Polyphasic evidence supporting the reclassification of *Bradyrhizobium japonicum* group Ia strains as *Bradyrhizobium diazoefficiens* sp. nov.

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Bradyrhizobium japonicum was described from soybean root-nodule bacterial isolates. Since its description, several studies have revealed heterogeneities among rhizobia assigned to this species. Strains assigned to *B. japonicum* group Ia have been isolated in several countries, and many of them are outstanding soybean symbionts used in inoculants worldwide, but they have also been isolated from other legume hosts. Here, we summarize published studies that indicate that group Ia strains are different from the *B. japonicum* type strain USDA 6^T and closely related strains, and present new morphophysiological, genotypic and genomic evidence to support their reclassification into a novel species, for which the name *Bradyrhizobium diazoefficiens* sp. nov. is proposed. The type strain of the novel species is the well-studied strain USDA 110^T (=IAM 13628^T =CCRC 13528^T =NRRL B-4361^T =NRRL B-4450^T =TAL 102^T =BCRC 13528^T =JCM 10833^T =TISTR 339^T =SEMIA 5032^T =3I1B110^T =ACCC 15034^T =CCT 4249^T =NBRC 14792^T =R-12974^T =CNPSo 46^T).

Biological fixation is the main source of nitrogen for natural and agricultural ecosystems. In agriculture, the symbioses of nitrogen-fixing bacteria, collectively known as rhizobia, with crops belonging to the family Leguminosae (=Fabaceae) are the most studied. Relatively high contributions to nitrogen nutrition have been demonstrated in pulses, fodders, green manures and trees (Ormeño-Orrillo *et al.*, 2013). Members of the genus *Bradyrhizobium* constitute an important group of rhizobia, some of which form symbioses with economically important crops, such as soybean [*Glycine max* (L.) Merr.].

A first systematic classification of rhizobia was proposed by Fred *et al.* (1932), based on the cross-inoculation concept with respect to the host legume, resulting in six species of *Rhizobium*: *Rhizobium meliloti*, *R. trifolii*, *R. phaseoli*, *R. lupini*, *R. leguminosarum* and *R. japonicum*, the last of which is a symbiont of soybean. Fifty years later, the taxonomy was redefined using numerical criteria, including several morphophysiological and genetic properties. A new genus was created, *Bradyrhizobium*, to accommodate rhizobia with the typical property of slow growth rates in culture media, with only one defined species, *Bradyrhizobium japonicum* (Jordan, 1982). It is noteworthy that the division of rhizobia into fast and slow growers *in vitro* had been proposed earlier by Löhnis & Hansen (1921).

It has been suggested that *Bradyrhizobium* is the ancestor of the alpha-rhizobia, probably originating in the tropics (Lloret & Martínez-Romero, 2005; Norris, 1965; Provorov & Vorob'ev, 2000). However, despite its evolutionary position, significantly fewer species have been described in *Bradyrhizobium* in comparison with *Rhizobium*. One possible explanation is that the 'backbone' of modern taxonomy relies on the analysis of 16S rRNA gene sequences (Woese, 1987), which are highly conserved in

Abbreviations: DDH, DNA–DNA hybridization; ITS, intergenic transcribed spacer; MLSA, multilocus sequencing analysis.

The GenBank/EMBL/DDBJ accession numbers for the sequences determined in this study are detailed in Table S2.

Four supplementary figures and five supplementary tables are available with the online version of this paper.

Bradyrhizobium (Germano *et al.*, 2006; Menna *et al.*, 2006; So *et al.*, 1994; Urtz & Elkan, 1996; van Berkum & Fuhrmann, 2000; Vinuesa *et al.*, 1998; Willems *et al.*, 2001b). However, high diversity in a variety of morpho-physiological and genetic properties within *Bradyrhizobium* has been reported (Boddey & Hungria, 1997; Minamisawa, 1989; Minamisawa *et al.*, 1998; Tian *et al.*, 2012; Urtz & Elkan, 1996; van Berkum & Fuhrmann, 2001; Vinuesa *et al.*,1998), and better resolution of species-level clades has been obtained by the analysis of other housekeeping genes using the MLSA (multilocus sequencing analysis) approach (e.g. Delamuta *et al.*, 2012; Menna *et al.*, 2009).

Since the subdivision of strains classified as *B. japonicum* in 1992, with the creation of *Bradyrhizobium elkanii* (Kuykendall *et al.*, 1992), no other change has been proposed for *B. japonicum*, despite several reports of subgroups within this species. These reports started in 1980 when DNA–DNA hybridization (DDH) analyses defined two relatedness groups for *B. japonicum* strains (Hollis *et al.*, 1981), I and Ia, later confirmed by Kuykendall *et al.* (1992). Urtz & Elkan (1996) also reported low DDH between the type strain USDA 6^T and the broadly studied group Ia strain USDA 110, proposed in our study as the type strain for a novel species. Other reports of heterogeneity within *B. japonicum* included differences in extracellular polysaccharide composition (Huber *et al.*, 1984), fatty acid profiles (fatty acid methyl esters) (Graham *et al.*, 1995; van Berkum & Fuhrmann, 2001), ribosomal and housekeeping genes (Delamuta *et al.*, 2012; Germano *et al.*, 2006; Menna *et al.*, 2006; van Berkum & Fuhrmann, 2000; Vinuesa *et al.*, 2008) and comparative genomics (Tian *et al.*, 2012), among others.

For the last two decades, our group has reported morpho-physiological, genetic and symbiotic diversity within *B. japonicum* (Boddey & Hungria, 1997; Delamuta *et al.*, 2012; Ferreira & Hungria, 2002; Germano *et al.*, 2006; Menna & Hungria, 2011; Menna *et al.*, 2006; Santos *et al.*, 1999), including a proposition, based on MLSA, that strains in group Ia might represent a novel species (Menna *et al.*, 2009).

In this study, we summarize differences reported by our group and other laboratories and, with additional analyses, we present new evidence to suggest that *B. japonicum* group Ia represents a novel species distinct from group I strains.

Four *Bradyrhizobium* strains used in this study, SEMIA 6059, SEMIA 5060, CPAC 7 and USDA 110 (Table 1), were chosen from a previous MLSA study by our group based on a high level of genetic similarity (Menna *et al.*, 2009). SEMIA 6059 was isolated from winged bean (*Psophocarpus tetragonolobus*), and the others from soybean growing in the USA, Japan and Brazil; other *Bradyrhizobium* strains used in this study are listed in Table 1. We always include comparisons with two strains well studied by our group because of their broad use in commercial inoculants in Brazil: group Ia strain CPAC 7, a natural variant of CB 1809 (Santos *et al.*, 1999), and group I strain CPAC 15, a natural variant of SEMIA 566 (Mendes *et al.*, 2004). CPAC 15 and SEMIA 566 belong to the same highly competitive serogroup as USDA 123 (Mendes *et al.*, 2004). All strains from this study are deposited at the Culture Collection of Diazotrophic and Plant Growth Promoting Bacteria of Embrapa Soja (Londrina, Brazil) and at the Center for Genomic Sciences Culture Collection (Cuernavaca, Mexico).

The rhizobia were grown on yeast extract-mannitol agar (YMA) at 28 °C. Stocks were prepared on YMA and kept for long-term storage at −80 and −150 °C (in 30 % glycerol) and lyophilized, and at 4 °C as source cultures.

Genomic DNA extraction and fingerprints with the BOX-A1R primer were performed as described by Kaschuk *et al.* (2006). Cluster analyses, performed with the Bionumerics 4.6 software using the UPGMA algorithm and Jaccard similarity coefficients, revealed that all four group Ia strains clustered at an 85 % similarity level (Fig. S1, available in IJSEM Online). This group was separated from the group I *B. japonicum* strains USDA 6^T, CPAC 15 and SEMIA 566, as well as from other species of *Bradyrhizobium*.

The 16S rRNA gene and fragments of the *atpD*, *glnII*, *recA*, *gyrB*, *rpoB* and *dnaK* genes and of the 16S–23S rRNA intergenic transcribed spacer (ITS) were amplified using primers and conditions indicated in Table S1, and were sequenced by using dideoxy termination as described before (Menna *et al.*, 2009). Accession numbers for the sequences used in our study are given in Table S2. Multiple sequence alignment, alignment editing and phylogenetic analyses were performed with MEGA5 (Tamura *et al.*, 2011). Neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) phylogenetic reconstructions gave similar results; therefore, only neighbour-joining phylograms are presented. For the ITS, a neighbour-joining dendrogram was reconstructed based on a matrix of uncorrected distances (Willems *et al.*, 2001a).

In the 16S rRNA gene phylogram, group Ia strains CPAC 7, SEMIA 6059, SEMIA 5060 and USDA 110 were clustered in a well-supported clade that was closely related to *Bradyrhizobium daqingense*, *B. canariense*, *B. yuanmingense*, *B. liaoningense* and *B. japonicum* (Fig. 1). The results confirm a cluster reported previously by our group with ten strains, all but SEMIA 6059 symbionts of soybean (Menna *et al.*, 2009). Sequences of group Ia strains were 99.8–100 % identical to each other and showed 96.1–99.6 % identity to those of other *Bradyrhizobium* strains (Table 2). Recently, two exact copies of the rRNA gene cluster (*rrn*) were identified on the chromosome of the group I strain USDA 6^T (Kaneko *et al.*, 2011) whereas, in group Ia strain USDA 110, a single *rrn* copy was present (Kaneko *et al.*, 2002). The same difference was observed in the genomes of the Brazilian strains CPAC 15 (*B. japonicum*) and CPAC 7 (group Ia); therefore, this might be another differential characteristic of the two groups.

High diversity within *B. japonicum* in analyses of the ITS sequence has been reported before (van Berkum &

Table 1. Strains used in this study

Strain	Other strain nomenclature	Host species	Geographical origin	Reference or source
B. diazoefficiens sp. nov.				
CPAC 7	SEMIA 5080, CNPSo 6	*Glycine max*	Brazil	Menna *et al.* (2009)
SEMIA 5060	J 507, CNPSo 1064	*Glycine max*	Japan	Menna *et al.* (2009)
SEMIA 6059	USDA 3309, CNPSo 1098	*Psophocarpus tetragonolobus*	USA	Menna *et al.* (2009)
USDA 110^T	TAL 102^T, TISTR 339^T, SEMIA 5032^T, CNPSo 46^T	*Glycine max*	USA	Kaneko *et al.* (2002)
B. japonicum				
USDA 6^T	ATCC 10324^T, CCUG 27876^T, CIP 106093^T, DSM 30131^T, HAMBI 2314^T, NBRC 14783^T, JCM 20679^T, LMG 6138^T, NRRL B-4507^T, NRRL L-241^T, VKM B-1967^T, CNPSo 158^T	*Glycine max*	USA	Jordan (1982)
CPAC 15	SEMIA 5079, DF 24, CNPSo 7	*Glycine max*	Brazil	Menna *et al.* (2009)
SEMIA 566	BR 40, CNPSo 17	*Glycine max*	USA	Menna *et al.* (2009)
B. betae LMG 21987^T	PL7HG1^T, CECT 5829^T, CNPSo 2079^T	*Beta vulgaris*	Spain	Rivas *et al.* (2004)
B. canariense LMG 22265^T	BTA-1^T, ATCC BAA-1002^T, CFNE 1008^T, CNPSo 2078^T	*Chamaecytisus proliferus*	Spain	Vinuesa *et al.* (2005)
B. yuanmingense LMG 21827^T	CCBAU 10071^T, CFNEB 101^T, NBRC 100594^T, CNPSo 2080^T	*Lespedeza* spp.	China	Yao *et al.* (2002)
B. cytisi CTAW11^T	LMG 25866^T, CECT 7749^T, CNPSo 2469^T	*Cytisus villosus*	Morocco	Chahboune *et al.* (2011)
B. huanghuaihaiense CCBAU 23303^T	LMG 26136^T, CGMCC 1.10948^T, HAMBI 3180^T, CNPSo 2458^T	*Glycine max*	China	Zhang *et al.* (2012)
B. iriomotense EK05^T	NBRC 102520^T, LMG 24129^T, CNPSo 2470^T	*Entada koshunensis*	Japan	Islam *et al.* (2008)
'*B. rifense*' CTAW71	LMG 26781, CECT 8066, CNPSo 2468	*Cytisus villosus*	Morocco	Chahboune *et al.* (2012)
B. liaoningense LMG 18230^T	2281^T, ATCC 700350^T, CIP 104858^T, NBRC 100396^T, CNPSo 2455^T	*Glycine max*	China	Xu *et al.* (1995)

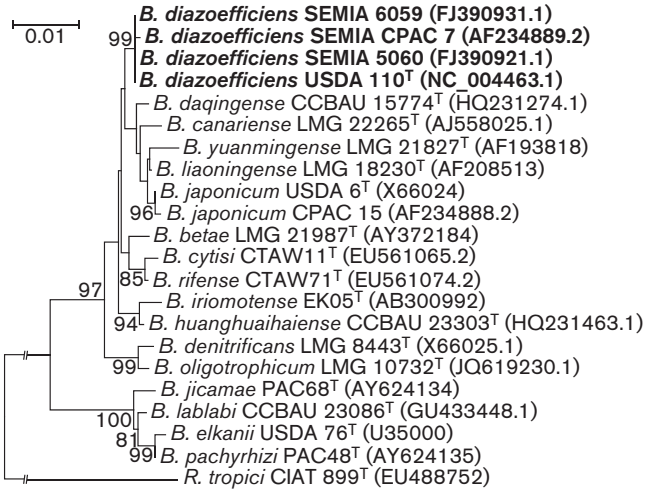

Fig. 1. Neighbour-joining phylogeny based on 16S rRNA gene sequences showing relationships between *Bradyrhizobium diazoefficiens* sp. nov. strains and other members of the genus *Bradyrhizobium*. Bootstrap values >70 % are indicated at nodes. *Rhizobium tropici* CIAT 899^T was used as an outgroup. Bar, 1 substitution per 100 nucleotide positions.

Fuhrmann, 2000; Willems *et al.*, 2001a). Accordingly, in previous studies by our group with RFLP-PCR (Germano *et al.*, 2006) and sequencing of the ITS genomic region (Menna *et al.*, 2009), differences between groups I and Ia were pointed out. Here, the dendrogram based on ITS sequences also revealed that group Ia strains were distinct from other bradyrhizobia (Fig. S2). ITS sequence similarity among group Ia strains was greater than 99 %, and less than 90 % with other bradyrhizobia; similarities between group I and Ia strains ranged from 83.5 to 84 % (Table 2). This observation is consistent with group Ia strains belonging to the same species; Willems *et al.* (2003) have shown that strains with more than 95.5 % ITS similarity usually show at least 60 % DDH and, therefore, belong to the same genospecies. Interestingly, group Ia strains were also characterized by a 37-nt insertion in the ITS (beginning 156 bp after the 16S rRNA gene in USDA 110) that was absent in group I strains. Therefore, this insertion may be characteristic of group Ia strains. Finally, it is noteworthy that, in a polyphasic analysis of the RFLP-PCR fragments of the 16S and 23S rRNA genes as well as the ITS, strains of groups I and Ia were positioned in different clusters (Germano *et al.*, 2006).

The MLSA approach has been used successfully in studies of *Bradyrhizobium* for over a decade (e.g. Menna *et al.*,

Table 2. Nucleotide identity (%) within *B. diazoefficiens* sp. nov. and between *B. diazoefficiens* strains and the type strains of other *Bradyrhizobium* species

Length of the aligned regions: 16S rRNA gene, 1303 bp; *atpD*, 435 bp; *glnII*, 513 bp; *recA*, 381 bp; ITS, 891 bp. Four strains of group Ia (*B. diazoefficiens* sp. nov.) were compared: CPAC 7, SEMIA 5060, SEMIA 6059 and USDA 110^T. NA, No available sequence for comparison.

Species comparison	16S rRNA	*atpD*	*glnII*	*recA*	ITS
Within *B. diazoefficiens*	99.8–100	99.5–100	99.4–100	95.5–99.7	99.4–99.7
Between *B. diazoefficiens* and:					
B. japonicum	99–99.2	95.8–96.2	97.4–97.6	94.7–96.5	83.5–84
B. betae	99.1–99.3	96.5–96.9	96.8–97.4	93.9–97.1	87.6–87.7
B. canariense	99–99.1	94.4–94.9	94.9–95.1	92.9–94.4	84.6–84.8
B. yuanmingense	98.6–98.7	95.1–95.6	93.5–93.7	91.3–92.6	82.7–82.9
B. liaoningense	99.1–99.3	94.9–95.3	95.7–95.9	93.1–94.4	85–85.3
B. jicamae	96.3–96.4	93.5–93.9	88.8–89	88.4–89.5	76.6–76.7
B. huanghuaihaiense	99.1–99.3	97.6–98.1	95.7–95.9	90.2–92.9	82.6–82.7
B. cytisi	99–99.2	94.2–94.6	94.9−95.1	91.8–94.2	NA
B. daqingense	99.4–99.6	95.8–96.2	94.5–94.7	90.5–91.8	85–85.3
B. iriomotense	98.9–99	94.6–95.1	94.9−95.1	89.5–90.5	80.3–80.5
'*B. rifense*'	99.3–99.4	97.2–97.6	95.5–95.7	91.3–92.9	NA
B. elkanii	96.1–96.3	93.5–93.9	89.2–89.6	91.8–93.4	73.9–74.2
B. lablabi	96.5–96.6	92.8–93.2	88.4–88.6	90.2−92.3	74.7–74.9
B. pachyrhizi	96.3−96.4	93.2–93.7	89–89.4	90.2−92.3	75.6–75.7

2009; Moulin *et al.*, 2004; Stepkowski *et al.*, 2005; Vinuesa *et al.*, 2008). Recently, sequences of at least three housekeeping genes were used for phylogenetic analysis and taxonomic classification of bradyrhizobia (Chahboune *et al.*, 2011, 2012; Chang *et al.*, 2011; Zhang *et al.*, 2012), and our results support the use of this methodology for such purposes. Group Ia strains formed distinct and well-supported clades in single-gene phylogenies of *atpD*, *recA* and *glnII*, for which sequences of all described *Bradyrhizobium* species are available (data not shown). They were closely related to *Bradyrhizobium betae* LMG 21987^T with *atpD* and *glnII* genes, and were most similar to *B. japonicum* group I for the *recA* gene. It should be noted that, in a previous study, we showed that *atpD* had limitations for the study of some strains of *B. elkanii*, but not with *B. japonicum* (Menna *et al.*, 2009). Now, in this study, group Ia strains shared higher identities in their *atpD* and *glnII* genes than with all other *Bradyrhizobium* species, but that was not the case for the *recA* gene (Table 2). Greater divergence in *recA* in comparison with other genes has also been found in the study of Chahboune *et al.* (2012). In the concatenated *atpD*+*glnII*+*recA* MLSA phylogram, group Ia strains also formed a distinct clade and were part of the group of species belonging to the *B. japonicum* 16S rRNA gene phylogenetic branch, having *B. betae* as its closest taxon (Fig. 2). Similar results were obtained when sequences of all six protein-encoding genes were analysed (*atpD*, *glnII*, *recA*, *gyrB*, *rpoB* and *dnaK*) (Fig. S3), confirming that sequences of three housekeeping genes seem to be sufficient for taxonomic classification of bradyrhizobia.

The draft genome sequence of group Ia strain CPAC 7, covering >99 % of the genome (our unpublished data), and the complete genome sequences of group Ia strain USDA 110 (Kaneko *et al.*, 2002) and *B. japonicum* USDA 6^T (Kaneko *et al.*, 2011) were used to estimate DDH,

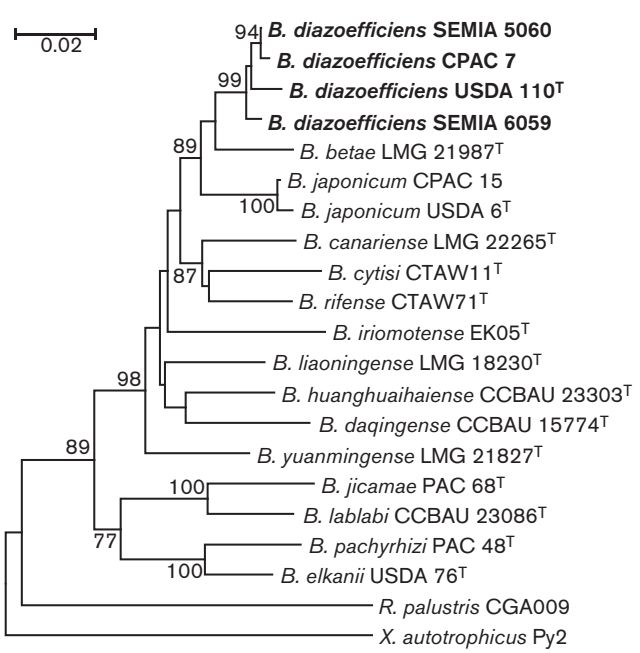

Fig. 2. Neighbour-joining phylogeny based on concatenated *atpD*, *glnII* and *recA* gene sequences showing relationships between strains from the novel species (shown in bold) and other members of the genus *Bradyrhizobium*. Bootstrap values >70 % are indicated at nodes. *Rhodopseudomonas palustris* CGA009 and *Xanthobacter autotrophicus* Py2 were used as outgroups. Bar, 2 substitutions per 100 nucleotide positions.

Table 3. Distinctive phenotypic features of *B. diazoefficiens* sp. nov. and phylogenetically related species

Strains: 1, SEMIA 5060; 2, CPAC 7; 3, SEMIA 6059; 4, USDA 110^T; 5, *B. japonicum* USDA 6^T; 6, *B. betae* LMG 21987^T; 7, *B. canariense* LMG 22265^T; 8, *B. yuanmingense* LMG 21827^T; 9, *B. cytisi* CTAW11^T; 10, *B. huanghuaihaiense* CCBAU 23303^T; 11, *B. iriomotense* EK05^T; 12, '*B. rifense*' CTAW71; 13, *B. liaoningense* LMG 18230^T. Data were obtained in this study unless indicated. +, Growth; −, no growth; w, weakly positive; ND, not determined. Carbon-source utilization was evaluated with the API 50CH kit (bioMérieux).

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Carbon-source utilization													
Glycerol	w	+	w	w	w	w	w	w	w	w	+	+	−
D-Arabinose	+	+	+	+	w	+	+	w	+	+	+	+	w
L-Arabinose	w	+	w	w	+	+	w	+	+	w	+	+	+
D-Ribose	+	+	+	+	+	+	+	+	w	+	+	+	+
D-Xylose	w	+	w	w	w	w	w	+	+	w	+	+	+
L-Xylose	+	+	+	+	w	+	+	+	+	+	+	+	w
D-Adonitol	−	w	w	−	−	−	w	−	w	−	w	+	−
Methyl β-D-xylopyranoside	w	w	w	w	w	−	w	w	w	w	w	w	−
D-Glucose	w	w	w	w	w	w	w	w	w	w	−	w	−
D-Fructose	w	w	w	w	w	w	w	w	w	w	+	w	−
D-Mannose	w	w	w	w	w	w	w	w	w	w	w	+	+
L-Rhamnose	w	w	w	w	w	w	+	w	w	w	w	w	w
Inositol	−	−	−	−	−	−	−	−	w	−	w	w	−
D-Mannitol	w	w	w	w	w	−	w	w	w	w	w	w	w
D-Sorbitol	w	w	w	w	w	−	w	−	w	−	−	−	−
N-Acetylglucosamine	−	−	−	−	−	−	−	−	−	−	−	w	−
Amygdalin	−	−	−	−	−	−	−	−	−	−	−	w	−
Arbutin	−	−	−	−	−	−	−	−	w	−	w	w	−
Aesculin ferric citrate	+	+	+	+	+	+	+	+	+	w	+	+	−
Salicin	−	−	−	−	−	−	−	−	w	−	w	w	−
Cellobiose	−	−	−	−	−	−	−	−	w	−	w	w	−
Maltose	−	−	−	−	−	−	−	w	−	−	−	−	−
Glycogen	−	−	−	−	−	+	+	−	+	+	+	+	−
Xylitol	−	w	w	−	−	−	−	−	−	−	w	w	−
Gentiobiose	−	−	−	−	−	−	−	−	w	−	w	+	−
L-Fucose	+	+	+	+	+	+	+	+	+	+	−	+	+
D-Arabitol	w	w	w	w	w	−	w	w	w	w	+	w	−
L-Arabitol	−	w	−	−	−	−	−	−	w	−	w	−	−
Potassium gluconate	−	+	+	−	−	−	−	−	−	+	+	−	w
Potassium 2-ketogluconate	−	−	−	−	−	+	−	−	−	−	−	−	−
Potassium 5-ketogluconate	−	−	+	−	−	−	−	−	+	+	−	−	−
Utilization as sole nitrogen source*													
Asparagine	+	+	+	+	−	−	w	w	+	+	+	+	+
Phenylalanine	+	+	+	+	−	w	w	−	w	+	+	+	−
Histidine	−	+	w	−	−	−	−	−	+	+	+	+	+
Leucine	+	+	+	+	w	−	−	w	+	+	+	+	w
Isoleucine	+	+	+	+	w	+	+	+	+	+	+	+	+
Growth in/at:													
37 °C	−	−	−	−	−	−	−	+	−	−	−	−	−
pH 4.5	+	+	+	+	+	+	+	w	+	+	+	w	w
pH 8	+	+	−	−	+	+	−	+	−	+	−	−	+
Resistance to (μg ml^{-1})													
Ampicillin (10)	−	+	w	w	−	−	w	−	+	+	+	+	−
Chloramphenicol (30)	+	+	+	+	+	+	+	+	−	+	−	+	w
Erythromycin (15)	+	+	+	−	+	+	+	+	+	+	+	+	−
Nalidixic acid (30)	+	+	+	+	+	+	+	+	+	+	w	+	+
Neomycin (30)	−	−	−	−	−	w	−	−	w	w	+	w	−
Penicillin G (10 U)	+	+	+	+	−	w	+	w	+	+	+	+	−
Tetracycline (30)	+	+	+	−	−	+	+	+	−	w	−	−	w
Streptomycin (10)	−	−	−	−	−	−	+	−	−	w	w	−	−

Table 3. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Carbenicillin (500)	−	−	−	−	−	−	+	−	ND	ND	ND	ND	−
Colony size (mm) after 7–10 days of incubation on YMA†	1.3	1.5	1.2	1.3	0.7	0.7	0.9	1.2	<1[a]‡	<1[b]	0.2–1[c]	<1[d]	0.3

*Growth (OD_{600}) was considered positive if $\geqslant$0.09, weak at 0.04–0.09, and negative at <0.04.
†Mean of three colonies.
‡Data taken from: *a*, Chahboune *et al.* (2011); *b*, Zhang *et al.* (2012); *c*, Islam *et al.* (2008); *d*, Chahboune *et al.* (2012).

average nucleotide identity (ANI) and G+C content. A digital DDH methodology using genomic sequences correlated well with experimental determinations (Auch *et al.*, 2010) and was used in our study to estimate the DDH of group Ia strains USDA 110 and CPAC 7, and of *B. japonicum* strains USDA 6^T and of CPAC 15, based on the draft genome sequence of this strain (>99 % of the genome; our unpublished data) (Table S3). Based on published recommendations (Auch *et al.*, 2010), we used the BLAT program and formula 3 for calculations. The estimated DDH between group Ia strains USDA 110 and CPAC 7 was 89 %, indicating that the two strains belong to the same species; in contrast, these two strains showed only 57.8 and 58.9 % DDH, respectively, with *B. japonicum* USDA 6^T, strongly indicating that the group Ia strains do not belong to the species *B. japonicum*. Experimental DDH values reported in the literature between group Ia strain USDA 110 and *B. japonicum* USDA 6^T are 44 % (Urtz & Elkan, 1996), 55.5 % (Hollis *et al.*, 1981), 65 % (Rivas *et al.*, 2004) and 65.5 % (Willems *et al.*, 2001a), all below the recommended 70 % threshold for species circumscription in prokaryotes (Stackebrandt & Goebel, 1994). In addition, low DDH values were also reported in the comparison of USDA 110 with the type strains of *B. liaoningense* (61 %) and *B. betae* (63 %) (Rivas *et al.*, 2004). Willems *et al.* (2001a) used a DDH value of 60 % as a threshold for genospecies delineation in *Bradyrhizobium*; however, close inspection of their published DDH data indicates that this 'relaxed' value was probably used to accommodate group I and Ia strains into a single genospecies and a group of heterogeneous *Aeschynomene* strains into genospecies VI. The same authors recognized that genospecies VI seems to comprise at least two highly related subgroups (Willems *et al.*, 2001a), and that group I and Ia strains most likely correspond to different taxa (Willems *et al.*, 2001b). Interestingly, by using the approach of hybridization with cosmid profiles, Kuykendall *et al.* (1992) reported a mean value for the coefficient of similarity of only 0.36 between group I and Ia strains; in addition, the coefficients among group Ia strains (USDA 110, USDA 62, 61A50, 5631 and 8) were homogeneous at 0.88–0.94.

ANI of genome sequences has been proposed as an alternative to DDH in prokaryotic taxonomy. First, it has been proposed that an ANI >94 % would correspond to 70 % DDH (Konstantinidis & Tiedje, 2004) and, lately, >95–96 % ANI has been accepted as the threshold for species delineation (Richter & Rosselló-Móra, 2009). Group Ia strains USDA 110 and CPAC 7 shared an ANI value of 98.7 %, indicating that this pair of strains belongs to the same species. *B. japonicum* strains USDA 6^T and CPAC 15 also shared a high ANI value (98.4 %), characteristic of isolates from the same species. In contrast, when group Ia strains were compared with *B. japonicum* strains, low values were obtained (~91 %), showing that these groups constitute different species (Table S3).

In conclusion, the results presented here emphasize DDH values lower than the 70 % threshold, and, in the comparison of USDA 6^T and USDA 110, include compiled data from experiments reported in the literature ranging from 44 to 65.5 %, as well as *in silico* results from our study for DDH (57.8 %) and ANI (91.1 %).

The G+C content was estimated by analysing genomic sequences with BioEdit (Hall, 1999). First, the sequences of 13 contigs representing the CPAC 7 genome were concatenated. The calculated DNA G+C content of group Ia strain CPAC 7 was 63.98 mol%, which falls within the 60–64 mol% range of experimental values reported for *Bradyrhizobium* species (Xu *et al.*, 1995), and is closer to the 64.1 mol% value for group Ia strain USDA 110 than to the 63.67 mol% value for *B. japonicum* USDA 6^T.

It is also interesting to comment on the symbiotic component of these strains. In a previous study from our group, Menna & Hungria (2011) reported high similarity of common (*nodA*) and host-specific (*nodZ*) nodulation genes for symbionts of soybean and belonging to groups I and Ia. In addition, group I and Ia soybean strains showed high similarity of nitrogen fixation genes, including *nifD* (Koppell & Parker, 2012; Parker *et al.*, 2002) and *nifH* (Menna & Hungria, 2011). However strains from groups I and Ia but symbionts of other hosts, such as SEMIA 6059, isolated from *P. tetragonolobus* and unable to nodulate soybean, were positioned in different clusters in the study of Menna & Hungria (2011), and similar results were obtained for other strains used by Parker *et al.* (2002) and Koppell & Parker (2012); in this last case, divergence was also associated with biogeography. Therefore, these results indicate the existence of additional symbiovars besides sv. glycinerarum in both group I and Ia strains.

To compare group Ia strains USDA 110, CPAC 7, SEMIA 6059 and SEMIA 5060 further with *B. japonicum* strains USDA 6^T, CPAC 15 and SEMIA 566, fatty acids were analysed. Profiles were determined using the MIDI Sherlock Microbial Identification System (MIDI, 2001) with the TSBA6 database after growth of strains on YMA for 5 days. All strains analysed showed $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) as major fatty acids (Table S4), a typical characteristic of *Bradyrhizobium* (Tighe *et al.*, 2000). However, group Ia strains were distinguished from *B. japonicum* strains by having larger relative amounts of summed feature 8 and lacking $C_{16:1}\omega5c$ and 11-methyl $C_{18:1}\omega7c$ (Table S4). These results confirm differences reported previously in the composition of fatty acids for group Ia strain USDA 110 and group I strain USDA 6^T (Graham *et al.*, 1995; van Berkum & Fuhrmann, 2001).

Several phenotypic characteristics were determined for group Ia strains and compared with those of strains of *B. japonicum* and *B. betae*, the two most closely related species. *B. canariense*, *B. yuanmingense*, *B. cytisi*, *B. huanghuaihaiense*, *B. iriomotense*, *B. liaoningense* and '*B. rifense*' strains were also included as representatives of more distant bradyrhizobial groups within the *B. japonicum* 16S rRNA gene phylogenetic branch. Unless indicated otherwise, all tests were performed at 28 °C. Colony morphology and size, acid/alkaline production and tolerance to pH and temperature were determined in liquid YM while tolerance to 1 % NaCl on YMA, as described before (Hungria *et al.*, 2001). Ability to growth in liquid Luria–Bertani (LB) medium was also tested. Antibiotic resistance was evaluated using the disc diffusion method on YMA plates, or in liquid to test for high-level resistance to carbenicillin. Carbon-source utilization was determined using the API 50CH kit (bioMérieux), according to the manufacturer's instructions, using YM-minus-mannitol as the basal medium. Nitrogen source utilization was analysed in the defined medium of Brown & Dilworth (Bergersen, 1980) supplied with 0.25 % glucose as the carbon source. Dissolved compounds were filter-sterilized and added to a final concentration of 0.7 %. All tests were performed in triplicate. Group Ia strains analysed in this study showed similar reactions in most morphophysiological tests, although levels of carbon-source utilization were relatively variable (Table 3). We observed that, in general, bradyrhizobia showed weak carbon-source utilization when evaluated with the API 50CH kit. Nevertheless, at least five phenotypic differences were observed between group Ia strains and all tested *Bradyrhizobium* species (Table 3). Colony size was larger for group Ia strains than for the other species, except for *B. yuanmingense* (Table 3). A cluster analysis of strains analysed based on the phenotypic data showed that group Ia strains are more similar to each other than to strains of other species (Fig. S4).

In addition to the four strains from this study, several others from our previous studies, all symbionts of soybean, also fitted into group Ia (Chueire *et al.*, 2003; Germano *et al.*, 2006; Menna *et al.*, 2009) (Table S5). Although strain USDA 6^T was not included in many studies from Japan and Thailand, undoubtedly several isolates from soybean also fit into group Ia (Minamisawa, 1989, 1990; Yokoyama *et al.*, 1999). Other group Ia strains from the pioneer studies of Hollis *et al.* (1981), Huber *et al.* (1984), Kuykendall *et al.* (1992) and van Berkum & Fuhrmann (2001) are listed in Table S5, together with strains from other recent studies.

Based on all the genotypic and phenotypic evidence presented in this study, we propose the reclassification of former *B. japonicum* group Ia strains into a novel species named *Bradyrhizobium diazoefficiens* sp. nov. The novel species includes several strains, the great majority isolated from soybean. It is worth mentioning that two of the strains used in our study are outstanding in their capacity to fix nitrogen with soybean: CPAC 7 is the most efficient strain in nitrogen fixation capacity used in commercial inoculants in Brazil (Hungria *et al.*, 2006; Mendes *et al.*, 2004), and USDA 110 has been used in commercial inoculants in USA and in other countries and is now being broadly evaluated in Africa, so far with excellent results (http://www.n2africa.org/). Other strains in this group are also very effective in fixing nitrogen, with especially high rates with the soybean crop.

Description of *Bradyrhizobium diazoefficiens* sp. nov.

Bradyrhizobium diazoefficiens (di.a.zo.ef.fi′ci.ens. L. inseparable particle *dis* twice, doubly; N.L. n. *azotum* nitrogen; N.L. pref. *diazo-* pertaining to dinitrogen; L. part. adj. *efficiens* efficient; N.L. part. adj. *diazoefficiens* dinitrogen efficient, referring to the efficiency in nitrogen fixation displayed by several strains of the species).

Cells are Gram-negative, aerobic rods, as for other species of the genus *Bradyrhizobium*. Colonies are 1.2–1.5 mm in diameter, circular, convex, opaque and slightly pink on YMA containing Congo red after 7 days of growth at 28 °C. All known strains produce an alkaline reaction in YM. The strains grow at pH 4.5, with optimum growth at pH 6.8. They grow optimally at 28 °C, and are unable to grow at 37 °C. They do not grow on YMA in the presence of 1 % NaCl or in LB broth. Carbon-source utilization API tests were positive for D-arabinose, D-ribose, L-xylose, aesculin ferric citrate, starch, D-lyxose, D-fucose and L-fucose as carbon sources, weakly positive with glycerol, L-arabinose, D-xylose, methyl β-D-xylopyranoside, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, D-mannitol, D-sorbitol and D-arabitol and weak or negative with D-adonitol and xylitol. Strains can assimilate asparagine, glutamine, phenylalanine, leucine, methionine, isoleucine, proline, threonine, tryptophan and valine as nitrogen sources. Strains are resistant (μg per disc unless indicated otherwise) to the antibiotics nalidixic acid (30 μg), chloramphenicol (30 μg) and penicillin G (10 U), and sensitive to neomycin (30 μg), cefuroxime (30 μg), streptomycin (10 μg) and carbenicillin (500 μg

ml^{-1}). Several strains are very effective in nodulating and fixing nitrogen when in symbiosis with soybean [*Glycine max* (L.) Merr.], but the species also includes symbionts of other host legumes.

The type strain is USDA 110^T (=IAM 13628^T =CCRC 13528^T =NRRL B-4361^T =NRRL B-4450^T =TAL 102^T =BCRC 13528^T =JCM 10833^T =TISTR 339^T =SEMIA 5032^T =3I1B110^T =ACCC 15034^T =CCT 4249^T =NBRC 14792^T =R-12974^T =CNPSo 46^T), isolated from an effective nodule of soybean [*Glycine max* (L.) Merr.] in 1959 in Florida, USA. Its genomic DNA G+C content is 63.98 mol%.

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Table S1. Primers and DNA amplification conditions used in this study.

Primer	Sequence (5`- 3`)[a]	Target gene (position)	PCR cycling	Reference
BRdnaKf	TTCGACATCGACGCSAACGG	*dnaK* (1411–1430)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1.5 min 72°C), 7 min 72°C	Menna *et al*. (2009)
BRdnaKr	GCCTGCTGCKTGTACATGGC	*dnaK* (1905–1885)		
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)		
FGPS1490	TGCGGCTGGATCACCTCCTT	16S rRNA (1490-1510)	3 min 94°C, 35 X (1 min 94°C, 1 min 55°C, 2 min 72°C and 6 min 72°C	Laguerre *et al*. (1996)
FGPS130	CCGGGTTTCCCCATTCGG	23S rRNA (148-130)		

[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

Laguerre, G., Mavingui, P., Allard, M. R., Charnay, M. P., Louvrier, P., Mazurier, S. I., Rigottier-Gois, L. & Amarger, N. (1996). Typing of rhizobia by PCR DNA fingerprinting and PCR-restriction fragment length polymorphism analysis of chromosomal and symbiotic gene regions: application to *Rhizobium leguminosarum* and its different biovars. *Appl Environ Microbiol* **62**, 2029-2036.

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Table S2. Accession numbers of the sequences used in this study. Sequences obtained in this study are shown in bold.

Strain	Genome	ITS	*atpD*	*dnaK*	*glnII*	*recA*	*gyrB*	*rpoB*
B. diazoefficiens								
CPAC 7		FJ391069.1	FJ390957.1	FJ390997.1	FJ391037.1	FJ391157.1	**JX867246**	**JX867243**
SEMIA 5060		FJ391122.1	**JX867237**	**JX867240**	**JX867241**	**JX867239**	**JX867245**	**JX867242**
SEMIA 6059		FJ391068.1	FJ390961.1	FJ391001.1	FJ391041.1	FJ391161.1	**JX867247**	**JX867244**
USDA 110^T	NC_004463.1	AF338865.1						
B. japonicum USDA 6^T		HQ143390.1	AM168320	AM168362	AF169582	AM182158	AM418801	AM295349
CPAC 15		FJ911085.1	FJ390956.1		FJ391036.1	FJ391156.1		
B. betae LMG 21987^T		AJ631967.1	FM253129	AY923046.1	AB353733.1	AB353734.1	FM253217	FM253260
B. canariense LMG 22265^T		AY386708.1	AY386739.1	AY923047.1	AY386765.1	FM253177	FM253220	FM253263
B. yuanmingense LMG 21827^T		AY386734.1	AY386760.1	AY923039.1	AY386780.1	AM168343	FM253226	FM253269
B. liaoningense LMG 18230^T		AJ279301.1	AY386752.1	AY923041.1	AY386775.1	AY591564.1	FM253223	FM253266
B. jicamae PAC 68^T		AY628094.1	FJ428211	JF308945.1	FJ428204	HM047133.1	HQ873309.1	HQ587647.1
B. huanghuaihaiense CCBAU 23303^T		HQ428043.1	HQ231682.1		HQ231639.1	HQ231595.1		
B. cytisi CTAW11^T			GU001613.1		GU001594.1	GU001575.1		
B. daqingense CCBAU 15774^T		HQ231312.1	HQ231289.1		HQ231301.1	HQ231270.1		
B. iriomotense EK05^T		AB300993.1	AB300994.1	JF308944.1	AB300995	AB300996	AB300997	HQ587646.1
B. rifense CTAW71^T			GU001617.1		GU001604.1	GU001585.1		
B. elkanii USDA 76^T		U35000	AY386758.1	AY328392.1	AY599117.1	AY591568.1	AM418800	AM295348
B. lablabi CCBAU 23086^T		GU433583.1	GU433473.1		GU433498.1	GU433522.1		
B. pachyrhizi PAC 48^T		AY628092.2	FJ428208	JF308946.1	FJ428201.1	HM047130.1	HQ873310.1	HQ587648.1
Rhodopseudomonas palustris CGA009	CP000283							
Xanthobacter autotrophicus Py2	AE005673.1							

Table S3. Digital DNA-DNA hybridization (DDH) and average nucleotide identity (ANI) values (%) obtained by the analysis of the genomes of Group Ia strains USDA 110^T and CPAC 7 and *B. japonicum* strains USDA 6^T and CPAC 15. Digital DDH analysis was performed as described by Auch *et al*. (2010), with the BLAT program and formula 3 for calculations. ANI was calculated with the JSpecies program (Richter & Rosello-Mora, 2010).

	DDH (lower diagonal) /ANI (upper diagonal)			
	Bradyrhizobium japonium		Group Ia	
	USDA 6^T	CPAC 15	USDA 110^T	CPAC 7
USDA 6^T	-	98.4	91.1	91
CPAC 15	86.4	-	91.2	91.1
USDA 110^T	58.9	57.4	-	98.7
CPAC 7	57.8	56.7	89	-

Auch, A. F., von Jan, M., Klenk, H. P. & Goker, M. (2010). Digital DNA-DNA hybridization for microbial species delineation by means of genome-to-genome sequence comparison. *Stand Genomic Sci* **2**, 117-134.

Richter M, Rossello-Mora R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. *Proc Natl Acad Sci USA* **106,** 19126-19131.

Table S4. Fatty acids profiles obtained with the MIDI system using FAME library TSBA6 of *Bradyrhizobium diazoefficiens* and *Bradyrhizobium japonicum* strains grown for 5 dyas at 28 °C on yeast extract-mannitol agar plates. Analyses performed in a GC Agilent model 6850 with an Ultra 2 column (25 m length, ID 0.2 mm, film f 0.33 µm), detector FID, carrier gas hydrogen (30 mL/min), and make-up gas nitrogen (30mL/min), automatic injector series 7683 (liner 19251-60540), injection of 2 µL, run of 25 min. Injector temperature of 250ºC and of the detector of 300 ºC. Program of the oven temperature: initial of 170ºC, raising 5 ºC/ mín till 260ºC (hold for 18 m), then raised at e 40ºC/min. till 310ºC (hold for 1.5 min).

B. diazoefficiens: 1, CPAC 7; 2, SEMIA 5060; 3, SEMIA 6059; 4, USDA 110^{T}; *B. japonicum*: 5, USDA 6^{T}; 6, CPAC 15; 7, SEMIA 566.

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**
C16:0	13.11	14.39	13.78	14.09	13.05	16.17	13.57
C16:1ω5c					3.63	1.80	1.78
C18:0					0.79		
C18:1ω7c 11-methyl					6.65	15.98	11.98
Summed features							
3[†]					1.07		
8[*]	86.89	85.61	86.22	85.91	74.81	66.04	72.67

[†] Summed feature 3= C16:1ω6c/C16:1ω7c
[*] Summed feature 8= C18:1ω6c/C18:1ω7c

Table S5. Other strains besides the ones used in this study (Table 1) that belong to Group Ia.

Origin of the strain[†]	**Method for classification**	**Reference**	**Strain**
USA, Brazil	Hybridization	Hollis *et al.* (1981); Kuykendall *et al.* (1992)	8, USDA 140, 5631, USDA 62 and 61A50.
USA, Brazil	EPS composition (gas liquid chromatography)	Huber *et al.* (1984)	8, USDA 140, 5631, USDA 62 and 61A50.
USA	FAME	van Berkum & Fuhrmann (2001)	USDAs 62, 91, 92, 122, 124, 125, 126, 129, 135.
USA	16S rRNA	Chueire *et al.* (2003)	SEMIA 586 (=CB 1809, =SEMIA 586, =USDA 136b, =TAL 379, =3I1b123, CNPSo 10), USDA 122 (CNPSo 58), USDA 136
USA, Brazil	RFLP-PCR of 16S rRNA, ITS, 23S rRNA	Germano *et al.* (2006)	SEMIAs 580 (CNPSo 972), 581 (=CNPSo 973), 586 (=CB 1809, =SEMIA 586, =USDA 136b, =TAL 379, =3I1b123, CNPSo 10), 5021 (=CNPSo 1035), 5024 (=TAL 378, =CC 709, CNPSo 1038), 5036 (=CNPSo 1047), 5059 (=USDA 143, =3I1B143, =ACCC 15039, =CNPSo 1063), 5084 (=CPAC 45, =CNPSo 1085)
Nepal	MLSA of *atpD*, *glnII*, *recA* and *rpoB*	Vinuesa *et al.* (2008)	NeMas 01, 02, 10, 11, 12, 16, NeRas 01, 02, 03, 04, 05, 06, 07, 08, 11, 12, 15
USA, Brazil, Japan	16S rRNA, ITS and MLSA of *dnaK*, *glnII* and *recA*	Menna *et al.* (2009)	SEMIAs 510 (=SEMIA 516, =SEMIA 5033, =UW 510, =USDA 510; CNPSo 162), 5020 (=BR 95, =965, =J 5033, CNPSo 12), 5021 (=CNPSo 1035), 5036 (=CNPSo 1047), 5043 (=8-T, CNPSo 1052), 5083 (=CPAC 44, CNPSo 1084)
USA	16S rRNA	Parker *et al.* (2002)	USDAs 62, 122, 129
USA	MLST of *asd, gap, gyrB, ilvI, lepA, mdh* and *purC*	van Berkum *et al.* (2012)	USDAs 122, 62, 126, 454, 439, 445, 444, 129, 422

[†] For many strains the precise origin of the strains is not completely clear.

Chueire, L. M. O., Bangel, E., Mostasso, F. L., Campo, R. J., Pedrosa, F. O. & Hungria, M. (2003). Classificação taxonômica das estirpes de rizóbio recomendadas para as culturas da soja e do feijoeiro baseada no seqüenciamento do gene 16S rRNA. *Rev Bras Ci Solo* **27,** 833-840.

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Hollis, A. B., Kloos, W. E. & Elkan, G. H. (1981). DNA-DNA hybridization studies of *Rhizobium japonicum* and related Rhizobiaceae. *J Gen Microbiol* **123**, 215-222.

Huber, T. A., Agarwal, A. K. & Keister, D. L. (1984). Extracellular polysaccharide composition, ex planta nitrogenase activity, and DNA homology in *Rhizobium japonicum*. *J Bacteriol* **158**, 1168-1171.

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.

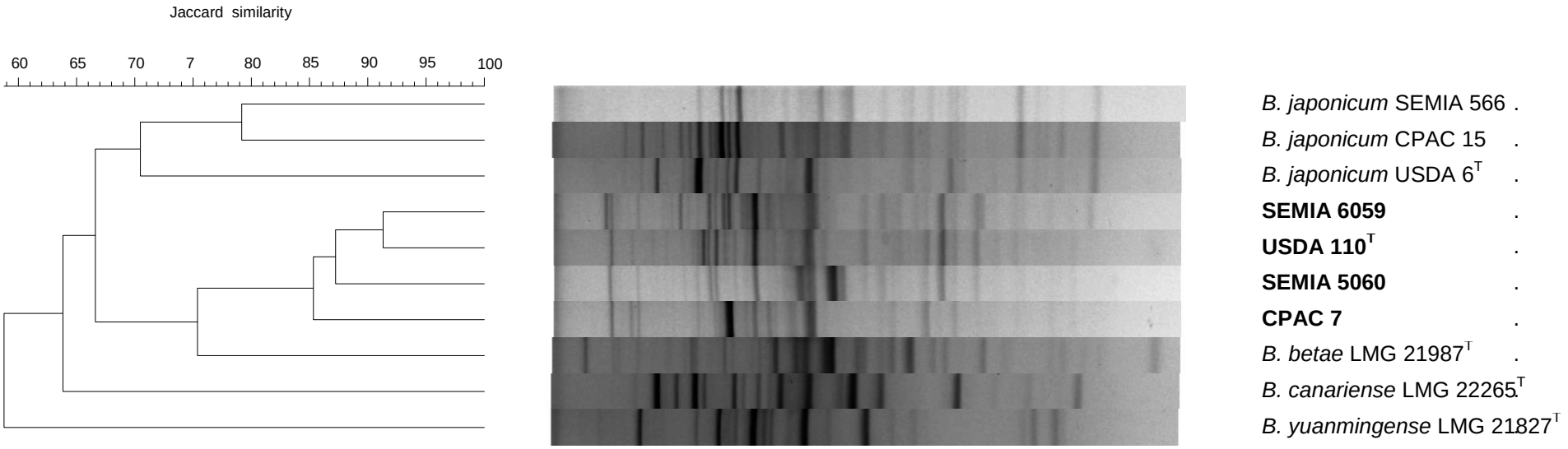
Menna, P., Barcellos, F. G. & Hungria, M. (2009). Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII*, *recA*, *atpD* and *dnaK* genes. *Int J Syst Evol Microbiol* **59**, 2934-2950.

Parker, M. A., Lafay, B., Burdon, J. J. & van Berkum, P. (2002). Conflicting phylogeographic patterns in rRNA and *nifD* indicate regionally restricted gene transfer in *Bradyrhizobium*. *Microbiology* **148**, 2557-2565.

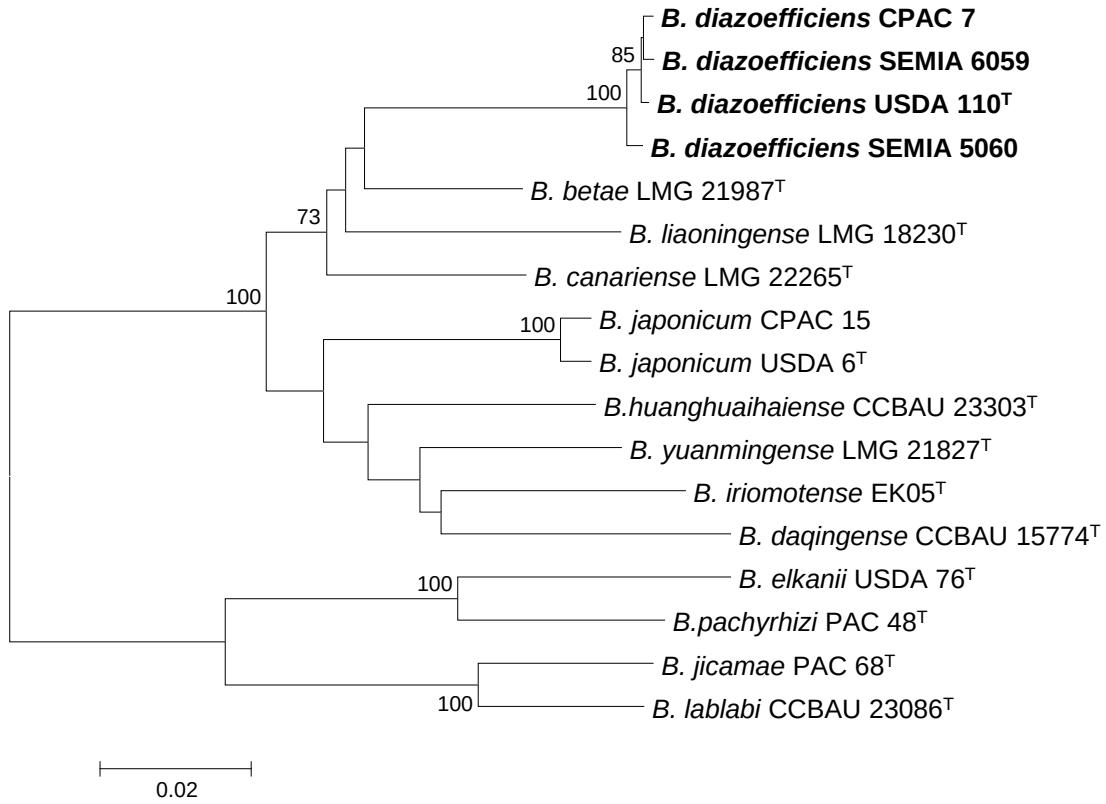
van Berkum, P. & Fuhrmann, J. J. (2001). Characterization of soybean bradyrhizobia for which serogroup affinities have not been identified. *Can J Microbiol* **47**, 519-25.

van Berkum, P., Elia, P., Song, Q. & Eardly, B. D. (2012). Development and application of a multilocus sequence analysis method for the identification of genotypes within genus *Bradyrhizobium* and for establishing nodule occupancy of soybean (*Glycine max* L. Merr). *Mol Plant-Microbe Interact* **25**, 321-330.

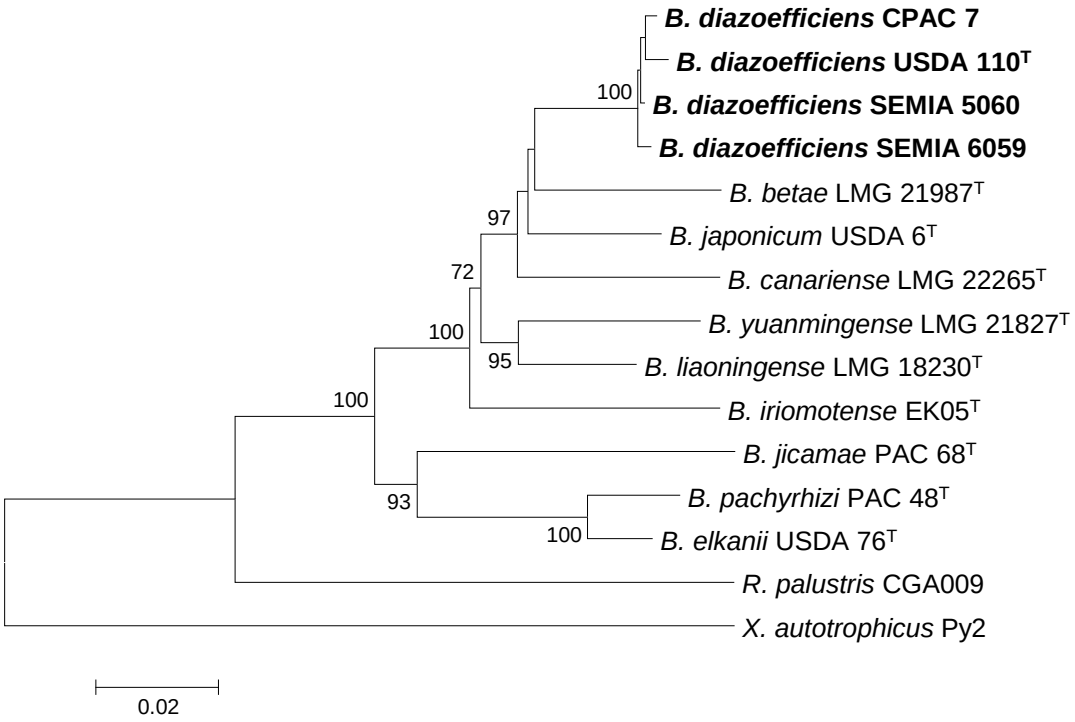
Vinuesa, P., Rojas-Jiménez, K., Contreras-Moreira, B., Mahna, S. K., Prasad, B. N., Moe, H., Selvaraju, S. B., Thierfelder, H. & Werner, D. (2008). Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the Asiatic continent. *Appl Environ Microbiol* **74**, 6987-6996.



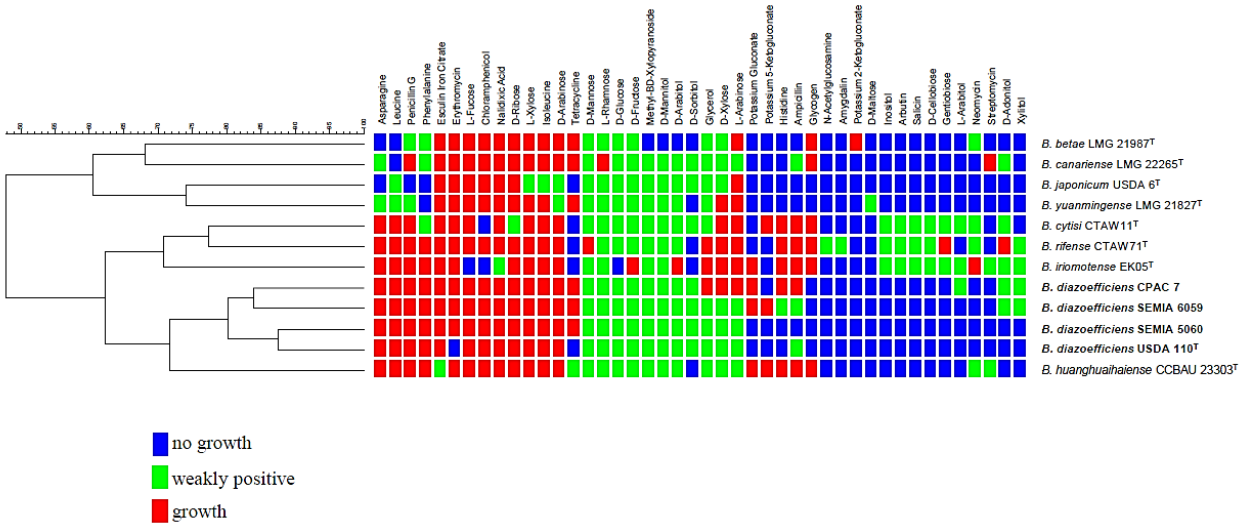
Supplementary Fig. S1. Cluster analysis (UPGMA algorithm with the Jaccard coefficient) of products obtained by BOX-A1R-PCR of *Bradyrhizobium diazoefficiens* strains (shown in bold) and related bradyrhizobia. Analysis performed with software Bionumerics v. 4.6.



Supplementary Figure S2. Dendrogram based on 16S-23S rRNA intergenic spacer (ITS) sequences showing the relationships of *Bradyrhizobium diazoefficiens* strains and other bradyrhizobia.



Supplementary Fig. S3. Neighbor joining phylogeny based on concatenated *atpD+glnII+recA+gyrB+rpoB+dnaK* gene sequences showing the relationships between *Bradyrhizobium diazoefficiens* and other members of the *Bradyrhizobium* genus. Bootstrap values >70 % are indicated at the nodes. *Rhodopseudomonas palustris* CGA009 and *Xanthobacter autotrophicus* Py2 were used as outgroups. Bar, 2 substitutions per 100 nucleotide positions.



Supplementary Fig. S4. Dendrogram (UPGMA algorithm and similarity coefficient of categorical values) based on phenotypic features of *Bradyrhizobium diazoefficiens* sp. nov. and phylogenetically related species. Dendrogram built software Bionumerics v. 7.0.