Bradyrhizobium tropiciagri sp. nov. and *Bradyrhizobium embrapense* sp. nov., nitrogen-fixing symbionts of tropical forage legumes

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Biological nitrogen fixation is a key process for agricultural production and environmental sustainability, but there are comparatively few studies of symbionts of tropical pasture legumes, as well as few described species of the genus *Bradyrhizobium*, although it is the predominant rhizobial genus in the tropics. A detailed polyphasic study was conducted with two strains of the genus *Bradyrhizobium* used in commercial inoculants for tropical pastures in Brazil, CNPSo 1112^T, isolated from perennial soybean (*Neonotonia wightii*), and CNPSo 2833^T, from desmodium (*Desmodium heterocarpon*). Based on 16S-rRNA gene phylogeny, both strains were grouped in the *Bradyrhizobium elkanii* superclade, but were not clearly clustered with any known species. Multilocus sequence analysis of three (*glnII*, *gyrB* and *recA*) and five (plus *atpD* and *dnaK*) housekeeping genes confirmed that the strains are positioned in two distinct clades. Comparison with intergenic transcribed spacer sequences of type strains of described species of the genus *Bradyrhizobium* showed similarity lower than 93.1 %, and differences were confirmed by BOX-PCR analysis. Nucleotide identity of three housekeeping genes with type strains of described species ranged from 88.1 to 96.2 %. Average nucleotide identity of genome sequences showed values below the threshold for distinct species of the genus *Bradyrhizobium* (<90.6 %), and the value between the two strains was also below this threshold (91.2 %). Analysis of *nifH* and *nodC* gene sequences positioned the two strains in a clade distinct from other species of the genus *Bradyrhizobium*. Morphophysiological, genotypic and genomic data supported the description of two novel species in the genus *Bradyrhizobium*, *Bradyrhizobium tropiciagri* sp. nov. (type strain CNPSo 1112^T=SMS 303^T=BR 1009^T=SEMIA 6148^T=LMG 28867^T) and *Bradyrhizobium embrapense* sp. nov. (type strain CNPSo 2833^T=CIAT 2372^T=BR 2212^T=SEMIA 6208^T=U674^T=LMG 2987).

Abbreviations: ANI, average nucleotide identity; ITS, intergenic transcribed spacer; ML, maximum-likelihood; MLSA, multilocus sequence analysis; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers for the *nifH*, *dnaK* and *nodC* gene sequences of *B. embrapense* CNPSo 2833^T and the *nodC* gene sequence of *B. tropiciagri* CNPSo 1112^T are KP234518, KP234519, KP234521 and KP234520, respectively.

Four supplementary figures are available with the online Supplementary Material.

Biological nitrogen fixation, performed by prokaryotes, mostly bacteria, with the ability to convert atmospheric nitrogen into ammonia and thereafter other nitrogen compounds that can be assimilated by plants, stands out as a key process for agricultural production and environmental sustainability (Hungria *et al.*, 2005). The most effective contribution occurs when the process is performed by bacteria collectively known as rhizobia in symbiosis with leguminous plants (Ormeño-Orrillo *et al.*, 2013). Brazil is a major producer of several grain legumes of economic

Table 1. Strains used in this study

Strain	Other strain names	Original host species	Geographical origin	Reference(s)
B. tropiciagri sp. nov. CNPSo 1112^T	SMS 303^T, BR 1009^T, SEMIA 6148^T, LMG 28867^T	*Neonotonia wightii*	Brazil	Delamuta *et al.* (2012)
B. embrapense sp. nov. CNPSo 2833^T	CIAT 2372^T, BR 2212^T, SEMIA 6208^T, U674^T LMG 29087	*Desmodium heterocarpon*	Colombia	Delamuta *et al.* (2012); Menna *et al.* (2009a)
B. elkanii USDA 76^T	ATCC 49852^T, DSM 11554^T, NBRC 14791^T, LMG 6134^T, CNPSo 62^T	*Glycine max*	USA	Kuykendall *et al.* (1992)
B. pachyrhizi PAC 48^T	LMG 24246^T, CECT 7396^T, CNPSo 2077^T	*Pachyrhizus erosus*	Costa Rica	Ramírez-Bahena *et al.* (2009)
B. jicamae PAC 68^T	LMG 24556^T, CECT 7395^T, CNPSo 2076^T	*Pachyrhizus erosus*	Honduras	Ramírez-Bahena *et al.* (2009)
B. lablabi CCBAU 23086^T	LMG 25572^T, HAMBI 3052^T, CNPSo 2585^T	*Lablab purpureus*	China	Chang *et al.* (2011)
B. retamae Ro19^T	LMG 27393^T, CECT 8261^T, CNPSo 2586^T	*Retama monosperma*	Morocco	Guerrouj *et al.* (2013)

importance such as soybean [*Glycine max* (L.) Merr.], but others are variously employed as green manures, in forestry, and for pastures, among other uses, all contributing to the improvement of soil quality and fertility (Hungria *et al.*, 2005; Ormeño-Orrillo *et al.*, 2013). Several efficient rhizobial strains for this broad range of applications have been isolated from Brazilian soils and are available for use in commercial inoculants; the great majority belong to the genus *Bradyrhizobium* (Binde *et al.*, 2009; Menna *et al.*, 2006, 2009a, b; Roma Neto *et al.*, 2010). In the last decade, our research groups have reported several studies showing high levels of genetic diversity among indigenous tropical rhizobia, including novel species and several groups that may represent novel species (Binde *et al.*, 2009; Dall'Agnol *et al.*, 2013, 2014; Delamuta *et al.*, 2012, 2013; Germano *et al.*, 2006; Menna *et al.*, 2006, 2009a, b; Ribeiro *et al.*, 2009, 2012; Roma Neto *et al.*, 2010).

The bradyrhizobial strains used in this study, CNPSo 1112^T and CNPSo 2833^T (Table 1), were identified as forming independent branches in a previous multilocus sequence analysis (MLSA) phylogeny (Delamuta *et al.*, 2012; Menna *et al.*, 2009a). These strains are effective symbionts of tropical pasture legumes. Strain CNPSo 1112^T was isolated from perennial soybean [*Neonotonia wightii* (Wight & Arn.) J. A. Lackey; formerly classified as *Glycine wightii*] by researchers of the Instituto Agronômico de Campinas (Collection SMS, Seção de Microbiologia do Solo), State of São Paulo, Brazil, and has been used in commercial inoculants for this legume since 1994. *Neonotonia wightii* is a perennial forage from Africa that grows well in several tropical countries including Brazil. Strain CNPSo 2833^T was isolated from *Desmodium heterocarpon (L.) DC.* subsp. *ovalifolium* (Prain) H. Ohashi (former *Desmodium ovalifolium* Merr.) by researchers of the International Center for Tropical Agriculture (CIAT), Colombia, and it has been used in commercial inoculants for this legume in Brazil since 1988. The use of commercial rhizobial inoculants should be emphasized, but globally is often concentrated in a small number of legumes (Hungria *et al.*, 2005), with a lack of elite strains for forage legumes, despite their key role in nitrogen cycling, helping to maintain soil fertility and contributing to animal nutrition. Due to the relevance of strains CNPSo 1112^T and CNPSo 2833^T as commercial inoculants for tropical forages, we performed a polyphasic analysis to determine their taxonomic positions.

Strains used in this study are listed in Table 1. Strains CNPSo 1112^T and CNPSo 2833^T are deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC collection # 1213 and WDCM collection # 1054), located at Londrina, State of Paraná, Brazil, and at the Center for Genomic Sciences Culture Collection (Cuernavaca, Mexico), in addition to other international 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 30 % glycerol at −80 and −150 °C, or by lyophilization.

A BOX-PCR genomic fingerprint was generated as described previously (Kaschuk *et al.*, 2006), using the closest species based on the 16S rRNA gene phylogeny. One cluster included strains CNPSo 1112^T and CNPSo 2833^T and the type strains of *Bradyrhizobium elkanii* and *Bradyrhizobium pachyrhizi*, with a similarity level of 81 % (Fig. S1, available in the online Supplementary Material). Another cluster including the type strains of *Bradyrhizobium jicamae* and *Bradyrhizobium lablabi* joined at 84 % similarity. These two clusters were 75.5 % similar among each other. The type strain of *Bradyrhizobium retamae* occupied an isolated position in relation to the other species, with a similarity level of 73.2 % (Fig. S1). Although BOX-PCR is suitable for revealing prokaryotic diversity, the results show that, within the *B. elkanii* superclade, the diversity is apparently lower than in other rhizobial superclades, especially those of fast growers, as observed previously (Menna *et al.*, 2009b).

For the 16S rRNA gene analyses, sequences were retrieved from the GenBank database and accession numbers are shown in the phylogram. Neighbour-joining (NJ) and

maximum-likelihood (ML) phylogenies were obtained with MEGA6 (Tamura *et al.*, 2013), using the Tamura–Nei model (Tamura & Nei, 1993) and a multiple sequence alignment constructed with MUSCLE (Edgar, 2004). Tree node support was evaluated with bootstrap analysis (Felsenstein, 1985) using 1000 pseudoreplicates. NJ and ML reconstructions gave similar results; therefore, only the ML phylogram is presented (Fig. 1). Species of the genus *Bradyrhizobium* described so far were included in the 16S rRNA gene tree and two groups were formed, the *Bradyrhizobium japonicum* superclade and the *B. elkanii* superclade, as reported by other authors (Delamuta *et al.*, 2012; Durán *et al.*, 2014a, b; Menna *et al.*, 2006, 2009a; Ramírez-Bahena *et al.*, 2009; Yao *et al.*, 2015). CNPSo 1112^T and CNPSo 2833^T were included in the *B. elkanii* superclade. *B. retamae* and *Bradyrhizobium valentinum* were isolated, and CNPSo 1112^T formed a clade that was closely related to *B. elkanii*, *B. pachyrhizi* and *Bradyrhizobium ferriligni*. CNPSo 2833^T clustered with *B. jicamae*, *Bradyrhizobium erythrophlei* and *Bradyrhizobium neotropicale* (Fig. 1). The 16S rRNA gene sequences of the two strains shared 99.6 % identity, and the level of similarity among all members of this genus ranged from 96.4 to 100 % (Table 2).

Although the 16S rRNA gene is used widely to assess phylogenetic relationships among bacteria, in some genera, including *Bradyrhizobium*, it is very highly conserved, thus limiting species definition (Delamuta *et al.*, 2012; Menna *et al.*, 2006, 2009a, b; Wang & Martínez-Romero, 2000). To improve our knowledge about the rRNA gene region, we also analysed the 16S–23S rRNA intergenic transcribed

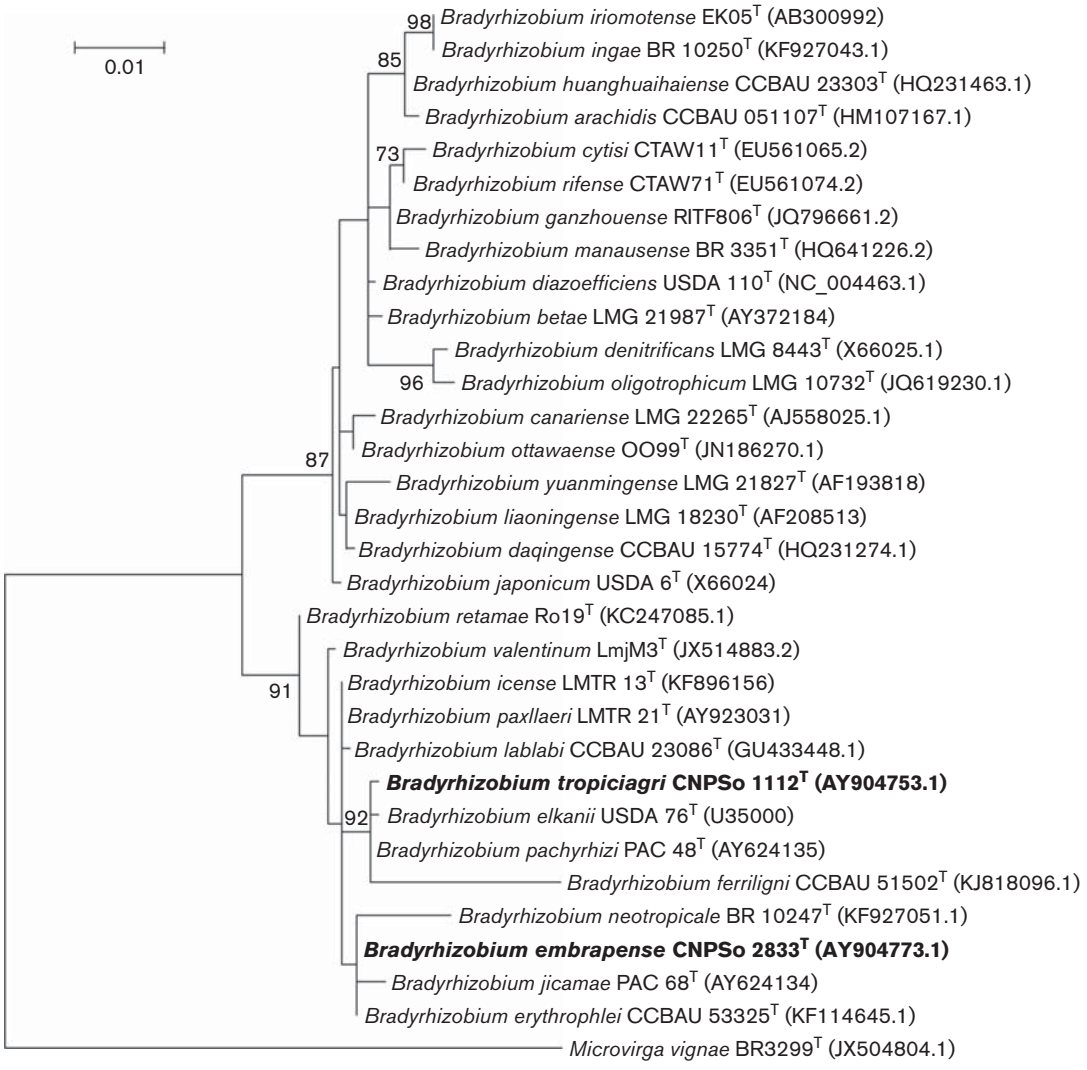

Fig. 1. ML phylogeny based on 16S rRNA gene sequences. Accession numbers are indicated in parentheses. Strains of the novel species are shown in bold. Bootstrap values >70 % are indicated at nodes. *Microvirga vignae* BR3299^T was used as an outgroup. Bar, 1 substitution per 100 nucleotide positions.

Table 2. Nucleotide sequence identity between strains CNPSo 1112^T and CNPSo 2833^T and the type strains of other species of the genus *Bradyrhizobium*

Length of the aligned regions: 16S rRNA gene, 1260 bp; ITS, 1091 bp; *atpD*, 432 bp; *glnII*, 505 bp; *recA*, 360 bp; *dnaK*, 238 bp; *gyrB*, 552 bp. For comparisons between the novel strains and the type strains of other species of the genus *Bradyrhizobium*, where the values are different, the value for CNPSo 2833^T is in bold. ND, Not determined.

Strain comparison	Identity (%)								
	16S rRNA	ITS	*atpD*	*glnII*	*recA*	*dnaK*	*gyrB*	*MLSA*	
								3 genes*	5 genes
Between CNPSo 1112^T and CNPSo 2833^T	99.6	82.7	96.2	96.8	96.3	93.6	96.1	96.4	96.1
Between CNPSo 1112^T and CNPSo 2833^T and the type strain of:									
B. elkanii	**99.6**–99.8	78.9–**93.1**	**95.3**–96.2	96.4–**97.6**	94.4	**93.2**–96.6	95.8–**96.1**	95.6–**96.2**	**95.7**–95.9
B. pachyrhizi	**99.6**–99.9	85.9–**93.1**	**95.6**–96.9	95.8–**96.6**	**94.4**–95.5	**93.2**–96.6	96.1	95.9	**95.5**–96.2
B. erythrophlei	99.6–**100**	75.4–**85.2**	ND	93.8–**94.0**	**92.5**–94.1	ND	**92.5**–93.2	**93.0**–93.7	ND
B. ferriligni	**97.6**–97.8	78.1–**92.1**	ND	95.8–**96.6**	95.2	ND	**92.2**–93.8	**94.5**–94.9	ND
B. jicamae	99.2–**99.6**	77.4–**88.0**	**93.2**–93.7	**89.7**–90.6	**90.8**–91.1	88.6–**90.3**	**90.3**–90.5	**90.2**–90.7	**90.8**–91.1
B. lablabi	99.5–**99.7**	73.3–**83.8**	**92.5**–94.4	90.4	94.1	88.6–**89.4**	89.1–**89.3**	90.8–**90.9**	**91.1**–91.3
B. retamae	99.1–**99.3**	72.7–**83.3**	**91.2**–91.6	**89.9**–90.8	93.0–**93.8**	88.2–**89.0**	**87.5**–87.6	**89.9**–90.1	**90.1**–90.2
B. icense	99.6–**99.8**	ND	92.3–**92.5**	90.2	91.9–**93.3**	89.9–**90.3**	**88.0**–88.5	90.0–**90.1**	90.5–**90.7**
B. paxllaeri	99.6–**99.8**	ND	92.5	**90.4**–90.8	**91.1**–91.3	89.4–**90.3**	89.8–**90.0**	**90.4**–90.6	90.8
B. valentinum	99.3–**99.6**	ND	**91.4**–91.8	**85.9**–86.5	93.0	87.8–**88.2**	**87.5**–88.2	**88.3**–88.8	**88.9**–89.3
B. diazoefficiens	96.9–**97.3**	70.3–**77.9**	**93.5**–95.1	89.3–**89.7**	91.3–**91.9**	87.8	**93.2**–94.0	91.6	**91.6**–91.9
B. canariense	96.9–**97.3**	67.2–**76.5**	**93.2**–93.9	87.3–**87.9**	**92.7**–93.8	86.5	**91.8**–92.3	**90.6**–90.9	**90.7**–91.0
B. betae	96.8–**97.2**	68.3–**75.8**	**92.1**–92.8	**87.9**–88.7	**91.9**–93.0	89.0–**90.3**	**91.4**–92.0	**90.3**–91.1	**90.7**–91.2
B. japonicum	97.3–**97.6**	68.5–**74.0**	**94.6**–95.3	**88.9**–89.1	**91.9**–92.5	**87.3**–88.6	**92.5**–93.4	**91.1**–91.6	**91.4**–92.0
B. yuanmingense	96.9–**97.3**	68.2–**75.4**	**95.3**–96.0	89.1	**91.9**–93.0	87.3	**91.3**–92.5	**90.6**–91.4	**91.2**–91.9
B. liaoningense	97.2–**97.6**	67.1–**74.3**	91.6–**91.8**	89.9–**90.0**	**93.3**–94.4	**87.3**–88.2	**92.2**–92.7	**91.7**–92.1	**91.2**–91.6
B. arachidis	96.5–**96.9**	68.0–**75.2**	**92.8**–93.2	89.7	**93.3**–93.6	**86.9**–87.8	**91.4**–92.3	**91.3**–91.7	**91.1**–91.6
B. huanghuaihaiense	96.7–**97.1**	67.8–**75.1**	**93.5**–94.2	88.5	**91.1**–91.9	**86.9**–88.6	**92.2**–92.9	**90.6**–91.1	**90.8**–91.4
B. cytisi	96.5–**96.9**	66.5–**75.5**	**93.5**–93.7	87.9	**90.8**–91.3	87.3–**87.8**	**90.0**–90.5	**89.4**–89.8	**90.1**–90.3
B. daqingense	97.1–**97.5**	67.6–**77.5**	91.8–**92.3**	89.3–**89.7**	**90.5**–91.9	88.2	**91.4**–92.3	**90.6**–91.1	**90.7**–90.9
B. iriomotense	96.4–**96.8**	**71.6**–75.2	**93.7**–95.3	86.7–**87.3**	**89.1**–90.2	89.4–**89.9**	**92.2**–92.3	**89.6**–89.8	**90.5**–90.9
B. rifense	96.5–**96.9**	67.3–**74.2**	**92.8**–93.5	88.9–**89.3**	**91.6**–92.2	88.2–**88.6**	**90.9**–92.2	**90.5**–91.0	**90.8**–91.2
B. ganzhouense	96.7–**97.1**	ND	**93.0**–93.5	88.3–**88.7**	90.5	**88.6**–89.0	**92.0**–92.5	**90.4**–90.5	**90.8**–90.9
B. ottawaense	97.1–**97.5**	ND	**92.1**–92.3	88.7–**89.5**	**91.6**–92.2	**86.5**–87.3	**90.9**–92.0	**90.6**–90.8	**90.4**–90.8
B. neotropicale	98.4–**98.8**	70.1–**79.7**	ND	88.7–**88.9**	**91.1**–92.2	ND	**92.2**–92.3	**90.7**–91.0	ND
B. ingae	96.4–**96.8**	ND	ND	89.1–**89.5**	**88.8**–90.0	ND	90.5	**89.7**–89.9	ND
B. manausense	96.4–**96.8**	ND	ND	88.5–**89.3**	**91.6**–92.7	ND	**91.4**–91.6	**90.7**–90.8	ND
B. denitrificans	96.7–**97.1**	**52.2**–53.2	**91.2**–92.1	86.9–**88.1**	89.4	87.3–**88.2**	89.1–**89.8**	88.4–**89.1**	89.0–**89.4**
B. oligotrophicum	96.7–**97.1**	**52.0**–54.3	**91.2**–91.6	86.1–**86.9**	90.8	88.2	88.2–**88.7**	88.1–**88.6**	88.8–**89.1**

**glnII*, *gyrB* and *recA*.

spacer (ITS). An NJ phylogram was build using a matrix of uncorrected distances (Willems *et al.*, 2001) and revealed that strains CNPSo 1112^T and CNPSo 2833^T were distinct from other bradyrhizobia (Fig. S2). The strains showed only 82.7 % identity to each other and less than 93.1 % identity to all other species of the genus *Bradyrhizobium* (Table 2). In a pioneering study, Willems *et al.* (2003) reported that strains of *Bradyrhizobium* with less than 95.5 % similarity in their ITS sequences belonged to different species, showing less than 60 % DNA–DNA hybridization.

To overcome the limitations of the 16S rRNA gene, other housekeeping genes with higher rates of evolution have been used to provide more information on phylogenetic relationships, in the MLSA approach (Azevedo *et al.*, 2015; Gevers *et al.*, 2005; Ribeiro *et al.*, 2009, 2012, 2013, 2015; Rivas *et al.*, 2009; Thompson *et al.*, 2005). MLSA phylograms were reconstructed as described for the 16S rRNA gene, first considering the *glnII*, *gyrB* and *recA* genes, since their sequences are available for all species of the genus *Bradyrhizobium*. In contrast to the 16S rRNA

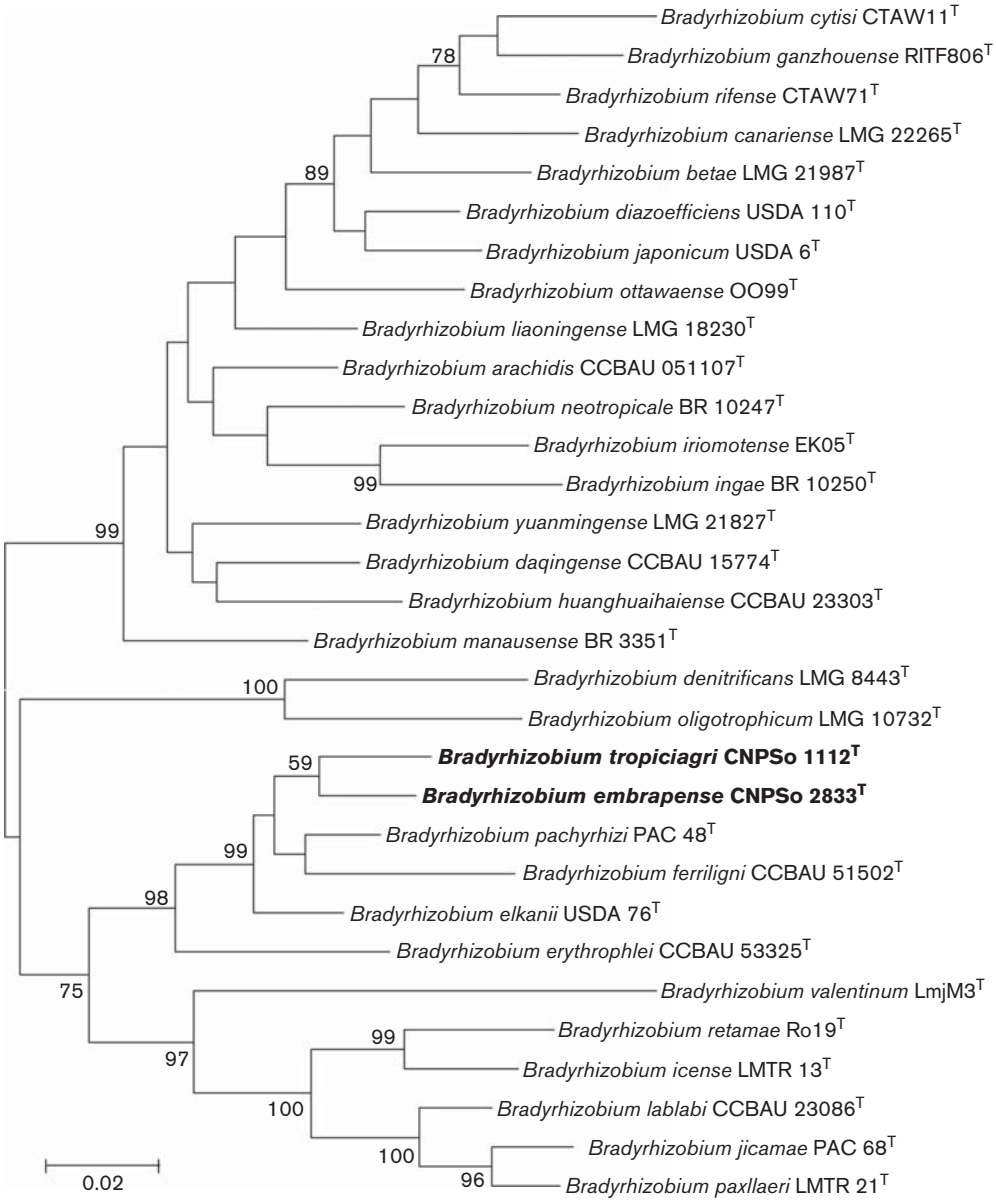

Fig. 2. ML phylogeny based on concatenated *glnII*+*gyrB*+*recA* gene sequences showing the relationships between strains of the novel species (in bold) and other members of the genus *Bradyrhizobium*. Accession numbers are listed in Table S2. Only bootstrap values >70 % are indicated at nodes, except for the novel species. Bar, 2 substitutions per 100 nucleotide positions.

gene phylogeny (Fig. 1), where strains CNPSo 1112^T and CNPSo 2833^T did not occupy well-defined positions, the MLSA phylogeny grouped both strains with *B. elkanii*, *B. pachyrhizi* and *B. ferriligni*, with 99 % bootstrap support (Fig. 2). It is worth mentioning that MLSA with three housekeeping genes has been used as support for the description of novel rhizobial species (Dall'Agnol *et al.*, 2014; Delamuta *et al.*, 2013; Ribeiro *et al.*, 2015). In addition, a phylogram was reconstructed with five protein-encoding genes (*glnII*, *gyrB*, *recA*, *atpD* and *dnaK*), and similar results were obtained (Fig. S3). Both phylograms show that strains CNPSo 1112^T and CNPSo 2833^T do not belong to any described species of *Bradyrhizobium*. In addition, although CNPSo 1112^T and CNPSo 2833^T were more related to each other than to *B. elkanii*, *B. pachyrhizi* or *B. ferriligni*, they formed a clade with low bootstrap support (75 % or less), suggesting that they do not belong to the same species (Figs 2 and S3).

The range of nucleotide identity between described species of *Bradyrhizobium* calculated from the three concatenated

Table 3. ANI of genome sequences of strains CNPSo 1112^T and CNPSo 2833^T and related type strains

ANI values were obtained using JSpecies (Richter & Rosselló-Móra, 2009) and Mummer for sequence alignment.

Strain used as query	ANI (%)	
	CNPSo 1112^T	CNPSo 2833^T
B. tropiciagri sp. nov. CNPSo 1112^T	(100)	91.2
B. embrapense sp. nov. CNPSo 2833^T	91.2	(100)
B. pachyrhizi PAC 48^T	90.6	90.3
B. elkanii USDA 76^T	90.6	90.6
B. jicamae PAC 68^T	85.3	85.4
B. lablabi CCBAU 23086^T	85.4	85.3
B. retamae Ro19^T	85.0	85.0
B. valentinum LmjM3^T	85.1	85.1

genes varied from 88.1 to 96.2 %, with CNPSo 1112^T and CNPSo 2833^T exhibiting 96.4 % identity to each other (Table 2). The closest described species to CNPSo 1112^T and CNPSo 2833^T were *B. elkanii*, with nucleotide identities to the type strain of 95.6 and 96.2 %, respectively, and *B. pachyrhizi*, with nucleotide identity to the type strain of 95.9 % for both strains. These values are lower than the 97.0 % suggested as a threshold for definition of species of the genus *Bradyrhizobium* (Durán *et al.*, 2014a), indicating that the two strains represent two novel, distinct species.

Average nucleotide identity (ANI) of genome sequences represents an alternative to DNA–DNA hybridization to estimate genome relatedness, and has been used recently in rhizobial taxonomy (Dall'Agnol *et al.*, 2013, 2014; Delamuta *et al.*, 2013; Durán *et al.*, 2014a, b). As suggested by Richter & Rosselló-Móra (2009), an ANI of 95–96 % corresponds to 70 % DNA–DNA hybridization, the standard level for prokaryotic species circumscription. ANI was estimated from the genome sequences of CNPSo 1112^T (accession no. SAMN03784761), CNPSo 2833^T (SAMN03782074) and *B. pachyrhizi* PAC 48^T (SAMN03782120), obtained in this study, and the genome sequences of *B. elkanii* USDA 76^T (NZ_ARAG00000000), *B. valentinum* LmjM3^T (SAMN 02688507), *B. retamae* Ro19^T (SAMN02689496), *B. lablabi* CCBAU 23086^T (SAMN02689497) and *B. jicamae* PAC 68^T (SAMN02689491). ANI values were calculated with JSpecies (Richter & Rosselló-Móra, 2009) and Mummer for sequence alignment. CNPSo 1112^T and CNPSo 2833^T showed ANI values below the species circumscription threshold when compared with each other (91.2 %) and in relation to all compared type strains (lower than 90.6 %), showing that these strains represent two novel species (Table 3).

To determine the DNA G+C contents of CNPSo 1112^T and CNPSo 2833^T, genome contigs were concatenated and the proportions of G+C bases were calculated with BioEdit (Hall, 1999). The genome of strain CNPSo 1112^T had a G+C content of 63.49 mol%, while that of CNPSo 2833^T was 62.81 mol%, which fall within the range reported for members of the genus *Bradyrhizobium* (Xu *et al.*, 1995).

The main agronomic feature of CNPSo 1112^T and CNPSo 2833^T is their high efficiency in fixing atmospheric N_2 with the hosts from which they have been isolated, perennial soybean and desmodium, respectively. As genes related to nodulation and nitrogen-fixation capacity may provide additional information about their symbiotic properties, we investigated both features. Under sterile substrate conditions, CNPSo 1112^T and CNPSo 2833^T had their effectiveness in nodulating and fixing nitrogen with their respective host legumes confirmed (data not shown). In addition, both strains were unable to nodulate soybean [*Glycine max* (L.) Merr.] and formed ineffective nodules on common bean (*Phaseolus vulgaris L.*). CNPSo 2833^T formed effective nodules when in symbiosis with siratro (*Macroptilium atropurpureum*), while CNPSo 1112^T formed ineffective nodules with this legume. CNPSo 2833^T was unable to nodulate perennial soybean (data not shown).

Sequences of the *nifH* and *nodC* genes were obtained in this study (according to Menna & Hungria, 2011) or retrieved from the GenBank database and accession numbers are shown in the phylograms. In phylogenetic trees reconstructed from both *nifH* (Fig. 3) and *nodC* (Fig. S4) gene sequences, CNPSo 1112^T and CNPSo 2833^T clustered together, but were separated from all other species of the genus *Bradyrhizobium*, indicating the evolutionary specificity of nitrogen-fixation-related genes.

Fatty-acid profiles were determined using the MIDI Sherlock Microbial Identification System (MIDI, 2001 with the TSBA6 database after growth on YMA (Delamuta *et al.*, 2013) to the end of the exponential growth phase (5 days). The main fatty acids of CNPSo 1112^T and CNPSo 2833^T were $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega6c$ and/or $C_{18:1}\omega7c$), typical of the genus *Bradyrhizobium* (Tighe *et al.*, 2000), but with different concentrations, and $C_{19:0}$ cyclo $\omega8c$, with CNPSo 2833^T strain exhibiting a larger proportion of this fatty acid in comparison with CNPSo 1112^T (Table S1).

Phenotypic tests were performed and the most relevant data are shown in Table 4. Type strains of *B. elkanii*,

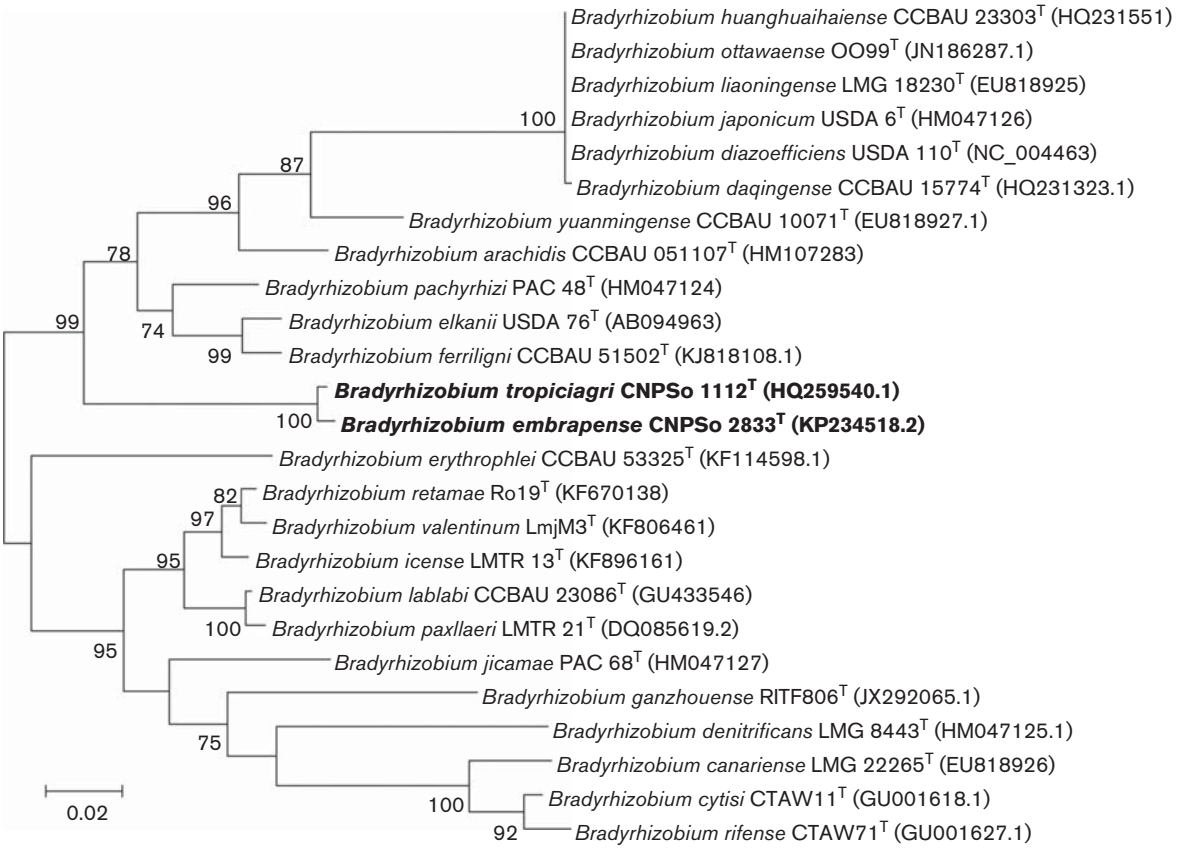

Fig. 3. ML phylogeny of *nifH* gene sequences (594 aligned positions). Accession numbers are indicated in parentheses. Strains of the novel species are shown in bold. Bootstrap values >70 % are indicated at nodes. Bar, 2 substitutions per 100 nucleotide positions.

B. pachyrhizi, *B. lablabi*, *B. jicamae* and *B. retamae*, representing the closest species, were also included in the analyses. Morphophysiological tests included utilization of carbon sources using the API 50CH kit (BioMérieux), according to the manufacturer's instructions, with YM-minus-mannitol used as the basal medium. Growth under distinct conditions of temperature, pH and salinity was verified in liquid YM. Other features evaluated were the capacity to grow in liquid Luria–Bertani (LB) medium, urease activity in YMA medium with red phenol, colony size and resistance to antibiotics using the disc diffusion method on YMA plates. CNPSo 1112^T and CNPSo 2833^T differed from each other primarily in relation to carbon-source utilization, growth at 37 °C and in LB and in colony size. We observed that, in general, CNPSo 1112^T, CNPSo 2833^T and the type strains of *B. elkanii* and *B. pachyrhizi* were more resistant to antibiotics when compared with related species of *Bradyrhizobium*. However, it is worth mentioning that phenotypic data may give limited information because, frequently, these characteristics are encoded on the accessory genome and can easily be lost, resulting in incongruence with genetic data (Ormeño-Orrillo & Martínez-Romero, 2013).

After an extensive polyphasic analysis comprising genotypic, phenotypic and phylogenetic analyses, our results strongly indicate that strains CNPSo 1112^T and CNPSo 2833^T represent two novel species distinct from all described species in the genus *Bradyrhizobium*. We propose the names *Bradyrhizobium tropiciagri* sp. nov. for CNPSo 1112^T and *Bradyrhizobium embrapense* sp. nov. for CNPSo 2833^T.

Description of *Bradyrhizobium tropiciagri* sp. nov.

Bradyrhizobium tropiciagri (tro.pi.ci.a′gri. L. adj. *tropicus* tropical; L. masc. gen. n. *agri* of a pasture; N.L. gen. n. *tropiciagri* of a tropical pasture, named for its nitrogen-fixation capacity with an important tropical pasture legume).

Cells are Gram-negative, non-spore-forming rods. Colonies are less than 1 mm in diameter, circular, convex and opaque, with low production of mucus and slightly pink, when grown on YMA medium containing Congo red after 7 days of growth at 28 °C. Produces an alkaline reaction in YMA containing bromothymol blue. The generation time is 7.42 h in YM broth. Grows at pH 4.5–8.0, with optimal growth at

Table 4. Distinctive phenotypic features of strains CNPSo 1112^T and CNPSo 2833^T and their closest relatives

Strains: 1, *B. tropiciagri* sp. nov. CNPSo 1112^T; 2, *B. embrapense* sp. nov. CNPSo 2833^T; 3, *B. elkanii* USDA 76^T; 4, *B. pachyrhizi* PAC 48^T; 5, *B. jicamae* PAC 68^T; 6, *B. lablabi* CCBAU 23086^T; 7, *B. retamae* Ro19^T. Data were obtained in this study. Carbon-source utilization was evaluated with the API 50CH kit (BioMérieux). +, Growth; −, no growth; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Carbon-source utilization							
L-Arabinose	+	+	+	+	+	w	+
D-Xylose	w	+	+	+	+	w	w
L-Xylose	w	+	+	+	+	+	+
D-Adonitol	w	−	w	w	−	w	−
D-Galactose	+	+	+	+	+	+	w
D-Glucose	−	+	w	w	w	w	w
D-Mannose	+	+	+	+	+	w	−
L-Rhamnose	w	w	w	w	+	+	w
Dulcitol	w	−	w	−	−	−	−
D-Mannitol	w	w	w	w	−	w	−
D-Sorbitol	w	w	w	w	−	−	−
Aesculin iron citrate	w	−	w	−	−	+	w
Melibiose	−	+	−	−	−	−	−
Starch	−	+	+	+	+	+	+
Glycogen	−	+	−	−	−	+	−
Xylitol	−	−	w	−	−	−	−
Gentiobiose	−	+	−	−	−	−	−
D-Lyxose	w	+	+	+	+	+	+
D-Fucose	+	+	+	+	+	+	w
L-Fucose	w	+	+	+	+	+	+
D-Arabitol	w	w	w	w	−	w	−
L-Arabitol	−	−	w	w	−	−	−
Potassium gluconate	−	+	−	−	−	−	−
Potassium 5-ketogluconate	−	+	−	−	−	−	−
Growth in/at:							
pH 4.5	+	+	−	w	−	−	−
37 °C	−	+	−	−	−	−	−
LB broth	−	+	−	−	−	−	−
Urease	+	+	+	+	−	+	−
Resistance to (μg per disc):							
Erythromycin (15)	+	+	+	+	w	+	+
Cefuroxime (30)	+	+	+	+	−	+	−
Neomycin (30)	w	w	−	−	−	−	w
Tetracycline (30)	+	+	+	+	+	−	−
Streptomycin (10)	+	−	+	+	−	−	−
Colony size (mm) on YMA*	<1	1.07	<1	1.37	<1	<1	<1

*Mean diameter of six colonies after 7 days of incubation.

pH 6.8. Not able to grow at or above 37 °C, in the presence of 1 % NaCl or in LB broth. Test for urease activity is positive. Assimilates D- and L-arabinose, D-ribose, D-galactose, D-mannose and D-fucose as carbon sources. Shows weak growth with D- and L-xylose, D-adonitol, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, aesculin iron citrate, D-lyxose, L-fucose, D-arabitol, glycerol and D-fructose and does not grow with erythritol, methyl β-D-xylopyranoside, L-sorbose, D-glucose, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, turanose, D-tagatose, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Resistant to the antibiotics erythromycin, cefuroxime, streptomycin, tetracycline, nalidixic acid, chloramphenicol and bacitracin and moderately sensitive to neomycin.

The type strain is CNPSo 1112^T (=SMS 303^T=BR 1009^T=SEMIA 6148^T=LMG 28867^T), isolated from a nodule of perennial soybean (*Neonotonia wightii*) in Brazil and very effective in fixing nitrogen with the host legume. Its DNA G+C content is 63.49 mol%.

Description of *Bradyrhizobium embrapense* sp. nov.

Bradyrhizobium embrapense [em.bra.pen′se. N.L. neut. adj. *embrapense* arbitrary name formed from the acronym Embrapa (Empresa Brasileira de Pesquisa Agropecuária)].

Cells are Gram-negative, non-spore-forming rods. Colonies are 0.93–1.15 mm in diameter, circular, convex, translucent, with low production of mucus and slightly pink when grown in YMA medium containing Congo red after 7 days of growth at 28 °C. Produces an alkaline reaction in YMA containing bromothymol blue. The generation time is 7.49 h in YM broth. Grows at pH 4.5–8.0, with optimal growth at pH 6.8. Able to grow at 37 °C and in LB broth but not at 40 °C or in the presence of 1 % NaCl. Test for urease activity is positive. Assimilates D- and L-arabinose, D-ribose, D- and L-xylose, D-galactose, D-glucose, D-mannose, melibiose, starch, glycogen, gentiobiose, D-lyxose, D- and L-fucose, potassium gluconate and potassium 5-ketogluconate as carbon sources. Shows weak growth with glycerol, D-fructose, L-rhamnose, D-mannitol, D-sorbitol and D-arabitol and does not grow with erythritol, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin iron citrate, salicin, cellobiose, maltose, lactose, sucrose, trehalose, inulin, melezitose, raffinose, xylitol, turanose, D-tagatose, L-arabitol or potassium 2-ketogluconate. Resistant to the antibiotics erythromycin, cefuroxime, nalidixic acid, tetracycline, chloramphenicol and bacitracin, moderately sensitive to neomycin and sensitive to streptomycin.

The type strain is CNPSo 2833^T (=CIAT 2372^T=BR 2212^T=SEMIA 6208^T=U674^T=LMG 29087), isolated from a nodule of *Desmodium heterocarpon* in Colombia and very effective in fixing nitrogen with the host legume. Its DNA G+C content is 62.81 mol%.

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References

Azevedo, H., Lopes, F., Silla, P. & Hungria, M. (2015). A database for the taxonomic and phylogenetic identification of the genus *Bradyrhizobium* using multilocus sequence analysis. *BMC Genomics* **16** (Suppl. 5), S10.

Binde, D. R., Menna, P., Bangel, E. V., Barcellos, F. G. & Hungria, M. (2009). rep-PCR fingerprinting and taxonomy based on the sequencing of the 16S rRNA gene of 54 elite commercial rhizobial strains. *Appl Microbiol Biotechnol* **83**, 897–908.

Chang, Y. L., Wang, J. Y., Wang, E. T., Liu, H. C., Sui, X. H. & Chen, W. X. (2011). *Bradyrhizobium lablabi* sp. nov., isolated from effective nodules of *Lablab purpureus* and *Arachis hypogaea*. *Int J Syst Evol Microbiol* **61**, 2496–2502.

Dall'Agnol, R. F., Ribeiro, R. A., Ormeño-Orrillo, E., Rogel, M. A., Delamuta, J. R. M., Andrade, D. S., Martínez-Romero, E. & Hungria, M. (2013). *Rhizobium freirei* sp. nov., a symbiont of Phaseolus vulgaris that is very effective at fixing nitrogen. *Int J Syst Evol Microbiol* **63**, 4167–4173.

Dall'Agnol, R. F., Ribeiro, R. A., Delamuta, J. R. M., Ormeño-Orrillo, E., Rogel, M. A., Andrade, D. S., Martínez-Romero, E. & Hungria, M. (2014). *Rhizobium paranaense* sp. nov., an effective N_2-fixing symbiont of common bean (*Phaseolus vulgaris* L.) with broad geographical distribution in Brazil. *Int J Syst Evol Microbiol* **64**, 3222–3229.

Delamuta, J. R. M., Ribeiro, R. A., Menna, P., Bangel, E. V. & Hungria, M. (2012). Multilocus sequence analysis (MLSA) of *Bradyrhizobium* strains: revealing high diversity of tropical diazotrophic symbiotic bacteria. *Braz J Microbiol* **43**, 698–710.

Delamuta, J. R. M., Ribeiro, R. A., Ormeño-Orrillo, E., Melo, I. S., Martínez-Romero, E. & Hungria, M. (2013). Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov. *Int J Syst Evol Microbiol* **63**, 3342–3351.

Durán, D., Rey, L., Mayo, J., Zúñiga-Dávila, D., Imperial, J., Ruiz-Argüeso, T., Martínez-Romero, E. & Ormeño-Orrillo, E. (2014a). *Bradyrhizobium paxllaeri* sp. nov. and *Bradyrhizobium icense* sp. nov., nitrogen-fixing rhizobial symbionts of Lima bean (*Phaseolus lunatus* L.) in Peru. *Int J Syst Evol Microbiol* **64**, 2072–2078.

Durán, D., Rey, L., Navarro, A., Busquets, A., Imperial, J. & Ruiz-Argüeso, T. (2014b). *Bradyrhizobium valentinum* sp. nov. *isolated from effective nodules of Lupinus mariae-josephae, a lupine endemic of basic-lime soils in Eastern Spain. Syst Appl Microbiol* **37**, 336–341.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**, 1792–1797.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.

Germano, M. G., Menna, P., Mostasso, F. L. & Hungria, M. (2006). RFLP analysis of the rRNA operon of a Brazilian collection of bradyrhizobial strains from 33 legume species. *Int J Syst Evol Microbiol* **56**, 217–229.

Gevers, D., Cohan, F. M., Lawrence, J. G., Spratt, B. G., Coenye, T., Feil, E. J., Stackebrandt, E., Van de Peer, Y., Vandamme, P., Thompson, F. L. & Swings, J. (2005). Re-evaluating prokaryotic species. *Nat Rev Microbiol* **3**, 733–739.

Guerrouj, K., Ruíz-Díez, B., Chahboune, R., Ramírez-Bahena, M. H., Abdelmoumen, H., Quiñones, M. A., El Idrissi, M. M., Velazquez, E., Fernandez-Pascual, M., Bedmar, E. J. & Peix, A. (2013). Definition of a novel symbiovar (sv. retamae) within *Bradyrhizobium retamae* sp. nov., nodulating. *Retama sphaerocarpa* and *Retama monosperma*. *Syst Appl Microbiol* **36**, 218–223.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.

Hungria, M., Loureiro, M. F., Mendes, I. C., Campo, R. J. & Graham, P. H. (2005). Inoculant preparation, production and application. In *Nitrogen Fixation: Origins, Applications and Research Progress*, vol. 4, pp. 223–254. Edited by W. E. Newton. Dordrecht, Amsterdam: Springer.

Kaschuk, G., Hungria, M., Andrade, D. S. & Campo, R. J. (2006). Genetic diversity of rhizobia associated with common bean (*Phaseolus vulgaris* L.) grown under no-tillage and conventional systems in Southern Brazil. *Appl Soil Ecol* **32**, 210–220.

Kuykendall, L. D., Saxena, B., Devine, T. E. & Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* **38**, 501–505.

Menna, P. & Hungria, M. (2011). Phylogeny of nodulation and nitrogen-fixation genes in *Bradyrhizobium*: supporting evidence for the theory of monophyletic origin, and spread and maintenance by both horizontal and vertical transfer. *Int J Syst Evol Microbiol* **61**, 3052–3067.

Menna, P., Hungria, M., Barcellos, F. G., Bangel, E. V., Hess, P. N. & Martínez-Romero, E. (2006). Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. *Syst Appl Microbiol* **29**, 315–332.

Menna, P., Barcellos, F. G. & Hungria, M. (2009a). Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII*, *recA*, *atpD* and *dnaK* genes. *Int J Syst Evol Microbiol* **59**, 2934–2950.

Menna, P., Pereira, A. A., Bangel, E. V. & Hungria, M. (2009b). rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool. *Symbiosis* **48**, 120–130.

MIDI (2001). *Sherlock Microbial Identification System Operating Manual, version 4.0.* Newark: MIDI, Inc.

Ormeño-Orrillo, E. & Martínez-Romero, E. (2013). Phenotypic tests in *Rhizobium* species description: an opinion and (a sympatric speciation) hypothesis. *Syst Appl Microbiol* **36**, 145–147.

Ormeño-Orrillo, E., Hungria, M. & Martínez-Romero, E. (2013). Dinitrogen-fixing prokaryotes. *In The Prokaryotes – Prokaryotic Physiology and Biochemistry*, pp. 427–451 Edited by E. Rosenberg et al. Berlin, Heidelberg: Springer-Verlag.

Ramírez-Bahena, M. H., Peix, A., Rivas, R., Camacho, M., Rodríguez-Navarro, D. N., Mateos, P. F., Martínez-Molina, E., Willems, A. & Velázquez, E. (2009). *Bradyrhizobium pachyrhizi* sp. nov. and *Bradyrhizobium jicamae* sp. nov., isolated from effective nodules of *Pachyrhizus erosus*. *Int J Syst Evol Microbiol* **59**, 1929–1934.

Ribeiro, R. A., Barcellos, F. G., Thompson, F. L. & Hungria, M. (2009). Multilocus sequence analysis of Brazilian *Rhizobium* microsymbionts of common bean (*Phaseolus vulgaris* L.) reveals unexpected taxonomic diversity. *Res Microbiol* **160**, 297–306.

Ribeiro, R. A., Rogel, M. A., López-López, A., Ormeño-Orrillo, E., Barcellos, F. G., Martínez, J., Thompson, F. L., Martínez-Romero, E. & Hungria, M. (2012). Reclassification of *Rhizobium tropici* type

A strains as *Rhizobium leucaenae* sp. nov. *Int J Syst Evol Microbiol* **62**, 1179–1184.

Ribeiro, R. A., Ormeño-Orrillo, E., Dall'Agnol, R. F., Graham, P. H., Martínez-Romero, E. & Hungria, M. (2013). Novel *Rhizobium* lineages isolated from root nodules of the common bean (*Phaseolus vulgaris* L.) in Andean and Mesoamerican areas. *Res Microbiol* **164**, 740–748.

Ribeiro, R. A., Martins, T. B., Ormeño-Orrillo, E., Delamuta, J. R. M., Rogel, M. A., Martínez-Romero, E. & Hungria, M. (2015). *Rhizobium ecuadorense* sp. nov., an indigenous N_2-fixing symbiont of the Ecuadorian common bean (*Phaseolus vulgaris* L.) genetic pool. *Int J Syst Evol Microbiol* **65**, 3162–3169.

Richter, M. & Rosselló-Móra, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci U S A* **106**, 19126–19131.

Rivas, R., Martens, M., de Lajudie, P. & Willems, A. (2009). Multilocus sequence analysis of the genus *Bradyrhizobium*. *Syst Appl Microbiol* **32**, 101–110.

Roma Neto, I. V., Ribeiro, R. A. & Hungria, M. (2010). Genetic diversity of elite rhizobial strains of subtropical and tropical legumes based on the 16S rRNA and *glnII* genes. *World J Microbiol Biotechnol* **26**, 1291–1302.

Tamura, K. & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol* **10**, 512–526.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30**, 2725–2729.

Thompson, F. L., Gevers, D., Thompson, C. C., Dawyndt, P., Naser, S., Hoste, B., Munn, C. B. & Swings, J. (2005). Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl Environ Microbiol* **71**, 5107–5115.

Tighe, S. W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G. & Jarvis, B. D. W. (2000). Analysis of cellular fatty acids and phenotypic relationships of *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* species using the Sherlock Microbial Identification System. *Int J Syst Evol Microbiol* **50**, 787–801.

Vincent, J. M. (1970). *Manual for the Practical Study of Root Nodule Bacteria (*IBP Handbook *no. 15)*. Oxford: Blackwell Scientific.

Wang, E. T. & Martínez-Romero, E. (2000). Phylogeny of root- and stem nodule bacteria associated with legumes. In *Prokaryotic Nitrogen Fixation: a Model System for Analysis of a Biological Process*, pp. 177–186. Edited by E. W. Triplett. Wymondham, UK: Horizon Scientific.

Willems, A., Coopman, R. & Gillis, M. (2001). Comparison of sequence analysis of 16S-23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in *Bradyrhizobium*. *Int J Syst Evol Microbiol* **51**, 623–632.

Willems, A., Munive, A., de Lajudie, P. & Gillis, M. (2003). In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations. *Syst Appl Microbiol* **26**, 203–210.

Xu, L. M., Ge, C., Cui, Z., Li, J. & Fan, H. (1995). *Bradyrhizobium liaoningense* sp. nov., isolated from the root nodules of soybeans. *Int J Syst Bacteriol* **45**, 706–711.

Yao, Y., Sui, X. H., Zhang, X. X., Wang, E. T. & Chen, W. X. (2015). *Bradyrhizobium erythrophlei* sp. nov. and *Bradyrhizobium ferriligni* sp. nov., isolated from effective nodules of *Erythrophleum fordii*. *Int J Syst Evol Microbiol* **65**, 1831–1837.