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JAKELINE RENATA MARÇON DELAMUTA

ANÁLISE POLIFÁSICA DE ESTIRPES DE *BRADYRHIZOBIUM* E DESCRIÇÃO DE NOVAS ESPÉCIES

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BANCA EXAMINADORA

Orientadora: Profª Dra. Mariangela Hungria
Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA

Prof. Dr. Jerri Édson Zilli
Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA

Prof. Dr. Douglas Fabiano Gomes
Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA

Prof. Dr. Renan Augusto Ribeiro
Empresa Brasileira de Pesquisa Agropecuária - EMBRAPA

Prof. Dr. Fernando Gomes Barcellos
Universidade Estadual de Londrina - UEL

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RESUMO

O Brasil sobressai no cenário agrícola por ser um grande produtor de diversas culturas de importância econômica, como por exemplo, a soja (*Glycine max*). Atualmente, tem-se buscado incrementar a produtividade das culturas, mas o manejo inadequado dos solos e das culturas pode limitar a qualidade dos solos e a sustentabilidade agropecuária. A fixação biológica de nitrogênio, realizada por bactérias diazotróficas conhecidas de modo geral como rizóbios, em simbiose com plantas leguminosas, destaca-se como uma importante prática na sustentabilidade agrícola, mas estima-se que a diversidade dessas bactérias ainda é pouco conhecida. A técnica polifásica, utilizada hoje na taxonomia e sistemática dos procariotos, engloba informações genotípicas, fenotípicas e filogenéticas para garantir uma classificação apropriada dos microrganismos. A essas informações deve-se destacar o incremento, na última década, na incorporação de dados de sequências genômicas e os avanços computacionais para analisar tais sequências, proporcionando uma verdadeira revolução nos estudos taxonômicos e filogenéticos de procariotos. Neste trabalho, foram conduzidos estudos polifásicos, incluindo a análise genômica, em grupos de estirpes de *Bradyrhizobium* com forte indicativo de representarem novas espécies. O primeiro estudo englobou duas estirpes, CNPSo 1112 e CNPSo 2833, microssimbiontes de soja perene (*Neonotonia wightii*) e desmodium (*Desmodium heterocarpon*), inicialmente relacionadas às espécies *Bradyrhizobium elkanii* e *Bradyrhizobium pachyrhizi*, respectivamente. A análise polifásica dos dados morfofisiológicos, genotípicos e genômicos suportam a proposta de duas novas espécies, para as quais estão sendo propostos os nomes *Bradyrhizobium tropiciagri* sp. nov. (CNPSo 1112) e *Bradyrhizobium embrapense* sp. nov. (CNPSo 2833). No segundo estudo, diferenças genéticas (rep-PCR, hibridação DNA-DNA), genômicas (identidade média de nucleotídeos, conteúdo C+G), fenotípicas (testes morfofisiológicos e perfil de ácidos graxos) e filogenéticas (análise do gene 16S RNAr e *multilocus sequence analysis* - MLSA) revelaram que um grupo representado por quatro estirpes relevantes para a agricultura e simbiontes de soja, classificadas como *Bradyrhizobium japonicum* grupo Ia deveriam ser reclassificadas na nova espécie *Bradyrhizobium diazoefficiens*, sendo a estirpe USDA 110 eleita como estirpe tipo. Os resultados obtidos contribuem para o maior conhecimento da diversidade de rizóbios, com ênfase no gênero *Bradyrhizobium*, que aparentemente representa a maior porcentagem de rizóbios nos trópicos. Também houve grande aporte de conhecimento sobre a aplicação da genômica em estudos de sistemática procariótica.

ABSTRACT

Brazil stands out in the agricultural scenario as a major producer of several economically important crops, such as soybean (*Glycine max*). Currently, there is need to increase crop yields, but inadequate soil and crop management can limit soil quality and agricultural sustainability. Biological nitrogen fixation, performed by nitrogen-fixing bacteria commonly known as rhizobia in symbiosis with leguminous plants, is highlighted as an important practice in agricultural sustainability, but it is estimated that the diversity of these bacteria is still poorly known. The polyphasic technique used today in taxonomy and systematics of prokaryotes includes genotypic, phenotypic and phylogenetic information to ensure proper classification of microorganisms. In the past decade, emphasis should also be given to the increasing incorporation of genomic data, as well as to the computational advances to analyze such sequences, providing a revolution in taxonomic and phylogenetic studies of prokaryotes. In this study, polyphasic analyses—including genomics—were performed in groups of *Bradyrhizobium* strains with strong indication of representing new species. The first study included two strains, CNPSo 1112 and CNPSo 2833, microsymbionts of perennial soybean (*Neonotonia wightii*) and desmodium (*Desmodium heterocarpon*), and originally classified in the species *Bradyrhizobium elkanii* and *Bradyrhizobium pachyrhizi*, respectively. The polyphasic analysis of morpho-physiological, phenotypic and genomic data supported the proposal of two new species, *Bradyrhizobium tropiciagri* sp. nov. (CNPSo 1112) and *Bradyrhizobium embrapense* sp. nov. (CNPSo 2833). In the second study, genetic (rep-PCR, DNA-DNA hybridization), genomic (average nucleotide identity - ANI, C+G content), phenotypic (morpho-physiological characteristics and fatty acid profiles) and phylogenetic (analysis of the 16S rRNA gene and multilocus sequencing analysis - MLSA) analyses revealed that a group represented by four strains relevant to agriculture, symbionts of soybean and classified as *Bradyrhizobium japonicum* group Ia should be reclassified into the new species *Bradyrhizobium diazoefficiens*, with the USDA 110 strain elected as the type strain. The results contribute to a better understanding of the diversity of rhizobia, with an emphasis on the genus *Bradyrhizobium*, which apparently is the dominant rhizobial species in the tropics. In addition, the results contribute to improve our knowledge on the application of genomic information in studies of systematics of prokaryotes.

SUMÁRIO

1 INTRODUÇÃO

É fato que a população mundial está crescendo em um ritmo acelerado. Estima-se que, até o final do século, a população aumente de 7,2 para 10,9 bilhões (UNITED NATIONS, 2013). Consequentemente, esse crescimento exorbitante demandará uma maior produção de alimentos, de aproximadamente 60%. Nesse contexto, um cenário preocupante que surge é o da necessidade de novas áreas para o cultivo, geralmente impróprias para a agricultura, uma vez que 33% dos solos mundiais encontram-se degradados (HUNGRIA; VARGAS, 2000; VARGAS, 2014). Assim, o grande desafio atual é o de produzir mais alimentos de forma sustentável, adotando práticas agrícolas que visam manter a qualidade do solo, sem trazer consequências negativas para a biodiversidade ali presente.

Em termos globais, a disponibilidade de água, nitrogênio (N), fósforo (P) e cálcio (Ca) são os principais recursos que limitam a produção agrícola (ANDREWS et al., 2009). O N é um nutriente essencial às plantas e requerido em grandes quantidades, pois faz parte da constituição de macromoléculas fundamentais à vida, os ácidos nucleicos e proteínas (HUNGRIA, MENDES, MERCANTE, 2013; NEWTON, 2008). A matéria orgânica do solo pode fornecer, durante alguns cultivos, o N necessário às plantas, porém, esse reservatório é limitado. Em regiões tropicais, o empobrecimento do solo é ainda mais acentuado devido às condições de temperatura e umidade elevadas, as quais aceleram a decomposição da matéria orgânica aumentado, assim, as perdas de N (HUNGRIA, MENDES, MERCANTE, 2013; HUNGRIA et al., 2001).

Outra forma de suprir a escassez de N nos ambientes agrícolas é a utilização de fertilizantes nitrogenados. As plantas assimilam rapidamente o N presente nos fertilizantes mas, infelizmente, essa prática tem um custo elevado. Calcula-se que, para produzir uma tonelada de amônia (NH_3), sejam necessários seis barris de petróleo, uma fonte não renovável de energia. Além disso, as plantas utilizam, no máximo, 50% do N fornecido pela fertilização, resultando em sérios problemas de poluição ambiental. Esse excesso pode ser perdido por lixiviação para o ambiente aquático, acelerando o processo de eutrofização, ou pode ser retornado à atmosfera em sua forma gasosa, quer seja por volatilização ou desnitrificação, resultando na emissão de gases de efeito estufa (ANDREWS et al., 2009; HUNGRIA; VARGAS, 2000; HUNGRIA, MENDES, MERCANTE, 2013).

Como resultado desses agravantes ambientais e financeiros causados pelo uso de fertilizantes, outras estratégias se fazem necessárias, notoriamente em países em desenvolvimento, que precisam importar grande parte dos fertilizantes químicos para atender a sua demanda. O N gasoso (N_2) representa quase 80% dos gases presentes na atmosfera, mas a tripla ligação entre seus átomos não permite que plantas e animais o utilizem diretamente na síntese de proteínas (HUNGRIA et al., 1994). Nesse cenário, a fixação biológica do nitrogênio (FBN), realizada exclusivamente por procariotos, se destaca como um processo chave na ciclagem de N. Apenas algumas bactérias e arqueobactérias conseguem converter biologicamente o N_2 em amônia (NH_3), devido à presença de um complexo enzimático altamente sensível à presença de oxigênio (O_2), a nitrogenase. Estima-se que a FBN contribua com 65% da entrada total de N na Terra e, juntamente com a fotossíntese, esses processos são considerados os mais relevantes para vida existente no planeta (NEWTON, 2008; ORMEÑO-ORRILLO; HUNGRIA; MARTÍNEZ-ROMERO, 2013).

O principal aporte de N na biosfera por processos naturais se dá pela associação simbiótica que ocorre entre microrganismos diazotróficos, conhecidos popularmente como rizóbios, e determinadas espécies da família Leguminosae (=Fabaceae). Não é difícil perceber a imponência dessa interação simbiótica, principalmente em sistemas agrícolas, onde a maior parte da produção mundial é baseada no cultivo de leguminosas (HERRIDGE et al., 2008; RIVAS et al., 2009) e os benefícios econômicos gerados pela redução do uso de fertilizantes nitrogenados são expressivos. No caso do Brasil, um grande produtor de diversas culturas de importância econômica, como, por exemplo, a soja (*Glycine max* L. (Merr.), a economia por utilizar a FBN é de cerca de 14 bilhões de reais/ano (HUNGRIA, MENDES, MERCANTE, 2013). Deve-se ressaltar que o sucesso dessa associação resulta da formação de estruturas altamente complexas nas raízes das leguminosas, os nódulos, onde o N_2 fixado é convertido em formas utilizáveis pelas plantas, em troca dos produtos da fotossíntese (ANDREWS et al., 2009; BERRADA; FIKRI-BENBRAHIM, 2014; HUNGRIA et al., 1994; ZAKHIA; de LAJUDIE, 2001).

Os microrganismos fixadores de N_2 são encontrados em mais de 100 filos do domínio *Bacteria* e, em *Archaea*, a FBN é restrita ao filo Euryarchaeota. Embora esse número pareça ser elevado, a lista de bactérias fixadoras de N_2 está longe de ser representativa, e ainda mais se forem considerados os procariotos que ainda não foram cultivados (ORMEÑO-ORRILLO; HUNGRIA; MARTÍNEZ-ROMERO, 2013). A maior parte dos fixadores de N_2 vive livremente no solo, na região rizosférica. Todavia, alguns grupos bacterianos se associam a diversas plantas em diferentes graus de especificidade, como associações endofíticas ou simbióticas, sendo encontrados no interior de raízes, caules e folhas (HUNGRIA et al., 2007).

Nos últimos anos, diversos estudos têm aumentado nosso conhecimento sobre a diversidade dos microrganismos facilitando, dessa forma, a identificação de bactérias nodulíferas em distintos gêneros. Dentro da classe *Alphaproteobacteria*, encontram-se representantes nos gêneros *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, *Sinorhizobium* (=*Ensifer*), *Neorhizobium*, *Methylobacterium*, *Devosia*, *Ochrobactrum*, *Aminobacter*, *Microvirga*, *Shinella* e *Phyllobacterium*. Notavelmente, algumas bactérias capazes de nodular leguminosas foram identificadas como pertencentes às classes *Betaproteobacteria* (*Burkholderia* e *Cupriavidus*) e *Gammaproteobacteria* (*Pseudomonas*) (BENHIZIA et al., 2004; BERRADA; FIKRI-BENBRAHIM, 2014; MOUSAVI et al., 2014; ORMEÑO-ORRILLO; HUNGRIA; MARTÍNEZ-ROMERO, 2013; SHIRAISHI; MATSUSHITA; HOUGETSU, 2010).

Apesar dos recentes avanços que buscam compreender a vasta diversidade do mundo microbiano em diferentes hábitats, ainda é escasso o conhecimento sobre os microrganismos que vivem no solo. Estima-se que um grama de solo contenha milhares de bactérias, sem falar da comunidade fúngica, que é muito pouco estudada. Além disso, os estudos sobre a diversidade do solo são limitados, muitas vezes, por questões consideradas simples, como amostragem e recuperação dos microrganismos; no caso dos procariotos, apenas 1% da população presente no solo pode ser recuperada utilizando-se os métodos de isolamento tradicionais (GILLER et al., 1997). Esse quadro se torna mais alarmante quando se leva em consideração os solos tropicais, que devido às condições climáticas, são mais expostos e estruturalmente mais frágeis, apresentam um baixo teor de matéria orgânica e elevada acidez, além de índices elevados de desmatamento (HUNGRIA; VARGAS, 2000; STRALIOTTO, 2005). Certamente, todos esses fatores citados acarretam em profundas alterações nos grupos microbianos ali existentes, levando à perda elevada da diversidade do solo, incluindo microrganismos ainda não identificados. Como já mencionado, a simbiose rizóbio-leguminosa é considerada uma das associações mais importantes para a agricultura e, por isso, tem sido objeto de investigação. Em razão disso, estudos sobre a diversidade dos rizóbios, com destaque para aqueles de regiões tropicais, têm aumentado nesses últimos anos (BINDE et al., 2009; GERMANO et al., 2006; DELAMUTA et al., 2012; MENNA et al., 2006; MENNA; BARCELLOS; HUNGRIA, 2009; PINTO; HUNGRIA; MERCANTE, 2007; RIBEIRO et al., 2009; 2013). Esses estudos têm facilitado a identificação de genótipos mais eficientes, competitivos e geneticamente estáveis, que toleram melhor as condições ambientais estressantes, auxiliando a esclarecer as complexas relações ecológicas e evolutivas envolvendo esses simbiontes e, não menos importante, apontando fortemente a existência de possíveis novas espécies (DELAMUTA et al., 2012; HUNGRIA et al., 2000; RIBEIRO et al., 2013).

A estimativa de que existam aproximadamente 4-6 x 10^{30} células procarióticas no planeta, correspondendo a mais de 50% de toda biomassa existente, torna necessário o emprego de um sistema taxonômico confiável, reprodutível e informativo, a fim de que toda biodiversidade seja classificada e catalogada corretamente (ROSSELLÓ-MÓRA; AMANN, 2001; THOMPSON et al., 2013). A ciência taxonômica começou a ser difundida a partir dos conhecimentos de Lineu no século XVIII e, ao longo dos anos, vem sofrendo várias mudanças. Geralmente, a taxonomia é utilizada como sinônimo de sistemática, mas, de acordo com Kämpfer e Glaeser (2012), seria mais adequado considerar a taxonomia como parte da sistemática. A sistemática se preocupa em estudar a diversidade dos organismos e as relações naturais que existem entre eles, e fornece à taxonomia o alicerce para a classificação, um pré-requisito para a identificação de qualquer organismo (ZAKHIA; de LAJUDIE, 2001). Portanto, o principal objetivo da taxonomia é criar um sistema hierárquico que reflita as relações evolutivas, possibilitando compreender a origem da vida (KÄMPFER, 2011; KÄMPFER; GLAESER, 2012).

Tradicionalmente, a taxonomia bacteriana engloba: a classificação dos organismos em categorias ou grupos taxonômicos ordenados, com base nas similaridades e diferenças apresentadas entre eles, a nomeação desses grupos taxonômicos de forma correta, seguindo as regras estabelecidas pelo Código Internacional de Nomenclatura de Bactérias, e a identificação de organismos desconhecidos, buscando alocá-los aos níveis taxonômicos validamente publicados. Isso só é possível se as principais características dos isolados forem determinadas por métodos padronizados; no caso dos isolados não pertencerem aos grupos conhecidos, eles serão descritos e classificados como um novo taxa (KÄMPFER; GLAESER, 2012; VANDAMME et al., 1996; ZHI et al., 2012). Recentemente, um quarto objetivo foi proposto por Staley (2010) para a taxonomia microbiana. O autor propõe que, além das áreas citadas acima, o campo “Compreender a Diversidade Microbiana” também deve ser explorado nos estudos taxonômicos, frente à quantidade de microrganismos que ainda não foram descobertos, cultivados e nomeados, e por não haver um consenso para o conceito de espécie microbiana em pleno século XXI.

A classificação bacteriana começou a ser desvendada no final do século XIX, quando Ferdinand Cohn, em 1872, classificou as bactérias em seis gêneros com base, principalmente, em características morfológicas, necessidades de crescimento e potencial patogênico. Deve-se observar que naquela época esses microrganismos eram classificados como plantas, evidenciando claramente que os avanços nessa área foram ocasionados pela disponibilidade de novas técnicas. Já na metade do século XX, um número cada vez maior de dados bioquímicos, fisiológicos, enzimáticos e metabólicos passou a ser utilizado na caracterização bacteriana, culminando no surgimento de uma taxonomia numérica, a qual permitiu uma análise conjunta de todos os dados empregando-se métodos estatísticos, fornecendo, assim, medidas quantitativas de similaridade entre as bactérias, de uma forma menos subjetiva e mais rigorosa. A taxonomia numérica também ganhou reforços com o advento dos marcadores quimiotaxonômicos, de modo que a análise da composição química dos componentes celulares aumentou o poder de resolução da classificação bacteriana (ROSSELLÓ-MÓRA; AMANN, 2001; SCHLEIFER, 2009; SENTAUSA; FOURNIER, 2013; ZHI et al., 2012).

Nas décadas seguintes, o conhecimento mais aprofundado das propriedades do DNA e o desenvolvimento de técnicas de biologia molecular, como a análise da porcentagem de guanina e citosina do genoma e experimentos de hibridação DNA-DNA (HDD; ou DNA-DNA *hybridization*, DDH), juntamente com as metodologias de PCR e sequenciamento, promoveram uma revolução na taxonomia bacteriana. O avanço mais notável foi em relação ao sequenciamento do gene ribossomal, mais especificamente da subunidade menor 16S para os procariotos, que se mostrou um excelente marcador molecular para estimar relações filogenéticas entre as bactérias (LUDWIG; SCHLEIFER, 1994). O marco que resultou na taxonomia moderna foi conduzido por Woese et al. (1990), ao introduzirem métodos mais rápidos de análise comparativa da sequência do RNAr para a construção de uma árvore filogenética universal, fornecendo, pela primeira vez, uma visão global sobre a filogenia de procariotos, e tornando-o, dessa forma, a espinha dorsal da classificação microbiana (KÄMPFER; GLAESER, 2011).

Diante da riqueza de dados gerados por diversas técnicas, a taxonomia bacteriana passou a integrar vários níveis de informação, desde ecológicos até moleculares, visando à construção de uma classificação multidimensional. Neste contexto, a taxonomia é referida atualmente como taxonomia polifásica e objetiva utilizar todos os dados disponíveis para garantir uma classificação apropriada. Tais dados incluem informações fenotípicas, abrangendo análises morfológicas, fisiológicas e quimiotaxonômicas, informações genotípicas, derivadas da análise dos ácidos nucleicos (DNA e RNA), como conteúdo G+C, hibridação DNA-DNA, padrões de restrição (RFLP, AFLP) e PCR *fingerprinting*, e informações filogenéticas, obtidas, sobretudo, a partir das sequências dos genes ribossomais e, em menor escala, de genes que codificam proteínas conservadas (*housekeeping*) (ROSSELLÓ-MÓRA; AMANN, 2001;

ROSSELLÓ-MÓRA, 2012; SENTAUSA; FOURNIER, 2013; STACKEBRANDT et al., 2002; THOMPSON et al., 2013; VANDAMME et al., 1996).

Não é de hoje que um dos assuntos mais debatidos entre os microbiologistas é que ainda não existe um conceito de espécie microbiana, ou seja, o que vem a ser uma espécie bacteriana. A comunidade científica busca alcançar uma definição de espécie que seja universal, todavia, o conceito aplicado aos organismos superiores, o qual considera mecanismos de especiação, como o isolamento reprodutivo, para delimitar as espécies, nitidamente não pode ser aplicado à comunidade microbiana. Em 2001, Rosselló-Móra e Amann propuseram um conceito filofenético para delinear uma espécie procariótica, que, mesmo não sendo fundamentado em uma teoria, é universalmente aplicável. Nele, uma espécie bacteriana é definida como um grupo de estirpes/isolados monofilético e genomicamente semelhante, que compartilham um elevado grau de similaridade em várias características independentes, sendo empiricamente fundamentada em três técnicas independentes (hibridação DNA-DNA, descrição fenotípica e análise filogenética do gene ribossomal 16S). No entanto, ela apresenta um inconveniente, pois é útil apenas para os isolados em cultura pura, excluindo, assim, os organismos ainda não cultivados, que constituem a maior proporção de procariotos existentes.

Na prática, um conjunto de parâmetros e níveis de corte (*cut-off*) foi definido para delimitar os grupos que compartilham semelhanças funcionais e filogenéticas. O parâmetro mais aceito para a circunscrição de espécie é a similaridade total do DNA. Os valores são expressos em % de similaridade, ou em diferença de $\triangle T_m$ (diferença na temperatura de desnaturação das fitas de DNA) e representam uma medida indireta do grau de similaridade entre dois genomas diferentes. Ao longo dos anos, observou-se que espécies bem delimitadas fenotipicamente, comumente incluem estirpes com 70% ou mais de similaridade por hibridação DNA-DNA e 5^oC, ou menos, nos valores de $\triangle T_m$. Entretanto, é digno de nota ressaltar que esses números são empíricos e não representam valores absolutos, podendo variar conforme os isolados estudados. Outro parâmetro considerado é a reconstrução filogenética a partir da análise do gene 16S RNAr. Admite-se que bactérias que apresentem uma similaridade genômica de 70% geralmente compartilham mais de 97% de similaridade na sequência desse gene ribossomal. Nitidamente, a extensa aceitação desses valores ao longo dos anos tornaram esses parâmetros como padrões de referência para descrever toda e qualquer espécie microbiana (ROSSELLÓ-MÓRA; AMANN, 2001; ROSSELLÓ-MÓRA, 2012; STACKEBRANDT; GOEBEL, 1994; TINDALL et al., 2010; VANDAMME et al., 1996).

Na última década, o desenvolvimento de métodos de sequenciamento de alto rendimento e mais econômicos tem tornado mais acessível o uso de sequências genômicas em estudos de taxonomia e filogenia microbiana. Dúvidas sobre a precisão da técnica de HDD nessa era dominada pela genômica têm sido levantadas, devido às variações dos resultados e metodologias quando realizados em diferentes laboratórios, além do alto custo e trabalho envolvidos, não permitindo, portanto, uma rápida identificação dos microrganismos. Além disso, a impossibilidade de se construir um banco de dados com os resultados gerados pela técnica tem-se tornado um grande problema, havendo a necessidade de se incluir muitas estirpes referência a cada novo experimento (CHUN; RAINEY, 2014; GORIS et al., 2007; RAMASAMY et al., 2014; RICHTER; ROSSELLÓ-MÓRA, 2009; ROSSELLÓ-MÓRA, 2012; STACKEBRANDT et al., 2002).

Esforços têm sido feitos para correlacionar os valores de HDD com os índices obtidos pela análise de múltiplos genes, ou mesmo do genoma inteiro. Notoriamente, a proposta mais promissora é a da técnica de ANI (*Average Nucleotide Identity*), fundamentada na identidade nucleotídica média que os genomas de duas estirpes compartilham. Inicialmente, um ANI dos genes ortólogos partilhados entre dois genomas foi sugerido como uma medida robusta da distância evolutiva, com valores de 94% correspondendo ao tradicional 70% da hibridação DNA-DNA (KONSTANTINIDIS; TIEDJE, 2005). Mais tarde, Goris et al. (2007) aperfeiçoou o método ao fragmentar artificialmente o genoma total, sugerindo um ANI de 95% para estirpes da mesma espécie. Atualmente, valores de ANI entre 95-96% podem ser empregados de forma simples e objetiva para delinear espécies procarióticas, e têm grande potencial para substituir a técnica de hibridação (CHUN; RAINEY, 2014; KÄMPFER, 2014; RICHTER; ROSSELLÓ-MÓRA, 2009). Além do mais, os genomas sequenciados podem ser depositados em bancos de dados públicos, tornando-os facilmente acessíveis.

Outros recursos lançados incluem o emprego de técnicas computacionais que comportem a comparação de genomas *in silico*, inferindo valores de HDD digitais que correspondem aos valores de HDD experimentais (AUCH et al., 2010; MEIER-KOLTHOFF et al., 2013a) e a iniciativa de se utilizar a análise completa de diversos parâmetros genéticos, abrangendo até a anotação do genoma, em um método denominado taxono-genômica (KOKCHA et al., 2012, LAGIER et al., 2013; RAMASAMY et al., 2014).

Ainda hoje, a análise filogenética do gene 16S RNAr é a primeira etapa a ser realizada na identificação de novas espécies, e é responsável por indicar a necessidade de se realizar a HDD em estirpes que compartilham um valor acima de 97% de similaridade em relação às espécies validadas. Contudo, uma reavaliação dos dados acumulados sobre a relação 16S/HDD sugere valores mais rigorosos, entre 98,2-99% de similaridade, para indicar o uso da metodologia de HDD (MEIER-KOLTHOFF et al., 2013b; STACKEBRANDT; EBERS, 2006).

Sob condições padronizadas, um estudo realizado por Kim et al. (2014) investigou a correlação 16S/ANI ao empregar mais de um milhão de comparações entre diversos genomas; um valor de 98,65% de similaridade do gene ribossomal sugere refletir os valores de ANI aceitos para delinear espécies. No entanto, em muitos casos, é difícil diferenciar duas espécies utilizando-se apenas o gene 16S RNAr. Em alguns gêneros procarióticos, a sequência é muito conservada e um único genoma pode abrigar mais de um operon ribossomal; além disso, há evidências de que ele pode sofrer transferência horizontal. Como consequência, as informações sobre as relações filogenéticas dos procariotos podem ser incoerentes (MENDE et al., 2013; OREN; GARRITY, 2014; ROSSELLÓ-MÓRA, 2012; SCHLEIFER, 2009).

As limitações do gene 16S RNAr ampliaram a busca por outras estratégias de análise filogenética. A princípio, outros genes ribossomais, como o 23S, e genes essenciais que codificam proteínas (*housekeeping*) foram sugeridos como alternativa ao 16S, porém, a análise também pode apresentar as mesmas limitações. Todavia, a análise conjunta de vários genes fornece um número maior de sítios nucleotídicos informativos e ameniza os efeitos de recombinação gênica e de transferência horizontal que podem ocorrer em um único gene (GEVERS et al., 2005; SCHLEIFER, 2009). Com base nesta proposta, a metodologia de MLSA (*Multilocus Sequence Analysis*) foi desenvolvida e se tornou uma importante ferramenta taxonômica, uma vez que a concatenação de, no mínimo, cinco genes *housekeeping* apresenta um poder de resolução superior aquele apresentado pela análise tradicional do gene ribossomal 16S (GEVERS et al., 2005; STACKEBRANDT et al., 2002; VANDAMME; PEETERS, 2014). Recentemente, Mende et al. (2013) desenvolveram um método mais refinado de MLSA, chamado specI, que utiliza 40 genes universais de cópia única presentes em uma infinidade de genomas. Segundo os autores, resultados precisos são obtidos de forma rápida e podem ser úteis em delinear espécies bacterianas.

Sem dúvida, a tarefa mais tediosa na classificação dos microrganismos é representada pela análise fenotípica dos isolados, exigindo tempo e habilidade para realizar todos os testes necessários. As estirpes devem ser caracterizadas tanto quanto possível e, para isso, existem orientações completas sobre as técnicas que devem ser utilizadas (ROSSELLÓ-MÓRA; AMANN, 2001; TINDALL et al., 2010; VANDAMME et al., 1996). Entretanto, a utilização de parâmetros fenotípicos na taxonomia vem sendo cada vez mais contestada. O principal problema está pautado na falta de reprodutibilidade dos dados, pois não há uma padronização dos métodos (são as famosas notas “nossos dados não confirmam os dados da literatura”, encontradas com frequência nos artigos científicos); nesse caso, o ideal seria a construção de bancos de dados fenotípicos, nos quais as informações podem ser recuperadas rapidamente, como ocorre com os bancos de dados genômicos (ROSSELLÓ-MÓRA, 2012).

Como resultado do crescimento exponencial de genomas sequenciados, é de se esperar que as características fenotípicas sejam obtidas diretamente dessas sequências, mas a simples presença ou ausência do gene não implica em sua expressão. Em outras palavras, o fenótipo não é apenas resultado da expressão gênica, ele também reflete as condições sob as quais os genes são expressos (KÄMPFER, 2012, 2014). Contudo, a coerência dos testes fenotípicos tem sido questionada, já que muitas características são codificadas pelo genoma acessório e podem ser facilmente perdidas, subestimando as diferenças fenotípicas entre as espécies e resultando em incongruências com os dados genéticos (ORMEÑO-ORRILLO; MARTÍNEZ-ROMERO, 2013). O valor do fenótipo dentro da classificação microbiana é inegável, mas está em curso uma revolução taxonômica. Nos próximos anos, a principal questão levantada será quanto à necessidade de se realizar todos os testes fenotípicos exigidos hoje, visto que o progresso na sistemática procariótica dependerá grandemente da necessidade de se adotar padrões mínimos de testes, ao invés de uma caracterização tão abrangente (SUTCLIFFE; TRUJILLO; GOODFELLOW, 2012).

Nas últimas duas décadas, houve um aumento acentuado no número de espécies procarióticas descritas a cada ano de tal forma que, hoje, existem aproximadamente 12.000 espécies validadas. Contudo, por mais que esse número seja elevado, ele representa menos de 1% de toda diversidade microbiana existente! A questão é mais preocupante porque, ainda que a taxa de descrição de espécies tenha incremento em cerca de dez vezes nos últimos anos, o desafio de se catalogar adequadamente toda diversidade existente pode levar outro século (SUTCLIFFE; TRUJILLO; GOODFELLOW, 2012). Mesmo diante desse quadro, um tema muito debatido se refere ao fato de que mais de 90% das descrições de novos taxa tem como base características de uma única estirpe. Sem dúvida, estudos que englobam múltiplos isolados da mesma espécie elevam nosso conhecimento tanto sobre o grau de variabilidade fenotípica quanto às propriedades genotípicas que existem no mundo microbiano. Por outro lado, deve-se considerar que haveria uma perda muito maior para a compreensão sobre a diversidade procariótica quando espécies com uma única estirpe não são descritas do que se a descrição tivesse esperado até que estirpes adicionais pudessem ser incluídas (OREN; GARRITY, 2014).

As recentes mudanças incididas sobre a ciência taxonômica têm impactado positivamente a classificação dos rizóbios. Até o início de 1980, as bactérias fixadoras de N_2 simbióticas de leguminosas estavam classificadas em um único gênero, *Rhizobium*, incluindo apenas seis espécies, de acordo com a leguminosa a qual nodulavam (FRED et al., 1932). No entanto, o conceito de inoculação cruzada deixou de ser empregado, visto que uma única leguminosa pode abrigar mais de uma espécie simbiótica. Além disso, vários trabalhos começaram a demonstrar a existência de grande variabilidade genética e fisiológica entre as espécies de *Rhizobium* e, como consequência, o gênero *Bradyrhizobium* foi descrito para alocar as estirpes de crescimento lento que alcalinizam o meio de cultura contendo manitol como fonte de carbono (JORDAN, 1982). Por muito tempo, esse gênero incluiu apenas uma única espécie, *B. japonicum*, agrupando todas as estirpes nodulantes de soja (*Glycine max*). No entanto, devido à elevada heterogeneidade entre as estirpes, elas foram divididas em três grupos – I, Ia e II. Posteriormente, dados de hibridação DNA-DNA levaram Kuykendall et al. (1992) a sugerirem a subdivisão de *Bradyrhizobium* em duas espécies: *B. japonicum*, alocando as estirpes dos grupos I e Ia, e *B. elkanii*, incluindo as estirpes do grupo II.

Após a introdução da taxonomia polifásica, centenas de espécies nodulantes alocadas em cerca de uma dezenas de gêneros já foram identificadas e descritas, e esse número deve se elevar nos próximos anos, uma vez que apenas cerca de um quarto das espécies de leguminosas já foram caracterizadas em relação aos seus microssimbiontes (BERRADA; FIKRI-BENBRAHIM, 2014); como exemplo, podemos citar o gênero *Bradyrhizobium*, com mais de 25 espécies descritas atualmente, sendo que algumas foram isoladas no Brasil. Aos poucos, as análises envolvendo o genoma total estão começando a ser empregadas na taxonomia dos rizóbios e têm levado ao delineamento de novas espécies; em alguns estudos, a HDD foi totalmente substituída pelos índices de similaridades derivados do genoma (por exemplo, o ANI) (ORMEÑO-ORRILLO et al., 2014). Considerando a facilidade para analisar milhares de genes de uma só vez, uma visão mais aprofundada sobre as relações filogenéticas existente neste grupo procariótico é plausível e, com certeza, trará contribuições importantes para os estudos envolvendo rizóbios.

É necessário reconhecer que o *boom* de informações genômicas fornecerá oportunidades nunca vistas para avaliar as relações taxonômicas entre os microrganismos. O sequenciamento do genoma total incrementará o conhecimento sobre a natureza genética, ecológica e fisiológica das espécies microbianas, e proporcionará um mecanismo mais eficiente para delinear a extensa biodiversidade encontrada no globo. O desafio futuro será admitir que a taxonomia procariótica baseada em técnicas polifásicas precisa ser modernizada, e o primeiro passo consiste em tornar a análise genômica um aspecto fundamental da microbiologia. Dada a relevância das bactérias diazotróficas ao sistema agrícola, pesquisas sobre a sua biodiversidade são essenciais no cenário atual de conscientização ambiental. A busca por espécies mais eficientes e adaptadas aos solos tropicais fortalecerá ainda mais a necessidade de avaliação sistemática dessa diversidade.

2 OBJETIVO GERAL

Aplicar a taxonomia polifásica visando encontrar diferenças genéticas, fenotípicas e filogenéticas em estirpes do gênero *Bradyrhizobium* de importância agrícola para o Brasil.

2.1 OBJETIVOS ESPECÍFICOS DO CAPÍTULO 01

Realizar um estudo polifásico detalhado, com ênfase em ferramentas genômicas, que permita definir duas novas espécies de *Bradyrhizobium*, esclarecendo as suas relações filogenéticas dentro do clado de *Bradyrhizobium elkanii*.

2.2 OBJETIVOS ESPECÍFICOS DO CAPÍTULO 02

Reclassificar estirpes classificadas como *Bradyrhizobium japonicum* grupo Ia em uma nova espécie.

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4 CAPÍTULO 01

Bradyrhizobium tropiciagri sp. nov. and *Bradyrhizobium embrapense* sp. nov., nitrogen-fixing symbionts of tropical forage legumes

Jakeline Renata Marçon Delamuta,[1,2] Renan Augusto Ribeiro,[3] Ernesto Ormeño-Orrillo,[4] Marcia Maria Parma,[5] Itamar Soares Melo,[5] Esperanza Martínez-Romero,[6] Mariangela Hungria,[1,2,3]

[1]Embrapa Soja, C.P. 231, 86001-970, Londrina, Paraná, Brazil.
[2]Universidade Estadual de Londrina, Dept. of Microbiology, C.P. 10.011, 86057-970, Londrina, Paraná, Brazil.
[3]Conselho Nacional de Desenvolvimento Científico e Tecnológico, SHIS QI 1 Conjunto B, Blocos A, B, C e D, Lago Sul, 71605-001, Brasília, Distrito Federal, Brazil.
[4]Universidad Nacional Agraria La Molina, Av. La Molina s/n La Molina, Lima, Peru.
[5]Embrapa Meio Ambiente, C.P. 69, 13820-000, Jaguariúna, São Paulo, Brazil.
[6]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico.

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*Corresponding author:
Mariangela Hungria
Embrapa Soja
Cx. Postal 231
86001-970 Londrina, Paraná, Brazil
Fax: (+55)4333716100
Telephone: (+55)4333716206
E-mail: mariangela.hungria@embrapa.br; biotecnologia.solo@hotmail.com

E-mail addresses:
J.R.M. Delamuta (jake_renata@hotmail.com); R.A. Ribeiro (renanribeiro83@hotmail.com); E. Ormeño-Orrillo (eormeno.orrillo@gmail.com); M.M. Parma (marcia.parma@embrapa.br); I.S. Melo (itamar.melo@embrapa.br); E. Martínez-Romero (emartine@ccg.unam.mx).

New sequences: *nifH* of *B. embrapense* CNPSo 2833^{T} (KP234518); *dnaK* of *B. embrapense* CNPSo 2833^{T} (KP234519); *nodC* of *B. embrapense* CNPSo 2833^{T} (KP234521); *nodC* of *B. tropiciagri* CNPSo 1112^{T} (KP234520).

Abstract

Biological nitrogen fixation is a key process for agriculture production and environmental sustainability, but there are comparatively few studies of symbionts of tropical pasture legumes, as well as few described *Bradyrhizobium* species, although it is the predominant genus in the tropics. A detailed polyphasic study was conducted with two *Bradyrhizobium* strains used in commercial inoculants for tropical pastures in Brazil, CNPSo 1112^T isolated from perennial soybean (*Neonotonia wightii*), and CNPSo 2833^T from desmodium (*Desmodium heterocarpon*). Based on 16S-rRNA phylogeny, both strains were grouped in the *B. elkanii* superclade, but were not clearly clustered with any known species. MLSA of three (*glnII*, *gyrB* and *recA*) and five (+ *atpD* and *dnaK*) housekeeping genes confirmed that the strains are positioned in two distinct clades. Comparison with ITS sequences of described *Bradyrhizobium* species showed similarity lower than 93.1%, and differences were confirmed by BOX-PCR analysis. Nucleotide identity of three housekeeping genes with described species ranged from 88.1% to 96.1%. ANI of genome sequences showed values below the threshold of *Bradyrhizobium* species (< 90.6%) and also between the two strains (91.2%). The analysis of *nifH* and *nodC* genes positioned the two strains in a distinct clade from other *Bradyrhizobium* species. Morpho-physiological, genotypic and genomic data supported the description of two novel clades in the genus *Bradyrhizobium*, *B. tropiciagri* sp. nov. (type strain CNPSo 1112^T =SMS 303^T =BR 1009^T =SEMIA 6148^T =LMG 28867^T) and *B. embrapense* sp. nov. (type strain CNPSo 2833^T =CIAT 2372^T =BR 2212^T =SEMIA 6208^T =U674^T).

Keywords: *Bradyrhizobium*; biological nitrogen fixation; inoculant; MLSA; ANI; nodulation.

Biological nitrogen fixation, performed by prokaryotes—mostly bacteria—with the ability to convert atmospheric nitrogen (N_2) into ammonia and thereafter other N compounds that can be assimilated by plants, stands out as a key process for agricultural production and environmental sustainability. The most effective contribution occurs when the process is performed by bacteria collectively known as rhizobia in symbiosis with leguminous plants (Ormeño-Orrillo *et al*., 2013). Brazil is a major producer of several grain legumes of economic importance such as soybean [*Glycine max* (L.) Merr.], but others are variously employed as green manures, in forestry, and for pastures, among other uses, all contributing to improvement of soil quality and fertility (Hungria *et al*., 2005; Ormeño-Orrillo *et al*., 2013). Several efficient rhizobial strains for this broad-range of applications have been isolated from Brazilian soils and are available for use in commercial inoculants; the great majority belongs to the genus *Bradyrhizobium* (Binde *et al*., 2009; Menna *et al*., 2006, 2009a, 2009b; Roma Neto *et al*., 2010). In the last decade, our research groups have reported several studies showing high levels of genetic diversity among indigenous tropical rhizobia, including new species and several groups that may represent new species (Binde *et al*., 2009; Dall'Agnol *et al*., 2013. 2014; Delamuta *et al*., 2012, 2013; Germano *et al*., 2006; Menna *et al*., 2006, 2009a, 2009b; Ribeiro *et al*., 2009, 2012; Roma Neto *et al*., 2010).

Bradyrhizobium strains used in this study, CNPSo 1112^T and CNPSo 2833^T (Table 1), were identified as forming independent branches in a previous MLSA phylogeny (Delamuta *et al*., 2012; Menna *et al*., 2009a). These strains are effective symbionts of tropical pasture legumes. Strain CNPSo 1112^T was isolated from perennial soybean [*Neonotonia wightii* (Wight & Arn.) J.A. Lackey; *formerly classified as Glycine wightii*] by researchers of the Instituto Agronômico de Campinas (Collection SMS, Seção de Microbiologia do Solo), State of São Paulo, Brazil, and has been used in commercial inoculants for this legume since 1994. *N. wightii* is a perennial forage from Africa that produces well in several tropical countries including Brazil. Strain CNPSo 2833^T was isolated from *Desmodium heterocarpon* (L.) DC. subsp. *ovalifolium* (Prain) H. Ohashi (former *D. ovalifolium* Merr.) by researchers of the International Center for Tropical Agriculture (CIAT), Colombia, and it has been used in commercial inoculants for this legume in Brazil since 1988. The use of commercial rhizobial inoculants should be emphasized, but globally is often concentrated in few legumes (Hungria *et al*., 2005), with a lack of elite strains for forage legumes, despite their key role in N cycling, helping to maintain soil fertility and contributing to animal nutrition. Due to the relevance of strains CNPSo 1112^T and CNPSo 2833^T as commercial inoculants for tropical forages, we performed a polyphasic analysis to determine their taxonomic positions.

Bradyrhizobium strains used in this study are listed in Table 1. Both strains are deposited at the Diazotrophic and Plant Growth Promoting Bacteria Culture Collection of Embrapa Soja (WFCC Collection # 1213, WDCM Collection # 1054), located in Londrina, State of Paraná, Brazil, and at the Center for Genomic Sciences Culture Collection (Cuernavaca, Mexico), besides other international collections. Unless otherwise indicated, strains were grown on yeast extract-mannitol agar (YMA) medium at 28^{o}C (Vincent, 1970). Stock cultures were maintained on YMA at 4^{o}C, while long-term preservation was performed in 30% glycerol at –80^{o}C and –150^{o}C, or by lyophilization.

A BOX-PCR genomic fingerprint was performed as previously described (Kaschuk *et al*., 2006), using the closest species based on the 16S rRNA phylogeny. One cluster included strains CNPSo 1112^T, CNPSo 2833^T and *B. elkanii* and *B. pachyrhizi* type strains with a similarity level of 81% (Fig. S1). Another cluster included *B. jicamae* and *B. lablabi* type strains joined with 84% of similarity. These two clusters were 75.5% similar among each other. *B. retamae* type strain occupied an isolated position in relation to the other species, with a similarity level of 73.2% (Fig. S1). Although BOX-PCR is suitable for revealing prokaryotic diversity, the results show that, within the *B. elkanii* superclade, the diversity is apparently lower than in other rhizobial superclades, especially those of fast growers, as previously observed (Menna *et al*., 2009b).

For the 16S rRNA gene analyses, the sequences were retrieved from the GenBank database and accession numbers are shown in the phylogram. Neighbor joining (NJ) and maximum likelihood (ML) phylogenies were obtained with MEGA 6 (Tamura *et al*., 2013) using the Tamura-Nei model (Tamura & Nei, 1993) and a multiple sequence alignment constructed with MUSCLE (Edgar, 2004). Tree node support was evaluated with bootstrap analysis (Felsenstein, 1985) using 1,000 pseudoreplicates. NJ and ML reconstructions gave similar results; therefore, only the ML phylogram is presented (Fig. 1). *Bradyrhizobium* species described so far were included in the 16S rRNA tree and two groups were formed—the *B. japonicum* superclade and the *B. elkanii* superclade—as reported by other authors (Delamuta *et al*., 2012; Durán *et al*., 2014a, 2014b; Menna *et al*., 2006; 2009a; Ramírez-Bahena *et al*., 2009; Yao *et al*., 2015). CNPSo 1112^T and CNPSo 2833^T were included in the *B. elkanii* superclade. *B. retamae* and *B. valentinum* were isolated, and CNPSo 1112^T formed a clade that was closely related to *B. elkanii*, *B. pachyrhizi* and *B. ferriligni*. CNPSo 2833^T clustered with *B. jicamae*, *B. erythrophlei* and *B. neotropicale* (Fig. 1). 16S rRNA sequences of the two new species shared 99.6% of identity, and the level of similarity among all species of this genus ranged from 96.4 to 100% (Table 2).

Although 16S rRNA is broadly used to appraise phylogenetic relationships among bacteria, in some genera, including *Bradyrhizobium*, it is highly conserved, thus limiting species definition (Delamuta *et al*., 2012; Menna *et al*., 2006, 2009a, 2009b; Wang & Martínez-Romero, 2000). To improve our knowledge about the ribosomal DNA region, we also analyzed the 16S–23S rRNA intergenic transcribed spacer (ITS). An NJ phylogram was build using a matrix of uncorrected distances (Willems *et al*., 2001) and revealed that CNPSo 1112^T and CNPSo 2833^T strains were distinct from other bradyrhizobia (Fig. S2). The strains showed only 82.7% of identity to each other and less than 93.1% of identity with all other *Bradyrhizobium* species (Table 2). In a pioneer study, Willems *et al.* (2003) reported that *Bradyrhizobium* strains with less than 95.5% of similarity in ITS sequences belonged to different species, showing less than 60% DNA-DNA hybridization.

Also to overcome 16S rRNA limitations, other housekeeping genes with higher rates of evolution have been used to provide more information on phylogenetic relationships, in the multilocus sequence analysis (MLSA) approach (Azevedo *et al.*, 2015; Gevers *et al.*, 2005; Ribeiro *et al.*, 2009, 2012, 2013, 2015; Rivas *et al.*, 2009; Thompson *et al.*, 2005). MLSA phylograms were constructed as described for the 16S rRNA gene, first considering *glnII*, *gyrB* and *recA* genes, since their sequences are available for all species of the genus *Bradyrhizobium*. In contrast to the 16S rRNA phylogeny (Fig 1), where CNPSo 1112^T and CNPSo 2833^T strains did not occupy well defined positions, the MLSA phylogeny grouped both strains with *B. elkanii*, *B. pachyrhizi* and *B. ferriligni*, with a bootstrap support of 99% (Fig. 2). It is worth mentioning that MLSA analysis with three housekeeping genes has been used as support for the description of novel rhizobial species (Dall´Agnol *et al.*, 2014; Delamuta *et al.*, 2013; Ribeiro *et al.*, 2015). In addition, a phylogram was built with five protein-coding genes (*glnII, gyrB*, *recA atpD* and *dnaK*) and similar results were obtained (Fig. S3). Both phylograms show that strains CNPSo 1112^T and CNPSo 2833^T do not belong to any described species of *Bradyrhizobium*. In addition, although CNPSo 1112^T and CNPSo 2833^T were more related to each other than to *B. elkanii*, *B. pachyrhizi* or *B. ferriligni*, they formed a clade with low bootstrap support (less than 75%), suggesting that they do not belong to the same species (Fig. 2, Fig. S3).

The range of nucleotide identity (NI) between described species of *Bradyrhizobium* calculated from the three concatenated genes varied from 88.1% to 96.1%, with CNPSo 1112^T and CNPSo 2833^T exhibiting 96.4% identity to each other (Table 2). The closest described species to CNPSo 1112^T and CNPSo 2833^T was *B. elkanii*, with nucleotide identities of 95.6% and 96.1%, and *B. pachyrhizi*, with nucleotide identities of 95.9% and 95.8%, respectively. These values are lower than the 97.0% suggested as a cut-off level for definition of *Bradyrhizobium* species (Durán *et al.*, 2014a), indicating that the CNPSo strains represent two new, distinct species.

Average nucleotide identity (ANI) of genome sequences represents an alternative to DNA-DNA hybridization (DDH) to estimate genome relatedness and has been recently used in rhizobial taxonomy (Dall'Agnol *et al.*, 2013, 2014; Delamuta *et al.*, 2013; Durán *et al.*, 2014a, 2014b). As suggested by Richter & Rosselló-Móra (2009), an ANI of 95–96% corresponds to 70% DDH, the standard level for prokaryotic species circumscription. ANI was estimated with the genome sequences of CNPSo 1112^T (SAMN03784761, obtained in this study), CNPSo 2833^T (SAMN03782074, obtained in this study) and *B pachyrhizi* PAC 48^T (SAMN03782120, obtained in this study), and the genomes of type strains of *B. elkanii* (NZ_ARAG00000000), *B. paxllaeri* LMTR 21^T, *B. icense* LMTR 13^T, *B. valentinum* LmjM3^T *(*SAMN02688507), *B. retamae* Ro19^T (SAMN02689496), *B. lablabi* CCBAU 23086^T (SAMN02689497) and *B. jicamae* PAC 68^T (SAMN02689491) (from the studies of Durán *et al.*, 2014a, 2014b, kindly supplied by the authors). ANI values were calculated with JSpecies (Richter & Rosselló-Móra, 2009) and Mummer for sequence alignment. Both CNPSo 1112^T and CNPSo 2833^T showed ANI values below the species circumscription threshold between each other (91.2%) and in relation to all type strains (lower than 90.6%), showing that these strains represent two novel species (Table 3).

To determine the DNA G+C content of CNPSo 1112^T and CNPSo 2833^T, genome contigs were concatenated and the proportions of G+C bases were calculated with BioEdit (Hall, 1999). The CNPSo 1112^T genome had a G+C content of 63.49 mol% while that of CNPSo 2833^T was of 62.81 mol%, which fit within the range reported for *Bradyrhizobium* species (Xu *et al.*, 1995).

The main agronomic feature of CNPSo 1112^T and CNPSo 2833^T is their high efficiency in fixing atmospheric N_2 with the hosts from which they have been isolated, perennial soybean and desmodium, respectively. As genes related to nodulation and N_2-fixation capacity may provide additional information about their symbiotic properties, we investigated both features. Under sterile substrate conditions, CNPSo 1112^T and CNPSo 2833^T had their effectiveness in nodulating and fixing nitrogen with their respective host legumes confirmed (data not shown). In addition, both strains were unable to nodulate soybean [*Glycine max* (L.) Merr.] and formed ineffective nodules on common bean (*Phaseolus vulgaris* L.). CNPSo 2833^T formed effective nodules when in symbiosis with siratro (*Macroptilium atropurpureum*), while CNPSo 1112^T formed ineffective nodules with this legume. CNPSo 2833^T was unable to nodulate perennial soybean (data not shown).

nifH and *nodC* genes were obtained in this study (according to Menna *et al*., 2011) or retrieved from the Genbank database and accession numbers are shown in the phylograms. In both *nifH* (Fig. 3) and *nodC* (Fig. S4) phylogenetic trees CNPSo 1112^T and CNPSo 2833^T clustered together, but separated from all other *Bradyrhizobium* species, indicating evolutionary specificity of nitrogen-fixing related genes.

Fatty-acid profiles were determined using the MIDI Sherlock Microbial Identification System (MIDI, 2001) with the TSBA6 database after growth on YMA (Delamuta *et al*., 2013) till the end of the exponential growth phase (5 days). The main fatty acids of CNPSo 1112^T and CNPSo 2833^T were $C_{16:0}$ and summed feature 8 ($C_{18:1}\omega6c/C_{18:1}\omega7c$), typical of the *Bradyrhizobium* genus (Tighe *et al.*, 2000), but with different concentrations, and $C_{19:0}$ cyclo ω8c, with CNPSo 2833^T strain exhibiting a higher proportion of this fatty acid in comparison with CNPSo 1112^T (Table S1).

Phenotypic tests were performed and the most relevant data are shown in Table 4. Type strains of *B. elkanii*, *B. pachyrhizi*, *B. lablabi*, *B. jicamae* and *B. retamae*, representing the closest species, were also included in the analyses. Morpho-physiological tests included utilization of carbon sources using the API 50CH kit (BioMérieux), according to the manufacturer’s instructions, with YM-minus-mannitol used as the basal medium. Growth in distinct conditions of temperature, pH and salinity were verified in liquid YM. Other features evaluated were the capacity to grow in liquid Luria-Bertani (LB) medium; urease activity in YMA medium with red phenol; colony size and resistance to antibiotics using the disc diffusion method in YMA plates. CNPSo 1112^T and CNPSo 2833^T differed from each other primarily in relation to carbon source utilization, growth at 37°C, in LB and in colony size. We observed that, in general, CNPSo 1112^T, CNPSo 2833^T, *B. elkanii* and *B. pachyrhizi* are more resistant to antibiotics when compared to the other related species of *Bradyrhizobium.* However, it is worth mentioning that phenotypic data may give limited information because, frequently, these characteristics are encoded on the accessory genome and can be easily lost, resulting in incongruence with genetic data (Ormeño-Orrillo & Martínez-Romero, 2013).

After an extensive polyphasic analysis comprising genotypic, phenotypic and phylogenetic data, our results strongly indicate that CNPSo 1112^T and CNPSo 2833^T strains represent two new species distinct from all described species in the *Bradyrhizobium* genus. We propose the names *Bradyrhizobium tropiciagri* sp. nov. for CNPSo 1112^T and *Bradyrhizobium embrapense* sp. nov. for CNPSo 2833^T.

Description of *Bradyrhizobium tropiciagri* sp. nov.

B. tropiciagri (tro.pi.ci.a'gri. L. adj. tropicus tropical; L. masc. n. ager pasture; N.L. gen. n. tropiciagri of a tropical pasture). Named after its nitrogen-fixation capacity with an important tropical pasture legume.

Cells are Gram-negative, non-spore-forming rods. Colonies are smaller than 1 mm in diameter, circular, convex, opaque, with low production of mucus and slightly pink, when grown in YMA medium containing Congo red after 7 days of growth at 28°C. Produces alkaline reaction in YMA containing bromothymol blue. The generation time is 7.42 h in YM broth. The type strain grows at pH values ranging from 4.5 to 8.0, with optimal growth at pH 6.8. It is not able to grow at 37°C or above, in the presence of 1% NaCl or in Luria-Bertani broth.

Urease activity is positive. It assimilates D-arabinose, L-arabinose, D-ribose, D-galactose, D-mannose and D-fucose as carbon-sources, shows weak growth with D-xylose, L-xylose, D-adonitol, L-rhamnose, dulcitol, D-mannitol, D-sorbitol, esculin iron citrate, D-lyxose, L-fucose, D-arabitol, glycerol and D-fructose and did not present growth with erythritol, methyl-βD-xylopyranoside, L-sorbose, D-glucose, inositol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-tagatose, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. It is resistant to the antibiotics erythromycin, cefuroxime, streptomycin, tetracycline, nalidixic acid, chloramphenicol and bacitracin and moderately sensitive to neomycin.

The type strain is CNPSo 1112^T (=SMS 303^T =BR 1009^T =SEMIA 6148^T =LMG 28867^T), isolated from a nodule of perennial soybean (*Neonotonia wightii*) in Brazil and very effective in fixing nitrogen with the host legume. Its G+C content is of 63.49 mol%.

Description of *Bradyrhizobium embrapense* sp. nov.

B. embrapense (em.brap.en'se. N.L. neut. adj. embrapense, of Embrapa (Empresa Brasileira de Pesquisa Agropecuária).

Cells are Gram-negative, non-spore-forming rods. Colonies are 0.93-1.15 mm in diameter, circular, convex, translucent, with low production of mucus and slightly pink when grown in YMA medium containing Congo red after 7 days of growth at 28°C. Produces an alkaline reaction in YMA containing bromothymol blue. The generation time is 7.49 h in YM broth. The type strain grows at pH values ranging from 4.5 to 8.0, with optimal growth at pH 6.8. It is able to grow at 37°C and in Luria-Bertani broth but not at 40°C or in the presence of 1% NaCl. Urease activity is positive. It assimilates D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-galactose, D-glucose, D-mannose, D-melibiose, starch, glycogen, gentiobiose, D-lyxose, D-fucose, L-fucose potassium gluconate and potassium 5-ketogluconate as carbon sources, shows weak growth with glycerol, D-fructose, L-rhamnose, D-mannitol, D-sorbitol and D-arabitol and did not present growth with erythritol, D-adonitol, methyl-βD-xylopyranoside, L-sorbose, dulcitol, inositol, methyl-αD-mannopyranoside, methyl-αD-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin iron citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-saccharose, D-trehalose, inulin, D-melezitose, D-raffinose, xylitol, D-turanose, D-tagatose, L-arabitol and potassium 2-ketogluconate. It is resistant to the antibiotics erythromycin, cefuroxime, nalidixic acid, tetracycline, chloramphenicol and bacitracin, moderately sensitive to neomycin and sensitive to streptomycin.

The type strain is CNPSo 2833^T (=CIAT 2372^T =BR 2212^T =SEMIA 6208^T =U674^T) isolated from a nodule of *Desmodium heterocarpon* in Colombia and very effective in fixing nitrogen with the host legume. Its G+C content is of 62.81 mol%.

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Table 1. Strains used in this study.

Species/strain name	**Other strain nomenclature**	**Original host species**	**Geographical origin**	**Reference**
B. tropiciagri CNPSo 1112^T	SMS 303^T, BR 1009^T, SEMIA 6148^T, LMG 28867 ^{T}	*Neonotonia wightii*	Brazil	Delamuta *et al*. (2012)
B. embrapense CNPSo 2833^T	CIAT 2372^T, BR 2212^T, SEMIA 6208^T, U674^T	*Desmodium heterocarpon*	Colombia	Delamuta *et al*. (2012); Menna *et al*. (2009a)
B. elkanii USDA 76^T	ATCC 49852^T, DSM 11554^T, NBRC 14791^T, LMG 6134^T, CNPSo 62^T	*Glycine max*	USA	Kuykendall *et al*. (1992)
B. pachyrhizi PAC 48^T	LMG 24246^T, CECT 7396^T, CNPSo 2077^T	*Pachyrhizus erosus*	Costa Rica	Ramírez-Bahena *et al*. (2009)
B. jicamae PAC 68^T	LMG 24556^T, CECT 7395^T, CNPSo 2076^T	*Pachyrhizus erosus*	Honduras	Ramírez-Bahena *et al*. (2009)
B. lablabi CCBAU 23086^T	LMG 25572^T, HAMBI 3052^T, CNPSo 2585^T	*Lablab purpureus*	China	Chang *et al*. (2011)
B. retamae Ro19^T	LMG 27393^T, CECT 8261^T, CNPSo 2586^T	*Retama monosperma*	Morocco	Guerrouj *et al*. (2013)

Table 2. Percentage nucleotide identity between *B. tropiciagri* CNPSo 1112^T and *B. embrapense* CNPSo 2833^T strains and the type strains of other *Bradyrhizobium* species in the 16S rRNA and protein-coding genes. ND, not determined.

Length of the aligned regions: 16S rRNA gene (1262 bp); ITS (1091 bp); *atpD* (434 bp); *glnII* (505 bp); *recA* (360 bp); *dnaK* (238 bp); *gyrB* (562 bp)

Species	**Gene**								
	16S rRNA	**ITS**	***atpD***	***glnII***	***recA***	***dnaK***	***gyrB***	**MLSA (3 genes)***	**MLSA (5 genes)**
Between CNPSo 1112^T and CNPSo 2833^T	99.6	82.7	96.2	96.6	96.3	93.6	96.1	96.4	96.0
Similarity of CNPSo 1112^T (normal) and CNPSo 2833^T (bold) with type strains of:									
B. elkanii	**99.6**-99.8	78.9-**93.1**	**95.3**-96.2	96.4-**97.4**	94.4	**93.2**-96.6	95.8-**96.1**	95.6-**96.1**	**95.6**-95.9
B. pachyrhizi	**99.6**-99.9	85.9-**93.1**	**95.5**-96.9	95.8-**96.4**	**94.4**-95.5	**93.2**-96.6	96.1	**95.8**-95.9	**95.4**-96.2
B. erythrophlei	99.6-**100**	75.4-**85.2**	ND	93.8	**92.5**-94.1	ND	**92.5**-93.2	**93**-93.7	ND
B. ferriligni	**97.6**-97.8	78.1-**92.1**	ND	95.8-**96.4**	95.2	ND	**92.2**-93.8	**94.4**-94.9	ND
B. jicamae	99.2-**99.6**	77.4-**88**	**93.2**-93.7	**89.5**-90.6	**90.8**-91.1	88.6-**90.3**	**90.3**-90.5	**90.1**-90.7	**90.8**-91.1
B. lablabi	99.5-**99.7**	73.3-**83.8**	**92.5**-94.4	**90.2**-90.4	94.1	88.6-**89.4**	89.1-**89.3**	90.8	**91**-91.3
B. retamae	99.1-**99.3**	72.7-**83.3**	**91.1**-91.6	**89.7**-90.8	93-**93.8**	88.2-**89**	**87.5**-87.6	**89.9**-90.1	**90**-90.2
B. icense	99.6-**99.8**	ND	92.3-**92.5**	**90**-90.2	91.9-**93.3**	89.9-**90.3**	**88**-88.5	90-**90.1**	90.5-**90.6**
B. paxllaeri	99.6-**99.8**	ND	92.5	**90.2**-90.8	**91.1**-91.3	89.4-**90.3**	89.8-**90**	**90.4**-90.6	90.8
B. valentinum	99.3-**99.6**	ND	**91.4**-91.8	**85.7**-86.5	93	87.8-**88.2**	**87.5**-88.2	**88.2**-88.8	**88.9**-89.3
B. diazoefficiens	96.9-**97.3**	70.3-**77.9**	**93.5**-95.1	89.3-**89.5**	91.3-**91.9**	87.8	**93.2**-94	91.6	**91.5**-91.9
B. canariense	96.9-**97.3**	67.2-**76.5**	**93.2**-93.9	87.3-**87.7**	**92.7**-93.8	86.5	**91.8**-92.3	**90.6**-90.9	**90.6**-91
B. betae	96.8-**97.2**	68.3-**75.8**	**92.1**-92.8	**87.7**-88.7	**91.9**-93	89-**90.3**	**91.4**-92	**90.2**-91.1	**90.6**-91.2
B. japonicum	97.3-**97.6**	68.5-**74**	**94.6**-95.3	**88.7**-89.1	**91.9**-92.5	**87.3**-88.6	**92.5**-93.4	**91**-91.6	**91.3**-92
B. yuanmingense	96.9-**97.3**	68.2-**75.4**	**95.3**-96	**88.9**-89.1	**91.9**-93	87.3	**91.3**-92.5	**90.6**-91.4	**91.2**-91.9
B. liaoningense	97.2-**97.6**	67.1-**74.3**	91.6-**91.8**	89.9	**93.3**-94.4	**87.3**-88.2	**92.2**-92.7	**91.6**-92.1	**91.2**-91.6
B. arachidis	96.5-**96.9**	68-**75.2**	**92.8**-93.2	**89.5**-89.7	**93.3**-93.6	**86.9**-87.8	**91.4**-92.3	**91.2**-91.7	**91**-91.6
B. huanghuaihaiense	96.7-**97.1**	67.8-**75.1**	**93.5**-94.1	**88.3**-88.5	**91.1**-91.9	**86.9**-88.6	**92.2**-92.9	**90.5**-91.1	**90.7**-91.4
B. cytisi	96.5-**96.9**	66.5-**75.5**	**93.5**-93.7	**87.7**-87.9	**90.8**-91.3	87.3-**87.8**	**90**-90.5	**89.4**-89.8	**90**-90.3

B. daqingense	97.1-**97.5**	67.6-**77.5**	91.8-**92.3**	89.3-**89.5**	**90.5**-91.9	88.2	**91.4**-92.3	**90.5**-91.1	**90.6**-90.9
B. iriomotense	96.4-**96.8**	**71.6**-75.2	**93.7**-95.3	86.7-**87.1**	**89.1**-90.2	89.4-**89.9**	**92.2**-92.3	**89.6**-89.8	**90.5**-90.9
B. rifense	96.5-**96.9**	67.3-**74.2**	**92.8**-93.5	88.9-**89.1**	**91.6**-92.2	88.2-**88.6**	**90.9**-92.2	**90.4**-91	**90.7**-91.2
B. ganzhouense	96.7-**97.1**	ND	**93**-93.5	88.3-**88.5**	90.5	**88.6**-89	**92**-92.5	**90.4**-90.5	**90.7**-90.9
B. ottawaense	97.1-**97.5**	ND	**92.1**-92.3	88.7-**89.3**	**91.6**-92.2	**86.5**-87.3	**90.9**-92	**90.5**-90.8	**90.4**-90.7
B. neotropicale	98.4-**98.8**	70.1-**79.7**	ND	88.7	**91.1**-92.2	ND	**92.2**-92.3	**90.6**-91	ND
B. ingae	96.4-**96.8**	ND	ND	89.1-**89.3**	**88.8**-90	ND	90.5	**89.6**-89.9	ND
B. manausense	96.4-**96.8**	ND	ND	88.5-**89.1**	**91.6**-92.7	ND	**91.4**-91.6	**90.6**-90.8	ND
B. denitrificans	96.6-**97.0**	**52.2**-53.2	**91.1**-92.1	86.9-**87.9**	89.4	87.3-**88.2**	89.1-**89.8**	88.4-**89**	89-**89.4**
B. oligotrophicum	96.7-**97.1**	**52**-54.3	**91.1**-91.6	86.1-**86.7**	90.8	88.2	88.2-**88.7**	88.1-**88.5**	88.8-**89**

*Three genes (*glnII*, *gyrB* and *recA*).

Table 3. Percentages of average nucleotide identity (ANI) of genome sequences of *B. tropiciagri*, *B. embrapense* and related species. ANI values were obtained using JSpecies (Richter & Rosselló-Móra, 2009) and Mummer for sequence alignment.

	Strain used as reference	
Strain used as query	*B. tropiciagri* CNPSo 1112^T	*B. embrapense* CNPSo 2833^T
B. tropiciagri CNPSo 1112^T	100	91.2
B. embrapense CNPSo 2833^T	91.2	100
B. pachyrhizi PAC 48^T	90.6	90.3
B. elkanii USDA 76^T	90.6	90.6
B. jicamae PAC 68^T	85.3	85.4
B. paxllaeri LMTR 21^T	85.4	85.4
B. lablabi CCBAU 23086^T	85.4	85.3
B. retamae Ro19^T	85	85
B. icense LMTR 13^T	85.1	85.1
B. valentinum LmjM3^T	85.1	85.1

Table 4. Distinctive phenotypic features of *B. tropiciagri*, *B. embrapense* and their closest relatives.

Strains: 1, *B. tropiciagri* CNPSo 1112^T; 2, *B. embrapense* CNPSo 2833^T; 3, *B. elkanii* USDA 76^T; 4, *B. pachyrhizi* PAC 48^T; 5, *B. jicamae* PAC 68^T; 6, *B. lablabi* CCBAU 23086^T; 7, *B. retamae* Ro19^T. Data were obtained in this study. +, Growth; −, no growth; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Carbon source utilization*							
L-Arabinose	+	+	+	+	+	w	+
D-Xylose	w	+	+	+	+	w	w
L-Xylose	w	+	+	+	+	+	+
D-Adonitol	w	−	w	w	−	w	−
D-Galactose	+	+	+	+	+	+	w
D-Glucose	−	+	w	w	w	w	w
D-Mannose	+	+	+	+	+	w	−
L-Rhamnose	w	w	w	w	+	+	w
Dulcitol	w	−	w	−	−	−	−
D-Mannitol	w	w	w	w	−	w	−
D-Sorbitol	w	w	w	w	−	−	−
Esculin Iron Citrate	w	−	w	−	−	+	w
D-Melibiose	−	+	−	−	−	−	−
Starch	−	+	+	+	+	+	+
Glycogen	−	+	−	−	−	+	−
Xylitol	−	−	w	−	−	−	−
Gentiobiose	−	+	−	−	−	−	−
D-Lyxose	w	+	+	+	+	+	+
D-Fucose	+	+	+	+	+	+	w
L-Fucose	w	+	+	+	+	+	+
D-Arabitol	w	w	w	w	−	w	−
L-Arabitol	−	−	w	w	−	−	−
Potassium Gluconate	−	+	−	−	−	−	−
Potassium 5-Ketogluconate	−	+	−	−	−	−	−
Growth in/at:							
pH 4.5	+	+	−	w	−	−	−
37 °C	−	+	−	−	−	−	−
LB broth	−	+	−	−	−	−	−
Enzyme activity							
Urease	+	+	+	+	−	+	−
Resistance to (µg per disc)							
Erythromycin (15)	+	+	+	+	w	+	+
Cefuroxime (30)	+	+	+	+	−	+	−
Neomycin (30)	w	w	−	−	−	−	w
Tetracycline (30)	+	+	+	+	+	−	−
Streptomycin (10)	+	−	+	+	−	−	−

Colony size (mm) after 7 d of incubation in YMA[a]	< 1	1.07	< 1	1.37	< 1	< 1	< 1

* Carbon source utilization was evaluated with the API 50CH kit (bioMérieux)

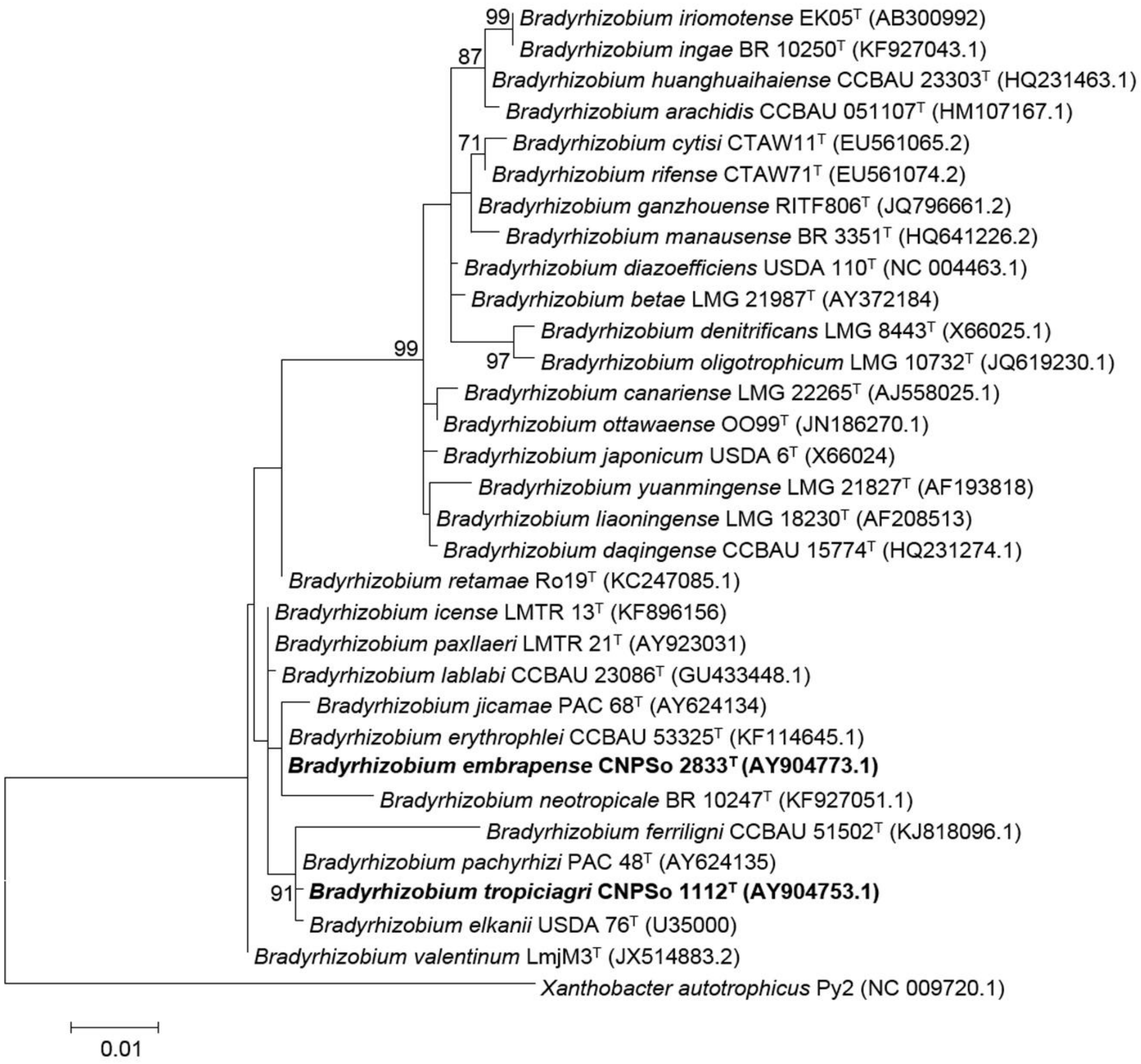
[a] mean of six colonies

Figure legends

Figure 1. Maximum likelihood phylogeny based on 16S rRNA gene sequence. Accession numbers are indicated within parenthesis. Strains of the novel species are shown in boldface. Bootstrap values >70% are indicated at the nodes. *Xanthobacter autotrophycus* Py2 was used as outgroup. Bar indicates 1 substitution per 100 nucleotide positions.

Figure 2. Maximum likelihood phylogeny based on concatenated *glnII*+*gyrB*+recA gene sequences showing the relationships between strains of the novel species (shown in bold) and other members of the *Bradyrhizobium* genus. Accession numbers are indicated in Table S2. Only bootstrap values >70 % are indicated at the nodes, except for the new *Bradyrhizobium* species. *Xanthobacter autotrophycus* Py2 was used as outgroup. Bar indicates 2 substitutions per 100 nucleotide positions.

Figure 3. Maximum likelihood phylogeny of *nifH* (594 aligned positions) gene sequence. Accession numbers are indicated within parenthesis. Strains of the novel species are shown in boldface. Bootstrap values >70% are indicated at the nodes. Bar, 2 substitutions per 100 nucleotide positions.


99
87
71
99
97
91
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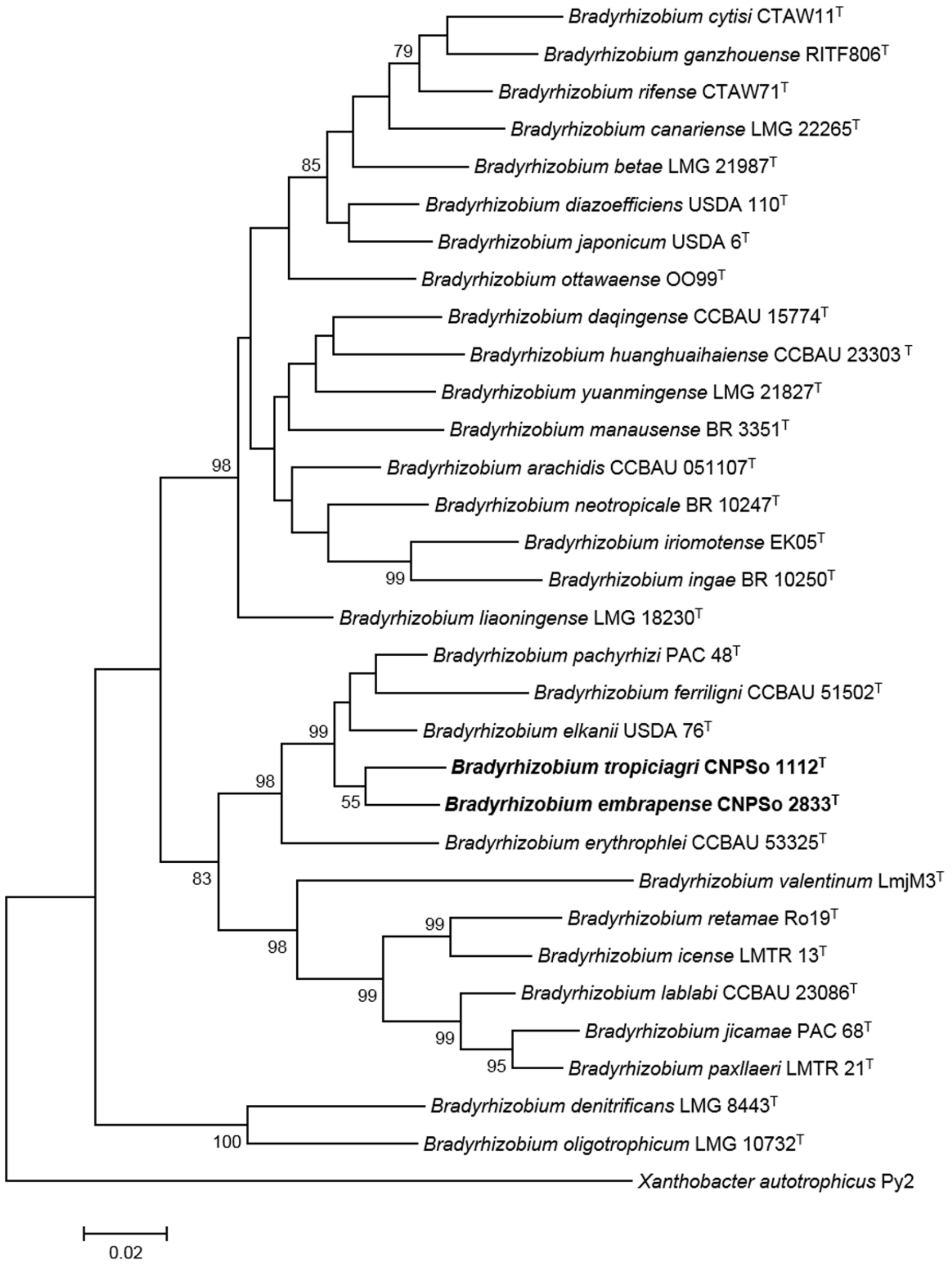
Bradyrhizobium ingae BR 10250^T (KF927043.1)
Bradyrhizobium huanghuaihaiense CCBAU 23303^T (HQ231463.1)
Bradyrhizobium arachidis CCBAU 051107^T (HM107167.1)
Bradyrhizobium cytisi CTAW11^T (EU561065.2)
Bradyrhizobium rifense CTAW71^T (EU561074.2)
Bradyrhizobium ganzhouense RITF806^T (JQ796661.2)
Bradyrhizobium manausense BR 3351^T (HQ641226.2)
Bradyrhizobium diazoefficiens USDA 110^T (NC 004463.1)
Bradyrhizobium betae LMG 21987^T (AY372184)
Bradyrhizobium denitrificans LMG 8443^T (X66025.1)
Bradyrhizobium oligotrophicum LMG 10732^T (JQ619230.1)
Bradyrhizobium canariense LMG 22265^T (AJ558025.1)
Bradyrhizobium ottawaense OO99^T (JN186270.1)
Bradyrhizobium japonicum USDA 6^T (X66024)
Bradyrhizobium yuanmingense LMG 21827^T (AF193818)
Bradyrhizobium liaoningense LMG 18230^T (AF208513)
Bradyrhizobium daqingense CCBAU 15774^T (HQ231274.1)
Bradyrhizobium retamae Ro19^T (KC247085.1)
Bradyrhizobium icense LMTR 13^T (KF896156)
Bradyrhizobium paxllaeri LMTR 21^T (AY923031)
Bradyrhizobium lablabi CCBAU 23086^T (GU433448.1)
Bradyrhizobium jicamae PAC 68^T (AY624134)
Bradyrhizobium erythrophlei CCBAU 53325^T (KF114645.1)
Bradyrhizobium embrapense CNPSo 2833^T (AY904773.1)
Bradyrhizobium neotropicale BR 10247^T (KF927051.1)
Bradyrhizobium ferriligni CCBAU 51502^T (KJ818096.1)
Bradyrhizobium pachyrhizi PAC 48^T (AY624135)
Bradyrhizobium tropiciagri CNPSo 1112^T (AY904753.1)
Bradyrhizobium elkanii USDA 76^T (U35000)
Bradyrhizobium valentinum LmjM3^T (JX514883.2)
Xanthobacter autotrophicus Py2 (NC 009720.1)
0.01

Fig. 01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Bradyrhizobium ganzhouense RITF806^{T}
79
Bradyrhizobium rifense CTAW71^{T}
Bradyrhizobium canariense LMG 22265^{T}
85
Bradyrhizobium betae LMG 21987^{T}
Bradyrhizobium diazoefficiens USDA 110^{T}
Bradyrhizobium japonicum USDA 6^{T}
Bradyrhizobium ottawaense OO99^{T}
Bradyrhizobium daqingense CCBAU 15774^{T}
Bradyrhizobium huanghuaihaiense CCBAU 23303 ^{T}
Bradyrhizobium yuanmingense LMG 21827^{T}
Bradyrhizobium manausense BR 3351^{T}
98
Bradyrhizobium arachidis CCBAU 051107^{T}
Bradyrhizobium neotropicale BR 10247^{T}
Bradyrhizobium iriomotense EK05^{T}
99
Bradyrhizobium ingae BR 10250^{T}
Bradyrhizobium liaoningense LMG 18230^{T}
Bradyrhizobium pachyrhizi PAC 48^{T}
Bradyrhizobium ferriligni CCBAU 51502^{T}
99
Bradyrhizobium elkanii USDA 76^{T}
Bradyrhizobium tropiciagri CNPSo 1112^{T}
98
55
Bradyrhizobium embrapense CNPSo 2833^{T}
Bradyrhizobium erythrophlei CCBAU 53325^{T}
83
Bradyrhizobium valentinum LmjM3^{T}
99
Bradyrhizobium retamae Ro19^{T}
98
Bradyrhizobium icense LMTR 13^{T}
Bradyrhizobium lablabi CCBAU 23086^{T}
99
Bradyrhizobium jicamae PAC 68^{T}
99
95
Bradyrhizobium paxllaeri LMTR 21^{T}
Bradyrhizobium denitrificans LMG 8443^{T}
100
Bradyrhizobium oligotrophicum LMG 10732^{T}
Xanthobacter autotrophicus Py2
0.02

Fig. 02

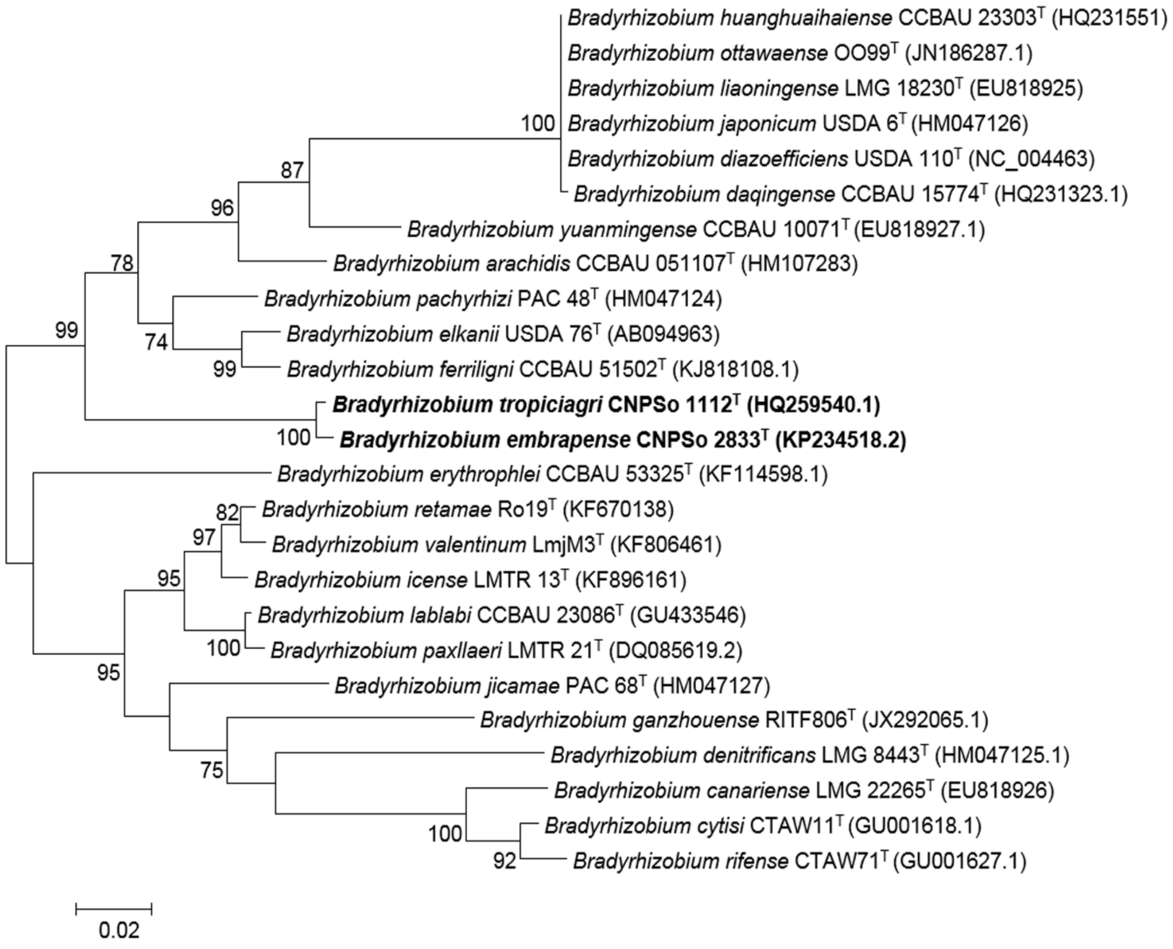
Bradyrhizobium huanghuaihaiense CCBAU 23303T (HQ231551)
Bradyrhizobium ottawaense OO99T (JN186287.1)
Bradyrhizobium liaoningense LMG 18230T (EU818925)
Bradyrhizobium japonicum USDA 6T (HM047126)
Bradyrhizobium diazoefficiens USDA 110T (NC_004463)
Bradyrhizobium daqingense CCBAU 15774T (HQ231323.1)
Bradyrhizobium yuanmingense CCBAU 10071T (EU818927.1)
Bradyrhizobium arachidis CCBAU 051107T (HM107283)
Bradyrhizobium pachyrhizi PAC 48T (HM047124)
Bradyrhizobium elkanii USDA 76T (AB094963)
Bradyrhizobium ferriligni CCBAU 51502T (KJ818108.1)
Bradyrhizobium tropiciagri CNPSo 1112T (HQ259540.1)
Bradyrhizobium embrapense CNPSo 2833T (KP234518.2)
Bradyrhizobium erythrophlei CCBAU 53325T (KF114598.1)
Bradyrhizobium retamae Ro19T (KF670138)
Bradyrhizobium valentinum LmjM3T (KF806461)
Bradyrhizobium icense LMTR 13T (KF896161)
Bradyrhizobium lablabi CCBAU 23086T (GU433546)
Bradyrhizobium paxllaeri LMTR 21T (DQ085619.2)
Bradyrhizobium jicamae PAC 68T (HM047127)
Bradyrhizobium ganzhouense RITF806T (JX292065.1)
Bradyrhizobium denitrificans LMG 8443T (HM047125.1)
Bradyrhizobium canariense LMG 22265T (EU818926)
Bradyrhizobium cytisi CTAW11T (GU001618.1)
Bradyrhizobium rifense CTAW71T (GU001627.1)
100
87
96
78
99
74
99
100
82
97
95
100
95
75
100
92
0.02

Fig. 03

Table S1. Fatty acid profile (%) of *B. tropiciagri* CNPSo 1112^T and *B. embrapense* CNPSo 2833^T grown for 5 days at 28°C on yeast extract-mannitol agar plates. Data obtained with the MIDI system using FAME library TSBA6. Analyses performed in a GC Agilent model 6850 with an Ultra 2 column (25 m length, ID 0.2 mm, film f 0.33 µm), detector FID, carrier gas hydrogen (30 mL/min), and make-up gas nitrogen (30 mL/min), automatic injector series 7683 (liner 19251-60540), injection of 2 µL, run of 25 min. Injector temperature of 250ºC and of the detector of 300ºC. Program of the oven temperature: initial of 170ºC, raising 5ºC/min till 260ºC (hold for 18 min), then raised at 40ºC/min. till 310ºC (hold for 1.5 min).

Strains: 1, *B. tropiciagri* CNPSo 1112^T; 2, *B. embrapense* CNPSo 2833^T; 3, *B. elkanii* USDA 76^T; 4, *B. pachyrhizi* PAC 48^T; 5, *B. lablabi* CCBAU 23086^T; 6, *B. retamae* Ro19^T; 7, *B. jicamae* PAC 68^T; 8, *B. paxllaeri* LMTR 21^T; 9, *B. icense* LMTR 13^T; 10, *B. valentinum* LmjM3^T.

Fatty acid	**1**	**2**	**3**	**4**	**5**	**6**	**7**[a]	**8**[a]	**9**[a]	**10**[b]
C16:0	15.83	16.51	11.07	5.97	15.21	15.44	13.4	17.8	14	15.87
C18:1 ω7c 11-methyl					4.41	19.22	2.8	7.7	14.8	13.4
C19:0 cyclo ω8c	15.09	26.51	13.67	4.77		8.19		1.4	7.3	1.11
Summed features										
8*	69.08	56.99	75.26	89.26	80.39	57.15	70.9	70.6	45.5	64.52

* Summed feature 8= C18:1ω6c/C18:1ω7c

[a] Data from Durán *et al*. (2014a)
[b] Data from Durán *et al*. (2014b)

Table S2. Accession numbers of the sequences of the housekeeping genes used in this study. Sequence obtained in this study is shown in bold. Primers and DNA amplification conditions used in this study according to Delamuta *et al*. (2013). 16S rRNA and ITS accession numbers are shown in the phylogenetic trees.

Strain	Genome	*atpD*	*dnaK*	*glnII*	*recA*	*gyrB*
B. tropiciagri CNPSo 1112^{T}		FJ390968	FJ391008.1	FJ391048	FJ391168	HQ634890
B. embrapense CNPSo 2833^{T}		HQ634875	**KP234519.2**	GQ160500	HQ634899	HQ634891
B. elkanii USDA 76^{T}		AY386758.1	AY328392.1	AY599117.1	AY591568.1	AM418800
B. jicamae PAC 68^{T}		FJ428211	JF308945.1	FJ428204	HM047133.1	HQ873309.1
B. lablabi CCBAU 23086^{T}		GU433473.1	KF962687.1	GU433498.1	GU433522.1	JX437670.1
B. pachyrhizi PAC 48^{T}		FJ428208	JF308946.1	FJ428201.1	HM047130.1	HQ873310.1
B. retamae Ro19^{T}		KC247101.1	KF896184.1	KC247108.1	KF962711.1	KF896204.1
B. paxllaeri LMTR 21^{T}		KF896186	AY923038	KF896169	JX943617	KF896195
B. icense LMTR 13^{T}		KF896192	KF896182	KF896175	JX943615	KF896201
B. valentinum LmjM3^{T}		JX518561.2		JX518575	JX518589.2	
B. erythrophlei CCBAU 53325^{T}		NA	NA	KF114693.1	KF114669.1	KF114717.1
B. ferriligni CCBAU 51502^{T}		NA	NA	KJ818099.1	KJ818112.1	KJ818102.1
B. diazoefficiens USDA 110^{T}	NC_004463.1					
B. canariense LMG 22265^{T}		AY386739.1	AY923047.1	AY386765.1	FM253177	FM253220
B. betae LMG 21987^{T}		FM253129	AY923046.1	AB353733.1	AB353734.1	FM253217
B. japonicum USDA 6^{T}		AM168320	AM168362	AF169582	AM182158	AM418801
B. daqingense CCBAU 15774^{T}		HQ231289.1	KF962684.1	HQ231301.1	HQ231270.1	JX437669.1
B. denitrificans LMG 8443^{T}		FM253153.1	KF962685.1	HM047121.1	FM253196.1	FM253239.1
B. oligotrophicum LMG 10732^{T}		JQ619232.1	KF962688.1	JQ619233.1	JQ619231.1	KF962697.1
B. yuanmingense LMG 21827^{T}		AY386760.1	AY923039.1	AY386780.1	AM168343	FM253226
B. liaoningense LMG 18230^{T}		AY386752.1	AY923041.1	AY386775.1	AY591564.1	FM253223
B. iriomotense EK05^{T}		AB300994.1	JF308944.1	AB300995	AB300996	AB300997
B. cytisi CTAW11^{T}		GU001613.1	JQ945184.1	GU001594.1	GU001575.1	JN186292.1
B. huanghuaihaiense CCBAU 23303^{T}		HQ231682.1	JX437665.1	HQ231639.1	HQ231595.1	JX437672.1
B. rifense CTAW71^{T}		GU001617.1	JQ945187.1	GU001604.1	GU001585.1	KC569466.1
B. arachidis CCBAU 051107^{T}		HM107217.1	JX437668.1	HM107251.1	HM107233.1	JX437675.1
B. ingae BR 10250^{T}		NA	KF927055.1	KF927067.1	KF927061.1	KF927079.1
B. manausense BR 3351^{T}		NA	KF786001.1	KF785986.1	KF785992.1	KF786000.1
B. neotropicale BR 10247^{T}		NA	KJ661693.1	KJ661700.1	KJ661714.1	KJ661707.1
B. ganzhouense RITF806^{T}		JX277182.1	KP420023.1	JX277110.1	JX277144.1	KP420022.1

B. ottawaense OO99^T		HQ455212.1	JF308816.1	HQ587750.1	HQ587287.1	HQ873179.1
Xanthobacter autotrophicus Py2	AE005673.1					

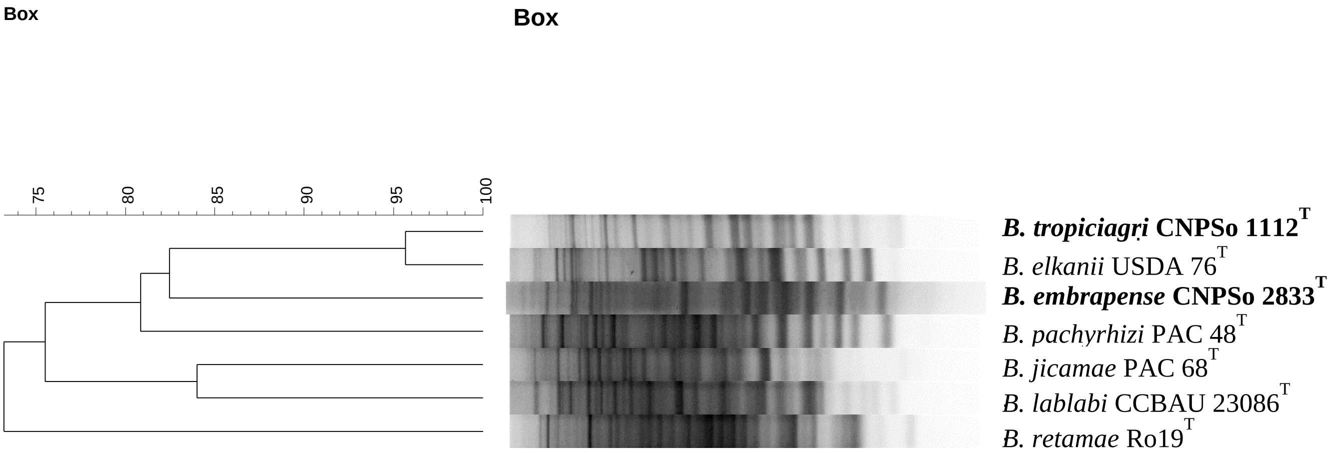

Fig. S1. Cluster analysis (UPGMA algorithm with the Jaccard coefficient) of products obtained by BOX- PCR of *Bradyrhizobium tropiciagri* CNPSo 1112^{T} and *Bradyrhizobium embrapense* CNPSo 2833^{T} strains (shown in bold) and closer bradyrhizobial species. Analysis performed with software Bionumerics v.7.1.

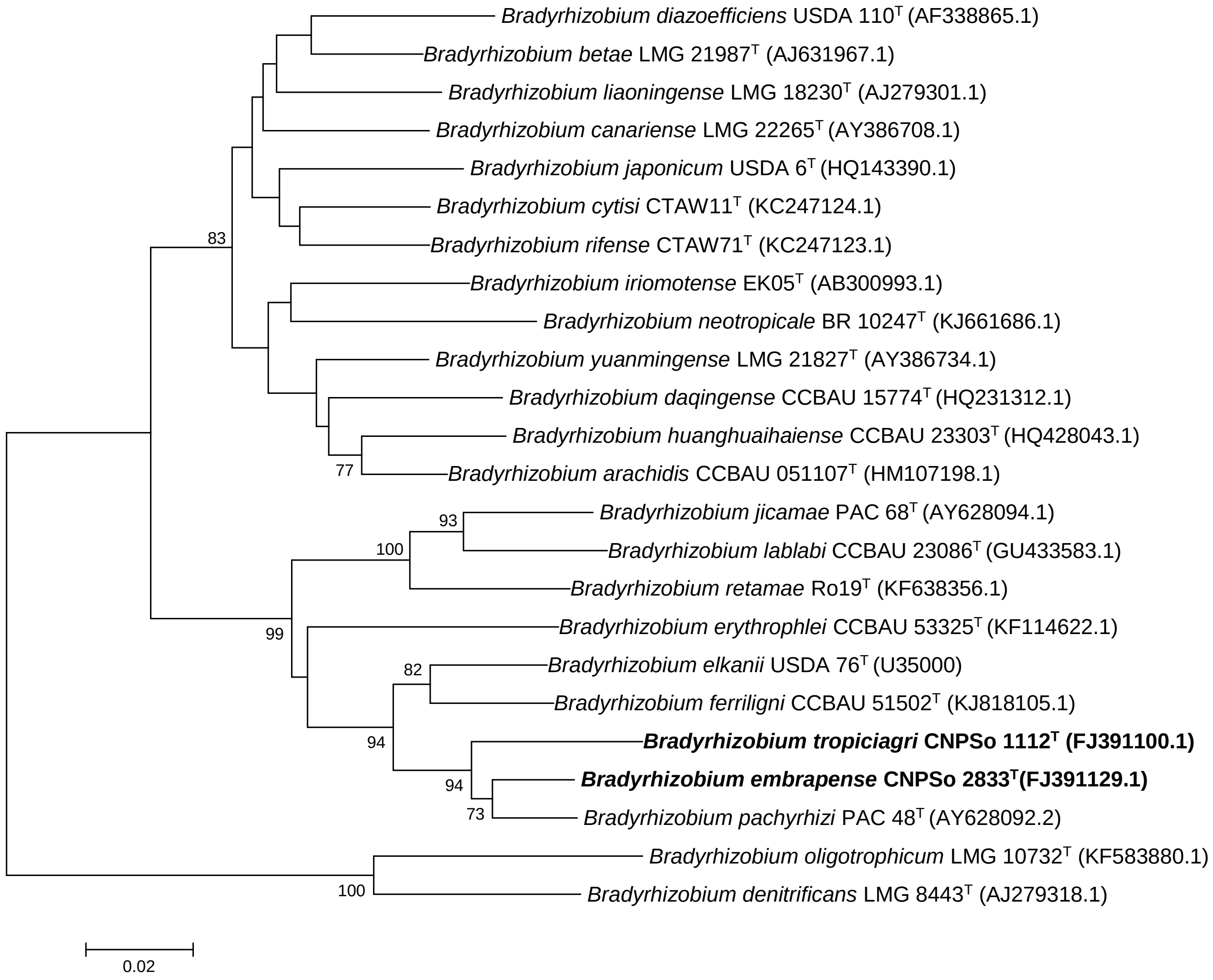

Fig. S2. Neighbor joining phylogeny based on the 16S-23S rRNA intergenic spacer (ITS) gene sequences showing the relationships between *Bradyrhizobium tropiciagri* CNPSo 1112^{T} and *Bradyrhizobium embrapense* CNPSo 2833^{T} and other species of the *Bradyrhizobium* genus. Bootstrap values >70 % are indicated at the nodes. Bar indicates 2 substitutions per 100 nucleotide positions. Accession numbers are shown in parenthesis.

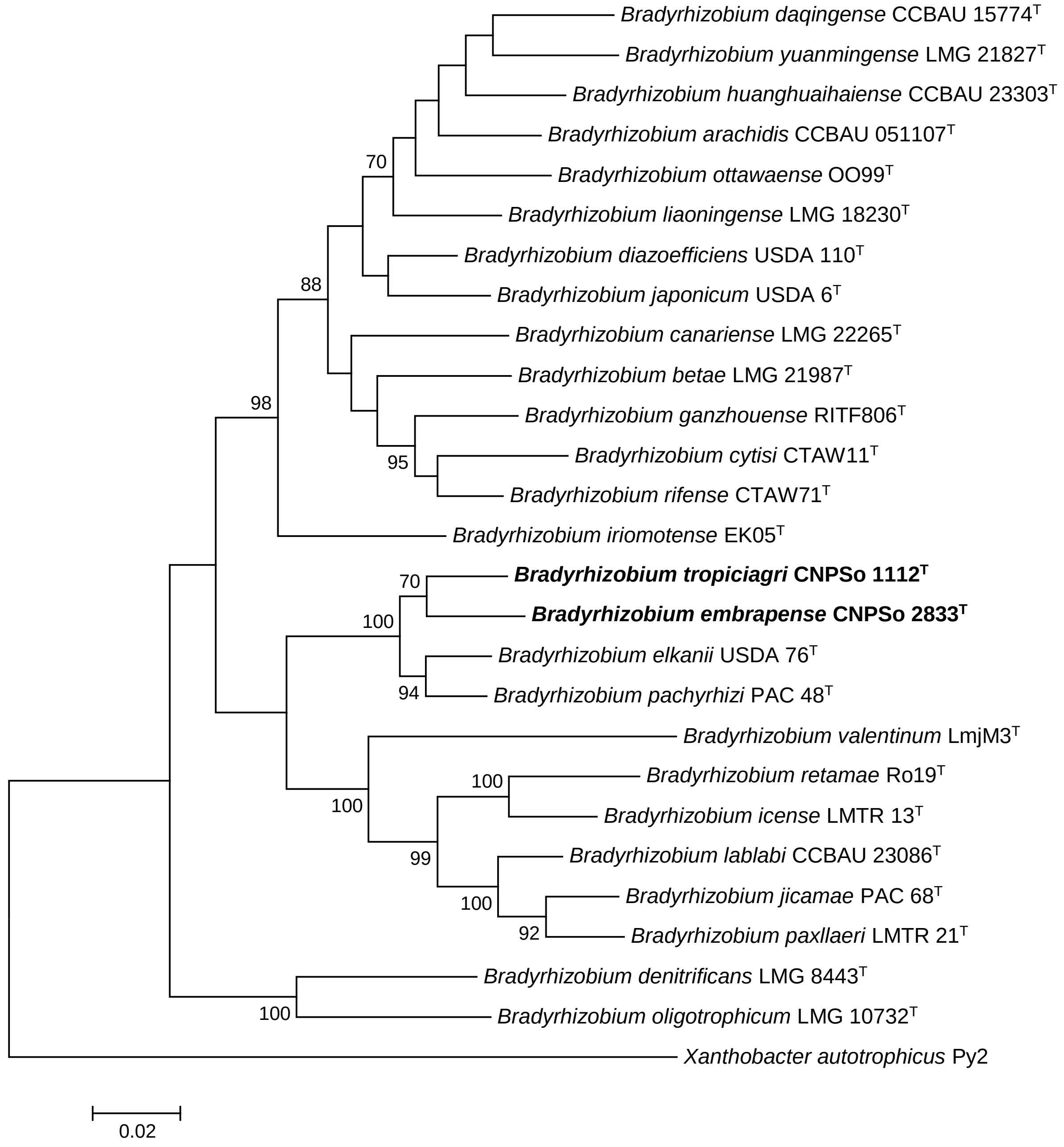

Fig. S3. Maximum likelihood phylogeny based on concatenated *atpD+dnaK+glnII+gyrB+recA* gene sequences showing the relationships between *Bradyrhizobium tropiciagri* CNPSo 1112^{T} and *Bradyrhizobium embrapense* CNPSo 2833^{T} and other members of the *Bradyrhizobium* genus. Only bootstrap values >70 % are indicated at the nodes, except for the node of the new species. *Xanthobacter autotrophicus* Py2 was used as outgroup. Bar indicates 2 substitutions per 100 nucleotide positions.

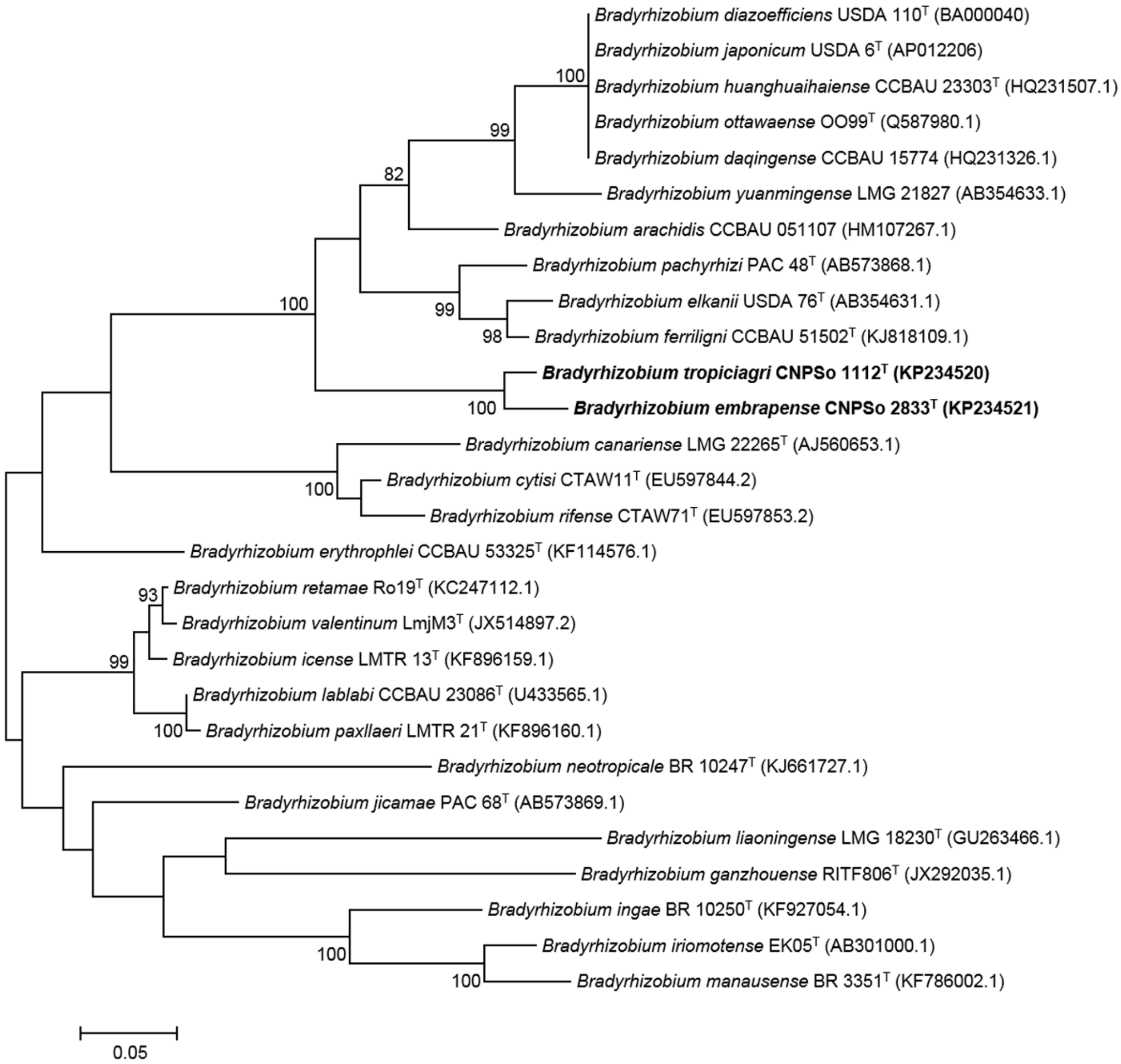

Fig. S4. Maximum likelihood phylogeny based on *nodC* (441 aligned positions) gene sequences showing the relationships between *Bradyrhizobium tropiciagri* CNPSo 1112^{T} and *Bradyrhizobium embrapense* CNPSo 2833^{T} and other members of the *Bradyrhizobium* genus. Bootstrap values >70 % are indicated at the nodes. Bar indicates 5 substitutions per 100 nucleotide positions. Accession numbers of *nodC* genes are shown in parenthesis.

5 CAPÍTULO 02

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

Jakeline Renata Marçon Delamuta,[1,2] Renan Augusto Ribeiro,[1,2], Ernesto Ormeño-Orrillo,[3] Itamar Soares Melo[4], Esperanza Martínez-Romero,[3] Mariangela Hungria[1,2]

[1]*Embrapa Soja, C.P. 231, 86001-970, Londrina, Paraná, Brazil.*
[2]*Universidade Estadual de Londrina, Dept. of Microbiology, Cx. Postal 60001, 86051-990, Londrina, Paraná, Brazil.*
[3]*Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico.*
[4]*Embrapa Meio Ambiente, C.P. 69, 13820-000, Jaguariúna, São Paulo, Brazil.*

Number of text pages	18
Number of Tables	03
Number of Figures	02
Number of Supplementary Tables	05
Number of Supplementary Figures	04

*Corresponding author:
Mariangela Hungria
Embrapa Soja
Cx. Postal 231
86001-970 Londrina, Paraná, Brazil
Fax: (+55)4333716100
Telephone: (+55)4333716206
E-mail: mariangela.hungria@embrapa.br; biotecnologia.solo@hotmail.com

E-mail addresses:
J.R.M. Delamuta (jake_renata@hotmail.com); R.A. Ribeiro (renan.ribeiro@embrapa.br; renanribeiro83@hotmail.com); E. Ormeño-Orrillo (eormeno.orrillo@gmail.com; eormeno@ccg.unam.mx); Itamar S. Melo (itamar.melo@embrapa.br); E. Martínez-Romero (emartine@ccg.unam.mx; esperanzaeriksson@yahoo.com.mx).

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

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Ernesto Ormeño-Orrillo,[3] Itamar Soares Melo,[4]
Esperanza Martínez-Romero[3] and Mariangela Hungria[1,2]

[1]Embrapa Soja, C.P. 231, 86001-970 Londrina, Paraná, Brazil

[2]Universidade Estadual de Londrina, Dept of Microbiology, C.P. 60001, 86051-990 Londrina, Paraná, Brazil

[3]Centro de Ciencias Genómicas, Universidad Nacional Autónoma de México, Cuernavaca, Morelos, Mexico

[4]Embrapa Meio Ambiente, C.P. 69, 13820-000 Jaguariúna, São Paulo, Brazil

Correspondence
Mariangela Hungria
mariangela.hungria@embrapa.br
or biotecnologia.solo@hotmail.com

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Table 1. Strains used in this study

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

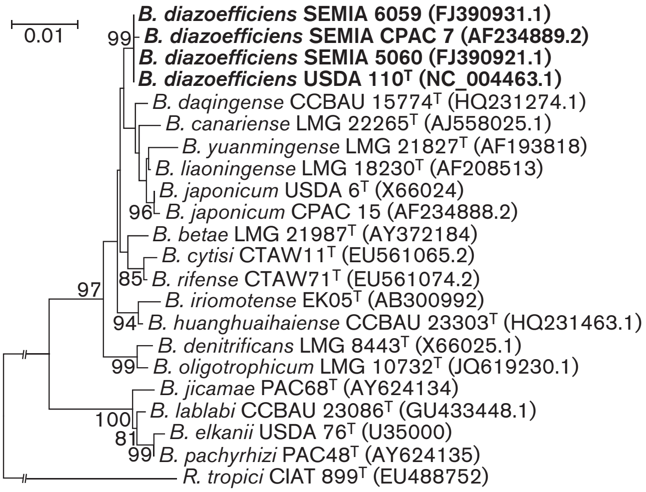

Fig. 1. Neighbour-joining phylogeny based on 16S rRNA gene sequences showing relationships between *Bradyrhizobium diazoefficiens* sp. nov. strains and other members of the genus *Bradyrhizobium*. Bootstrap values >70 % are indicated at nodes. *Rhizobium tropici* CIAT 899^{T} was used as an outgroup.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

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

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

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

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

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

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

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

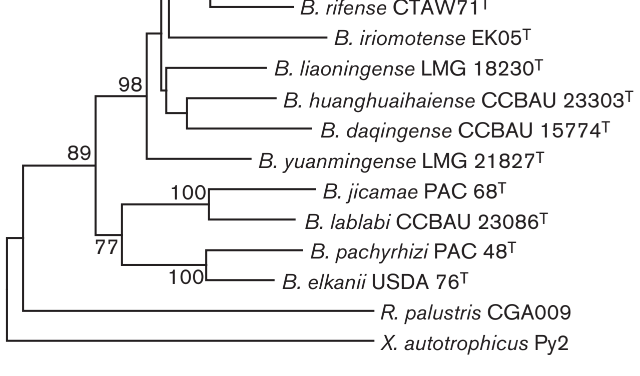

Fig. 2. Neighbour-joining phylogeny based on concatenated *atpD*, *glnII* and *recA* gene sequences showing relationships between strains from the novel species (shown in bold) and other members of the genus *Bradyrhizobium*. Bootstrap values >70 % are indicated at nodes. *Rhodopseudomonas palustris*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

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

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

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

Table 3. cont.

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

*Growth (OD_{600}) was considered positive if $\geqslant$0.09, weak at 0.04–0.09, and negative at <0.04.

†Mean of three colonies.

‡Data taken from: *a*, Chahboune *et al.* (2011); *b*, Zhang *et al.* (2012); *c*, Islam *et al.* (2008); *d*, Chahboune *et al.* (2012).

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

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

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

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

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

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

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

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

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

Description of *Bradyrhizobium diazoefficiens* sp. nov.

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

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

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

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

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

Primer	Sequence (5`- 3`)[a]	Target gene (position)	PCR cycling	Reference
BRdnaKf	TTCGACATCGACGCSAACGG	*dnaK* (1411–1430)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1.5 min 72°C), 7 min 72°C	Menna *et al.* (2009)
BRdnaKr	GCCTGCTGCKTGTACATGGC	*dnaK* (1905–1885)		
TSrecAf	CAACTGCMYTGCGTATCGTCGAAGG	*recA* (8-32)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1.5 min 72°C and 7 min 72°C	Stepkowski *et al.* (2005)
TSrecAr	CGGATCTGGTTGATGAAGATCACCATG	*recA* (620-594)		
TSatpDf	TCTGGTCCGYGGCCAGGAAG	*atpD* (189-208)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1.5 min 72°C and 7 min 72°C	Stepkowski *et al.* (2005)
TSatpDr	CGACACTTCCGARCCSGCCTG	*atpD* (804-784)		
TSglnIIf	AAGCTCGAGTACATCTGGCTCGACGG	*glnII* (13-38)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1.5 min 72°C and 7 min 72°C	Stepkowski *et al.* (2005)
TSglnIIr	SGAGCCGTTCCAGTCGGTGTCG	*glnII* (681-660)		
gyrB343F	TTCGACCAGAAYTCCTAYAAGG	*gyrB* (343-364)	5 min 95°C, 5X (2 min 94°C, 2 min 58°C, 1 min 72°C) 28 X (30s 94°C, 1 min 58°C, 1 min 72°C and 5 min 72°C	Martens *et al.* (2008)
gyrB1043R	AGCTTGTCCTTSGTCTGCG	*gyrB* (1061-1043)		
rpoB83F	CCTSATCGAGGTTCACAGAAGGC	*rpoB* (83-103)	5 min 95°C, 3X (2 min 94°C, 2 min 58°C, 1 min 72°C) 30 X (30s 94°C, 1 min 58°C, 1 min 72°C and 5 min 72°C	Martens *et al.* (2008)
rpoB1061R	AGCGTGTTGCGGATATAGGCG	*rpoB* (1081-1061)		
fD1	AGAGTTTGATCCTGGCTCAG	16S rRNA (9-29)	2 min 95°C, 30 X (15s 94°C, 45s 93°C, 45s 55°C, 2 min 72°C and 5 min 72°C	Weisburg *et al.* (1991)
rD1	CTTAAGGAGGTGATCCAGCC	16S rRNA (1474-1494)		
FGPS1490	TGCGGCTGGATCACCTCCTT	16S rRNA (1490-1510)	3 min 94°C, 35 X (1 min 94°C, 1 min 55°C, 2 min 72°C and 6 min 72°C	Laguerre *et al.* (1996)
FGPS130	CCGGGTTTCCCCATTCGG	23S rRNA (148-130)		

[a] Mixtures of bases used at certain positions are given as: K, T or G; S, G or C; Y, C or T; R, A or G; M, A or C

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

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Weisburg, W. G., Barns, S. M., Pelletier, D. A. & Lane, D. J. (1991). 16S ribossomal DNA amplification for phylogenetic study. *J Bacteriol* **173**, 697-703.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Germano, M. G., Menna, P., Mostasso, F. L. & Hungria, M. (2006). RFLP analysis of the RNA operon of a Brazilian collection of bradyrhizobial strains from thirty-three legume species. *Int J Syst Evol Microbiol* **56**, 217-229.

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

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

Kuykendall, L. D., Saxena, B., Devine, T. E. & Udell, S. E. (1992). Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and a proposal for *Bradyrhizobium elkanii* sp. nov. *Can J Microbiol* **38**, 501-505.

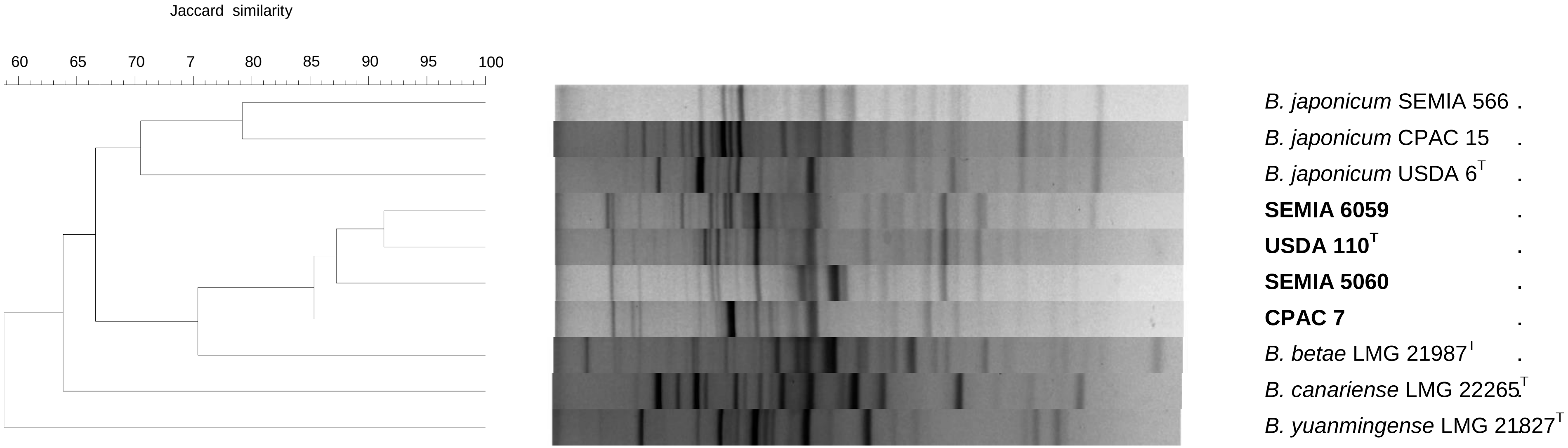
Menna, P., Barcellos, F. G. & Hungria, M. (2009). Phylogeny and taxonomy of a diverse collection of *Bradyrhizobium* strains based on multilocus sequence analysis of the 16S rRNA gene, ITS region and *glnII*, *recA*, *atpD* and *dnaK* genes. *Int J Syst Evol Microbiol* **59**, 2934-2950.

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

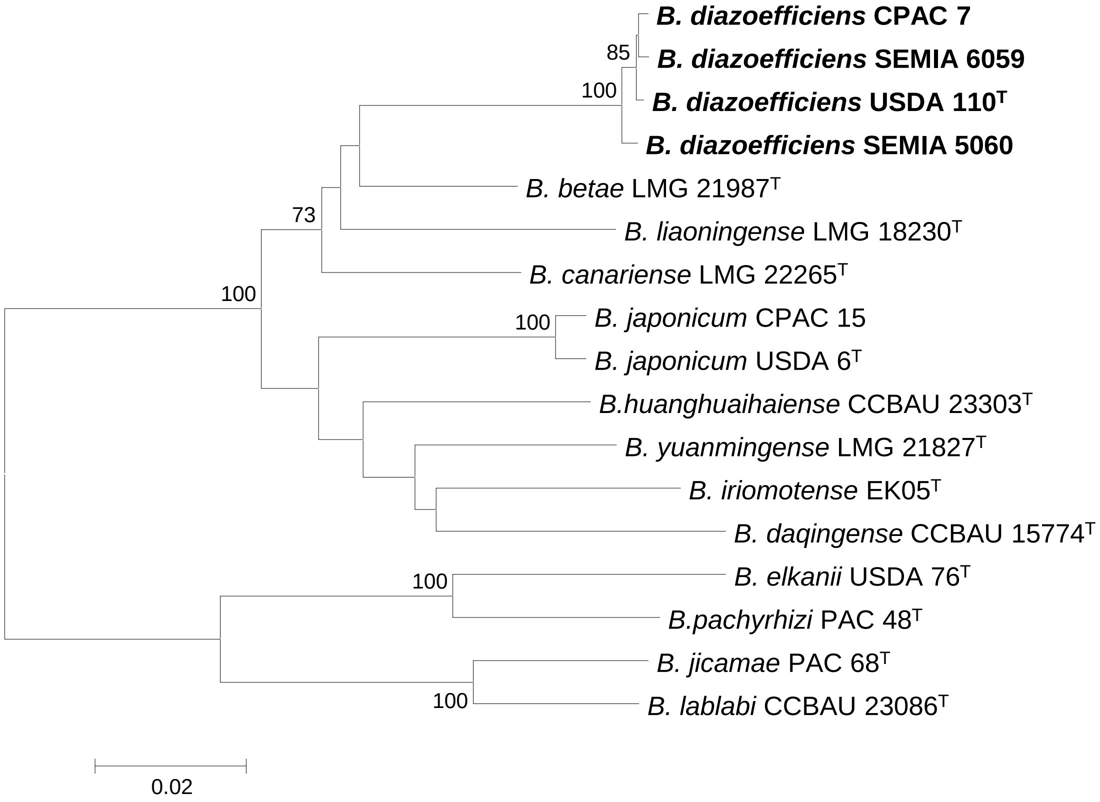
van Berkum, P. & Fuhrmann, J. J. (2001). Characterization of soybean bradyrhizobia for which serogroup affinities have not been identified. *Can J Microbiol* **47**, 519-25.

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

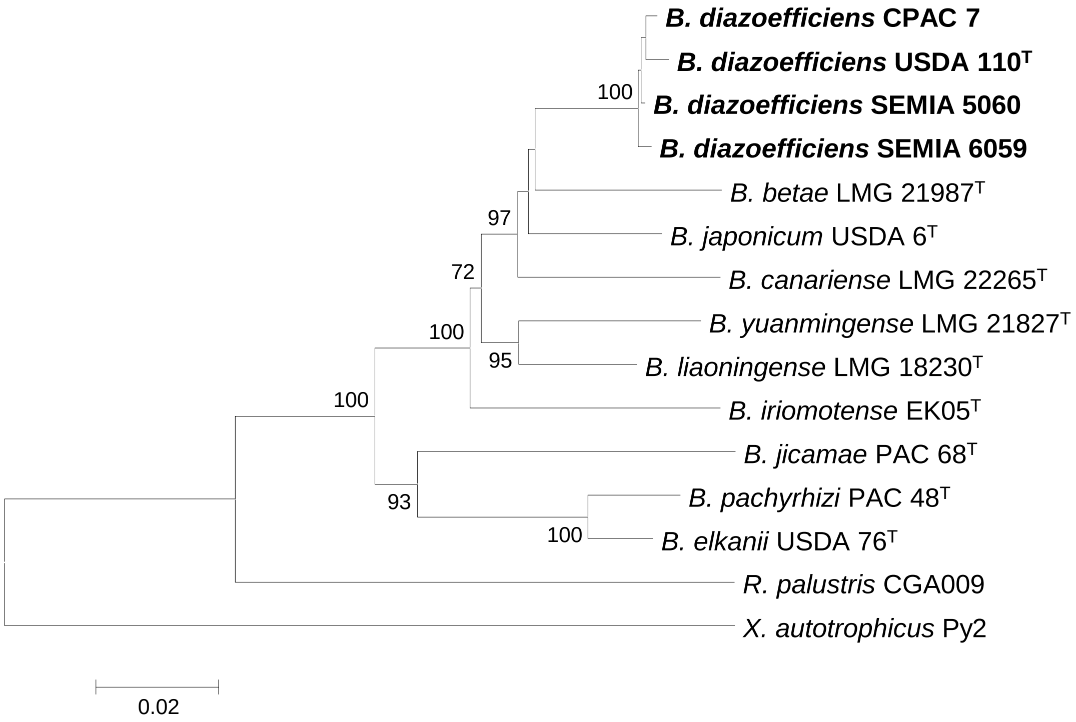
Vinuesa, P., Rojas-Jiménez, K., Contreras-Moreira, B., Mahna, S. K., Prasad, B. N., Moe, H., Selvaraju, S. B., Thierfelder, H. & Werner, D. (2008). Multilocus sequence analysis for assessment of the biogeography and evolutionary genetics of four *Bradyrhizobium* species that nodulate soybeans on the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



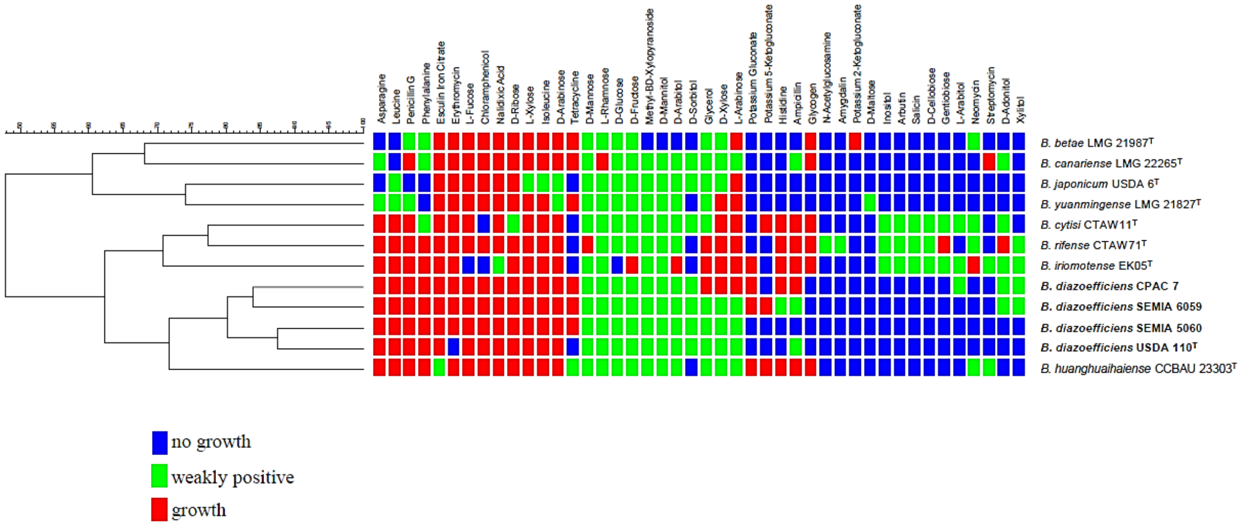
Supplementary Fig. S1. Cluster analysis (UPGMA algorithm with the Jaccard coefficient) of products obtained by BOX-A1R-PCR of *Bradyrhizobium diazoefficiens* strains (shown in bold) and related bradyrhizobia. Analysis performed with software Bionumerics v. 4.6.



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



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



	Asparagine	Leucine	Penicillin G	Phenylalanine	Esculin Iron Citrate	Erythromycin	L-Fucose	Chloramphenicol	Nalidixic Acid	D-Ribose	L-Xylose	Isoleucine	D-Arabinose	Tetracycline	D-Mannose	L-Rhamnose	D-Glucose	D-Fructose	Methyl-BD-Xylopyranoside	D-Mannitol	D-Arabitol	D-Sorbitol	Glycerol	D-Xylose	L-Arabinose	Potassium Gluconate	Potassium 5-Ketogluconate	Histidine	Ampicillin	Glycogen	N-Acetylglucosamine	Amygdalin	Potassium 2-Ketogluconate	D-Maltose	Inositol	Arbutin	Salicin	D-Cellobiose	Gentiobiose	L-Arabitol	Neomycin	Streptomycin	D-Adonitol	Xylitol
B. betae LMG 21987^T	🟦	🟦	🟩	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦	🟩	🟩	🟥	🟦	🟦	🟦	🟦	🟥	🟦	🟦	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟩	🟦	🟦	🟦
B. canariense LMG 22265^T	🟩	🟦	🟥	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟩	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟥	🟩	🟦
B. japonicum USDA 6^T	🟦	🟩	🟦	🟦	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟦	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
B. yuanmingense LMG 21827^T	🟩	🟩	🟩	🟦	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟩	🟥	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟩	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
B. cytisi CTAW11^T	🟥	🟥	🟥	🟩	🟥	🟥	🟥	🟦	🟥	🟩	🟥	🟥	🟥	🟦	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟥	🟥	🟦	🟥	🟥	🟥	🟥	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟩	🟦
B. rifense CTAW71^T	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟦	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟥	🟥	🟥	🟦	🟦	🟥	🟥	🟥	🟩	🟩	🟦	🟦	🟩	🟩	🟩	🟩	🟥	🟦	🟩	🟦	🟥	🟩
B. iriomotense EK05^T	🟥	🟥	🟥	🟥	🟥	🟥	🟦	🟦	🟩	🟥	🟥	🟥	🟥	🟦	🟩	🟩	🟦	🟥	🟩	🟩	🟥	🟦	🟥	🟥	🟥	🟥	🟦	🟥	🟥	🟥	🟦	🟦	🟦	🟦	🟩	🟩	🟩	🟩	🟩	🟩	🟥	🟩	🟩	🟩
B. diazoefficiens **CPAC 7**	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟥	🟥	🟥	🟥	🟦	🟥	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟩	🟦	🟦	🟩	🟩
B. diazoefficiens **SEMIA 6059**	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟥	🟥	🟩	🟩	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟩	🟩
B. diazoefficiens **SEMIA 5060**	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
B. diazoefficiens **USDA 110^T**	🟥	🟥	🟥	🟥	🟥	🟦	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟦	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟦	🟦	🟩	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦
B. huanghuaihaiense CCBAU 23303^T	🟥	🟥	🟥	🟥	🟩	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟥	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟩	🟦	🟩	🟩	🟩	🟥	🟥	🟥	🟥	🟥	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟦	🟩	🟩	🟦	🟦

🟦 no growth

🟩 weakly positive

🟥 growth

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