UNIVERSIDADE ESTADUAL DE LONDRINA

PÂMELA MENNA PEREIRA

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

Londrina
2005

PÂMELA MENNA PEREIRA

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

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

Londrina
2005

PÂMELA MENNA PEREIRA

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

BANCA EXAMINADORA

_____

Dra. Mariangela Hungria
(Orientadora)
EMBRAPA-Soja

_____

Dr. Fernando Gomes Barcellos
(Pesquisador)
EMBRAPA-Soja

_____

Profa. Dra. Márcia Cristina Furlaneto
Universidade Estadual de Londrina - UEL

Londrina, 11 de março de 2005.

DEDICATÓRIA

A Deus, pelo seu infinito amor.
Aos meus pais, Juldimar e Eva, pelo incentivo e compreensão durante todos os momentos de minha vida.
Aos meus irmãos Wagner e Lindsay pelo carinho.
Ao meu noivo, Wander, pela ajuda e principalmente pelo amor dedicado.

Muito Obrigada.

AGRADECIMENTOS

Agradeço a todos que diretamente ou indiretamente influenciaram para a concretização deste trabalho cientifico.

Á Dr. Mariangela Hungria, pela orientação, apoio, dedicação e principalmente pelo carinho e amizade dedicada.

Á Embrapa Soja– Empresa Brasileira de Pesquisa Agropecuária, por permitir a realização deste trabalho.

Á CAPES, pela concessão da bolsa de mestrado, a qual tornou possível a realização do presente trabalho.

Ao Dr. Fernando Gomes Barcellos, pela paciência, orientação, disponibilidade em ajudar e companheirismo.

Á Ligia M. O Chueire, pela disponibilidade em ajudar, pela paciência em ensinar, pelo seu incentivo nos momentos difíceis e pela amizade.

Á Fabiana G. S. Pinto, pela sua amizade, apoio durante os momentos difíceis.

À Luciana Grange, pela sua amizade, conselhos e pelo incentivo todos os dias.

Á Marisa F. Nicolás, por ajudar em minhas dúvidas, pelas idéias, conselhos e muitas vezes soluções dos problemas enfrentados.

Ao Fábio Luis Mostasso, pela atenção dispensada, pela ajuda e pelos momentos de incentivo e descontração.

Aos professores Halha e Marco pela ajuda, idéias e conhecimentos adquiridos possibilitando assim, a concretização deste trabalho.

Aos amigos de laboratório, Jesiane, Fabinho, Emilene, Maria Aparecida, Lílian, Alan, Ednéia, Rodrigo, Eriana, Rinaldo, Manuel e Leny, pela amizade e incentivo.

Aos professores do Curso de Microbiologia da Universidade Estadual de Londrina, Jacinta, Marilda, Sergio, Galdino, Márcia, Shiduca, Carlos, e Vitor, pelos conhecimentos adquiridos.

A coordenação do Curso de Microbiologia e principalmente a Rosilda de Alvarenga, pela disponibilidade em ajudar.

Muito Obrigada.

PEREIRA. Pámela Menna. **“Filogenia de rizóbios utilizados em inoculantes comerciais brasileiros, com base no seqüenciamento do gene ribossomal 16s”**. 2005. 70f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina, 2005.

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 seqüênciamento 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 acidas 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.

PEREIRA. Pámela Menna. **"Filogenia de rizóbios utilizados em inoculantes comerciais brasileiros, com base no seqüenciamento do gene ribossomal 16s"**. 2005. 70f. Dissertação (Mestrado em Microbiologia) – Universidade Estadual de Londrina, 2005.

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 N_2-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 N_2-fixation genes.

Keywords: Rhizobium. Leguminosae.

SUMÁRIO

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 nucléicos, 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 ºC) 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 escorrimento 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 N_2 (FBN), processo realizado por determinados procariontes, denominados organismos fixadores de N_2 ou diazotróficos. As bactérias capazes de fixar biologicamente o N_2 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 N_2, 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 N_2 à NH_3 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 N_2 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* (Garrity & 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 N_2 (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 simbiôntica 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 simbiônticas 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-quitinooligossacarideos 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 N_2 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 cromossomais 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, spécies 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 N_2 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 simbiônticas com plantas da família Leguminosae. As espécies simbiônticas eram *Rhizobium leguminosarum* (espécie tipo), *Rhizobium japonicum*, *Rhizobium lupini*, *Rhizobium meliloti*, *Rhizobium phaseoli* e *Rhizobium trifoli*. Espécies de *Agrobaterium 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 conseqüência, 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 seqüência do gene 16S RNAr, o qual tem demostrado 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 *Agrobcterium* 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 copias 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, contudos 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 simbiontes 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 *Agrobacterum* biovar-2. O segundo subgrupo correspondente ao gênero *Sinorhizobium,* inclui as espécies: *S*. *fredii* e *S*. *xinjiagensis* (Chen *et al*., 1988), *S*. *meliloti*, *S*. *terangae* 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*. *plurifarium* (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, è 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 O_2 (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 N_2, 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 *Afipia* sp., *Blastobacter denitrificans* e *Rhodopseudomonas*. O parentesco filogenético entre *Bradyrhizobium japonicum*, *Afipia 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 simbiônticas 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 simbiônticas. 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

- Caractertizar 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

Pâmela Menna[a,b,c], Mariangela Hungria[a,d,*], Fernando G. Barcellos[a,d], Eliane V. Bangel[e]

[a]Embrapa Soja, Cx. Postal 231, 86001-970, Londrina, PR, Brazil; [b]M.Sc. in Microbiology at the Universidade Estadual de Londrina; [c]CAPES, Brazil; [d]CNPq, Brazil; [e]FEPAGRO, Rua Gonçalves Dias 579, 90130-060, Porto Alegre, RS, Brazil.

**Author for correspondence: Tel. +55-43-33716206, Fax: +55-43-33716100, E-mail: hungria@cnpso.embrapa.br.*

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 N_2-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 N_2-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 N_2-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 (Garrity 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 N_2 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 thehost 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^{o}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 µL: dNTPs (300 *µM* of each); PCR-buffer (Tris-base 20 *mM* pH 8.4 and KCl 50 *mM*); $MgCl_2$ (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 95ºC for 2 min; thirty cycles of denaturation at 94ºC for 15 s, 93 ºC for 45 s, annealing at 55ºC for 45 s, and extension at 72ºC for 2 min; a final extension cycle of 72ºC for 5 min.

For the purification of the PCR-products, 48 µL of each PCR reaction were added to each 300 µL-capacity well of a 96-well U-plate. Each well also received 5 µL of sterile ammonium acetate (7.5 *M*) and 165 µL 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 µL 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 37ºC for 15 min, followed by the addition of 15 µL of milli-Q water, homogenized, and kept at -20ºC. After 24 h, the concentration of the samples was verified in 1.5% agarose gels, adjusted to 40 ng DNA μL^{-1} and kept at -20ºC.

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 µL 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 95ºC for 2 min; thirty cycles of denaturation at 95ºC for 10 s, 50ºC for 4 s, and extension at 60ºC for 4 min; final soak at 4ºC.

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 37ºC for 15 min, resuspended in 7 *µ*l 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 µL: dNTPs (200 *µM* of each); PCR-buffer (Tris-base 20 *mM* pH 8.4 and KCl 50 *mM*); $MgCl_2$, (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 94ºC for 90 s, annealing at 67ºC for 30s, and extension at 72ºC for 80 s; and a final extension cycle of 72ºC for 3 min.

The *nodC* amplification was performed with primers nodCIf (5´-GTCGATTGCMRGTCAAGACTACG-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, recomended 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 lebbeck (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 bootstroop 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, respectivelly, 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 caesalpiniifolia* (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 caesalpiniifolia*, 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 aparently 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 lebbeck* (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-N_2-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). N_2-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 *Crolalaria* 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 caesalpiniifolia* 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 N_2-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.

Acknowledgments

The authors thank Ligia Maria O. Chueire (Embrapa Soja) for help in several steps of this work, and to Dr. Maria Monteros (University of Georgia, USA) for helpful discussion. Research described herein was partially supported by CNPq (Conselho Nacional de

Desenvolvimento Científico e Tecnológico, Brazil) (PRONEX-41.96.0884.00 and 35116/1992-3) and by ANPII (Associação Nacional dos Produtores e Importadores de Inoculantes, Brazil). Finally, the authors wish to emphasize the relevant studies of Dr. Sergio M. Faria (Embrapa Agrobiologia, Seropédica, Brazil), who selected most of the tree legume rhizobia.

5. References

Aguilar, O.M., Lópes, M.V., Riccillo, P.M., González, R.A., Pagano, M., Grasso, D.H., Pühler, A., Favelukes, G., 1998. Prevalence of the *Rhizobium-etli* like allele in genes coding for 16S rRNA among the indigenous rhizobial populations found associated with wild beans from the Southern Andes in Argentina. Appl. Environ. Microbiol. 64, 3520-3524.

Allen, O.N., Allen, E.K., 1981. The Leguminosae: a source book of characteristics, uses, and nodulation. Univ. Wisconsin Press, Madison, USA.

Bernal, G., Graham, P.H., 2001. Diversity of the rhizobia associated with *Phaseolus vulgaris* L. in Ecuador, and comparisons with Mexican bean rhizobia. Can. J. Microbiol. 47, 526-534.

Chen, L.S., Figueredo, A., Pedrosa, F.O., Hungria, M., 2000. Genetic characterization of soybean rhizobia in Paraguay. Appl. Environ. Microbiol. 66, 5099-5103.

Chen, W.-M., Moulin, L., Bontemps, C., Vandamme, P., Béna, G., Boivin-Masson, C., 2003. Legume symbiotic nitrogen fixation by β-Proteobacteria is widespread in nature. J. Bacteriol. 185, 7266-7272.

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

Ewing, B., Green, P., 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. 8, 186-194.

Ewing, B., Hillier, L., Wendl, M. C., Green, P., 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. 8, 175-185.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783-791.

FEPAGRO (Fundação Estadual de Pesquisa Agropecuária), 1999. Culture Collection Catalogue. 8th ed. FEPAGRO, Porto Alegre, RS, Brazil.

Fernandes, M.F., Fernandes, R.P.M, Hungria, M., 2003. Caracterização genética de rizóbios nativos dos tabuleiros costeiros eficientes em culturas do guandu e caupi. Pesq. Agropec. Bras. 38, 911-920.

Fred, E.B., Baldwin, I.L., McCoy, E., 1932. Root Nodule Bacteria of Leguminous Plants. Univ. Wisconsin Press, Madison, USA.

Garrity, G.M., Holt, J.G., 2001. The road map to the manual. In: Boone, D.R., Castenholz, R.W. (Eds.), Manual of Systematic Bacteriology, the Archaea and the Deeply Branching and Phototrophic Bacteria. 2nd. Springer-Verlag, New York, USA, v. 1, pp. 119-166

Germano, M.G., Hungria, M., 2005. Ribosomal phylogeny of a Brazilian collection of Bradyrhizobium symbionts of thirty-three legume species. Int. J. Syst. Evol. Microbiol. (submitted).

Gordon, D., Abajian, C., Green, P., 1998. Consed: a graphical tool for sequence finishing. Genome Res. 8, 195-202.

Grange, L., Hungria, M., 2004. Genetic diversity of indigenous common bean (*Phaseolus vulgaris*) rhizobia in two Brazilian ecosystms. Soil Biol. Biochem. 36, 1389-1398.

Herendeen, P.S., Crepet, W.L., Dilcher, D.L., 1992. The fossil record. In: Herendeen, P.S., Dilcher, D.L (Eds.), Advances in Legume Systematics. Royal Botanic Gardens, Kew, UK, v. 4, pp. 303–316.

Hernandez-Lucas, I., Segovia, L., Martínez-Romero, E., Pueppke, S.G., 1995. Phylogenetic relationships and host range of *Rhizobium* spp. that nodulate *Phaseolus vulgaris* L. Appl. Environ. Microbiol. 61, 2775-2779.

Hungria, M., Araujo, R.S., 1995. Relato da VI Reunião de laboratórios para recomendação de estirpes de *Rhizobium* e *Bradyrhizobium* In: Hungria, M.; Balota, E.L.; Colozzi-Filho, A.; Andrade, D.S. (Eds.), Microbiologia do Solo**:** Desafios para o Século XXI. IAPAR/EMBRAPA-CNPSo, Londrina, Brazil, pp.476-489.

Hungria, M., Campo, R.J., Mendes, I.C., Graham, P.H., 2005a. Contribution of biological nitrogen fixation to the N nutrition of grain crops in the tropics: the success of soybean (*Glycine max* L. Merr.) in South America. In: Singh, R.P., Shankar, N., Jaiwal, P.K. (Eds.), Nitrogen Nutrition and Sustainable Plant Productivity. Studium Press, Houston, USA (in press).

Hungria, M., Loureiro, M.F., Mendes, I.C., Campo, R.J., Graham, P.H., 2005b. Inoculant preparation, production and application. In: Werner, D., Newton, W.E. (Eds.), Nitrogen Fixation in Agriculture, Forestry, Ecology and the Environment. Springer, Dordrecht, the Netherlands. (in press).

ILDIS (International Legume Database & Information Service), 2004. Retrieved January 1st, 2005, from http://www.ildis.org.

Jordan, D.C., 1984. Rhizobiaceae. In: Krieg, N.R., Holt, J.R. (Eds.), Bergey's Manual of systematic bacteriology. Williams & Wilkens Co, Baltimore, USA, v. 1, pp. 234-244.

Khbaya, B., Neyra, M., Normand, P., Zerhari, Z., Filali-Maltouf, A., 1998. Genetic diversity and phylogeny of rhizobia that nodulate *Acacia* spp. in Morocco assessed by analysis of rRNA genes. Appl. Environ. Microbiol. 64, 4912-4917.

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

Martinez Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P., Pardo, M.A., 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. Int. J. Syst. Bacteriol. 41, 417-426.

Mhamdi, R., Jebara, M., Aouani, M.E., Ghrir, R., Mars, M., 1999. Genotypic diversity and symbiotic effectiveness of rhizobia isolated from root nodules of *Phaseolus vulgaris* L. in Tunisian soils. Biol. Fert. Soils 28, 313-320.

Moreira, F.M.S., Haukka, K., Young, J.P.W., 1998. Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. Mol. Ecol. 7, 889-895.

Moreira, F.M.S., Haukka, K., Young, J.P.W., 1998. Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. Mol. Ecol. 7, 889-895.

Molouba, F., Lorquin, J., Willems, A., Hoste, B., Giraud, E., Dreyfus, B., Gillis, M., de Lajudie, P., Masson-Boivin, C., 1999. Photosynthetic bradyrhizobia from *Aeschynomene* ssp. are specific to stem-nodulate species and form a separate 16S ribosomal DNA restriction fragment length polymorphism group. Appl. Environ. Microbiol. 65, 3084-3089.

Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C., 2001. Nodulation of legumes by members of the β-subclass of Proteobacteia. Nature 411, 948-950.

Novikova, N. Y., Safronova, V., 1992. Transconjugants of *Agrobacterium radiobacter* harbouring sym genes of *R. galegae* can form an effective symbiosis with *Medicago sativa*. FEMS Microbiol. Lett. 93, 262-268.

Oyaizu, H., Naruhashi, N., Gamou, T., 1992. Molecular methods of analysing bacterial diversity: The case of rhizobia. Biodivers. Conserv. 1, 237-249.

Polhill, R.M., Raven, P.H. (Eds.), 1981. Advances in Legume Systematics. Royal Botanic, Gardens, Kew, U.K.

Segovia, L., Young, J.P.W., Martínez-Romero, E., 1993. Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I strains as *Rhizobium etli sp. nov.* Int. J. Syst. Bacteriol. 43, 374-377.

Silva, C., Vinuesa, P., Eguiarte, L.E., Martinez-Romero, E., Souza, V., 2003. *Rhizobium etli* and *Rhizobium gallicum* nodulate commom bean (*Phaseolus vulgaris*) in a traditionally managed milpa plot in México: population genetics and biogeographic implications. Appl. Environ. Microbiol. 69, 884-893.

Sneath, P.B.A., Sokal, R.R, 1973. Numerical taxonomy. W.H. Freeman & Co, San Francisco, USA.

Sterner, J.P., Parker, M.A., 1999. Diversity and relationships of bradyrhizobia form *Amphicarpaeae bracteata* based on partial *nod* and ribossomal sequences. Syst. Appl. Microbiol. 22, 387-392.

Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., de Lajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., Dreyfus, B., 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol. 183, 214-220.

Terefework, Z., Nick, G., Suomalaine, S., Paulin, L., Lindström, K., 1998. Phylogeny of *Rhizobium galegae* with respect to other rhizobia and agrobacteria. Int. J. Syst. Bacteriol. 48, 349-356.

van Berkum, P., Fuhrmann, J.J., 2000. Evolutionary relationships among the soybean bradyrhizobia reconstructed from 16S rRNA gene and internally transcribed spacer region sequence divergence. Int. J. Syst. Bacteriol. 50, 2165-2172.

Vincent, J.M., 1970. Manual for the Practical Study of Root-Nodule Bacteria. Blackwell Scientific, Oxford, UK. (IBP Handbook No. 15).

Vinuesa, P., Rademaker, J.L.W., de Bruijn, F.J., Werner, D., 1998. Genotypic characterization of *Bradyrhizobium* strains nodulating endemic woody legumes of the Canary Islands by PCR-Restriction Fragment Length Polymorphism analysis of genes encoding 16S rRNA (16S rDNA) and 16S-23S rRNA intergenic spacers, repetitive extragenic palindomic PCR genomic fingerprinting, and partial 16S rRNA sequencing. Appl. Environ. Microbiol. 64, 2096-2104.

Vinuesa, P., Silva, C., Werner, D., Martinez-Romero, E., 2005. Population genetics and phylogenetic inference in bacterial molecular systematics: the roles of migration and recombination in Bradyrhizobium species cohesion and delineation. Mol. Phylogenet. Evol. 34, 29-54.

Wang, E.T., Martinez-Romero, E., 2000. Phylogeny of root- and stem-nodule bacteria associated with legumes. In: Triplett, E.W. (Eds.), Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process. Horizon Scientific Press, Madison, USA, pp. 177-186.

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

Wernegreen, J.J., Riley, M.A., 1999. Comparison of the evolucionary dynamics and symbiotic and housekeeping loci: a case for the genetic coherence of rhizobial lineages. Mol. Biol. Evol. 16, 98-113.

Willems, A., Coopman, R., Gillis, M., 2001. Comparison of sequence analysis of 16S-23S rDNA spacer regions, AFLP analysis and DNA-DNA hybridizations in *Bradyrhizobium*. Int. J. Syst. Evol. Microbiol. 51, 623-632.

Willems, A., Munive, A., de Lajudie, P., Gillis, M., 2003. In most *Bradyrhizobium* groups sequence comparison of 16S-23S rDNA internal transcribed spacer regions corroborates DNA-DNA hybridizations.Syst. Appl. Microbiol. 26, 203-210.

Young, J.M., Kuykendall, L.D., Martínez-Romero, E., Kerr, A., Sawada, H., 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter, R. rhizogenes, R. rubi, R. undicola* and *R. vitis*. Int. J. Syst. Evol. Microbiol. 51, 89-103.

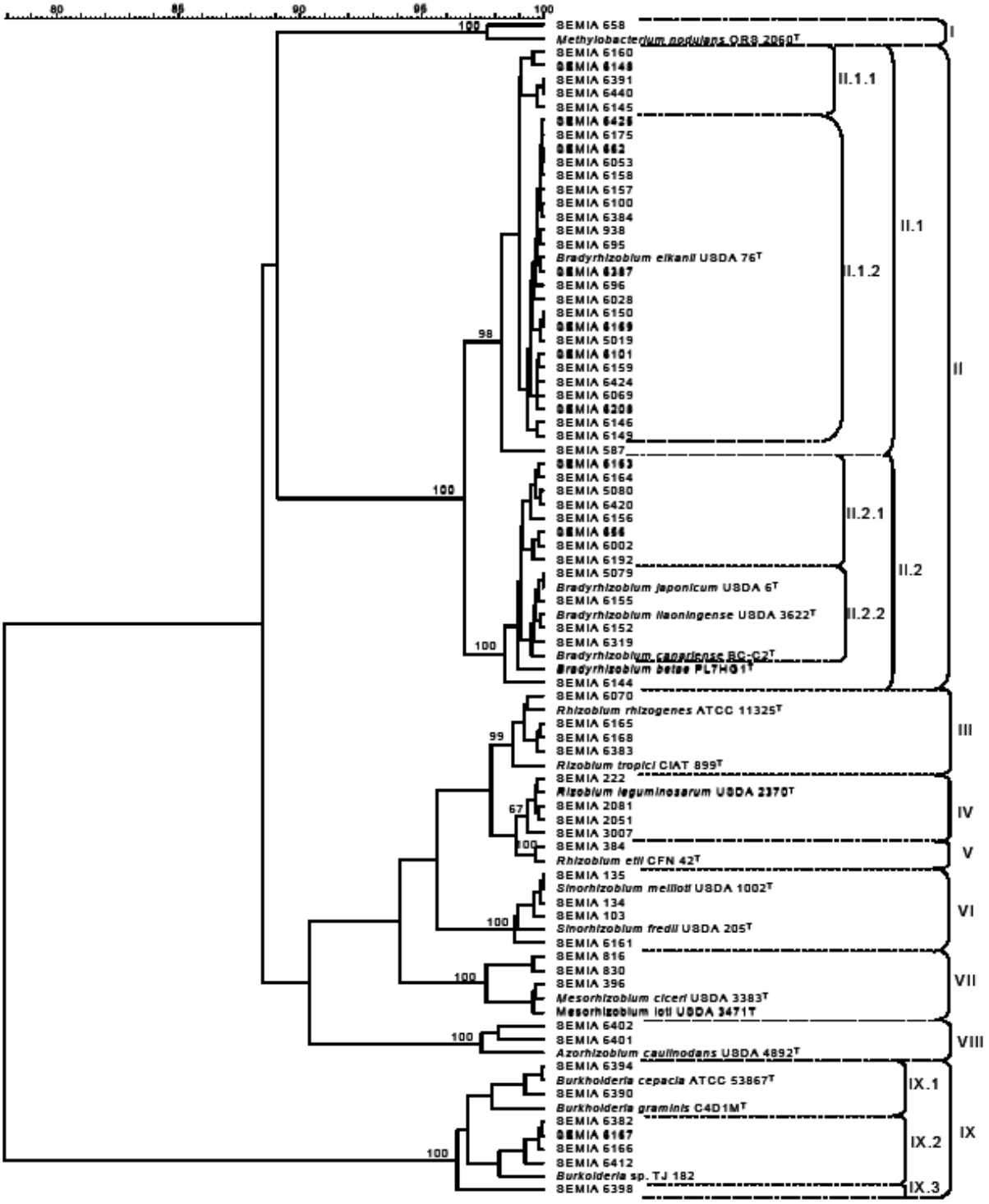
Young, J.P.W., Downer, H.L., Eardly, B.D., 1991. Phylogeny of the phototrophic rhizobium strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. 173, 2271-2277.

Zakhia, F., de Lajudie, P., 2001. Taxonomy of rhizobia. Agronomie 21, 569-576.

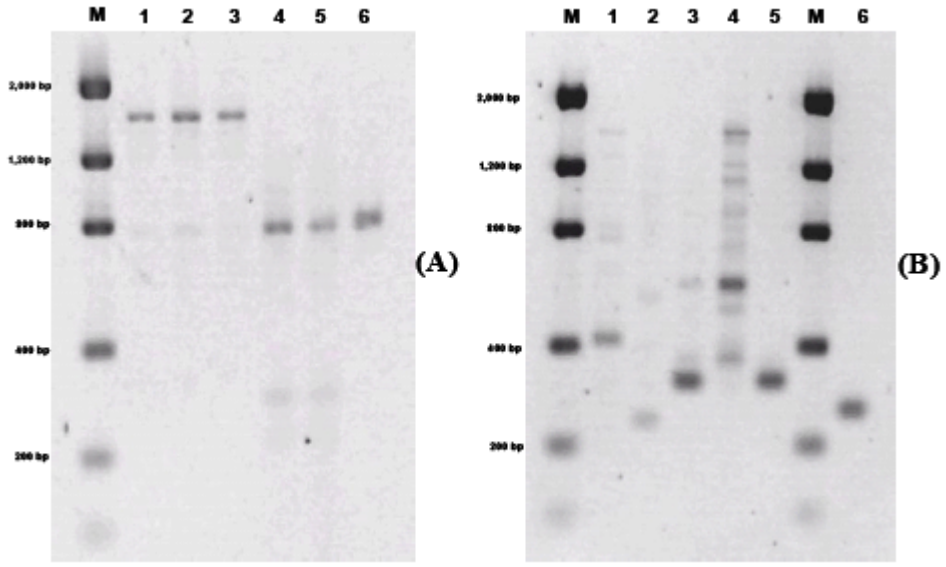
6. Legend of Figures

Fig. 1. Phylogenetic tree based on the 16S rRNA sequences of sixty-eight strains, N_2-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.


80
85
90
95
100
100
98
100
99
67
100
100
100
100
100
SEMIA 658
Methylobacterium populiens ORS 2060T
SEMIA 6160
SEMIA 6148
SEMIA 6391
SEMIA 6440
SEMIA 6145
SEMIA 6428
SEMIA 6175
SEMIA 662
SEMIA 6053
SEMIA 6158
SEMIA 6157
SEMIA 6100
SEMIA 6384
SEMIA 938
SEMIA 695
Bradyrhizobium elkanii USDA 76T
SEMIA 6387
SEMIA 696
SEMIA 6028
SEMIA 6150
SEMIA 6169
SEMIA 5019
SEMIA 6101
SEMIA 6159
SEMIA 6424
SEMIA 6069
SEMIA 6208
SEMIA 6146
SEMIA 6149
SEMIA 587
SEMIA 6163
SEMIA 6164
SEMIA 5080
SEMIA 6420
SEMIA 6156
SEMIA 666
SEMIA 6002
SEMIA 6192
SEMIA 5079
Bradyrhizobium japonicum USDA 6T
SEMIA 6155
Bradyrhizobium liaoningense USDA 3622T
SEMIA 6152
SEMIA 6319
Bradyrhizobium canariense BC-C2T
Bradyrhizobium betae PL7HG1T
SEMIA 6144
SEMIA 6070
Rhizobium rhizogenes ATCC 11325T
SEMIA 6165
SEMIA 6168
SEMIA 6383
Rizobium tropici CIAT 899T
SEMIA 222
Rizobium leguminosarum USDA 2370T
SEMIA 2081
SEMIA 2051
SEMIA 3007
SEMIA 384
Rhizobium etli CFN 42T
SEMIA 135
Sinorhizobium meliloti USDA 1002T
SEMIA 134
SEMIA 103
Sinorhizobium fredii USDA 205T
SEMIA 6161
SEMIA 816
SEMIA 830
SEMIA 396
Mesorhizobium ciceri USDA 3383T
Mesorhizobium loti USDA 3471T
SEMIA 6402
SEMIA 6401
Azorhizobium caulinodans USDA 4892T
SEMIA 6394
Burkholderia cepacia ATCC 53867T
SEMIA 6390
Burkholderia graminis C4D1MT
SEMIA 6382
SEMIA 6167
SEMIA 6166
SEMIA 6412
Burkolderia sp. TJ 182
SEMIA 6398
II.1.1
II.1.2
II.1
II.2.1
II.2.2
II.2
II
I
III
IV
V
VI
VII
VIII
IX.1
IX.2
IX.3
IX

6.1 – Figure 1


M 1 2 3 4 5 6
2,000 bp
1,200 bp
800 bp
400 bp
200 bp
(A)
M 1 2 3 4 5 M 6
2,000 bp
1,200 bp
800 bp
400 bp
200 bp
(B)

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,g]	Some common names[c]	Subfamily[b]	Tribe[b]	Descriptor[c]	Main use in Brazil	Applications worldwide[c]	SEMIA number	Other designations[f,g]	Use in inoculants[g]	Source of the strain[g]	Original host plant[g]	Institution[h] recommending[g]	Basis[i]
Acacia auriculiformis Benth.	Earpod wattle, blackwattle, acacia[d]	Mimosoideae	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
Acacia decurrens Willd.	Green wattle, acacia-da-Australia[d]	Mimosoideae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6164	BR 3608, LMG 9960	Actual	Brazil	*Acacia mearnsii* De Wild.	Embrapa Agrobiologia	II
							6390	BR 3614	Actual	Brazil	Same	Embrapa Agrobiologia	II
Acacia mangium Willd.	Mangium, silk leaf acacia, akasia, acacia mangium[d]	Mimosoideae	Acacieae	Perennial non-climbing tree	Tree	Environment, wood	6387	BR 3609 LMG 9961	Actual	Brazil	*Acacia auriculiformis* Benth.	Embrapa Agrobiologia	IV
							6420	BR 3617, LMG 9965	Actual	Brazil	Same	Embrapa Agrobiologia	IV
Acacia mearnsii De Wild.	Black wattle, acacia negra[d]	Mimosoideae	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
Albizia lebbeck (L.) Benth.	Rain tree, woman's tongue, bois noir, baile de caballero, lengua de mujer, coração-de-negro[d], faveiro[e], pau-preto[e], acacia[d]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, chemical, toxins, wood	6160	BR 5610	Actual	Brazil	Same	Embrapa Agrobiologia	III
Arachis hypogaea L.	Peanut, groundnut, cacahuete, 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
Arachis pintoi 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
Cajanus cajan (L.) Millsp.	Pigeonpea, Congo pea, pois cajan, frijol de arbol, lenteja, guandu[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	*Crotalaria spectabilis* Roth.	Embrapa Cerrados	IV
							6157	BR 2801	Actual	Brazil	Same	Embrapa Agrobiologia	II
Calopogonium	Calopo,	Papilionoide	Phaseoleae	Perennial	Green	Cover crop,	6152	BR 1602	Actual	Brazil	*Calopogo-*	Embrapa	IV

spp.	calopogônio[4]	ae		climbing or non-climbing herb	manure	green manure, forage					*nium* sp.	Agrobiologia	
Canavalia ensiformis (L.) DC.	Jackbean, swordbean, horsebean, pois de sabre, dólique, cocorico, haba criolla, feijão-de-porco[4]	Papilionoideae	Phaseoleae	Annual/perennial, climbing or non-climbing herb or shrub	Green manure	Cover crop, green manure, forage, food	6156	CPAC-U, DF F-2	Actual	Brazil	*Crotalaria spectabilis* Roth	Embrapa Cerrados	III
							6158	CPAC 42	Actual	Brazil	*Crotalaria spectabilis* Roth	Embrapa Cerrados	III
Centrosema spp.	Centrosema, fleur langouste, pois pianta, chonchо, conchitas, centrosema[4]	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment	6146	BR 1808	Actual	Brazil	*Centrosema* sp.	Embrapa Agrobiologia	III
							6424	CPAC L36	Actual	Brazil	*Centrosema pubescens* Benth.	Embrapa Cerrados	IV
							6425	CIAT 2380	Actual	Brazil	*Centrosema pubescens* Benth.	Embrapa Cerrados	IV
Cicer arietinum L.	Chickpea, pois chiche, garbanzo, grão-de-bico[4]	Papilionoideae	Cicereae	Annual non-climbing herb	Grain	Food, forage, medicine	396	TAL 1148, USDA 3100, Nit 27A3	Actual	USA	Not known	FEPAGRO/ UFRGS	IV
Clitoria fairchildiana R. Howard	Butterfly pea tree, faveira, palheteira, palheiro[4], sombreiro[4]	Papilionoideae	Phaseoleae	Perennial non-climbing tree	Tree	Environment, medicine, wood	6412	BR 8003	Actual	Brazil	Same	Embrapa Agrobiologia	IV
Clitoria ternatea L.	Pigeon wings, pois sauvage, blue pea, anulejo, campanilla, conchita blanca o azul, zapatico de la reina, clitoria[4]	Papilionoideae	Phaseoleae	Perennial climbing herb or shrub	Tree	Environment, food, chemical, wood, forage, toxins	6053	TAL 827, UMKL I28	Till 1999	Malasya	Same	Embrapa Agrobiologia	II
Crotalaria juncea L.	Sunnhemp, cascavelle, grand sonnette, grand tcha-tcha, crotalaria[4]	Papilionoideae	Crotalarieae	Annual non-climbing herb	Green manure	Fibre, environment, firewood	6145	BR 2001	Actual	Libia	*Arachis hypogaea* L.	Embrapa Agrobiologia	II
Crotalaria juncea L.	Sunnhemp, cascavelle, grand sonnette, grand tcha-tcha, crotalaria[4]	Papilionoideae	Crotalarieae	Annual non-climbing herb	Green manure	Fibre, environment, firewood	6156	CPAC-U, DF F-2	Actual	Brazil	*Crotalaria spectabilis* Roth	Embrapa Cerrados	IV
Crotalaria spectabilis Roth	Rattle Box, showy rattlepod, cascavelle jaune, 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
							6158	CPAC 42	Actual	Brazil	Same	Embrapa Cerrados	IV

	maraquita, crotalaria[4]			shrub									
Cyamopsis tetragonoloba (L.) Taubert	Chusterbean, guar bean, guar gum, feijão-guarda[4], guar[4]	Papilionoideae	Indigoferese	Annual non-climbing herb	Green manure	Environment, chemical, food, forage	6145	BR 2001	Actual	Brazil	*Arachis hypogaea* L.	Embrapa Agrobiologia	II
							6319	NC 92, SMS 561	Actual	Bolivia	*Arachis* sp.	IAC	II
Dalbergia nigra (Vell. Conc.) Benth.	Brazilian rosewood, jacaranda, palisandre, cabiúna[4], jacarandá-da-Bahia[4]	Papilionoideae	Dalbergiease	Perennial non-climbing tree	Tree	Environment, chemical, wood	6101	BR 8404	Actual	Brazil	Same	Embrapa Agrobiologia	III
Desmodium incanum DC. *(D. canum)*	Spanish clover; wild peanut, colle-colle, herbe gallon, cepa de caballo, collaut, pega pega; desmodio[4]	Papilionoideae	Desmodiease	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage, medicine	6028	TAL 569, MAR 472	Actual	Zimbabwe	*Desmodium uncinatum* (Jacq.) DC.	FEPAGRO/ UFRGS	II
Desmodium intortum (Miller) Urban	Beggarlice, copal de cocha, pega pega, zarza blanca, amor seco, desmódio[4]	Papilionoideae	Desmodiease	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage	656	SEMIA original	Actual	Brazil	*Neonotonia wightii* (Wight & Arn.) Lackey	FEPAGRO/ UFRGS	II
Desmodium heterocarpon *(L.) DC. subsp.* ovalifolium *(Prain) Ohashi*	*Desmodium ovalifolium*, desmodium, desmodio[4]	Papilionoideae	Desmodiease	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage	6208	CIAT 2372	Actual	Colombia	Same	CEPEC	III
Desmodium uncinatum (Jacq.) DC.	Silverleaf desmodium, desmodio[4]	Papilionoideae	Desmodiease	Perennial non-climbing herb or shrub	Forage (tropical)	Environment, forage	696	CB 627	Till 1999	Australia	Same	FEPAGRO/ UFRGS	III
Enterolobium contortisiliquum (Vell. Conc.) Morong	Earpod tree, orelja de negro, timbó[4], tamboril-do-campo[4], orelha-de-negro[4], orelha-de-macaco[4], timba-tiba[4], timbaúva[4]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, medicine, toxin, wood	6159	BR 4406	Actual	Brazil	*Enterolobium ellipticum* Benth.	Embrapa Agrobiologia	III
Enterolobium cyclocarpum (Jacq.) Griseb.	Devil's ear, earpod tree, elephant's ear, monkey ear, bois tenniste rouge, oreille d'éléphant,	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, medicine, pasture, wood, food	6159	BR 4406	Actual	Brazil	*Enterolobium ellipticum* Benth.	Embrapa Agrobiologia	IV

	guanacaste, orelha-de-elefante[6]												
Enterolobium timbouva Martius	Timbor, tamboril[4], timbaúva[4], timbouva[4]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, medicine, tanin, wood	6159	BR 4460	Actual	Brasil	*Enterolobium ellipticum* Benth.	Embrapa Agrobiologia	IV
Erythrina verna Vell. Conc.	Mulungu, cristi-galli, imbungu[6], suinã[6], suinã-da-serra[4]	Papilionoideae	Phaseoleae	Perennial, non-climbing tree	Tree	Environment, wood	6100	BR 5609	Actual	Brasil	*Falcataria moluccana* (Miq.) Barneby & Grimes	Embrapa Agrobiologia	II
Falcataria moluccana (Miq.) Barneby & Grimes	*Albizia falcataria*, molucca albizia, batai wood, sau, molucca, albizia[6]	Mimosoideae	Ingeae	Perennial non-climbing tree	Tree	Environment, wood, food	6100	BR 5609	Actual	Brasil	Same	Embrapa Agrobiologia	IV
							6169	BR 5612	Actual	Brasil	Same	Embrapa Agrobiologia	III
Galactia striata (Jacq.) Urban	Galactia, guatabito, frijolillo, galtezia[6], galactia[4], amendoim-de-veado[6]	Papilionoideae	Phaseoleae	Perennial climbing herb	Forage (tropical)	Forage, environment	6149	CB 627, SMS 138	Actual	Australia	Same	LAC	II
							6150	SMS 300	Actual	Brasil	*Acacia mearnsii* De Wild.	LAC	II
Gliricidia sepium (Jacq.) Walp.	Grow stick, cacahuananche, madricacao, gliricidia[4]	Papilionoideae	Robinieae	Perennial non-climbing tree	Tree	Environment, wood	6168	BR 8801, LMG 10132	Actual	Brasil	Same	Embrapa Agrobiologia	IV
Glycine max (L.) Merr.	Soybean, soya bean, Manchurian bean, soja[4]	Papilionoideae	Phaseoleae	Annual climbing or non-climbing herb	Grain	Food, medicine	387	BR 96	Actual	Brasil	Same	FEPAGRO/ UFRGS	IV
							5019	29W, BR 29	Actual	Brasil	Same	FEPAGRO/ UFRGS	IV
							5079	CPAC 15, DF 24	Actual	Brasil	Same	Embrapa Cerrados	IV
							5080	CPAC 7	Actual	Brasil	Same	Embrapa Cerrados	IV
Indigofera hirsuta L.	Hairy indigo, anil bravo[4], anileira[4], indigofera[4]	Papilionoideae	Indigoferae	Annual/per e-nnial, non-climbing herb	Forage (tropical)	Forage, environment, medicine	6156	CPAC-II, DF F-2	Actual	Brasil	*Crotalaria spectabilis* Roth.	Embrapa Cerrados	III
							6158	CPAC 42	Actual	Brasil	*Crotalaria spectabilis* Roth.	Embrapa Cerrados	III
Lablab purpureus (L.) Sweet	Black bean antaqua, pois antaqua, bonavist, lablab[6]	Papilionoideae	Phaseoleae	Annual/per e-nnial, climbing or non-climbing	Forage (tropical)	Forage, chemical, environment, food, medicine,	662	CB 188	Actual	Australia	*Vigna unguiculata* (L.) Walp.	FEPAGRO/ UFRGS	II
							695	QA 922, E 83, SU	Actual	Australia	*Neonotonia wightii*	FEPAGRO/ UFRGS	II

				herb		toxins		422, NA 630			(Wight & Arn.) Lacke		
Leucaena diversifolia (Schldl.) Benth.	Wild tamarind, guash, guache, guaje, guaje blanco, leucena[d]	Mimosoideae	Mimoseae	Perennial non-climbing shrub, tree	Tree	Environment, food	6168	BR 8801, LMG 10132	Till 2002	Brasil	*Gliricidia sepium* (Jacq.) Walp.	Embrapa Agrobiologia	III
							6169	BR 5612	Actual	Brasil	*Falcataria moluccana* (Miq.) Barneby & Grimes	Embrapa Agrobiologia	III
Leucaena leucocephala (Lam.) De Wit v. Cunningham	Jumbie bean, lead tree, leucaena, cowbush, bois bourro, cassie blanc, graines de lin, grandillo bobo, leucena[d]	Mimosoideae	Mimoseae	Perennial non-climbing shrub, tree	Tree	Environment, food	6069	DF 10, BR 414	Actual	Brasil	*Leucaena leucocephala* (Lam.) De Wit	Embrapa Cerrados	IV
							6070	DF 15	Actual	Brasil	*Leucaena leucocephala* (Lam.) De Wit	Embrapa Cerrados	IV
Lotononis bainesii Baker	Miles lotononis, lotononis[d]	Papilionoideae	Crotalarieae	Annual non-climbing herb	Forage (tropical)	Forage	658	CB 376	Actual	South Africa	Same	FEPAGRO/ UFRGS	III
Lotus corniculatus L.	Cat's clover, birds' foot trefoil, broadleaf trefoil, cornichilo[d]	Papilionoideae	Loteae	Perennial non-climbing herb, shrub	Forage (subtropical)	Forage, environment	816	SEMIA original	Actual	Brasil	Same	FEPAGRO/ UFRGS	IV
Lotus glaber Miller (*L. tenuis* Willd.)	*Lotus tenuis*, narrow trefoil, birds' foot trefoil, cornichilo[d]	Papilionoideae	Loteae	Perennial non-climbing herb, shrub	Forage (subtropical)	Forage, environment	830	Hansen inoculant	Actual	USA	Not known	FEPAGRO/ UFRGS	II
Lupinus albus L.	White lupine, Egyptian lupine, chochos, lupino blanco, tremoso, 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
Macroptilium atropurpureum (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	Brasil	*Neonotonia wightii* (Wight & Arn.) Lackey	FEPAGRO/ UFRGS	II
Macrotyloma axillare (E. Mayer) Verdc.	Macrotyloma, perennial horse, macrotiloma[d]	Papilionoideae	Phaseoleae	Perennial climbing or non-climbing herb	Forage (tropical)	Forage, environment	6149	CB 627, SMS 138	Actual	Australia	*Galactia striata* (Jacq.) Urban	IAC	II
Medicago polymorpha L.	Bur medic, purple medick,	Papilionoideae	Trifolieae	Annual non-	Forage (subtro-	Forage, environment,	103	SEMIA original	Actual	Brasil	Same	FEPAGRO/ UFRGS	II

	California burclover, lucerne, clover, trevo carretilha[d]			climbing herb	pical)	medicine, food							
Medicago sativa L.	Alfalfa, lucerne, luzerne, alfafa[4]	Papilionoideae	Trifolieae	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
Mimosa acutistipula Benth.	Mimosa, jurema-preta[d], espinheiro[d]	Mimosoideae	Mimoseae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6383	BR 3407	Actual	Brazil	*Mimosa caesalpinii-folia* Benth.	Embrapa Agrobiologia	III
							6384[e]	BR 3446[e]	Actual	Brazil	*Mimosa obovata* Benth. (=*M. laticifera*)	Embrapa Agrobiologia	III
Mimosa caesalpiniifolia Benth.	Mimosa, jupiunan, mimosa[d], unha-de-gata[d], sabiá[d], sansão-do-campo[d]	Mimosoideae	Mimoseae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6166[e]	BR 3446[e]	Till 1999	Brazil	Same	Embrapa Agrobiologia	II
							6167	BR 3452	Till 1999	Brazil	Same	Embrapa Agrobiologia	II
							6382	BR 3405	Actual	Brazil	Same	Embrapa Agrobiologia	IV
Mimosa scabrella Benth.	Abaracaatinga, bracaatinga, paracaatinga, bracatinga[d]	Mimosoideae	Mimoseae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6165	BR 3454	Actual	Brazil	Same	Embrapa Agrobiologia	III
Neonotonia wightii (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
Ormosia nitida Vogel	Ormosia[d], tento-macanaiba[d]	Papilionoideae	Sophoreae	Perennial non-climbing shrub or tree	Tree	Environment, wood	6394	BR 4103	Actual	Brazil	Same	Embrapa Agrobiologia	II
Piptadenia gonoacantha (Martius) Macbr.	Piptadenia, pau-jacaré[d], angico-jacaré[d], canivete-iro[d], icarapé[d]	Mimosoideae	Mimoseae	Perennial non-climbing tree	Tree	Wood, environment, chemical	6398	BR 4812, UFC 832.55	Actual	Brazil	*Piptadenia stipulacea* (Benth.) Ducke	UFC	III
Piptadenia stipulacea (Benth.) Ducke	Piptadenia, jurema-branca[d]	Mimosoideae	Mimoseae	Perennial non-climbing tree	Tree	Wood, environment	6398	BR 4812 UFC 832.59	Till 1999	Brazil	Same	UFC	II
Pisum sativum L.	Field pea, petit pois, alverja, guisante, ervilha[d]	Papilionoideae	Vicieae	Annual, climbing, herb	Grain	Food, forage	3007	B 11A	Actual	Mexico	Same	FEPAGRO/ UFRGS	IV

Prosopis juliflora (Sw.) DC.	Mesquite, cashaw, bayarone, epinard bayahonda blanca, algaroba[4]	Mimosoideae	Mimoseae	Perennial non-climbing, tree, shrub	Tree	Environment, wood	6161	BR 4002, PRJ B4	Actual	Brazil	Same	Embrapa Agrobiologia	IV
Pueraria phaseoloides (Roxb.) Benth.	Tropical kudzu, grande feuille, puero, cudzu 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
Sclerolobium paniculatum Vogel	Velame, veludo, taxi-branco[4], ajusta-contas[4], anga[4], arapoçu[4], carvoeiro[4]	Caesalpinioideae	Caesalpinieae	Perennial non-climbing tree	Tree	Environment, wood	6160	BR 3610	Actual	Brazil	*Albizia lebbeck* (L.) Benth.	Embrapa Agrobiologia	II
							6420	BR 3617 LMG 9965	Actual	Brazil	*Acacia mangium* Willd.	Embrapa Agrobiologia	II
Sesbania virgata (Cav.) Pers.	Acacia, cibil, cumand, sesbania[4]	Papilionoideae	Robinieae	Perennial non-climbing shrub, tree	Tree	Environment, wood, forage, medicine	6401	BR 5401, LMG 9993	Actual	Brazil	Same	Embrapa Agrobiologia	II
							6402	BR 5404 LMG 9994	Actual	Brazil	Same	Embrapa Agrobiologia	II
Stizolobium aterrimum Piper & Tracy	Velvet-, Bengal-bean, Portuguese coffee, black mucuna, cafe Brazilii, pois mascate, feijão-mucuna[4], mucuna-preta[4]	Papilionoideae	Phaseoleae	Perennial non-climbing, shrub	Green manure	Environment, green manure, forage	6158	CPAC 42	Actual	Brazil	*Crotalaria spectabilis* Roth	Embrapa Cerrados	IV
Stylosanthes spp.	Stylo, Brazilian lucerne, stylosanthes, estilosantes[4]	Papilionoideae	Aeschynomeneae	Perennial non-climbing herb or shrub	Forage (tropical),	Forage, environment	6155	BR 502	Actual	Brazil	*Stylosanthes* sp.	Embrapa Agrobiologia	III
Tipuana tipu (Benth.) Kuntze	Tipa, palo montero, tipa blanca, tipuana[4], tipa- branca[4], tipu[4]	Papilionoideae	Dalbergieae	Perennial non-climbing tree	Tree	Environment, wood, chemical, forage	6192	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	II
Trifolium pratense 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	*Trifolium subterraneum* L.	FEPAGRO/ UFRGS	IV
							2081	EEL 1285	Actual	Brazil	Same	EPAGRI	IV
Trifolium repens 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	*Trifolium subterraneum* L.	FEPAGRO/ UFRGS	IV
Trifolium subterraneum L.	Subclover, subterranean	Papilionoideae	Trifolieae	Annual non-	Forage (subtro-	Forage, environment,	222	TA 1, CB 1165	Actual	Australia	Same	FEPAGRO/ UFRGS	IV

	clover, trevo subterrâneo[d]			climbing, herb	pical)	medicine							
Trifolium vesiculosum Savi	Arrowleaf clover, trevo yuchi, trevo vesiculoso[d]	Papilionoideae	Trifolieae	Annual non-climbing herb	Forage (subtro-pical)	Forage, environment, medicine	2051	SEMIA original	Actual	Brazil	Same	FEPAGRO/ UFRGS	II
Vicia sativa L.	Common vetch, pois France, arveja, ervilhaca[d]	Papilionoideae	Viciaeae	Annual climbing or non-climbing herb	Forage (subtro-pical)	Forage, environment, medicine	384	SEMIA original	Actual	Brazil	*Vicia* sp.	FEPAGRO/ UFRGS	II
Vigna unguiculata (L.) Walp.	Cowpea, black eye pea, barbati, boeme, pois manger cochon, caupi[d], feijão-de-corda[d], feijão miúdo[d]	Papilionoideae	Phaseoleae	Annual/per e-nnial, 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	*Arachis hypogaea* 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.cnip.org.br/edalcin/arvores/; umbuzeiro.cnip.org.br/db/forrag/vernac.shtml; www.arboretos.cnpm.embrapa.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/clitoriaternatea.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 Microbiologia 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 Queensalnd, 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, Sidney, 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, Malasya); 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 Agronômico de Campinas); FEPAGRO (Fundação Estadual de Pesquisa Agropecuária); UFRGS (Universidade Federal do Rio Grande do Sul); CEPEC (Centro de Pesquisas do Cacau); IAPAR (Instituto Agronômico 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)	*Sinorhizobium meliloti*
134	Acid	AY904727	1426/1430 (USDA 1002^T)	*Sinorhizobium meliloti*
135	Acid	AY904728	1429/1430 (USDA 1002^T)	*Sinorhizobium meliloti*
222	Acid	AY904729	1369/1376 (USDA 2370^T)	*Rhizobium leguminosarum*
384	Acid	AY904730	1426/1431 (CFN42^T)	*Rhizobium etli*
396	Acid	AY904731	1411/1417 (USDA3383^T)	*Mesorhizobium ciceri*
587	Alkaline	AF234890[b]	1384/1388 (USDA 76^T)	*Bradyrhizobium elkanii*
656	Alkaline	AY904732	1434/1449 (USDA 6^T)	*Bradyrhizobium japonicum*
658	Alkaline	AY904733	1393/1428 (ORS 2060^T)	*Methylobacterium* sp.
662	Alkaline	AY904734	1441/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
695	Alkaline	AY904735	1440/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
696	Alkaline	AY904736	1439/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
816	Acid	AY904737	1406/1430 (USDA3471^T)	*Mesorhizobium* sp.
830	Acid	AY904738	1399/1430 (USDA3471^T)	*Mesorhizobium* sp.
938	Alkaline	AY904739	1441/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
2051	Acid	AY904740	1417/1423 (USDA 2370^T)	*Rhizobium leguminosarum*
2081	Acid	AY904741	1414/1421 (USDA 2370^T)	*Rhizobium leguminosarum*
3007	Acid	AY904742	1411/1422 (USDA 2370^T)	*Rhizobium leguminosarum*
5019	Alkaline	AF237422[b]	1406/1411 (USDA 76^T)	*Bradyrhizobium elkanii*
6002	Alkaline	AY904743	1438/1449 (USDA 6^T)	*Bradyrhizobium japonicum*
5079	Alkaline	AF234888[b]	1409/1411 (USDA 6^T)	*Bradyrhizobium japonicum*
5080	Alkaline	AF234889[b]	1391/1403 (USDA 6^T)	*Bradyrhizobium japonicum*
6028	Alkaline	AY904744	1439/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
6053	Alkaline	AY904745	1443/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
6069	Alkaline	AY904746	1438/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
6070	Acid	AY904747	1434/1447(ATCC11325^T)	*Rhizobium* sp.
6100	Alkaline	AY904748	1440/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
6101	Alkaline	AY904749	1438/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
6144	Alkaline	AY904750	1412/1427 (USDA 6^T)	*Bradyrhizobium japonicum*
6145	Alkaline	AY904751	1437/1448 (USDA 76^T)	*Bradyrhizobium elkanii*
6146	Alkaline	AY904752	1429/1434 (USDA 76^T)	*Bradyrhizobium elkanii*
6148	Alkaline	AY904753	1419/1434 (USDA 76^T)	*Bradyrhizobium elkanii*
6149	Alkaline	AY904754	1426/1436 (USDA 76^T)	*Bradyrhizobium elkanii*
6150	Alkaline	AY904755	1442/1448 (USDA 76^T)	*Bradyrhizobium elkanii*

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

[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 (Chueire et al., 2003).

9. Table 3

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

SEMIA strain	*nifH*	*nodB*	*nodC*
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 simbiôntica 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 simbionte 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.

REFERÊNCIAS

Amarger, N., Macheret, V., Laguerre, G. 1997. *Rhizobium galicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. International Journal of Systematic Bacteriology. 47, 996-1006.

ASM-American Society for Microbiology, 1994. Bacterial Diversity Research Priorities. American Society for Microbiology. Washington DC. 7.

Brenner, D.J., Staley, J.T. & Krieg, N.R. Classification of Procaryotic Organisms and the Concept of Bacterial Speciation. In: Boone, D.R., Castenholz, R.W. (Eds.) Bergey's Manual of Systematic Bacteriology. Springer-Verlag, New York, pp.27-31.

Chen W. X., Wang E. T., Wang S. Y., Ly Y. B., Chen, X. Q., Li Y. 1995. Characteristics of *Rhizobium tianshanense* sp. nov., a moderately and slowly growing root nodule bacterium isolated from an acid saline environment in Xingiian. People´s Republic of China. International Journal of Systematic Bacteriology. 45, 153-159.

Chen, W. X., Ly. G. S., Qi, Y. L., Wang, E. T., Yuan, H. L., Li, J. L. 1991. *Rhizobium huakuii* sp. nov. isolated from the root nodules of *Astragalus sinicus*. International Journal of Systematic Bacteriology. 41, 275-280.

Chen, W.X., Yan, G.H., and Li, J.L., 1988. Numerical taxonomic study of fast-growing soybean rhizobia and a proposal that *Rhizobium fredii* be assigned to *Sinorhizobium* gen. nov. International Journal of Systematic Bacteriology, 38, 392-397.

Coutinho, H.L.C., 2003. Biodiversidade: Perspectivas e Oportunidades Tecnológicas. Disponível em <http//www.bdtfat.org.br/publicações/padct/bio/cap9/1/> Acesso em 12 de setembro de 2003.

Cowan, S.T., 1968. A dictionary of microbial taxonomic usage. Oliver & Boyd, Edinburgh.

De Lajudie, P., Laurent-Fulele, E., Willems, A., Torck, U., Coopman, R., Collins, M.D., Kersters, K., Dreyfus, B. L., Gillis, M., 1998b. *Allorhizobium undicola* gen. nov. sp. nov., nitrogen-fixing bacteria that efficiently nodulate *Neptunia natans* in Senegal. International Journal of Systematic Bacteriology, 48, 1277-1290.

De Lajudie, P., Willems, A., Nick, G., Moreira, F., Molouba, F., Hoste, B., Torck, U., Neyra, M., Collins, M. D., Lindström, K., Dreyfus, B., and Gillis, M., 1998a. Characterization of tropical tree rhizobia and description of *Mesorhizobium plurifarium* sp. nov. International Journal of Systematic Bacteriology, 48, 369-382.

De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, M. D., Dreyfus, B., Kersters, K., and Gillis, M., 1994. Polyphasic taxonomy of rhizobia: emendation of the genus *Sinorhizobium* and description of *Sinorhizobium meliloti* comb. Nov., *Sinorhizobium saheli* sp. nov., and *Sinorhizobium teranga* sp. nov. Internationa Journal of Systematic. Bacteriology, 44, 715-733.

Dreyfus, B., Garcia, J.L., Gillis, M., 1988. Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., astem-nodulating nitrogen-fixing bacterium isolated from Sesbania rostrata. International Journal Systematic Bacteriology. 38, 89-98.

Drozdowicz, A., 1997. Bacterias do solo. In: Vargas, M.A.T. & Hungria, M. (Eds.), Biologia dos solos do cerrados. EMBRAPA, Planaltina, pp.17-67.

Dupuy, N., Willems, A., Pot, B., Dewettinck, D., Wandenbruaene, I., Maestrojuan, G., Drefus, B., Kersters, K., Collins M.D. Gillis M., 1994. Phenotypic and genotypic characterization of bradyrhizobia nodulating the leguminous tree Acacia albida. International Journal Systematic Bacteriology. 44, 461-473.

Elliot, L.F., Lynch, J.M., 1994. Biodiversity and soil resilience. In: Greenland, D.J., Szaboles, I. (Eds.), Soil Resilience and Sustainable Land Use. CAB International, Wallingford, pp. 353-364.

Garrity, G.M., Holt, J.G., 2001. The road map to the Manual. In: Garrity, G.M., Boone, D.R., Castenholz, R.W. (Eds.), Bergey's manual of systematic bacteriology, 1, 2ed, pp. 119-154.

Gewin, V.L., Kennedy, A.C., Veseth, R., Miller, B.C., 1998. Soil quality changes in eastern Washington with Conservation Reserve Program (CRP) take-out. Journal of Soil Water Conservation, 54, 41-47.

Hawksworth, D.L., 1991. The Biodiversity of Microorganisms and Invertebrates. It's Role In Sustainable Agriculture. In: Hawkswort, D.L. (Eds.), Importance of Invertebrates and Microorganisms as Components of Biodiversity. CAB International, Redwood Press, Melksham, pp. 302.
Hungria, M., 1994. Sinais moleculares envolvidos na nodulação das leguminosas por rizobio. Revista Brasileira de Ciência do Solo. 18, nº3, 339-364.

Hungria, M., Campo, R.J., Mendes, I.C., 2001. Fixação biológica do nitrogênio na cultura da soja. Londrina: Embrapa Soja. 11-48. (Embrapa Soja. Circular Técnica, 35; Embrapa Cerrados. Circular Técnica, 13).

Hungria, M., Vargas, M.A.T., Suhet, A.R., Peres, J.R.R., 1994. Fixação biológica do nitrogênio em soja. In: Araujo, R.S.; hungria, M. (Ed.) Microrganismos de importância agrícola. Brasília: EMBRAPA-SPI, cap.2, .9-89.

Jarvis, B. D. W. Pankhurst, C. E., Patel, J. J. 1982. *Rhizobium loti* a new species of legume root nodule bacteria. International Journal of Systematic Bacteriology. 32, 378-380.

Jarvis, B.D.W., Wan Berkum, P., Chen, W.X., Nour, S.M. Fernandez, M.P., Cleyet-Marel, J.C., Gillis, M., 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesohizobium* gen. nov. International Journal of Systematic Bacteriology. 47, 895-898.

Jordan, D.C. 1982., Transfer of *Rhizobium japonicum* Buchan 1980 to *Bradyrhizobium* gen. nov., a genus of slow growing root-nodule bacteria from leguminous plants. Internatinal Systematic Bacteriology. 32, 136-139.

Kuykendall, L.D., Saxena, B., Devine, T.E., Udell, S.E., 1992. Genetic diversity in *Bradyrhizobium japonicum* Jordan 1982 and proposal for *Bradyrhizobium elkanii* sp. nov. Canadian Journal of Microbiology. 38, 501-505.

Lindström K., 1989. *Rhizobium galegae*, a new species of legume root nodule bactéria. International Journal of Systematic Bacteriology. 39, 365-367.

Martinez-Romero, E., Sergovia, L., Martins, F., Franco, A. A., Graham, P., Pardo, M. A., 1991. *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. Trees. International Systematic Bacteriology. 41, 417-426

Morgante, P.G., 2003. Fixação Biológica e Assimilação de Nitrogênio. Disponível em <http//www.ciagri.usp.br/~lazaropp/FisioVegGrad/NetNitro.htm> acesso em 14 de setembro de 2003.

Moulin, L., Munive, A., Dreyfus, B., Boivin-Masson, C., 2001. Nodulation of legumes by members of the β-subclass of Proteobacteia. Nature. 411, 948-950.

Nick, G., de Lajudie, P., Eardly, B. D., Suomalaimen, S., Paulin, L., Zhang, X., Gillis M., Lindström, k. 1999. *Sinorhizobium arboris* sp. nov. and *Sinorhizobium kostiense* sp. nov., isolated from leguminous trees in Sudan and Kenya. International Journal of Systematic Bacteriology. 49, 1359-1368.

Nour, S. M. Cleyet-Marel, J. C., Normand, P., Fernandez, M. P. 1995. Genomic heteogencity of strains nodulating chickpeas (*Cicer arietinum* L.) and description of *Rhizobium mediterraneum* sp. nov.. International Journal of Systematic Bacteriology. 45, 640-648.

Nour, S. M. Fernandez, M. P., Normand, P., Cleyet-Marel, J. C. 1994. *Rhizobium ciceri* sp. nov. consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). International Journal of Systematic Bacteriology. 44, 511-522.

Provorov, N.A., Vorob'ev, N.I., 2000. Evolutionary Genetics of Nodule Bacteria: Molecular and Population Aspects. Journal of Genetics. 36, 1323-1335.

Rivas, R., Willems, A., Palomo, J.L., García-Benavides, P., Mateos, P.F., Martinez-Molina, E., Gillis, M., Velázquez, E., 2004. *Bradyrhizobium betae* sp. nov. isolated from roots of *Beta vulgaris* affected by tumor-like deformations. International Journal Systematic Evolution Microbiology (In press).

Rome, S., Fernandez, M. P., Brunel, B., Normand, P., Cleyet-Marel, J. C., 1996. *Sinorhizobium medicae* sp. nov., isolated from annual *Medicago* spp. International of Systematic Bacteriology. 46, 972-980.

Segovia, L., Young, J. P. W., Martinez-Romero, E., 1993. Reclassification of American *Rhizobium leguminosarum* biovar *phaseoli* typeI strains as *Rhizobium etli* sp. nov.. International of Systmatic Bacteriology, 4, 374-377.

Skerman, V.B.D., McGowan, V., Sneath, P. H. A., 1980. Approved lists of bacterial names. International Journal of Systematic Bacteriology. 30, 225-420.

Sneath, P.H.A. 1992. International Code of Nomenclature of Bacteria (1900 Revision), American Society for Microbiology, Washington, DC.
Sy, A., Giraud, E., Jourand, P., Garcia, N., Willems, A., deLajudie, P., Prin, Y., Neyra, M., Gillis, M., Boivin-Masson, C., Dreyfus, B., 2001. Methylotrophic *Methylobacterium* Bacteria Nodulate and Fix Nitrogen in Symbiosis with Legumes. Journal of Bacteriology. 183, 214-220.

Terefework, Z., Lortet, G., Suominen, L., Lindström, K., 2000. Molecular Evolution of Interactions Between Rhizobia and Their Legume Hosts. 187-206, cap 13. In: Prokaryotic Nitrogen Fixation A Model System for Analysis of a Biological Process. Horizon Scientific Press, Wymondham, UK.

Thomas, V.C., Kevan, P., 1993. Basic principles of agroecology and sustainable agriculture. Journal ofAgricultural and Environmental Ethics. 5, 1-19.

Thórsson, A.E., Sverrisson, H., Anamthawat-Jónsson, K., 2000. Genotyping icelandic isolates of rhizobia based on rDNA-RFLP. Icelandic Agricultural Science. 13, 17-25.

Truper, H.G., Schleifer, K.H., 1991 Prokaryote characterization and identification. In: Balows, A., Truper, H. G., Dworkin, M., Harder, W., Schleifer, K. H., (Eds.), The Prokaryotes, Springer, New York, 2nd edn, 1, pp. 126-148.

Van Berkum, P., Beyene, D., Bao, G., Campbell, T. A., Eardly, B. D. 1998. *Rhizobium mongolense* sp. nov. is one of three rhizobial genotypes identified which nodulate and form nitrogen-fixing symbioses with *Medicago ruthenica* [(L.) Ledebour]. International Journal of Systematic Bacteriology. 48, 13-22.

Vinuesa, P., Leon-Barrios, M., Silva, C., Willems, A., Jarabo-Lorenzo, A., Perez-Galdona, R., Werner, D. Martinez-Romero, E., 2005. *Bradyrhizobium canariense* sp. nov., an acid-tolerant endosymbiont isolated from the nodules of endemic genistoid legumes (Papilionoideae: Genisteae) growing in the Canary Islands. Unpublished.

Wang, E. T., Van Berkum, P., Beyene, D., Sui, X. H., Dorado, O., Chen, W. X., Martinez-Romero, E. 1998. *Rhizobium huautlense* sp. nov., a symbiont of *Sesbania herbaceae* that has a close phylogenetic relationship with *Rhizobium galegae*. International Journal of Systematic Bacteriology. 48, 687-699.

Wang, E. T., Van Berkum, P., Sui, X. H., Beyene, D., Chen, W. X., Martinez-Romero, E. 1999. Diversity of rhizobia associated with *Amorpha fruticosa* isolated from Chinese soils and description of *Mesorhizobium amorphae* sp. nov.. International Journal of Systematic Bacteriology. 49, 51-65.

Wang, E.T., Martinez-Romero, E., 2000. Phylogeny of root- and stem-nodule bacteria associated with legumes. In: Triplett, E. W. (Eds.), Prokaryotic Nitrogen Fixation: A Model System for Analysis of a Biological Process. Horizon Scientific Press, Madison, pp. 177-186.

Willems, A., Coopman, R., Gillis, M., 2001. Comparison of sequence analysis of 16S-23S rRNA spacer regions, AFLP analysis and DNA-DNA hybridization in *Bradyrhizobium*. International Journal of Systematic Evolutionary Microbiology. 51, 623-632.

Xu, L.M., Ge, C., Cui, Z., Li, J., Fan, H., 1995. *Bradyrhizobium liaoningense* sp. Nov., isolated from the root nodules of soybeans. International Journal of Systematic Bacteriology. 45, 706-711.

Yao, Z.Y., Kan F.L., Wang, E.T., Wei, G.H., Chen, W.X., 2002. Characterization of rhizobia that nodulate legume species of the genus *Lespedeza* and description of *Bradyrhizobium yuanmingense* sp. nov. International Journal of Systematic and Evolutionary Microbiology. 52, 2219-2230.

Yap, W.H., Zhang, Z., Wang, Y., 1999. Distinct types of RNAr operons exist in the genome of the actinomycete *Thermomonospora chromogena* and evidence for transfer of an entire rRNA operon. Journal of Bacteriology. 181, 5201-5209.

Young, J.P.W., and Haukka, K.E., 1996. Diversity and phylogeny of rhizobia. New Phytologist. 133, 87-94.

Young, J.P.W., Kuykendall, L.D., Martinez-Romero, E., Kerr, A., Sawada, H., 2001. A revision of *Rhizobium* Frank 1889, with an emended description of the genus, and the inclusion of all species of *Agrobacterium* Conn 1942 and *Allorhizobium undicola* de Lajudie et al. 1998 as new combinations: *Rhizobium radiobacter*, *R. rizogenes*, *R. rubi*, *R. undicola* and *R. vitis*. International Journal of Systematic Evolutionary Microbiology. 51, 89-103.

Zaklha, F. & de Lajudie, P. 2001. Taxonomy of rhizobia. Agronomic, 21, 569-576.

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

Mariangela Hungria
Embrapa Soja
Cx. Postal 231
86001-970, Londrina, Parana, Brazil
fax:+55-43-33716206
e-mail: hungria@cnpso.embrapa.br; hungria@sercomtel.com.br

Elsevier Editorial System(tm) for Molecular Phylogenetics and Evolution Manuscript

Draft

Manuscript Number:

Title: Molecular phylogeny of rhizobial strains used in Brazilian commercial inoculants based on the sequencing of the 16S rRNA gene

Article Type: Research

Paper Section/Category:

Keywords: biological nitrogen fixation; Azorhizobium; Bradyrhizobium; Burkholderia; inoculant; Leguminosae; Methylobacterium; nodulation; Rhizobium; Sinorhizobium; 16S rRNA gene

Corresponding Author: Dr Mariangela Hungria, PhD

Corresponding Author's Institution: Embrapa, Centra Nacional de Pesquisa do Soja

First Author: Pamela Menna, M.Sc.

Order of Authors: Pamela Menna, M.Sc; Mariangela Hungria, PhD; Fernando G. Barcellos, PhD; Eliane V. Bangel, M.Sc.

Manuscript Region of Origin:

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.