Universidade Estadual de Londrina

PAMELA MENNA PEREIRA

"TAXONOMIA E DIVERSIDADE GENÉTICA DE RIZÓBIOS MICROSSIMBIONTES DE DISTINTAS LEGUMINOSAS COM BASE NA ANÁLISE POLIFÁSICA (BOX-PCR E 16S RNAr) E NA METODOLOGIA DE MLSA"

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

Orientadora: Dra. Mariangela Hungria
Co-Orientador: Dr. Fernando Gomes Barcellos

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Londrina, 27 de junho de 2008.

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RESUMO

O termo genérico "rizóbio" se aplica às bactérias capazes de fixar nitrogênio atmosférico (N_2) e convertê-lo a uma forma assimilável pela planta, quando estas se encontram em simbiose com determinadas plantas da família *Leguminosae.* Contudo, apesar da importância ecológica e econômica, os rizóbios têm sido relativamente pouco estudados. Baseado nos dados de seqüenciamento do gene ribossomal 16S, existem, atualmente, cinco gêneros de rizóbios descritos, *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium (=Ensifer) e Mesorhizobium,* todos pertencentes à subclasse alfa das Proteobacterias. Além disso, existem outras bactérias recentemente descritas e também denominadas como rizóbios, por serem microssimbiontes de leguminosas, e que pertencem a gêneros bastante distintos, tanto na subclasse alfa *(Devosia, Methylobacterium),* como na subclasse beta *(Ralstonia, Burkholderia).* Embora o gene ribossomal 16S seja hoje a molécula mais utilizada para estimar relações filogenéticas em procariotos, a elevada conservação na seqüência nucleotídica deste gene observada entre diferentes grupos bacterianos é uma limitação para a determinação das espécies. Assim, vários estudos vêm sendo conduzidos, a fim de determinar metodologias alternativas para estudos de diversidade, bem como filogenia e taxonomia rizobiana. Uma metodologia amplamente utilizada em estudos de diversidade tem sido a técnica de BOX-PCR. Com base nessa técnica, é possível obter, através de reações de PCR, perfis genômicos de bactérias. Por outro lado, recentemente foi proposta a metodologia de MLSA ("multi locus sequencing analysis"), como uma nova estratégia para os estudos de filogenia e taxonomia em procariontes. Tendo em vista a elevada diversidade genética de rizóbios tropicais ainda pouco estudada, o objetivo do presente trabalho foi caracterizar a diversidade genética de estirpes de rizóbios simbiontes de distintas leguminosas de importância agrícola e ambiental, e avaliar o potencial das metodologias de BOX-PCR e MLSA em estudos de diversidade, filogenia e taxonomia. Em um primeiro estudo, o dendrograma resultante do BOX-PCR de 68 SEMIAs isoladas de 47 distintas leguminosas apresentou uma baixa correlação (7,6%) com à árvore filogenética construída com o gene ribossomal 16S. Todavia, quando uma análise polifásica utilizando BOX-PCR e as seqüências do gene ribossomal 16S foi analisada em uma proporção de 2:8 (proporção para o peso relativo de cada análise, 16SrRNA:BOX-PCR), foi possível observar agrupamentos com elevada similaridade (90%) aos grupos taxonómicos propostos pela arvore filogenética do gene ribossomal 16S. Em um segundo estudo utilizando a metodologia de MLSA com os genes 16S RNAr, *atpD, dnaK, glnII, recA* e a região ITS, em uma coleção de estirpes de 169 *Bradyrhizobium* isoladas de 43 distintas leguminosas, foi possível observar a divisão de dois grupos bem definidos, sendo que o primeiro incluiu as estirpes tipo de *B. japonicum, B. betae, B. liaoningense, B. canariense, B. yuanmingense* e *B. japonicum* USDA 110, já o segundo grupo incluiu estirpes relacionadas à *B. elkanii* USDA 76^T. Uma elevada variabilidade foi observada no gene *atpD,* sendo que cinco estirpes relacionadas à *B. elkanii* apresentaram uma variabilidade ainda não constatada para esse gene. Outra importante observação foi que o grupo composto pelas estirpes USDA 110, SEMIAs 5080 e 6059 de *B. japonicum,* todas isoladas de soja, foram sempre reunidas em todas as seis árvores construídas e formaram um grupo distinto do agrupamento formado pela estirpe tipo de *B. japonicum* USDA 6^T. Desse

modo, foi observado que tanto a análise polifásica, utilizando BOX-PCR e as seqüências do gene ribossomal 16S, quanto a técnica de MLSA possibilitaram acessar a diversidade genética em rizóbios bem como determinar relações filogenéticas e identificar possíveis novas espécies de rizóbios.

Palavras-chave: Rizóbio. Genética vegetal. Taxonomia vegetal.

PEREIRA, Pâmela Menna. **Taxonomia e diversidade genética de rizóbios microssimbiontes de distintas leguminosas com base na análise polifásica (BOX-PCR e 16S RNAr) e na metodologia de MLSA.** 2008. 100f. Tese (Doutorado em Microbiologia) – Universidade Estadual de Londrina, Londrina, 2008.

ABSTRACT

The term "rhizobia" is generically applied to bacteria capable of fixing the atmospheric nitrogen (N_2) and converting it to a form assimilable by the plant, when in symbiosis with plants of the *Leguminosae* family. However, besides their ecological and economic importance, the rhizobia have not been subject of many studies. Based on the data obtained with the 16S ribosomal gene sequences, five rhizobial genera have been described so far, *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium (=Ensifer)* and *Mesorhizobium,* all of them belonging to the alpha subclass of *Proteobacteria.* In addition, there are other bacteria described recently and also referred as rhizobia, due to be symbiosis of leguminous plants, and that belong to quite different genera, either in the alpha *(Devosia, Methylobacterium)* or in the beta *(Ralstonia, Burkholderia)* subclasses. Even though the 16S ribosomal gene is nowadays the molecule more used to estimate the phylogenetics relationships in prokaryotes, the high conservation in the nucleotide sequences of this gene, as observed among the different bacterial groups, is a limitation to its use to species determination. Therefore, several studies have been conducted to determine alternative methodologies to evaluate diversity genetic as well as to rhizobial phylogeny and taxonomy. A methodology widely used in the diversity studies has been the BOX-PCR technique. With this technique it is possible to obtain genomic fingerprints of bacterial strains. More recently, the MLSA (Multi Locus Sequence Analysis) methodology has been proposed as a new strategy to phylogenetic and taxonomic studies in prokaryotes. Due to the high genetic diversity of the tropical rhizobial strains still not well characterized, the aim of this study was to characterize the genetic diversity of rhizobial strains symbionts of several tropical leguminous plants of environmental and agricultural importance. Also, we have evaluated the potential utility of using the BOX-PCR and the MLSA technology in diversity, phylogeny and taxonomy studies. In a first study, the analysis of the dendrograma resulting from the BOX-PCR of 68 SEMIAs isolated from 47 distinct legumes was possible to observe a low correlation (7, 6%) to the phylogenetic tree based in the 16S ribosomal gene sequences. However, when the polyphasic analysis using the BOX-PCR and the 16S ribosomal gene sequences was used at a proportion of 2:8 (proportion for the relative weight of each analysis), it was possible to observe clusters with high similarities (90%) to the taxonomic groups obtained with the phylogenetic trees based in the 16S ribosomal gene sequences. In a second study using the MLSA methodology with the genes 16S RNAr, *atpD, dnaK, glnII, recA* and the ITS region in a collection of 169 *Bradyrhizobium* isolated of 43 distinct legumes, was possible to observe a division of the strains into two well defined clusters, where the first cluster included the type strains from *B. japonicum, B. betae, B. liaoningense, B. canariense, B. yuanmingense* and the *B. japonicum* USDA 110 strain, and the second cluster included the strains related to the *B. elkanii* USDA 76^T. A high variability was observed among the *atpD* gene sequences analyzed, and five strains related to *B. elkanii* showed variability to this gene not detected before. Another important observation was that the cluster composed by the strains USDA 110, SEMIA 5080 and 6059, all isolated from soybean, were always clustered in all trees obtained from the six different housekeeping gene sequences analyzed. Also, these strains were always distinct from the cluster containing the *B.*

japonicum USDA 6^{T}. Therefore, it was observed that the polyphasic analysis, using the BOX-PCR and the 16S ribosomal gene sequences, and the MLSA technique, allowed to access the genetic diversity as well as to infer the phylogenetic relationships of the rhizobia strains analyzed and also to identify possible new species of rhizobia.

Keywords: Rhizobium. Plant genetics. Plant taxonomy.

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1 INTRODUÇÃO

A fixação biológica do nitrogênio (FBN) constitui um dos processos de maior importância na biosfera, sendo responsável pela produção de um dos nutrientes essenciais para o crescimento das plantas, o nitrogênio (N). Esse processo é realizado, em grande parte, por determinados microrganismos, denominados organismos fixadores de N ou diazotróficos (DROZDOWICZ, 1997).

Dentre os sistemas conhecidos que realizam o processo de FBN, o melhor caracterizado é o da simbiose rizóbio-leguminosa. Rizóbios constituem um grupo de bactérias Gram-negativas que inclui os gêneros *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium (=Ensifer)* e *Mesorhizobium,* e cerca de 50 espécies, na subclasse alfa das Proteobacterias (GARRITY; HOLT, 2001). Além disso, existem outras bactérias recentemente descritas e também denominadas como rizóbios, por serem microssimbiontes de leguminosas, e que pertencem a gêneros bastante distintos, tanto na subclasse alfa, tais como *Devosia* (RIVAS et al., 2003) e *Methylobacterium* (SAMBA et al., 1999; SY et al., 2001; JOURAND et al., 2004) como na subclasse beta, tais como *Ralstonia* (CHEN et al., 2001) e *Burkholderia* (VANDAMME et al., 2002; CHEN et al., 2003, 2005, 2006).

Para que a relação simbiótica entre planta e rizóbio possa se estabelecer, essas bactérias precisam possuir, em seu genoma, genes responsáveis pelo processo de FBN. Estudos têm demonstrado que esses genes encontram-se localizados em elementos genômicos potencialmente transferíveis. Esses elementos podem ser plasmídeos ou megaplasmídeos em espécies de *Rhizobium, Sinorhizobium, Mesorhizobium amorphae* e *Mesorhizobium huakuii,* ou regiões cromossomais, em *Mesorhizobium loti, Bradyrhizobium japonicum* e *Bradyrhizobium elkanii* (WANG; MARTÍNEZ-ROMERO, 2000).

Quando a simbiose se estabelece, as bactérias induzem à formação de órgãos altamente especializados, os nódulos. A formação dos 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 FBN, enquanto que as mudanças na planta hospedeira visam assimilar a amônia produzida pelas bactérias (HUNGRIA et al., 1994a).

Estima-se que a FBN seja um processo cuja importância ecológica só possa ser comparada à da fotossíntese, e que os organismos que a realizam podem suplementar

ecossistemas naturais com novas quantidades de N, aproveitando a grande reserva de N2 presente na atmosfera (DELWICHE, 1970; BURNS; HARDY, 1975; BRILL, 1979; POSTGATE; HILL, 1979; SMIL, 1999; NEWTON, 2000; GRAHAM; VANCE, 2003).

Nos solos brasileiros, caracterizados pelo baixo teor de N e em geral insuficientes para sustentar a demanda de N das plantas, a FBN se torna fundamental (HUNGRIA et al., 1994b, HUNGRIA; CAMPO, 2007).

Assim, visando o estabelecimento de estirpes altamente eficientes no processo de FBN, rizóbios têm sido um dos grupos de microrganismos amplamente estudados, devido não apenas a sua grande importância aplicada, mas também por possibilitar o entendimento sobre os mecanismos genéticos envolvidos com a simbiose principalmente em paises tropicais (MENNA et al., 2006, GERMANO et al., 2006, BARCELLOS et al., 2007; BATISTA et al., 2007; GRANGE et al., 2007; PINTO et al., 2007).

Como em muitas outras bactérias, a taxonomia e o estudo da diversidade dos rizóbios têm sido derivadas, principalmente, da análise de seqüências nucleotídicas do gene ribossomal 16S (16S RNAr) (WEISBURG et al., 1991; VANDAMME et al., 1996; GARRITY; HOLT, 2001; MENNA et al., 2006). O uso desse gene se deve à constatação de que ele é suficientemente conservado, o que permite o estabelecimento de relações evolutivas (WOESE et al., 1990; WEISBURG et al., 1991; WANG; MARTINEZ-ROMERO, 2000).

No entanto, conforme discutido por Garrity e Holt (2001), somente níveis taxonómicos mais elevados, tais como filo, classe, ordem, família e gênero têm sido classificados com base na análise das seqüências do gene ribossomal 16S. Não existe, ainda, um consenso sobre a determinação de espécies em procariotos, sendo que análises adicionais de hibridização DNA-DNA, características fisiológicas, entre outras, vêm sendo utilizadas como ferramentas complementares para a caracterização de espécies (GARRITY; HOLT, 2001; VANDAMME et al., 1996; COENYE et al., 2005).

Diversas técnicas moleculares têm sido utilizadas como ferramentas práticas e úteis para a identificação, classificação bacteriana e nos estudos de diversidade. Uma metodologia amplamente empregada nos estudos de populações bacterianas tem sido a análise por PCR de regiões repetitivas no genoma (REP-PCR), tais como a região conhecida como "BOX" (ALBERTON et al., 2006; KASCHUK et al., 2006a,b; MOSTASSO et al., 2002; GRANGE; HUNGRIA, 2004; HUNGRIA et al., 2006; BATISTA et al. 2007).

Embora a técnica de BOX-PCR seja hoje utilizada em estudos de diversidade (VERSALOVIC et al., 1991, 1994; de BRUIJIN, 1992), a identificação de espécie procariótica utilizando essa metodologia, ainda é considerada limitada e insuficiente

(VERSALOVIC et al., 1994). Estudos demonstram que um grande agravante dessa técnica, está no fato de que elevadas taxas de transferência horizontal bem como duplicações e deleções genômicas podem dificultar sua análise (VERSALOVIC et al., 1991, 1994; de BRUIJIN, 1992).

Como conseqüência dessa limitação, a técnica de BOX-PCR somente tem sido utilizada em estudos de diversidade, e não empregada como ferramenta para a identificação na taxonomia bacteriana (VERSALOVIC et al., 1991, 1994; De BRUIJIN, 1992).

Atualmente, a espécie procariótica é definida como um grupo de bactérias com elevada similaridade (>97%) das seqüências do gene ribossomal 16S, valores de hibridização DNA-DNA superiores a 70% ou 5°C ou menos de AT_m (diferença da temperatura média de desnaturação da dupla fita de DNA entre os híbridos e os homólogos formados em condições padrões) e características morfofisiológicas similares à estirpe tipo da espécie (VANDAMME et al., 1996; COENYE et al., 2005; GEVERS et al., 2005).

Todavia, o método de hibridização DNA-DNA representa uma grande limitação à descrição de novas espécies, particularmente devido ao fato de ser um processo laborioso, dificultando a rápida identificação de procariotos e incapaz de identificar microrganismos não cultiváveis (GEVERS et al., 2005).

Como alternativa ao uso do método de hibridização DNA-DNA, estudos têm demonstrado que a análise do espaço intergênico entre os genes ribossomais 16S e 23S (ITS1) pode ser utilizado em complementação ao gene ribossomal 16S (Van BERKUM; FUHRMANN, 2000; TAN et al., 2001; Van BERKUM; EARDLY, 2002; WILLEMS et al., 2001a, 2001b, 2003; GERMANO et al., 2006). Em rizóbios, as seqüências da região ITS1 têm demonstrado elevada correlação com os dados obtidos utilizando a técnica de hibridização DNA-DNA (WILLEMS et al., 2003).

Além dos genes ribossomais, alguns estudos evidenciaram que outros genes também conservados e com função de manutenção do metabolismo basal da célula procariótica, podem dar grande suporte para a determinação de relações filogenéticas e taxonômicas (COENYE; VANDAMME, 2003). Esses genes têm, como principal vantagem, o fato de apresentarem uma taxa de evolução mais rápida do que os genes ribossomais (GEVERS et al., 2005), fato que possibilita estudos de filogenia envolvendo indivíduos intimamente relacionados.

A análise conjunta de vários genes também representa uma estratégia útil, pois funciona como um "tampão" contra efeitos de recombinação ou transferência horizontal

ocorrido quando apenas um gene é utilizado (GEVERS et al., 2005). Desse modo, os resultados encontrados em estudos polifásicos com genes ribossomais e não-ribossomais, indicam uma nova tendência em taxonomia e filogenia bacteriana, que consiste na análise conjunta de múltiplos genes (loci) (GEVERS et al., 2005). Com base nessa estratégia foi desenvolvida, para muitos grupos bacterianos, principalmente em bactérias patogênicas, a metodologia de "Multi locus Sequence Analysis" (MLSA) (GEVERS et al. 2005). O MLSA consiste no seqüenciamento e análise conjunta (como uma única seqüência concatenada) de, no mínimo, cinco genes conservados ("*housekeeping")* (STACKEBRANDT et al., 2002).

Recentemente, o MLSA tem sido utilizado em diversos estudos de procariotos, tais como *Bradyrhizobium, Bulkholderia, Bacillus, Vibrio, Mycobacterium* e *Ensifer* e os dados obtidos têm permitido elucidar dogmas sobre a filogenia bem como a taxonomia dessas estirpes (GEVERS et al., 2005; THOMPSON et al., 2005; STEPKOWSKI et al., 2005; PARKER 2004; STEPKOWSKI et al., 2003; MARTENS et al., 2007).

Assim, devido ao grande número de microrganismos que ainda necessitam ser identificados e classificados, a utilização de dados filogenéticos tais como os obtidos pela metodologia de MLSA, tem adquirido maior importância dentro da microbiologia, principalmente em estudos de diversidade e taxonomia rizobiana.

A diversidade bacteriana se encontra diretamente relacionada a alterações ambientais tais como os causados pela agricultura, poluição e outros estresses (ELLIOT; LYNCH, 1994). Desse modo, o conhecimento da diversidade rizobiana, pode contribuir para o entendimento da qualidade do solo, o desenvolvimento de agroecossistemas sustentáveis, além de possibilitar identificar novas estirpes com elevados potenciais agronômicos (THOMAS; KEVAN, 1993).

2 OBJETIVOS

2.1 OBJETIVO GERAL

Caracterizar a diversidade genética de estirpes de rizóbios simbiontes de distintas leguminosas de importância agrícola e ambiental, e identificar regiões gênicas adequadas para o desenvolvimento de marcadores moleculares para o uso em estudos de diversidade, filogenia e taxonomia.

2.1.1 Objetivos específicos estudo 1

a) Utilizar a metodologia de BOX-PCR para inferir diversidade genética de 68 estirpes de rizóbios recomendadas como inoculantes comerciais brasileiros, previamente classificadas com base no gene ribossomal 16S por Menna et al. (2006).

b) Comparar dados obtidos pela análise de regiões *box* com dados obtidos pelas seqüências do gene ribossomal 16S previamente analisados por Menna et al. (2006).

c) Realizar uma análise polifásica utilizando conjuntamente os dados obtidos pela técnica de BOX-PCR e dados obtidos com as seqüências do gene ribossomal 16S e assim, verificar a viabilidade do uso desta estratégia como ferramenta para estudos de taxonomia.

2.1.2 Objetivos específicos estudo 2

a) Utilizar a metodologia de BOX-PCR para inferir diversidade genética entre 169 estirpes de *Bradyrhizobium* isoladas de distintas leguminosas e distintos países.

b) Selecionar representantes dos agrupamentos obtidos pela metodologia de BOX-PCR para análise dos genes *"housekeeping", atpD, dnaK, glnII, recA* e 16S RNAr e região intergênica entre os genes ribossomais 16S e 23S (ITS1).

b) Utilizar a metodologia de MLSA com os genes *"housekeeping"* e a região ITS1, descritos no item acima, a fim de verificar relações filogenéticas bem como possíveis novas espécies de *Bradyrhizobium.*

3 REVISÃO BIBLIOGRÁFICA

3.1 VISÃO GLOBAL DO APORTE DE NUTRIENTES

O crescimento da população mundial, que deve atingir oito bilhões de pessoas em 2030, demanda uma maior oferta de alimentos, que vem sendo atendida tanto pelo cultivo em áreas novas, freqüentemente marginais e impróprias para a agricultura, como pelo aprimoramento de tecnologias, por exemplo, a maior produção e utilização de insumos e a melhor exploração de recursos genéticos. Desse modo, o crescimento da população e do consumo de alimentos contrasta com a falta de novas áreas disponíveis para a agricultura. Evidencia-se, assim, a necessidade de desenvolvimento e utilização de tecnologias que garantam a segurança alimentar, mas preservando os recursos naturais, pois, somente nas últimas cinco décadas, estima-se que cerca de dois dos 8,7 bilhões de hectares de terras agricultáveis, pastagens permanentes e florestas do mundo tenham sido degradadas (CASSMAN, 1999; FRESCO, 2003; NORSE, 2003; PINSTRUP-ANDERSEN, 2003).

Como resultado do empobrecimento físico e químico do solo, o hábitat para a vida biológica é desfavorecido e, como esses organismos desempenham um papel fundamental nos processos de estruturação dos solos, de ciclagem e de aporte de nutrientes, estabelece-se um ciclo de empobrecimento dos solos (GILLER, 2001; HUNGRIA; CAMPO, 2005). A situação é ainda mais grave em países tropicais e em desenvolvimento, como é o caso do Brasil: a degradação física é acentuada pelo manejo intensivo e inadequado do solo, a química pela natureza dos solos, suscetíveis à erosão e com baixos teores de nutrientes e, tanto as propriedades físicas, como as químicas são desfavorecidas pelos estresses ambientais freqüentes, como temperaturas elevadas e longos períodos de estiagem (HUNGRIA; VARGAS, 2000; HUNGRIA; CAMPO, 2005).

Em termos globais, consideram-se a disponibilidade de água e dos nutrientes, nitrogênio (N) e fósforo (P), como os principais fatores limitantes à produção agrícola. Em caso de deficiência hídrica, a água precisa ser fornecida às culturas por meio de irrigação. Em relação ao P, deve ser suprido como fertilizante, mas diversos microrganismos, como os fungos micorrízicos, podem incrementar a absorção do nutriente, enquanto outros, como espécies de *Aspergillus,* são capazes de solubilizar fontes fosfatadas com baixa disponibilidade para as plantas. Já o N pode ser, em diversos casos, fornecido, parcial ou

totalmente, pelo processo da fixação biológica do nitrogênio atmosférico (N2) (HUNGRIA; CAMPO, 2007).

Como os solos brasileiros são, em geral, pobres em N e P, o aporte contínuo desses nutrientes é necessário para a manutenção da fertilidade do solo e para a nutrição das plantas. A produção dos fertilizantes nitrogenados envolve um consumo elevado de derivados de petróleo, portanto, com oscilações constantes de preço. Além disso, a maior parte dos fertilizantes nitrogenados e dos fosfatados mais solúveis utilizados no Brasil é importada, também com preços atrelados ao dólar. Como resultado, em períodos de preços baixos dos produtos agrícolas, o agricultor não consegue repor todos os nutrientes que foram retirados pelas culturas, resultando no empobrecimento gradual do solo. Nesse contexto, particularmente nas últimas décadas, vem sendo dada cada vez, maior importância aos processos biológicos envolvendo os microrganismos do solo capazes de aportarem nutrientes como o N e permitirem o melhor aproveitamento de fontes de P, entre outros, e, desse modo, enriquecendo o solo e diminuindo as perdas de nutrientes, por lixiviação ou na forma de gases (HUNGRIA; CAMPO, 2007).

3.2 FIXAÇÃO BIOLÓGICA DE NITROGÊNIO E BACTÉRIAS SIMBIÓTICAS

O N, por ser um constituinte dos ácidos nucléicos e proteínas, moléculas fundamentais para todos os processos biológicos, é o quarto nutriente mais abundante nas plantas, sendo superado apenas pelo carbono (C), pelo oxigênio (O) e pelo hidrogênio (H). As fontes para o fornecimento desse elemento são: i) o solo, principalmente pela decomposição da matéria orgânica; ii) o processo de fixação não-biológica, resultante de descargas elétricas, combustão e vulcanismo; iii) os fertilizantes nitrogenados; e iv) o processo de fixação biológica do nitrogênio atmosférico (N2) (BRILL, 1979; POSTGATE; HILL, 1979; POSTGATE, 1982; NEWTON, 2000).

Na natureza o N presente na forma de N_2, constitui cerca de 80% do gás atmosférico, todavia, organismos eucariontes tais como as plantas, são incapazes de absorver N_2 e convertê-lo a uma forma assimilável, devido à tripla ligação existente entre os átomos do N_2 que é uma das mais fortes que se tem conhecimento na natureza (HUNGRIA et al., 1994b).

No solo, o reservatório de N presente na matéria orgânica é limitado, podendo ser rapidamente esgotado após alguns cultivos. Além disso, as condições de

temperatura e umidade predominantes no território brasileiro aceleram os processos de decomposição da matéria orgânica, bem como de perdas gasosas e por lixiviação de N, resultando em baixos teores desse nutriente. Por essas razões os solos brasileiros são capazes de fornecer, em média, apenas 10 a 15 kg de N.ha^{-1}.safra^{-1}. Deve-se considerar, ainda, que a preservação da matéria orgânica com uma relação C/N adequada é importante para a manutenção dos microrganismos do solo, sem os quais a sustentabilidade dos sistemas agrícolas se torna inviável (HUNGRIA et al., 2001b; HUNGRIA; CAMPO, 2005).

O processo de fixação não biológica assim como o reservatório natural de N na matéria orgânica, também é limitado. Já a produção de fertilizantes nitrogenados resultantes do processo químico é responsável por transformar N_2 atmosférico em amónia (NH_3). O NH_3 representa a forma assimilada com maior rapidez pelas plantas, todavia representa em termos ecológicos um custo elevado, pois o processo químico o qual transforma N2 em amónia, requer hidrogênio (derivado do gás de petróleo), catalisador contendo ferro, altas temperaturas (300 a 600 °C) e altas pressões (200 a 800 atm.) (HUNGRIA et al., 2001b). 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 da utilização dos fertilizantes nitrogenados reside, na baixa eficiência de sua utilização pelas plantas, raramente ultrapassando 50%. Deve-se ressaltar, 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., 2001b).

A terceira fonte de N citada 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 um complexo enzimático denominado dinitrogenase, que é formado por duas unidades protéicas, a ferro-proteína (Fe-proteína) e a molibdênio-ferro-proteína (MoFe-proteína), ambas capazes de transportar 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 representa a principal via de incorporação do N_2 à biosfera, perfazendo aproximadamente 65% da entrada de N no planeta, ou 96% da fixação por processos naturais, e é considerada após a fotossíntese, como o processo biológico mais importante, sendo fundamental para a vida na Terra. Em termos quantitativos, estima-se que de 44 a 66 milhões de toneladas métricas de N_2 são fixados,

anualmente, por leguminosas de importância agrícola, com outros 3 a 5 milhões de toneladas métricas sendo fixados por leguminosas em ecossistemas naturais, fornecendo cerca de metade de todo o N usado na agricultura (DELWICHE, 1970; BURNS; HARDY, 1975; BRILL, 1979; POSTGATE; HILL, 1979; SMIL, 1999; NEWTON, 2000; GRAHAM; Vance, 2003).

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, coletivamente denominados rizóbios (DROZDOWICZ, 1997). Rizóbios constituem um grupo de bactérias Gram-negativas que incluem os gêneros *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium(=Ensifer)* e *Mesorhizobium* e cerca de 50 espécies, na subclasse alfa das Proteobacterias (GARRITY; HOLT, 2001). Além disso, existem outras bactérias recentemente descritas e também denominadas como rizóbios, por serem microssimbiontes de leguminosas, e que pertencem a gêneros bastante distintos, tanto na subclasse alfa, tais como *Devosia* (RIVAS et al., 2003) e *Methylobacterium* (SAMBA et al., 1999; SY et al., 2001; JOURAND et al., 2004) como na subclasse beta, tais como *Ralstonia* (CHEN et al., 2001) e *Burkholderia* (VANDAMME et al., 2002; CHEN et al., 2003, 2005, 2006).

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, et al., 1994a).

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., 1994a).

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, que fazem com que as bactérias simbiônticas sejam atraídas em direção às raízes da planta, por quimiotactismo positivo (DROZDOWICZ, 1997).

Os flavonóides são moléculas responsáveis por induzir a transcrição de genes de nodulação *(nod, nol* e *noe)* em bactérias simbiônticas, conduzindo à síntese e a secreção dos fatores Nod. Os fatores Nod são lipo-quitinooligossacarídeos determinantes para o processo de reconhecimento entre bactéria e planta hospedeira e pela indução de uma intensa divisão celular no córtex da raiz. Bactérias atraídas por quimiotactismo a rizosfera da planta, se multiplicam e colonizam 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. As bactérias invadem o tricoma, utilizando o canal formado pela invaginação do plasmalema, dando origem ao cordão de infecção (MORGANTE, 2003; HUNGRIA et al., 1994a).

O cordão de infecção migra 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, ocupam agora, o citoplasma das células vegetais do nódulo primário. A partir do estabelecimento do nódulo radicular, as bactérias, presentes nas células radiculares hospedeiras param de se multiplicar, aumentam de tamanho e sofrem várias alterações bioquímicas se transformando em bactérias especializadas na fixação de nitrogênio também chamadas de bacteróides (MORGANTE, 2003).

Rizóbios, portanto, 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 uma relação simbiótica entre planta e rizóbio possa se estabelecer, essas bactérias precisam dispor de genes 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 esses genes simbióticos estejam 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 amplamente estudados no campo da genética (PROVOROV; VOROB'EV, 2000).

3.3 CLASSIFICAÇÃO DAS BACTÉRIAS FIXADORAS DE NITROGÊNIO

3.1.1 Taxonomia dos rizóbios

A sistemática bacteriana é 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, utilizada como um sinônimo para sistemática e consisti 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 sendo o maior, chamado de Domínio. Todos os procariotos são localizados dentro de dois Domínios, *Archaea* e *Bacteria.* Para cada Domínio são descritos os Filos, Classes, Ordens, Famílias, Gêneros, espécies e subespécies (GARRITY; HOLT, 2001).

Primeiramente, bactérias capazes de nodular e fixar nitrogênio em relações simbiônticas com plantas da família *Leguminosae* eram representadas apenas pelo gênero *Rhizobium,* o qual foi pela primeira vez, descrito por Kirchner em 1896. Fred et al. (1932) em um critério baseado, principalmente, em grupos de inoculação cruzada, entre o microssimbionte e a planta hospedeira, descreveu seis espécies: *Rhizobium leguminosarum* (espécie tipo), *R. japonicum, R. lupini, R. meliloti, R. phaseoli* e *R. trifoli.*

Características fisiológicas, bioquímicas e genéticas, além da inoculação cruzada e do crescimento lento com reação alcalina em meio de cultura contendo manitol como fonte de carbono, passaram a ser consideradas nas décadas seguintes, permitindo a divisão das bactérias simbióticas em dois grupos, de crescimento rápido e de crescimento lento, contudo, continuaram sendo classificadas no gênero *Rhizobium* (JORDAN; ALLEN, 1974; BUCHANAN, 1980).

Foi somente em 1982, que bactérias pertencentes à espécie *Rhizobium japonicum,* foram reclassificadas em um novo gênero, *Bradyrhizobium,* nessa época apenas com uma espécie definida, *Bradyrhizobium japonicum* (JORDAN, 1982, 1984). O nome *Bradyrhizobium* é alusivo às taxas de crescimento dessa bactéria, pois "bradus" vem do grego, significando lento e *Rhizobium* é o nome genérico da bactéria. Na primeira edição do Manual Bergey de Bacteriologia Sistemática, as demais estirpes de crescimento rápido continuaram a ser classificadas em cinco espécies pertencentes ao gênero *Rhizobium* (JORDAN, 1984).

Na década de 1980 e início da década de 1990, o emprego de técnicas de biologia molecular resultou em uma verdadeira revolução na taxonomia procariótica. Nessa época estudos filogenéticos passaram a ser conduzidos com os genes ribossomais (RNAr), levando a alteração na taxonomia atual. Os resultados coerentes obtidos pela análise do gene ribossomal 16S, resultou na escolha preferencial dessa molécula para estimar relações filogenéticas e assim determinar posições taxonómicas em procariotos (GARRITY; HOLT, 2001).

Conforme Brenner et al. (2001) somente 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 ainda permanece incerta, sendo que análises de similaridade DNA-DNA, bem como características fisiológicas também vêm sendo utilizadas a fim de caracterizar uma determinada espécie procariótica (GARRITY; HOLT, 2001). Contudo, não há, até o presente momento, uma determinação precisa quanto à especiação em bactérias.

Com base na taxonomia atual, os rizóbios encontram-se classificados como pertencentes a: Domínio: *Bacteria;* Filo: *Proteobacteria;* Classe: *Alfaproteobacteria;* Ordem: *Rhizobiales;* e distribuído nas Famílias *Rhizobiaceae, Phyllobacteriaceae*, *Bradyrhizobiaceae* e *Methylobacteriaceae* (GARRITY; HOLT, 2001). Recentemente, algumas espécies de bactérias também denominadas como rizóbios, por serem microssimbiontes de leguminosas e que pertencem a gêneros bastante distintos, também têm sido identificadas, tais como *Devosia* (RIVAS et al., 2003) e *Methylobacterium* (SAMBA et al., 1999; SY et al., 2001; JOURAND

et al., 2004) pertencentes à classe das *Alfaproteobacterias,* bem como *Ralstonia* (Chen et al., 2001) e *Burkholderia* (VANDAMME et al., 2002; CHEN et al., 2003, 2005, 2006) pertencentes à classe das *Betaproteobacterias.*

A grande diversidade entre bactérias fixadoras de N_2, observada em recentes estudos, tem levado a questionamentos sobre a taxonomia atual desse grupo de bactérias, bem como a descrição de novas espécies de rizóbios (ZAKHIA; de LAJUDIE, 2001; VINUESA et al., 2005a, 2005b, MARTENS et al., 2007).

3.1.2 Atuais ferramentas de análises para determinação da diversidade genética, filogenia e taxonomia dos rizóbios

Diversas técnicas moleculares têm sido utilizadas visando determinar ferramentas práticas e úteis para a determinação da diversidade microbiana, sendo a metodologia de BOX-PCR utilizada principalmente para analisar populações onde há elevada diversidade (De BRUIJN, 1992; JUDD et al., 1993; LAGUERRE et al., 1997).

A técnica de BOX-PCR, consiste da amplificação de elementos repetitivos presente no genoma (REP-PCR), provavelmente localizados em distintas posições intergênicas e com ambas as orientações no cromossomo (VERSALOVIC et al., 1991, 1994; de BRUIJIN, 1992).

A metodologia de BOX-PCR foi primeiramente proposta por Martin et al. (1992) para inferir diversidade genética entre microrganismos denominados Gram-positivos *(Streptococcus pneumoniae),* e desde então esta técnica tem sido aplicada em estudos de diversos grupos de microrganismos, incluindo espécies de Gram-negativas (KOEUTH et al., 1995).

Embora a metodologia de BOX-PCR seja hoje amplamente utilizada para inferir diversidade em procariotos (VERSALOVIC et al., 1991, 1994; de BRUIJIN, 1992), a determinação de espécie utilizando essa técnica ainda é considerada limitada e insuficiente (VERSALOVIC et al., 1994). Estudos demonstram que um grande agravante dessa técnica, está no fato de que elevadas taxas de transferência horizontal bem como duplicações e deleções genômicas podem dificultar sua análise (VERSALOVIC et al., 1991, 1994; de BRUIJIN, 1992).

Uma poderosa ferramenta utilizada pela taxonomia para a determinação de espécie procariótica, tem sido o estudo da filogenia, a qual é amplamente utilizada 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 seus ancestrais (GARRIT; HOLT, 2001).

Na década de 70, a determinação de espécie bacteriana era baseada, principalmente, em propriedades morfológicas, fisiológicas e bioquímicas (BUCHANAN; GIBBONS, 1974). Quase duas décadas depois, com desenvolvimento de técnicas de amplificação gênica, particularmente a PCR ("polymerase chain reaction", reação em cadeia da polimerase, (SAIKI et al., 1988) e das técnicas de seqüênciamento direto do DNA amplificado (INNES et al., 1988; WINSHIP, 1989), teve início uma verdadeira revolução na taxonomia bem como na filogenia dos procariotos. Foram iniciados estudos de busca de genes que pudessem ser utilizados em modelos evolucionários, o que resultou na identificação das vantagens da utilização de genes ribossomais. Esses genes apresentam uma série de características favoráveis em estudos filogenéticos, pois: *i)* são encontrados em todos os organismos vivos, uma vez que a síntese de proteínas ribossomais é obrigatória; *ii)* são componentes principais da estrutura dos ribossomos e, portanto, abundantes nas células, facilitando a sua identificação e *iii)* possuem regiões que evoluem em taxas diferentes, permitindo que análises filogenéticas sejam realizadas em vários níveis de resolução taxonómica (WOESE, 1987; DELONG et al., 1989; AMANN et al., 1990, 1995; WOESE et al., 1990; WEISBURG et al., 1991; WILLEMS; COLLINS, 1993; OLSEN et al., 1994; VANDAMME et al., 1996; MARTÍNEZ-ROMERO; CABALLERO-MELLADO, 1996; PACE, 1997; Van BERKUM; EARDLY, 1998; GARRITY; HOLT, 2001; CANNONE et al., 2002).

Devido a essas propriedades, as seqüências do gene ribossomal 16S, passaram a ser consideradas como boas escolhas para comparar organismos e inferir filogenias (BROSIUS et al., 1981; WEISBURG et al., 1991; GARRITY; HOLT, 2001).

Embora os genes ribossomais, particularmente o gene ribossomal 16S, seja hoje a molécula mais utilizada para estimar relações filogenéticas entre bactérias, em alguns casos, conforme constatado no gênero *Burkholderia* e *Bradyrhizobium,* a magnitude da divergência na seqüência do gene ribossomal 16S é limitada e insuficiente para distinguir claramente as espécies (OLSEN; WOESE, 1993; LUDWIG; SCHLEIFER, 1994; BARRERA et al., 1997; WANG; MARTÍNEZ-ROMERO, 2000; COENYE et al., 2001; COENYE; VANDAMME, 2003).

3.1.3 Refinamento da especiação de procariotos e diversidade de rizóbios

Pela definição atual de taxonomia, a seqüência completa do gene ribossomal 16S permite classificar procariotos somente até nível de gênero (GARRITY; HOLT, 2001). Assim, a definição atual de uma espécie continua sendo definida como um grupo de bactérias com elevada similaridade (>97%) das seqüências do gene ribossomal 16S, valores de hibridização DNA-DNA superiores a 70% ou 5°C ou menos de AT_m (diferença da temperatura média de desnatauração da dupla fita de DNA entre os híbridos e os homólogos formados em condições padrões) e características morfofisiológicas similares à estirpe tipo da espécie (VANDAMME et al., 1996; COENYE et al., 2005; GEVERS et al., 2005).

Todavia, o método de hibridização DNA-DNA representa uma grande limitação à descrição de novas espécies, particularmente devido ao fato de ser um processo laborioso, dificultando a rápida identificação de procariotos, e incapaz de identificar microrganismos não cultiváveis (GEVERS et al., 2005).

Como metodologias alternativas visando substituir os dados de hibridização DNA-DNA, a análise do gene ribossomal 23S, bem como a análise da região intergenica entre o 16S RNAr e o 23S RNAr (ITS1), também têm sido utilizadas em complementação ao 16S RNAr. Estudos têm demonstrado que tanto o 23S rRNA como o ITS1 apresentam maior variabilidade nas seqüências nucleotídicas, principalmente quando espécies distintas são analisadas (TESFAYE; HOLL, 1998; VINUESA et al., 1998; Van BERKUM; FURHMANN, 2000; BOYER et al., 2001; GERMANO, 2003; GERMANO et al., 2006).

Uma limitação do uso das seqüências ITS1 e 23S RNAr pode residir no fato de que muitos gêneros de bactérias contêm cópias múltiplas do operon ribossomal (16S, ITS, 23S, 5S), e os alelos em cada cópia podem divergir, em maior ou menor extensão, devido à recombinação intragênica localizada e à transferência horizontal, parcial ou total, dos operons, ou, ainda, devido a uma estrutura completamente diferente da seqüência da região intergênica (HAUKKA et al., 1996; YAP et al., 1999; BOYER et al., 2001; TAN et al., 2001). A transferência horizontal de genes, porém, já foi verificada também no gene ribossomal 16S (Van BERKUM et al., 2003), todavia quando vários genes ribossomais são analisados em uma análise polifásica, é possível considerar a contribuição de todos eles para inferir a diversidade microbiana (VINUESA et al., 1998; GERMANO et al., 2006).

Além dos genes ribossomais, alguns estudos identificaram outros genes capazes de fornecer suporte nas relações filogenéticas entre procariotos, por exemplo, os

genes *gyrB, rpoD, recA, sodA* e *dnaK* (COENYE; VANDAMME, 2003). Esses genes têm, como principal vantagem, o fato de apresentarem uma taxa de evolução mais rápida do que os genes ribossomais (GEVERS et al., 2005).

A análise conjunta de vários genes representa uma estratégia útil pela idéia de funcionar como um "tampão" contra efeitos de recombinação ou tranferência horizontal ocorridos em um gene específico. Desse modo, os resultados encontrados em estudos polifásicos com genes ribossomais e não-ribossomais, indicam para uma nova tendência em taxonomia e filogenia bacteriana, que consiste na análise conjunta de múltiplos genes (loci) (GEVERS et al., 2005). Com base nessa estratégia foi desenvolvida e implementada, para muitos grupos bacterianos, principalmente em bactérias patogênicas, a metodologia de "Multi Locus Sequencing Analysis" (MLSA) (GEVERS et al. 2005). O MLSA consiste no seqüenciamento e análise conjunta (como uma única seqüência concatenada) de, no mínimo, cinco genes conservados *(housekeeping)* (STACKEBRANDT et al., 2002).

De acordo com Zeigler (2003) e Thompson et al. (2005), os genes utilizados como marcadores filogenéticos alternativos, além de serem conservados para o grupo em estudo, precisam obedecer alguns critérios como: *i)* estar distribuídos no genoma com uma distância mínima entre os genes de 100 kb; *ii)* estar presentes no genoma em uma única cópia; *iii)* apresentar uma extensão nucleotídica suficiente para permitir o seqüênciamento; *iv)* conter informações suficientes para a análise; e *v)* estar correlacionados com dados obtidos com o gene ribossomal 16S e com os percentuais de similaridade obtidos por hibridização DNA-DNA.

No caso dos rizóbios a técnica de MLSA tem sido utilizada em estudos com *Bradyrhizobium* e os dados obtidos têm permitido elucidar dogmas sobre sua filogenia e taxonomia (STEPKOWSKI et al., 2005; PARKER 2004; STEPKOWSKI et al., 2003). A recente descrição de *B. canariense* encontra-se embasada em dados filogenéticos obtidos através da análise dos genes *"housekeeping" atpD, glnII, recA* e da região ITS1 (VINUESA et al., 2005a). Similarmente, Martens et al. (2007) também confirmaram a importância da utilização do MLSA para determinação de espécies de *Sinorhizobium=Ensifer.*

Assim, utilizando essa metodologia novas espécies de rizóbios vêm sendo descritas, refletindo o número crescente de grupos de pesquisa envolvidos em estudos de diversidade de rizóbios (VINUESA et al., 2005; MARTENS et al. 2007). A maior parte das novas espécies de rizóbios tem sido 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 conhecimento e exploração racional da diversidade de rizóbios (COUTINHO, 2007).

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4 ESTUDO 1

rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool

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Running title: Biodiversity and phylogeny of tropical rhizobia

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rep-PCR of tropical rhizobia for strain fingerprinting, biodiversity appraisal and as a taxonomic and phylogenetic tool

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Abstract. With more than 30 million doses of rhizobial inoculants marketed per year, it is probable that Brazilian agriculture benefits more than that of any other country from symbiotic N_2 fixation. As a result of strain-selection programs, 142 strains of rhizobia are officially recommended for use in commercial inoculants for ninety-six leguminous crops. In this study, sixty-eight of these elite strains were characterized by rep-PCR with the BOX-primer. Reproducibility of the DNA profiles was confirmed, suggesting efficacy of BOX-PCR both for control of quality of inoculants and for preliminary characterization of rhizobial culture collections. Strains of different species never showed similarity higher than 70% in the BOX-PCR analysis, however, some strains of the same species fit into more than one cluster, and correlation between BOX-PCR products and 16S rRNA sequences was low (7.6%). On the other hand, a polyphasic approach—20%:80% of BOX-PCR:16S rRNA which correlated well with the 16S rRNA analysis (95%), and provided higher definition of the genotypes, resulting in clearer indications of the taxonomic groups—might expedite rhizobial diversity studies.

Keywords*:* Bacterial fingerprinting. Bacterial taxonomy. Biological nitrogen fixation. Rhizobiales. Inoculants. 16S rRNA.

Introduction

Many bacteria belonging to several genera, collectively known as "rhizobia," are capable of establishing symbiotic associations with thousands of plant species in the family Leguminosae. Rhizobia were first isolated and characterized in 1888 by the Dutch

microbiologist Martinus Willem Beijerinck (Fred et al., 1932), but progress towards understanding their diversity has been achieved mainly in the past two decades, largely due to development of molecular techniques, in particular DNA analysis by rep-PCR, and by RFLP-PCR and sequence analysis of the 16S rRNA gene (e.g., Young et al., 1991; de Bruijn, 1992; Laguerre et al., 1994, 1997; Vinuesa et al., 1998; Germano et al., 2006; Menna et al., 2006).

Rhizobia were first classified based on the capacity to nodulate certain host plants, in "cross inoculation" groups (Fred et al., 1932), whereas 50 years later their taxonomy was redefined using numerical criteria, considering several morpho-physiological and genetic properties (Jordan, 1984). Several studies since the 1980s have demonstrated that the 16S rRNA gene is an especially useful molecular marker for assessing phylogeny and taxonomy of prokaryotes (Woese, 1987; Woese et al., 1990; Weisburg et al., 1991; Vandamme et al., 1996; Garrity & Holt, 2001). When this criterion was applied to the rhizobia, it resulted in profound changes in taxonomy (de Lajudie et al., 1994; Garrity & Holl, 2001; Young et al., 2001). However, although precise for the definition of kingdoms and genera, 16S rRNA provides poor resolution of species and subspecies (Woese, 1987; Fox et al., 1992; Stackebrandt & Goebel, 1994; Garrity & Holt, 2001). Therefore, the classification of known species usually requires additional information, such as morpho-physiological and other genetic properties (Garrity & Holt, 2001), and the definition of new species usually involves labor- and time-consuming DNA-DNA hybridization (Vandamme et al., 1996; Coenye et al., 2005).

Other molecular techniques may be useful for prospecting bacterial biodiversity and fingerprinting strains, and rep-PCR has proven to be adequate with a variety of bacteria, including rhizobia (e.g., de Bruijn, 1992; Judd et al., 1993; Laguerre et al., 1997). The technique consists of amplification of repetitive DNA elements dispersed in the genome and probably located in distinct, intergenic positions all over and in both orientations of the chromosome (Versalovic et al., 1991, 1994; de Bruijin, 1992). Three main sets of repetitive DNA elements have been used for typing bacteria: the 35- to 40-pb repetitive extragenic palindromic (REP) sequences (Stern et al., 1984), the 124- to 127-bp enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton et al., 1991), and the BOX elements (Martin et al., 1992). This third class consists of differentially conserved subunits, named boxA, boxB, and boxC (Martin et al., 1992), though only the boxA-like subunit appears highly conserved across diverse bacteria (Koeuth et al., 1995). BOX elements were first identified in a Gram-positive microorganism *(Streptococcus pneumoniae)* (Martin et al., 1992), but soon proved to

be useful in fingerprinting other bacteria including Gram-negative species (Koeuth et al., 1995). REP- and ERIC-sequences were first identified in the Gram-negative *Escherichia coli* and *Salmonella typhimurium* (Gilson et al., 1984; Hulton et al., 1991) and then were found to be conserved in all related Gram-negative enteric bacteria, as well as in many unrelated bacteria from multiple phyla (Versalovic et al., 1994; Olive & Bean, 1999).

Species biodiversity in the tropics, albeit poorly characterized, is often claimed as a major global treasure, of which the rhizobia are an important part (Oyaizu et al., 1992; Vinuesa et al., 1998; Germano et al., 2006; Menna et al., 2006). Indeed, indigenous and naturalized rhizobia in Brazilian soils have been found to exhibit high levels of genetic diversity using ERIC- (Hungria et al., 1998, 2000, 2006; Santos et al., 1999; Ferreira & Hungria, 2002; Mostasso et al., 2002; Grange & Hungria, 2004; Pinto et al., 2007), REP- (Hungria et al., 1998; Santos et al., 1999; Hungria et al., 2000, 2006; Mostasso et al., 2002; Barcellos et al., 2007; Pinto et al., 2007), and BOX-PCR (Fernandes et al., 2003; Galli-Terasawa et al., 2003; Alberton et al., 2006; Kaschuk et al., 2006a,b; Barcellos et al., 2007; Batista et al., 2007; Pinto et al., 2007).

A polyphasic approach, combining ERIC-, REP- and BOX-PCR profiles, has been used to improve the precision of genetic discrimination (Louws et al., 1994; de Bruijn et al., 1996); it has also proven to be useful in surveys of diversity of tropical rhizobia (Hungria et al., 2000, 2006; Mostasso et al., 2002; Pinto et al., 2007). Although congruence among the three sets of rep-primers has rarely been evaluated, using *Xanthomonas* spp. as a model, Rademaker et al (2000) showed high correlations between profiles obtained with the three sets of primers. Also among *Xanthomonas* spp., a high correlation between rep-PCR and DNA-DNA hybridization was found (Rademaker et al., 2000), but, apparently, there is poor congruence between rep-PCR and the taxonomy of rhizobial species (Laguerre et al., 1997; Mostasso et al., 2002; Fernandes et al., 2003; Grange & Hungria, 2004; Hungria et al., 2006).

Brazil may benefit from symbiotic N2 fixation more than any other country; more than 30 million doses of rhizobial inoculants are applied to legume crops per year (Hungria & Campo, 2007). As a result of government-funded strain-selection programs, 142 strains are officially recommended for application in inoculants to ninety-six leguminous hosts. Maintenance of these bacteria as well as their distribution to the industry is the responsibility of the "*Rhizobium* Culture Collection SEMIA" (Seção de Microbiologia Agrícola) (IBP Catalogue of *Rhizobium* Collections n°. 443 in the WFCC World Data Center on Microorganisms). However, despite accrual over many years of a great deal of data on the

symbiotic properties of those strains, evaluations of their genetic diversity and taxonomic position are just beginning (Germano et al., 2006; Menna et al., 2006). Furthermore, strain fingerprinting is not a component of quality control of the inoculants commercialized in Brazil (Hungria & Campo, 2007).

A first step towards the characterization of this valuable Brazilian collection consisted of determination of 16S rRNA sequences of sixty-eight strains, which fit into seven described genera and nine known species, with indications of several putative new species (Menna et al., 2006). This set of strains represents valuable material to both confirm if rep-PCR has high discriminatory power and reproducibility for fingerprinting rhizobia, and to determine if the technique has taxonomic utility.

Material and methods

Strains

Sixty-eight elite strains from the "Brazilian *Rhizobium* Culture Collection SEMIA", of the FEPAGRO-MIRCEN [Fundação Estadual de Pesquisa Agropecuária (Rio Grande do Sul, Brazil)-Microbiological Resources Center] recommended for use in commercial inoculants in Brazil are listed on Table 1, including the host legumes of origin and the legumes for which the strains are recommended. Taxonomic classification of the strains, based on the sequence analysis of the 16S rRNA gene, previously determined by Menna et al. (2006), is also included in Table 1. Other information about the strains and their host legumes is available elsewhere (Menna et al., 2006).

Fourteen reference strains were used as follows: *Bradyrhizobium japonicum* USDA 6^T, *Bradyrhizobium liaoningense* USDA 3622^T, *Bradyrhizobium elkanii* USDA 76^T, *B. japonicum* USDA 110, *Rhizobium tropici* type A CFN 299, *R. tropici* type B CIAT 899^T, *Rhizobium leguminosarum* USDA 2370^T, *Rhizobium giardini* $H152^T$, *Sinorhizobium (Ensifer) fredii* USDA 205^T, *Sinorhizobium (Ensifer) meliloti* USDA 1002^T, *Mesorhizobium loti* USDA 3471^T, *Mesorhizobium ciceri* USDA 3383^T, *Azorhizobium caulinodans* USDA 4892^T and *Rhizobium etli* CFN 42^T. They were provided by USDA, Beltsville, USA, and by the Centro de Ciências Genómicas, Cuernavaca, Mexico, and are deposited at the "Diazotrophic and PGPR Culture Collection" of Embrapa Soja. Preparation of stock cultures and strains maintenance were as described before (Menna et al., 2006).

DNA extraction and rep-PCR (BOX) genomic fingerprinting

Total genomic DNA of each strain was extracted as described by Kaschuk et al. (2006a), and amplified by PCR with the primer BOX A1R (Versalovic et al., 1994; Koeuth et al., 1995). Amplification procedures were performed as described by Kaschuk et al. (2006a), and the 1-kb DNA marker (Invitrogen™) was included on the left, right and in the centre of each gel. The amplified fragments were separated by horizontal electrophoresis on 1.5% agarose, as described before (Kaschuk et al., 2006a). Gels were stained with ethidium bromide, visualized under UV radiation and photographed.

Cluster analyses

First, the sizes of the fragments in all analyses were normalized according to the sizes of the DNA marker. Cluster analyses of the BOX-PCR profiles and of the 16S rRNA sequences were performed using the Bionumerics program (Applied Mathematics, Kortrijh, Belgium, version 4.6), with the UPGMA algorithm (unweighted pair-group method, with arithmetic mean) (Sneath & Sokal, 1973). In the BOX-PCR, the Jaccard coefficient (Jaccard, 1912) was used, considering the optimum values indicated by the Bionumerics program for the tolerance and the optimization parameters.

After cluster analyses of each experiment (16S rRNA and BOX-PCR) and of the polyphasic analysis (16S rRNA + BOX-PCR), matrices were constructed. In the polyphasic analysis, different ratios (5:5; 6:4; 7:3; 8:2 and 9:1; 16S rRNA:BOX-PCR) were used. Congruence of each experiment was calculated according to the Bionumerics program.

Results

The sixty-eight elite strains used in this study had been isolated from forty-seven leguminous hosts; no information was available on the original host of one strain (SEMIA 396). They are recommended for use in commercial inoculants in Brazil, for application to sixty-three legumes (Table 1). Complex fingerprinting patterns with multiple distinct bands of various intensities were obtained for the strains in the BOX-PCR analysis, with the smallest number of bands (seven) observed for SEMIA 658, a *Methylobacterium* sp. isolated from *Lotononis bainesii,* whereas the others showed an average of twenty bands (Figure 1). All

individual profiles are now available at the homepage of the "Brazilian Microbiology Resource Center" (http://www.bmrc.lncc.br/).

To confirm the reproducibility of the BOX-PCR fingerprinting protocol, the strains were analyzed three times, starting from the DNA extraction. Visual comparisons of the band profiles revealed good agreement, and coefficients of similarity for profiles of the same strain were estimated at 94% or higher.

Although we usually adopt a tolerance of 2% in clustering analyses of BOX-PCR products (e.g., Alberton et al., 2006; Kaschuk et al., 2006a,b), in this study we considered the levels of tolerance and optimization indicated by the Bionumerics program, which were more strict, of 1.0% and 0.4, respectively. The resulting dendrogram identified sixty-two distinct profiles for the sixty-eight strains analyzed, and, together with the type and reference strains, were clustered at a very low level of similarity, only 25% (Figure 1). Interestingly, some strains that had been selected based exclusively on their symbiotic properties showed identical or very similar profiles. In this category are strains isolated from and recommended for the same host legume, such as *Sinorhizobium meliloti* strains SEMIAs 134 and 135, isolated from alfalfa *(Medicago sativa),* and *Burkholderia* sp. SEMIAs 6166, 6167 and 6383, isolated from *Mimosa caesalpiniifolia.* In addition, highly similar profiles were obtained from strains isolated from hosts belonging to the same genus, such as *Bradyrhizobium* sp. SEMIAs 6163 *(Acacia mearnsii)* and 6420 *(Acacia mangium).* However, other strains showing identical or almost identical profiles had been isolated from hosts belonging to different genera: *Rhizobium* sp. SEMIAs 6165 *(Mimosa scabrella),* 6168 *(Gliricidia sepium)* and 6382 *(M. caesalpiniifolia); Bradyrhizobium elkanii* SEMIAs 662 *(Vigna unguiculata),* 695 *(Neonotonia wightii)* and 938 *(Lupinus albus);* and *Bradyrhizobium* sp. SEMIAs 6160 *(Albizia lebbeck)* and *B. elkanii* 6169 *(Falcataria moluccana)* (Figure 1). Strains sharing similar BOX-PCR profiles also showed strong similarity—99% or higher—in the sequences of the ribosomal gene 16S RNA, In general, strains positioned in the same BOX-PCR group were not related in terms of the original host of derivation.

In the BOX-PCR dendrogram, some strains belonging to the same species were positioned in more than one cluster, e.g. *B. japonicum* and *S. meliloti* (Figure 1). However, considering a cut off of 70%, as suggested for diversity studies of tropical rhizobia using rep-PCR (Grange and Hungria, 2004; Alberton et al., 2006; Kaschuk et al., 2006a,b), although a species might be found in more than one cluster, strains belonging to different species never showed similarity higher than 70% (Figure 1).

The BOX-PCR technique was thus confirmed to be powerful both in fingerprinting and in detecting high genetic diversity among rhizobial strains. However, the results obtained also illustrate difficulties of using the profiles for grouping or defining species and even genera.

On the other hand, the dendrogram resulting from the analysis of the 16S rRNA (Menna et al., 2006) was probably very conservative in showing putative new species. Therefore, in this study we also investigated the possibility of using a combined analysis of the sequences of the 16S rRNA gene and of the BOX-PCR products for a more accurate taxonomic classification of the strains.

After transforming the 16S rRNA sequencing data into a similarity matrix, it was possible to perform a polyphasic analysis and to determine the correlations between the dendrograms built with the BOX-PCR profiles, with the 16S rRNA sequences and with the combination of 16S rRNA + BOX-PCR. First, the correlation between BOX-PCR products and 16S rRNA sequences was very low, 7.6%. Then, several ratio values were given to the matrices and, as expected, the correlation with the 16S rRNA clustering analysis obtained by Menna et al. (2006) decreased as the weight of the BOX-PCR analysis increased (Table 2). In contrast, when the weight of the BOX-PCR analysis was decreased, genetic diversity was lower in comparison to the BOX-PCR dendrogram (data not shown). We found that, starting from a proportion of 6:4 (60% 16S rRNA:40% BOX-PCR), good correlations were obtained between the combined dendrogram and the cluster analysis of the 16S rRNA (Table 2), which allowed detection of higher genetic diversity in comparison to the clustering based on the 16S rRNA sequences.

Although few differences were detected in the clusters formed in the dendrograms obtained at the ratios of 6:4, 7:3, 8:2 and 9:1% of 16S rRNA:BOX, we chose the combined analysis at a ratio of 8:2, which showed a high correlation, almost 95%, with the 16S rRNA genes (Table 2). Considering a cutoff of 90% in the combined analysis, fifty-one distinct profiles were defined (Figure 2), therefore showing considerably higher genetic diversity than the 16S rRNA analysis alone (Menna et al., 2006).

The polyphasic analysis of BOX-PCR profiles and 16S rRNA sequences also defined some major clusters (Figure 2), and, in general, they were similar to those observed in the phylogenetic tree of the 16S rRNA (Menna et al., 2006). The two first clusters included strains belonging to the genera *Sinorhizobium* (I) and *Mesorhizobium* (II). Fourteen strains were linked in cluster III, thirteen of them of the genus *Rhizobium;* the exception was strain SEMIA 103 of *S. meliloti.* The *R. giardinii* type strain occupied an isolated position and was

not included in cluster III. High genetic diversity was observed among the three *Azorhizobium* strains, which were linked in cluster IV with relatively low similarity, 84%. Forty-six *Bradyrhizobium* strains were linked in the major cluster V, but several subclusters were also observed. In the upper part of cluster V, the first subclusters included thirty-four strains showing higher 16S rRNA gene similarity with *B. elkanii* or *Bradyrhizobium* sp. In the lower part of cluster V, twelve strains had stronger 16S rRNA similarity with *B. japonicum,* except for the type strain of *B. liaoningense,* clustered with *Bradyrhizobium* sp strains SEMIAs 656 and 6192, and three other *Bradyrhizobium* sp. strains, SEMIAs 6144, 6156, and 6164. The only strain of genus *Methylobacterium* was linked to all previous strains at a low level of similarity, of 76%. Finally, cluster VI linked seven strains belonging to the genus *Burkholderia,* and the cluster was joined to the others at a level of similarity of 70%. When compared to the phylogenetic tree based on the 16S rRNA (Menna et al., 2006), the clusters and subclusters obtained in the combined analysis of BOX and 16S rRNA resulted in better definition of the genotypes.

Discussion

Particularly in the last 15 years, DNA-typing methods employed in diversity studies have greatly improved our understanding of bacterial phylogeny and taxonomy. Among those methods, rep-PCR analysis might be highlighted for being relatively quick, easy and inexpensive, therefore useful in the screening of large numbers of isolates (Hulton et al., 1991; Versalovic et al., 1991, 1994; de Bruijn, 1992; Martin et al., 1992; Gilson et al., 1984; Koeuth et al., 1995; Olive & Bean, 1999). rep-PCR is also useful for fingerprinting rhizobia (e.g., de Bruijn, 1992; Judd et al., 1993; Laguerre et al., 1997); it has been used in our laboratory largely for typing indigenous or naturalized strains (Hungria et al., 1998, 2000; 2006; Santos et al., 1999; Chen et al., 2000; Ferreira & Hungria, 2002; Fernandes et al., 2003; Galli-Terasawa et al., 2003; Grange & Hungria, 2004; Alberton et al., 2006; Kaschuk et al., 2006a,b; Barcellos et al., 2007; Batista et al., 2007; Pinto et al., 2007).

In this study, profiles of BOX-PCR for sixty-eight rhizobial strains were obtained, and their reproducibility confirmed, corroborating previous reports (Schneider & de Bruijn, 1996). In contrast with diversity studies, the SEMIA strains from this study represent elite diazotrophic symbiotic bacteria identified in selection programs performed in the last 40 years in Brazil. The strains had been isolated from forty-seven leguminous species, including hosts

belonging to all three subfamilies of the Leguminosae, and, therefore, were expected to be diverse. Among the strains from this study collectively called rhizobia, were eight belonging to two newly described symbiotic genera, *Methylobacterium* and *Burkholderia.* These sixty-eight elite strains are officially recommended for the production of inoculants for sixty-three legumes, and as reliable BOX-PCR profiles were easily obtained for all of them, we suggest that the method should be included as an important tool for control of inoculant quality, not only in Brazil, but also in other countries where rhizobial inoculants are produced commercially.

Another critical issue in the determination of diversity and its biotechnological potential is the taxonomy of elite microorganisms. Since the 1970s, when Carl R. Woese and coworkers began sequencing 16S rRNA genes, our knowledge of phylogeny and taxonomy of bacteria has greatly increased, resulting in profound changes (Woese, 1987; Woese et al., 1990). Nevertheless, as highlighted by Garrity & Holt (2001), only Phylum, Class, Order, Family and Genera can be defined by sequencing 16S rRNA genes. Species determined still depended on a polyphasic approach that includes both phenotypic (e.g., biochemical properties, fatty-acid composition) and genotypic (e.g., rRNA gene sequences, DNA fingerprints) data. Isolates differing by 3% or more of the 16S rRNA bases are probably different species, but DNA-DNA-hybridization experiments are still required for definition of new species (Garrity & Holt, 2001). In the DNA-DNA hybridization method, isolates with more than 70% DNA-DNA-binding values and less than 5% difference in their melting temperature (**D**Tm) should belong to the same species, whereas those sharing less than 50% of DNA-DNA-binding values belong to different species (Vandamme et al., 1996; Stackebrandt, et al., 2002; Coenye et al., 2005; Gevers et al., 2005). However, few laboratories routinely perform time-consuming, complex DNA-DNA hybridization analyses, representing a major limitation to microbial diversity studies (Rademaker et al., 2000).

The apparent evolutionary conservation of the repetitive elements used in rep-PCR methods is the main property enabling their use for fingerprinting bacteria (de Bruijn et al., 1996; Koeuth et al., 1995), but the application of rep-PCR in taxonomy studies remains to be confirmed. An exhaustive study was performed with a *Xanthomonas* collection, in which rep-PCR profiles were compared to DNA-DNA homology groups (Rademaker et al., 2000). Interestingly, the authors found high correlation between both methods, such that ERIC-REP-PCR fingerprint similarity values above 0.5 correlated with values exceeding 70% of DNA-DNA homology, and above 0.8 correlated with values exceeding 90% (Rademaker et al.,

2000). In our study, we opted for BOX-PCR analysis because in several studies we found that it results in more bands than with ERIC and REP primers. In the cluster analysis, strains belonging to different species were never grouped at a level of similarity higher than 70%; however, some strains belonging to the same species were positioned in more than one cluster. Additionally, the correlation between the BOX-PCR and the 16S rRNA was very low, 7.6%. Previous reports have mentioned lack of cohesion between rep-PCR grouping and taxonomic classification (Laguerre et al., 1997; Mostasso et al., 2002; Fernandes et al., 2003; Grange & Hungria, 2004; Hungria et al., 2006), but those studies did not include detailed comparisons. Our results indicate that the use of rep-PCR for taxonomy of rhizobia is indeed very limited. Differences between BOX-PCR and 16S rRNA can be attributed to several issues; for example, high rates of horizontal gene transfer that have been reported in rhizobia (e.g., Sullivan et al., 1995; Barcellos et al., 2007; Batista et al., 2007), potentially affecting the rep-PCR profiles.

When the 16S rRNA sequences of the sixty-eight elite strains were determined, despite a high level of genetic diversity and strong indications of several new species, there were indications that diversity was underestimated in some genera (Menna et al., 2006), for example *Bradyrhizobium* (Menna et al., 2006), consistent with previous indications of its low diversity in terms the 16S rRNA gene (e.g., Vinuesa et al., 1998; Chen et al., 2000; Willems et al., 2001). The analysis of other ribosomal regions, such as the 23S rRNA and the intergenic space between the 16S and the 23S rRNA can greatly improve definition of species, at least in *Bradyrhizobium* (Germano et al., 2006), and other conserved genes might also be useful. Besides using rep-PCR profiles for fingerprinting strains, we have investigated if it might also be helpful in taxonomy and phylogeny studies.

When adopting a polyphasic approach, the topology of the dendrograms resulting from the combined analysis of 6:4, 7:3, 8:2, 9:1 (16S rRNA:BOX-PCR) correlated well with the 16S rRNA analysis (from 77 to 99%), and also showed higher definition of the genotypes, resulting in clearer indication of the taxonomic groups. One major example was observed in the genus *Bradyrhizobium:* much higher genetic diversity was obtained in the polyphasic approach in comparison to the 16S rRNA analysis. Therefore we suggest the use of this polyphasic analysis—good results were obtained with the ratio 8:2 (16S rRNA:BOX-PCR)—as a rapid and more precise method of determining taxonomic diversity and phylogenetic structure of large collections of rhizobial isolates.

In conclusion, most of the large collections of rhizobia that exist worldwide are poorly characterized. Interest in biological nitrogen fixation should increase in the next decade, not only due to concerns about the indiscriminate use of N-fertilizers and their greenhouse effects, but also because prices of these fertilizers are expected to increase considerably. Therefore, we suggest, as an initial step in comparing strains from rhizobial collections, that they be fingerprinted with BOX-PCR. Later, in addition to sequencing of the 16S rRNA of representative or elite strains, polyphasic analyses of BOX-PCR and 16S rRNA may improve our understanding of the phylogeny and taxonomy of rhizobia.

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Figure 1. Cluster analysis (UPGMA with the coefficient of Jaccard) of the products obtained by BOX-PCR analysis. Reference and type strains are labeled and SEMIA strains are classified according to the sequencing analysis of the 16S rRNA gene, as described by Menna et al. (2006). More information about the strains is given on Table 1.

Figure 2. Polyphasic cluster analysis considering a ratio of 80%:20%, 16S rRNA sequences:BOX-PCR products. Reference and type strains are labeled and SEMIA strains are classified according to the sequencing analysis of the 16S rRNA gene, as described by Menna et al. (2006). More information about the strains is given on Table 1.

Table 1 – Strains used in this study

SEMIA strain	Original Host	Host for which the strain is recommended	Source of the strain	Taxonomy based 16S rRNA[a]	Gene Bank access number[a]
103	*Medicago polymorpha*	*Medicago polymorpha*	Brazil	*Sinorhizobium meliloti*	AY904726
134	*Medicago sativa*	*Medicago sativa*	Brazil	*Sinorhizobium meliloti*	AY904727
135	*Medicago sativa*	*Medicago sativa*	Brazil	*Sinorhizobium meliloti*	AY904728
222	*Trifolium subterraneum*	*Trifolium pratense; Trifolium repens; Trifolium subterraneum*	Australia	*Rhizobium leguminosarum*	AY904729
384	*Vicia* sp.	*Vicia sativa*	Brazil	*Rhizobium etli*	AY904730
396	not known	*Cicer arietinum*	USA	*Mesorhizobium ciceri*	AY904731
587	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium elkanii*	AF234890
656	*Neonotonia wightii*	*Desmodium intortum; Macroptilium atropurpureum; Neonotonia wightii*	Brazil	*Bradyrhizobium* sp.	AY904732
658	*Lotononis bainesii*	*Lotononis bainesii*	South Africa	*Methylobacterium* sp.	AY904733
662	*Vigna unguiculata*	*Lablab purpureus; Vigna unguiculata*	Australia	*Bradyrhizobium elkanii*	AY904734
695	*Neonotonia wightii*	*Lablab purpureus; Neonotonia wightii*	Australia	*Bradyrhizobium elkanii*	AY904735
696	*Desmodium uncinatum*	*Desmodium uncinatum*	Austrália	*Bradyrhizobium elkanii*	AY904736
816	*Lotus corniculatus*	*Lotus corniculatus*	Brazil	*Mesorhizobium* sp.	AY904737
830	*Lotus corniculatus*	*Lotus glaber*	USA	*Mesorhizobium* sp.	AY904738
938	*Lupinus albus*	*Lupinus albus*	USA	*Bradyrhizobium elkanii*	AY904739
2051	*Trifolium vesiculosum*	*Trifolium vesiculosum*	Brazil	*Rhizobium leguminosarum*	AY904740
2081	*Trifolium pratense*	*Trifolium pratense*	Brazil	*Rhizobium leguminosarum*	AY904741
3007	*Pisum sativum*	*Pisum sativum*	México	*Rhizobium leguminosarum*	AY904742
5019	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium elkanii*	AF237422
5079	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium japonicum*	AF234888
5080	*Glycine max*	*Glycine max*	Brazil	*Bradyrhizobium japonicum*.	AF234889
6002	*Vigna unguiculata*	*Vigna unguiculata*	Zimbabwe	*Bradyrhizobium japonicum*	AY904743
6028	*Desmodium uncinatum*	*Desmodium incanum*	Zimbabwe	*Bradyrhizobium elkanii*	AY904744
6053	*Clitoria ternatea*	*Clitoria ternatea*	Malaysia	*Bradyrhizobium elkanii*	AY904745
6069	*Leucaena leucocephala*	*Leucaena leucocephala*	Brazil	*Bradyrhizobium elkanii*	AY904746
6070	*Leucaena leucocephala*	*Leucaena leucocephala*	Brazil	*Rhizobium* sp.	AY904747
6100	*Falcataria moluccana*	*Erythrina verna; Falcataria moluccana*	Brazil	*Bradyrhizobium elkanii*	AY904748
6101	*Dalbergia nigra*	*Dalbergia nigra*	Brazil	*Bradyrhizobium elkanii*	AY904749
6144	*Arachis hypogaea*	*Arachis hypogaea*	Zimbabwe	*Bradyrhizobium* sp.	AY904750
6145	*Arachis hypogaea*	*Crotalaria juncea; Cyamopsis tetragonoloba Vigna unguiculata*	Líbia	*Bradyrhizobium* sp.	AY904751
6146	*Centrosema* sp.	*Centrosema* spp.	Brazil	*Bradyrhizobium elkanii*	AY904752
6148	*Neonotonia wightii*	*Neonotonia wightii*	Brazil	*Bradyrhizobium* sp.	AY904753
6149	*Galactia striata*	*Galactia striata; Macrotyloma axillare*	Australia	*Bradyrhizobium elkanii*	AY904754
6150	*Acacia mearnsii*	*Galactia striata*	Brazil	*Bradyrhizobium elkanii*	AY904755
6152	*Calopogonium* sp.	*Calopogonium* spp.	Brazil	*Bradyrhizobium japonicum*	AY904756
6155	*Stylosanthes* sp.	*Stylosanthes* spp.	Brazil	*Bradyrhizobium japonicum*	AY904757
6156	*Crotalaria spectabilis*	*Cajanus cajan; Canavalia ensiformis; Crotalaria juncea; Crotalaria spectabilis; Indigofera hirsuta*	Brazil	*Bradyrhizobium* sp.	AY904758
6157	*Cajanus cajan*	*Cajanus cajan*	Brazil	*Bradyrhizobium elkanii*	AY904759
6158	*Crotalaria spectabilis*	*Canavalia ensiformis; Crotalaria spectabilis;*	Brazil	*Bradyrhizobium elkanii*	AY904760

		Indigofera hirsuta; Stizolobium aterrimum			
6159	*Enterolobium ellipticum*	*Enterolobium contortisiliquum; Enterolobiumcyclocarpum Enterolobium timbouva*	Brazil	*Bradyrhizobium elkanii*	AY904761
6160	*Albizia lebbeck*	*Albizia lebbeck; Sclerolobium paniculatum*	Brazil	*Bradyrhizobium* sp.	AY904762
6161	*Prosopis juliflora*	*Prosopis juliflora*	Brazil	*Sinorhizobium* sp.	AY904763
6163	*Acacia mearnsii*	*Acacia mearnsii*	Brazil	*Bradyrhizobium* sp.	AY904764
6164	*Acacia mearnsii*	*Acacia decurrens; Acacia mearnsii*	Brazil	*Bradyrhizobium* sp.	AY904765
6165	*Mimosa scabrella*	*Mimosa scabrella*	Brazil	*Rhizobium* sp.	AY904766
6166	*Mimosa caesalpiniifolia*	*Mimosa caesalpiniifolia*	Brazil	*Burkholderia* sp.	AY904767
6167	*Mimosa caesalpiniifolia*	*Mimosa caesalpiniifolia*	Brazil	*Burkholderia* sp.	AY904768
6168	*Gliricidia sepium*	*Gliricidia sepium; Leucaena diversifolia*	Brazil	*Rhizobium* sp.	AY904769
6169	*Falcataria moluccana*	*Falcataria moluccana; Leucaena diversifolia*	Brazil	*Bradyrhizobium elkanii*	AY904770
6175	*Pueraria phaseoloides*	*Pueraria phaseoloides*	Brazil	*Bradyrhizobium elkanii*	AY904771
6192	*Tipuana tipu*	*Tipuana tipu*	Brazil	*Bradyrhizobium* sp.	AY904772
6208	*Desmodium heterocarpon*	*Desmodium heterocarpon* subsp. ovalifolium	Colombia	*Bradyrhizobium elkanii*	AY904773
6319	*Arachis* sp.	*Cyamopsis tetragonoloba*	Bolivia	*Bradyrhizobium* sp.	AY904774
6382	*Mimosa caesalpiniifolia*	*Mimosa caesalpiniifolia*	Brazil	*Burkholderia* sp.	AY904775
6383	*Mimosa caesalpiniifolia*	*Mimosa acutistipula*	Brazil	*Rhizobium* sp.	AY904776
6384	*Mimosa obovata*	*Mimosa acutistipula*	Brazil	*Bradyrhizobium elkanii*	AY904777
6387	*Acacia auriculiformis*	*Acácia auriculiformis; Acacia mangium*	Brazil	*Bradyrhizobium elkanii*	AY904778
6390	*Acacia decurrens*	*Acacia decurrens*	Brazil	*Burkholderia cepacia*	AY904779
6391	*Acacia auriculiformis*	*Acácia auriculiformis*	Brazil	*Bradyrhizobium* sp.	AY904780
6394	*Ormosia nitida*	*Ormosia nitida*	Brazil	*Burkholderia cepacia*	AY904781
6398	*Piptadenia stipulacea*	*Piptadenia gonoacantha Piptadenia stipulacea*	Brazil	*Burkholderia* sp.	AY904782
6401	*Sesbania virgata*	*Sesbania virgata*	Brazil	*Azorhizobium* sp.	AY904783
6402	*Sesbania virgata*	*Sesbania virgata*	Brazil	*Azorhizobium* sp.	AY904784
6412	*Clitoria fairchildiana*	*Clitoria fairchildiana*	Brazil	*Burkholderia* sp.	AY904785
6420	*Acacia mangium*	*Acacia mangium; Sclerolobium paniculatum*	Brazil	*Bradyrhizobium* sp.	AY904786
6424	*Centrosema pubescens*	*Centrosema* spp.	Brazil	*Bradyrhizobium elkanii*	AY904787
6425	*Centrosema pubescens*	*Centrosema* spp.	Brazil	*Bradyrhizobium elkanii*	AY904788
6440	*Arachis pintoi*	*Arachis pintoi*	Brazil	*Bradyrhizobium* sp.	AY904789

[a] After Menna et al. (2006).

Table 2 – Correlation between the 16S rRNA sequencing analysis and the polyphasic analysis of 16S rRNA+ BOX-PCR considering different ratios of each one.

Polyphasic dendrogram 16S rRNA:BOX-PCR	**16S rRNA dendrogram**
50%:50%	64.7%
60%:40%	76.9%
70%:30%	87.3%
80%:20%	94.8%
90%:10%	98.8%

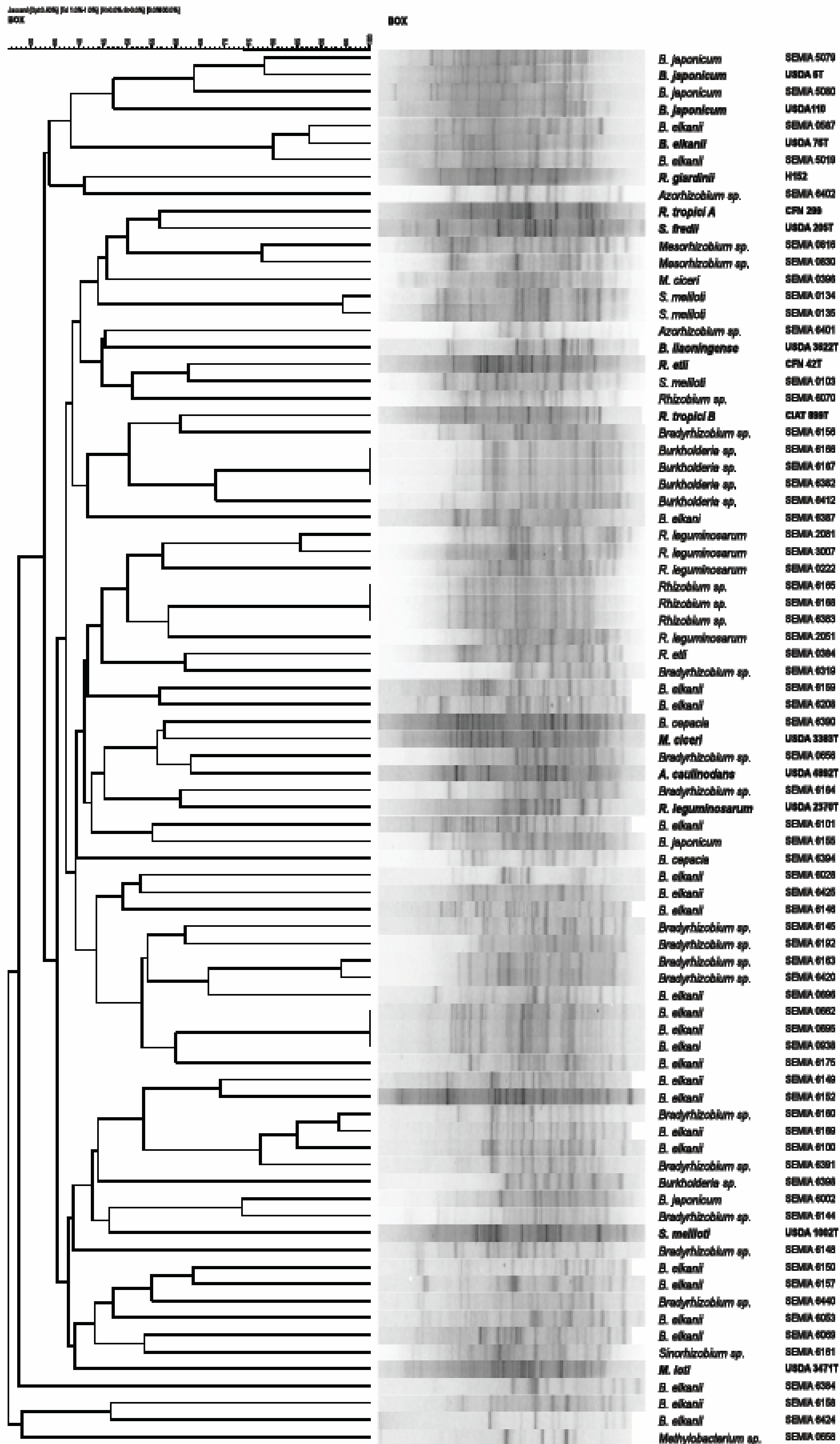

Fig 1.

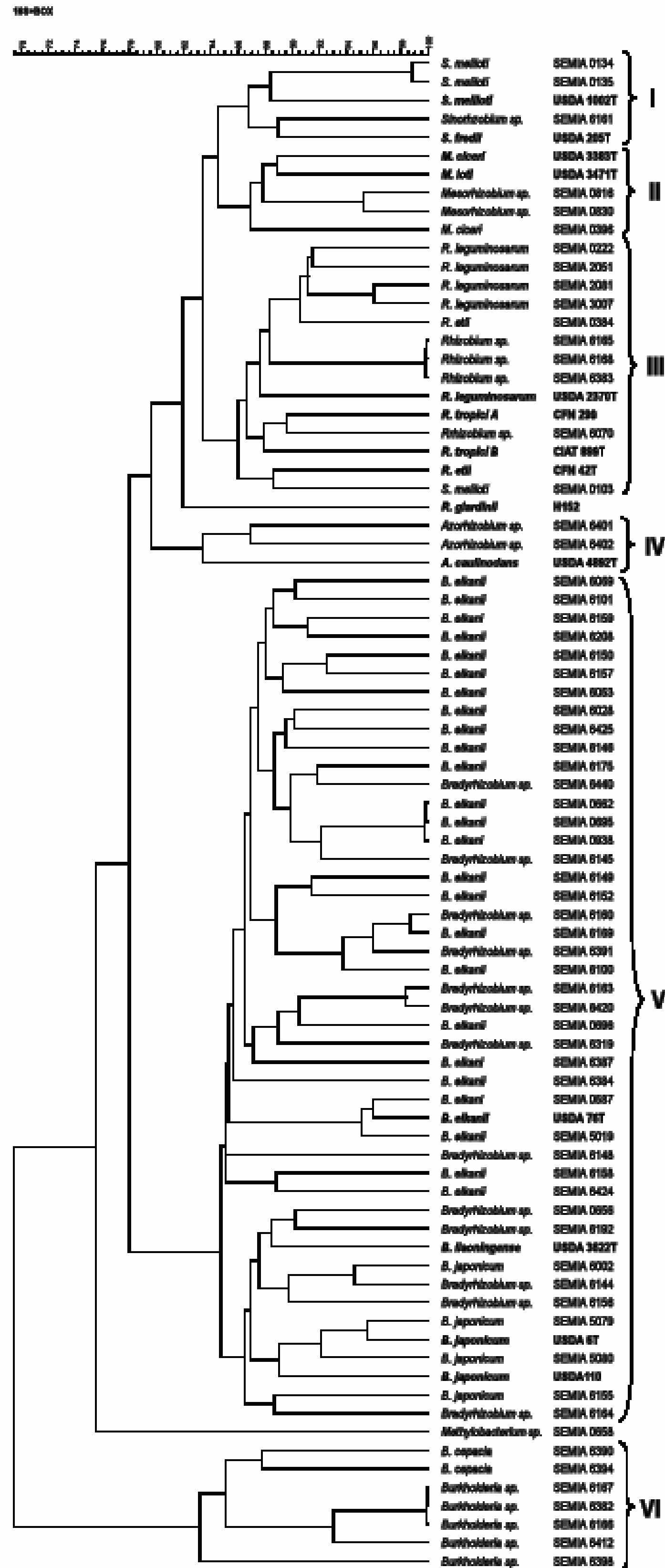

Fig. 2.

5 ESTUDO 2

Phylogeny and taxonomy of a broad collection of *Bradyrhizobium* based on multilocus sequence analysis of 16S rRNA, ITS, *glnII, recA, atpD* and *dnaK* genes

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Phylogeny and taxonomy of of a broad collection of *Bradyrhizobium* based on multilocus sequence analysis of 16S rRNA, ITS, *glnII, recA, atpD* and *dnaK* genes

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Abstract. The genus *Bradyrhizobium* accommodates a variety of symbiotic and endophitic bacteria in association with both legumes and non-legumes, and characterized by physiological and symbiotic versatility and a broad-range distribution. However, despite indications of great genetic variability within the genus, there are today only six described species, mainly due to the highly conserved nature of the 16S rRNA gene within the genus. In this study a collection of 169 strains isolated from 43 distinct legumes was analyzed by rep-PCR with the BOX primers and by MLSA with the 16S RNA gene, the 16S-23S rRNA intergenic transcribed spacer (ITS) and four additional housekeeping genes, *glnII, recA, atpD* and *dnaK.* Considering a cut off at a level of similarity of 70%, 80 different rep-PCR profiles were distinguished, and together with the type strains were clustered at a very low level of similarity, of only 24%. In the 16S rRNA and ITS analyses, two great groups were formed, both with bootstrap support of 100% in the concatenated analysis. The first one included type strains of *B. japonicum, B. betae, B. liaoningense, B. canariense, B. yuanmingense* and *B. japonicum* strain USDA 110, and the second great group included strains related to *B. elkanii* USDA 76^T. Similar results were obtained with the MLSA considering all genes. The greatest variability was observed with *atpD* gene, and five strains related to *B. elkanii* have shown variability never reported before. Another important observation was that a group composed by strains USDA110, SEMIA5080 and SEMIA6059, all isolated from soybean, clustered in all six trees with a high bootstrap support and were quite distinct from the clusters including *B. japonicum* USDA 6^T. The MLSA has proven to provide a fast and reliable way of improving information about phylogenetic relationships and of identifying strains potentially representative of new species.

Keywords: Biological nitrogen fixation, *Bradyrhizobium,* Leguminosae, multilocus sequence analysis; phylogeny of bacteria, taxonomy of bacteria.

INTRODUCTION

The genus *Bradyrhizobium* was created to accommodate bacteria capable of establishing N_2-fixing symbioses with several leguminous plants and characterized by slow-growth rate and alkaline reaction in culture medium containing mannitol as carbon source (Jordan, 1982). Based on 16S rRNA gene sequences, *Bradyrhizobium* forms a clade in the a subclass of the Proteobacteria, along with oligotrophic soil and aquatic bacteria as *Rhodopseudomonas palustris, Rhodoplanes roseus, Nitrobacter winogradskyi, Blastobacter denitrificans* and pathogens as *Afipia* spp., among others (Saito *et al.,* 1998; Sawada *et al.,* 2003; Willems *et al.,* 2001b; van Berkum & Eardly, 2002). Symbiotic *Bradyrhizobium* strains have been isolated from the nodules of highly divergent legume tribes, including herbaceous and woody species of tropical and temperate origin, aquatic legumes as *Aeschynomene* species, and also from the non-legume *Parasponia andersonii* (Sprent, 2001; Menna *et al.,* 2006). In addition, bacteria belonging to this genus have been reported as endophytes of wild and modern rice *(Oriza sativa* L.) species (e.g. Tan *et al.,* 2001), and *Bradyrhizobium betae* refers to endophytic isolates of sugar beet *(Beta vulgaris* L.) roots affected by tumor-like deformations and which do not nodulate legumes (Rivas *et al.,* 2004).

In the last few years more attention has been given to the genus *Bradyrhizobium,* with reports showing high genetic diversity among strains, demonstrated with several molecular markers (van Berkum & Fuhrmann, 2000; Tan *et al.,* 2001; van Berkum & Eardly, 2002; Willems *et al.,* 2003; Liu *et al.,* 2005; Vinuesa *et al.,* 2005; Germano *et al.,* 2006; Giongo *et al.,* 2008; Menna *et al.,* 2008). Intriguing evolutionary and ecological questions rely on the versatility of *Bradyrhizobium,* however, despite impressive diversity and a worldwide distribution, only six species have been described so far, three symbionts of *Glycine* sp. [B. *japonicum* (Jordan, 1982), *B. elkanii* (Kuykendall *et al,* 1992) and *B. liaoningense* (Xu *et al.,* 1995)], one nodulating *Lespedeza cuneata [B. yuanmingense* (Yao *et al.,* 2002)], another nodulating endemic shrubs of the tribes *Genisteae* and *Loteae [B. canariense* (Vinuesa *et al,* 2005)], and the already cited *B. betae* (Rivas *et al.,* 2004); *B. denitrificans* is a proposed new species (van Berkum *et al,* 2006) still not confirmed by the taxonomic committee. A variety of other strains are commonly referred to as *Bradyrhizobium* sp., followed by the name of the host

legume. Apparently the definition of species in the genus is limited by the low diversity in the 16S rRNA sequences investigated so far *(e.g.,* van Berkum & Fuhrmann, 2000; Willems *et al.,* 2001a; Qian *et al.,* 2003).

Although the DNA-DNA hybridization is still required to define new species (Garrity & Holt, 2001), an increasing number of arguments against its obligatory adoption has been raised, including the high cost and intensive work required in the method development (Vandamme *et al.,* 1996; Coenye *et al.,* 2005), the existence of more accurate approaches (Konstantinidis & Tiedje, 2004), and doubts about its adequacy to the genomic era (Achtman & Wagner, 2008). The multilocus sequence analysis (MLSA) consists on the analysis of several conserved housekeeping genes dispersed in at least 100 kb of the genome, and has been proposed as an interesting and more accessible tool for accessing phylogeny and taxonomy of prokaryotes (Brett *et al.,* 1998; Maiden *et al.,* 1998; Godoy *et al,* 2003; Cooper *et al.,* 2004). Using MLSA, Moulin *et al.* (2004) observed high genetic diversity among *Bradyrhizobium* spp. strains, and Ribeiro *et al.* (2008) found high concordance between the MLSA with five housekeeping genes and the DNA-DNA hybridization in *Rhizobium* species microsymbionts of common bean *(Phaseolus vulgaris* L.).

In this study, the genetic diversity of a collection of 169 *Bradyrhizobium* strains isolated from distinct legumes and countries was analyzed using the amplification of repetitive and conserved DNA elements (BOX-PCR), and the sequencing analysis of the 16S rRNA, the 16S-23S rRNA intergenic transcribed spacer (ITS), and four additional conserved housekeeping genes *(glnll, recA, atpD* and *dnaK).* The objective was to apply a polyphasic approach to access genetic diversity, phylogenetic relations and taxonomic position of these strains, gaining a better knowledge of the genus *Bradyrhizobium.*

METHODS

Strains. One-hundred and sixty-nine *Bradyrhizobium* strains from the "Brazilian *Rhizobium* Culture Collection SEMIA", of the FEPAGRO-MIRCEN [Fundação Estadual de Pesquisa Agropecuária (Rio Grande do Sul, Brazil)-Microbiological Resources Center] (IBP World Catalogue of *Rhizobium* Collections n°443 in the WFCC World Data Center on Microorganisms) were used in this study and are listed on Table 1. The strains have been isolated from the three subfamilies and twelve tribes of the family Leguminosae, as shown in Table 1. Forty of those strains are recommended for the use in commercial inoculants in

Brazil and have been previously analyzed by BOX-PCR (Menna *et al,* 2008) and 16S rRNA (Menna *et al,* 2006). Preparation of stock cultures, strains growth conditions and maintenance were performed as described before (Menna *et al.,* 2006).

Description of type strains used as control in all experiments. Three type strains were used: *B. japonicum* USDA 6^T, *B. liaoningense* USDA 3622^T and *B. elkanii* USDA 76^T, provided by USDA, Beltsville, USA and deposited at the "Culture Collection of Diazotrophic and PGPR Bacteria" of Embrapa Soja, with information available at http://www.bmrc.lncc.br.

DNA extraction and rep-PCR (BOX) genomic fingerprinting. Total genomic DNA of the 169 strains was extracted as described by Kaschuk *et al.* (2006a), and amplification by PCR with the primer BOX A1R (Versalovic *et al,* 1994; Koeuth *et al.,* 1995) was performed as described by Kaschuk *et al.* (2006a), with the 1-kb DNA marker (Invitrogen™) being included on the left, right and in the centre of each gel. The amplified fragments were separated by horizontal electrophoresis on 1.5% agarose gels (Kaschuk *et al.,* 2006a), which were then stained with ethidium bromide, visualized under UV radiation and photographed.

Sequencing analysis of the 16S rRNA and ITS rRNA. The DNA of 80 strains representative of the rep-PCR groups were submitted to the amplification with primers for the 16S rRNA and ITS regions, as listed on Table 2. The PCR products were purified with the PureLink™ PCR Purification Kit (Invitrogen), and the reactions were performed as described by Menna *et al.* (2006). The sequencing was performed on a MEGA BACE 1000 (Amersham Biosciences) capillary sequencer, as described before (Menna et al., 2006).

Sequencing analysis of *glnII, recA, atpD* and *dnaK* genes. The DNAs of 40 strains selected after the analysis of the 16S rRNA and ITS sequences were amplified for the regions coding for four housekeeping genes: *glnII, recA, atpD* and *dnaK.* The primers, amplification conditions and references are listed on Table 2. The purification and sequencing analysis were performed as described in the previous item.

Cluster analyses. In the rep-PCR analysis, the sizes of the fragments were normalized according to the sizes of the DNA marker. Cluster analyses were performed with the Bionumerics program version 4.6 (Applied Mathematics, Kortrijk, Belgium), using the UPGMA algorithm (unweighted pair-group method, with arithmetic mean) (Sneath & Sokal, 1973) and the coefficient of Jaccard (J) (Jaccard, 1912), with the optimum values indicated by the Bionumerics program for the tolerance and the optimization parameters.

The 16S rRNA, ITS, *glnll, recA, atpD* and *dnaK* sequences generated were analyzed with the programs Phred (Ewing & Green, 1998; Ewing *et al,* 1998), Phrap (www.phrap.org) and Consed (Gordon *et al,* 1998). All sequences obtained were analyzed individually and concatenated using the MEGA (Molecular Evolutionary Genetics Analysis) software version 4.0 with the default parameters, K2P distance model (Kimura, 1980), and the Neighbor-Joining algorithm (Saitou & Nei, 1987). A site is parsimony-informative if it contains at least two types of nucleotides (or amino acids), and at least two of them occur with a minimum frequency of two, and the parameter was estimated using the MEGA program. Statistical support for tree nodes was evaluated by bootstrap (Felsenstein, 1985) analyses with 1000 samplings (Hedges, 1992).

For the alignment and comparison, the following type strains were added to the analyses (accession numbers of the GenBank Data Library in parentheses): *B. betae* PL74H1^{T} (16S rRNA, AY372184; *dnaK,* AY923046.1; *glnll,* AB353733.1; ITS, AJ631967.1; *recA,* AB353734.1); *B. canariense* BC-C2^{T} (16S rRNA, AY577427; *atpD,* AY386739; *glnll,* AY386762.1; *dnaK,* AY923047.1; ITS, AY386704.1; *recA,* AY591541.1); *B. yuanmingense* B071^{T} (16S rRNA, AF208513; *atpD,* AY386760.1; *dnaK,* AY923039.1; *glnII,* AY386780.1; ITS, AY386734.1; *recA,* AM168343), *B. liaoningense* LMG 18230^{T} (16S rRNA, AF208513.1; *atpD,* AY386752.1; *dnaK,* AY923041.1; *glnll,* AY386775.1; ITS, AF345256.1; *recA,* AY591564.1), *B. elkanii* USDA76 ^{T}, (16S rRNA, U35000.3; *atpD,* AY386758.1, *dnaK,* AY328392.1, *glnll,* AY599117.1, ITS U35000, *recA,* AY591568.1), *B. japonicum* USDA6 ^{T}, (16S rRNA, U69638; *atpD,* AM168320, *dnaK,* AM168362, *glnll,* AM182979, ITS, U69638, *recA,* AM168341) and *B. japonicum* USDA 110 (genome, NC004463). *Caulobacter crescentus* strain CB15 (genome, AE005673), was used as an outgroup.

RESULTS

Morpho- physiological properties. All 169 strains were characterized by slow growth and alkaline reaction in culture medium containing mannitol as carbon source after five to seven days of growth, typical morpho-physiological proprieties of *Bradyrhizobium.* Mucus production *in vitro* varied from low to high after this period, with no correlation with the host plant or the ecosystem from which the strain had been isolated (data not shown).

rep-PCR genomic fingerprinting. DNA profiles after the amplification with BOX primer were obtained for all 169 strains, with an average of twenty bands differing in intensity and

varying from 200 to 5,000 bp in size (Fig. 1). The profiles of each strain are now available at the homepage of the Culture Collection of Embrapa Soja (http://www.bmrc.lncc.br).

The cluster analysis allowed the identification of numerous but well-defined groups of bradyrhizobia, with a high level of intraspecific diversity. Considering a cut off at 70% of similarity, as suggested for diversity studies of rhizobia using rep-PCR (Grange & Hungria, 2004; Alberton *et al,* 2006; Kaschuk *et al,* 2006a,b), 80 different profiles were distinguished, and together with the type strains, all strains were clustered at a very low level of similarity, of only 24% (Fig. 1). In some of the groups, strains isolated from the same host legume and in the same country were clustered with similarities higher than 70%, *e g,* SEMIAs 6163, 6420 and 6189, isolated form *Acacia* spp. in Brazil. However, in other groups, strains isolated from distinct legumes and countries have shown similarity as high as 100%, e.g., SEMIAs 662, 695 and 938, isolated from *Vigna unguiculata, Neotonia wightii* and *Lupinus albus,* respectively.

Diversity in the 16S rRNA and ITS rRNA. When the DNA of the 80 strains representative of each rep-PCR group were submitted to the sequencing analysis of the 16S rRNA and ITS regions, unique bands were obtained in each amplification, with approximately 1,500 and 700 bp, respectively. For the alignment of the 16S rRNA, a region of 1,480 bp was considered. No statistical differences among the strains were detected on the contents of thymine (T), cytosine (C), adenine (A), and guanine (G), and the estimated average frequencies for those nucleotides were of 20.1, 24.1, 24.3 and 31.5%, respectively (Table 3). In the consensus sequence of all strains, there was a conservation of 1,370, a variability of 114 and a parsimony-informative site of 67 nucleotides.

The phylogenetic tree built with the 16S rRNA sequences split the strains in two great groups, with bootstrap supports of 96 and 99%, respectively (Fig. 2A). The first group included 48 SEMIAs and the following reference strains: *B. japonicum* USDA 6^T, *B. betae* PL7HG1^T, *B. liaoningense* USDA 3622^T, *B. canariense* BCC2^T, B. *yuanmingense* B071^T and *B. japonicum* USDA 110. Still in this first great group it was possible to observe eleven subgroups, six of which without any type strain, in addition to two isolated strains *(B. betae* PL7HG1^T and SEMIA 839). Bootstrap support for these subgroups ranged from 52 to 94%. The second great group assembled 32 SEMIAs and *B. elkanii* USDA 76^T, and was split in five subgroups and three isolated SEMIAs (SEMIA 695, SEMIA 6056 and SEMIA 6093). Whithin this great group twenty SEMIAs were clustered with *B. elkanii* USDA76^T in the fourth subgroup, with a bootstrap support of 85% (Figure 2A).

When compared to the 16S rRNA, higher variability was detected in the analysis of the ITS region (Fig. 2B). Similar to the results obtained with the 16S rRNA, no statistical variation was observed in the percentages of T, C, A and G, and considering the gaps and insertion of nucleotides observed in the some strains, the consensus sequence had 790 bp, with a conservation of 581, a variability of 367 and a parsimony-informative site of the 292 nucleotides (Table 3).

The phylogenetic tree of the ITS sequences resulted in two great groups with a bootstrap support of 100 and 75%, respectively, and the SEMIA 6434 that belong isolated (Fig. 2B). The first great group joined 47 SEMIAs and all type and reference strains except for *B. elkanii* USDA 76^T. This first great group could be split into more subgroups than observed in the 16S rRNA — thirteen — nine of which without any type strain, in addition to six isolated strains (SEMIAs 6163, 6187, 6014, 656, 511 and *B. betae* PL7HG1^T). The second great group assembled 32 SEMIAs and *B. elkanii* USDA 76^T; the great group could be split into eight subgroups. Nine SEMIAs were clustered with *B. elkanii* USDA 76^T in the first subgroup, and the strain SEMIA 587 shown high genetic variability in comparison to the type strain. All the other subgroups were phylogenetic distant of the first subgroup in that type strains was positioned (Figure 2B).

Aiming at improving the phylogenetic information based on ribosomal regions, an analysis of the concatenated sequences of the 16S rRNA and ITS was realized. The concatenated sequence presented 2,270 nucleotide, 1,942 of which conserved, with a variability of 488 and a parsimony-informative site of 361 nucleotides (Table 3). In the resulting NJ tree, again two great groups were well defined, both with maximum bootstrap support — 100% (Fig. 3). The first great group was split in eleven subgroups, with six SEMIAs (SEMIA 839, 511, 6179, 6014, 656 and 6434) and *B. betae* PL7HG1^T remaining isolated. All type and reference strains were included in this great group, except for USDA 76^T. The second great group assembled 32 strains and *B. elkanii* USDA 76^T, and could be split into six subgroups and two isolated strains (SEMIAs 6101 and 587) (Fig. 3).

In general, in both isolated trees built with the 16S rRNA and ITS sequences, it was not possible to establish a clear relation between the clustering of the strains and their host plant or origin.

Diversity in the *atpD, dnaK, glnII* and *recA* genes. The additional housekeeping genes selected to refine the phylogeny analysis in this study are highly conserved among the

bacteria of the order Rhizobiales, and are dispersed in the genome USDA 110. The genes encode for important proteins, as follows: *atpD,* ATP-synthase beta-chain; *glnII,* glutamine synthetase II; *recA,* recombination protein RecA; and *dnaK,* 70-kDa chaperone protein. Forty strains representative of the subgroups of the concatenated tree built with the 16S rRNA and the ITS sequences, in addition to the type and reference strains, were select for the analysis. The amplification resulted in fragments of 577 bp for *atpD,* 370 bp for *dnaK,* 603 bp for *glnII,* and 524 bp for *recA* and the sequence characteristics are provided in Table 3. The conserved regions of each housekeeping gene were of 38.3% for *atpD,* 72.9% for *dnaK,* 71.8% for *glnII* and 71.9% for *recA,* these last two very similar to the 72.2% detected for the ITS, and all considerably lower than the 93.1% observed for 16S rRNA. For each housekeeping gene, phylogenetic trees were constructed using NJ methods, and resulted in distinct groups (Figs. 4A, 4B, 5A and 5B). When NJ trees were built with the amino acids sequences, clustering was not as clear as with the nucleotides (data not shown).

The greatest variability was observed with *atpD* gene (Table 3, Fig. 4A), and five strains (SEMIAs 5026, 5027, 587, 5011 and SEMIA 5019) have cleared formed a different cluster from the others. These strains have been isolated from soybean plants *(Glycine max* L.) in Brazil, USA and Thailand (Table 1), and in the 16S rRNA and ITS they have shown higher similarity with *B. elkanii* USDA 76^{T}. Interestingly, the PCR product obtained with *atpD* primers for these five strains were larger (s 700 pb) than the other strains. The highest similarity of *atpD* was detected with USDA 76^{T}, but was of only 37%. Furthermore, BLASTN had no significant similarity with other sequences deposited in the NCBI database. The other subgroups formed with *atpD* were also observed in the 16S rRNA tree, e.g., the clusters with *B. canariense* $BCC2^{T}$, *B. liaoningense* USDA 3622^{T}, *B. yuanmingense* $B071^{T}$ and *B. japonicum* USDA 6^{T}. We would like to draw the attention to the observation that strains SEMIAs 5080 and 6059 were clustered with *B. japonicum* USDA 110 in a different group from that of the type strain USDA 6^{T} (Fig. 4A), similarly to what had also been observed for the 16S rRNA and the ITS (Figs. 2A, 2B, 3).

The two great groups observed in the 16S rRNA and ITS trees were also detected in the *dnaK* and *glnII* trees, but were not well defined in the *atpD* and *recA* trees. However, three subgroups were observed in all six trees and two clustered type strains, *B. japonicum* USDA 6^{T} and the SEMIAs 512, 5079, both isolated from soybean, and *B. canariense* $BCC2^{T}$ and

SEMIA 928, this last one isolated from *Lupinus* spp. and of unknown origin. Another noticeable group present in all trees was that including *B. japonicum* USDA110, and SEMIAs

5080 and 6059, all isolated from soybean. Following, we can point out that the subgroup including *B. elkanii* USDA76 ^{T} and SEMIAs 5026, 587, 5027, 5019 and 5011, also isolated from soybean, was present in all trees except for with *atpD*. *B. yuanimingense* B071^{T} was also always clustered with SEMIA 6319, isolated from *Arachis* spp. in Bolivia, except in the tree with *glnII*. *B. liaoningense* USDA 3622^{T} and SEMIA 5025, isolated from soybean in Thailand, were also always clustered, except for with *glnII,* and SEMIA 5062 was within the same cluster, except for with *glnII* and *recA*. Interesting, SEMIAs 511 and 5045 were clustered with USDA 6^{T} considering all four housekeeping genes, but not with 16S rRNA and ITS.

Concatenated analysis of *atpD, dnaK, glnII, recA,* 16S rRNA and ITS rRNA. Assuming that each tree represents a partial view of the information contained in the genome, all six sequences were concatenated to get a better knowledge about the strains. The concatenation resulted in 4,348 bp, with a conservation of 3,275 bp, a 1,259 bp variable but informative sequence and a parsimony-informative site of 1,000 bp (Table 3).

The tree built with the concatenated genes resulted in two great groups, with a bootstrap support of 100% (Fig. 6). Once more the first great group assembled 23 SEMIAs together with the type strains of *B. japonicum, B. betae, B. liaoningense, B. canariense, B. yuanmingense* and with *B. japonicum* USDA 110. However, within this great group some SEMIAs were clearly distantly related to the type strains, as those clustered in subgroups 1, 3 and 4, in addition to three isolated strains, SEMIAs 560, 6179 and 6434. The second great group assembled 17 SEMIAs and *B. elkanii* USDA 76^{T}, in this great group, four subgroups were observed and the SEMIA 6101, SEMIA695 and the type strain remaining isolated. The fourth subgroup, with SEMIAS 5027, 587, 5011, 5026 and 5019 was noticeably more distant than the others (Fig. 6), what might be attributed to the variability in *atpD* gene. We have then decided to build another tree without the *atpD* gene (Fig. 7). The consensus sequence resulted in 3,770 bp, with a conservation of 3,057 bp, a 867 bp variable but informative sequence and a parsimony-informative site of 649 bp (Table 3). The first great group (Fig. 7) was similar to that observed in the tree built with all genes (Fig. 6). However, differences were observed in the second great group, in which SEMIAs 5026, 587, 5027, 5019 and 5011 were clustered with *B. elkanii* USDA 76 ^{T} (Fig. 7).

DISCUSSION

The 169 SEMIA strains used in this study had been isolated from 43 distinct host legumes, 104 from soybean. Forty of those strains have been obtained from various selection programs aiming at identifying very effective rhizobial strains, and today they are officially recommended as commercial inoculants for 64 legumes. A previous analysis of these elite strains has been realized by Menna *et al.* (2006, 2008), and pointed out to a level of genetic diversity beyond to that described for the *Bradyrhizobium* genus *(e.g.,* van Berkum & Fuhrmann, 2000; Willems *et al.,* 2001a, 2001b; Qian *et al.,* 2003; Vinuesa et al., 2005), demanding further analyses to refine their phylogenetic relations and taxonomic classification. Additional strains and housekeeping genes were then included in this study and confirmed high intra and interspecific genetic diversity within bacteria belonging to the genus *Bradyrhizobium.*

Genetic characterization and evaluation of *Bradyrhizobium* strains has greatly improved with several molecular techniques (van Berkum & Fuhrmann, 2000; Willens *et al.,* 2003; Stepkowski *et al.,* 2005; Rodríguez-Navarro *et al.,* 2004). One example is the amplification of repetitive regions dispersed in the genome of eubacteria, such as the repetitive sequences known as BOX, which result in highly characteristic bacterial fingerprints, (Hungria *et al.,* 2006; Mostasso et al., 2002; Saldana *et al.,* 2003; Wang *et al,* 2006; Pinto *et al.,* 2007; Menna *et al.,* 2008) and have been used for accessing genetic diversity of strains isolated from soybean (Abaidoo *et al.,* 2000; Chen *et al.,* 2000; Loureiro *et al.,* 2007), *Lupinus* spp. (Barrera *et al.,* 1997), *Acacia albida* (Dupuy & Dreyfus, 1992), *Aeschynomene* spp. (Wong *et al.,* 1994), and other legumes (Parker & Lunk, 2000; Willems *et al.,* 2003; Wolde-Meskel et al., 2004). Similarly, our BOX-PCR profiles have shown remarkable diversity among *Bradyrhizobium* strains isolated from distinct legumes and countries, such that all strains were joined at a low level of similarity, only 24%.

The BOX-PCR fingerprinting is important for identification of those elite strains, with immediate application in the quality control of both the culture collection and of the commercial inoculants carrying the strains. However in relation to the taxonomy, the current definition of new species as well as classification, has been strongly based on a polyphasic classification, taking into account phenotypic characteristics as well as genetic characteristics (Vandamme *et al.,* 1996), with the 16S rRNA providing the backbone of the classification (Willems & Collins, 1993; Young & Haukka, 1996; Yanagi & Yamasato, 1993; Garrit & Holt, 2001; Young *et al.,* 2001). In *Bradyrhizobium,* as a result of the limited diversity on the

16S rRNA gene (Olsen & Woese, 1993; Ludwig & Schleifer, 1994; Barrera *et al,* 1997; Wang & Martinez-Romero, 2000), only six species have been described and accepted so far (Jordan, 1982; Kuykendall *et al,* 1992; Xu *et al.,* 1995; Yao *et al.,* 2002; Rivas *et al.,* 2004; Vinuesa *et al,* 2005). A proposed strategy to improve definiton of species, is the analysis of additional conserved ribosomal genes, as the 23S rRNA and the ITS (Willems *et* al., 2001a, 2003), which may detect higher genetic diversity in *Bradyrhizobium* (Doignon-Bourcier *et al.,* 2000; Willems *et al.,* 2001a, 2003; van Berkum & Eardly, 2002; Stepkowski *et al.,* 2005; Germano *et al.,* 2006), as confirmed with the ITS in our study (Fig. 2B). Willems *et al.* (2003), using ITS sequencing and DNA-DNA hybridization observed a high correlation between the results obtained in both methods, such that strains with more than 95.5% of similarity in the ITS sequences belong to the same genospecies, showing more than 60% DNA-DNA hybridization values. Based on these value, in our study some clusters with high bootstrap support in the ITS analysis should represent new species, with an emphasis on strains 6187, 6163, 6014, 511 and the subgroups 5, 6 and 8 in the great group I, and subgroups 3-8 in the great group II (Fig. 2B). However, althougt there are still controversies about the use of ITS to properly resolve the evolutionary relationships among strains of *Bradyrhizobum* (Vinuesa et al., 2005), undoubtedly, in this study, when the ITS sequence was associated with 16S rRNA, resulted in a much better phylogenetic definition (Fig. 3).

Notable is also the recent proposal of the use of at least four housekeeping genes for the phylogenetic analysis and taxonomic classification of bradyrhizobia (Stepkowski *et al.,* 2003, 2005; Vinuesa *et al.,* 2005). This approach called multilocus sequence analyze (MLSA) has the objective of combining congruent genomic data, excluding partitions with a significant level of incongruence, and leading to a reliable genotypic characterization (Bull *et al.,* 1993; Miyamoto & Fitch, 1995). Among the housekeeping genes used in the MLSA for phylogenetic reconstructions of the order *Rhizobiales* are *atpD, dnaK, glnII, gltA, glnA, recA, rpoB* and *thrC* (Gaunt *et al,* 2001; Turner & Young, 2000; Vinuesa *et al,* 2005; Weir *et al.,* 2004; Stepkowski *et al.,* 2005; Martens *et al.,* 2007; Ribeiro *et al.,* 2008), and the approach has already been used in studies with *Bradyrhizobium* (Stepkowski *et al.,* 2003, 2005; Parker, 2004). One important example is the recent description of *B. canariense,* based on the phylogeny of *atpD, glnII, recA* and ITS rRNA (Vinuesa *et al.,* 2005). Likewise, the application of the MLSA in our study has clearly contributed to a better phylogeny definition, as well as to the identification of new subgroups, highly indicative of new species. One new group that should be emphasized in our study is that composed by *B. japonicum* strains

USDA 110, SEMIAs 5080 and 6059, isolated from soybean, which were clustered in all six trees in a different group from that of *B. japonicum* USDA 6^T, clearly indicating a new species. Complementary studies are now in progress in our laboratory to describe this new species.

The study has also detected a still unknown diversity on the *atpD* gene (Table 3), on the strains SEMIA 5011, SEMIA 5019, SEMIA 587, SEMIA 5027 and SEMIA 5026, all symbionts of soybean. The strains with these genes were isolated in Brazil, USA, and Thailand, therefore showing a broad geographic distribution. Searching on the NCBI database has shown no significant similarity to any genome or gene sequenced so far. In other studies with rhizobia, the use of *atpD* for phylogenetic inference and taxonomic classification has proven to be highly correlated with the phylogeny based on the 16S rRNA (Gaunt et al., 2001, Vinuesa et al., 2005, Stepkowski et al., 2005, Wang et al., 2007, Santillana et al., 2008), and the differences detected in this study had never been detected before. For *atpD* gene, an insertion of the 15 bp in *Azorhizobium caulinodans* and *Brevundimonas bullata* and of 12 bp in *B. japonicum* has been reported, and these insertions provided good support for the monophyly of these three species (Gaunt et al., 2001). However in this study the high genetic heterogeneity of *atpD* could have resulted from genetic recombination induced for insertions or deletions peculiar to this gene. Genetic information from such genes can be very useful to determine the genetic nature of microbial species (Achtman & Wagner, 2008). Therefore at this moment we suggest more studies with *atpD,* because the gene could be an excellent marker to trace evolution of *Bradyrhizobium,* but should be used with caution in other studies with bacteria of the order Rhizobiales.

An important consideration is that in general all subgroups formed in the trees built with the housekeeping genes *dnaK, glnII, recA* and ITS rRNA were similar to those obtained with the 16S rRNA, confirming the usefulness of these genes for phylogenetic reconstruction of the genus *Bradyrhizobium.* In our study the geographical localization did not affect the patterns of housekeeping genes, thus reinforcing the belief of a common origin for *Bradyrhizobium* with subsequent diffusion of the strains by soil-contaminated seeds (Perez-Ramirez et al., 1998), or by artificial inoculation (Strijdom 1998).

In conclusion, the concatenation of the housekeeping genes has been suggested as a powerful method to improve phylogenetic analysis *Bradyrhizobium* (Martens *et al.* 2007; Stepkowski *et al.* 2005; Vinuesa *et al.,* 2005), and indeed in our study the species tree build with ITS, 16S

rRNA, *dnaK, recA* and *glnII* detected high intra and interspecies genetic variability. Therefore the MLSA has proven to be a reliable technique applicable to phylogeny and taxonomy studies of *Bradyrhizobium,* improving the definition of new species within the genus. Finally, the analysis of four housekeeping in addition to the 16S rRNA and ITS has clearly indicated putative new species.

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Table 1 – Information about the strains used in this study.

SEMIA	Host plant [a]	Source of the strain [b]	Other designation [c]	*Subfamily* [b]	*Tribe* [b]
501	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
509	*Glycine max*	USA	UW 509, USDA 487	Papilionoideae	Phaseoleae
510	*Glycine max*	USA	SEMIA 516, SEMIA 5033, UW 510 (USDA510)	Papilionoideae	Phaseoleae
511	*Glycine max*	USA	UW 511, USDA 500	Papilionoideae	Phaseoleae
512	*Glycine max*	USA	3I1b73 (USDA- Dr. Erdman)	Papilionoideae	Phaseoleae
513	Not know	USA	3I1b6 (USDA- Dr. Erdman	not determined	not determined
515	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
516	*Glycine max*	USA	SEMIA 510, SEMIA 5033, UW 510 (USDA Univ. Wisconsin)	Papilionoideae	Phaseoleae
518	*Glycine max*	USA	UW 518, USDA 10, Nit 3I1b10	Papilionoideae	Phaseoleae
527	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
528	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
538	*Glycine max*	USA	not known	Papilionoideae	Phaseoleae
542	*Glycine max*	Brazil	R 1	Papilionoideae	Phaseoleae
543	*Glycine max*	Brazil	R 2	Papilionoideae	Phaseoleae
549	*Glycine max*	USA	not known	Papilionoideae	Phaseoleae
556	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
560	*Glycine max*	Brazil	no synonyms	Papilionoideae	Phaseoleae
565	*Glycine max*	Brazil	no synonyms	Papilionoideae	Phaseoleae
566	*Glycine max*	USA	BR 40	Papilionoideae	Phaseoleae
567	*Glycine max*	USA	no synonyms	Papilionoideae	Phaseoleae
568	*Glycine max*	USA	no synonyms	Papilionoideae	Phaseoleae
571	*Glycine max*	USA	no synonyms	Papilionoideae	Phaseoleae
574	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
576	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
577	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
579	*Glycine max*	Brazi	Original SEMIA	Papilionoideae	Phaseoleae
580	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
581	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
583	*Glycine max*	Thailand	CB 1786	Papilionoideae	Phaseoleae
584	*Glycine max*	USA	CB 1795, BR 46	Papilionoideae	Phaseoleae
587**	*Glycine max*	Brazil	BR 96	Papilionoideae	Phaseoleae
589	*Glycine max*	Brazil	SM1c	Papilionoideae	Phaseoleae
590	*Glycine max*	Brazil	RT-2a	Papilionoideae	Phaseoleae
591	*Glycine max*	Brazil	A Ia	Papilionoideae	Phaseoleae
593	*Glycine max*	Brazil	R-15a	Papilionoideae	Phaseoleae
598	*Glycine max*	Brazil	R-5a	Papilionoideae	Phaseoleae
613	*Vigna unguiculata*	USA	3I6n (USDA Dr. Erdman)	Papilionoideae	Phaseoleae
621	*Lespedeza striata*	USA	UW 621, USDA 610	Papilionoideae	Desmodieae
635	*Vigna unguiculata*	Brazil	C 57	Papilionoideae	Phaseoleae
637	*Acacia mearnsii*	Brazil	Original SEMIA	Mimosoideae	Acacieae
656**	*Neonotonia wightii*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
662**	*Vigna unguiculata*	Australia	CB 188	Papilionoideae	Phaseoleae
695**	*Neonotonia wightii*	Australia	E 85, QA 922, SU 422, NA 630	Papilionoideae	Dalbergieae
696*	*Desmodium uncinatum*	Australia	CB 627	Papilionoideae	Loteae

839*	*Lotus pedunculatus*	USA	NZP 2021, CC8 29, TAL 925	Papilionoideae	Loteae
905*	*Ornithopus sativus*	not know	Original SEMIA	Papilionoideae	Loteae
928*	*Lupinus spp.*	not know	W-72	Papilionoideae	Cytiseae
929*	*Ornithopus sativus*	not know	Original SEMIA	Papilionoideae	Loteae
938**	*Lupinus albus*	USA	no synonyms	Papilionoideae	Cytiseae
5000	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5002	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5003	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5005	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5010	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5011	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5012	*Glycine max*	Brazil	no synonyms	Papilionoideae	Phaseoleae
5013	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5014	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5015	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5016	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5018	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5019**	*Glycine max*	Brazil	29 W, BR 29	Papilionoideae	Phaseoleae
5020	*Glycine max*	Brasil	Original SEMIA	Papilionoideae	Phaseoleae
5021	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5022	*Glycine max*	Thailand	TAL 205	Papilionoideae	Phaseoleae
5023	*Glycine max*	Thailand	TAL 211, THA 136	Papilionoideae	Phaseoleae
5024	*Glycine max*	not known	TAL 378, CC 709	Papilionoideae	Phaseoleae
5025	*Glycine max*	Tailândia	TAL 411, Tha3	Papilionoideae	Phaseoleae
5026	*Glycine max*	Thailand	TAL 415, THA 9	Papilionoideae	Phaseoleae
5027	*Glycine max*	USA	TAL 183, 61a76	Papilionoideae	Phaseoleae
5028	*Glycine max*	USA	TAL 377, USDA 138	Papilionoideae	Phaseoleae
5029	*Glycine max*	USA	TAL 103	Papilionoideae	Phaseoleae
5030	*Glycine max*	Thailand	TAL 216, THA 106	Papilionoideae	Phaseoleae
5032	*Glycine max*	USA	TAL 102, USDA 110, TISTR 339	Papilionoideae	Phaseoleae
5034	*Glycine max*	USA	USDA 10, 3I1b10	Papilionoideae	Phaseoleae
5036	*Glycine max*	not know	no synonyms	Papilionoideae	Phaseoleae
5037	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5038	*Glycine max*	not know	no synonyms	Papilionoideae	Phaseoleae
5039	*Glycine max*	Brazil	532c, G3	Papilionoideae	Phaseoleae
5042	*Glycine max*	Brazil	S 89, 535Re	Papilionoideae	Phaseoleae
5043	*Glycine max*	Brasil	Original SEMIA	Papilionoideae	Phaseoleae
5044	*Glycine max*	USA	8-O	Papilionoideae	Phaseoleae
5045	*Glycine max*	not know	Nit 123P35	Papilionoideae	Phaseoleae
5046	*Glycine max*	USA	123P44	Papilionoideae	Phaseoleae
5048	*Glycine max*	USA	Nit 123P67	Papilionoideae	Phaseoleae
5051	*Glycine max*	USA	USDA 73, 3Iib73	Papilionoideae	Phaseoleae
5052	*Glycine max*	Not know	USDA 6, ATCC 10324, RCR 3435	Papilionoideae	Phaseoleae
5055	*Glycine max*	Brazil	532Re	Papilionoideae	Phaseoleae
5056	*Glycine max*	not known	USDA 6, 3I1B6, ATCC 10324, RCR 3425	Papilionoideae	Phaseoleae
5057	*Glycine max*	USA	USDA 59	Papilionoideae	Phaseoleae
5058	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5059	*Glycine max*	Indonesia	USDA 143, 3I1B143, ACCC 15039	Papilionoideae	Phaseoleae
5060	*Glycine max*	Japan	J 507	Papilionoideae	Phaseoleae
5061	*Glycine max*	Argentina	INPA 037, AR2A	Papilionoideae	Phaseoleae
5062	*Glycine max*	Brazil	SVJ-04	Papilionoideae	Phaseoleae

5063	*Glycine max*	Uruguay	U 284	Papilionoideae	Phaseoleae
5064	*Glycine max*	Uruguay	U 287	Papilionoideae	Phaseoleae
5065	*Glycine max*	Uruguay	U 391	Papilionoideae	Phaseoleae
5066	*Glycine max*	Nigeria	IRJ2 133b	Papilionoideae	Phaseoleae
5067	*Glycine max*	Nigeria	IRJ2 177a	Papilionoideae	Phaseoleae
5068	*Glycine max*	Nigeria	IRC 2180a	Papilionoideae	Phaseoleae
5069	*Glycine max*	Brazil	CPAC BW	Papilionoideae	Phaseoleae
5070	*Glycine max*	Brazil	CPAC 74K	Papilionoideae	Phaseoleae
5071	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5072	*Glycine max*	USA	NC 1005	Papilionoideae	Phaseoleae
5073	*Glycine max*	USA	NC 1005, Ery SPCA.1	Papilionoideae	Phaseoleae
5074	*Glycine max*	USA	USDA 123, Ery STRA.1	Papilionoideae	Phaseoleae
5075	*Glycine max*	Finland	HAMBI 1135, ATCC 1032	Papilionoideae	Phaseoleae
5079**	*Glycine max*	Brazil	CPAC 15, DF 24	Papilionoideae	Phaseoleae
5080**	*Glycine max*	Brazil	CPAC 7	Papilionoideae	Phaseoleae
5081	*Glycine max*	Brazil	S 372	Papilionoideae	Phaseoleae
5082	*Glycine max*	Brazil	CPAC 42	Papilionoideae	Phaseoleae
5083	*Glycine max*	Brazil	Original SEMIA	Papilionoideae	Phaseoleae
5084	*Glycine max*	Brazil	CPAC 45	Papilionoideae	Phaseoleae
5085	*Glycine max*	Argentina	E 109	Papilionoideae	Phaseoleae
5086	*Glycine max*	USA	USDA 31	Papilionoideae	Phaseoleae
5087	*Glycine max*	USA	USDA 76	Papilionoideae	Phaseoleae
5090	*Glycine max*	USA	USDA 122	Papilionoideae	Phaseoleae
6002**	*Vigna unguiculata*	Zimbabwe	CB 756, TAL 309, RCR 3824	Papilionoideae	Phaseoleae
6014	*Stylosanthes guianensis*	Peru	CIAT 1316, USM 128, TAL 405	Papilionoideae	Aeschynomeneae
6028**	*Desmodium uncinatum*	Zimbabwe	TAL 569, SPRL 472, MAR 472	Papilionoideae	Dalbergieae
6053**	*Clitoria ternatea*	Malasya	TAL 827, UMKI 28	Papilionoideae	Phaseoleae
6056	*Arachis hypogaea*	not know	TAL 1371, SMS 128	Papilionoideae	Aeschynomeneae
6057	*Psophocarpus tetragonolobus*	not Know	not know	Papilionoideae	Phaseoleae
6059**	*Psophocarpus tetragonolobus*	USA	USDA 3309	Papilionoideae	Phaseoleae
6069*	*Leucaena leucocephala*	Brazil	DF 10	Mimosoideae	Mimoseae
6071	*Stylosanthes spp.*	Brazil	SMS 55	Papilionoideae	Aeschynomeneae
6077	*Stylosanthes spp*	Australia	CB 82	Papilionoideae	Aeschynomeneae
6093	*Aeschynomene americana*	USA	USDA 3331	Papilionoideae	Aeschynomeneae
6099*	*Dimorphandra exaltata*	Brazil	BR 5004, LMG 9989	Caesalpinioideae	Caesalpinieae
6100**	*Albizia falcataria*	Brazil	BR5 608	Mimosoideae	Ingeae
6101**	*Dalbergia nigra*	Brazil	BR 8404	Papilionoideae	Dalbergieae
6118	*Stylosanthes sp.*	Colombia	CIAT308	Papilionoideae	Aeschynomeneae
6144**	*Arachis hypogaea*	Zimbabwe	SMS 400, USDA 3187, MAR 11	Papilionoideae	Aeschynomeneae
6145**	*Crotalaria juncea*	Brazil	BR 2001	Papilionoideae	Crotalarieae
6146**	*Centrosema spp.*	Brazil	BR 1808	Papilionoideae	Phaseoleae
6148**	*Neonotonia wightii*	Brazil	SMS 303	Papilionoideae	Phaseoleae
6149**	*Galactia striata*	Australia	CB 627, SMS 138	Papilionoideae	Phaseoleae
6150**	*Acacia mearnsii*	Brazil	SMS 300	Mimosoideae	Acacieae
6152**	*Calopogonium spp*	Brazil	BR-1602	Papilionoideae	Phaseoleae

6155**	*Stylosanthes spp.*	Brazil	BR 502	Papilionoideae	Aeschynomeneae
6156**	*Crotalaria spectabilis*	Brazil	CPAC IJ	Papilionoideae	Crotalarieae
6157**	*Cajanus cajans*	Brazil	BR 2801	Papilionoideae	Phaseoleae
6158**	*Crotalaria spectabilis*	Brazil	CPAC 42	Papilionoideae	Crotalarieae
6160**	*Albizia lebbek*	Brazil	BR 5610	Mimosoideae	Ingeae
6163**	*Acacia mearnsii*	Brazil	BR 3607	Mimosoideae	Acacieae
6164**	*Acacia mearnsii*	Brazil	BR 3608, LMG 9960	Mimosoideae	Acacieae
6169**	*Albizia falcataria*	Brazil	BR 5612	Mimosoideae	Ingeae
6175**	*Pueraria phaseoloides*	Brazil	DF Q-1	Papilionoideae	Phaseoleae
6179	*Acacia miersii*	Brazil	Original SEMIA	Mimosoideae	Acacieae
6181	*Melanoxylum brauna*	Brazil	Original SEMIA	Caesalpinioideae	Caesalpinieae
6186	*Acacia miersii*	Brazil	Original SEMIA	Mimosoideae	Acacieae
6187	*Acacia miersii*	Brazil	Original SEMIA	Mimosoideae	Acacieae
6189	*Acacia miersii*	Brazil	Original SEMIA	Mimosoideae	Acacieae
6192**	*Tipuana tipa*	Brazil	Original SEMIA	Papilionoideae	Dalbergieae
6208**	*Desmodium ovalifolium*	Colombia	CIAT 2372	Papilionoideae	Dalbergieae
6319**	*Arachis spp*	Bolivia	NC 92	Papilionoideae	Aeschynomeneae
6368	*Centrosema plumieri*	Colombia	CIAT 3101	Papilionoideae	Phaseoleae
6374	*Arachis pintoi*	not know	not know	Papilionoideae	Aeschynomeneae
6384**	*Mimosa acutistipula*	Brazil	BR 3446	Mimosoideae	Mimoseae
6388*	*Acacia podalyriaefolia*	not Know	not know	Mimosoideae	Acacieae
6391**	*Acacia auriculiformis*	Brazil	BR 3624	Mimosoideae	Acacieae
6420**	*Acacia mangium*	Brazil	BR 3617, LMG 9965	Mimosoideae	Acacieae
6424**	*Centrosema pubescens*	Brazil	CPAC 36	Papilionoideae	Phaseoleae
6425**	*Centrosema pubescens*	Colombia	CIAT 2380	Papilionoideae	Phaseoleae
6426*	*Erythrina poeppigeana*	not know	not know	Papilionoideae	Phaseoleae
6434*	*Inga sp.*	not Know	not know	Mimosoideae	Ingeae
6440**	*Arachis pintoi*	Brazil	MGAP 13	Papilionoideae	Aeschynomeneae
6443*	*Acosmium nitens*	not Know	not know	Papilionoideae	Sophoreae

* strains recommended to use in commercial inoculants in Brazil and ** analyzed for Menna et al. (2006 and 2008).

[a] Taxonomy based on ILDIS, www.ildis.org/LegumeWeb/6.00/taxa/1621.shtml.

[b] Source on the strains were isolated.

[c] 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 Australia, 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).

Table 2 – Primers and DNA amplification conditions used in this study[a]

Primer	Sequence (5`- 3`)*	Target gene (position)	PCR cycling	Reference
TSrecAf	CAACTGCMYTGCG TATCGTCGAAGG	*recA* [b] (8–32)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	Stepkowski et al. (2005)
TSrecAr	CGGATCTGGTTGA TGAAGATCACCAT G	*recA* [b] (620–594)		
TSatpDf	TCTGGTCCGYGGC CAGGAAG	*atpD* [b] (189–208)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	Stepkowski et al. (2005)
TSatpDr	CGACACTTCCGAR CCSGCCTG	*atpD* [b] (804–784)		
TSglnIIf	AAGCTCGAGTACA TCTGGCTCGACGG	*glnII* [b] (13–38)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	Stepkowski et al. (2005)
TSglnIIr	SGAGCCGTTCCAG TCGGTGTCG	*glnII* [b] (681–660)		
BRdnaKf	TTCGACATCGACG CSAACGG	*dnaK* [b] (1411-1430)	2 min 95°C, 35 X (45s 95°C, 30s 58°C, 1,5 min 72°C and 7 min 72°C.	This study
BRdnaKr	GCCTGCTGCKTGT ACATGGC	*dnaK* [b] (1905-1885)		
fD1	AGAGTTTGATCCT GGCTCAG	16S rRNA [b] (9-29)	2 min 95°C, 30 X (15s 94°C, 45s 93°C, 45s 55°C, 2 min 72°C and 5 min 72°C.	Weisburg et al. (1991)
rD1	CTTAAGGAGGTGA TCCAGCC	16S rRNA [b] (1474-1494)		
FGPS1490	TGCGGCTGGATCA CCTCCTT	*16S rRNA* [b] *(1490-1510)*	3 min 94°C, 35 X (1 min. 94°C, 1 min. 55°C, 2 min 72°C and 6 min 72°C.	Laguerre et al. (1996)
FGPS130	CCGGGTTTCCCCA TTCGG	*23S rRNA* [b] *(148-130)*		

[a] All primers were used for amplification and sequencing of PCR products.
[b] Position of the primer in the corresponding sequence of *Bradyrhizobium japonicum* USDA 110.
* Mixtures of bases used at certain positions are given as: K, T or G; S, G or C; Y, C or T; R, A or G; M, A or C.

Table 3 – Sequence information

Locus	N° strains analyzed	Conserved region (nt)	Variable region (nt)	Parsimony Informative (nt)	No. nt	Frequency T/C/A/G (%)
ITS rRNA	87[b]	581	367	292	790	26.1/ 24.7/ 20.1/ 29.1
16S rRNA	87[b]	1370	114	67	1480	20.1/ 24.1/ 24.3/ 31.5
16S rRNA+ITS rRNA	87[b]	1942	488	361	2270	22.2/ 24.3/ 22.8/ 30.7
atpD	47[a]	221	378	350	577	18.6/ 32.2/ 16.1/ 33.2
dnaK	47[a]	270	107	74	370	10.3/ 31.1/ 24.6/ 34.0
glnII	47[a]	433	171	126	603	17.3/ 33.1/ 19.9/ 29.7
recA	47[a]	377	148	108	524	15.9/ 30.9/ 17.2/ 36.0
ITS rRNA	47[a]	571	362	263	790	26.0 /24.8/ 20.0/ 29.1
16S rRNA	47[a]	1378	106	64	1480	20.1/ 24.0/ 24.3/ 31.5
Concatenated genes	47[a]	3275	1259	1000	4348	19.2/ 27.9/ 21.0/ 31.8
Concatenated genes without *atpD*	47[a]	3057	867	649	3770	19.3/ 27.3/ 21.8/ 31.6

[a b] 40 and 80 strains from this study and six reference strains, as described in the material and methods section.

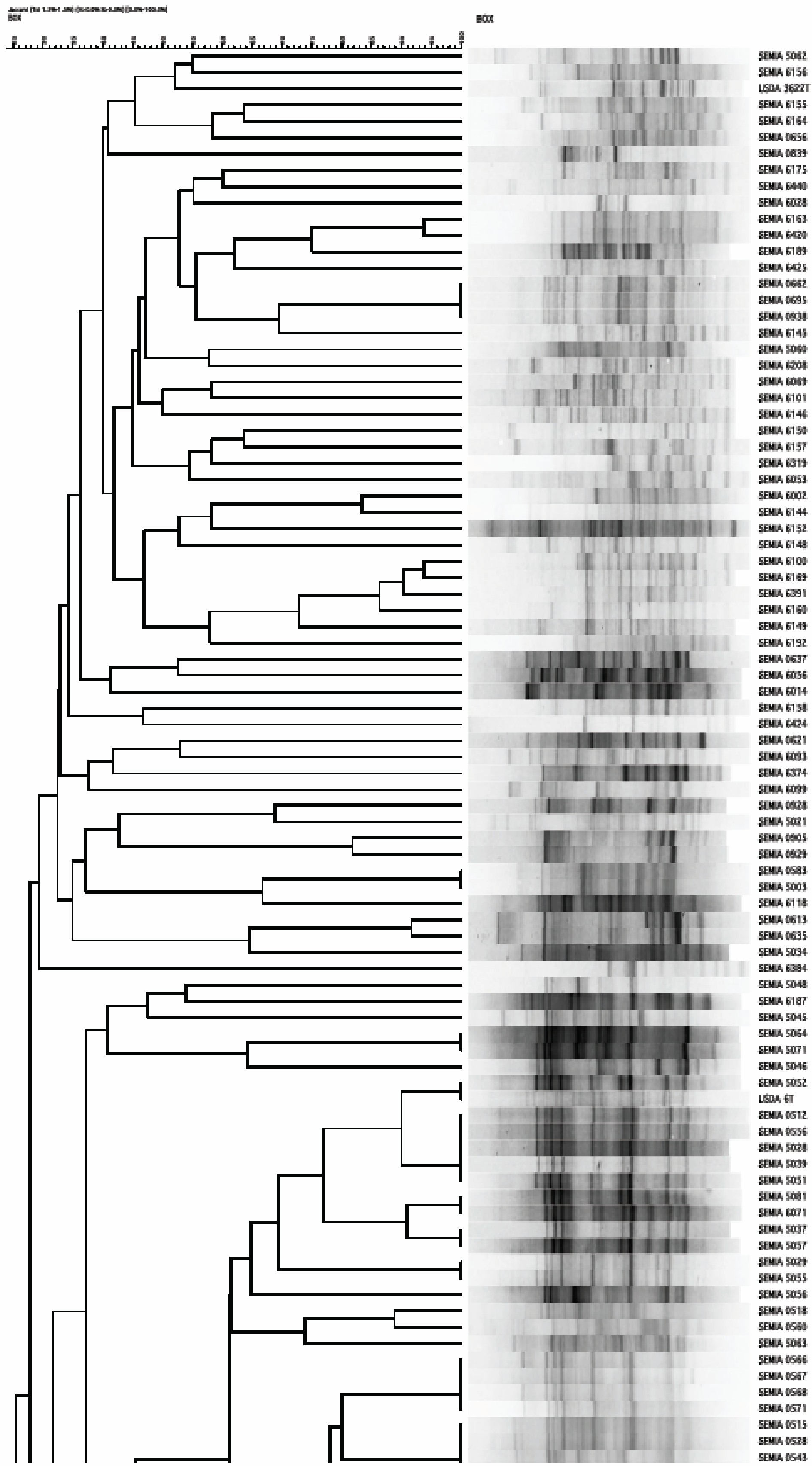
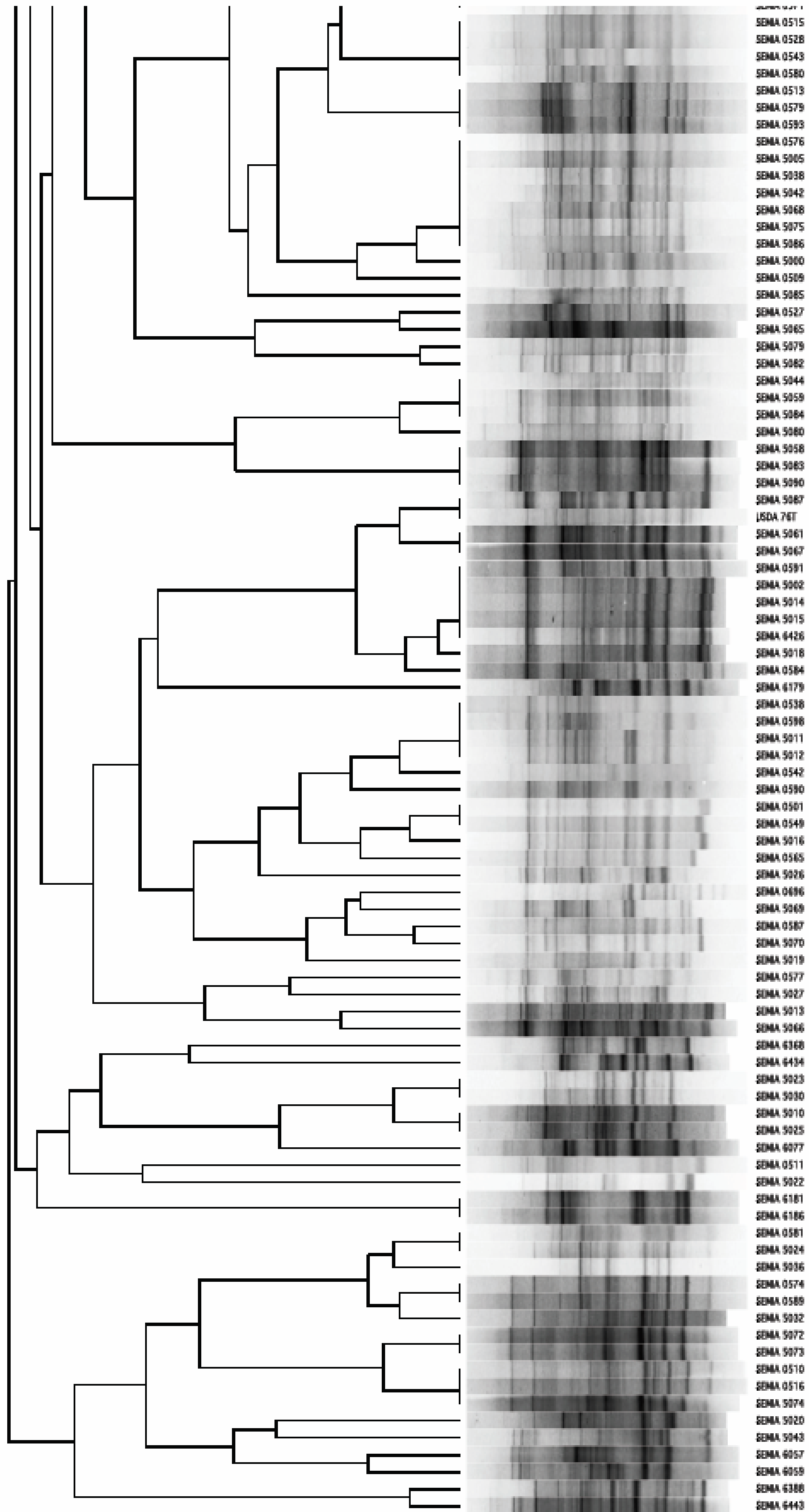


Figure 1

Cont. Figure 1



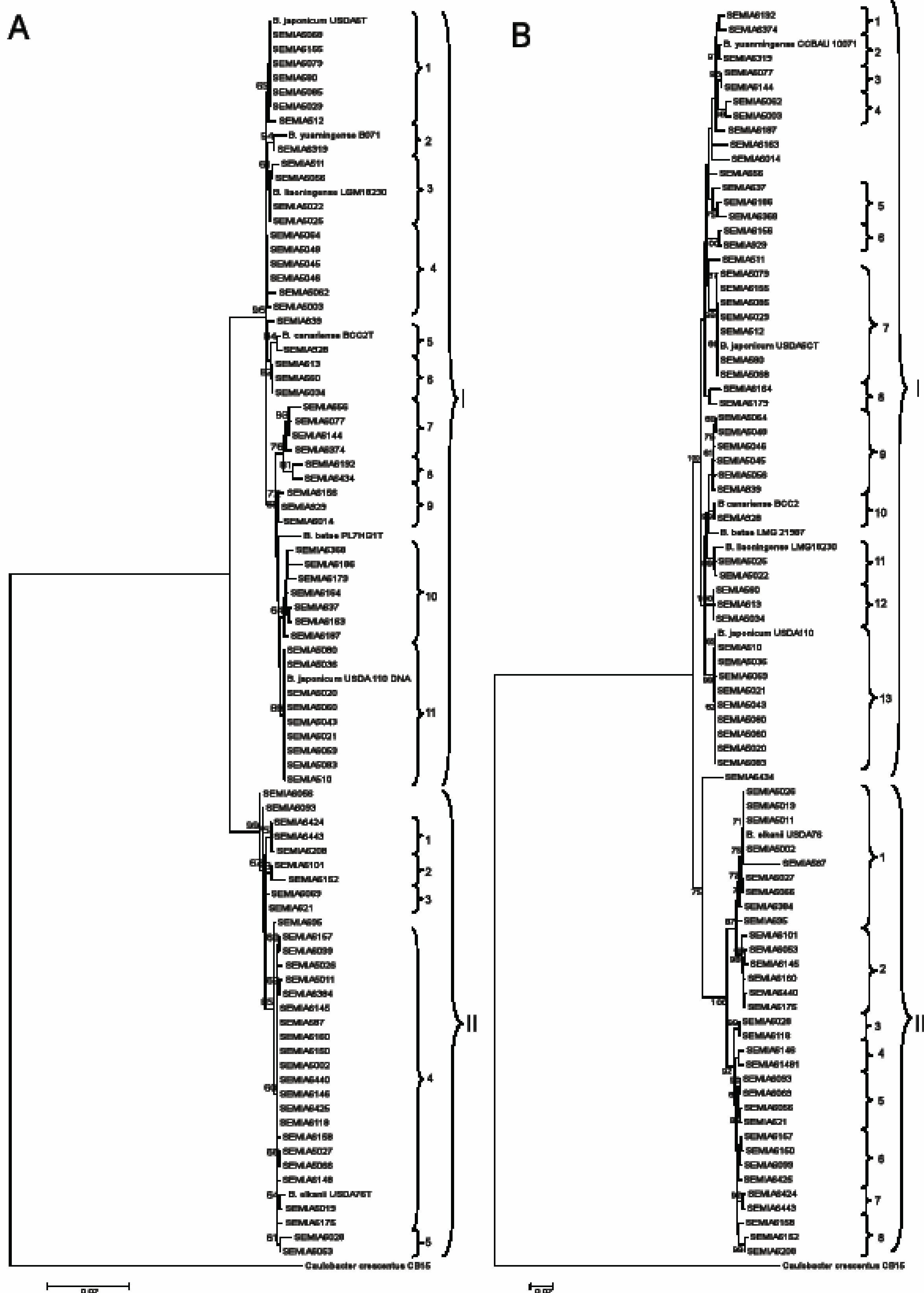


Figure 02: Phylogenetic relationships of 88 taxa based on the 16S rRNA (A) and on the ITS rRNA (B). Phylogeny was inferred using the Neighbor-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

Figure03: Phylogenetic relationships of 88 taxa based on polyphasic 16S+ITS rRNA. Phylogeny history was inferred using the Neighbor - Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4. * Strains selected for MLSA analysis.

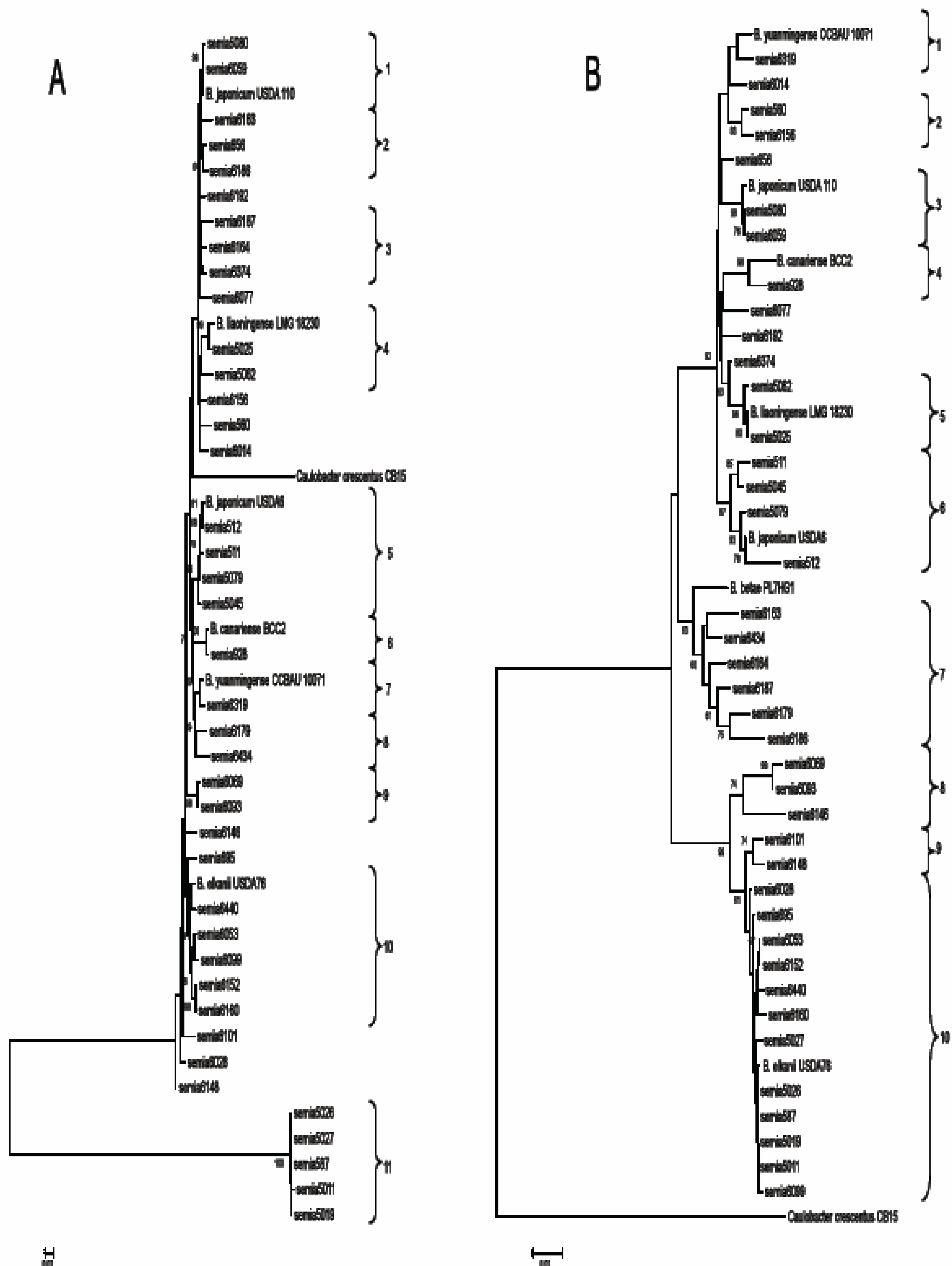


Figure04: Phylogenetic trees using the Neighbor-Joining method for 47 strains based on *atpD* (A) and *dnaK* (B). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic trees. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

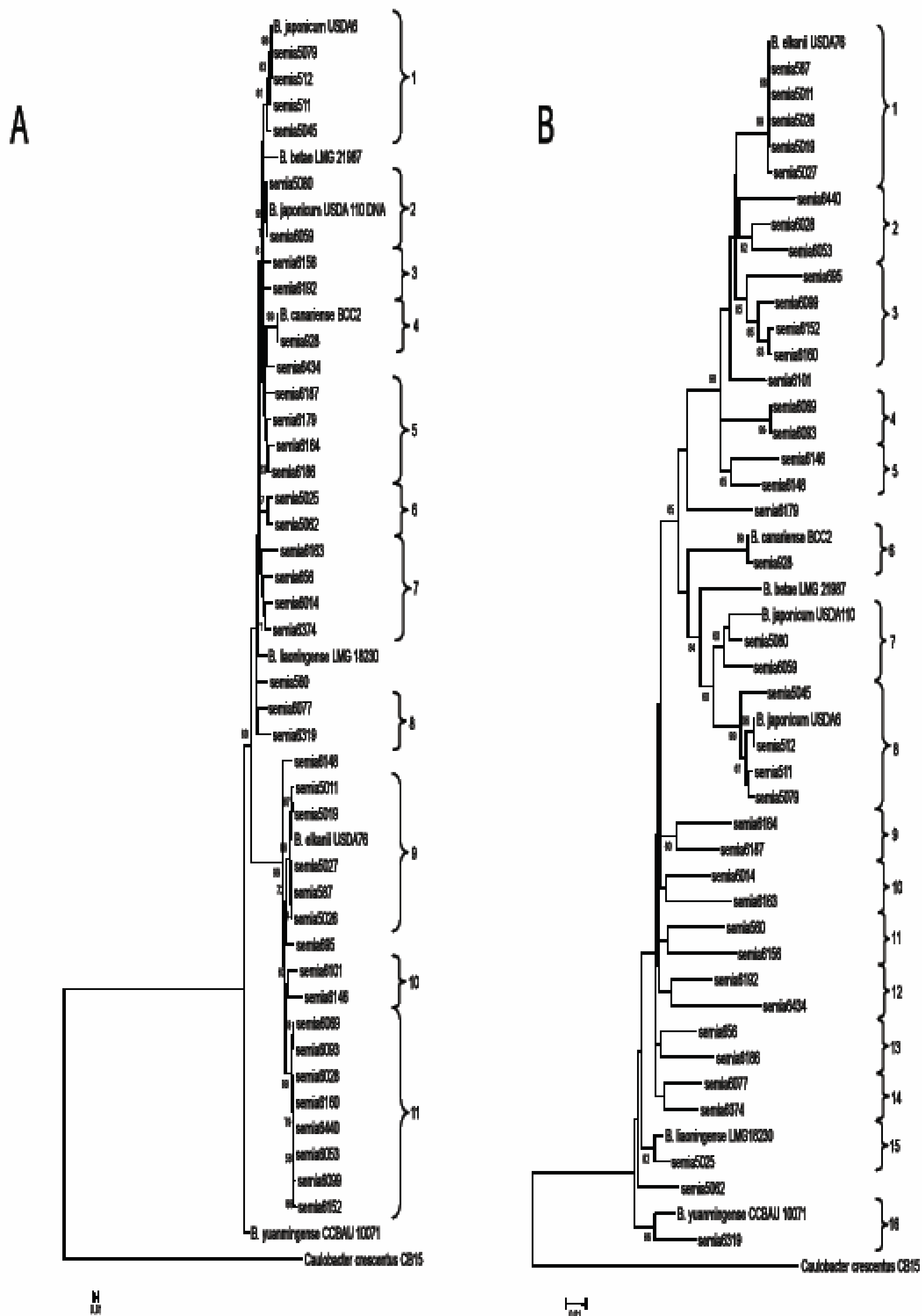


Figure05: Phylogenetic trees inferred using the Neighbor-Joining method for 47 strains based on *glnII* (A) and *recA* (B). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The phylogenetic distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

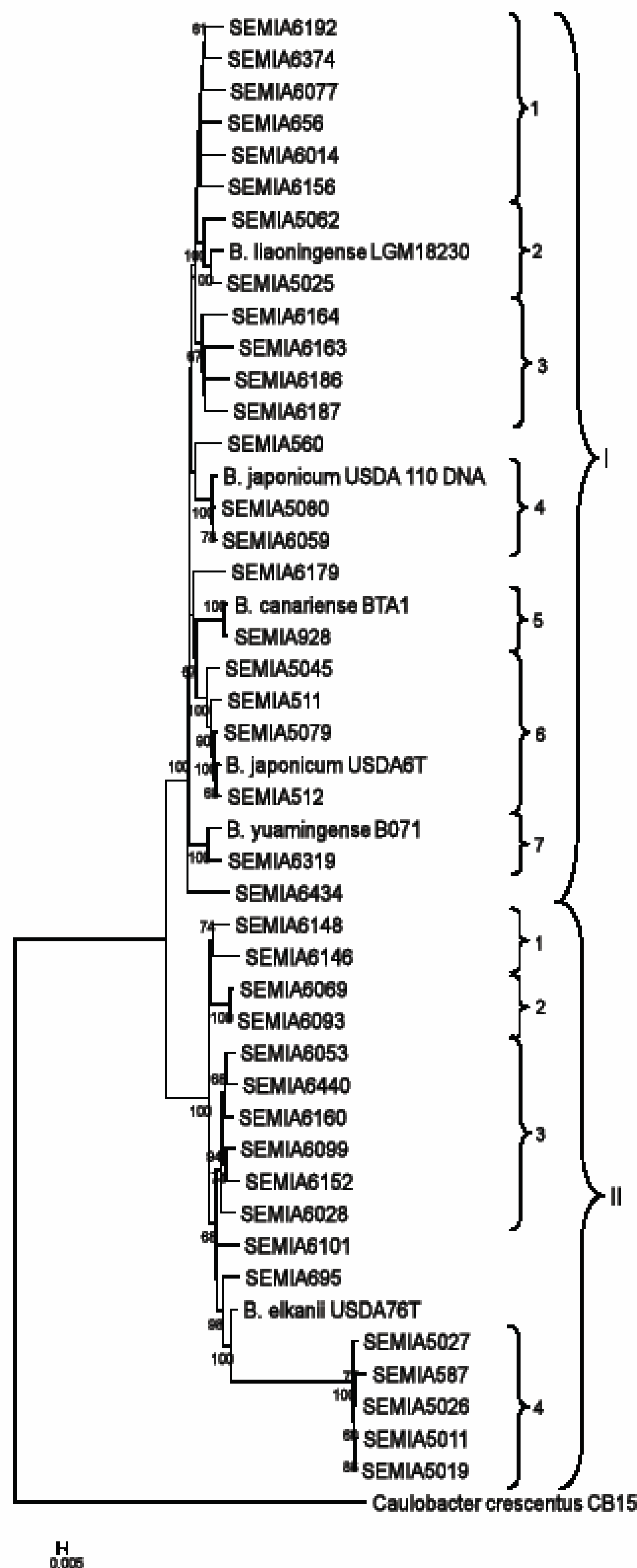


Figure06: Evolutionary tree inferred using the Neighbor-Joining method for 47 strains based on concatenated genes *(16S rRNA, atpD, dnaK, GlnII,recA* and *ITS rRNA)* The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

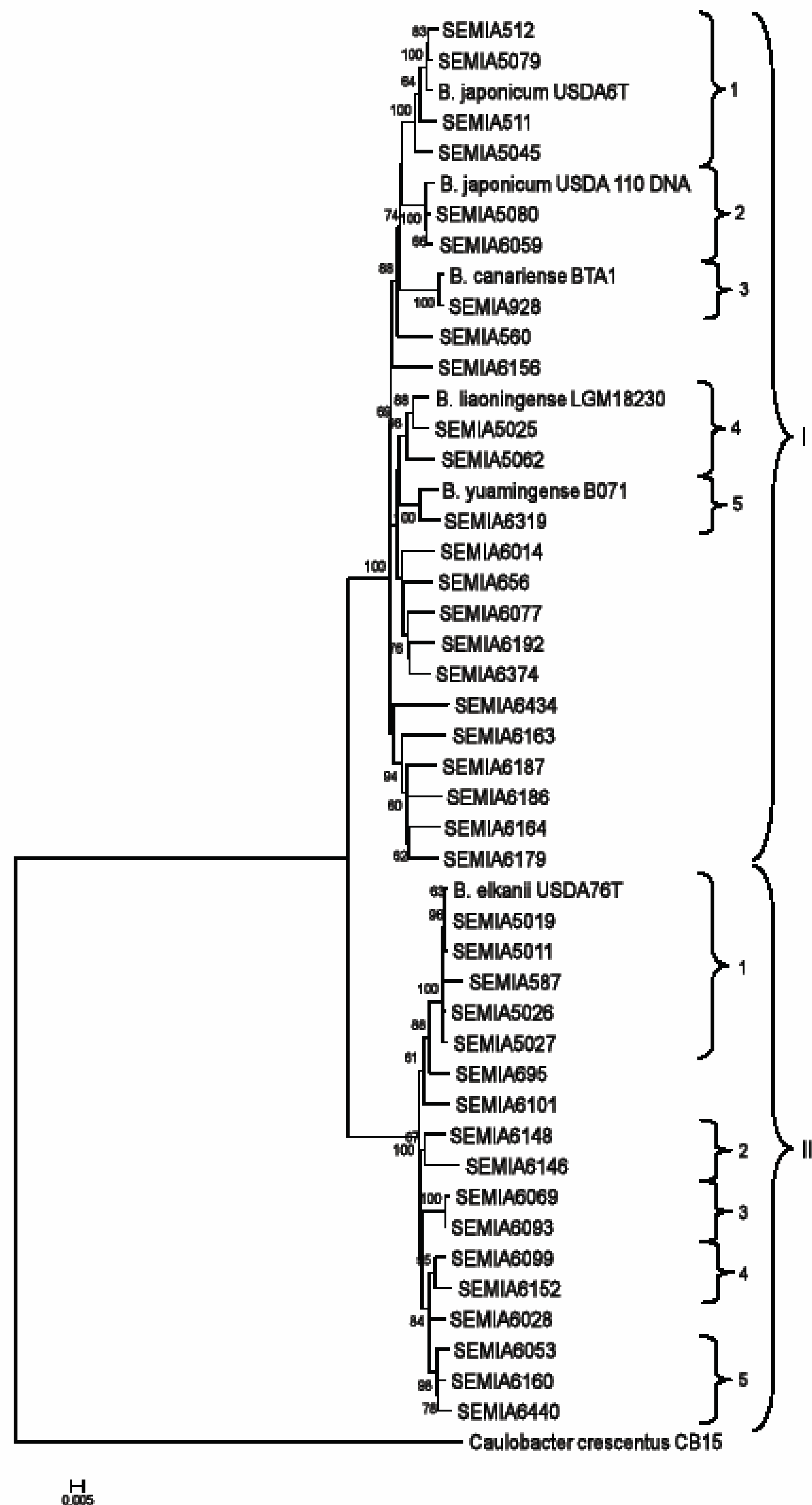


Figure07: Evolutionary tree inferred using the Neighbor-Joining method for 47 strains based on concatenated genes *(16S rRNA, dnaK, GlnII,recA* and *ITS rRNA)* The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4.

6 CONCLUSÕES

- Somente a análise da região *box* é limitada para determinação de grupos taxonómicos.
- A análise polifásica utilizando um peso de 8:2 (16S RNAr:BOX-PCR) demonstrou elevada definição dos genotipos e clara indicação de grupos taxonómicos em rizóbios.
- Análise polifásica demonstrou ser uma rápida e precisa metodologia para determinar, diversidade taxonómica e estrutura filogenética de grandes coleções de rizóbios.
- A análise dos genes *"housekeeping" (atpD, dnaK, glnlI* e *recA)* em estirpes de *Bradyrhizobium* demonstraram elevada intra e inter-específica diversidade genética, não observada pela análise do 16S RNAr.
- A análise da seqüência concatenada (ITS1 e 16S RNAr) permitiu identificar possíveis novas espécies de *Bradyrhizobium.*
- Os subgrupos formados com os genes *"housekeeping" dnaK, glnlI* e *recA,* foram similar aos observados com as seqüências do 16S RNAr, confirmando a utilização desses em filogenia e taxonomia do gênero *Bradyrhizobium.*
- O gene *atpD* apresentou elevada variabilidade e desse modo pode não ser útil como ferramenta taxonómica em espécies de *Bradyrhizobium,* mais úteis em estudos de diversidade genética.
- A técnica de MLSA com os genes *dnaK, glnlI, recA,* 16S RNAr e a região ITS1 permitiu estabelecer relações filogenéticas e taxonómicas no gênero *Bradyrhizobium.*
- A localização geográfica não afetou o padrão dos genes *"housekeeping"* reforçando a teoria de origem comum do gênero *Bradyrhizobium.*