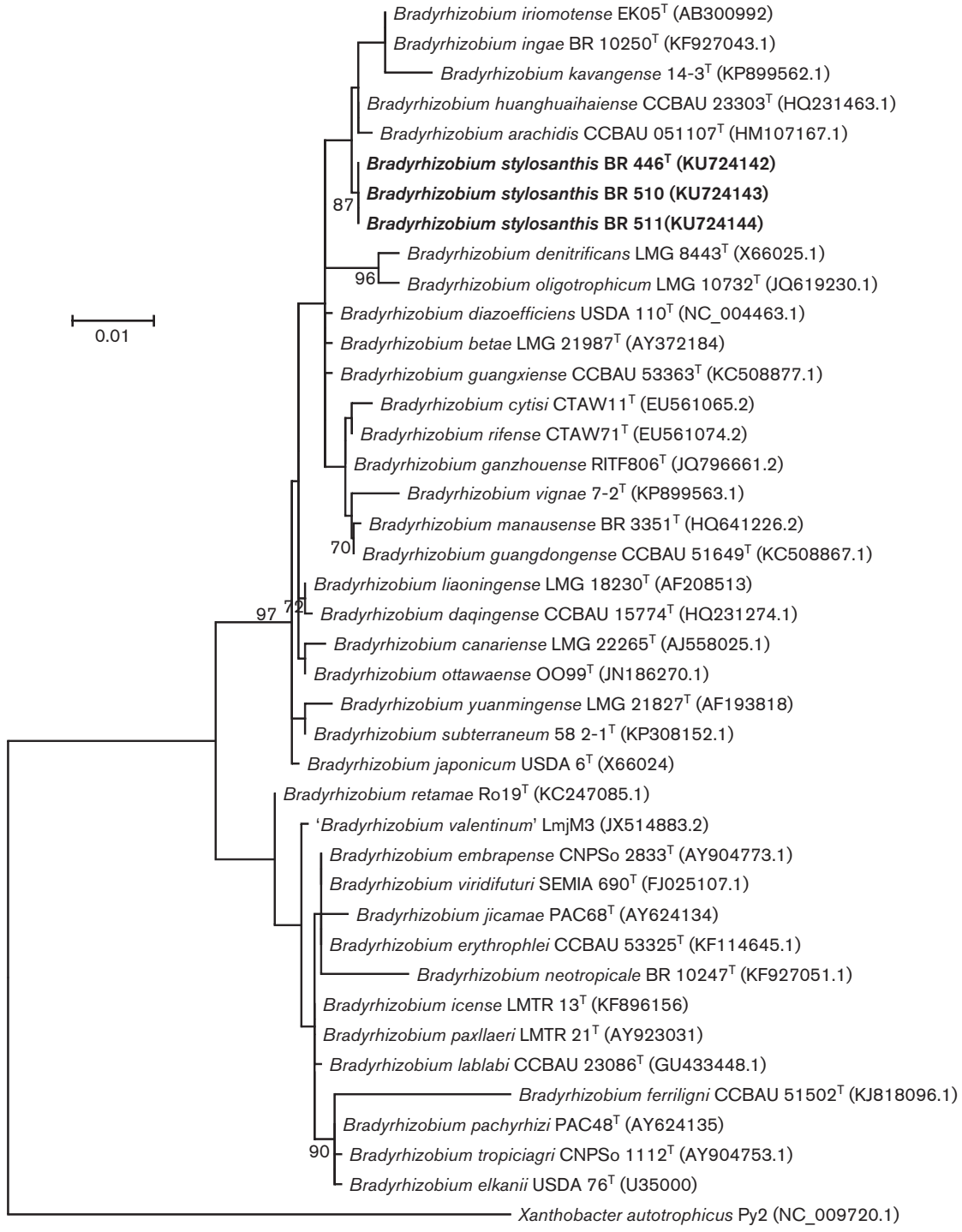

Fig. 1. Maximum-likelihood phylogeny based on 16S rRNA gene sequences. Accession numbers are indicated within parentheses. Strains of the novel species are shown in boldface. Bootstrap values >70 % are indicated at the nodes. *Xanthobacter autotrophicus* Py2 was used as an outgroup. Bar, 1 substitution per 100 nucleotide positions.

sequence of *B. huanghuaihaiense* CCBAU 23303^T (SAMN02927902) were used to calculate the ANI with the JSpecies (Richter & Rosselló-Móra, 2009) and Mummer for sequence alignment. Strain BR 446^T and *B. huanghuaihaiense* CCBAU 23303^T shared an ANI value of 90.7 %, indicating that these strains do not belong to the same species.

Table 2. Percentage nucleotide identity between *Bradyrhizobium stylosanthis* sp. nov. strains and the type strains of other species of the genus *Bradyrhizobium* in the 16S rRNA gene, ITS and multilocus sequence analysis

Species	Gene				
	16S rRNA	ITS	MLSA (3 genes)*	MLSA (4 genes)†	MLSA (5 genes)
Within *B. stylosanthis*	100	99.5–100	99.5–100	99.2–100	99–100
Between *B. stylosanthis* and:					
B. huanghuaihaiense	99.8	87.8–87.9	96.5–96.6	96.7–96.9	96.2–96.5
B. arachidis	99.6	92.8-92.9	95.4	95.5–95.7	95.7–96.1
B. diazoefficiens	99.5	81.4	95.1–95.2	95.4	95.2–95.3
B. japonicum	99.1	86–86.2	95.6	95.1–95.2	95.5–95.6
B. vignae	98.8	89.9–90	94.4–94.5	94.6–94.8	ND
B. kavangense	98.9	84.3–84.4	94.8–95.3	95.1	ND
B. guangdongense	99.2	ND	93.2–93.3	93.9–94.2	94.4
B. guangxiense	99.5	ND	95.4–95.5	95.1	94.7
B. canariense	99.1	86.6	94.3	93.3–93.5	93.2–93.3
B. betae	99.5	83.1	94.2–94.3	93.9–94.1	93.4
B. yuanmingense	98.6	93.3–93.4	94.7–95	95–95.1	94.7–94.9
B. liaoningense	99.1	84.4–84.5	95.8–95.9	95.7–95.9	95.6
B. cytisi	99.2	86.5–86.6	92.8–92.9	93–93.2	92.7–92.9
B. daqingense	99.1	88.2–88.3	94.8–94.9	94.9	94.6
B. iriomotense	99.5	78.5–78.6	93.1–93.4	92.5–92.7	93.1–93.3
B. rifense	99.2	86–86.1	93.9–94	94.1	93.6–93.8
B. ganzhouense	99.3	ND	93.9–94	ND	ND
B. ottawaense	99.1	ND	94.5–94.6	94.6–94.8	94.2–94.4
B. neotropicale	96.9	82.2–82.3	93.5–93.6	93.5–93.6	94
B. ingae	99.5	ND	93.5–93.6	92.7–92.9	93–93.1
B. subterraneum	99	86.9–87.1	93.1–93.4	93.7–93.8	ND
B. manausense	99.1	ND	94.2–94.3	94.6–94.9	94.2–94.3
B. tropiciagri	96.5	67.3	90.8–90.9	90.1–90.2	90.9
B. embrapense	96.9	75.9	90.8–90.9	90.2–90.4	90.7–90.8
B. viridifuturi	96.9	ND	91–91.2	90.4–90.5	90.9–91.1
B. elkanii	96.5	75.7–75.9	90.4–90.5	89.9	90.4–90.6
B. pachyrhizi	96.6	72.8–72.9	90–90.1	89.5–89.6	90.2
B. erythrophlei	96.9	78.4–78.5	ND	ND	ND
B. ferriligni	94.7	76.3–76.4	ND	ND	ND
B. jicamae	96.5	74.4–74.7	89.1–89.3	88.7–88.8	89–89.1
B. lablabi	96.7	76.7–77	90.6	89.8	89.6
B. retamae	97.1	73.4–73.6	89.1–89.4	88.2–88.5	88
B. icense	96.8	ND	90.2	ND	ND
B. paxllaeri	96.8	ND	89.4–89.6	ND	ND
B. valentinum	96.7	ND	88.7	ND	ND
B. denitrificans	98.6	55.4–55.5	88.3–88.4	88.6–88.7	88.9
B. oligotrophicum	98.9	53.1–53.4	88.3–88.4	89–89.1	89–89.2

ND, Not determined.
**dnaK+glnII+recA.*
†dnaK+glnII+recA+rpoB.

To determine the DNA G+C content of BR 446^T, genome contigs were concatenated and the proportions of G+C were calculated with JSpecies (Richter & Rosselló-Móra, 2009). The BR 446^T genome had a DNA G+C content of 63.9 mol%, similar to the values reported for species of the genus *Bradyrhizobium* (Xu *et al.*, 1995).

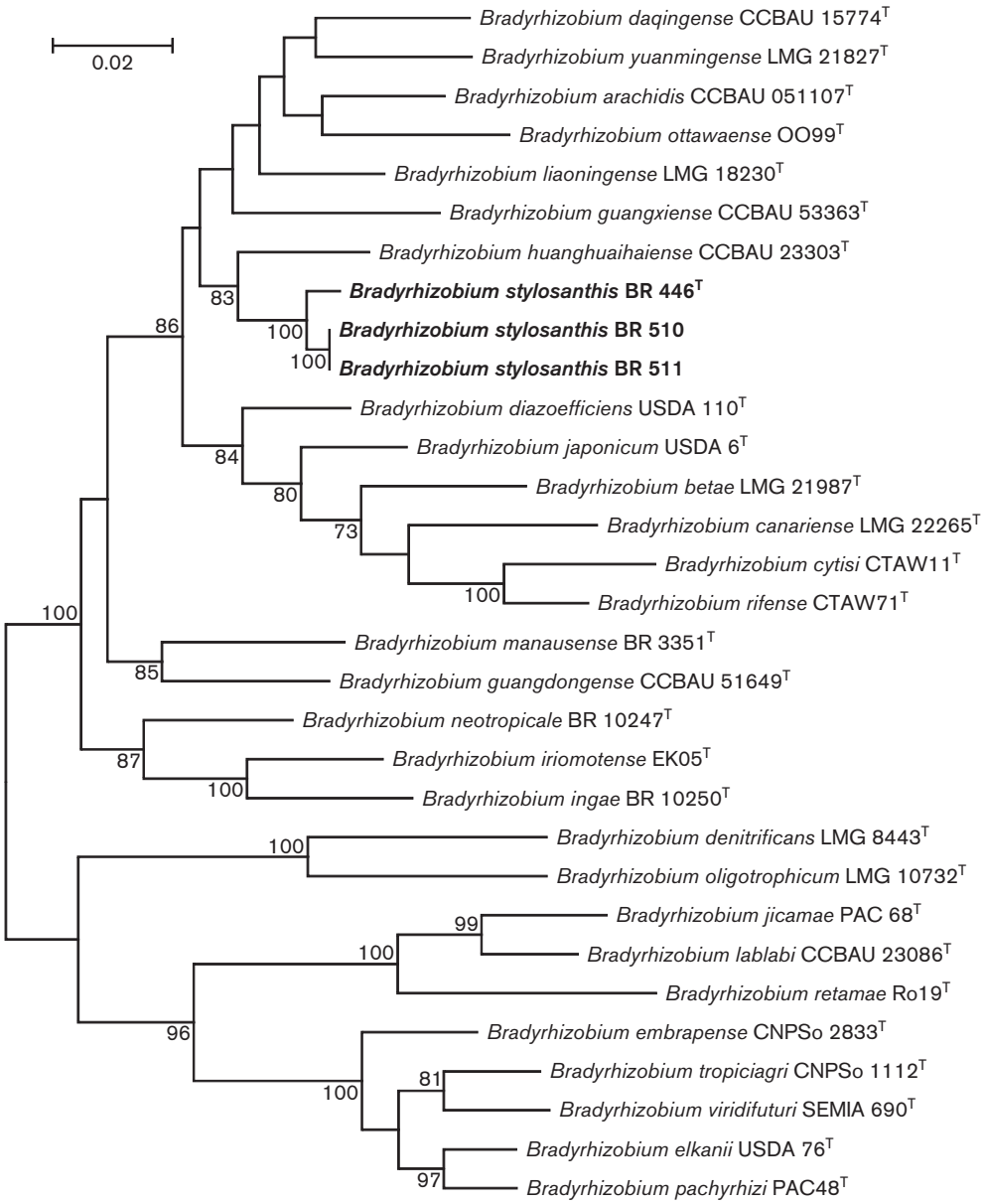

Fig. 2. Maximum-likelihood phylogeny based on concatenated *dnaK+glnII+recA+rpoB+gyrB* gene sequences showing the relationships between strains of the novel species (in boldface) and other members of the genus *Bradyrhizobium*. Accession numbers are indicated in Table S1. Only bootstrap values >70 % are indicated at the nodes. Bar, 2 substitutions per 100 nucleotide positions.

Knowing that the BR strains are very effective in fixing nitrogen with species of the genus *Stylosanthes*, we investigated genes related to nodulation and N_2-fixation capacity. *nodC* and *nifH* sequences were either obtained in this study using the primers and amplification conditions described by Menna & Hungria (2011), or retrieved from the Genbank database. The BR strains were clustered in a well-supported clade in both *nifH* (Fig. 3) and *nodC* (Fig. S5) phylograms and were positioned in a different branch from all other species of the genus *Bradyrhizobium*. Interestingly, they showed greater similarity in both analyses to *Bradyrhizobium iriomotense* and also *Bradyrhizobium manausense* which, similar to strains BR 510 and 511, was isolated in Manaus, in the Amazon region. This indicates a different evolutionary history for the conserved and the nitrogen-fixation genes.

We also evaluated the nodulation and nitrogen fixation abilities of the BR strains in different host legumes. The BR strains formed effective nodules in *S. guianensis*, siratro (*Macroptilium atropurpureum*), centrosema (*Centrosema pubescens*), Bambara groundnut (*Vigna subterranea*) and desmodium (*Desmodium heterocarpon*), but were unable to

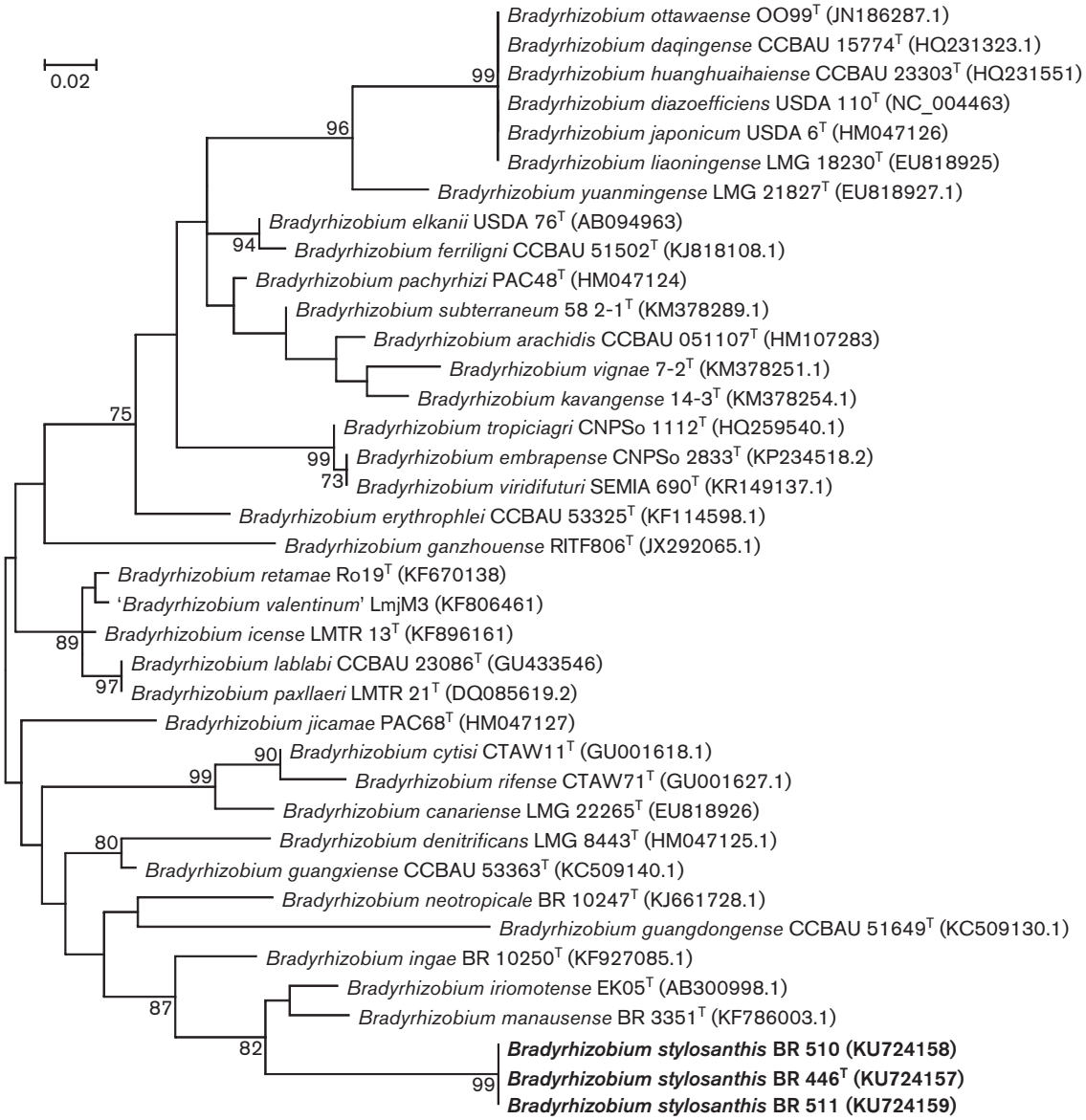

Fig. 3. Maximum-likelihood phylogeny of *nifH* (203 aligned positions) gene sequences. Accession numbers are indicated within parentheses. Strains of the novel species are shown in boldface. Bootstrap values >70 % are indicated at the nodes. Bar, 2 substitutions per 100 nucleotide positions.

nodulate both a restricted (BRS 360RR) and a promiscuous (TGX1845-10E) genotype of soybean (*Glycine max*).

Fatty acids profiles were determined using the MIDI Sherlock Microbial Identification System (MIDI, 2001) with the TSBA6 database after growing the BR strains, *B. huanghuaihaiense* and *B. arachidis* on YMA until the end of the exponential growth phase (5 days). All the BR strains exhibited, in different proportions, $C_{16:0}$, $C_{16:1}\omega5c$ and summed feature 8 ($C_{18:1}\omega6c$/$C_{18:1}\omega7c$) as major fatty acids; BR 446^T also presented a small amount of $C_{19:0}$ cyclo $\omega8c$ (Table S3). These results are consistent with those found in bradyrhizobia (Tighe *et al.*, 2000). It is noteworthy that these strains were different from *B. huanghuaihaiense* by having larger relative amounts of summed feature 8 and from *B. arachidis* by lacking $C_{16:1}\omega5c$.

Several phenotypic characteristics were evaluated for the BR strains, as well as for *B. huanghuaihaiense* and *B. arachidis*. In addition, we made comparisons with previous data from our laboratory for *Bradyrhizobium diazoefficiens* and *Bradyrhizobium japonicum*. Morpho-physiological tests included utilization of carbon sources by fermentation using the API 50CH kit (BioMérieux), according to the manufacturer's instructions, with YM-minus-mannitol used as the basal medium. Growth under distinct temperature and pH conditions was verified in liquid YM, while evaluation of tolerance to 1 %

Table 3. Distinctive phenotypic features of the novel species and their closest relatives

Strains: 1, *B. stylosanthis* sp. nov. BR 446^T; 2, *B. stylosanthis* sp. nov. BR 510; 3, *B. stylosanthis* sp. nov. BR 511; 4, *B. huanghuaihaiense* CCBAU 23303^T; 5, *B. arachidis* CCBAU 051107^T; 6, *B. diazoefficiens* USDA 110^T; 7, *B. japonicum* USDA 6^T. Data were obtained in this study. +, Growth; −, no growth; w, weakly positive.

Characteristic	1	2	3	4	5	6*	7*
Carbon source utilization‡							
D-Arabinose	w	+	+	+	+	+	w
L-Arabinose	w	+	+	w	+	w	+
D-Ribose	w	+	w	w	+	+	+
L-Xylose	+	+	+	+	+	+	w
Methyl β-D-xylopyranoside	w	w	w	w	−	w	w
D-Sorbitol	−	−	−	−	w	w	w
Aesculin iron citrate	+	+	+	w	+	+	+
Glycogen	+	+	+	+	+	−	−
D-Fucose	w	w	w	+	+	+	+
Potassium gluconate	+	+	+	+	+	−	−
Potassium 5-ketogluconate	+	+	+	+	+	−	−
Growth at pH 8	+	+	+	−	+	−	+
Resistance to (μg per disc):							
Erythromycin (15)	+	+	w	+	+	−	+
Ampicillin (10)	−	+	+	−	−	w	−
Neomycin (30)	−	−	−	w	w	−	−
Tetracycline (30)	−	w	w	w	+	−	−
Nalidixic acid (30)	w	w	−	+	+	+	+
Colony size (mm) after 7 days of incubation in YMA†	1	1.27	1.34	1.19	1.37	1.3	0.7

*Data from Delamuta *et al.* (2013).
†Mean of six colonies.
‡Carbon source utilization was evaluated with the API 50CH kit (BioMérieux).

NaCl was performed on YMA. We also evaluated the ability of the strains to grow in liquid Luria-Bertani (LB) medium; urease activity in YMA medium with phenol red; and colony size and resistance to antibiotics using the disc diffusion method on YMA plates. In general, the BR strains analysed in this study showed similar properties in most tests, with the resistance to antibiotics and colony size being more variable. Results that were different among the BR strains, or that were different among species are shown in Table 3.

In conclusion, by using a polyphasic approach comprising genotypic, phenotypic and phylogenetic analyses, the results indicated that the BR strains represent a novel species distinct from all previously described species of the genus *Bradyrhizobium*, for which the name *Bradyrhizobium stylosanthis* sp. nov. is proposed, with BR 446^T nominated as the type strain.

Description of *Bradyrhizobium stylosanthis* sp. nov.

Bradyrhizobium stylosanthis (sty.los.an′this. N.L. gen. n. *stylosanthis* of *Stylosanthes*, a botanical genus name; referring to the isolation source of the strains, root nodules of the genus *Stylosanthes*).

Cells are Gram-stain-negative, non-spore-forming rods. Colonies are 1 to 1.34 mm in diameter, circular, convex, opaque, with low production of mucus and slightly pink, after 7 days of growth at 28 °C on plates containing YMA medium with Congo Red. All strains produce an alkaline reaction in YMA. Grows at pH 4.5 and 8, with optimum growth at pH 6.8. Grows optimally at 28 °C, and is unable to grow at 37 °C or above. Does not grow on YMA in the presence of 1 % NaCl or in LB broth. Urease activity is positive. In general, all strains ferment the carbohydrates D-arabinose, L-arabinose, L-xylose, aesculin iron citrate, starch, glycogen, D-lyxose, L-fucose, potassium gluconate and potassium 5-ketogluconate. They weakly ferment glycerol, D-ribose, D-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-fucose and D-arabitol, and they fail to ferment erythritol, D-adonitol, L-sorbose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose,

xylitol, gentiobiose, turanose, D-tagatose, L-arabitol and potassium 2-ketogluconate. Most strains are resistant to the antibiotics penicillin G, chloramphenicol, erythromycin, ampicillin and bacitracin, moderately sensitive to tetracycline and nalidixic acid, and sensitive to cefuroxime, streptomycin and neomycin.

The type strain is BR 446^T (=CNPSo 2823^T=HAMBI 3668^T=H-8^T), isolated from an effective nodule of *Stylosanthes guianensis* in Brazil. Its genomic DNA G+C content is 63.9 mol%.

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