MURDOCH RESEARCH REPOSITORY

This is the author's final version of the work, as accepted for publication following peer review but without the publisher's layout or pagination.
The definitive version is available at
http://dx.doi.org/10.1099/ijs.0.063727-0

da Silva, K., De Meyer, S.E., Rouws, L.F.M., Farias, E.N.C., dos Santos, M.A.O., O'Hara, G., Ardley, J.K., Willems, A., Pitard, R.M. and Zilli, J.E. (2014) Bradyrhizobium ingae sp. nov., isolated from effective nodules of Inga laurina grown in Cerrado soil. International Journal of Systematic and Evolutionary Microbiology, 64 (Pt 10). pp. 3395-3401.

http://researchrepository.murdoch.edu.au/24978/

Copyright: © Society for General Microbiology

It is posted here for your personal use. No further distribution is permitted.

IJSEM Papers in Press. Published July 10, 2014 as doi:10.1099/ijs.0.063727-0

1 **Title**

2 *Bradyrhizobium ingae* sp. nov., isolated from effective nodules of *Inga laurina* grown
3 in Cerrado soil of Amazonia, Brazil.

4

5 **Short title**

6 *Bradyrhizobium ingae* sp. nov.

7 **Contents category**

8 New taxa

9 **Subsection**

10 Proteobacteria

11

12 Krisle da Silva[1], Sofie E. De Meyer[2,4], Luc F. M. Rouws[5], Eliane N. C. Farias[1] Marco
13 A. O. dos Santos[3], Graham O'Hara[2], Julie K. Ardley[2], Anne Willems[4], Rosa Maria
14 Pitard[5] and Jerri E. Zilli[5].

15

16 [1]Embrapa Roraima, Rodovia BR 174 km 08, Boa Vista, Roraima 69301-970, Brazil.
17 [2]Centre for Rhizobium Studies, Murdoch University, 90 South Street, Murdoch 6150,
18 Western Australia, Australia.
19 [3]Universidade Estadual de Roraima, Rua Sete de Setembro, 231, Canarinho, Boa Vista -
20 RR, 69306-530, Brazil.
21 [4]Laboratory of Microbiology, Department of Biochemistry and Microbiology (WE10),
22 Ghent University, K. L. Ledeganckstraat 35, B-9000 Ghent, Belgium.
23 [5]Embrapa Agrobiologia, Rodovia BR 465 km 07, Seropédica, Rio de Janeiro 23891-
24 000, Brazil.

25

26 ***Corresponding author**

27 e-mail: jerri.zilli@embrapa.br

28 Phone: ++ 55 21 3441-1611

29 Fax: ++ 55 21 2682-1230

30

31 The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *dnaK*, *glnII*, *gyrB*
32 *recA*, *rpoB*, *nodC* and *nifH* gene sequences of *Bradyrhizobium ingae* sp. nov. BR
33 10250^T are KF927043, KF927055, KF927067, KF927079, KF927061, KF927073,
34 KF927054 and KF927085, respectively. The accession numbers for all other strains are
35 listed in Table S2.

36

37 **Abstract**

38 Root nodule bacteria were isolated from *Inga laurina* (Sw.) Willd. growing in the
39 Cerrado Amazon region, State of Roraima (Brazil). The 16S rRNA gene sequences of
40 six strains (BR 10250^T, BR 10248, BR 10249, BR 10251, BR 10252 and BR 10253)
41 isolated from the nodules showed low similarities with currently described
42 *Bradyrhizobium* species. Phylogenetic analyses of five housekeeping genes (*dnaK*,
43 *glnII*, *gyrB*, *recA* and *rpoB*) revealed *Bradyrhizobium iriomotense* strain $EK05^T$ (=LMG
44 24129^T) to be the closest type strain (97.4% sequence similarity or less).
45 Chemotaxonomic data, including fatty acid profiles (with majority being $C_{16:0}$ and
46 Summed feature 8), the slow growth rate and carbon compound utilization patterns
47 supported the assignment of our strains to the genus *Bradyrhizobium.* Results from
48 DNA-DNA hybridisations and physiological traits differentiated our strains from the
49 closest validly named *Bradyrhizobium* species. Symbiosis-related genes for nodulation
50 (*nodC*) and nitrogen fixation (*nifH*) grouped together with *B. iriomotense* strain $EK05^T$

51 and *Bradyrhizobium* strain SEMIA 6434 (used as commercial inoculant for *I. marginata*
52 in Brazil) and TUXTLAS-10 (previously observed in Central America). Based on the
53 data, these six strains represent a novel species for which the name *Bradyrhizobium*
54 *ingae* sp. nov. (BR 10250^T = HAMBI 3600^T), is proposed.
55

56 *Inga* Mill. (Leguminosae, Mimosoideae), tribe *Ingeae* is considered an exclusive
57 neotropical genus containing around 300 species, some native to the Amazon region.
58 However, several species are also found in Mexico, Antilles and other South American
59 countries (Possette & Rodrigues, 2010; Pennington, 1997).

60 The pods of this genus contain seeds covered by a white sweet pulp that is rich
61 in minerals and is used for animal as food (Possette & Rodrigues, 2010; Pennington,
62 1997). In addition, some *Inga* species are used in agriculture for nitrogen input
63 especially in alley-cropping or agroforestry systems, and also for land reclamation
64 because the plants tolerate poorly drained, acid soils and other major growth constraints
65 (Franco & de Faria, 1997, Romero-Alvarado *et al.*, 2002; (Kurppa *et al.*, 2010).

66 In general, *Inga* spp. are recognized as efficient nitrogen fixers in association
67 with root nodule bacteria, and several countries have selected efficient inoculant strains
68 for certain species in this genus (Franco & de Faria, 1997; Kurppa *et al.*, 2010).
69 However, very little is known about the diversity of root nodule bacteria associated with
70 this genus.

71 Previous authors have suggested that bacteria which nodulate *Inga* spp. Are part
72 of the "cowpea miscellany" group of root nodule bacteria, because the rhizobial strains
73 isolated from nodules also nodulate and fix nitrogen efficiently with other legumes
74 including species of *Cajanus*, *Acacia*, *Erythrina* and *Vigna* (Allen & Allen, 1939;
75 Grossman *et al.*, 2005). Additionally, it has been reported that slow-growing strains,

76 including *Bradyrhizobium* are characteristic root nodule bacteria for *Inga* spp. as for
77 other tropical legumes (Grossman *et al.*, 2005).

78 During a field study in 2008, 30 root nodules were collected from *Inga laurina*
79 (Sw.) Willd. growing in natural conditions in two sites in the Cerrado (locally known as
80 Lavrado, State of Roraima, Brazil), including Monte Cristo Experimental Field of
81 Embrapa Roraima and a site located in the Boa Vista city (2°50'21''N, 60°
82 40'32,25''W; 2°57'00''**N**, 60°42'25''W, respectively). The climate in this region is
83 classified as Aw (Köppen) with average rainfall of 1,600 mm year^{-1} and an average
84 temperature of 27°C (Araújo, *et al.*, 2001). *I. laurina* is a common species naturally
85 occurring in the Cerrado and other ecosystems in Brazil (Condé & Tonini, 2013; Filardi
86 *et al.*, 2008).

87 To collect the nodules, adult *I. laurina* plants were located and young seedlings
88 of *I. laurina* growing under these trees were manually uprooted. Nodules presented
89 were collected from intact roots and transported to the laboratory. Later, the nodules
90 were superficially disinfected (Zilli *et al.*, 2004) and individually crushed and the
91 exudate streaked onto the YMA medium (Fred & Waksman, 1928). Following
92 purification from single colonies, 17 isolates were obtained. All strains presented typical
93 *Bradyrhizobium* characteristics: white colonies, alkaline reaction in medium and slow-
94 growth. Partial 16S rRNA sequencing confirmed this observation.

95 For the present study, six representative strains (BR 10250^{T}, BR 10248, BR
96 10249, BR 10251, BR 10252 and BR 10253) were selected and subjected to a more
97 detailed polyphasic taxonomic study, including gene sequence analysis (16S rRNA,
98 *glnII*, *gyrB*, *recA*, *rpoB*, *dnaK*, *nodC* and *nifH*), as well as DNA-DNA relatedness, fatty
99 acid profiles and phenotypic characterization. The strains were deposited in the
100 Diazothrophic Microbial Culture Collection -CRB-Johanna Döbereiner- (Embrapa

101 Agrobiologia, Rio de Janeiro, Brazil); strain BR 10250^T, was also deposited at the
102 Hambi Collection (http://www.helsink.fi/hambi) as HAMBI 3600^T. All strains were
103 cultured on YMA medium at 28°C and for long-term storage the cultures were
104 lyophilized and kept at -80°C.

105 For PCR, genomic DNA was prepared using the RBC Bioscience kit
106 (cat.YGB300) and the BOX PCR analysis was performed as described previously
107 (Versalovic *et al.*, 1994). Fingerprint analysis was performed with the BioNumerics
108 7.01 software package (Applied Maths, Sint-Martens Latem, Belgium) using the
109 UPGMA algorithm and Pearson correlation index. The cluster analysis showed that the
110 six strains grouped together with 75% similarity level in three sub-groups, indicating
111 that they represent genetically distinct strains (Fig. S1, available in IJSEM Online).

112 Nearly full length sequences of the 16S rRNA gene (1318bp) were obtained for
113 all strains using the primers and conditions described previously (Radl *et al.*, 2013).
114 Sequence alignment, alignment editing and phylogenetic analyses were performed using
115 the MEGA5 software package (Tamura *et al.*, 2011). Phylogenetic trees were
116 constructed using the Neighbor-joining (NJ) (Saitou & Nei, 1987) and Maximum
117 Likelihood (ML) (Felsenstein, 1981) reconstructions. The strength of each topology was
118 verified using 1000 bootstrap replications. The overall topologies of the phylogenetic
119 trees obtained with the NJ and ML methods were very similar (data not shown) and the
120 ML tree is provided (Fig 1).

121 The six strains formed a separate branch within the genus *Bradyrhizobium*
122 together with *B. iriomotense* EK05^T isolated from *Entada koshunensis* (Leguminosae,
123 Mimosoideae) in Japan (Islam e*t al.*, 2008) (Fig. 1). They shared 100% sequence
124 similarity with each other, and 98% with other *Bradyrhizobium* type strains. We, also
125 observed that our strains clustered together with SEMIA 6434 (BR 6610) used as a

126 commercial inoculant for *Inga marginata* in Brazil (Franco & de Faria, 1997; Menna *et*
127 *al.*, 2006) and the strain TUXTLAS-10 isolated in Mexico, which are referred to be part
128 of the “BCI Bradyrhizobium lineage” common in Central America (Parker, 2003;
129 Ormeño-Orrillo *et al.*, 2012).

130 Although high similarity percentages were observed for 16S rRNA, previous
131 reports have suggested that closely related *Bradyrhizobium* species do not necessarily
132 belong to the same species (Menna *et al.*, 2009, Willems *et al.*, 2001). Therefore, Multi
133 Locus Sequence Analysis (MLSA) was performed for *dnaK* (238bp), *glnII* (537bp),
134 *gyrB* (592bp), *recA* (423bp) and *rpoB* (525bp) genes following previous reports
135 (Martens *et al.*, 2008; Menna *et al.*, 2009; Vinuesa *et al.*, 2005). Before concatenating
136 the sequences for the genes *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*, the congruence existence
137 (tree topology) and partition homogeneity tests were evaluated (Farris, *et al.*, 1994). The
138 phylogenetic tree based on the concatenated sequences of the five housekeeping genes
139 (Fig. 2) revealed that our strains belonged to a monophyletic cluster with high bootstrap
140 support (100%). Sequence similarities among our strains were 99% or 100% for all
141 investigated genes (Table S1, available in IJSEM Online). The closest type strain in the
142 16S rRNA analysis, *B. iriomotense* EK05^T, showed 97.4% or less sequence similarity
143 with strain BR 10250^T for all investigated genes (Fig. 2; Table S1; Supplementary Fig.
144 S2, Fig. S3 and Fig. S4, available in IJSEM Online). These figures also showed that our
145 strains belonged to a different group than the commercial strain SEMIA 6434 and
146 TUXTLAS-10, even though they are closely related to *B. iriomotense* EK05^T.

147 For phenotypic characterization, the strains were Gram stained and were grown
148 for 7 days on YMA at different temperatures (15, 20, 25, 28, 30, 32, and 37°C), pH
149 values (4, 5, 6, 7, 8, 9, 10 and 11) and NaCl concentrations (0.1, 0.3, 0.5, 1.0, 1.5, 2.0
150 and 2.5%). Cell motility was observed by light microscopy of a wet preparation and cell

151 morphology by transmission and scanning electron microscopy. Oxidase activity was
152 detected by immersion of cells in 1% N,N,N′,N′-tetramethyl-p-phenylenediamine
153 solution and catalase activity was determined by flooding a colony with 10% (v/v) H_2O_2
154 and checking for the presence of bubbles. Other biochemical tests were performed by
155 inoculating API 20NE strips (BioMérieux, France) and Biolog GN2 microplates (Biolog
156 Inc, CA, USA) according to the manufacturer's instructions and incubating for 8 days at
157 28°C. The antibiotic susceptibility tests were performed on YMA using the antibiotic
158 Sensi-disc dispenser system (Oxoid) with bio-discs (Oxoid) containing ampicillin (10
159 µg and 25 µg), chloramphenicol (30 µg and 50 µg), erythromycin (30 µg), gentamicin
160 (10 µg), kanamycin (30 µg), neomycin (10 µg), penicillin (10 µg), streptomycin (10 µg
161 and 25 µg) and tetracycline (30 µg). The plates were incubated at 28°C and read after
162 10 days.

163 Discriminating phenotypic characteristics of our strains are given in Table 1 and
164 the details of carbon source utilization are presented in the Supplementary Table S3,
165 available in IJSEM Online. Our strains were able to grow between 15 and 32 °C and at a
166 pH between 4 to 8, which are common characteristics for the genus *Bradyrhizobium*.
167 The optimum growth was verified at 28-30°C and pH 5-7 (Table 1). All strains were
168 resistant to erythromycin, gentamicin and neomycin and sensitive to ampicillin,
169 chloramphenicol, kanamycin, streptomycin and tetracycline. Additionally, the closest
170 type strain EK05^T showed chloramphenicol and streptomycin resistance. Enzymatic
171 reactions were positive for catalase, oxidase, urease and hydrolysis of esculin, and
172 negative for nitrate reduction, tryptophan deaminase, glucose fermentation, arginine
173 dihydrolase, hydrolysis of gelatine and β-galactosidase. The *Inga* strains differed also
174 from EK05^T in the β-galactosidase and urease reaction (Table 1).

175 Whole-cell fatty acid methyl esters of strain BR 10250^T were extracted
176 according to the MIDI protocol (http://www.microbialid.com/PDF/TechNote_101.pdf,
177 (Delamuta *et al.*, 2013). Cultures were grown for 5 days at 28°C on YMA prior to
178 extraction. The profiles were generated using a chromatograph Agilent model 6850 and
179 identified using the TSBA database version 6.10 (Microbial Identification System -
180 MIDI Inc.). The most abundant cellular fatty acids detected were $C_{16:0}$ (17.51%) and
181 Summed Feature (SF) 8 ($C_{18:1}$ w7c) (70.78%). Moderate amounts of $C_{18:1}$ w7c 11-
182 methyl (10.8%) and $C_{19:0}$ cyclo w8c (11.71%) were also found. The presence of $C_{16:0}$
183 and SF 8 supports the placement of these strains in the genus *Bradyrhizobium* (Tighe *et*
184 *al.*, 2000) and revealed some differences between BR 10250^T and *B. iriomotense*
185 $EK05^T$, especially the lower abundance of $C_{16:0}$ (14.7%) and higher levels of $C_{18:1}$ w7c
186 (70.78%) (Islam *et al.*, 2008).

187 For DNA-DNA hybridization and for the determination of the DNA G+C
188 content, high-molecular weight DNA was prepared as described by Pitcher *et al.* (1989).
189 DNA-DNA hybridizations were performed using a microplate method and biotinylated
190 probe DNA (Ezaki *et al.,* 1989). The hybridization temperature was 50°C ± 1°C.
191 Reciprocal reactions (A x B and B x A) were performed for each DNA pair and their
192 variation was within the limits of this method (Goris et *al.,* 1998). The DNA-DNA
193 relatedness between BR 10250^T and the closest type strain $EK05^T$ was 65.7%,
194 confirming that our strains belong to a new species, since the threshold recommended is
195 70% (Lindström & Gyllenberg, 2007). The G+C content of DNA was determined by
196 HPLC according to the method of Mesbah *et al.* (1989) using a Waters Breeze HPLC
197 system and XBridge Shield RP18 column thermostabilised at 37°C. The solvent was
198 0.02M $NH_4H_2PO_4$ (pH 4.0) with 1.5% (v/v) acetonitrile. Non-methylated lambda phage
199 (Sigma) and *E. coli* DNA were used as calibration reference and control, respectively.

200 The DNA G+C content of strain BR 10250^T, was 63.4 mol% (Table 1), differentiating it
201 from the closest type strain EK05^T for which the G+C mol% was 61.2 (Islam *et al*.,
202 2008).

203 Nodulation and nitrogen fixation genes are required for effective legume
204 symbiosis, therefore *nodC* and *nifH* genes were analysed according to Laguerre *et al*.,
205 (2001) and Ueda *et al*., (1995), respectively. Phylogenetic trees were constructed as
206 described previously and the results are given in Figs. S5 and S6 (available in IJSEM
207 Online) for *nodC* and *nifH*, respectively. Both, *nodC* and n*ifH* gene sequences analyses
208 clustered strain BR 10250^T in the same branch as *B. iriomotense* EK05^T, but with low
209 similarity (Table S1, available in IJSEM Online). The maximum identity observed for BR
210 10250^T *nodC* sequence by BLAST search (Altschul *et al*., 1990) was 92% with a strain
211 isolated from *Ormosia fastigiata* (Leguminosae, Papilionoideae; accession n° KF031520).
212 The BLAST and phylogenetic analysis of *nifH* gene revealed 98% sequence similarity with
213 strain SEMIA 6434 isolated in Brazil (Fig S5, available in IJSEM Online).

214 To confirm the nodulation ability of the strains investigated in this study, two
215 glasshouse experiments were performed. In the first trial the six strains were tested on
216 *Inga edulis*, because no viable seeds of *I. laurina*, their original host, could be found.
217 These experiments were performed in Leonard jars containing N-free nutrient solution
218 according to Radl *et al.* (2013). Thereafter, host plant tests were performed with strain
219 BR 10250^T on 14 different legume species using the axenic sand-culture system
220 described previously (Howieson *et al*., 2013). For both experiments the seeds were
221 surface sterilized and inoculated with 1 mL of YM broth suspension containing 10^9
222 bacterial cells grown for 5 days at 28°C. All treatments, plus an uninoculated control,
223 were replicated four times in a split-plot design (Howieson et al., 2013). Nodulation was
224 evaluated 60 days and 35 days after inoculation in the first and the second experiment,

225 respectively. Results showed that the six strains were able to nodulate *I. edulis* (Table
226 S4, available in IJSEM Online). Strain BR 10250^T also effectively nodulated *Arachis*
227 *hypogaea*, *Macroptillium atropurpureum, Vigna radiata* and *V. unguiculata*, and
228 formed ineffective root nodules on *Glycine max*. No nodulation was observed for
229 *Acacia ligulata*, *Cajanus cajan*, *Crotalaria juncea*, *Lupinus angustifolius*, *Ornithopus*
230 *compressus*, *Phaseolus vulgaris*, *Pisum sativum, Vicia faba* and *Vigna angularis*.

231 The genotypic and phenotypic data presented in this study demonstrate that the
232 strains isolated from *Inga laurina* root nodules collected in the Cerrado of the Amazonia
233 region represent a novel species, for which the name *Bradyrhizobium ingae* sp. nov. is
234 proposed, with BR 10250^T (=HAMBI 3600^T) as the type strain.

235

236 **Description of the *Bradyrhizobium ingae* sp. nov.**

237 *Bradyrhizobium ingae* [in'gae. N.L. gen. n. ingae, of Inga, referring to the fact that the
238 bacterium was isolated from root nodules of *Inga laurina* (Sw.) Willd].

239

240 The cells are motile with polar flagella, Gram-negative rods (approximately 1.5 x 0.6
241 μm), aerobic, non-spore-forming (Supplementary Fig. S7). Colonies on YMA medium
242 are circular and translucent, and have a diameter of 1 mm within 7–8 days of incubation
243 at 28 °C. The generation time is 9.5 h in YM broth. The pH range for growth in YMA is
244 4–8, with optimum growth at pH 5.0-7.0. Growth occurs between 15°C and 32°C, with
245 optimum growth at 28-30°C. Does not grow in the presence of 0.5% (w/v) NaCl or
246 higher. Resistance to erythromycin (30 μg), gentamicin (10 μg) and neomycin (10 μg),
247 and sensitive to ampicillin (10 μg), chloramphenicol (50 μg), kanamycin (30 μg),
248 streptomycin (10 μg) and tetracycline (30 μg) were observed. Positive reactions were

249 recorded for the utilization of the carbohydrates, D-arabitol, D-fructose, D-galactose, D-
250 mannitol, D-mannose, D-sorbitol, L-arabinose, L-fucose, L-rhamnose, m-inositol, N-
251 acetyl-D-glucosamine, xylitol and α-D-Glucose. Oxidase, catalase and urease were also
252 positive, while nitrate reduction and β-galactosidase are negative. The most dominant
253 cellular fatty acids were $C_{16:0}$ and summed feature 8 ($C_{18:1}$ w7c). DNA G+C content of
254 the strain BR 10250^T is 63.4 mol%. The type strain BR 10250^T (=HAMBI 3600^T) was
255 isolated from *Inga laurina* nodules collected in a Cerrado area of Amazon, from
256 Roraima State-Brazil.

257

258 **Acknowledgements**

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

264

265 **References**

266 **Allen, O. N. & Allen, E. K. (1939).** Root Nodule Bacteria of Some Tropical
267 Leguminous Plants: II. Cross-Inoculation Tests Within the Cowpea Group. *Soil Science*
268 **47**, 63-76.

269 **Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990).** Basic
270 Local Alignment Search Tool. *J Mol Biol* **215**, 403-410.

271 **Araújo, W. F., Andrade Júnior, A. S. d., Medeiros, R. D. d. & Sampaio, R. A.**
272 **(2001).** Precipitação pluviométrica mensal provável em Boa Vista, Estado de Roraima,
273 Brasil. *Rev Bras Eng Agric Ambient* **5**, 563-567.

274 **Condé, T. M. & Tonini, H. (2013).** Fitossociologia de uma Floresta Ombrófila Densa
275 na Amazônia Setentrional, Roraima, Brasil. *Acta Amazonica* **43**, 247-259.

276 **Delamuta, J. R. M., Ribeiro, R. A., Ormeño-Orrillo, E., Melo, I. S., Martínez-**
277 **Romero, E. & Hungria, M. (2013).** Polyphasic evidence supporting the reclassification
278 of *Bradyrhizobium japonicum* Group Ia strains as *Bradyrhizobium diazoefficiens* sp.
279 nov. *Int J Syst Evol Microbiol*. In press.

280 **Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989).** Fluorometric deoxyribonucleic
281 acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to
282 membrane filter hybridization in which radioisotopes are used to determine genetic
283 relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.

284 **Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. (1994).** Testing significance of
285 incongruence. *Cladistics* **10**, 315-319.

286 **Felsenstein, J. (1981).** Evolutionary trees from DNA sequences: A maximum
287 likelihood approach. *J Mol Evol* **17**, 368-376.

288 **Filardi, F. L. R., da Costa Rodrigues, I. M., Garcia, F. C. P. & de Carvalho-Okano,**
289 **R. M. (2008).** Padrões de distribuição geográfica de espécies arbóreas de Leguminosae
290 ocorrentes no cerrado. *Rev Bras de Biocien* **5**, pg. 1116-1118.

291 **Franco, A. A. & de Faria, S. M. (1997).** The contribution of N2-fixing tree legumes to
292 land reclamation and sustainability in the tropics. *Soil Biol Biochem* **29**, 897-903.

293 **Fred, E. B. & Waksman, S. A. (1928).** *Yeast extract-manitol agar. Laboratory Manual*
294 *of General Microbiology.* New York: McGraw Hill.

295 **Goris, J., Suzuki, K., De Vos, P., Nakase, T. & Kersters, K. (1998).** Evaluation of a
296 microplate DNA - DNA hybridization method compared with the initial renaturation
297 method. *Can J Microbiol* **44**, 1148–1153.

298 **Grossman, J. M., Sheaffer, C., Wyse, D. & Graham, P. H. (2005).** Characterization
299 of slow-growing root nodule bacteria from Inga oerstediana in organic coffee
300 agroecosystems in Chiapas, Mexico. *Appl Soil Ecol* **29**, 236-251.

301 **Howieson, J. G.; De Meyer S. E.; Vivas-Marfisi, A.; Ratnayake S.; Ardley, J. K.;
302 Yates, R. J. (2013).** Novel *Burkholderia* bacteria isolated from *Lebeckia ambigua* - A
303 perennial suffrutescent legume of the fynbos, *Soil Biol Biochem* **60**, 55-64.

304 **Islam, M. S., Kawasaki, H., Muramatsu, Y., Nakagawa, Y. & Seki, T. (2008).**
305 *Bradyrhizobium iriomotense* sp. nov., Isolated from a tumor-Like root of the legume
306 *Entada koshunensis* from Iriomote Island in Japan. *Biosci Biotechnol Biochem* **72**,
307 1416-1429.

308 **Kurppa, M., Leblanc, H. & Nygren, P. (2010).** Detection of nitrogen transfer from
309 N2-fixing shade trees to cacao saplings in 15N labelled soil: ecological and
310 experimental considerations. *Agroforest Syst* **80**, 223-239.

311 **Laguerre, G., Nour, S. M., Macheret, V., Sanjuan, J., Drouin, P. & Amarger, N.
312 (2001).** Classification of rhizobia based on *nodC* and *nifH* gene analysis reveals a close
313 phylogenetic relationship among *Phaseolus vulgaris* symbionts. *Microbiol* **147**, 981-
314 993.

315 **Lindström, K. & H. Gyllenberg (2007).** The species paradigm: proposal for a cross-
316 disciplinary species concept. 11the International Congress on Culture Collections. I.
317 Kurtböke. Goslar, Germany. World Federation of Culture Collections.

318 **Martens, M., Dawyndt, P., Coopman, R., Gillis, M., De Vos, P. & Willems, A.
319 (2008).** Advantages of multilocus sequence analysis for taxonomic studies: a case study

320 using 10 housekeeping genes in the genus *Ensifer*. *Int J Syst Evol Microbiol* **58**, 200–
321 214.

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

326 **Menna, P., Hungria, M., Barcellos, F. G., Bangel, E. V., Hess, P. N. & Martínez-**
327 **Romero, E. (2006).** Molecular phylogeny based on the 16S rRNA gene of elite
328 rhizobial strains used in Brazilian commercial inoculants. *Syst Appl Microbiol* **29**, 315-
329 332.

330 **Mesbah, M., Premachandran, U. & Whitman, W. B. (1989).** Precise measurement of
331 the G+C content of deoxyribonucleic-acid by highperformance liquid-chromatography.
332 *Int J Syst Bacteriol* **39**, 159–167.

333 **Ormeño-Orrillo, E., Rogel-Hernández, M., Lloret, L., López-López, A., Martínez,**
334 **J., Barois, I. & Martínez-Romero, E. (2012).** Change in land use alters the diversity
335 and composition of *Bradyrhizobium* communities and led to the introduction of
336 *Rhizobium etli* into the tropical rain forest of Los Tuxtlas (Mexico). *Microb Ecol* **63**,
337 822-834.

338 **Parker, M. A. (2003).** A widespread neotropical Bradyrhizobium lineage associated
339 with *Machaerium* and *Desmodium* (Papilionoideae). *Plant Soil* **254**, 263–268.

340 **Pennington, T. D. (1997).** *The genus Inga: botany*: Royal Botanic Gardens.

341 **Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989).** Rapid extraction of bacterial
342 genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.

343 **Possette, R. F. d. S. & Rodrigues, W. A. (2010).** O gênero Inga Mill. (Leguminosae -
344 Mimosoideae) no estado do Paraná, Brasil. *Acta Bot Brasil* **24**, 354-368.

345 **Radl, V., Simões-Araújo, J. L., Leite, J., Passos, S. R., Martins, L. M. V., Xavier,**
346 **G. R., Rumjanek, N. G., Baldani, J. I. & Zilli, J. E. (2013).** *Microvirga vignae* sp.
347 nov., a root nodule symbiotic bacterium isolated from cowpea grown in the semi-arid of
348 Brazil. *Int J Syst Evol Microbiol*. In press.

349 **Romero-Alvarado, Y., Soto-Pinto, L., García-Barrios, L. & Barrera-Gaytán, J. F.**
350 **(2002).** Coffee yields and soil nutrients under the shades of Inga sp. vs. multiple species
351 in Chiapas, Mexico. *Agroforest Syst* **54**, 215-224.

352 **Saitou, N. & Nei, M. (1987).** The neighbor-joining method: a new method for
353 reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406-425.

354 **Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011).**
355 MEGA5: Molecular evolutionary genetics analysis using maximum likelihood,
356 evolutionary distance, and maximum parsimony methods. *Mol Biol Evol **28**, 2731-2739.*

357 **Tighe, S. W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G. & Jarvis, B. D.**
358 **W. (2000).** Analysis of cellular fatty acids and phenotypic relationships of
359 *Agrobacterium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and Sinorhizobium
360 species using the Sherlock Microbial Identification System. *Int J Syst Evol Microbiol*
361 **50**, 787-801.

362 **Ueda, T., Suga, Y., Yahiro, N. & Matsuguchi, T. (1995).** Remarkable N_2-fixing
363 bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene
364 sequences. *J Bacteriol* 177, 1414-1417.

365 **Versalovic, J., Schneider, M., De Bruijn, F. & Lupski, J. R. (1994).** Genomic
366 fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction.
367 *Method Mol Cell Biol* **5**, 25-40.

368 **Vinuesa, P., Silva, C., Werner, D. & Martinez-Romero, E. (2005)** Population
369 genetics and phylogenetic inference in bacterial molecular systematics: the roles of

370 migration and recombination in *Bradyrhizobium* species cohesion and delineation. *Mol*
371 *Phylogenet Evol* **34**, 29–54.

372 **Willems, A., Coopman, R. & Gillis, M. (2001).** Phylogenetic and DNA-DNA
373 hybridization analyses of *Bradyrhizobium* species. *Int J Syst Evol Microbiol* **51**, 111-
374 117.

375 **Zilli, J. E., Valisheski, R. R., Freire Filho, F. R., Prata Neves, M. C. & Rumjanek,**
376 **N. G. (2004).** Assessment of cowpea rhizobium diversity in cerrado areas of
377 northeastern Brazil. *Braz J Microbiol* **35**, 281–287.

378 **Table 1.** Different features of *Bradyrhizobium ingae* sp. nov. strains and closest related *Bradyrhizobium iriomotense* strain EK05$^{T(1)}$.

Characteristic	BR 10250^T	BR 10248	BR 10249	BR 10251	BR 10252	BR 10253	EK05^T
C source utilization							
Gentiobiose	-	-	-	-	-	-	+
m-Inositol	+	+	+	+	+	+	-
L-Rhamnose	+	+	+	+	+	+	-
Xylitol	+	+	+	+	+	+	-
Succinic acid	+	+	+	+	+	+	-
p-Hydroxyphenylacetic acid	-	-	-	-	-	-	+
Malonic acid	-	-	-	-	-	-	+
Sebacic acid	-	-	-	-	-	-	+
L-glutamic acid	-	-	-	-	-	-	+
Glycyl-L-aspartic acid	+	+	+	+	+	+	-
L-Threonine	+	+	+	+	+	+	-
D,L-Carnitine	-	-	-	-	-	-	+
Urocanic acid	-	-	-	-	-	-	+
Inosine	+	+	+	+	+	+	-
Uridine	+	+	+	+	+	+	-
Thymidine	+	+	+	+	+	+	-
L-Alaninamide	-	-	-	-	-	-	+
Enzymatic reaction							
β-galactosidase	-	-	-	-	-	-	+
Nitrate reduction	-	-	-	-	-	-	+
Antibiotic resistance							
Chloramphenicol (50 μg)	-	-	-	-	-	-	+
Penicillin (10 μg)	-	+	+	-	-	-	+
Streptomycin (10 μg)	-	-	-	-	-	-	+

Temperature Growth range (°C)	15-32	15-32	15-32	15-32	15-32	15-32	15-32
pH growth range	4-8	4-8	4-8	4-8	4-8	4-8	4,5-9
Generation Time (h)	7.8	Nd	Nd	Nd	Nd	Nd	7-9
NaCl tolerance (%)	0.5	0.5	0.5	0.5	0.5	0.3	1.0[(2)]
DNA G+C content (% mol)	63.4	ND	ND	ND	ND	ND	61.2

379 (1) It was used the strain LMG 24129^T (formal deposit of the strain EK05^T) obtained from the LMG culture collection.

380 (2) Less than 1% (Islam *et al.*, 2008)

381

382

383

384

385

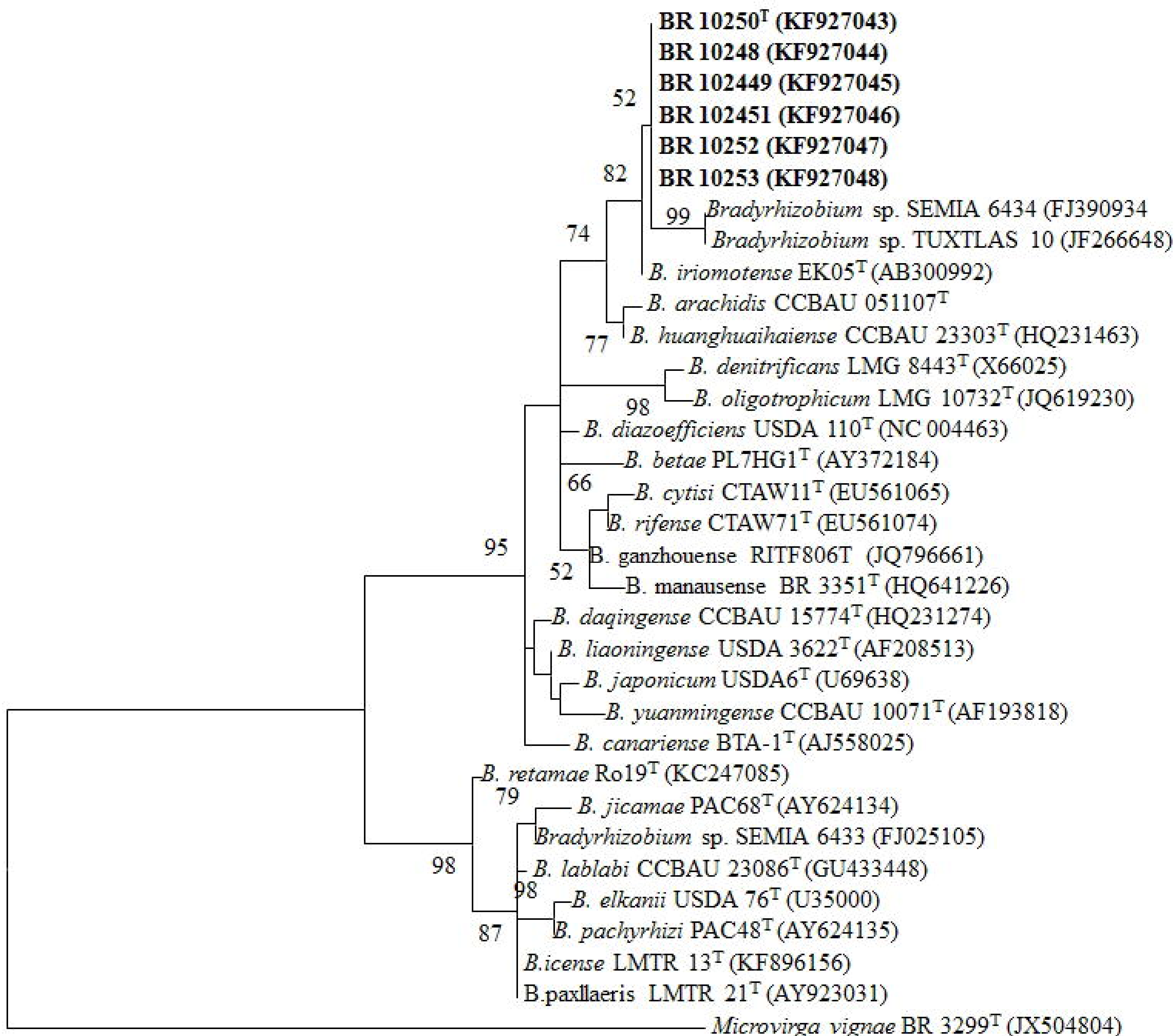
386

387

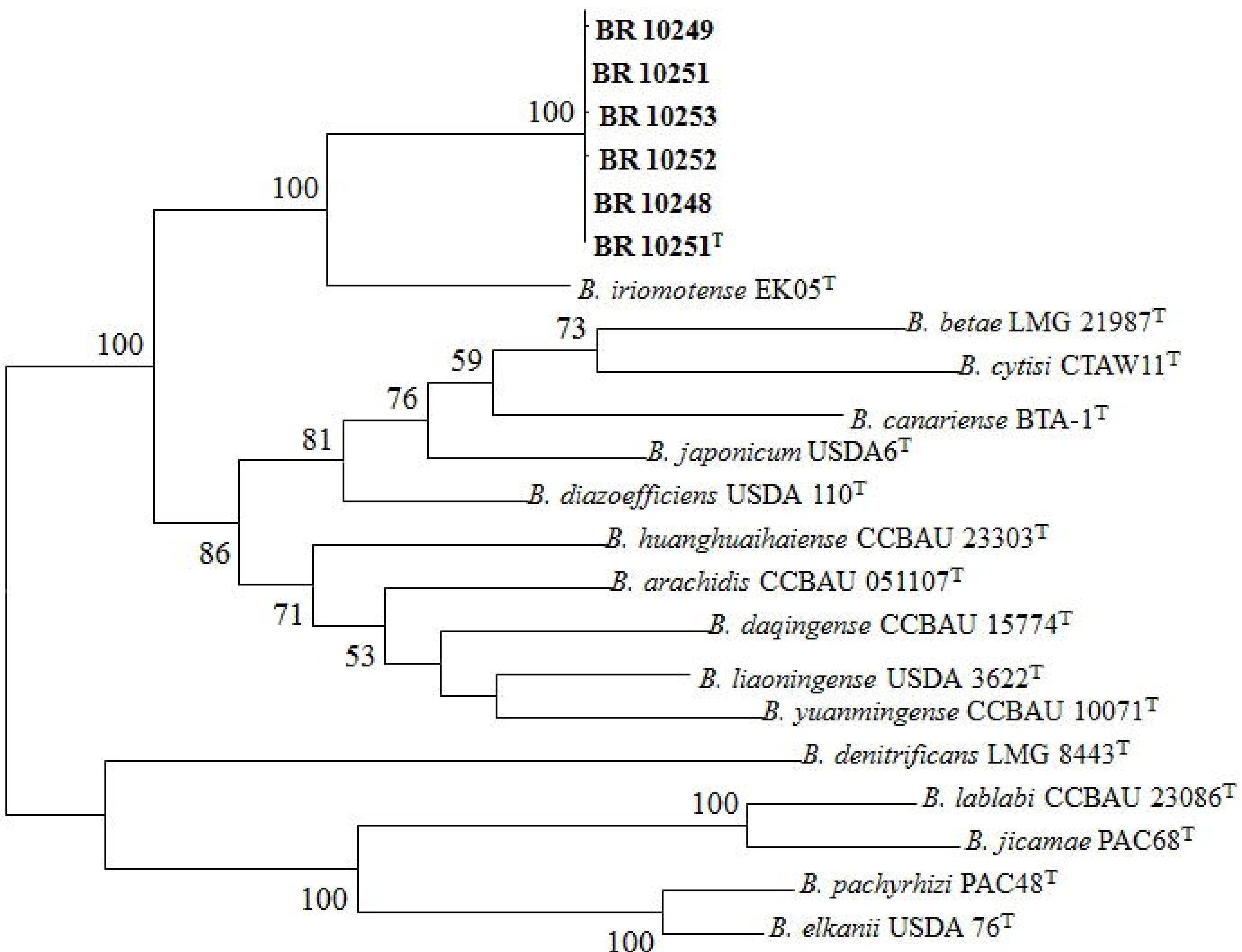
388

389 **Fig. 1** - Maximum likelihood phylogeny based on 16S rRNA gene sequences showing the
390 relationships between *Bradyrhizobium ingae* strains (shown in bold) and other members of the
391 *Bradyrhizobium* genus. The strains SEMIA 6433 and SEMIA 6434 are commercial inoculants in
392 Brazil for *Inga marginata*. The significance of each branch is indicated by a bootstrap value (greater
393 than 50% showed) calculated for 1000 subsets. Bar, 1 substitution per 100 nucleotide positions.
394 Sequence accession numbers of the 16S rRNA genes are presented in parenthesis.
395

396 **Fig. 2.** Maximum likelihood phylogeny based on concatenated *dnaK*, *glnII*, *gyrB*, *recA* and *rpoB*
397 gene sequences showing the relationships between strains from the novel species (shown in bold)
398 and other members of the *Bradyrhizobium* genus. The significance of each branch is indicated by a
399 bootstrap value (greater than 50% showed) calculated for 1000 subsets. Bar, 1 substitution per 100
400 nucleotide positions.



BR 10250T (KF927043)
BR 10248 (KF927044)
BR 102449 (KF927045)
BR 102451 (KF927046)
BR 10252 (KF927047)
BR 10253 (KF927048)
Bradyrhizobium sp. SEMIA 6434 (FJ390934
Bradyrhizobium sp. TUXTLAS 10 (JF266648)
B. iriomotense EK05T (AB300992)
B. arachidis CCBAU 051107T
B. huanghuaihaiense CCBAU 23303T (HQ231463)
B. denitrificans LMG 8443T (X66025)
B. oligotrophicum LMG 10732T (JQ619230)
B. diazoefficiens USDA 110T (NC 004463)
B. betae PL7HG1T (AY372184)
B. cytisi CTAW11T (EU561065)
B. rifense CTAW71T (EU561074)
B. ganzhouense RITF806T (JQ796661)
B. manausense BR 3351T (HQ641226)
B. daqingense CCBAU 15774T (HQ231274)
B. liaoningense USDA 3622T (AF208513)
B. japonicum USDA6T (U69638)
B. yuanmingense CCBAU 10071T (AF193818)
B. canariense BTA-1T (AJ558025)
B. retamae Ro19T (KC247085)
B. jicamae PAC68T (AY624134)
Bradyrhizobium sp. SEMIA 6433 (FJ025105)
B. lablabi CCBAU 23086T (GU433448)
B. elkanii USDA 76T (U35000)
B. pachyrhizi PAC48T (AY624135)
B.icense LMTR 13T (KF896156)
B.paxllaeris LMTR 21T (AY923031)
Microvirga vignae BR 3299T (JX504804)
52
82
99
74
77
98
66
95
52
79
98
98
87
0.01



BR 10249
BR 10251
BR 10253
BR 10252
BR 10248
BR 10251T
B. iriomotense EK05T
B. betae LMG 21987T
B. cytisi CTAW11T
B. canariense BTA-1T
B. japonicum USDA6T
B. diazoefficiens USDA 110T
B. huanghuaihaiense CCBAU 23303T
B. arachidis CCBAU 051107T
B. daqingense CCBAU 15774T
B. liaoningense USDA 3622T
B. yuanmingense CCBAU 10071T
B. denitrificans LMG 8443T
B. lablabi CCBAU 23086T
B. jicamae PAC68T
B. pachyrhizi PAC48T
B. elkanii USDA 76T
100
100
100
73
59
76
81
86
71
53
100
100
100
0.01