Bradyrhizobium neotropicale sp. nov., isolated from effective nodules of Centrolobium paraense

Jerri E. Zilli,1 Alexandre C. Baraúna,2 Krisle da Silva,3 Sofie E. De Meyer,4,5 Eliane N. C. Farias,3 Paulo E. Kaminski,3 Ismaele B. da Costa,3 Julie K. Ardley,4 Anne Willems,5 Natália N. Camacho,1 Fernanda dos S. Dourado1 and Graham O’Hara4

1Embrapa Agrobiologia, Rodovia BR 465 km 07, Seropédica, Rio de Janeiro 23891-000, Brazil
2Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465 km 07, Seropédica, Rio de Janeiro 23890-000, Brazil
3Embrapa Roraima, Rodovia BR 174 km 08, Boa Vista, Roraima 69301-970, Brazil
4Centre for Rhizobium Studies, Murdoch University, 90 South Street, Murdoch 6150, Western Australia, Australia
5Laboratory of Microbiology, Department of Biochemistry and Microbiology (WE10), Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium

Root nodule bacteria were isolated from Centrolobium paraense Tul. grown in soils from the Amazon region, State of Roraima (Brazil). 16S rRNA gene sequence analysis of seven strains (BR 10247T, BR 10296, BR 10297, BR 10298, BR 10299, BR 10300 and BR 10301) placed them in the genus Bradyrhizobium with the closest neighbours being the type strains of Bradyrhizobium paxllaeri (98.8 % similarity), Bradyrhizobium icense (98.8 %), Bradyrhizobium lablabi (98.7 %), Bradyrhizobium jicamae (98.6 %), Bradyrhizobium elkanii (98.6 %), Bradyrhizobium pachyrhizi (98.6 %) and Bradyrhizobium retamae (98.3 %). This high similarity, however, was not confirmed by the intergenic transcribed spacer (ITS) 16S–23S rRNA region sequence analysis nor by multi-locus sequence analysis. Phylogenetic analyses of five housekeeping genes (dnaK, glnII, gyrB, recA and rpoB) revealed Bradyrhizobium iromotense EK05 (=LMG 24129T) to be the most closely related type strain (95.7 % sequence similarity or less). Chemotaxonomic data, including fatty acid profiles [major components being C16:0 and summed feature 8 (18:1ω6c/18:1ω7c)], DNA G+C content, slow growth rate and carbon compound utilization patterns, supported the placement of the novel strains in the genus Bradyrhizobium. Results of DNA–DNA relatedness studies and physiological data (especially carbon source utilization) differentiated the strains from the closest recognized species of the genus Bradyrhizobium. Symbiosis-related genes for nodulation (nodC) and nitrogen fixation (nifH) placed the novel species in a new branch within the genus Bradyrhizobium. Based on the current data, these seven strains represent a novel species for which the name Bradyrhizobium neotropicale sp. nov. is proposed. The type strain is BR 10247T (=HAMBI 3599T).

Centrolobium paraense Tul. (Leguminosae, Papilionoideae), tribe Dalbergieae, locally known as ‘pau-rainha’, is a nodulating neotropical leguminous tree occurring from the northern Brazilian Amazonia to Panama (Pirie et al., 2009). It grows in semi-deciduous forest, gallery forest, forest islands in cerrado and transition cerrado/forest, and has several ecological roles including nutrient input through symbiotic nitrogen fixation, protecting soils against erosion and being a pioneer or early secondary plant (Marques et al., 2001; Dahmer et al., 2009). This species also has economic and social importance because its wood is used by...
indigenous communities and in industry as timber or fuel (Dahmer et al., 2009; Pedreira, 2010).

Centrolobium paraense is capable of forming nodules with rhizobia native to the soil of Amazonia (Souza et al., 1994; Baraúna et al., 2014), and bacteria belonging to the genera Bradyrhizobium and Rhizobium have been reported as symbionts of Centrolobium species. However, previous studies have not identified these bacteria at the species level (Moreira et al., 1998; Pagano, 2008; Baraúna et al., 2014).

A recent investigation of the ecology of root-nodulating bacteria isolated from Centrolobium paraense grown in soils collected from different areas in Roraima State, Brazil, found that about 90% of the 178 isolates exhibited phenotypic characteristics similar to species of the genus Bradyrhizobium (Baraúna et al., 2014). Analysis of partial 16S rRNA gene sequences confirmed these results, but placed these isolates in branches different from previously described species. Nine of the new isolates also show high efficiency in nitrogen fixation associated with Centrolobium paraense (Baraúna et al., 2014).

Here we report the results from a polyphasic taxonomic study of seven isolates (BR 10247 T, BR 10296, BR 10297, BR 10298, BR 10299, BR 10300 and BR 10301). This polyphasic study included gene sequence analysis [16S rRNA, intergenic transcribed spacer (ITS), glnII, gyrB, recA, rpoB, nodC and nifH], DNA–DNA relatedness studies, fatty acid profiling and phenotypic characterization. The strains were obtained from Centrolobium paraense grown in soil samples collected in the Mucajai municipality of Roraima (2° 27 12.9 N 60° 54 11.2 W) (Baraúna et al., 2014). The climate in this region is classified as Aw (Köppen) with average annual rainfall of 1600 mm and an average temperature of 27 °C (Araújo, et al., 2001). The strains were deposited in the Diazotrophic Microbial Culture Collection – CRB – Johanna Döbereiner (Embrapa Agrobiologia, Rio de Janeiro, Brazil); strain BR 10247 T was also deposited at the Hambi Collection (http://www.helsinki.fi/hambi) as HAMBHI 3599 T. All strains were cultured on 79 medium (Fred & Waksman, 1928) at 28 °C and for long-term storage the cultures were lyophilized and maintained at −80 °C.

For PCR, genomic DNA was prepared using the Promega genomic DNA purification kit (cat. A1120), according to the manufacturer’s instructions. Nearly full-length sequences of the 16S rRNA gene (1336 bp) were obtained for all strains using the primers and conditions described by Radl et al., (2014). The ITS sequences were also obtained for the new strains following the conditions presented by Menna et al. (2009). Sequence alignment, alignment editing and phylogenetic analyses were performed using the MEGAS5 software package (Tamura et al., 2011). Phylogenetic trees were reconstructed using the maximum-likelihood (ML) (Felsenstein, 1981) method as recommended by Tindall et al. (2010). The strength of each topology was verified using 1000 bootstrap replications and the ML trees are provided (Figs 1 and 2).

The 16S rRNA gene phylogenetic analysis showed that the seven strains shared more than 99.5% sequence similarity with each other and formed a separate branch within the genus Bradyrhizobium, with Bradyrhizobium jicamae PAC68 T as the closest neighbour (Fig. 1). The 16S rRNA gene sequence similarity between strain BR 10247 T and other Bradyrhizobium sp. nov. types strains was between 96.0 and 98.8% (Table S1, available in the online Supplementary Material). Thus, even though the 16S rRNA gene is highly conserved in members of the genus Bradyrhizobium (Menna et al., 2009; Willems et al., 2001b), the analysis showed that our strains form a branch separate from other species of the genus.

ITS phylogenetic analysis (891 bp) showed less than 86.6% similarity between the novel strains and other recognized Bradyrhizobium type strains, and the similarity between the novel strains was greater than 98% (Table S1). The ITS phylogenetic reconstruction placed the new strains in a separate branch with Bradyrhizobium jicamae EKO5 T as the closest neighbour (Fig. 2). Previous studies have demonstrated that ITS sequences are a suitable marker to separate species of the genus Bradyrhizobium and 95.5% similarity or more indicates strains belonging to the same genospecies, corresponding to about 60% DNA–DNA relatedness (Willems et al., 2001a, 2003).

To confirm the ITS results we performed a multi-locus sequence analysis (MLSA), using housekeeping genes that have previously been used for Bradyrhizobium species delimitation, and produce phylogenies that are supported by ITS sequence and DNA–DNA hybridization data (Menna et al., 2009; Rivas et al., 2009). We obtained sequences and performed the analyses for dnaK (238 bp), glnII (534 bp), gyrB (591 bp), recA (418 bp) and rpoB (408 bp) genes following previous reports (Vinuesa et al., 2005; Martens et al., 2008; Menna et al., 2009; Rivas et al., 2009). Congruence between the different gene sequences was first checked using partition homogeneity tests (Farris, et al., 1994) performed with PAUP software version 4.0b10 (Swofford, 2002). As congruence (P > 0.01) was found only between the genes gyrB, recA and rpoB, the concatenation (performed by the software SeaView version 4.0; Gouy et al., 2010) was done for these three genes, and the other two genes (dnaK and glnII) were analysed individually.

The phylogenetic tree based on the concatenated sequences of the three genes confirmed that the novel strains belonged to a monophyletic cluster with high bootstrap support (100%) (Fig. 3). Similar relationships were also obtained for the dnaK and glnII genes when analysed individually (Figs S1 and S2). In addition, sequence similarities between the strains were more than 99% for all investigated genes (Table S1). The closest type strain in the 16S rRNA gene sequence analysis, B. jicamae PAC68 T, showed less than 90% similarity with strain BR 10247 T in MLSA. However, the closest type strain from the ITS analysis, B. jicamae EKO5 T, had a similarity between 92.6 and 95.7% for all investigated genes in comparison with strain BR 10247 T.
It is of note that our strains presented discordance in the 16S rRNA gene sequence phylogeny compared with the ITS phylogeny, which was also confirmed by MLSA. While in the 16S rRNA gene sequence analysis the closest strain was *B. jicamae* (subgroup I); the ITS and concatenated MLSA trees placed the novel strains together with *B. iriomotense*, belonging to subgroup II (Figs 1–3). The *Bradyrhizobium* subgroup division (I and II) is based on DNA–DNA hybridization and was used to separate the *Bradyrhizobium japonicum* division (I and II) is based on DNA–DNA hybridization gene recombination event, leading to a reticulate evolutionary history (van Berkum et al., 2003, 2009; Parker, 2003). This may indicate that strains belonging to this novel species of the genus *Bradyrhizobium* are distributed from northern Brazil to Panama or Mexico.

For phenotypic characterization, the strains were Gram stained and were incubated for 7 days on 79 at different temperatures (15, 20, 25, 28, 30, 32 and 37 °C), pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0) and NaCl concentrations (0.1, 0.3, 0.5, 1.0, 1.5, 2.0 and 2.5 %, w/v). Cell motility was observed by light microscopy of wet preparations (0.1, 0.3, 0.5, 1.0, 1.5, 2.0 and 2.5 %, w/v). Cell motility was observed by light microscopy of wet preparations of the strains grown in YM medium, and cell morphology by transmission (Philips CM100 BioTwin) and scanning electron microscopy (Philips XL20). Oxidase activity was evaluated by touching a colony with a paper impregnated in 1 % *N,N,N',N''-tetramethyl-p-phenylenediamine* solution and observing the colour change; catalase activity was determined by flooding a colony with 10 % (v/v) H₂O₂ and checking for the presence of bubbles.

Other biochemical tests were performed by inoculating API 20NE strips (bioMérieux) and Biolog GN2 microplates (Biolog) according to the manufacturers’ instructions followed by incubation for 8 days at 28 °C. Antibiotic susceptibility tests were performed on 79 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 the novel strains are given in Table 1 and the details of carbon source utilization are presented in Table S3. The strains grew between 15 and 37 °C and in the pH range 4.0–10.0, common characteristics within the genus Bradyrhizobium. Optimum growth occurred at 28–32 °C and pH 5–7 (Table 1). All strains were resistant to chloramphenicol (50 μg) but sensitive to ampicillin (10 μg), penicillin (10 μg), streptomycin (10 and 25 μg) and tetracycline (30 μg), while the closest type strain, B. iriomotense EK05T, showed resistance to penicillin and streptomycin (Table 1). Enzymic reactions were positive for catalase, oxidase, urease, arginine dihydrolase and hydrolysis of asescul, but negative for nitrate reduction, tryptophan deaminase, glucose fermentation, hydrolysis of gelatin and β-galactosidase. The novel strains differed from B. iriomotense LMG 24129T in β-galactosidase, arginine dihydrolase and nitrate reduction (Table 1).

Whole-cell fatty acid methyl esters of strain BR 10247T were extracted according to the MIDI protocol (http://www.microbialid.com/PDF/TechNote_101.pdf; Delamuta et al., 2013). Cultures were grown for 5 days at 28 °C on 79 prior to extraction. The profiles were generated using an Agilent model 6850 chromatograph and identified using the TSBA database version 6.10 (Microbial Identification System; MIDI). The most abundant cellular fatty acids detected were C_{16:0} (13.56 %) and summed feature 8 (18:1ω6c/18:1ω7c) (66.52 %). Moderate amounts of C_{18:1ω7c} 11-methyl (11.77 %), C_{19:0} cyclo ω8c (7.14 %) and C_{18:0} (1.04 %) were also found. The presence of C_{16:0} and summed feature 8 supports the placement of these strain in the genus Bradyrhizobium (Tighe et al., 2000), and differences were noted between strain BR 10247T and B. iriomotense EK05T, especially the higher abundance of C_{16:0} (14.7 %) and lower C_{18:1ω7c} (80.1 %) detailed by Islam et al. (2008).

For DNA–DNA hybridization and 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 in triplicate for each DNA pair and their variation was within the limits reported for this method (Goris et al., 1998). The level of
DNA–DNA relatedness between strain BR 10247\textsuperscript{T} and the closest type strain, \textit{B. iriomotense} EK05\textsuperscript{T}, was 63.8\%, confirming that the Amazonia strains belong to a novel species, given the recommended threshold of 70\% (Lindström & Gyllenberg, 2007; Tindall \textit{et al.}, 2010). The G\,+\,C content of the DNA was determined by HPLC according to the method of Mesbah \textit{et al.} (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} (pH 4.0) with 1.5\% (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and \textit{Escherichia coli} DNA were used as calibration reference and control, respectively. The DNA G\,+\,C content of strain BR 10247\textsuperscript{T} was 63.9 mol\% (Table 1), differentiating it from the closest related type strain, \textit{B. iriomotense} LMG 24129\textsuperscript{T} (61.2\%; Islam \textit{et al.}, 2008).

Nodulation and nitrogen fixation genes are required for effective legume symbiosis, and therefore \textit{nodC} and \textit{nifH} genes were analysed according to Sarita \textit{et al.} (2005) and Ueda \textit{et al.} (1995), respectively. Phylogenetic trees were reconstructed as described above and the results are given in Figs S3 and S4 for \textit{nodC} and \textit{nifH}, respectively. \textit{nodC} analysis placed strain BR 10247\textsuperscript{T} in a separate branch in relation to other species of the genus \textit{Bradyrhizobium} and it was grouped outside the four recognized symbiovars (Cobo-Díaz \textit{et al.}, 2014) (similarity <80\%), possibly indicating a new symbiovar within this genus (Fig. S3, Table S1). \textit{nifH} gene sequence analysis clustered strain BR 10247\textsuperscript{T} in the same branch as \textit{B. iriomotense} EK05\textsuperscript{T} (closest strain in the previous analyses), but the level of similarity observed between the two was lower than 90\% (Fig. S4, Table S1), indicating again that it might be a new symbiovar.

To confirm the nodulation ability of the strains investigated in this study, two glasshouse experiments were performed. In the first experiment, the seven strains were tested on their original host \textit{Centrolobium paraense} and this was performed using Leonard jars containing N-free nutrient solution according to Radl \textit{et al.} (2014).

Secondly, host plant tests with strain BR 10247\textsuperscript{T} were performed on 14 different legume species using the axenic sand-culture system described by Howieson \textit{et al.} (2013). For both experiments the seeds were surface sterilized with H\textsubscript{2}O\textsubscript{2} (5\%, v/v; 5 min) and inoculated with 1 ml of YM broth suspension containing approximately 10\textsuperscript{9} bacterial

![Fig. 3. ML phylogeny based on concatenated \textit{gyrB}, \textit{recA} and \textit{rpoB} gene sequences showing the relationships between strains of the novel species (shown in bold type) and other members of the genus \textit{Bradyrhizobium}. The significance of each branch is indicated by a bootstrap value (only values >50\% are shown) calculated for 1000 subsets. Bar, 2 substitutions per 100 nucleotide positions.](image-url)
Table 1. Differential features between strains of *Bradyrhizobium neotropicale* sp. nov. and the closest related type strain, *Bradyrhizobium iriomotense* EK05<sup>T</sup>

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<th>Characteristic</th>
<th>BR 10247&lt;sup&gt;T&lt;/sup&gt;</th>
<th>BR 10296</th>
<th>BR 10297</th>
<th>BR 10298</th>
<th>BR 10299</th>
<th>BR 10300</th>
<th>BR 10301</th>
<th>EK05&lt;sup&gt;T&lt;/sup&gt;*</th>
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<td>L-Rhamnose</td>
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<td>DNA G+C content (mol%)</td>
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<td>ND</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>61.2†</td>
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*LMG* 24129<sup>T</sup> (formal deposit of strain EK05<sup>T</sup>) was obtained from the LMG culture collection.

†Data from Islam et al. (2008).

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 70 and 35 days after inoculation in the first and the second experiment, respectively. The seven strains nodulated *Centrolobium paraense* (Table S4). Strain BR 10247<sup>T</sup> formed effective nitrogen-fixing nodules on roots of *Arachis hypogaea*, *Acacia ligulata*, *Cajanus cajan*, *Crotalaria juncea*, *Macroptillium atropurpureum*, *Vigna unguiculata*, *Vigna angularis* and *Vigna radiata*, and ineffective (non-fixing) root nodules on *Ornithopus compressus* and *Phaseolus vulgaris*. No nodulation was observed for *Glycine max*, *Lupinus angustifolius*, *Pisum sativum* or *Vicia faba*.

The genotypic and phenotypic data presented in this study demonstrate that the strains isolated from *Centrolobium paraense* root nodules collected in Amazonia represent a novel species, for which the name *Bradyrhizobium neotropicale* sp. nov. is proposed.

**Description of *Bradyrhizobium neotropicale* sp. nov.**

*Bradyrhizobium neotropicale* [ne.o.tro.pi.ca’le. Gr. adj. neos new; N.L. adj. tropicalis (from L. masc. adj. tropicus tropical), referring to the tropics; N.L. neut. adj. neotropicale of the neotropics].

Cells are motile with polar flagella, Gram-stain-negative (approx. 2.4 × 0.6 μm), aerobic, non-spore-forming rods (Fig. S5). Colonies on 79 medium are circular and translucent, and have a diameter of 1 mm within 7–8 days of incubation at 28 °C. The generation time is 10.8 h in YM broth. The pH range for growth on 79 is pH 4.0–10.0, with optimal growth at pH 5.0–7.0. Growth occurs between 15 and 37 °C, with optimal growth at 28–32 °C. Does not grow in the presence of 1.5 % (w/v) NaCl or higher. Positive reactions for carbon source utilization are recorded for L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, D-mannitol, D-mannose, L-rhamnose, acetic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketoglutaric acid, DL-lactic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, glycerol, methyl pyruvate, monomethyl succinate, D-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-leucine, L-phenylalanine, urocanic acid, succinamic acid, glucuronamide.
and bromosuccinic acid. Oxidase, catalase, arginine dihydrolase, hydrolysis of aesculin and urease are also positive, while nitrate reduction, tryptophan deaminase, glucose fermentation, β-galactosidase and hydrolysis of gelatin are negative. The most abundant cellular fatty acids are C₁₆:₀ and summed feature 8 (18:1ω6c/18:1ω7c).

The type strain, BR 10247T (=HAMBI 3599T), was isolated from nodules of *Centrolobium paraense* grown in soils of Amazonia, Roraima State, Brazil. The DNA G+C content of the type strain is 63.9 mol%.

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**References**


