Bradyrhizobium ingae sp. nov., isolated from effective nodules of Inga laurina grown in Cerrado soil

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Root-nodule bacteria were isolated from Inga laurina (Sw.) Willd. growing in the Cerrado Amazon region, State of Roraima, Brazil. The 16S rRNA gene sequences of six strains (BR 10250T, BR 10248, BR 10249, BR 10251, BR 10252 and BR 10253) showed low similarities with currently described species of the genus Bradyrhizobium. Phylogenetic analyses of sequences of five housekeeping genes (dnaK, glnII, gyrB, recA and rpoB) revealed Bradyrhizobium iriomotense EK05T to be the closest type strain (97.4% sequence similarity or less). Chemotaxonomic data, including fatty acid profiles [with the major components C16:0 and summed feature 8 (C18:1ω6c/C18:1ω7c)], the slow growth rate and carbon compound utilization patterns supported the assignment of our strains to the genus Bradyrhizobium. Results from DNA–DNA hybridizations and physiological traits differentiated our strains from the closest related species of the genus Bradyrhizobium with validly published names. Sequences of symbiosis-related genes for nodulation (nodC) and nitrogen fixation (nifH) grouped together with those of B. iriomotense EK05T and Bradyrhizobium sp. strains BR 6610 (used as a commercial inoculant for Inga marginata in Brazil) and TUXTLS-10 (previously observed in Central America). Based on these data, the six strains represent a novel species, for which the name Bradyrhizobium ingae sp. nov. is proposed. The type strain is BR 10250T (=HMBI 3600T).

Inga Mill. (Leguminosae, Mimosoideae), tribe Ingeae, is considered to be an exclusively neotropical genus, containing around 300 species, some native to the Amazon region. However, several species are also found in Mexico, the Antilles and other South American countries (Possette & Rodrigues, 2010; Pennington, 1997). The pods of members of this genus contain seeds that are covered by a white, sweet pulp that is rich in minerals and is used as a food for animals (Possette & Rodrigues, 2010; Pennington, 1997). In addition, some species of the genus Inga are used in agriculture for nitrogen input, especially in alley-cropping or agroforestry systems, and also for land reclamation, because the plants tolerate poorly drained, acid soils and other major growth constraints (Franco & de Faria, 1997; Romero-Alvarado et al., 2002; Kurppa et al., 2010). In general, members of the genus Inga are recognized as efficient nitrogen fixers in association with root-nodule bacteria, and several countries have selected efficient inoculant strains for certain species in this genus (Franco & de Faria, 1997; Kurppa et al., 2010). However, very little is known about the diversity of root-nodule bacteria associated with this genus.
Previous authors have suggested that bacteria that nodulate members of the genus *Inga* are part of the ‘cowpea miscellany’ group of root-nodule bacteria, because the rhizobial strains isolated from nodules also nodulate and fix nitrogen efficiently with other legumes including species of *Cajanus*, *Acacia*, *Erythrina* and *Vigna* (Allen & Allen, 1939; Grossman et al., 2005). Additionally, it has been reported that slow growing strains, including strains of *Bradyrhizobium*, are characteristic root-nodule bacteria for members of the genus *Inga*, as they are for other tropical legumes (Grossman et al., 2005).

During a field study in 2008, 30 root nodules were collected from *Inga laurina* (Sw.) Willd. growing in natural conditions at two sites in the Cerrado (locally known as Lavrado, State of Roraima, Brazil), the Monte Cristo experimental field of Embrapa Roraima and a site located for members of the genus *Inga* (Conde & Tonini, 2013; Filardi et al., 2008). To collect the nodules, adult *I. laurina* plants were located and fix nitrogen efficiently with other legumes including species of *Cajanus*, *Acacia*, *Erythrina* and *Vigna* (Allen & Allen, 1939; Grossman et al., 2005). Additionally, it has been reported that slow growing strains, including strains of *Bradyrhizobium*, are characteristic root-nodule bacteria for members of the genus *Inga*, as they are for other tropical legumes (Grossman et al., 2005).

To collect the nodules, adult *I. laurina* plants were located and seedlings of *I. laurina* growing under these trees were manually uprooted. Nodules presented were collected in Boa Vista city (2° 50′ 21″ N 60° 40′ 32.25″ W and 2° 57′ 00″ N 60° 42′ 25″ W, respectively). The climate in this region is classified as Aw (Koppén), with mean rainfall of 1600 mm year −1 and a mean temperature of 27 °C (Araújo et al., 2001). *I. laurina* is a common species naturally occurring in the Cerrado and other ecosystems in Brazil (Conde & Tonini, 2013; Filardi et al., 2008).

For PCR, genomic DNA was prepared using the RBC Bioscience kit (catalogue no. YGB300) and BOX PCR analysis was performed as described previously (Versalovic et al., 1994). Fingerprint analysis was performed with the BioNumerics 7.01 software package (Applied Maths) using the UPGMA algorithm and Pearson’s correlation index. Cluster analysis showed that the six strains grouped together at a similarity level of 75 % in three subgroups, indicating that they represent genetically distinct strains (Fig. S1, available in the online Supplementary Material).

Nearly full-length sequences of the 16S rRNA gene (1318 bp) were obtained for all strains using primers and conditions described previously (Radl et al., 2014). Sequence alignment, alignment editing and phylogenetic analyses were performed using the MEGAS software package (Tamura et al., 2011). Phylogenetic trees were reconstructed using the neighbour-joining (NJ) (Saitou & Nei, 1987) and maximum-likelihood (ML) (Felsenstein, 1981) methods. The strength of each topology was verified using 1000 bootstrap replications. The overall topologies of the phylogenetic trees obtained with the NJ and ML methods were very similar (not shown), so only the ML tree is provided (Fig. 1).

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The six strains formed a separate branch within the genus *Bradyrhizobium* together with *Bradyrhizobium iriomotense* EK05T, isolated in Japan from *Entada koshunensis* (Leguminosae, Mimosoideae) (Islam et al., 2008) (Fig. 1). They shared 100 % sequence similarity with each other, and 98 % or less with type strains of other species of the genus *Bradyrhizobium*. We also observed that our strains clustered together with BR 6610, used as a commercial inoculant for *Inga marginata* in Brazil (Franco & de Faria, 1997; Menna et al., 2006), and strain TUXTLAS-10, isolated in Mexico, which are referred to as part of the ‘BCI Bradyrhizobium lineage’ common in Central America (Parker, 2003; Ormeño-Orrillo et al., 2012).

Although high similarity was observed for the 16S rRNA gene sequence, previous reports have suggested that closely related members of the genus *Bradyrhizobium* do not necessarily belong to the same species (Menna et al., 2009; Willems et al., 2001). Therefore, multilocus sequence analysis was performed with the dnaK (238 bp), glnII (537 bp), gyrB (592 bp), recA (423 bp) and rpoB (525 bp) genes following previous reports (Martens et al., 2008; Menna et al., 2009; Vinuesa et al., 2005). Before the sequences for the dnaK, glnII, gyrB, recA and rpoB genes, the congruence existence (tree topology) and partition homogeneity tests were evaluated (Farris et al., 1994). The phylogenetic tree based on the concatenated sequences of the five housekeeping genes (Fig. 2) revealed that our strains belonged to a monophyletic cluster with high bootstrap support (100 %). Sequence similarities among our strains were 99 or 100 % for all investigated genes (Table S1). The closest type strain in the 16S rRNA gene sequence analysis, *B. iriomotense* EK05T, showed ≤97.4 % sequence similarity with strain BR 10250T for all investigated genes (Figs 2 and S–S4 and Table S1). These trees also showed that our strains belonged to a different group than the commercial strain SEMIA 6434 and strain TUXTLAS-10, even though they are closely related to *B. iriomotense* EK05T.

For phenotypic characterization, the strains were Gram-stained and were grown for 7 days on 79 medium at 15, 20,
25, 28, 30, 32 and 37 °C, pH 4, 5, 6, 7, 8, 9, 10 and 0.1, 0.3, 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v) NaCl. Cell motility was observed by light microscopy of a wet preparation and cell morphology by transmission and scanning electron microscopy. Oxidase activity was detected by immersion of cells in 1% N,N,N′,N″-tetramethyl p-phenylenediamine solution and catalase activity was determined by flooding a colony with 10% (v/v) H2O2 and checking for the formation of bubbles. Other biochemical tests were performed by inoculating API 20NE strips (bioMérieux) and Biolog GN2 microplates according to the manufacturers’ instructions and incubating for 8 days at 28 °C. Antibiotic susceptibility tests were performed on YMA using the antibiotic Sensi-disc dispenser system (Oxoid) with bio-discs (Oxoid) containing ampicillin (10 and 25 μg), chloramphenicol (30 and 50 μg), erythromycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (10 μg), penicillin (10 μg), streptomycin (10 and 25 μg) and tetracycline (30 μg). The plates were incubated at 28 °C and read after 10 days.

Discriminatory phenotypic characteristics of our strains are given in Table 1 and the details of carbon source utilization are presented in Table S3. Our strains were able to grow at 15–32 °C and pH 4–8, which are common characteristics for the genus *Bradyrhizobium*. Optimum growth was verified at 28–30 °C and pH 5–7. All strains were resistant to erythromycin, gentamicin and neomycin and sensitive to ampicillin, chloramphenicol, kanamycin, streptomycin and tetracycline. Additionally, the closest related type strain, *B. iriomotense* LMG 24129T, showed chloramphenicol and streptomycin resistance. Enzymic reactions were positive for catalase, oxidase, urease and hydrolysis of aesculin and negative for nitrate reduction, glucose fermentation, activities of tryptophan deaminase, arginine dihydrolase and β-galactosidase and hydrolysis of gelatin. The *Inga* strains differed from *B. iriomotense* LMG 24129T in β-galactosidase and urease reactions (Table 1).

Whole-cell fatty acid methyl esters of strain BR 10250T were extracted according to the MIDI protocol (http://ijs.sgmjournals.org/3397)
www.microbialid.com/PDF/TechNote_101.pdf, Delamuta et al., 2013). Cultures were grown for 5 days at 28 °C on YMA prior to extraction. Profiles were generated using an Agilent model 6850 chromatograph and identified using the TSBA database version 6.10 (Microbial Identification System, MIDI Inc.). The most abundant cellular fatty acids detected were C16:0 (17.51 %) and summed feature 8 (C18:1ω6c/C18:1ω7c) (70.78 %). Moderate amounts of 11-methyl C18:0ω7c (10.8 %) and C19:0 cyclo ω8c (11.71 %) were also found. The presence of C16:0 and summed feature 8 supports the placement of these strains in the genus Bradyrhizobium (Tighe et al., 2000). Some differences were evident between the fatty acid profiles of BR 10250T and B. iriomotense EK05T, especially the lower abundance of C16:0 (14.7 %) and higher levels of summed feature 8 (70.78 %) (Islam et al., 2008).

For DNA–DNA hybridization and the determination of DNA G+C content, high-molecular-mass DNA was prepared as described by Pitcher et al. (1989). DNA–DNA hybridizations were performed using a microplate method and biotinylated probe DNA (Ezaki et al., 1989). The hybridization temperature was 50 ± 1 °C. Reciprocal reactions (A × B and B × A) were performed for each DNA pair, and! their variation was within the limits of this method (Goris et al. 1998). The DNA–DNA relatedness between BR 10250T and the closest type strain, B. iriomotense LMG 24129T, was 65.7 %, conﬁrming that our strains belong to a novel species, since the threshold recommended is 70 % (Lindström & Gyllenberg, 2007). The G+C content of DNA was determined by HPLC according to the method of Mesbah et al. (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M NH4H2PO4 (pH 4.0) with 1.5 % (v/v) acetonitrile. Non-methylated lambda phage DNA (Sigma) and Escherichia coli DNA were used as calibration reference and control, respectively. The DNA G+C content of strain BR 10250T was 63.4 mol% (Table 1), slightly higher than the closest type strain B. iriomotense EK05T, for which the DNA G+C content was reported to be 61.2 mol% (Islam et al., 2008).

Nodulation and nitrogen-fixation genes are required for effective legume symbiosis; therefore, nodC and nifH genes were analysed according to Laguerre et al. (2001) and Ueda et al. (1995), respectively. Phylogenetic trees were reconstructed as described previously and the results are given in Figs S5 and S6. Both nodC and nifH gene sequence analyses clustered strain BR 10250T in the same branch as B. iriomotense EK05T, but with low similarity (Table S1). The maximum identity observed for the nodC sequence of strain BR 10250T by BLAST search (Altschul et al., 1990) was 92 % with a strain isolated from Ormosia fastigiata (Leguminosae, Papilionoideae; GenBank accession no. KF031520). BLAST and phylogenetic analyses of nifH gene sequences revealed 98 % sequence similarity with strain SEMIA 6434 isolated in Brazil (Fig. S5).

To confirm the nodulation ability of the strains investigated in this study, two glasshouse experiments were performed.
In the first trial, the six strains were tested on *Inga edulis*, because no viable seeds of *I. laurina*, their original host, could be found. These experiments were performed in Leonard jars containing nitrogen-free nutrient solution according to Radl et al. (2014). Thereafter, host plant tests were performed with strain BR 10250T on 14 different legume species using the axenic sand-culture system described previously (Howieson et al., 2013). For both experiments, the seeds were surface-sterilized and inoculated with 1 ml YM broth suspension containing 10⁹ bacterial cells grown for 5 days at 28°C. All treatments, plus an uninoculated control, were replicated four times in a split-plot design (Howieson et al., 2013). Nodulation was evaluated 60 and 35 days after inoculation in the first and second experiments, respectively. Results showed that the six strains were able to nodulate *I. edulis* (Table S4). Strain BR 10250T also effectively nodulated *Arachis hypogaea*, *Macroptillium atropurpureum*, *Vigna radiata* and *V. unguiculata*, and formed ineffective root nodules on *Glycine max*.

No nodulation was observed for *Acacia ligulata*, *Cajanus cajan*, *Crotalaria juncea*, *Lupinus angustifolius*, *Ornithopus compressus*, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia faba* and *Vigna angularis*.

The genotypic and phenotypic data presented in this study demonstrate that the strains isolated from *I. laurina* root nodules collected in the Cerrado of the Amazonia region represent a novel species, for which the name *Bradyrhizobium ingae* sp. nov. is proposed.

### Description of *Bradyrhizobium ingae* sp. nov.

*Bradyrhizobium ingae* [in’gae. N.L. gen. n. ingae of Inga, a botanical genus name, referring to the isolation of the first strains from root nodules of Inga laurina (Sw.) Willd.]

Cells are Gram-negative, aerobic, non-spore-forming rods (approx. 1.5 x 0.6 μm) that are motile by means of polar flagella (Fig. S7). Colonies on YMA are circular and translucent and 1 mm in diameter within 7–8 days of

Table 1. Differential features of strains of *Bradyrhizobium ingae* sp. nov. and the closest related type strain

<table>
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<td>ND</td>
<td>ND</td>
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*Less than 1 % according to Islam et al. (2008).
†Data from Islam et al. (2008).
incubation at 28°C. The generation time is 9.5 h in YM broth. The pH range for growth in YMA is 4–8, with optimum growth at pH 5.0–7.0. Growth occurs at 15–32°C, with optimum growth at 28–30°C. Does not grow in the presence of 0.5% (w/v) NaCl or higher. Resistant to erythromycin (30 μg), gentamicin (10 μg) and neomycin (10 μg) and sensitive to ampicillin (10 μg), chloramphenicol (50 μg), kanamycin (30 μg), streptomycin (10 μg) and tetracycline (30 μg). Positive reactions are recorded for the utilization of D-arabitol, D-fructose, D-galactose, D-mannitol, D-mannose, D-sorbitol, L-arabinose, L-fucose, L-rhamnose, myo-inositol, N-acetyl-D-glucosamine, xylitol and 2-D-glucose. Oxidase, catalase and urease tests are positive, while nitrate reduction and β-galactosidase are negative. The most dominant cellular fatty acids are C₁₆:₀ and summed feature 8 (C₁₈:₁ω₆c/C₁₈:₁ω₇c).

The type strain, BR 10250T (=HMBI 3600T), was isolated from nodules collected from Inga laurina in a Cerrado area of the Amazon, from Roraima State, Brazil. The DNA G+C content of the type strain is 63.4 mol%.

Acknowledgements

The authors would like to thank Fernanda Dourado and Natalia Camacho (Embrapa Agrobiologia), Regina Carr and Rebecca Swift (Murdock University) and Liesbeth Lebbe (Ghent University) for technical assistance. We also thank Dr Itamar Soares Melo (Embrapa Meio Ambiente) for bacterial fatty acid analysis. This study was supported financially by CNPq, Embrapa and Murdoch University.

References


