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

Short title
Bradyrhizobium neotropicale sp. nov.

Contents category
New taxa

Subsection
Proteobacteria

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, ITS 16S-23S rRNA, dnaK, glnII, gyrB recA, rpoB, nodC and nifH gene sequences of Bradyrhizobium neotropicale sp. nov. BR 10247T are KF927051, KJ661686, KJ661693, KJ661700, KJ661707, KJ661714, KF983829, KJ661727 and KJ661728, respectively. The accession numbers for all other strains are listed in Table S2.

Abstract
Root nodule bacteria were isolated from Centrolobium paraense Tul. grown in soils from the Amazon region, State of Roraima (Brazil). The 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 into the genus Bradyrhizobium with the closest neighbouring species B. paxllaeri (98.8%), B. icense (98.8%), B. lablabi (98.7%), B. jicamae (98.6%), B. elkanii (98.6%), B. pachyrhizi (98.6%) and B. retamae (98.3%). This high similarity, however, was not confirmed by the ITS 16S-23S rRNA analysis nor by MLSA. Phylogenetic analyses of five housekeeping genes (dnaK, glnII, gyrB, recA and rpoB) revealed Bradyrhizobium riomotense strain EK05T (=LMG 24129T) to be the closest type strain (95.7% sequence similarity or less). Chemotaxonomic data,
including fatty acid profiles (with majority being C\textsubscript{16:0} and Summed Feature 8 (C\textsubscript{18:1} w7c), DNA G+C content (% mol), the slow growth rate and carbon compound utilization patterns supported the placement of our strains in the genus \textit{Bradyrhizobium}. Results of DNA-DNA relatedness and physiological data (especially C source utilization) differentiated our strains from the closest validly named \textit{Bradyrhizobium} species. Symbiosis-related genes for nodulation (\textit{nodC}) and nitrogen fixation (\textit{nifH}) placed the new species in a new branch within the \textit{Bradyrhizobium} genus. Based on the current data, these seven strains represent a novel species for which the name \textit{Bradyrhizobium neotropicalesp. nov.} (BR 10247\textsuperscript{T} = HAMBI 3599\textsuperscript{T}) is proposed.

\textit{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 \textit{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 \textit{et al.}, 2001; Dahmer \textit{et al.}, 2009). This species has also economic and social importance because its wood is used by indigenous communities and in the industry as timber or fuel (Dahmer \textit{et al.}, 2009; Pedreira, 2010).

\textit{C. paraense} is capable of forming nodules with rhizobia native to the soil of Amazonia (Souza \textit{et al.}, 1994; Baraúna, 2013) and bacteria belonging to the genera \textit{Bradyrhizobium} and \textit{Rhizobium} have been reported as symbionts of \textit{Centrolobium} spp. However, previous studies have not identified the bacteria at species level (Moreira \textit{et al.}, 1998; Pagano, 1995, Baraúna \textit{et al.}, 2013).
A recent investigation of the ecology of root-nodulating-bacteria isolated from *C. paraense* grown in soils collected from different areas in Roraima State-Brazil found that about 90% of the 178 isolates exhibit phenotypic characteristics similar to species of *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 *C. paraense* (Baraúna *et al.*, 2014).

Here we report the results from a polyphasic taxonomic study of seven isolates (BR 10247T, BR 10296, BR 10297, BR 10298, BR 10299, BR 10300 and BR 10301). This polyphasic study included gene sequence analysis (16S rRNA, ITS, *dnaK*, *glnII*, *gyrB*, *recA*, *rpoB*, *nodC* and *nifH*), DNA-DNA relatedness, fatty acid profiling and phenotypic characterization. The strains were obtained from *C. paraense* grown in soil samples collected in the Mucajáí municipality-State of Roraima (2º 27 12.9 N; 60º 54 11.2 W) (Baraúna *et al.*, 2014). The climate in this region is classified as Aw (Köppen) with average rainfall of 1600 mm year\(^{-1}\) and an average temperature of 27°C (Araújo, *et al.*, 2001). The strains were deposited in the Diazothrophic Microbial Culture Collection -CRB-Johanna Döbereiner- (Embrapa Agrobiologia, Rio de Janeiro, Brazil); strain BR 10247T, was also deposited at the Hambi Collection (http://www.helsinki.fi/hambi) as HAMBI 3599T. All strains were cultured on YMA 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 (1336bp) were obtained for all strains using the primers and conditions described previously (Radl *et al.*, 2013). The intergenic transcribed spacer (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 MEGA5 software package
(Tamura et al., 2011). Phylogenetic trees were constructed using the Maximum-
Likelihood (ML) (Felsenstein, 1981) reconstruction 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 (Fig. 1 and Fig. 2).

The 16S rRNA 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 *B. jicamae* PAC68<sup>T</sup> as the closest neighbour (Fig. 1). The
16S rRNA gene sequence similarity between strain BR 10247<sup>T</sup> and other
*Bradyrhizobium* type strains was between 96.0-98.8% (Table S1, available in IJSEM
online). Thus, even though the 16S rRNA is highly conserved in *Bradyrhizobium*
(Menna et al., 2009, Willems et al., 2001b), the analysis showed that our strains form a
separate branch compared to other species.

ITS phylogenetic analysis (891bp) showed less than 86.6% similarity between
our strains and other recognized *Bradyrhizobium* type strains, and the similarity
between our strains was greater than 98% (Table S1, available in IJSEM Online). The
ITS phylogenetic reconstruction placed the new strains in a different branch with
*B. iriomotense* EK05<sup>T</sup> as the closest neighbor (Fig. 2). Previous studies have
demonstrated that ITS sequences are a suitable marker to separate *Bradyrhizobium*
species and 95.5% similarity value or more indicates strains belonging to the same
genospecies, corresponding to about 60% DNA: DNA hybridization (Willems et al.,
2001a, Willems et al., 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 (Rivas et al., 2009; Menna et al., 2009). We obtained
sequences and performed the analyses for dnaK (238bp), glnII (534bp), gyrB (591bp),
recA (418bp) and rpoB (408bp) genes following previous reports (Martens et al., 2008;
Rivas et al., 2009; Menna et al., 2009; Vinuesa et al., 2005). Congruence between the
different gene sequences was firstly checked using the partition homogeneity tests
(Farris, et al., 1994) performed with PAUP software v. 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 v. 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 our strains belonged to a monophyletic cluster with high bootstrap support
(100%) (Fig. 3). Similar relationships were also obtained for the genes dnaK and glnII
when individually analysed (Fig. S1 and S2, available in IJSEM Online). In addition,
the sequence similarities between our strains were more than 99% for all investigated
genes (Table S1, available in IJSEM Online). The closest type strain in the 16S rRNA
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. iriomotense
EK05$^T$, had a similarity between 92.6-95.7% for all five investigated genes in
comparison with strain BR 10247$^T$.

It is interesting to emphasize that our strains presented a discordance in the 16S
rRNA phylogenetic compared to ITS, which was also confirmed by MLSA analysis.
While in the 16S rRNA gene analysis the closest strain was B. jicamae, closely related
to B. elkanii (subgroup I), the ITS and concatenated MLSA tree placed our strains
together with *B. iriomotense*, belonging to subgroup II (Fig. 1, Fig 2 and Fig. 3). The
*Bradyrhizobium* subgroup division (I and II) is based on DNA: DNA hybridisation and
was used to separate the *B. japonicum* type strain from the *B. elkanii* type strain (Hollis
*et al.*, 1981; Willems *et al.*, 2001). The discordance found for our strains may indicate
either a lateral gene transfer or a gene recombination event, leading to a reticulate
evolutionary history (van Berkum *et al.*, 2003; van Berkum *et al.*, 2009; Parker *et al.*, 2003; Parker *et al.*, 2008).

BLAST search (Altschul *et al.*, 1990) revealed, in general, low similarity of the
sequences of our strains with deposited sequences. However, high similarity was
obtained with gene sequences from strain TUXTLAS-14 (16S rRNA, ITS, *dnaK*, *recA*
and *glnII*), isolated from *Vigna unguiculata* in Mexico (Ormeño-Orrillo *et al.*, 2012)
and strain Cp5-3 (16S rRNA, *recA*), isolated from *Centrosema pubescens*, in Panama
(Parker, 2003), and both strains TUXTLAS-14 and Cp5-3 have a phylogenetic pattern
similar to our strains with a discordance between 16S rRNA and housekeeping genes
(Ormeño-Orrillo *et al.*, 2012). This may indicate that strains belonging to this novel
*Bradyrhizobium* species are distributed from Northern Brazil to Panama or Mexico.

For phenotypic characterization, the strains were Gram stained and were
incubated for 7 days on YMA at different temperatures (15, 20, 25, 28, 30, 32, and
37°C), pH values (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 of the strains grown in YM medium, and cell
morphology by transmission and scanning electron microscopy. 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) H$_2$O$_2$ and checking for the presence of bubbles.

Other biochemical tests were performed by inoculating API 20NE strips (BioMérieux, France) and Biolog GN2 microplates (Biolog Inc, CA, USA) according to the manufacturer’s instructions followed by incubation for 8 days at 28°C. The antibiotic susceptibility tests were performed on YMA using the antibiotic Sensi-disc dispenser system (Oxoid) with bio-discs (Oxoid) containing ampicillin (10 μg and 25 μg), chloramphenicol (30 μg and 50 μg), erythromycin (30 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (10 μg), penicillin (10 μg), streptomycin (10 μg and 25 μg) and tetracycline (30 μg). The plates were incubated at 28°C and read after 10 days.

Discriminating phenotypic characteristics of our strains are given in Table 1 and the details of carbon source utilization are presented in the Supplementary Table S3, available in IJSEM Online. Our strains grew between 15 and 37 °C and in pH range 4.0 to 10.0, common characteristics within the *Bradyrhizobium* genus. The optimum growth was verified at 28-32°C and pH 5-7 (Table 1). All strains were resistant to chloramphenicol (50 μg) and sensitive to ampicillin (10 μg), penicillin (10 μg), streptomycin (10 μg and 25 μg) and tetracycline (30 μg), while the closest type strain *B. iriomotense* EK05$^T$ showed penicillin and streptomycin resistance (Table 1). Enzymatic reactions were positive for catalase, oxidase, urease, arginine dihydrolase and hydrolysis of esculin, and negative for nitrate reduction, tryptophan deaminase, glucose fermentation, hydrolysis of gelatine and β-galactosidase. The *Centrolobium* strains differed from LMG 24129$^T$ 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 YMA prior to extraction. The profiles were generated using a chromatograph Agilent model 6850 and identified using the TSBA database version 6.10 (Microbial Identification System - MIDI Inc.). The most abundant cellular fatty acids detected were C\textsubscript{16:0} (13.56%) and Summed Feature (SF) 8 (C\textsubscript{18:1} w7c) (66.52%). Moderate amounts of C\textsubscript{18:1} w7c 11-methyl (11.77%) and C\textsubscript{19:0 cyclo w8c} (7.14%) and C\textsubscript{18:0} (1.04%) were also found. The presence of C\textsubscript{16:0} and SF 8 supports the placement of these strain in the genus *Bradyrhizobium* (Tighe et al., 2000) and revealed some differences between BR 10247T and *B. iriomotense* EK05T, especially the higher abundance of C\textsubscript{16:0} (14.7%) and lower C\textsubscript{18:1} w7c (80.1%) as was first presented by Islam et al. (2008).

For DNA-DNA hybridization and determination of DNA G+C content, high-molecular weight 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°C ± 1°C. Reciprocal reactions (A x B and B x A) were performed in triplicate for each DNA pair and their variation was within the limits of this method (Goris et al., 1998). The DNA-DNA relatedness between BR 10247T and the closest type strain *B. iriomotense* EK05T was 63.8%, confirming that our strains belong to a new species, since the threshold recommended is 70% (Lindström & Gyllenberg, 2007; Tindall et al., 2010). 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 thermostabilised at 37°C. The solvent was 0.02M NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} (pH 4.0) with 1.5% (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *E. coli* DNA were used as
calibration reference and control, respectively. The DNA G+C content of strain BR 10247^T, was 63.9 mol% (Table 1), differentiating it from the closest type strain LMG 24129^T for which the G+C mol% was 61.2 (Islam et al., 2008).

Nodulation and nitrogen fixation genes are required for effective legume symbiosis, therefore nodC and nifH genes were analysed according to Sarita et al., (2001) and Ueda et al., (1995), respectively. Phylogenetic trees were constructed as described previously and the results are given in Figs. S3 and S4 (available in IJSEM Online) for nodC and nifH, respectively. NodC analysis placed strain BR 10247^T in a separate branch in relation to other Bradyrhizobium species and it was grouped outside the four recognized symbiovars (Cobo-Díaz et al., 2014) (similarity less than 80%), possibly indicating a new symbiovar within this genus (Fig. S3, Table S1). NifH gene sequence analysis clustered strain BR 10247^T in the same branch as B. iriomotense EK05^T (closest strain in the previous analyses), but the similarity observed was lower than 90% between both strains (Fig. S4, Table 1), 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 C. paraense and this was performed using Leonard jars containing N-free nutrient solution according to Radl et al. (2013). Secondly, host plant tests with strain BR 10247^T were performed 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 with H_2O_2 (5%; v/v); 5 min) and inoculated with 1 mL of YM broth suspension containing approximately 10^9 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 70 days and 35 days after inoculation in the first and the second experiment, respectively. The seven strains nodulated *C. paraense* (Table S4, available in IJSEM Online). Strain BR 10247T formed effective nitrogen fixing nodules on roots of *Arachis hypogaea, Acacia ligulata, Cajanus cajan, Crotalaria juncea, Macroptillium atropurpureum, Vigna unguiculata, V. angularis, V. radiata*, and ineffective (non-fixing) root nodules on *Ornithopus compressus* and *Phaseolus vulgaris*. No nodulation was observed for *Glycine max, Lupinus angustifolius, Pisum sativum* and *Vicia faba*.

The genotypic and phenotypic data presented in this study demonstrate that the strains isolated from *Centrolobium paraense* root nodules collected in the Amazonia represent a novel species, for which the name *Bradyrhizobium neotropicale* sp. nov. is proposed, with BR 10247T (= HAMBI 3599T) as the type strain.

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


Cells are motile with polar flagella, Gram-negative rods (approximately 2.4 x 0.6 µm), aerobic, non-spore-forming (Supplementary Fig. S5). Colonies on YMA 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 YMA is 4.0–10.0, with optimum growth at pH 5.0-7.0. Growth occurs between 15°C and 37°C, with optimum growth at 28-30°C. Does not grow in the presence of 1.5% (w/v) NaCl or higher. Positive reactions for carbon source utilization were 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-glucuronic acid, D-glucosaminic acid, $\alpha$-hydroxybutyric acid, $\beta$-hydroxybutyric acid, $\gamma$-hydroxybutyric acid, p-hydroxyphenylacetic acid, $\alpha$-ketobutyric acid, $\alpha$-ketoglutaric acid, D.L-lactic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, glycerol, methyl pyruvate, mono-methyl-succinnate, D-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-leucine, L-phenylalanine, urocanic acid, succinamic acid, glucuronamide and bromo succinic acid. Oxidase, catalase, arginine dihydrolase, hydrolysis of esculin and urease were also positive, while nitrate reduction, tryptophan deaminase, glucose fermentation, $\beta$-galactosidase and hydrolysis of gelatin were negative. The most abundant cellular fatty acids are C$_{16:0}$ and summed feature 8 (C$_{18:1}$ w7c). The DNA G+C content of the type strain BR 10247$^T$ is 63.9 mol%. The type strain BR 10247$^T$ (=HAMBI 3599$^T$) was isolated from nodules of *Centrolobium paraense* grown in soils of Amazonia, Roraima State-Brazil.

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nov., a root nodule symbiotic bacterium isolated from cowpea grown in the semi-arid of


rhizobial nodC sequences from soil total DNA and comparison to nodC diversity of root


MEGA5: Molecular evolutionary genetics analysis using maximum likelihood,

Tighe, S. W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G. & Jarvis, B. D.
Agrobacterium, Bradyrhizobium, Mesorhizobium, Rhizobium and Sinorhizobium
50, 787-801.


Table 1. Different features of *Bradyrhizobium neotropicae* sp. nov. strains and closest related strain *Bradyrhizobium iriomotense* EK05<sup>T</sup>.

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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Streptomycin (10)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>pH growth range</strong></td>
<td>4-10</td>
<td>4-10</td>
<td>4-10</td>
<td>4-10</td>
<td>4-10</td>
<td>4-10</td>
<td>4-10</td>
<td>4-10&lt;sup&gt;(2)&lt;/sup&gt;</td>
</tr>
<tr>
<td>Generation Time (h)</td>
<td>10.8</td>
<td>ND</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>Nd</td>
<td>7.9&lt;sup&gt;(2)&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaCl tolerance (%)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>&lt;1.0&lt;sup&gt;(2)&lt;/sup&gt;</td>
</tr>
<tr>
<td>DNA G+C content (% mol)</td>
<td>63.9</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>61.2&lt;sup&gt;(2)&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

(1) The strain LMG 24129<sup>1</sup> (formal deposit of the strain EK05<sup>T</sup>) were obtained from the LMG culture collection.
(2) Data from (Islam *et al.*, 2008); ND = not determined
**Fig. 1** - Maximum likelihood phylogeny based on 16S rRNA gene sequences showing the relationships between *Bradyrhizobium neotropicale* strains (shown in bold) and other members of the *Bradyrhizobium* genus. The significance of each branch is indicated by a bootstrap value (greater than 50% showed) calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions. Sequence accession numbers of the 16S rRNA genes are presented in parenthesis.

**Fig. 2.** Maximum likelihood phylogeny based on intergenic transcribed spacer (ITS) sequences showing the relationships between strains from the novel species (shown in bold) and other members of the *Bradyrhizobium* genus. The significance of each branch is indicated by a bootstrap value (greater than 50% showed) calculated for 1000 subsets. Bar, 5 substitutions per 100 nucleotide positions. Sequence accession numbers of the ITS are presented in parenthesis.

**Fig. 3.** Maximum likelihood phylogeny based on concatenated *gyrB*, *recA* and *rpoB* gene sequences showing the relationships between strains from the novel species (shown in bold) and other members of the *Bradyrhizobium* genus. The significance of each branch is indicated by a bootstrap value (greater than 50% showed) calculated for 1000 subsets. Bar, 2 substitutions per 100 nucleotide positions.