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PÂMELA MENNA PEREIRA

**“FILOGENIA DE RIZÓBIOS UTILIZADOS EM INOCULANTES
COMERCIAIS BRASILEIROS, COM BASE NO
SEQÜENCIAMENTO DO GENE RIBOSSOMAL 16S”**

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Dissertação apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina, como requisito parcial à obtenção do título de Mestre em Microbiologia

Orientadora: Dra. Mariangela Hungria

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RESUMO

Rizóbios são bactérias capazes de fixar nitrogênio atmosférico e convertê-lo á uma forma assimilável pela planta, quando estes se encontram em simbiose com determinadas plantas da família Leguminosae. Contudo, apesar da importância ecológica e econômica, os rizóbios têm sido relativamente pouco estudados. Baseado nos dados de seqüenciamento do gene 16S RNAr, existem, atualmente cinco gêneros de rizóbios descritos, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* e *Sinorhizobium* e mais de 40 espécies, compreendidos na subclasse alfa das Proteobacterias. Vários estudos vêm sendo conduzidos, a fim de determinar a diversidade existente entre os rizóbios, visando assim estabelecer relações filogenéticas com bactérias relacionadas e estruturar hipóteses quanto à sua evolução simbiótica. Nesse contexto, uma coleção de 68 estirpes de rizóbios isoladas de 63 diferentes espécies de leguminosas, todas recomendadas como inoculante, foram analisadas a fim de determinar diferenças morfológicas e fisiológicas entre as estirpes “SEMIAS” e determinar a variabilidade genética e suas relações filogenéticas com base na análise das seqüências do gene 16S RNAr. As estirpes foram isoladas de leguminosas pertencentes as três subfamílias e 17 tribos. Foi possível observar que 25% das estirpes apresentaram reações ácidas em meio YMA e em geral foram caracterizadas como altas produtoras de muco, 70.58% das estirpes apresentaram reações alcalinas em YMA, sendo a maioria produtora de pouco muco, e apenas três estirpes não alteraram o pH do meio YMA. Nenhum padrão contratante foi observado em relação aos parâmetros morfológicos avaliados (cor, transparência, bordas e elevação). O dendrograma resultante após análises das seqüências do 16S RNAr, revelou heterogeneidade e dividiu assim, as estirpes em nove principais grupos reunidos em um nível de similaridade de 77,85%, sendo sete deles relatados aos gêneros/espécies: *Bradyrhizobium japonicum*, *B. elkanii*, *Rhizobium tropici*/*Agrobacterium* (reclassificado como *Rhizobium*), *R. leguminosarum*, *Sinorhizobium meliloti*/*S. fredii*, *Mesorhizobium ciceri*/*M. loti*, e *Azorhizobium caulinodans*. Contudo algumas estirpes diferiram em mais de 35 nucleotídeos das espécies tipo, sugerindo que essas podem representar novas espécies. Dois outros grupos incluíram bactérias mostrando baixa similaridade aos gêneros *Methylobacterium* e *Burkholderia*, e a presença dos genes *nifH* e/ou *nodC* foi confirmada para essa estirpes. Diversas estirpes foram capazes de nodular leguminosas de diferentes tribos e subfamílias. O alto grau de diversidade observado enfatiza que os trópicos são um importante reservatório de genes fixadores de nitrogênio.

Palavras-chave: Rizóbio. Leguminosa.

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ABSTRACT

Nitrogen is often a limiting nutrient, therefore the sustainability of food crops, forages and green manure legumes is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating N₂-fixing rhizobia. The selection, identification and maintenance of elite strains for each host is critical. Decades of research in Brazil resulted in a list of strains officially recommended for several legumes, but their genetic diversity is poorly known. This study aimed at gaining a better understanding of phylogenetic relationships of sixty-eight rhizobial strains recommended for sixty-three legumes, based on the sequencing of the 16S rRNA genes. The strains were isolated from a wide range of legumes, including all three subfamilies and seventeen tribes. Nine main clusters were defined, joined with a similarity of 77.8%, seven of them related to rhizobial genera/species: *Bradyrhizobium japonicum*, *B. elkanii*, *Rhizobium tropici*/*Rhizobium* resembling *agrobacteria*, *R. leguminosarum*, *Sinorhizobium meliloti*/*S. fredii*, *Mesorhizobium ciceri*/*M. loti*, and *Azorhizobium caulinodans*. However, some strains differed by up to thirty-five nucleotides from the type strains, which suggests that they may represent new species. Two other clusters included bacteria showing similarity with the genera *Methylobacterium* and *Burkholderia*, and the presence of *nifH* and/or *nodC* was confirmed in these strains. Several strains were capable of nodulating legumes of different tribes and subfamilies. The great diversity observed emphasizes that tropics are an important reservoir of N₂-fixation genes.

Keywords: Rhizobium. Leguminosae.

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1 REVISÃO BIBLIOGRÁFICA

1.1 FIXAÇÃO BIOLÓGICA DE NITROGÊNIO E BACTÉRIAS SIMBIÔNTICAS

O nitrogênio (N) é o quarto elemento mais abundante nas plantas, sendo superado apenas pelo carbono (C), pelo oxigênio (O) e pelo hidrogênio (H). O N é constituinte essencial de aminoácidos, proteínas, bases nitrogenadas, ácidos nucleicos, hormônios, clorofila, entre outros (Morgante, 2003).

O N é abundante na natureza, constituindo cerca de 80% do gás atmosférico, na forma de N_2 . No entanto, organismos eucariontes são incapazes de absorver N_2 e convertê-lo a uma forma assimilável devido à tripla ligação existente entre os átomos do N_2 , o qual constitui uma das mais fortes que se tem conhecimento na natureza (Hungria *et al.*, 1994).

As principais fontes de N assimilável pelas plantas são: 1) N do solo, proveniente principalmente da decomposição da matéria orgânica; 2) N obtido pela fixação não-biológica, resultante de descargas elétricas, combustão e vulcanismo; 3) N fornecido pelos fertilizantes; e 4) N fornecido pelo processo de fixação biológica (Hungria *et al.*, 2001).

Entretanto, o N presente na matéria orgânica do solo é limitado, podendo ser esgotado rapidamente após alguns cultivos. Já o processo químico o qual transforma N_2 em amônia, representa a forma assimilada com maior rapidez pelas plantas, mas a um custo elevado, pois o processo químico o qual transforma N_2 em amônia, requer hidrogênio (derivado do gás de petróleo), catalisador contendo ferro, altas temperaturas (300 a 600 °C) e altas pressões (200 a 800 atm.) (Hungria *et al.*, 2001). Conseqüentemente, o gasto de fontes energéticas não renováveis é elevado e estima-se que sejam necessários, aproximadamente, seis barris de petróleo por tonelada de NH_3 sintetizada. Um outro agravante na utilização dos fertilizantes nitrogenados reside na baixa eficiência de sua utilização pelas plantas, raramente ultrapassando 50%. Deve-se considerar, ainda, que o uso indiscriminado de fertilizantes

nitrogenados resulta em poluição ambiental, pois a lixiviação do N e o escoamento desse nutriente pela superfície do solo resultam em acúmulo de formas nitrogenadas nas águas dos rios, lagos e lençóis de água subterrâneos, podendo atingir níveis tóxicos a peixes e ao homem (Hungria *et al.*, 2001).

A terceira fonte de N é representada pela fixação biológica do N₂ (FBN), processo realizado por determinados procariontes, denominados organismos fixadores de N₂ ou diazotróficos. As bactérias capazes de fixar biologicamente o N₂ possuem uma enzima chamada dinitrogenase, que é formada por duas unidades protéicas, a Ferro-proteína (Fe-proteína) e a Molibdênio-Ferro-proteína (MoFe-proteína), ambas capazes de transportar elétrons. Durante a reação de redução do N₂, a dinitrogenase é auxiliada por uma terceira molécula transportadora de elétrons, a ferridoxina, a qual, na sua forma reduzida, transfere um elétron para a unidade Fe-proteína que, então, reduzida, doa o elétron recebido para a MoFe-proteína, a qual acumula os elétrons até que ocorram oito transferências concentrando oito elétrons, os quais são necessários para que a redução completa do N₂ à NH₃ ocorra (Morgante, 2003).

Em termos globais, estima-se que a FBN contribua com 65% da entrada anual de N na Terra, enquanto que a produção industrial contribui com 24% e a fixação não-biológica com cerca de 10% (Hungria *et al.*, 2001).

As bactérias fixadoras de N₂ se associam a diversas plantas em diferentes graus de especificidade, levando a sua classificação como bactérias associativas, por exemplo: *Azospirillum* sp., endofíticas, por exemplo: *Acetobacter diazotrophicus* e *Burkholderia* sp., e simbióticas, por exemplo: rizóbios (Drozdowicz, 1997), os quais constituem um grupo de bactérias Gram negativas que incluem os gêneros *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium* e *Allorhizobium* (Garrrity & Holt, 2001).

Rizóbios são bactérias que formam simbioses específicas com determinadas plantas da família Leguminosae (Fabaceae nos Estados Unidos), conduzindo à formação de órgãos altamente especializados, os nódulos, nos quais ocorre a fixação biológica do N₂ (Hungria, 1994).

A formação de nódulos é um processo complexo, que ocorre em várias etapas, e envolve mudanças fisiológicas e morfológicas tanto na célula hospedeira,

como na bactéria. As mudanças na bactéria visam, principalmente, o recebimento de fontes de carbono da planta hospedeira, para prover o ATP e poder redutor necessário para o processo de fixação biológica, enquanto que as mudanças na planta hospedeira visam assimilar a amônia produzida pelas bactérias (Hungria *et al.*, 1994).

Para que ocorra a formação dos nódulos, ambos, bactéria simbiótica e planta hospedeira, desenvolveram um complexo sistema para interagirem mantendo, assim, uma comunicação molecular. Esse sistema faz com que bactérias simbióticas vivendo saprofiticamente no solo percebam sinais químicos sintetizados pela planta hospedeira, geralmente flavonóides, os quais fazem com que bactérias simbióticas sejam atraídas em direção às raízes da planta, por quimiotactismo positivo (Drozdowicz, 1997).

Os flavonóides irão induzir a transcrição de genes de nodulação (*nod* *nol* e *noe*) nas bactérias, conduzindo à síntese e secreção dos fatores Nod. Os fatores Nod são lipo-quitinooligossacarídeos responsáveis, principalmente, pelo reconhecimento entre bactéria e planta hospedeira e pela indução de uma intensa divisão celular no córtex da raiz. As bactérias, então, serão atraídas por quimiotactismo a rizosfera, onde vão se multiplicar, colonizando os tricomas (pêlos) radiculares. Os tricomas enrolam-se, envolvendo grupos de bactérias, que em seguida, degradam uma porção da parede celular do tricoma, levando a invaginação do plasmalema. A seguir, as bactérias invadem o tricoma, utilizando o canal formado pela invaginação do plasmalema, dando origem ao cordão de infecção (Morgante, 2003; Hungria *et al.*, 1994).

O cordão de infecção irá migrar em direção as células em divisão no córtex da raiz da planta que recebe o nome de nódulo primário, nessa etapa, as bactérias presentes no interior do cordão de infecção, continuam se multiplicando. Ao chegar às proximidades do nódulo primário, o cordão de infecção se ramifica para invadir as células vegetais. Pequenos grupos de bactérias, contidas no interior de vesículas membranosas, são liberados dentro do citoplasma das células vegetais do nódulo primário. A partir do estabelecimento do nódulo radicular, as bactérias, que se encontram dentro das células radiculares hospedeiras param de se multiplicar,

aumentam de tamanho e sofrem várias alterações bioquímicas para se transformarem em bactérias especializadas na fixação de nitrogênio, os bacteróides (Morgante, 2003).

Rizóbios, entretanto, são bactérias de grande importância para a agricultura, sendo responsáveis por aproximadamente metade da fixação global de nitrogênio (Wang & Martinez-Romero, 2000). Estima-se que a FBN seja um processo cuja importância ecológica só pode ser comparada à fotossíntese, e que os organismos que a realizam podem suplementar ecossistemas naturais com novas quantidades de N, aproveitando a reserva inesgotável de N₂ presente na atmosfera. Rizóbios também são bactérias telúricas de vida livre e, quando não se encontram em simbiose, possuem a capacidade de sobreviverem *in situ* à custa de compostos nitrogenados do solo (Drozdowicz, 1997).

Para que esta relação simbiótica entre planta e rizóbio possa se estabelecer, essas bactérias precisam, carregar genes, os quais são responsáveis pelo processo de FBN (genes *nif* e *fix*) e genes responsáveis pelo processo de nodulação (genes *nod* *nol* e *noe*). Existem evidências de que os genes simbióticos estão localizados em elementos genômicos potencialmente transferíveis. Esses elementos podem ser plasmídeos ou megaplasmídeos em todas as espécies de *Rhizobium*, *Sinorhizobium*, *Mesorhizobium amorphae* e *Mesorhizobium huakuii*, ou regiões cromossômicas transferíveis, em *Mesorhizobium loti* e *Bradyrhizobium japonicum* (Wang & Martínez-Romero, 2000).

Desse modo, devido não apenas a sua grande importância prática, mas também pelo seu interessante mecanismo genético de simbiose, rizóbios têm sido um dos grupos de microrganismos mais amplamente estudados no campo da genética (Provorov & Vorob'ev, 2000).

1.2 TAXONOMIA DOS RIZÓBIOS

A sistemática bacteriana pode ser definida como o estudo científico da diversidade e inter-relações com o objetivo de caracterizar e arranjar, de uma maneira

ordenada, as bactérias (Truper & Schleifer, 1991). A taxonomia é, freqüentemente, usada como um sinônimo para sistemática e consiste na classificação, nomenclatura e identificação de um organismo (Cowan, 1968).

A classificação consiste no arranjo dos organismos em grupos (táxons) com base em similaridades, enquanto que a nomenclatura é a determinação de nomes para os grupos taxonômicos, de acordo com as regras internacionais descritas pelo “*International Code of Nomenclature of Bacteria*” (Sneath, 1992). Já a identificação é o uso prático da classificação a fim de determinar a identidade de um isolado como membro, ou não, de uma unidade. A classificação bacteriana consiste de diversos níveis. O maior nível é chamado de Domínio. Todos os procariotos são localizados dentro de dois Domínios, *Archae* e *Bacteria*. Para cada Domínio são descritos os Filos, Classes, Ordens, Famílias, Gêneros, espécies e subespécies.

Conforme Brenner *et al.* (2001), níveis mais altos tais como: Filo, Classe, Ordem, Família e Gênero têm sido classificados com base em análise das seqüências do gene ribossomal 16S. A determinação de espécies, entretanto, permanece incerta, sendo que análises de similaridades entre DNA-DNA, seqüência do 16S RNAr e características fisiológicas vêm sendo utilizadas a fim de caracterizar uma dada espécie. Contudo, não há, até o presente momento, uma determinação precisa quanto à especiação em bactérias.

Os rizóbios encontram-se classificados como pertencentes a: Domínio: *Bactéria*; Filo: *Proteobacteria*; Classe: *Alfaproteobacteria*; Ordem: Rhizobiales; e distribuído nas Famílias Rhizobiaceae, Phyllobacteriaceae, Bradyrhizobiaceae e Methylobacteriaceae, (Garrity & Holt, 2001), Além disso, algumas espécies de bactérias capazes de nodular e fixar N₂ em simbiose com leguminosas foram recentemente identificadas como pertencentes à classe das *Betaproteobacteria*; Ordem: Burkholderiales; Família: Burkholderiaceae; gênero: *Burkholderia* (Moulin *et al.*, 2001) e também pertencente à classe das *Alfaproteobacterias*; Ordem Rhizobiales; Família: *Methylobacteriaceae*; gênero: *Methylobacterium* (Sy *et al.*, 2001).

Durante recentes anos, estudos têm demonstrado grande diversidade entre rizóbios, especialmente em zonas tropicais e mediterrâneas, onde a diversidade é

ainda pouco conhecida, levando a mudanças em sua classificação (Zaklha & de Lajudie, 2001).

Primeiramente, a família Rhizobiaceae era representada apenas pelo gênero *Rhizobium*, o qual era constituído por bactérias capazes de nodular e fixar nitrogênio em relações simbióticas com plantas da família Leguminosae. As espécies simbióticas eram *Rhizobium leguminosarum* (espécie tipo), *Rhizobium japonicum*, *Rhizobium lupini*, *Rhizobium meliloti*, *Rhizobium phaseoli* e *Rhizobium trifoli*. Espécies de *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Agrobacterium rubi* e espécies de *Phyllobacterium myrsinacearum* e *Phyllobacterium rubiacearum*, todas causando hipertrofias em plantas, foram também incluídas na família Rhizobiaceae (Skerman et al., 1980). Os rizóbios foram assim classificados, com base nas espécies de leguminosas com as quais fossem capazes de formar nódulos e fixar nitrogênio. Portanto, *Rhizobium phaseoli* nodulava o feijão (*Phaseolus vulgaris* L.); *R. japonicum* a soja (*Glycine max* L.); *R. meliloti* a alfafa (*Medicago sativa*); *R. viciae* o feijão fava (*Vicia faba*) e *R. trifolii* o trevo (*Trifolium repens*) (Coutinho, 2003).

O conceito de planta hospedeira, porém, foi mudada, após a observação de muitas reações cruzadas entre as planta hospedeiras e os microrganismos, pois uma única leguminosa, por exemplo, *Acácia*, *Glycine max* ou *Leucaena* poderiam abrigar diferentes simbiontes (Terefework et al., 2000).

A taxonomia rizobiana, entretanto, revolucionou com o advento de metodologias avançadas, tais como a sistemática molecular e quimiotaxonômica, para a caracterização de microrganismos.

Como primeira consequência, Jordan (1982), com base em análises fenotípicas, reassociação DNA-DNA e dados fisiológicos, descreveu o gênero *Bradyrhizobium*, sendo a espécie padrão a estirpe USDA 6^T (ATCC 10324). *Bradyrhizobium*, que do grego *Bradus* significa lento, foi assim classificado e diferenciado de *Rhizobium*, principalmente, pelo crescimento mais lento e produção de álcali em meio de cultura.

A partir de então, todas as demais estirpes de crescimento lento passaram a receber a denominação de *Bradyrhizobium* sp., seguido do nome da planta entre parênteses.

Já na década de 80, vários trabalhos começaram a demonstrar a existência de grande variabilidade genética e fisiológica entre as estirpes de *Bradyrhizobium*, levando Kuykendall *et al.* (1992) a sugerirem a subdivisão de *Bradyrhizobium* em duas espécies: *B. japonicum*, com as estirpes do grupo I, e *B. elkanii*, com as estirpes do grupo II.

Desde então, técnicas moleculares têm sido desenvolvidas, a fim de melhor caracterizar os rizóbios, sendo que, atualmente utiliza-se como ferramenta indispensável à taxonomia bacteriana a análise de genes ribossomais, mais especificamente da sequência do gene 16S RNAr, o qual tem demonstrado suficiente conservação estrutural, possibilitando a descrição de muitas espécies novas. Contudo, como citado anteriormente, as classificações, principalmente em nível de espécies, não devem ser baseadas apenas em características moleculares, mas também em propriedades fisiológicas e, no caso de rizóbios, simbióticas. É o que chamamos de classificação polifásica (Coutinho, 2003).

1.3 ANÁLISES FILOGENÉTICAS MOLECULARES PARA DETERMINAÇÃO DA DIVERSIDADE DE RIZÓBIO

Uma poderosa ferramenta para a taxonomia de um determinado organismo é o estudo da filogenia, o qual é amplamente utilizado para determinar a relação existente entre os organismos, indicando seu possível grupo, suas relações com outros grupos e seu lugar nas famílias e reinos, bem como auxiliando no reconhecimento dos ancestrais. Em bactérias a filogenia é baseada, principalmente, em dados de seqüenciamento de macromoléculas biológicas. Nesse contexto, moléculas mais conservadas ajudam a comparar organismos relacionados distantemente, enquanto que moléculas que mudam rapidamente permitem identificar mudanças pequenas e recentes (Wang & Martínez-Romero, 2000).

Os genes ribossomais (RNAr) que codificam moléculas de RNA ribossomal são essenciais para a sobrevivência de todos os organismos e são

altamente conservados em bactérias e outros organismos, uma vez que todos realizam síntese de proteínas. Assim, a conservação desses genes, devido à reserva estrutural dos ribossomos, e à existência de variabilidade em alguns domínios, torna as seqüências dos genes ribossomais (5S, 16S e 23S) boas escolhas para comparar organismos e inferir filogenias (Thórsson *et al.*, 2000).

Como em muitas outras bactérias, a filogenia dos rizóbios tem sido derivada, principalmente, da análise de seqüências nucleotídicas do gene que codifica a região do gene 16S do RNA ribossomal. O seqüenciamento direto e completo do produto de PCR obtido pela amplificação da região do DNA que codifica o gene 16S RNAr tem sido o método mais comumente utilizado para estimar filogenia, devido ao fato de que há, nesta região gênica, suficiente conservação, o que permite o estabelecimento de relações evolucionárias universais (Wang & Martinez-Romero, 2000).

Seqüências dos genes ribossomais 5S, 23S e da região intergênica (IGS) também têm sido utilizadas para estimar relações entre espécies de rizóbios. Uma comparação parcial do gene 23S DNAr revelou que a relação entre *Agrobacterium vitis* com *Rhizobium leguminosarum*, *R. etli* e *R. galegae* indicou que estes gêneros não estão tão relacionados à *Agrobacterium* como quando se analisam seqüências do gene 16S RNAr. Esse fato indica que a taxa de mudanças nas seqüências do gene 23S RNAr, assim como também já foi constatado para os genes 5S RNAr e IGS RNAr, são maiores e mais rápidas que as observadas para o gene 16S RNAr, sendo assim, esses genes (23S e 5S e IGS) poderiam ser utilizados com o propósito de identificação e tipagem entre espécies, mais do que para inferir relações filogenéticas (Wang & Martinez-Romero, 2000).

Uma grande complicação em análises filogenéticas das seqüências do operon RNAr (16S, 23S, 5S e IGS) é o fato de que muitos gêneros de bactérias possuem múltiplas cópias do operon RNAr, e os alelos RNAr podem divergir em cada cópia, em maior ou em menor extensão, devido à recombinação intragênica localizada e transferência lateral parcial, ou total, dos operons RNAr (Yap *et al.*, 1999).

No entanto, para as estirpes do gênero *Bradyrhizobium*, tal problema pode ser negligenciado, devido ao fato de que, até o momento, observou-se que

estirpes deste gênero, possuem apenas uma única copia do operon RNAr, enquanto que para as estirpes dos gêneros *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Allorhizobium* e *Azorhizobium*, já foram identificadas múltiplas cópias, contudo são cópias incompletas, sendo assim possíveis de identificação em análises de gel de eletroforese, ou são cópias idênticas não alterando assim, as análises (Willems *et al.*, 2001).

Com base nas seqüências do 16S RNA, os simbiossiontes de plantas leguminosas encontram-se divididos em três grupos filogenéticos (Zakha & de Lajudie, 2001).

O primeiro grupo consiste de diversos subgrupos, sendo que cada subgrupo representa um gênero de rizóbio, são eles: *Rhizobium*, *Sinorhizobium*, *Mesorhizobium* e *Allorhizobium*. Neste grupo também se encontram algumas bactérias patogênicas à planta (*Agrobacterium* e *Phyllobacterium*), bactérias do solo (*Mycoplana*) e algumas bactérias clínicas (*Brucella*, *Ochrobactrum* e *Bartonella*). O primeiro subgrupo correspondente ao gênero *Rhizobium*, inclui *R. leguminosarum*, como espécie tipo, *R. tropici* (Martinez-Romero *et al.*, 1991), *R. etli* (Segovia *et al.*, 1993), *R. galilicum* (Amarger *et al.*, 1997), *R. mongolense* (Van Berkum *et al.*, 1998) e *Agrobacterium* biovar-2. O segundo subgrupo correspondente ao gênero *Sinorhizobium*, inclui as espécies: *S. fredii* e *S. xinjiagensis* (Chen *et al.*, 1988), *S. meliloti*, *S. teranga* e *S. sahelense* (de Lajudie *et al.*, 1994), *S. medicae* (Rome *et al.*, 1996), *S. kostiense* e *S. arboris* (Nick *et al.*, 1999), essas espécies, entretanto, se encontram mais relacionados ao subgrupo dos *Rhizobium*, sendo assim, provavelmente dividem um ancestral comum. O terceiro subgrupo corresponde ao gênero *Mesorhizobium* (Jarvis *et al.*, 1997) e inclui as espécies: *M. loti* (Jarvis *et al.*, 1982), *M. huakuii* (Chen *et al.*, 1991), *M. ciceri* (Nour *et al.*, 1994), *M. mediterraneum* (Nour *et al.*, 1995), *M. tianshanense* (Chen *et al.*, 1995), *M. plurifarum* (de Lajudie *et al.*, 1998a), *M. amorphae* (Wang *et al.*, 1999). Espécies desse gênero e algumas bactérias metilotróficas pertencentes aos gêneros *Aminobacter* e *Chelatobacter*, isoladas de rizosfera de plantas, e o gênero *Phyllobacterium*, também se encontram relacionados na árvore filogenética do 16S RNAr, com maior parentesco com as espécies de *Rhizobium* do que com espécies dos gêneros *Azorhizobium* e *Bradyrhizobium*. O quarto subgrupo inclui, espécies

pertencentes ao gênero *Agrobacterium* biovar-1, mas também, *R. galegae* (Lindström, 1989), *R. giardinii* (Amarger et al., 1997), *R. huautlense* (Wang et al., 1998) e *Allorhizobium undicola* (de Lajudie et al., 1998b). Este parentesco levou recentemente a mudança do gênero *Agrobacterium* para o gênero *Rhizobium* (Young et al., 2001).

O segundo grupo filogenético, é representado, pelo gênero *Azorhizobium*. O qual consiste de apenas uma espécie, *A. caulinodans*, proposto para as bactérias que nodulam o caule e raízes de *Sesbania rostrata*. Esse gênero exibe características muito especiais entre os rizóbios, tais como a fixação “in vitro” do nitrogênio e crescimento sob baixa tensão de O₂ (3%) (Zakha & de Lajudie, 2001).

A. caulinodans forma um grupo bem distinto dos outros rizóbios, estando mais intimamente relacionado a espécies de *Xanthobacter*, que são também bactérias associadas a plantas e incluem alguns membros fixadores de N₂, isolado da rizosfera de plantas, tais como o arroz (Dreyfus et al., 1988).

O gênero *Bradyrhizobium*, representado por espécies de crescimento lento e reação alcalina em meio contendo manitol como fonte de carbono, forma o terceiro grupo filogenético (Zakha & de Lajudie, 2001). Existem atualmente quatro espécies conhecidas, *B. japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall et al., 1992), *B. liaoningense* (Xu et al., 1995), *B. yuanmingense* (Yao et al., 2002), *Bradyrhizobium canariense* (Vinuesa et al., 2005) e *Bradyrhizobium betae* (Rivas et al., 2004) e ainda muitas linhagens não nomeadas, incluindo alguns fotossintéticos e algumas linhagens nodulantes de caule. Nesse grupo também se encontram espécies de *Afixia* sp., *Blastobacter denitrificans* e *Rhodopseudomonas*. O parentesco filogenético entre *Bradyrhizobium japonicum*, *Afixia* sp., *Blastobacter denitrificans* e *Rhodopseudomonas palustris* é maior que entre *B. japonicum* e *B. elkani*. Portanto, esse gênero aceito como *B. elkanii*, já foi questionado (Dupuy et al., 1994). Através de um filograma baseado no gene 16S RNAr de diversas seqüências de bactérias da subclasse alfa das Proteobacterias, foi possível observar que *Bradyrhizobium* está mais relacionado a bactérias não simbióticas do que a outros rizóbios (Wang & Martinez-Romero, 2000).

Este fato levou cientistas a sugerirem que os gêneros de rizóbios foram formados em diferentes estágios da evolução e que eles possuem estreitas relações com bactérias não simbióticas. Desse modo, *Bradyrhizobium* teria divergido de

Rhizobium muito antes dos nódulos evoluírem e os genes de nodulação, os quais são, claramente relacionados, provavelmente foram adquiridos por transferência lateral (Young & Haukka, 1996; Wang & Martinez-Romero, 2000).

Desse modo, as análises filogenéticas fornecem informações sobre o processo de vida e evolução bacteriana, além de fornecerem uma importante ferramenta para estudos de diversidade bacteriana, permitindo um aumento no entendimento de interações biológicas, auxiliando grandemente na conservação e restauração biológica. Bactérias representam uma das formas de vida mais diversa na Terra, podendo consistir de mais de um milhão de espécies (ASM, 1994). No entanto, apenas uma fração dessas espécies, foram identificadas, e ainda, poucas são estudadas ou estão em coleções de cultura (Hawksworth, 1991, Gewin *et al.*, 1998).

Bactérias são sensíveis a distúrbios, como os causados pela agricultura, poluição e outros stress (Elliot & Lynch, 1994), sendo assim, entender o efeito dos distúrbios na diversidade bacteriana pode contribuir, amplamente, para o entendimento da qualidade do solo e o desenvolvimento de agroecossistemas sustentáveis (Thomas & Kevan, 1993).

Novas espécies de rizóbios vêm sendo descritas nos últimos anos, refletindo o número crescente de grupos de pesquisa envolvidos em estudos da diversidade de rizóbios. A maior parte das novas espécies de rizóbios foi isolada de regiões tropicais, realçando a importância dos trópicos como fonte de biodiversidade. Se considerarmos os sistemas de agricultura sustentável como pré-requisito para a melhoria de qualidade de vida nos países tropicais, bem como a importância da FBN para a sustentabilidade dos agroecossistemas, percebe-se a grande relevância e potencial benéfico do entendimento e exploração racional da diversidade de rizóbios (Coutinho, 2003).

2 JUSTIFICATIVAS

Organismos unicelulares existem no planeta há 2-3 bilhões de anos, antes mesmo de qualquer outra forma de vida celular complexa. As bactérias são as mais diversas formas de vida na Terra e consistem em mais de um milhão de espécies (ASM, 1994). Estima-se que, somente de 1 a 10% das espécies bacterianas da Terra já foram identificadas, deixando uma vasta porção da biota desconhecida e, conseqüentemente, não estudada (Hawksworth, 1991).

Coleções de culturas atuam como centros de recursos microbiológicos e reservatórios notáveis de genes microbianos, além de fornecem serviços de identificação, preservação, testes de viabilidade, pureza e autenticidade das estirpes que são, continuamente, monitoradas a fim de disponibilizar culturas puras, em estado viável e geneticamente estável para a educação e a pesquisa. Sendo assim, o estudo destas coleções pode contribuir para a descoberta de genótipos superiores ou, até mesmo, novas espécies.

Em rizóbios vários estudos vêm sendo conduzidos, sobretudo através de análises das seqüências dos genes ribossomais 16S RNAr, a fim de determinar relações filogenéticas com bactérias relacionadas e estruturar hipóteses quanto a sua evolução simbiótica. Vários trabalhos, entretanto, têm focado a diversidade de estirpes de rizóbio, principalmente as de importância agrícola e, como resultado, novas espécies vêm sendo descritas. Seqüências do gene 16S RNAr têm sido utilizadas principalmente devido a sua estrutura conservada e essencialidade em todas as bactérias, possibilitando, assim, estimar suas posições no domínio *Bacteria* (Wang & Martinez-Romero, 2000).

No Brasil, diferentes instituições de pesquisa são depositárias de coleções de culturas de rizóbios, sendo que a “Coleção de Culturas SEMIA” do Centro de Pesquisa de Fixação Biológica do Nitrogênio, da Fundação Estadual de Pesquisa Agropecuária (FEPAGRO) é a responsável pela manutenção e distribuição de estirpes recomendadas para o uso em inoculantes comerciais para diversas leguminosas, sendo considerada a coleção de referência nacional para rizóbios. Algumas dessas

leguminosas são de grande importância econômica nacional ou regional, como a soja, o feijoeiro, a ervilha, o feijão-de-corda, o amendoim, a alfafa, os trevos e diversas leguminosas arbóreas utilizadas em programas de recuperação de áreas degradadas e de reflorestamento. Assim, torna-se essencial realizar a caracterização molecular das culturas da coleção SEMIA, principalmente para o controle de qualidade das estirpes recomendadas para uso em inoculantes comerciais, e para analisar características diferenciais entre as estirpes, possibilitando assim a identificação de novas espécies ou novos gêneros.

3 OBJETIVOS

- Caracterizar as diferenças morfológicas e fisiológicas de uma coleção de estirpes de rizóbio proveniente de diferentes leguminosas, todas de grande importância econômica nacional ou de importância para programas de reflorestamento e recuperação de áreas degradadas, e a relação destas estirpes com a distribuição geográfica e suas plantas hospedeiras.

- Determinar a filogenia molecular e assim classificar esta coleção, por meio do estudo da variabilidade encontrada na região conservada do gene ribossomal 16S DNAr.

4. Artigo. Molecular phylogeny of rhizobial strains used in Brazilian commercial inoculants based on the sequencing of the 16S rRNA gene

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Abstract

Nitrogen is often a limiting nutrient, therefore the sustainability of food crops, forages and green manure legumes is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating N₂-fixing rhizobia. The selection, identification and maintenance of elite strains for each host is critical. Decades of research in Brazil resulted in a list of strains officially recommended for several legumes, but their genetic diversity is poorly known. This study aimed at gaining a better understanding of phylogenetic relationships of sixty-eight rhizobial strains recommended for sixty-three legumes, based on the sequencing of the 16S rRNA genes. The strains were isolated from a wide range of legumes, including all three subfamilies and seventeen tribes. Nine main clusters were defined, joined with a similarity of 77.8%, seven of them related to rhizobial genera/species: *Bradyrhizobium japonicum*, *B. elkanii*, *Rhizobium tropici*/*Rhizobium* resembling *agrobacteria*, *R. leguminosarum*, *Sinorhizobium meliloti*/*S. fredii*, *Mesorhizobium ciceri*/*M. loti*, and *Azorhizobium caulinodans*. However, some strains differed by up to thirty-five nucleotides from the type strains, which suggests that they may represent new species. Two other clusters included bacteria showing similarity with the genera *Methylobacterium* and *Burkholderia*, and the presence of *nifH* and/or *nodC* was confirmed in these strains. Several strains

were capable of nodulating legumes of different tribes and subfamilies. The great diversity observed emphasizes that tropics are an important reservoir of N₂-fixation genes.

Keywords: Biological nitrogen fixation; *Azorhizobium*; *Bradyrhizobium*; *Burkholderia*; Inoculant; Leguminosae; *Methylobacterium*; Nodulation; *Rhizobium*; *Sinorhizobium*; 16S rRNA gene

1. Introduction

Divergence within the family Leguminosae (Fabaceae in the USA) is estimated to have occurred over 65 million years, such that over 18,000 species are now classified into around 650 genera, occupying nearly all terrestrial biomes (Polhill and Raven, 1981; Herendeen et al., 1992). The wide use of legumes as food crops, forages and green manures is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating N₂-fixing bacteria, collectively called rhizobia (Allen and Allen, 1981). These bacteria are among the most intensely studied groups of microorganisms, mainly due to their potential to replace N-fertilizers, with emphasis on their key role in achieving sustainability of tropical N-poor soils.

Nodules generally occur in the subfamilies Mimosoideae and Papilionoideae, and are rare in the Caesalpinioideae; recent information indicates that about 3,000 taxa are capable of nodulating and 400 taxa are not; however, information is lacking for nearly 40% of the genera (ILDIS, 2005). Until the early 1980s, all bacteria isolated from root nodules were classified into the genus *Rhizobium*, and speciation was based on nodulation with certain host plants, establishing the “cross-inoculation group” concept (Fred et al., 1932). After that, numerical taxonomy considering several properties led to the definition of a new genus, *Bradyrhizobium*, and the renomination of some other species (Jordan, 1984). Particularly in the last decade, ribosomal sequences with emphasis on the 16S rDNA, have become the basis of bacterial molecular phylogeny and taxonomy (Garritty and Holl, 2001), leading to the description of new rhizobial genera and species. Currently, rhizobia are positioned in four deep branches,

Azorhizobium, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium-Sinorhizobium-Allorhizobium*, and non-symbiotic relatives within those branches may indicate common ancestry for rhizobial species and other parasitic or soil-borne bacteria (Wang and Martínez-Romero, 2000; Garrity and Holl, 2001). However, in comparison to the number of nodulating-legume species, very few rhizobial species have been described.

In Brazil, economical and agronomic benefits have been achieved with several legumes due to research efforts focused on N₂ fixation. Since 1975, the government demands that inoculants commercialized in the country contain only strains recommended by Brazilian public research institutions. To enforce the recommendation, the RELARE (=Rede de Laboratórios para a Recomendação de Estirpes de *Rhizobium*) was created in 1985, by the initiative of the Microbial Resources Centre Network (MIRCEN), establishing a net of laboratories with the objective of identifying the most effective rhizobial strains for each legume species. Since then, the maintenance of the strains and their distribution to the inoculant industry has been a responsibility of the “*Rhizobium* Culture Collection SEMIA” (Seção de Microbiologia Agrícola) (IBP World Catalogue of *Rhizobium* Collections #443 in the WFCC World Data Center on Microorganisms), at the Fundação Estadual de Pesquisa Agropecuária (FEPAGRO).

Searching for the most effective rhizobial strains is a time-consuming process involving the production of thousands of rhizobial cultures, and many greenhouse experiments and field trials. In the case of soybean (*Glycine max*) alone, the benefits resulting from the use of inoculants with selected superior strains is equivalent to about US\$ 3 billion per cropping season, that otherwise would be spent on the purchase, transportation and application of N-fertilizers (Hungria et al., 2005a). However, despite the considerable effort expended in selecting effective strains for almost a hundred legumes, their genetic characterization and taxonomic position is still poor. In this context, this study aimed at gaining better understanding of phylogenetic relationships, based on the sequencing of the whole 16S rRNA gene, of sixty-eight rhizobial strains recommended for sixty-three legumes in Brazil.

2. Material and methods

2.1. Strains

Sixty-eight strains from the Brazilian (SEMIA) culture collection of rhizobia were selected. Table 1 provides information of the strains, as well as of the host plants from which they were isolated and for which they are recommended. In total there are ninety-four recommendations for commercial inoculants. Strains were provided by FEPAGRO and their purity was verified on yeast extract-mannitol agar (YMA) medium (Vincent, 1970) containing Congo red (0.00125%). Stocks were prepared on YMA and kept at -70°C (under 50% glycerol) for long-term storage and at 4°C as source cultures.

2.2. Morpho-physiological characterization

Colony morphology (color, mucosity, diameter, transparency, borders, elevation) and acid/alkaline reaction were evaluated according to Vincent (1970), after 7 days of growth in the dark, at 28°C , on YMA containing either Congo red or bromothymol blue (0.00125%) as a pH-change indicator.

2.3. DNA extraction

Total genomic DNA of each strain was extracted from bacterial batch cultures grown in YM broth until late exponential phase (10^9 cells mL^{-1}). Extraction of DNA was performed as described before (Fernandes et al., 2003) and to obtain clean DNA samples the extraction procedure included the addition, for each 400 mL of bacteria resuspended in TE 50/20, of 50 μL of 10% SDS, 5 μL of proteinase-K (20 mg mL^{-1}), 10 μL of lysozyme (5 mg mL^{-1}), and 1 μL of RNase (10 mg mL^{-1}). After two steps of purification with ethanol at 96% and at 70%, the pellet was resuspended in 50 μL of TE 10/1 to estimate the concentration of the DNA. Samples were then diluted to 20 ng of DNA μL^{-1} and were kept at -20°C .

2.4. Amplification of the DNA region coding for the 16S rRNA gene and purification of the PCR-products

The DNA of each bacterial strain was amplified with the universal primers described by Weisburg et al. (1991), fD1 (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3'). Each replicate contained, in a volume of 50 μ L: dNTPs (300 μ M of each); PCR-buffer (Tris-base 20 mM pH 8.4 and KCl 50 mM); MgCl₂ (1.5 mM); primers (15 pmol of each); Taq DNA polymerase (1.2 U); DNA (20 ng) and DMSO (5%). The reaction was carried out in an MJ Research Inc. PTC 200 thermocycler, using an initial cycle of denaturation at 95°C for 2 min; thirty cycles of denaturation at 94°C for 15 s, 93 °C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min; a final extension cycle of 72°C for 5 min.

For the purification of the PCR-products, 48 μ L of each PCR reaction were added to each 300 μ L-capacity well of a 96-well U-plate. Each well also received 5 μ L of sterile ammonium acetate (7.5 M) and 165 μ L of 99.5% ethanol (room temperature). The plate was sealed, homogenized and the mixture was centrifuged at 4,000 rpm for 45 min. The supernatant was discarded and the plate was inverted on absorbent paper to dry. After drying, the pellet received 150 μ L of freshly prepared 70% ethanol, the plate was sealed and the suspension was homogenized and centrifuged again at 4,000 rpm for 10 min. The supernatant was discarded as described previously and the plate was inverted on absorbent paper and centrifuged at 300 rpm for 65 s. The pellet was dried at room temperature for 30 min, or at 37°C for 15 min, followed by the addition of 15 μ L of milli-Q water, homogenized, and kept at -20°C. After 24 h, the concentration of the samples was verified in 1.5% agarose gels, adjusted to 40 ng DNA μ L⁻¹ and kept at -20°C.

2.5. Sequencing analysis of the 16S rRNA gene

The PCR-reactions were carried out in 96-well-full-skirt-PCR microplates. Purified PCR products of each bacterial culture (80 ng per reaction) received a mixture of 3 μ L of dye (DYEnamic ET terminator reagent premix for the MegaBACE, Amersham

Biosciences), and 3 pmol of each primer. To obtain the complete sequence of the 16S rRNA gene, five reactions were carried out, with the following primers: fD1, Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3', Young et al., 1991) and the following primers designed by Prof. Leonardo M. Cruz (Dept. of Biochemistry, UFPR, Curitiba, PR, Brazil): 362f (5'-CTCCTACGGGAGGCAGCAGTGGGG-3'), 786f (5'-CGAAAGCGTGGGGAGCAAACAGG-3') and 1203f (5'-GAGGTGGGGATGACGTCAAGTCCTC-3'). The same program was used with all primers, as follows: denaturation at 95°C for 2 min; thirty cycles of denaturation at 95°C for 10 s, 50°C for 4 s, and extension at 60°C for 4 min; final soak at 4°C.

After amplification, to 20 µL of each sample (7 µL of each PCR reaction + 20 µL of milli-Q water) were added 2 µL of sterile ammonium acetate (7.5 M) and 65 µL of 99.5% ethanol (room temperature). The plate was sealed, homogenized and centrifuged at 4,000 rpm for 45 min. The supernatant was discarded and the plate was inverted on absorbent paper to dry. After drying, the pellet received 165 µL of freshly prepared 70% ethanol, the plate was sealed, homogenized, centrifuged again at 4,000 rpm for 10 min, and the supernatant was discarded. The plate was inverted on absorbent paper and centrifuged at 300 rpm for 65 s. The pellet was dried at room temperature for 30 min, or at 37°C for 15 min, resuspended in 7 µL of milli-Q water or in buffer (70% formamide, 1 mM EDTA), and submitted for sequencing analysis in a MegaBACE 1000 DNA Analysis System (Amersham Biosciences). In general, the electrophoresis parameters used were: sample injection voltage, 1 kV; sample injection time, 40 s; run voltage, 5 kV; run time, 240 min.

The high-quality sequences obtained for each strain were assembled into contigs using the programs phred (Ewing and Green, 1998; Ewing et al., 1998), phrap (www.phrap.org) and Consed (Gordon et al., 1998). Sequences confirmed in the 3' and 5' directions were submitted to the GenBank database (<http://www.ncbi.nlm.nih.gov/blast>) to seek significant alignments. Accession numbers from AY904726 to AY904789 were given to the 16S rRNA sequences of sixty-four strains (Table 2). The sequences of the four strains recommended for the soybean crop were reported before (Chueire et al., 2003) and were also confirmed and used in this

study: *B. japonicum* strains SEMIA 5079 (AF234888) and SEMIA 5080 (AF234889), and *B. elkanii* strains SEMIA 587 (AF234890) and SEMIA 5019 (AF237422).

2.6. Phylogeny and taxonomic position based on the 16S rRNA gene

The sequences obtained were aligned pairwise and compared to those of the following type/reference strains (accession numbers of the GenBank Data Library in parentheses): *Azorhizobium caulinodans* USDA 4892^T (X67221); *Bradyrhizobium betae* PL74H1^T (AY372184); *Bradyrhizobium canariense* BC-C2^T (AY577427); *Bradyrhizobium elkanii* USDA 76^T (U35000); *Bradyrhizobium japonicum* USDA 6^T (U69638); *Bradyrhizobium liaoningense* USDA 3622^T (AF208513); *Burkholderia cepacia* ATCC 53867^T (AY741356); *Burkholderia graminis* C4D1M^T (U96939); *Burkholderia* sp. TJ182 (AJ505301); *Burkholderia* sp. BR 3405 (AY773186); *Burkholderia* sp. BR 3407 (AY773186); *Burkholderia* sp. tpig4.4 (AY691396); *Burkholderia* sp. hpud10.4 (AY691394); *Mesorhizobium ciceri* USDA 3383^T (U07934); *Mesorhizobium loti* USDA 3471^T (X67229); *Methylobacterium nodulans* ORS 2060^T (AF220763); *Rhizobium etli* CFN 42^T (U28916); *Rhizobium leguminosarum* USDA 2370^T (U29386); *Rhizobium rhizogenes* ATCC 11325^T (D14501.1); *Rhizobium tropici* CIAT 899^T (U89832); *Sinorhizobium fredii* USDA 205^T (X67231); *Sinorhizobium meliloti* USDA 1002^T (X67222).

A phylogenetic tree was obtained with the UPGMA (unweighted pair-group method, with arithmetic mean) algorithm (Sneath and Sokal, 1973) using the Bionumerics program (Applied Mathematics, Kortrijk, Belgium). The robustness of the tree was inferred by bootstrap analysis (Felsenstein, 1985) with 500 replicates, also using the Bionumerics program.

2.7. PCR-amplification of the DNA region coding for the *nodB*, *nodC* and *nifH* genes

The DNAs of the bacteria showing similarity with the genera *Methylobacterium* and *Burkholderia* were used for amplification of the regions coding for the genes *nodB*, *nodC* and *nifH*.

For the amplification of the *nodB* gene region, the primers used were *nodB3f* (5'-TACCTGACSTTYGACGACGGTCC-3') (Wernegreen and Riley, 1999) and *nodCRR* (5'-GAGACGGCGRCRCTGGTTG-3') (Silva et al., 2003). Each replicate contained, in a volume of 50 μ L: dNTPs (200 μ M of each); PCR-buffer (Tris-base 20 mM pH 8.4 and KCl 50 mM); MgCl₂, (2.4 mM); primers (30 pmol of each); Taq DNA polymerase (1.5 U); DNA (40 ng). The reaction was carried out with thirty cycles of denaturation at 94°C for 90 s, annealing at 67°C for 30s, and extension at 72°C for 80 s; and a final extension cycle of 72°C for 3 min.

The *nodC* amplification was performed with primers *nodClf* (5'-GTCGATTGCMRGTCAGACTACG-3') and *nodCp8* (5'-GCCAGGTCTIGTTGCGATTGCTC-3'), used for *Bradyrhizobium*, as described by Sterner and Parker (1999). For the DNA region coding for *nifH*, amplification was performed with primers *nifHF* (5'-TACGGNAARGGSGGNATCGGCAA-3') and *nifHI* (5'-AGCATGTCYTCSASYTCNTCCA-3') used in a collection of rhizobial strains by Laguerre et al. (2001); the analysis was performed as described by those authors.

3. Results

3.1. Morpho-physiological haracterization of SEMIA strains

Seventeen strains showed acid reactions on YMA medium (Table 2), and in general they were characterized by medium to high production of mucus (data not shown). Forty-eight strains showed alkaline reactions on YMA, most with low to medium production of mucus (data not shown). In relation to the other morphological parameters evaluated (color, transparency, borders, elevation), no consistent patterns emerged within the acid- or alkali-producing group (data not shown). Only three strains, SEMIA 103, SEMIA 6382 and SEMIA 6412 showed neutral reaction on YMA (Table 2), all of which with medium production of mucus and colonies of 2 to 3 mm in diameter after 7 d.

3.2. Phylogeny and taxonomic position based on the 16S rRNA gene

Figure 1 shows the dendrogram obtained with the 16S rRNA sequences of all of the strains, including type and reference strains; they were clustered at a final level of similarity of 77.8%. Strains were grouped into nine phylogenetic branches or main clusters, with some subclusters (Fig. 1). We have defined that strains differing in more than fifteen nucleotides from the closest type strain could represent new species and were therefore designated in this paper as “sp.” (Table 2).

In the first cluster (I), strain SEMIA 658, isolated from nodules of *Lotononis bainesii* in South Africa and very effective for the same legume species in Brazil, showed 97.7% of similarity with *Metylobacterium nodulans* ORS 2060^T (Fig. 1) isolated from root nodules of *Crotalaria podocarpa* in Senegal (Samba et al., 1999; Sy et al., 2001). Considering 500 replicates, cluster I was fully supported (100%) by bootstrap analysis.

The second branch (II) grouped *Bradyrhizobium* strains at a level of similarity of 96.7% and two main clusters (II.1 and II.2) were observed (Fig. 1). Cluster II.1 included twenty-eight SEMIA strains and *B. elkanii* USDA 76^T, joined at a level of similarity of 99.0%; with the addition of SEMIA 587, recommended for soybean, a final level of similarity of 98.2% was achieved. Within cluster II.1, a first subdivision (II.1.1) included five strains with a similarity of 99.1%, isolated from legumes of the subfamilies Papilionoideae and Mimosoideae and belonging to four different tribes (Ingeae, Phaseoleae, Acacieae and Aeschynomeneae). Twenty-three SEMIA strains were clustered within II.1.2 (99.3 similarity), 50% of which isolated from legumes of the tribe Phaseoleae. Subcluster II.1.2 included three out of the four strains from this study that had been isolated from tribe Desmodieae, as well as one from the Dalbergieae and the only strain from the Cytiseae; the subcluster included seven strains isolated from the Mimosoideae and belonging to three tribes. The SEMIA strains positioned in the cluster II.1 are officially recommended for thirty-four host legume species, with some (SEMIAs 6160, 6100, 6169, 6387 and 6149) being the most effective for two different legume species, SEMIA 6145 and 6159 the most effective for three and SEMIA 6158 the most effective for four different host species. Some of those strains were very effective with legumes of distinct subfamilies, e.g., SEMIA 6160, recognized as the most effective for

Albizia lebbbeck (Mimosoideae, Ingeae) and for *Sclerolobium paniculatum* (Caesalpinioideae, Caesalpinieae); similarly, SEMIA 6100 was the most effective for a legume of the Papilionoideae and another of Mimosoideae (Fig. 1, Table 1). Considering 500 bootstrap replicates, a value of 98% was obtained for subcluster II.1.

Cluster II.2 grouped thirteen SEMIA with type strains of *B. japonicum*, *B. liaoningense*, *B. canariense* and *B. betae* with a final level of similarity of 98.4% (Fig. 1). All SEMIA strains showed typical morpho-physiological properties of *B. japonicum*, therefore they were classified as this species (Table 2). The majority (38%) was isolated from legumes of the Papilionoideae tribe Phaseoleae, followed by 23% from the Mimosoideae tribe Acacieae. Promiscuity was also observed within this group, e.g., SEMIA 6156, isolated from *Crotalaria spectabilis*, was identified as the most effective for five species (*C. spectabilis*, *C. juncea*, *Cajanus cajan*, *Canavalia ensiformis*, *Indigofera hirsuta*), and SEMIA 656, isolated from *Neonotonia wightii*, was also the most effective for two other species, *Desmodium intortum* and *Macroptilium atropurpureum* (Fig. 1, Table 1). Subcluster II.2 was also strongly bootstrap supported (100%).

Four SEMIA strains fit into cluster III, a branch of *Rhizobium tropici*-*R. rhizogenes* (*Agrobacterium*), at a final level of similarity of 98.7% (Fig. 1), and considering 500 replicates, a bootstrap of 99% was obtained for this grouping. The comparison of the four SEMIA with the type strains showed higher similarity of bases with *R. rhizogenes* ATCC 11325^T, but differing in eleven to fifteen nucleotides. The highest blast (seven to nine different nucleotides) was with another strain (163C) of *R. rhizogenes* (access # AY206687), isolated from tumors of *Prunus persica*. High similarity (eleven to thirteen different nucleotides) was also observed with strain p1-7 (AY206687), isolated from nodules of common beans (*Phaseolus vulgaris*) and classified as "*R. lusitanum*". Finally, a lower but still high similarity of nucleotides was observed with several *R. tropici* strains isolated from common bean, some of them from Brazil. In the discussion section we explain why, at this moment, based on symbiotic properties and on the 16S rRNA genes, we find it more appropriate to designate these four strains as *Rhizobium* sp. (Table 2).

The *R. leguminosarum* type strain was positioned in cluster IV, together with four SEMIA strains, with a similarity of 99.3% (Fig. 1). All these strains establish symbioses

with clovers, but the bootstrap value obtained in 500 replicates, 67%, was the lowest observed in this study.

Strain SEMIA 384, symbiont of *Vicia sativa* and isolated in Brazil, was highly related to the *R. etli* type strain in cluster V (99.6%) (Fig. 1, Table 1), with a 100% support in the bootstrap analysis for this grouping. However, due to the symbiotic properties, further investigation of the plasmids and other genes of SEMIA 384 should be performed to confirm its taxonomic position.

Sinorhizobium species were positioned in cluster VI with 98.8% of similarity (Fig. 1). *Medicago* is the host genus of SEMIA strains 135, 134 and 103, and the strains were highly related to *S. meliloti* USDA 1002^T (Fig. 1, Table 1). SEMIA 6161 was isolated from *Prosopis juliflora* and was distinct from type strains of *S. meliloti* (seventeen nucleotides) and *S. fredii* (twenty-five nucleotides); it might represent another species, thus was nominated as *Sinorhizobium* sp. (Table 2). Clusters III, IV, V and VI were joined in a deep branch with a similarity of 97.8% (Fig. 1).

Three SEMIA strains were positioned in *Mesorhizobium* cluster VII, with a final similarity of 97.6% (Fig. 1). Strains SEMIA 816 and 830 are symbionts of *Lotus corniculatus* and are highly related (99.5%) (Fig. 1, Table 1); however, they differed from type strain of *M. loti* in twenty-four and thirty nucleotides, respectively, therefore they may represent another species and were nominated as *Mesorhizobium* sp. (Table 2). SEMIA 396 is a symbiont of *Cicer arietinum* and was highly related (99.6%) to the *M. ciceri* type strain (Fig. 1, Table 1, Table 2). *Mesorhizobium* cluster was also strongly supported (100%) by the bootstrap analysis.

Strains SEMIA 6402 and 6401, isolated from stem nodules of *Sesbania virgata* (Table 1), were linked to the *Azorhizobium caulinodans* type strain with a similarity of 97.4% in cluster VIII and the bootstrap analysis indicated very strong support for this group (100%) (Fig. 1). However, although both SEMIAs were highly similar, they differed in more than thirty nucleotides from the type strain, therefore at this moment they are nominated as *Azorhizobium* sp., as they might represent another species (Table 2).

Finally, the most divergent group of strains fit into cluster IX, with a final level of similarity of 96.4% (Fig. 1). This group was confirmed in 100% of the 500 bootstrap replicates and included seven SEMIA strains, all isolated in Brazil, with type and

reference strains belonging to the genus *Burkholderia*. Two subclusters were defined, joined to one isolated strain. Subcluster IX.1 included strains SEMIA 6394, isolated from *Ormosia nitida* (Papilionoideae, Sophoreae) and SEMIA 6390, isolated from *Acacia decurrens* (Mimosoideae, tribe Acacieae), showing higher similarity (99.9%) with *Burkholderia cepacia* ATCC53867^T. Strains SEMIA 6382, 6167, 6166 were highly similar (99.8%) and were all isolated from *Mimosa caesalpiniiifolia* (Mimosoideae, Mimoseae). Strain SEMIA 6412, isolated from *Clitoria fairchildiana* (Papilionoideae, Phaseoleae) was joined to those strains at a level of similarity of 99.3%, and the group was then related to *Burkholderia* sp. strain TJ 182 with 98.1% of similarity. Finally, strain SEMIA 6398 from *Piptadenia gonoacantha* (Mimosoideae, Mimoseae) occupied an isolated position (Fig. 1, Table 1, Table 2). At the SEMIA collection, strains SEMIA 6382 and SEMIA 6383 should be the same as BR 3405 and BR 3407, respectively, however, differences in the some nucleotides were observed when compared with previous sequences deposited (AY773185 and AY773186, respectively). We still have to compare the strains in relation to other characteristics.

3.3. Amplification of *nod* and *nif* genes of *Methylobacterium* and *Burkholderia* strains

The eight SEMIA strains classified as *Burkholderia* and *Methylobacterium* were examined for *nod* and *nif* genes. In relation to the former, using the primers nodB3f (Wernegreen and Riley, 1999) and nodCRR (Silva et al., 2003), reported to amplify *nodB* genes of *Rhizobium*, a product of about 300 bp was obtained exclusively with *Burkholderia* sp. SEMIA 6398 (Fig. 2, Table 3). For the *nodC* gene, using a set of primers that resulted in a product of 243 bp in *Bradyrhizobium* strains (Sterner and Parker, 1999), amplification was obtained with *Burkholderia* sp. strains SEMIA 6398 and 6412, with *B. cepacia* strains SEMIA 6390 and SEMIA 6394 and with *Methylobacterium* sp. SEMIA 658 (Fig. 2, Table 3). The primers used to detect *nifH* resulted in products between 780 and 890 bp in analyses of several rhizobial strains (Laguerre et al., 2001), and the PCR products of *Methylobacterium* and both *B. cepacia* had approximately 700 bp, while the products of *Burkholderia* sp. SEMIAs 6166, 6167 and 6382, all isolated

from *Mimosa caesalpinifolia*, had about 1,500 bp (Fig. 2, Table 3); amplification with those primers was not observed with strains SEMIA 6398 and 6412 (Fig. 2, Table 3).

4. Discussion

Nitrogen is often the most limiting nutrient for plant growth worldwide. The situation is especially critical in the tropics, where the usually fragile soil structure and the low levels of soil organic matter have resulted in the depletion of this nutrient. In addition, the high cost of N-fertilizers in countries like Brazil has resulted in the need for actions emphasizing biological nitrogen fixation (BNF) (Hungria et al., 2005a). Successful approaches begin with long-term programs of rhizobia selection, and the identification of elite strains for each legume host of interest. Countries differ in their policies concerning the commercialization of rhizobial inoculants, and in Brazil, they must contain elite strains evaluated and recommended by an official committee of rhizobiologists (Hungria et al., 2005b). The Brazilian *Rhizobium* Culture Collection (SEMIA) was created in 1985 by the Microbial Resources Centre Network (MIRCEN), with the purpose of maintain the recommended rhizobial strains and distribute the cultures to the inoculant industry (Hungria et al., 2005b). Nowadays, SEMIA strains are classified as *R. meliloti*, *R. leguminosarum*, *B. japonicum*, *Bradyrhizobium* sp., *R. fredii*, or *R. loti* (FEPAGRO, 1999), based exclusively on their ability to produce alkaline or acid reaction in YM medium and on the cross-inoculation group (Vincent, 1970). However, some of those species were reclassified long ago and several new ones have been described. Therefore, although the SEMIA collection is a reservoir of rhizobia resulting from decades of selection, the genetic knowledge about the strains in the collection is very poor. Furthermore, the variety of legumes from which the strains have been isolated offers a unique opportunity to better understand the phylogenetic relationships of tropical rhizobia.

This study evaluated sixty-eight SEMIA strains that were isolated from sixty-three legume hosts, the great majority from Brazil, and represents ninety-four rhizobial recommendations. Host legumes were from a wide range, covering all three subfamilies and seventeen tribes. Phylogeny was based on the sequencing of the 16S rRNA, as this

gene has become the method of choice for tracing bacterial phylogenies and defining taxonomy (Weisburg et al., 1991; Garrity and Holt, 2001). A high level of genetic diversity was observed, and the strains were grouped into nine main clusters and several subclusters, with a final level of similarity of 77.8%. Many SEMIA strains had a broad host range, and apparently we found no evidence of evolutionary correlation with the host plants. Similar results have been observed with other tropical rhizobia (Moreira et al., 1998; Germano & Hungria, 2005). Some of the strains were very effective in fixing N_2 with legumes of distinct tribes and even subfamilies, e.g., *B. elkanii* SEMIA 6160, recognized as the most effective for both *Albizia lebbbeck* (Mimosoideae, Ingeae) and *Sclerolobium paniculatum* (Caesalpinioideae, Caesalpinieae), and *B. japonicum* SEMIA 656, recommended for plants of the subfamily Papilionoideae: *Desmodium* (Desmodieae), *Macroptilium* (Phaseoleae), and *Neonotonia* (Phaseoleae).

The main clusters defined rhizobial genera and species and were strongly (98-100%) supported by bootstrap analysis. The only exception to this was the *R. leguminosarum* cluster, with four symbionts of subtropical clovers from Brazil and Australia, and one strain from field pea isolated in Mexico, grouped with a similarity of 99.2%, but with a lower bootstrap support (67%). A further inclusion of other strains and genes should help to clarify this cluster.

R. etli species has been reported as the main symbiont of common beans in the centers of origin of this legume: Mesoamerica (Segovia et al., 1993), and Northern (Bernal and Graham, 2001) and Southern (Aguilar et al., 1998) Andean South America. However, recent reports indicate a wide distribution of *R. etli* associated with common bean in Brazil (Grange and Hungria, 2004). In our study, Brazilian strain SEMIA 384 from *Vicia sativa* was classified as *R. etli*, a symbiotic relationship that had not been previously reported (Hernandez-Lucas et al., 1995). Therefore, theories about the coevolution of *R. etli* with common beans in the genetic centers of origin of this legume (Segovia et al., 1993; Hernandez-Lucas et al., 1995; Silva et al., 2003) should be reviewed, especially in the light of the recent reports indicating the absence of lateral transfer of symbiotic plasmids between *R. etli* and other common bean rhizobia (*R. gallicum*) in a cropped area in Mexico (Silva et al., 2003).

Four SEMIA strains were clustered with plant-pathogenic non-N₂-fixing agrobacteria. It has been known that *Agrobacterium* spp. share several characteristics and are genetically closely related to some rhizobial species (*R. tropici*, *Rhizobium* genomic species Q, *R. galegae*, *R. huautlense*, and *Allorhizobium undicola*) (Martínez-Romero et al., 1991; Terefework et al., 1998; Wang and Martínez-Romero, 2000; Young et al., 2001; Zakhia and de Lajudie, 2001). Consequently, based on 16S rRNA gene sequences, agrobacteria were recently reclassified into the genus *Rhizobium* (Young et al., 2001). N₂-fixing rhizobia resembling agrobacteria were isolated from root nodules of *Acacia* spp. (Khbaya et al., 1998) and common bean (Mhamdi et al., 1999) in Africa, but the isolates were not able to maintain the symbiotic effectiveness. However, when isolated from soybean nodules in Paraguay (Chen et al., 2000), as well as in our study, when isolates were obtained from *Mimosa scabrella*, *Gliricida sepium*, and *Leucaena leucocephala*, effectiveness and genetic stability of symbiotic properties were confirmed. Therefore, based on the symbiotic properties and on the 16S rRNA sequences, we believe it is more appropriate to designate those four strains as *Rhizobium* spp. In the future, efforts should focus in understanding the evolution and ecological importance of these effective tropical rhizobia closely related to agrobacteria.

Symbionts of *Medicago* isolated from subtropical Brazil were confirmed as *S. meliloti*, but SEMIA 6161 from tropical *Prosopis juliflora* differed considerably from both *S. meliloti* and *S. fredii* type strains, which suggests that it may represent a new *Sinorhizobium* species. A deep branch clustered *R. rhizogenes*, *R. tropici*, *R. leguminosarum*, *R. etli*, *S. meliloti* and *S. fredii* with a similarity of 97.8% (92% of bootstrap), confirming phylogenetic relationships reported in other studies (Wang and Martínez-Romero, 2000).

Putative new species were also observed in two other genera. Strains SEMIAs 816 and 830, symbionts of *Lotus corniculatus*, isolated in Brazil and USA, respectively, differed from type strain of *Mesorhizobium loti* by twenty-four and thirty nucleotides, respectively. Also two strains isolated from stem nodules of *Sesbania virgata* in Brazil, SEMIAs 6401 and 6402, differed by more than thirty nucleotides from type strain of *Azorhizobium caulinodans*.

The majority of the strains from this study (forty-two) were classified into the genus *Bradyrhizobium* and two major subclusters were observed. The *B. elkanii* cluster included symbionts of all three subfamilies and several tribes, but clearly it could be subdivided into at least one new group (II.1.1. Fig. 1). Another new group (II.2.1) was verified in the *B. japonicum* cluster. Type strains of *B. japonicum*, *B. liaoningense* and *B. canariense* were clustered into II.2.2, and the similarity of the sequences of these three species has been previously reported (Willems et al., 2001; Zakhia & de Lajudie, 2001; Vinuesa et al., 2005). Although high diversity in morphological, physiological, and genetic properties within *Bradyrhizobium* strains has been reported, the differences are not reflected in diversity of the 16S rRNA genes (Vinuesa et al., 1998; Molouba et al., 1999; Chen et al., 2000; van Berkum and Fuhrmann, 2000; Willems et al., 2001). However, the results obtained in our study show variability in the 16S rRNA genes within *Bradyrhizobium*, and in addition to other reports (Willems et al., 2003; Germano and Hungria, 2005), may indicate the presence of new species to be revealed.

Strain SEMIA 658 (= CB 376) isolated from *Lotononis bainesii* in South Africa and also effective under Brazilian conditions was clustered with the type strain of the α -proteobacterium *Methylobacterium nodulans*, isolated from *Crotalaria* spp. (Sy et al., 2001). The strains studied by Sy et al. (2001) showed similarity of *nodA* genes with *Bradyrhizobium*, which suggests horizontal gene transfer. SEMIA 658 should be the same as CB 376, cited as belonging to the genus *Methylobacterium* (Sy et al., 2001), and in our study amplification was achieved with the primers designed for *nodC* region of *Bradyrhizobium*.

Seven strains from Brazil were grouped with β -proteobacteria of the genus *Burkholderia*. N_2 -fixing symbiotic associations of burkholderia with legume plants, preferentially from the Mimosoideae, have been reported (Moulin et al., 2001; Chen et al., 2003). In our study, SEMIA 6394 isolated from *Ormosia nitida* (Papilionoideae), and SEMIA 6390, isolated from *Acacia decurrens* (Mimosoideae), showed higher similarity of nucleotides (99.9%) with *Burkholderia cepacia* ATCC53867^T. Three other strains (SEMIA 6382, 6167, 6166) isolated from *Mimosa caesalpiniiifolia* were highly similar with each other in the 16S rRNA analysis, but we were unable to get amplification of those strains with the *nodB* or *nodC* primers. However, DNA amplification for the *nifH* region of

these three strains produced PCR-products of similar size (~1,500 bp), but differed from the products obtained with the other SEMIA classified as *Burkholderia* (~700bp). Strain SEMIA 6412 isolated from *Clitoria fairchildiana* (Papilionoideae) was grouped in the same cluster with these three strains and amplified with the *nodC* primers. The grouping of these four strains was genetically closer to the diazotrophic *Burkholderia* strain TJ 182, isolated from *Mimosa diplotricha* in Taiwan (Chen et al., 2003). Finally, SEMIA 6398 from *Piptadenia gonoacantha* (Mimosoideae) occupied an isolated position in the cluster and the strain did not amplify with *nifH* primers, but PCR-products were obtained with both *nodB* primers of common bean rhizobia and *nodC* primers of soybean bradyrhizobia. It is also noteworthy that PCR-products obtained with *nodC* primers varied in size among the strains, and were different from those obtained with *Bradyrhizobium* by Sterner & Parker (1999). The results obtained in our study indicate higher variability in relation to the host plant, to the ribosomal 16S gene, and to the *nif* and *nod* genes among burkholderia capable of nodulating legumes than previously thought (Moulin et al., 2001; Chen et al., 2003). Further studies may reveal additional members of symbiotic bacteria, differences in ribosomal and symbiotic genes, and help to clarify the origin of *nif* and *nod* genes in burkholderia.

It has been suggested that tropical rhizobia are poorly documented (Zakhia & de Lajudie, 2001), although reports indicate that rhizobia diversity may be greater in tropical than in temperate regions (Oyaizu et al., 1992). The strains included in our study are recognized as the most effective for their host legumes in Brazil, and therefore represent an important source of N₂-fixation genes. The promiscuous nature of some strains, the wide range of symbiotic associations with α - and β -proteobacteria, and the detection of several putative new species emphasize the great diversity of rhizobial strains that still remains to be discovered in the tropics.

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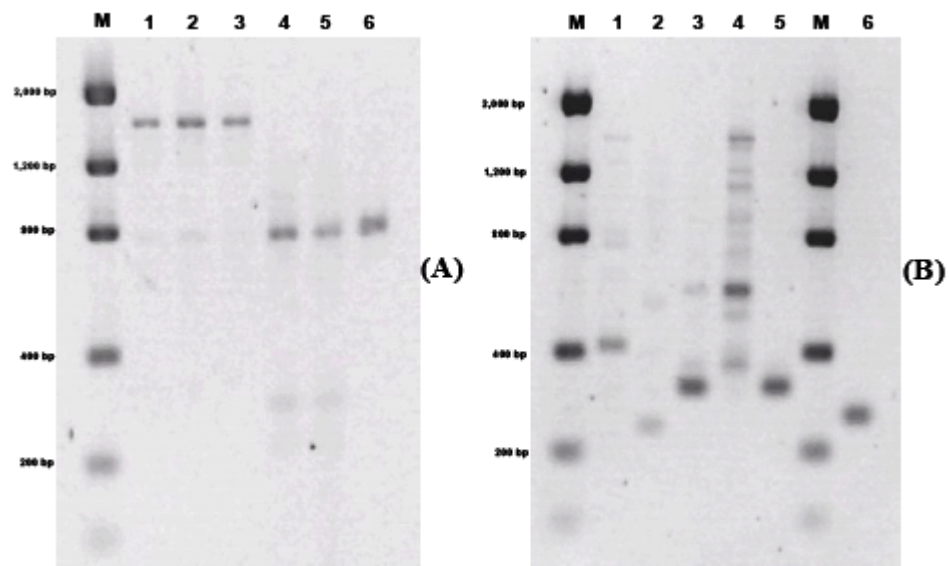
6. Legend of Figures

Fig. 1. Phylogenetic tree based on the 16S rRNA sequences of sixty-eight strains, N₂-fixing symbionts isolated from sixty-three legumes and officially recommended for the use in Brazilian commercial inoculants. GeneBank accession numbers of SEMIA strains (Table 2) and of type strains (material and methods) are given in the text. Cluster analysis using the UPGMA algorithm and numbers in the main branches indicate bootstrap values obtained with 500 replicates.

Fig. 2. Amplification of *nif* and *nod* genes of bacteria classified in the genera *Methylobacterium* and *Burkholderia*. (A) *nifH*-PCR-products of strains (lanes): SEMIA 6382 (1), SEMIA 6167 (2), SEMIA 6166 (3), SEMIA 6394 (4) , SEMIA 6390 (5) and SEMIA 658 (6); (B) *nodC*-PCR-products of strains: SEMIA 6412 (1), SEMIA 6398 (2), SEMIA 6394 (3), SEMIA 6390 (4), SEMIA 658 (5); and *nodB*-PCR-products of SEMIA 6398 (6). Strains are described in Table 1.



6.1 – Figure 1



6.2 – Figure 2

7. Table 1

Information about the strains recommended for the use in Brazilian commercial inoculants and sequenced in this study

Plant species ^{a,b}	Some common names ^c	Subfamily ^b	Tribe ^b	Descriptor ^a	Main use in Brazil	Applications worldwide ^c	SEMIA number	Other designations ^{a,c}	Use in inoculants ^a	Source of the strain ^b	Original host plant ^a	Institution ^b recommending ^a	Biost ^d
<i>Acacia auriculiformis</i> Benth.	Earpod wattle, blackwattle, acácia ^d	Mimosoidae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6387	BR 3609, LMG 9961	Actual	Brazil	Same	Embrapa Agrobiologia	IV
							6391	BR 3624	Actual	Brazil	Same	Embrapa Agrobiologia	IV
<i>Acacia decurrens</i> Willd.	Green wattle, acácia-da-Austrália ^d	Mimosoidae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6164	BR 3608, LMG 9960	Actual	Brazil	<i>Acacia nearnsi</i> De Wild.	Embrapa Agrobiologia	II
							6390	BR 3614	Actual	Brazil	Same	Embrapa Agrobiologia	II
<i>Acacia mangium</i> Willd.	Mangium, silk leaf acacia, akasia, acácia mangium ^d	Mimosoidae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6387	BR 3609, LMG 9961	Actual	Brazil	<i>Acacia auriculiformis</i> Benth.	Embrapa Agrobiologia	IV
							6420	BR 3617, LMG 9965	Actual	Brazil	Same	Embrapa Agrobiologia	IV
<i>Acacia nearnsi</i> De Wild.	Black wattle, acácia negra ^d	Mimosoidae	Acacieae	Perennial non-climbing shrub or tree	Tree	Environment, chemical, wood	6163	BR 3607	Actual	Brazil	Same	Embrapa Agrobiologia	II
							6164	BR 3608	Actual	Brazil	Same	Embrapa Agrobiologia	II
<i>Albizia lebbek</i> (L.) Benth.	Rain tree, woman's tongue, bois noir, baile de caballero, lengua de mujer, coração-de-negro ^d , fraxino ^d , pau-preto ^d , acácia ^d	Mimosoidae	Ingeae	Perennial non-climbing tree	Tree	Environment, chemical, toxins, wood	6160	BR 5610	Actual	Brazil	Same	Embrapa Agrobiologia	III
<i>Arachis hypogaea</i> L.	Peanut, groundnut, cacahoute, amendoim ^d	Papilionoideae	Aeschynomeneae	Annual non-climbing herb	Grain	Food, medicine, forage, environment	6144	SMS 400, USDA 3187, MAR 11	Actual	Zimbabwe	Same	IAC	IV
<i>Arachis pintoi</i> Krapov. & W. Gregory	Forage peanut, amendoim forrageiro ^d	Papilionoideae	Aeschynomeneae	Perennial non-climbing herb	Forage (tropical)	Forage, environment	6440	MGAP 13	Actual	Brazil	Same	EPAMIG/Embrapa Cerrados	IV
<i>Cajanus cajan</i> (L.) Millsp.	Pigeonpea, Congo pea, pois cajan, frijol de arbol, lentilha, gramim ^d	Papilionoideae	Phaseoleae	Annual/perennial, non-climbing herb or shrub	Forage (tropical)	Forage environment, food, medicine, wood	6156	CPAC-U, DF F-2	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	IV
							6157	BR 2801	Actual	Brazil	Same	Embrapa Agrobiologia	II
<i>Calopogonium</i>	Calopo,	Papilionoideae	Phaseoleae	Perennial	Grass	Cover crop,	6152	BR 1602	Actual	Brazil	<i>Calopogon</i>	Embrapa	IV

spp.	calopogônio ^d	ze		climbing or non-climbing herb	manure	green manure, forage					man sp.	Agrobiologia	
<i>Canavalia ensiformis</i> (L.) DC.	Jackbean, swordbean, horsebean, pois de sabre, doliqua, cocorico, haba criolla, feijão-de-porco ^d	Papilionoideae	Phaseoleae	Annual/perennial, climbing or non-climbing herb or shrub	Green manure	Cover crop, green manure, forage, food	6136	CPAC-U, DF F-2	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	III
							6138	CPAC 42	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	III
<i>Centrosema</i> spp.	<i>Centrosema</i> , fleur languette, pois pinxto, choncho, conchitas, centrosema ^d	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment	6146	BR 1808	Actual	Brazil	<i>Centrosema</i> sp.	Embrapa Agrobiologia	III
							6424	CPAC L36	Actual	Brazil	<i>Centrosema pubescens</i> Benth.	Embrapa Cerrados	IV
							6425	CIAT 2380	Actual	Brazil	<i>Centrosema pubescens</i> Benth.	Embrapa Cerrados	IV
<i>Cicer arvense</i> L.	Chickpea, pois chiche, garbanzo, grão-de-bico ^d	Papilionoideae	Ciceraceae	Annual non-climbing herb	Grain	Food, forage, medicine	396	TAL 1148, USDA 3100, Nix 27A3	Actual	USA	Not known	FEPAGRO/ UFRGS	IV
<i>Clitoria fairchildiana</i> R. Howard	Butterfly pea tree, faveira, palheira, palheiro ^d , sombreiro ^d	Papilionoideae	Phaseoleae	Perennial non-climbing tree	Tree	Environment, medicine, wood	6412	BR 8003	Actual	Brazil	Same	Embrapa Agrobiologia	IV
<i>Clitoria ternatea</i> L.	Pigeon wings, pois savana, blue pea, azulajo, campenilla, conchita blanca o azul, apatico de la reina, clitoria ^d	Papilionoideae	Phaseoleae	Perennial climbing herb or shrub	Tree	Environment, food, chemical, wood, forage, toxins	6053	TAL 827, UMEL 128	Till 1999	Malaysia	Same	Embrapa Agrobiologia	II
<i>Crotalaria juncea</i> L.	Sunn hemp, cascavelle, grand sonnette, grand tcha-tcha, crotalaria ^d	Papilionoideae	Crotalariaeae	Annual non-climbing herb	Green manure	Fibre, environment, firewood	6145	BR 2001	Actual	Libia	<i>Arachis hypogaea</i> L.	Embrapa Agrobiologia	II
<i>Crotalaria juncea</i> L.	Sunn hemp, cascavelle, grand sonnette, grand tcha-tcha, crotalaria ^d	Papilionoideae	Crotalariaeae	Annual non-climbing herb	Green manure	Fibre, environment, firewood	6156	CPAC-U, DF F-2	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	IV
<i>Crotalaria spectabilis</i> Roth	Kattle Box, showy rattlespod, cascavelle jauna, sonnette,	Papilionoideae	Phaseoleae	Annual non-climbing herb or	Green manure	Fibre, environment, firewood, toxins	6156	CPAC-U, DF F-2	Actual	Brazil	Same	Embrapa Cerrados	IV
							6138	CPAC 42	Actual	Brazil	Same	Embrapa Cerrados	IV

	marraquim, crotalaria ⁴			shrub									
<i>Cyanopsis tetragonoloba</i> (L.) Taubert	Clusterbean, guar bean, guar gum, feijão-guarda ⁴ , guar ⁴	Papilionoide ae	Indigoferaceae	Annual non- climbing herb	Green manure	Environment, chemical, food, forage	6145 6319	BR 2001 NC 92, SMS 561	Actual Actual	Brazil Bolivia	<i>Arachis hypogaea</i> L. <i>Arachis</i> sp.	Embrapa Agrobiologia IAC	II II
<i>Dalbergia nigra</i> (Vell. Conc.) Benth.	Brazilian rosewood, jacaranda, palisandre, cabinha ⁴ , jacaranda-da- Bahia ⁴	Papilionoide ae	Dalbergiaceae	Perennial non- climbing tree	Tree	Environment, chemical, wood	6101	BR 8404	Actual	Brazil	Same	Embrapa Agrobiologia	III
<i>Desmodium incanum</i> DC. (<i>D. canum</i>)	Spanish clover, wild peanut, colle-colle, herbe gallon, cepa de caballo, colleur, paga paga; desmodio ⁴	Papilionoide ae	Desmodiaceae	Perennial non- climbing herb or shrub	Forage (tropi- cal)	Environment, forage, medicine	6028	TAL 569, MAR 472	Actual	Zimbabwe	<i>Desmodium uncinatum</i> (Jacq.) DC.	FEPAGRO/ UFRGS	II
<i>Desmodium intortum</i> (Müller) Urban	Beggarlice, copal de cocha, paga paga, sarna blanca, amor seco, desmodio ⁴	Papilionoide ae	Desmodiaceae	Perennial non- climbing herb or shrub	Forage (tropi- cal)	Environment, forage	656	SEMIA original	Actual	Brazil	<i>Neonotonia wrightii</i> (Wight & Arn.) Lackey	FEPAGRO/ UFRGS	II
<i>Desmodium heterocarpum</i> (L.) DC. subsp. <i>ovalifolium</i> (Pratt) Ohashi	<i>Desmodium ovalifolium</i> , <i>desmodium</i> , <i>desmodio</i> ⁴	Papilionoide ae	Desmodiaceae	Perennial non- climbing herb or shrub	Forage (tropi- cal)	Environment, forage	6208	CIAT 2372	Actual	Colombia	Same	CEPEC	III
<i>Desmodium uncinatum</i> (Jacq.) DC.	Silverleaf desmodium, desmodio ⁴	Papilionoide ae	Desmodiaceae	Perennial non- climbing herb or shrub	Forage (tropi- cal)	Environment, forage	696	CB 627	Till 1999	Australia	Same	FEPAGRO/ UFRGS	III
<i>Enterolobium contortisiliquum</i> (Vell. Conc.) Marong	Earpod tree, oreja de negro, timbo ⁴ , tumboril-do- campo ⁴ , oreilha- de-negro ⁴ , oreilha- de-macaco ⁴ , timba-tiba ⁴ , timbeira ⁴	Mimosoideae	Ingeae	Perennial non- climbing tree	Tree	Environment, medicine, toxin, wood	6159	BR 4406	Actual	Brazil	<i>Enterolo- bium ellipticum</i> Benth.	Embrapa Agrobiologia	III
<i>Enterolobium cyclocarpum</i> (Jacq.) Griseb.	Devil's ear, earpod tree, elephant's ear, monkey ear, bois tauiste rouge, oreille d'elephant,	Mimosoideae	Ingeae	Perennial non- climbing tree	Tree	Environment, medicine, pasture, wood, food	6159	BR 4406	Actual	Brazil	<i>Enterolo- bium ellipticum</i> Benth.	Embrapa Agrobiologia	IV

	gumacasta, orelha-de-elefante ⁶												
<i>Enterolobium timbova</i> Martius	Timbor, timboril ⁴ , timbova ⁴ , timbova ⁴	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, medicine, toxin, wood	6139	BR 4460	Actual	Brazil	<i>Enterolobium ellipticum</i> Benth.	Embrapa Agrobiologia	IV
<i>Erythrina verna</i> Vell. Conc.	Mulunga, cristigalli, umhugu ⁴ , suinã ⁴ , suinã-da-terra ⁴	Papilionoideae	Phaseoleae	Perennial non-climbing tree	Tree	Environment, wood	6100	BR 5609	Actual	Brazil	<i>Falcata mahuana</i> (Miq.) Barneby & Grimes	Embrapa Agrobiologia	II
<i>Falcata mahuana</i> (Miq.) Barneby & Grimes	<i>Albizia falcata</i> , mahuana albiza, batui wood, sau, malecia, albiza ⁴	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, wood, food	6100	BR 5609	Actual	Brazil	Same	Embrapa Agrobiologia	IV
							6169	BR 5612	Actual	Brazil	Same	Embrapa Agrobiologia	III
<i>Galactia striata</i> (Jacq.) Urban	Galactia, guatabito, frijolillo, galactia ⁴ , galactia ⁴ , amandoin-da-terra ⁴	Papilionoideae	Phaseoleae	Perennial climbing herb	Forage (tropical)	Forage, environment	6149	CB 627, SMS 138	Actual	Australia	Same	IAC	II
							6130	SMS 300	Actual	Brazil	<i>Acacia mearnsii</i> De Wild.	IAC	II
<i>Glinicidia septima</i> (Jacq.) Walp.	Grow stick, cacabunanca, madricacao, glinicia ⁴	Papilionoideae	Robinieae	Perennial non-climbing tree	Tree	Environment, wood	6168	BR 8801, LMG 10132	Actual	Brazil	Same	Embrapa Agrobiologia	IV
<i>Glycine max</i> (L.) Merr.	Soybean, soya bean, Manchurian bean, soja ⁴	Papilionoideae	Phaseoleae	Annual climbing or non-climbing herb	Grain	Food, medicine	387	BR 96	Actual	Brazil	Same	FEPAGRO/ UFRGS	IV
							3019	29W, BR 29	Actual	Brazil	Same	FEPAGRO/ UFRGS	IV
							3079	CPAC 15, DF 24	Actual	Brazil	Same	Embrapa Cerrados	IV
							3080	CPAC 7	Actual	Brazil	Same	Embrapa Cerrados	IV
<i>Indigofera hirsuta</i> L.	Hairy indigo, unibezvo ⁴ , unileira ⁴ , indigofera ⁴	Papilionoideae	Indigoferae	Annual/perennial, non-climbing herb	Forage (tropical)	Forage, environment, medicine	6136	CPAC-U, DF F-2	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	III
							6138	CPAC 42	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	III
<i>Labiab purpureus</i> (L.) Sweet	Black bean antaqua, pois antaqua, bonavist, labiab ⁴	Papilionoideae	Phaseoleae	Annual/perennial, climbing or non-climbing	Forage (tropical)	Forage, chemical, environment, food, medicine	662	CB 188	Actual	Australia	<i>Vigna unguiculata</i> (L.) Walp.	FEPAGRO/ UFRGS	II
							695	QA 922, E 83, SU	Actual	Australia	<i>Neonotonia wightii</i>	FEPAGRO/ UFRGS	II

				herb		toxins		422, NA 630			(Wight & Arn.) Lacke		
<i>Leucaena diversifolia</i> (Schild.) Benth.	Wild tamarind, guachá, guacha, guaje, guaje blanco, leucena ^d	Mimosoideae	Mimosaceae	Perennial non-climbing shrub, tree	Tree	Environment, food	6168	BR 8801, LMG 10132	Till 2002	Brazil	<i>Girardinia sapium</i> (Jacq.) Walp.	Embrapa Agrobiologia	III
							6169	BR 5612	Actual	Brazil	<i>Falcataria mahuacana</i> (Miq.) Burnaby & Grimes	Embrapa Agrobiologia	III
<i>Leucaena leucocephala</i> (Lam.) De Wit v. Cunningham	Jumbie bean, lead tree, leucena, cowbrush, bois bourro, cassie blanc, graine de lin, granadillo bobó, leucena ^d	Mimosoideae	Mimosaceae	Perennial non-climbing shrub, tree	Tree	Environment, food	6069	DF 10, BR 414	Actual	Brazil	<i>Leucaena leucocephala</i> (Lam.) De Wit	Embrapa Cerrados	IV
							6070	DF 15	Actual	Brazil	<i>Leucaena leucocephala</i> (Lam.) De Wit	Embrapa Cerrados	IV
<i>Lotononis bainesii</i> Baker	Miles lotononis, lotononis ^d	Papilionoideae	Crotalariaeae	Annual non-climbing herb	Forage (tropical)	Forage	638	CB 376	Actual	South Africa	Same	FEPAGRO/ UFRGS	III
<i>Lotus corniculatus</i> L.	Cat's clover, birds' foot trefoil, broadleaf trefoil, cornichão ^d	Papilionoideae	Lotaeae	Perennial non-climbing herb, shrub	Forage (subtropical)	Forage, environment	816	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	IV
<i>Lotus glaber</i> Miller (<i>L. tenuis</i> Willd.)	<i>Lotus tenuis</i> , narrow trefoil, birds' foot trefoil, cornichão ^d	Papilionoideae	Lotaeae	Perennial non-climbing herb, shrub	Forage (subtropical)	Forage, environment	830	Hansen inoculant	Actual	USA	Not known	FEPAGRO/ UFRGS	II
<i>Lupinus albus</i> L.	White lupine, Egyptian lupine, chochos, lupino blanco, tremoro, tremoço ^d	Papilionoideae	Cytiseae	Annual non-climbing herb	Green manure	Forage, environment, green manure, medicine, toxin, food	938	Hansen inoculant	Actual	USA	Same	FEPAGRO/ UFRGS	III
<i>Macroptilium atropurpureum</i> (DC.) Urban	Purple bean, conchito, siratro ^d	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment, medicine, food	656	SEMIA original	Actual	Brazil	<i>Neonotonia wightii</i> (Wight & Arn.) Lackey	FEPAGRO/ UFRGS	II
<i>Macrotyloma axillare</i> (E. Meyer) Vardc.	Macrotyloma, perennial horse, macrotiloma ^d	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment	6149	CB 827, SMS 138	Actual	Australia	<i>Galactia striata</i> (Jacq.) Urban	LAC	II
<i>Medicago polymorpha</i> L.	Bar medic, purple medick,	Papilionoideae	Trifolieae	Annual non-	Forage (subtro-	Forage, environment,	103	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	II

	California barclover, hazarna, clover, trevo carretilha ^d			climbing herb	pical)	medicine, food							
<i>Medicago sativa</i> L.	Alfalfa, lucerna, hazarna, alfafa ^d	Papilionoide ae	Trifoliales	Perennial non- climbing herb	Forage (subtro- pical)	Forage, environment, medicine, food	134	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	IV
							135	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	IV
<i>Mimosa acutistipula</i> Benth.	Mimosa, jurema- preta ^d , espinheiro ^d	Mimosoideae	Mimosaceae	Perennial non- climbing shrub or tree	Tree	Environment, wood	6383	BR 3407	Actual	Brazil	<i>Mimosa caesalpinji- folia</i> Benth.	Embrapa Agrobiologia	III
							6384 ^e	BR 3446 ^e	Actual	Brazil	<i>Mimosa obovata</i> Benth. (=M. laticifera)	Embrapa Agrobiologia	III
<i>Mimosa caesalpinji- folia</i> Benth.	Mimosa, jupimura, mimosa ^d , umba- de-gata ^d , saboa ^d , sancho-do- campo ^d	Mimosoideae	Mimosaceae	Perennial non- climbing shrub or tree	Tree	Environment, wood	6166 ^e	BR 3446 ^e	Till 1999	Brazil	Same	Embrapa Agrobiologia	II
							6167	BR 3432	Till 1999	Brazil	Same	Embrapa Agrobiologia	II
							6382	BR 3403	Actual	Brazil	Same	Embrapa Agrobiologia	IV
<i>Mimosa scabrella</i> Benth.	Abracastanga, bracastanga, paracastanga, bracastanga ^d	Mimosoideae	Mimosaceae	Perennial non- climbing shrub or tree	Tree	Environment, wood	6165	BR 3434	Actual	Brazil	Same	Embrapa Agrobiologia	III
<i>Neonotonia wrightii</i> (Wight & Arn.) Lackey	Glycine, perennial soybean, soja perene ^d	Papilionoideae	Phaseoleae	Perennial climbing herb	Forage (tropi- cal)	Forage, environment, food	656	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	III
							695	E85, QA 922, SU 422, NA 630	Till 1999	Australia	Same	FEPAGRO/ UFRGS	II
							6148	SMS 303	Actual	Brazil	Same	IAC	II
<i>Ormosia nitida</i> Vogel	Ormosia ^d , tecto- macauba ^d	Papilionoideae	Sophoreae	Perennial non- climbing shrub or tree	Tree	Environment, wood	6394	BR 4103	Actual	Brazil	Same	Embrapa Agrobiologia	II
<i>Piptadenia gonocantha</i> (Martius) Macbr.	Piptadenia, per- jacara ^d , angico- jacara ^d , canive- teiro ^d , icarape ^d	Mimosoideae	Mimosaceae	Perennial non- climbing tree	Tree	Wood, environment, chemical	6398	BR 4812, UFC 832.55	Actual	Brazil	<i>Piptadenia stipulacea</i> (Benth.) Ducke	UFC	III
<i>Piptadenia stipulacea</i> (Benth.) Ducke	Piptadenia, jurema-branca ^d	Mimosoideae	Mimosaceae	Perennial non- climbing tree	Tree	Wood, environment	6398	BR 4812 UFC 832.59	Till 1999	Brazil	Same	UFC	II
<i>Pisum sativum</i> L.	Field pea, petit pois, shirazi, guisante, ervilha ^d	Papilionoideae	Viciaeae	Annual, climbing, herb	Grain	Food, forage	3007	B 11A	Actual	Mexico	Same	FEPAGRO/ UFRGS	IV

<i>Prosopis juliflora</i> (Sw.) DC.	Mesquite, cashew, bayarona, epinard bayahonda blanca, algaroba ⁴	Mimosoideae	Mimosaceae	Perennial non-climbing tree, shrub	Tree	Environment, wood	6161	BR 4002, PRJ B4	Actual	Brazil	Same	Embrapa Agrobiologia	IV
<i>Pueraria phaseoloides</i> (Roxb.) Benth.	Tropical kudzu, grande feijão, pueru, kudzu tropical ⁴ , kudzu ⁴	Papilionoideae	Phaseoleae	Perennial climbing herb	Green manure	Green manure, forage, environment	6175	DF Q-1	Actual	Brazil	Same	Embrapa Cerrados	III
<i>Sclerobolium paniculatum</i> Vogel	Velame, veludo, taxi-branco ⁴ , ajustu-coutas ⁴ , anga ⁴ , arapaçu ⁴ , carvoeiro ⁴	Caesalpiniaceae	Caesalpiniaceae	Perennial non-climbing tree	Tree	Environment, wood	6160	BR 5610	Actual	Brazil	<i>Albizia lebbek</i> (L.) Benth.	Embrapa Agrobiologia	II
							6420	BR 3617 LMG 9965	Actual	Brazil	<i>Acacia mangium</i> Willd.	Embrapa Agrobiologia	II
<i>Sesbania virgata</i> (Cav.) Pers.	Acacia, cibil, cumand, sesbania ⁴	Papilionoideae	Robiniaceae	Perennial non-climbing shrub, tree	Tree	Environment, wood, forage, medicine	6401	BR 3401, LMG 9993	Actual	Brazil	Same	Embrapa Agrobiologia	II
							6402	BR 3404 LMG 9994	Actual	Brazil	Same	Embrapa Agrobiologia	II
<i>Stizolobium aterrimum</i> Piper & Tracy	Velvet-, Bengal-bean, Portuguese coffee, black macuna, café Brazil, pois mascato, feijão-macuna ⁴ , macuna-preta ⁴	Papilionoideae	Phaseoleae	Perennial non-climbing shrub	Green manure	Environment, green manure, forage	6158	CPAC 42	Actual	Brazil	<i>Crotalaria spectabilis</i> Roth	Embrapa Cerrados	IV
<i>Stylosanthes</i> spp.	Stylo, Brazilian lucerne, stylosanthes, estilosantes ⁴	Papilionoideae	Aeschynomeneae	Perennial non-climbing herb or shrub	Forage (tropical),	Forage, environment	6155	BR 502	Actual	Brazil	<i>Stylosanthes</i> sp.	Embrapa Agrobiologia	III
<i>Tipuana tipu</i> (Benth.) Kuntze	Tipa, palo mortero, tipa blanca, tipuana ⁴ , tipa-branca ⁴ , tipu ⁴	Papilionoideae	Dalbergiaceae	Perennial non-climbing tree	Tree	Environment, wood, chemical, forage	6192	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	II
<i>Trifolium pratense</i> L.	Red clover, trebol de los prados, trebol rojo, trevo vermelho ⁴	Papilionoideae	Trifolieae	Perennial non-climbing herb	Forage (subtropical)	Forage, environment, medicine	222	SU 329, TA-1, NA 14	Actual	Australia	<i>Trifolium subterraneum</i> L.	FEPAGRO/ UFRGS	IV
							2081	EEL 1283	Actual	Brazil	Same	EPAGRI	IV
<i>Trifolium repens</i> L.	Dutch-, ladino-, white-clover, trebol blanco, trevo branco ⁴	Papilionoideae	Trifolieae	Perennial non-climbing herb	Forage (subtropical)	Forage, environment, medicine	222	SU 329, TA-1, NA 14	Actual	Australia	<i>Trifolium subterraneum</i> L.	FEPAGRO/ UFRGS	IV
<i>Trifolium subterraneum</i> L.	Subclover, subterranean	Papilionoideae	Trifolieae	Annual non-	Forage (subtro-	Forage, environment,	222	TA 1, CB 1165	Actual	Australia	Same	FEPAGRO/ UFRGS	IV

	clower, trevo subterrâneo ^a			climbing, herb	pical)	medicine							
<i>Trifolium vesiculosum</i> Savt	Arrowleaf clover, trevo yuchi, trevo vesiculoso ^a	Papilionoide ae	Trifoliaceae	Annual non- climbing herb	Forage (subtro- pical)	Forage, environment, medicine	2051	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	II
<i>Vicia sativa</i> L.	Common vetch, pois Franca, arveja, ervilhaca ^a	Papilionoide ae	Viciaceae	Annual climbing or non- climbing herb	Forage (subtro- pical)	Forage, environment, medicine	384	SEMIA original	Actual	Brazil	Vicia sp.	FEPAGRO/ UFRGS	II
<i>Pisum sativum</i> (L.) Walp.	Cowpea, black eye pea, barbati, boansa, pois manga cochoa, caupi ^a , feijão-de- corda ^a , feijão arrozado ^a	Papilionoide ae	Phaseoleae	Annual/per ennial, climbing, non- climbing herb	Grain	Grain, forage	662	CB 188	Till 1999	Australia	Same	FEPAGRO/ UFRGS	III
							6002	CB 756, TAL 309, RCR 3824	Till 1999	Zimbabwe	Same	FEPAGRO/ UFRGS	III
							6145	BR 2001	Actual	Brazil	<i>Arachis hyzogaia</i> L.	Embrapa Agrobiologia	IV

^a Plant species for which the strain is commercially recommended.

^b Taxonomy based on ILDIS (2005).

^c Information obtained from the following sites: juazeiro.cnp.org.br/edalcin/arvores/; umbuzeiro.cnp.org.br/db/forrag/vernac.shtml; www.arboretos.cnpemembrapa.br/faz_sm/especies.html; www.arvores.brasil.nom.br/listacient.htm; www.fao.org/ag/AGA/AGAP/FRG/AFRIS/Data/215.HTM; www.fao.org/ag/agp/agpc/doc/Gbase/latinsearch.htm; www.hear.org/pier/species/; www.ildis.org/LegumeWeb/6.00/taxa/1621.shtml; www.ipef.br/identificacao/nativas/detalhes.asp?codigo=65; www.newcrops.uq.edu.au/listing/clitoriateratea.htm; www.rain-tree.com/plants.htm

^d Common names used in Brazil. Information obtained from the sites cited on (3).

^e Different numbers in FEPAGRO (1999).

^f Culture collections: ATCC (The American Type Culture Collection, Manassas, USA); BR (Brazil, Embrapa Agrobiologia, Seropédica, Brazil); CB (Commonwealth Scientific and Industrial Research Organization – CSIRO, Canberra, Australia); CIAT (Centro Internacional de Agricultura Tropical, Cali, Colombia); DF (Distrito Federal, Embrapa Cerrados, Planaltina, Brazil); E (Instituto Nacional de Tecnología Agropecuaria – INTA – Castelar, Argentina); H (Embrapa Cerrados, Planaltina, Brazil); LMG (Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium); MAR (Marondera, Grasslands *Rhizobium* Collection, Soil Productivity Research Laboratory, Marondera, Zimbabwe; also called SPRL); MGAP (Ministerio de Ganadería, Agricultura y Pesca, Laboratorio de Microbiología y Suelos, Montevideo, Uruguay) CPAC (Centro de Pesquisa Agropecuária dos Cerrados, Embrapa Cerrados, Planaltina, Brazil); NA (New South Wales Dept. of Agriculture, NSW Dept. of Primary Industries – Agriculture, Victoria, Australia); NC (North Caroline, University of North Caroline, Raleigh, USA); Nit (**Nitragin, Inc.**, Brookfield, USA); PRF (Paraná Feijão, Embrapa Soja, Londrina, Brazil); QA (Queensland Austrália, University of Queensland, St. Lucia, Australia); RCR (Rothamsted *Rhizobium* Collection, Harpenden, UK); SEMIA (Seção de Microbiologia Agrícola, FEPAGRO, Porto Alegre, Brazil); SMS (Seção de Microbiologia do Solo, IAC, Campinas, Brazil); SU (The University of Sydney, Sydney, Australia); TA (Tasmania Dept. of Agriculture, The Department of Primary Industries, Water and Environment, Tasmanian State Government Agency, Tasmania, Australia); TAL (NifTAL, Nitrogen Fixation by Tropical Agricultural Legumes Project, University of Hawaii, Paia, USA); UMKL (University of Malaya – Kuala Lumpur, Dept. of Genetics and Cellular Biology, Kuala Lumpur, Malaysia); UMR (University of Minnesota *Rhizobium*, St. Paul, USA); USDA (United States Department of Agriculture, Beltsville, USA).

^g After Hungria & Araujo (1995); FEPAGRO (1999) and actas of RELARE (unpublished).

^h Embrapa (Empresa Brasileira de Pesquisa Agropecuária); EPAMIG (Empresa de Pesquisa Agropecuária de Minas Gerais); IAC (Instituto Agrônomo de Campinas); FEPAGRO (Fundação Estadual de Pesquisa Agropecuária); UFRGS (Universidade Federal do Rio Grande do Sul); CEPEC (Centro de Pesquisas do Cacau); IAPAR (Instituto Agrônomo do Paraná); UFC (Universidade Federal do Ceará).

ⁱ Basis for strain recommendation: I) selection in glass tubes or bags under axenic conditions; II) selection in jars under axenic conditions; III) selection in non-sterile soil; IV) field experiments.

8. Table 2

Acid/Alkaline reaction in yeast extract-mannitol agar (YMA) medium, accession number of the 16S rRNA sequence, identity of nucleotides in relation to type or reference strains and taxonomic position of sixty-eight strains officially recommended for the use in inoculants for sixty-three legume species in Brazil

SEMIA strain	Acid/Alkaline reaction in YMA	Gene Bank access #	Identity of nucleotides with type or reference strain ^a	Taxonomic position
103	Neutral	AY904726	1424/1430 (USDA 1002 ^T)	<i>Sinorhizobium meliloti</i>
134	Acid	AY904727	1426/1430 (USDA 1002 ^T)	<i>Sinorhizobium meliloti</i>
135	Acid	AY904728	1429/1430 (USDA 1002 ^T)	<i>Sinorhizobium meliloti</i>
222	Acid	AY904729	1369/1376 (USDA 2370 ^T)	<i>Rhizobium leguminosarum</i>
384	Acid	AY904730	1426/1431 (CFN42 ^T)	<i>Rhizobium etli</i>
396	Acid	AY904731	1411/1417 (USDA3383 ^T)	<i>Mesorhizobium ciceri</i>
587	Alkaline	AF234890 ^b	1384/1388 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
656	Alkaline	AY904732	1434/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
658	Alkaline	AY904733	1393/1428 (ORS 2060 ^T)	<i>Methylobacterium</i> sp.
662	Alkaline	AY904734	1441/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
695	Alkaline	AY904735	1440/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
696	Alkaline	AY904736	1439/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
816	Acid	AY904737	1406/1430 (USDA3471 ^T)	<i>Mesorhizobium</i> sp.
830	Acid	AY904738	1399/1430 (USDA3471 ^T)	<i>Mesorhizobium</i> sp.
938	Alkaline	AY904739	1441/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
2051	Acid	AY904740	1417/1423 (USDA 2370 ^T)	<i>Rhizobium leguminosarum</i>
2081	Acid	AY904741	1414/1421 (USDA 2370 ^T)	<i>Rhizobium leguminosarum</i>
3007	Acid	AY904742	1411/1422 (USDA 2370 ^T)	<i>Rhizobium leguminosarum</i>
5019	Alkaline	AF237422 ^b	1406/1411 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6002	Alkaline	AY904743	1438/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
5079	Alkaline	AF234888 ^b	1409/1411 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
5080	Alkaline	AF234889 ^b	1391/1403 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6028	Alkaline	AY904744	1439/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6053	Alkaline	AY904745	1443/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6069	Alkaline	AY904746	1438/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6070	Acid	AY904747	1434/1447(ATCC11325 ^T)	<i>Rhizobium</i> sp.
6100	Alkaline	AY904748	1440/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6101	Alkaline	AY904749	1438/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6144	Alkaline	AY904750	1412/1427 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6145	Alkaline	AY904751	1437/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6146	Alkaline	AY904752	1429/1434 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6148	Alkaline	AY904753	1419/1434 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6149	Alkaline	AY904754	1426/1436 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6150	Alkaline	AY904755	1442/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>

6152	Alkaline	AY904756	1439/1450 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6155	Alkaline	AY904757	1446/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6156	Alkaline	AY904758	1442/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6157	Alkaline	AY904759	1443/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6158	Alkaline	AY904760	1443/1449 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6159	Alkaline	AY904761	1438/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6160	Alkaline	AY904762	1439/1447 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6161	Acid	AY904763	1413/1430 (USDA 1002 ^T) 1405/1420 (USDA 205 ^T)	<i>Sinorhizobium</i> sp.
6163	Alkaline	AY904764	1426/1441 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6164	Alkaline	AY904765	1438/1451 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6165	Acid	AY904766	1433/1448(ATCC11325 ^T)	<i>Rhizobium</i> sp.
6166	Alkaline	AY904767	1447/1451 (BR 3405) 1447/1453 (BR 3407)	<i>Burkholderia</i> sp.
6167	Alkaline	AY904768	1449/1451 (BR 3405) 1446/1450 (BR 3407)	<i>Burkholderia</i> sp.
6168	Acid	AY904769	1421/1436(ATCC11325 ^T)	<i>Rhizobium</i> sp.
6169	Alkaline	AY904770	1442/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6175	Alkaline	AY904771	1443/1447 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6192	Alkaline	AY904772	1433/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6208	Alkaline	AY904773	1438/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6319	Alkaline	AY904774	1439/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6382	Neutral	AY904775	1424/1425 (BR 3405) 1437/1443 (BR 3407)	<i>Burkholderia</i> sp.
6383	Acid	AY904776	1432/1447(ATCC11325 ^T)	<i>Rhizobium</i> sp.
6384	Alkaline	AY904777	1440/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6387	Alkaline	AY904778	1436/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6390	Acid	AY904779	1412/1413(ATCC53867 ^T)	<i>Burkholderia cepacia</i>
6391	Alkaline	AY904780	1440/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6394	Alkaline	AY904781	1412/1414(ATCC53867 ^T)	<i>Burkholderia cepacia</i>
6398	Acid	AY904782	1430/1438 (tpig4.4) 1429/1438 (hpud10.4)	<i>Burkholderia</i> sp.
6401	Alkaline	AY904783	1396/1431 (USDA 4892 ^T)	<i>Azorhizobium</i> sp.
6402	Alkaline	AY904784	1333/1366 (USDA 4892 ^T)	<i>Azorhizobium</i> sp.
6412	Neutral	AY904785	1432/1439 (BR 3405) 1431/1440 (BR 3407)	<i>Burkholderia</i> sp.
6420	Alkaline	AY904786	1440/1449 (USDA 6 ^T)	<i>Bradyrhizobium japonicum</i>
6424	Alkaline	AY904787	1436/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6425	Alkaline	AY904788	1442/1448 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>
6440	Alkaline	AY904789	1440/1449 (USDA 76 ^T)	<i>Bradyrhizobium elkanii</i>

^a Accession numbers of the GenBank Data Library available on the material and methods section.

^b The nucleotides confirmed a previous submission of our group (Chusire et al., 2003).

9. Table 3

^a
Amplification of *nif* and *nod* genes of SEMIA strains classified in the genera *Methylobacterium* and *Burkholderia*

SEMIA strain	<i>nifH</i>	<i>nodB</i>	<i>nodC</i>
658	+	-	+
6390	+	-	+
6394	+	-	+
6166	+	-	-
6167	+	-	-
6382	+	-	-
6398	-	+	+
6412	-	-	+

^a (+) amplified and (-) not amplified with the primers described in the material and methods section.

5 CONCLUSÕES

- A comparação de seqüências do gene ribossomal 16S constitui uma ferramenta poderosa para deduzir relações filogenéticas, evolucionárias e definir posições taxonômicas entre rizóbios de origem tropical.

- Foi possível observar alguns agrupamentos distintos, com estirpes apresentando discrepância em mais de 15 nucleotídeos das estirpes-tipo, sugerindo que essas podem estar relacionadas a novas espécies.

- Foi confirmada a presença de genes *nifH* e/ou *nodC* relacionados a *Rhizobium* e/ou *Bradyrhizobium*, em espécies classificadas como pertencentes aos gêneros *Methylobacterium* e *Burkholderia*.

- Foi possível identificar uma relação simbiótica entre *Vicia sativa* e SEMIA 384 classificada como *Rhizobium etlii*, não descrita previamente, visto que, até o momento, essa espécie de *Rhizobium*, havia sido identificada como principal simbiote de *Phaseolus vulgaris*.

- Não houve correlação evolucionária entre as estirpes “SEMIAs” analisadas e as plantas hospedeiras.

- O elevado grau de diversidade genética observada entre as estirpes, com base em análises do gene ribossomal 16S, enfatiza que regiões tropicais são um importante reservatório de genes fixadores de nitrogênio, os quais necessitam ser explorado.

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ANEXOS

Cover Letter

To the
Editors of "Molecular Phylogenetics and Evolution"

Londrina, 31 st of January of 2005

Dear Editors

I would like to submit the paper "Molecular phytogeny of rhizobial strains used in Brazilian commercial inoculants based on the sequencing of the 16S rRNA gene", written by Pamela Menna, Mariangela Hungria, Fernando G. Barcellos and Eliane V. Bangel, to the appreciation of the reviewers of "Molecular Phylogenetics and Evolution". Thanks a lot for your attention.

Yours sincerely

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Abstract: Nitrogen is often a limiting nutrient, therefore the sustainability of food crops, forages and green manure legumes is mainly associated with their ability to establish symbiotic associations with stem and root-nodulating N₂-fixing rhizobia. The selection, identification and maintenance of elite strains for each host is critical. Decades of research in Brazil resulted in a list of strains officially recommended for several legumes, but their genetic diversity is poorly known. This study aimed at gaining a better understanding of phylogenetic relationships of sixty-eight rhizobial strains recommended for sixty-three legumes, based on the sequencing of the 16S rRNA genes. The strains were isolated from a wide range of legumes, including all three subfamilies and seventeen tribes. Nine main clusters were defined, joined with a similarity of 77.8%, seven of them related to rhizobial genera/species: *Bradyrhizobium japonicum*, *B. elkanii*, *Rhizobium tropici*/*Rhizobium* resembling *agrobacteria*, *R. leguminosarum*, *Sinorhizobium meliloti*/*S. fredii*, *Mesorhizobium ciceri*/*M. loti*, and *Azorhizobium caulinodans*. However, some strains differed by up to thirty- five nucleotides from the type strains, which suggests that they may represent new species. Two other clusters included bacteria showing similarity with the genera *Methylobacterium* and *Burkholderia*, and the presence of *nifH* and/or *nodC* was confirmed in these strains. Several strains were capable of nodulating legumes of different tribes and subfamilies. The great diversity observed emphasizes that tropics are an important reservoir of N₂-fixation genes.