JESIANE STEFÂNIA DA SILVA BATISTA

Caracterização da diversidade de estirpes de *Bradyrhizobium japonicum* e *B. elkanii* estabelecidas por inoculação em solos do Cerrados, isoladas de nódulos de soja *(Glycine max* (L.) Merr).

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Dissertação apresentada ao Programa de Pós-Graduação em Microbiologia da Universidade Estadual de Londrina como parte dos requisitos para a obtenção do título de Mestre em Microbiologia.

Orientadora: Dra. Mariangela Hungria

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2006

DEDICATÓRIA

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viver no topo da montanha, sem saber que a
verdadeira felicidade está na forma de subir a escarpa.
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aperta com seu pequeno punho, pela primeira
vez, o dedo do pai, o tem apanhado para sempre.
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o direito de olhar a outro com o olhar baixo quando
há de ajudar-lhe a se levantar.

Gabriel García Márquez

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1.1 INTRODUÇÃO

Considera-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, a água deve ser fornecida às culturas por meio de irrigação e o P deve ser suprido na forma de fertilizantes (Kochian, 2000).

O N é o nutriente requerido em maior quantidade pelas plantas e constitui cerca de 80% do gás atmosférico na forma de N_2. Apesar da abundante disponibilidade, o nitrogênio molecular (N_2) não pode ser assimilado por grande parte dos organismos devido à tripla ligação existente entre os dois átomos de N, que é altamente estável e possui uma elevada energia de ativação. Para tais organismos, o nitrogênio deve ser disponibilizado em formas passíveis de assimilação, como nitrato, amônia e nitrogênio orgânico (Tate, 1995).

As principais formas de disponibilização de N às plantas são: o solo, fertilização nitrogenada, fixação não-biológica e fixação biológica de N_2.

O reservatório de N presente no solo é devido à decomposição da matéria orgânica e é bastante limitado, podendo ser esgotado rapidamente após alguns cultivos. Além disso, condições de umidade e temperaturas elevadas, como no Brasil, tornam esse processo ainda mais acelerado devido à volatilização da amônia e desnitrificação (Sierra *et al.*, 2001).

O processo industrial de conversão do N_2 em amônia para a aplicação na forma de fertilizantes nitrogenados constitui a forma de assimilação mais fácil e com menor custo energético pelas plantas, porém, possui um elevado custo econômico e ambiental. Segundo Graham & Vance (2000), além da utilização de combustíveis fósseis, outros aspectos desvantajosos de grande importância associados à fertilização incluem a elevada perda de N por volatilização, ocorrência de chuvas ácidas, lixiviação de nutrientes do solo e conseqüente poluição de rios e lagos, alterações no ciclo global do N e poluição dos lençóis freáticos.

A fixação de N_2, ou seja, a conversão do gás N_2 atmosférico em formas assimiláveis por grande parte dos organismos, pode ocorrer de duas formas: não-biológica e

biológica. A fixação não-biológica é resultante de processos naturais, principalmente através de descargas elétricas, combustão e vulcanismo. Essas reações resultam na transformação do N_2 atmosférico em nitrato, que é adicionado ao solo e representa uma pequena contribuição para o balanço global de N (Hungria *et al.*, 1994).

A principal via de incorporação do N à biosfera é a fixação biológica de nitrogênio (FBN), um processo realizado por certos organismos de redução do N_2 a amônia, que é posteriormente utilizada para a síntese de diversos compostos nitrogenados. Esses organismos, denominados diazotróficos, são procariotos providos de um complexo enzimático denominado dinitrogenase que catalisa tal conversão, à custa de grande quantidade de energia.

Os organismos diazotróficos possuem diferentes tipos de associações com diversas plantas. Os de vida livre são capazes de fixar N_2 independente de um hospedeiro superior, utilizando energia disponível no ambiente. Essas bactérias podem estar presentes na região rizosférica (bactérias associativas), ou mesmo dentro dos tecidos das raízes, colmos e folhas das plantas (bactérias endofíticas) (Postgate, 1982).

As bactérias fixadoras de N_2 simbióticas, coletivamente denominadas de rizóbios, dependem de uma íntima relação com o hospedeiro e levam à formação de novos órgãos usualmente nas raízes, denominados nódulos. No interior desses órgãos, formas diferenciadas de rizóbios fixam nitrogênio, utilizando energia dos fotossintatos fornecidos pela planta hospedeira e liberam a amônia produzida diretamente no tecido vegetal (Schultze & Kondorosi, 1998).

Tal capacidade foi identificada em cerca de 87 espécies em 2 gêneros de arquea, 38 gêneros de bactérias e 20 gêneros de cianobactérias. Apesar da restrição no número de espécies de organismos diazotróficos, os mesmos são taxonomicamente diversos e encontram-se presentes em quase todos os nichos ecológicos. Devido à FBN ser uma fonte eficiente e ecologicamente benéfica de N, associada à vasta distribuição dos organismos responsáveis por esse processo, existe um reconhecido potencial na utilização de bactérias diazotróficas para aumento da incorporação de N a ecossistemas em recuperação e áreas de cultivo agrícola (Brockwell *et al.*, 1995; Tate, 1995).

1.2 A CULTURA DA SOJA NO BRASIL

Apesar de ser conhecida e explorada na China, seu berço genético, há mais de cinco mil anos, a soja (*Glycine Max* (L.) Merrill) chegou ao Brasil via Estados Unidos, em 1882 na Bahia e o primeiro registro de cultivo experimental data de 1914.

A primeira referência de produção comercial da soja no país data de 1941, na região Sul, sendo o verdadeiro estímulo à produção em larga escala dado na década de 50, com a decisão oficial de prover incentivos fiscais à produção de trigo. Devido à combinação das duas culturas, tanto por uma perspectiva técnica quanto econômica, tal política resultou em um crescimento significativo e consolidação da cultura da soja no Brasil (EMBRAPA, 2005).

Com a abertura de novas fronteiras agrícolas, a cultura expandiu-se, na década de 70, para outras regiões, como os Cerrados, uma das mais importantes áreas produtoras dessa leguminosa do Brasil, onde foram cultivados 10,251 milhões de hectares, com uma produção de mais de 29 milhões de toneladas na safra 2005/2006 (CONAB, 2006).

Atualmente, a quantidade em grãos de soja produzida no Brasil corresponde a 42% da produção agrícola nacional, sendo 72% dessa produção destinada à exportação e, no PIB, a cadeia produtiva da soja participa com 16% (CONAB, 2005). Devido ao seu elevado teor protéico, a soja também é destacada pelo seu importante papel nutricional.

Estimativas apontam que o custo de produção da soja nacional é cerca de 40% inferior ao dos Estados Unidos, e com uma produtividade superior, resultando em uma economia estimada em cerca de U$ três bilhões/ano. Esse incremento produtivo deve-se, especialmente, a pratica, no Brasil, da inoculação das sementes com rizóbios comprovadamente eficientes e competitivos. A simbiose entre rizóbios selecionados e plantas leguminosas através da inoculação constitui uma prática agronômica de suprimento de N para cultivos com baixo custo, segura e eficiente (Hungria *et al.*, 2005).

1.3 BACTÉRIAS MICROSSIMBIONTES DA SOJA

B. japonicum e B. elkanii são espécies de bactérias Gram-negativas do solo, pertencentes à subclasse *Proteobacteria*, classe *Alphaproteobacteria* (Garrity & Holt, 2001).

O critério inicial de classificação dos rizóbios baseava-se na capacidade de nodulação especifica com a planta hospedeira. Neste conceito, *Rhizobium japonicum* era a única espécie capaz de nodular a soja e se caracterizava por possuir crescimento lento e produção de reação alcalina em meio de cultura contendo manitol como fonte de carbono. Essa taxonomia se manteve até que as bactérias da espécie *R. japonicum* foram reclassificadas em um novo gênero, *Bradyrhizobium* ("bradus", do grego, lento) que apresentava uma única espécie definida, *Bradyrhizobium japonicum* (Jordan, 1982).

A partir da década de 80, vários trabalhos constataram grande variabilidade genética e fisiológica entre as estirpes de *B. japonicum,* o que levou Kuykendall *et al.* (1992) a sugerirem a subdivisão de *Bradyrhizobium* em duas espécies, *B. japonicum* e *B. elkanii*, que são as principais espécies de rizóbios noduladores da soja. Atualmente, encontram-se descritas outras quatro espécies de *Bradyrhizobium*: *B. liaoningense* (Xu *et al.*, 1995), *B. yuanmingense* (Yao *et al.*, 2002), *B. betae* (Rivas *et al.*, 2004) e *B. canariense* (Vinuesa et al, 2005). Supõe-se que o número de espécies deste gênero seja ainda maior, sendo sua taxonomia aprimorada através do isolamento de estirpes a partir de leguminosas pouco estudadas e aplicação de novas metodologias de análise filogenética.

Atualmente, a análise de seqüências nucleotídicas do gene ribossomal 16S rDNA foi estabelecida como padrão de análise em estudos de taxonomia e filogenia (Garrity & Holt, 2001; Krieg, 2001; Heyndrickx *et al.*, 1996). A conservação dos genes rRNA, devido à sua função estrutural nos ribossomos, e a existência de variabilidade em alguns domínios torna as seqüências desses genes ideais para comparação de organismos e inferência filogenética (Garrity & Holt, 2001; Weisburg *et al.*, 1991).

1.4 ASPECTOS GENÉTICOS DA FBN

A formação dos nódulos é um processo complexo, que ocorre em várias etapas e envolve mudanças fisiológicas e morfológicas, tanto na célula hospedeira quanto na bactéria. As alterações na bactéria visam, especialmente, o recebimento de fontes de carbono da planta hospedeira, para prover o ATP e poder redutor, necessários para o processo da fixação biológica, enquanto as mudanças na planta hospedeira visam assimilar a amônia produzida pelas bactérias (Hungria *et al.*, 1994).

O estabelecimento da associação simbiótica entre rizóbios e planta hospedeira inicia-se com a liberação, pelas raízes da leguminosa, de compostos fenólicos, geralmente flavonóides, que agem como substâncias quimiotáticas, estimuladoras da multiplicação dos rizóbios e indutoras de genes bacterianos responsáveis pela nodulação (genes *nod, nol* e *noe*). Esses genes codificam a produção dos fatores de nodulação, ou fatores Nod, que são oligossacarídeos lipoquitínicos secretados pelos rizóbios. Os fatores Nod, mediante reconhecimento pela planta, induzem a diversas modificações, como o curvamento dos pêlos radiculares, seguido por invaginação da parede celular e formação de um cordão de infecção no interior do pêlo radicular. O cordão de infecção prolonga-se até atingir o primórdio do nódulo, que é desenvolvido devido à reativação da divisão de células diferenciadas do córtex da raiz (Schultze *et al.*, 1994).

Os rizóbios são liberados na extremidade interna do cordão de infecção por endocitose em uma célula cortical, circundados por uma membrana, no interior da qual serão diferenciados em bacteróides, formas capazes de fixar N_2. Essa interação simbiótica requer um alto grau de compatibilidade entre o rizóbio e a planta hospedeira, pois a sinalização molecular, através dos flavonóides e dos fatores Nod, é altamente específica e coordenada (Schultze & Kondorosi, 1998).

A proteína NodD dos rizóbios representa uma interface molecular entre a bactéria e a planta, visto que a mesma interage com os sinais moleculares da planta e coordena a transcrição de demais genes de nodulação, responsáveis pela síntese dos fatores implicados na formação do nódulo. Tal controle da expressão ocorre pois a mesma pertence à família dos reguladores transcricionais “LysR-like” e se liga a seqüências altamente conservadas de 47 pares de bases (pb) encontradas nas regiões promotoras de muitos loci de nodulação, denominadas "caixas *nod"* (Perret *et al.*, 2000). Duas proteínas NodD com padrões distintos de expressão e função foram identificadas em *B. japonicum*: NodD1, que age como ativador transcricional em resposta a flavonóides exsudados pelas raízes das plantas e NodD2, que reprime a expressão dos genes *nod* (Loh & Stacey, 2001).

A síntese da estrutura básica do fator Nod é codificada pelos genes *nodABC*, presentes em todos os rizóbios até hoje investigados e, por esse motivo, denominados genes *nod* comuns, juntamente com o gene regulador *nodD*. Moléculas de N-acetilglucosamina sintetizadas pela bactéria são polimerizadas em tri, tetra ou pentassacarídeos mediante a atividade transferase da proteína NodC. A proteína NodB age na desacetilação do resíduo de N-acetilglucosamina da extremidade redutora do fator Nod, enquanto a proteína NodA

promove a transferência de um grupo acil ao grupamento amina livre gerado na extremidade redutora (Manyani, 2000).

Tal estrutura comum do fator Nod pode sofrer modificações químicas como sulfatação, metilação e fucosilação, o que confere especificidade do rizóbio ao hospedeiro. A síntese e transferência de grupos químicos são determinadas por genes conhecidos como *hsn* (host specific *nodulation*), ou genes de nodulação específicos do hospedeiro (Moulin *et al.*, 2004).

A capacidade de fixar biologicamente N_2 pelos diazotróficos, incluindo os rizóbios, deve-se à síntese de 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. Durante a reação de redução do N_2, a dinitrogenase é auxiliada por uma terceira molécula transportadora de elétrons, a ferridoxina, a qual, em sua forma reduzida, transfere um elétron para a unidade Fe-proteína que, então, reduzida, doa um elétron para a MoFe-proteína, que acumula os elétrons.O processo ocorre até que oito transferências de elétrons sejam acumuladas, possibilitando a redução completa do N_2 à NH_3. Estes oito elétrons são necessários porque, obrigatoriamente, também ocorre a redução de prótons de hidrogênio (H^+) a H_2 (Morgante, 2003). A reação pode ser assim expressa: $N_2 + 8\ e^- + 8\ H^+ + 16\ ATP \rightarrow 2\ NH^3 + H^2 + 16\ ADP + 16\ Pi$.

Por ser um processo de alta demanda energética, há um rigoroso controle genético do metabolismo da FBN através dos genes de fixação, *nif* e *fix*. Em *B. japonicum,* estes genes são organizados em "clusters"; a síntese da nitrogenase é codificada pelos genes estruturais *nifD, K* e *H*. Sua expressão gênica está associada à síntese das duas metaloproteínas que compõem a dinitrogenase, sendo a Fe-proteina codificada pelo gene *nifH* e a MoFe-proteina codificada pelos genes *nifD* e *K* (Halbleib **&** Ludden**,** 2000).

Os genes de nodulação e fixação de nitrogênio das espécies pertencentes ao gênero *Bradyrhizobium* encontram-se numa região cromossomal definida como "ilha simbiótica" (Kaneko et al, 2002; Göttfert *et al.*, 2001). Minamisawa *et al.* (2002) demonstraram que esse elemento cromossomal é potencialmente transferível de certas estipes de *B. japonicum* para estirpes não-simbióticas da espécie *B. elkanii*, capacitando-as à nodulação.

1.5 SELEÇÃO DE ESTIRPES ADAPTADAS DE *B. JAPONICUM* E *B. ELKANII* PARA A CULTURA DA SOJA

Como já foi destacada, a simbiose entre estirpes selecionadas e adaptadas de rizóbios e leguminosas é uma prática agronômica de baixo custo, efetiva e ambientalmente segura para garantir o suprimento adequado de N para as culturas agrícolas, portanto vantajosa em comparação com a aplicação de fertilizantes nitrogenados. (Hungria *et al.*, 2005).

Desse modo, a busca por bactérias microssimbiontes da soja adaptadas às condições brasileiras iniciou-se concomitantemente com a expansão da cultura no país, pois alguns trabalhos pioneiros já demonstravam que existia um grande potencial para incrementar os níveis de produtividade via FBN através da seleção de estirpes mais eficientes nesse processo e adaptadas às condições ambientais locais. O Brasil possui, atualmente, uma legislação especifica para o controle de qualidade de inoculantes, que podem conter apenas estirpes recomendadas por um comitê de microbiologistas (Araújo, 2006).

O método que vem sendo empregado com maior sucesso no Brasil consiste na seleção de estirpes adaptadas aos solos e às condições ambientais brasileiras. Em uma área inoculada há vários anos, segue-se um período de tempo de adaptação, que pode ser de vários anos, após o qual procede-se o reisolamento das bactérias de nódulos radiculares e verificação da eficiência dessas estirpes. A utilização de tais estirpes em inoculantes comerciais contribuiu para que cultivares cada vez mais produtivas pudessem ter suas necessidades em N totalmente supridas pelo processo biológico (Vargas et al, 1993). Isso é necessário, uma vez que produtividades crescentes vêm sendo obtidas com a soja, por exemplo, o rendimento nacional de grãos, de 1500kg/ha da década de 60, para 2.627kg/ha na safra 2005/2006 (CONAB, 2006).

Uma das estirpes recomendadas à cultura da soja no Brasil é a CPAC 15 (=SEMIA 5079, =566a) pertencente ao mesmo sorogrupo da SEMIA 566, que foi utilizada em inoculantes comerciais na década de 60. Para sua obtenção foi utilizada a estratégia de reisolamento de estirpes, em solos dos Cerrados, vários anos após a última inoculação, na busca de estirpes adaptadas, com elevada capacidade de FBN e competitivas. Tais estipes variantes adaptadas podem diferir das parentais quanto a diversas características. Estudos conduzidos com variantes do sorogrupo da SEMIA 566 adaptadas aos solos dos Cerrados, por exemplo, foram constatadas diferenças na capacidade competitiva, que estariam relacionadas

com a habilidade de alterar as proteínas da membrana em resposta ao estímulo das raízes (Scotti *et al.*, 1993).

A estirpe CPAC 7 (=SEMIA 5080) foi obtida utilizando-se uma diferente estratégia de seleção. Subcolônias da estirpe CB 1809 tiveram sua eficiência de fixação de N_2 avaliada através da atividade da redução do acetileno (ARA) em meio de cultura sólido. A seguir, as estirpes mais eficientes tiveram a competitividade avaliada em casa de vegetação, a fim de se obter uma estirpe que combinasse ambas habilidades, sendo então selecionada a CPAC 7 (Hungria & Vargas, 2000; Mendes *et al.*, 2004).

A SEMIA 587 foi isolada em Santa Rosa, RS, numa região cultivada com sementes de soja, importada dos Estados Unidos. Provavelmente, estas sementes carregavam bradirrizóbios do local de origem, permitindo seu estabelecimento em solos brasileiros (Freire & Vernetti, 1999).

A grande importância da estirpe 29W (=SEMIA 5019) foi permitir o estabelecimento de bactérias fixadoras de N_2 em solos dos Cerrados através da superação de condições adversas locais, como pH ácido, altas temperaturas, alumínio tóxico e resistência a antibióticos (em especial à estreptomicina) produzidos por populações residentes de actinomicetos (Scotti *et al.*, 1982).

Lunge et al (1994), com base na técnica de RFLP com marcadores *nif* e *nod*, dividiram essas quatro estirpes recomendadas em dois grupos: grupo CPAC 15 – CPAC 7 e grupo SEMIA 587 – 29W. Esse agrupamento foi confirmado por Lemos (1994), através da análise de múltiplos locos enzimáticos, sorologia, morfologia de colônia e atividade da hidrogenase, e também por Sato *et al.* (1999), por RAPD (*Random Amplified Polimorphic DNA*). Com base no seqüenciamento completo da região 16S rRNA, Chueire *et al.* (2003) confirmaram que as estirpes CPAC 15 e CPAC 7 apresentavam similaridade genética com a espécie *B. japonicum,* enquanto as estirpes SEMIA 587 e 29W apresentaram similaridade genética com a espécie *B. elkanii,*.

Incrementos na FBN e no rendimento das culturas pela inoculação com estirpes mais eficientes e competitivas vêm sendo constatados tanto no Brasil, como na Argentina, dois dos principais produtores mundiais de soja. Como resultado, ambos os paises apresentaram um grande crescimento na comercialização de inoculantes, que praticamente dobrou nos últimos anos (Hungria *et al.*, 2005).

A resposta positiva à inoculação no Brasil contrasta com os relatos obtidos nos EUA, onde raramente se observam incrementos no rendimento em solos com população

estabelecida (Thies *et al.*, 1991). Brockwell & Bottomley (1995), em revisão sobre os avanços na tecnologia da inoculação, citam que a produção mundial de inoculantes para leguminosas encontra-se em declínio, enfatizando severas limitações ao uso de tal prática agrícola, destacando-se os baixos efeitos dos inoculantes atualmente comercializados sobre o rendimento das culturas. A explicação para tal quadro, segundo Catroux *et al.* (2001) é que, apesar da elevada tecnologia industrial disponível para produção e aplicação de inoculantes comerciais, especialmente na Europa e nos EUA, os métodos de seleção de estirpes e controle de qualidade dos inoculantes são bastante ineficientes, refletindo em rendimentos relativamente baixos.

1.6 MICRODIVERSIDADE DE B. JAPONICUM E B. ELKANII

Novas espécies de rizóbios vêm sendo descritas nos últimos tempos, refletindo tanto a coleta de novas estirpes, como os avanços metodológicos para detectar novas espécies. Grande parte das novas espécies de rizóbios foi isolada de regiões tropicais, realçando a importância dos trópicos como fonte de biodiversidade. Se considerarmos os sistemas de agricultura sustentável como pré-requisito para a melhoria de qualidade de vida nos paises tropicais, bem como a importância da FBN para a sustentabilidade dos agroecossistemas, percebe-se a grande relevância e o potencial benéfico do entendimento e exploração racional da diversidade de rizóbios (Coutinho, 2003).

As espécies bacterianas podem possuir distintos fenótipos e genótipos, refletindo em uma ampla diversidade funcional e estrutural além deste nível. Este novo nível de diversidade, denominado microdiversidade bacteriana, pode ser acessado através de técnicas moleculares de alta resolução. Os estudos moleculares de microdiversidade disponibilizam informações refinadas, que permitem o entendimento de grupos funcionais de microrganismos e, especialmente, suas relações com ambientes e condições particulares (Schloter *et al.*, 2000).

Os estudos filogenéticos para determinação de microdiversidade de rizóbios podem ser baseados em três critérios principais. Primeiro, na filogenia dos cromossomos, através da análise de genes comuns e conservados em todas as bactérias como o 16S rRNA e *dnaK*, a fim de estimar sua posição no domínio *Bacteria.* Segundo, na filogenia dos genes de fixação do nitrogênio, disponibilizando informações acerca das relações entre os rizóbios e

outras bactérias fixadoras de nitrogênio. E, terceiro, na filogenia os genes de nodulação, que são excelentes marcadores para determinar as relações entre os rizóbios e seus hospedeiros ou origens geográficas (Wang & Martinez-Romero, 2000).

Outra metodologia de avaliação da diversidade genética de rizóbios é uma variação da técnica de PCR, baseada na utilização de iniciadores correspondentes a seqüências repetitivas intergênicas, de consenso, dispersas pelo genoma bacteriano. Essas seqüências são conhecidas como ERIC (*Enterobacterial Repetitive Intergenic Consensus*), REP (*Repetitive Extragenic Palindromic*) e BOX (*Box elements*) e geram padrões altamente característicos após separação em gel de agarose (Lanoot *et al.*, 2004; Madrzak *et al.*, 1995; Galli-Terasawa *et al.*, 2003; Sikora & Redzepovic, 2003). Resultados de análises baseadas em BOX-PCR são congruentes com análises baseadas em VNTRs (*Variable Number of Tandem Repeats*), sugerindo uma forte correlação entre esses dois tipos de elementos repetitivos (Kim *et al.*, 2001).

Apesar de ainda não possuírem uma função definida, é sugerido que as seqüências BOX possam estar envolvidas na estabilização do RNA mensageiro e na terminação da transcrição (Jansen, 2002), na variação fenotípica (Saluja *et al.*, 1995) e na patogenicidade bacteriana (Krauss & Hakenbeck, 1997). Além disso, por serem associados a elevados graus de polimorfismo, têm provável participação em processos de evolução adaptativa, mediando a interação dos microrganismos com ambientes hostis (van Belkum, 1999).

Em levantamentos realizados em áreas previamente inoculadas e cultivadas com soja, no Brasil, usualmente foram constatadas estirpes que não reagiram com nenhum sorogrupo conhecido, além de ser observada uma grande variabilidade morfológica, fisiológica, genética e simbiótica entre estirpes reisoladas após um período de adaptação ao solo e as estirpes inicialmente inoculadas (Hungria *et al.*, 1996, 1998; Nishi *et al.*, 1996; Boddey & Hungria, 1997; Santos *et al.*, 1999; Ferreira & Hungria, 2002; Galli-Terasawa *et al.*, 2003).

Para exibir tal diversidade fenotípica e genotípica, tais bactérias devem possuir um genoma extremamente dinâmico, permitindo a aquisição, deleção e rearranjos de relevante informação genética (Dutta & Pan, 2002). Duas classes de mecanismos podem explicar tal variabilidade: modificações internas da informação genética, através de mutações ou recombinação (Feil & Spratt, 2001; Milkman *et al.*, 1999), e a transferência lateral de genes específicos, conferindo novas habilidades metabólicas ao genoma receptor, capacitando o microrganismo a explorar novos nichos ecológicos (Boucher *et al.*, 2003).

Através do seqüenciamento da ilha simbiótica de 410 kb (kilobases) da estirpe padrão USDA 110 de *B. japonicum,* Göettfert *et al.* (2001) constataram que cerca de 19% dos quadros de leitura abertos (ORFs – "*open reading frames*") identificados eram relacionados a genes envolvidos em eventos de recombinação e integração, dentre os quais os mais conservados eram as seqüências repetitivas RSα e as seqüências de inserção HRS1 (Rodriguez-Quiñones *et al.*, 1992; Judd & Sadowsky, 1993). A recombinação entre os elementos RSα pode levar à inserção e à deleção de genes (Han *et al.*, 1984), transposições e rearranjos genômicos (Flores *et al.*, 1987; Minamisawa *et al.*, 1998).

A ocorrência da segunda classe de mecanismos de variabilidade em rizóbios foi confirmada por Sullivan *et al.* (1998), que constataram a transferência lateral da ilha simbiótica de 500 kb da estirpe inoculante de *Mesorhizobium loti* para uma estirpe não-simbiótica, convertendo, assim, uma estirpe saprofítica em simbiótica. Posteriormente, Minamisawa *et al.* (2002) comprovaram a transferência de genes de nodulação, em solos e microcosmos, de uma estirpe de *B. japonicum* para uma estirpe de *B. elkanii*.

Em estudos de inoculação de leguminosas, os resultados concentram-se especialmente na resposta da planta em produtividade; poucos dados são disponíveis quanto à persistência, estrutura genética e dinâmica populacional de estirpes inoculadas em solos ausentes ou com baixa população de estirpes nativas, (Madrzak *et al.*, 1995).

1.7 HIPÓTESE

A região dos Cerrados é caracterizada pela distribuição pluviométrica irregular, temperaturas elevadas e por possuir solos com alta velocidade de infiltração, baixa fertilidade natural, pH ácido e elevados teores de alumínio. Assim, introdução de estirpes exógenas de *Bradyrhizobium japonicum* nesses solos e de seu respectivo hospedeiro, associada à sua potencial plasticidade genômica, podem resultar em alterações genéticas de grau elevado, bem como na transferência lateral de genes simbióticos para rizóbios nativos não-simbióticos.

2 OBJETIVOS

Este trabalho tem como objetivo a caracterização morfológica, sorológica e genética de estirpes de *B. japonicum* e *B. elkanii* isoladas de nódulos de soja, sete anos após a introdução das estirpes CPAC 15 e CPAC 7 em um solo da região dos Cerrados, originalmente isento de estirpes capazes de estabelecer simbiose efetiva com a soja.

2.1 OBJETIVOS ESPECÍFICOS

a. Classificar as estipes reisoladas através da caracterização do gene ribossomal 16S rDNA através da técnica de PCR-RFLP;

b. Caracterizar a variabilidade genética das estirpes reisoladas em relação às inicialmente inoculadas através do perfil de BOX-PCR, seqüenciamento parcial do gene *nod*C e perfil de PCR-RFLP do gene *nifH;*

c. Analisar comparativamente os parâmetros genéticos analisados com a caracterização morfológica e sorológica das estirpes.

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Variability in *Bradyrhizobium japonicum* and *B. elkanii* Seven Years After Introduction of Both the Exotic Microsymbiont and the Soybean Host in a Cerrados Soil

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Abstract

The plasticity of rhizobial genomes is far greater than previously thought, with complex genomic recombination events that may be accelerated by the often-stressful environmental conditions of the tropics. This study aimed at evaluating changes in soybean rhizobia due to adaptation to inhospitable environmental conditions (high temperatures, drought and acid soils) in the Brazilian Cerrados. Both the host plant and combinations of four strains of soybean *Bradyrhizobium* were introduced in an uncropped soil devoid of rhizobia capable of nodulating soybean. After the third year, seeds were not reinoculated. Two hundred and sixty-three isolates were obtained from nodules of field-grown soybean after the seventh year, and their morphological, physiological, serological and symbiotic properties determined, followed by genetic analysis of conserved and symbiotic genes. *B. japonicum* strain CPAC 15 (same serogroup as USDA 123) was characterized as having high saprophytic capacity and competitiveness, representing up to 70% of the population by the seventh year, in contrast to *B. japonicum* strain CPAC 7 (same serogroup as CB 1809). In general, adapted strains had increased mucoidy, and up to 43% of the isolates showed no serological reaction. High variability, presumably resulting from the adaptation to the harsh environmental conditions, was verified in *rep*-PCR profiles, being lower in strain CPAC 15, intermediate in *B. elkanii* and higher in CPAC 7. RFLP-PCR types of the 16S rDNA corresponded to: one type for *B. elkanii* species, two for *B. japonicum*, associated to CPAC 15 and CPAC 7, and unknown combinations of profiles. However, when *nodC* sequences and RFLP-PCR of the *nifH* region data were considered, only two clusters were observed with full congruence with *B. japonicum* and *B. elkanii* species. Combining the results, variability was such that even within a genetically more stable group, such as that of CPAC 15, only 6.4% of the isolates showed high similarity to the inoculant strain, whereas none was similar to CPAC 7. The genetic variability in our study seems to result from a variety and combination of events including strain dispersion, genomic recombination and horizontal gene transfer. Furthermore, the genetic variability appears to be mainly associated with adaptation, saprophytic capacity and competitiveness, and not with symbiotic effectiveness, as the similarity of symbiotic genes was higher than that of conserved regions of the DNA.

Introduction

It is broadly accepted that nitrogen (N_2) fixation is one of the most important biological processes on earth, with the highest contribution occurring from the symbiosis of legumes with root-nodule bacteria collectively called "rhizobia," representing the most efficient, well developed and intensively studied model of beneficial plant-microbe interaction. Although the biological process has been recognized almost 120 years ago, the last decade has been critical in breaking many established paradigms of rhizobial ecology. Improved understanding of relatedness between and within species has been achieved mainly due to the availability of new molecular tools, which have allowed the detection of a wide range of new N_2-fixing bacteria and the realization that rhizobial genomes have an enormous plasticity with important implications for ecology [14, 61].

Examination of diazotrophic symbiotic bacteria especially in the still poorly documented soils of the tropics has led to the identification of unsuspected new genera not only within the α-Proteobacteria, *e.g. Methylobacterium* [60] and *Devosia* [46], but also within the β-Proteobacteria, with a variety of *Burkholderia* capable of nodulating and fixing N_2 in association with several host legumes of the Mimosoideae [37] and Papilionoideae [34] subfamilies.

The plasticity of rhizobial genomes is far greater than what was previously thought; complex genomic recombination events, including horizontal gene transfer, deletion, insertion and integration of various DNA elements, mutations and rearrangements, have led to extensive rhizobial variability. This was emphasized with the complete sequencing of some rhizobial genomes and one major example was found in *Bradyrhizobium japonicum*, with a high number of transposases, repetitive RSα and insertion sequences HRS1 [27], which may lead to insertions and deletions of genes, as well as transposition and genomic rearrangements [13, 27, 35]. In addition, the genes related to nodulation and N_2 fixation in both *B. japonicum* [13, 27] and *Mesorhizobium loti* [26] are located in 500- to 600-bp regions of the chromosome called symbiotic islands, potentially transferable to other rhizobia [36, 57]. A major change in the paradigms came from the reports that rhizobia can persist in soil in the absence of the host legumes [51, 57] and may acquire the symbiotic island from an inoculant strain upon legume reintroduction [57, 58, 59].

The extreme plasticity of rhizobia is also impacting the boundaries of phylogeny and taxonomy. First, although the conserved nature of the ribosomal genes, with emphasis on 16S rRNA, has led to its use for tracing bacterial phylogenies and defining taxonomy [10, 75], recombination in 16S [67] and 23S rRNA [40] genes of rhizobia has also been reported. Second, horizontal gene transfer resulting in strains with housekeeping and symbiotic genes from different species—which has been suggested, *e.g.* in *Rhizobium mongolense* [71], *Methylobacterium* [60], and *Burkholderia* [34]—might be more common than previously thought. As Thompson noted [64], biologists often distinguish between evolutionary and ecological time, but for some evolutionary processes metapopulation structure can alter the genetic boundaries of species within mere decades.

Rhizobial diversity can also be affected by interactions with the host plant and agricultural practices [*e.g.*, 7, 28, 44, 58]; furthermore, environmental stresses typical of the tropics, such as high temperatures, may accelerate genetic recombination [*e.g.*, 53, 61]. Indeed, a high level of morphological, physiological, genetic and symbiotic variability has been reported after some years of adaptation of exotic soybean rhizobia in the harsh environment of the Brazilian Cerrados, an edaphic type of tropical savanna occupying 207 million hectares that is characterized by acid soils, aluminum toxicity, high temperatures and drought conditions [1, 9, 18, 20, 38, 49]. Consistent with the recognition that adaptation to environmental and cropping conditions can affect the diversity of soybean rhizobia, the magnitude of the variability detected in the Brazilian Cerrados is greater than under less-severe environmental conditions, *e.g.*, in *B. japonicum* in the USA [25] and in France [2, 39].

Our objective was to gain better understanding of the variability that occurs in conserved and symbiotic genes during adaptation of soybean rhizobia to the inhospitable conditions of the Brazilian Cerrados. For that, isolates from field-grown soybean nodules were collected 7 years after introduction both of soybean and of inoculants containing strains of *B. japonicum* and *B. elkanii* in an uncropped soil initially devoid of rhizobia capable of nodulating this host.

Material and Methods

Reference Strains. Reference strains of soybean [*Glycine max* (L.) Merrill] rhizobia were: *Bradyrhizobium japonicum* strains SEMIA 566 (very competitive, belongs to the same serogroup as USDA 123; used in Brazilian commercial inoculants from 1966 to 1978); CPAC 15 (=SEMIA 5079, natural variant of SEMIA 566 selected after adaptation to the Cerrados; used in commercial inoculants since 1992); CB 1809 (=SEMIA 586, very effective in fixing N_2, but it shows poor competitiveness; used in commercial inoculants in 1977); CPAC 7 (=SEMIA 5080, natural variant of CB 1809, selected for adaptability to the Cerrados soils and shows greater competitiveness than CB 1809; used in commercial inoculants since 1992); and *B. elkanii* strains SEMIA 587 (used in commercial inoculants from 1968 to 1975 and since 1979) and 29W (=SEMIA 5019, used in commercial inoculants since 1979). More information about the strains is available elsewhere [19, 49]. CPAC 15 and CPAC 7 represent most of the strains applied to areas of the Cerrados cropped to soybean [19]. Type strains included in the study, *B. japonicum* USDA 6^T and *B. elkanii* USDA 76^T, were provided by the United States Department of Agriculture (USDA, Beltsville, MD).

Rhizobial Isolation. For this study rhizobial isolates were obtained from nodules of field-grown soybean in an experiment that has been in progress since 1993 at the Experimental Station of the Brazilian Cerrados Research Center (Embrapa Cerrados), in Planaltina, Federal District, Brazil. The experiment is arranged in a completely randomized block design, in plots of 4 × 17 m, with fifteen treatments, each with three replicates; the experiment has been described in detail by Mendes *et al.* [33]. The soil is classified as a clay dark-red oxisol, initially covered with the indigenous Cerrados vegetation, that had never been inoculated with rhizobia. At the establishment of the experiment, tests showed that the soil was devoid of indigenous rhizobia capable of establishing an effective symbiosis with soybean. In the first year, each plot was inoculated with one of four serologically distinct strains: *B. japonicum* strains CPAC 15 and CPAC 7 and *B. elkanii* strains 29W and SEMIA 587. In the second year, each plot was subdivided and either not inoculated, or inoculated with CPAC 7 or CPAC 15. In the third year, the entire area was inoculated with CPAC 7, and subsequently none of the plots was inoculated, although cropping was continued. Every year

the plots were cropped with soybean in the summer season (November–December to April–May), except for the fifth year when maize (*Zea mays* L.) was planted [33]. In this study, we have chosen to focus only on the nine treatments that had been inoculated with *B. japonicum* strains CPAC 15 and CPAC 7, as shown in Table 1.

In the seventh year of the experiment, forty nodules per treatment were randomly collected from field-grown soybean plants of cultivar Doko and rhizobia were isolated using standard procedures [70]. Purity of the cultures was confirmed by repeatedly streaking the bacteria on yeast extract-mannitol-agar (YMA) medium [70] and verifying a single type of colony morphology, and uniform absorption of Congo red and Gram-stain reaction. Some isolates stopped growing after isolation, therefore from twenty-one to thirty-nine isolates were obtained from each treatment, totalling two hundred and sixty-three rhizobia (Table 1). Single colonies were individually transferred to YM liquid broth (YMB) and after growth were mixed with glycerol (25%) and stored at –80°C. Working cultures were maintained on YMA slants at 4°C. Rhizobia were cultured routinely at 28°C in YMB on a rotary shaker operating at sixty-five cycles per minute.

Colonies were characterized in relation to size, color, mucoidy and acid/alkaline reaction as described previously [70], after seven days of growth in the dark at 28^{o}Con YMA containing either Congo red or bromothymol blue as a pH-change indicator.

Serotyping of Rhizobial Isolates. Serotyping was done by immuno-agglutination [70]. Polyclonal antisera were prepared against the somatic thermo-stable antigens of strains 29W (serogroup 29W), SEMIA 587 (serogroup 587), CPAC 15 (same serogroup as SEMIA 566) and CPAC 7 (same serogroup as CB 1809), as described by Somasegaran and Hoben [54].

Extraction of DNA. DNA was extracted from reference strains and rhizobial isolates as described before [28]. To obtain clean DNA, the extraction procedure included the addition, for each 400 μL of bacteria resuspended in TE 50/20, of 50 μL of 10% SDS, 5 μL of proteinase K (20 mg mL^{-1}), 10 μL of lysozyme (5 mg mL^{-1}), and 2 μL of RNAse (10 mg mL^{-1}). After two steps of purification with ethanol at 99.5% and at 70%, the pellet was resuspended in 50 μL of TE 10/1 to estimate the concentration of the DNA. Samples were then diluted to 20 ng of DNA μL^{-1} and were kept at –20°C.

Restriction Fragment Length Polymorphism (RFLP) of the PCR-Amplified DNA Region Coding for the 16S rRNA Gene. A nearly full-length portion

of the 16S rDNA (1,500 bp) was amplified from each strain and isolate by PCR with rD1 (3'-cccgggatccaagcttAAGGAGGTGATCCAGCC-5') and fD1 (5'-ccgaattcgtcgacaacAGAGTTTGATCCTGGCTCAG -3') primers, which correspond to positions 8-27 and 1524-1540 respectively, of *Escherichia coli* 16S rRNA gene [73]. Volumes and cycles used in the reaction were as described before [11], except for a decreased concentration of Taq DNA polymerase (1.0 U), and the reaction was carried out in an MJ Research Inc. PTC 200 thermocycler. Six-µL aliquots of the PCR products were digested separately with each of the following restriction endonucleases: *Hpa*II (5' – C/CGG – 3'; 3' – GGC/C – 5'), *Hha*I (5' - GCG/C – 3'; 3' – C/GCG – 5') and *Dde*I (5' – C/TNAG – 3'; 3' – GANT/C – 5') (Invitrogen™), as recommended by the manufacturers. The fragments obtained were analyzed by electrophoresis in a gel (17 × 11 cm) with 3% agarose, and carried out at 90 V for 4 h. The 1 kb Plus DNA Ladder (Invitrogen™) was used as a molecular size marker at the right, left and central lanes of each gel. The gels were then stained with ethidium bromide and photographed under UV radiation with a Kodak Digital Science 120 apparatus. The profiles obtained were confirmed in triplicates.

rep-*PCR Fingerprinting with BOX Primer.* PCR amplification of repetitive regions of the DNA (*rep*-PCR) was carried out with BOX-A1R primer (5'-CTACGGCAAGGCGACGCTGACG-3', Invitrogen™) [69]. Volumes and amplification cycles were performed as described before [28] and the reaction was performed in an MJ Research Inc. PT 200 thermocycler. The amplified fragments were separated by electrophoresis on 1.5% agarose (low EEO, type I-A) gels (20 × 25 cm), at 120 V, for 7 h. The 1 kb Plus DNA Ladder (Invitrogen™) was used as a molecular marker at the right, left and central lanes of each gel. Gels were stained with ethidium bromide, visualized under UV radiation and photographed.

RFLP-PCR of the DNA Region Coding for the nifH *Gene.* Primers nifHF and nifHI [31] were used to amplify a region of the DNA of approximately 800 bp of the *nifH* gene. Volumes and amplification cycles were performed as described by Laguerre *et al.* [31]. Six-µL aliquots of the PCR products were digested separately with each of three restriction endonucleases: *Hpa*II, *Hind*III (5' – A/AGCTT – 3'; 3' – TTCGA/A– 5') and *Hae*III (5' – GG/CC – 3'; 3' – CC/GG – 5') (Invitrogen™), as recommended by the manufacturers. The fragments obtained were analyzed as described in the item of RFLP-PCR of the 16S rDNA.

Sequencing of nodC Gene. DNA amplification of *nodC*-gene region was carried out using nodCIF (5′- GTCGATTGCMRGTCAAGACTACG- 3′) and nodCp8 (5′- GCCAGGTCTIGTTGCGATTGCTC – 3′) primers [55]. The amplification consisted of an initial cycle of denaturation at 94ºC for 1 min and 10 s; thirty-five cycles of denaturation at 94ºC for 20 s; of annealing at 59ºC for 50 s; of extension at 72ºC for 1 min and 20 s; and a final extension step at 72ºC for 5 min. A PCR product of about 300 bp was obtained and was purified as described before [34]. Sequencing of the fragments was performed using the same primers (nodCIF and nodCp8) and the procedure described by Menna *et al.* [34], in a MegaBACE Amersham Biosciences automated sequencing system with dye terminator chemistry.

Data Analyses. The sizes of the fragments in each analysis were normalized according to the MW of the DNA markers. The fingerprintings obtained by both the RFLP-PCR of the 16S rDNA and *nifH* genes and the *rep*-PCR were analyzed using BioNumerics software (Applied Biosystems, Kortrijk, Belgium, version 1.50). In the RFLP-PCR analysis, first the pattern with each restriction enzyme was obtained and then the combined analysis of the three enzymes was performed. A tolerance of 3% was established in the Bionumerics for the BOX analysis. The unweighted pair-grouping method with arithmetic mean (UPGMA) algorithm [52] with the coefficient of Jaccard [22] were used in both RFLP-PCR and *rep*-PCR analyses.

For the *nodC* region, sequences confirmed in the 3′ and 5′ directions were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek significant alignments.

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For the *nodC* region, sequences confirmed in the 3´ and 5´ directions were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) to seek significant alignments.

The sequences obtained have been deposited in the GenBank database under accession numbers DQ376585 through DQ376617. Multiple alignments were performed with ClustalX version 1.83 [63]. Phylogenetic trees were generated using MEGA version 3.1 [30] with default parameters, K2P distance model [29] and the Neighbor-Joining algorithm [47]. The sequences obtained were aligned and compared to those of the following type/reference strains (accession numbers of the GenBank Data Library in parentheses): *B. japonicum* USDA 6^{T} (D28962), *Rhizobium tropici* CFN 299 (X98514), *B. elkanii* USDA 46 (D28963), *Rhizobium* sp. NGR234 (NC000914), *Sinorhizobium meliloti* 1021 (AE007237). *Azorhizobium caulinodans* ORS571 (L18897) was used as an out-group reference. Statistic support for tree nodes was evaluated by bootstrap analysis [5] with 2,000 samplings [16].

Results

Morphological Characterization and Serotyping. All but one of the two hundred and sixty-three isolates alkalinized the medium containing mannitol as carbon source, showing typical properties of *B. japonicum/B. elkanii in vitro*. Large variability was detected in mucoidy, ranging from dry colonies to copious production of mucus, and isolates varied also in relation to the mucus viscosity, from aqueous to sticky. Size of the colonies varied from 0.2 to 2.5 mm in CPAC 15, from 0.2 to 2.0 mm in CPAC 7, and from 0.2 to 1.5 mm in *B. elkanii* SEMIA 587 and 29W. Size and mucoidy were not associated with any of the treatments from which the isolates were obtained (data not shown).

In relation to the serotype CPAC 15, by the seventh year from 40.0 to 69.7% (treatments inoculated with CPAC 15 in the first and in the second year, respectively) of the isolates reacted with the antiserum of CPAC 15 (Table 2). It is also noteworthy that treatments 7, 8 and 9 showed the highest percentages of isolates belonging to serogroup CPAC 15, although the first two had never been inoculated with that strain. In contrast, only a few isolates from two treatments reacted with the antiserum of CPAC 7, representing 7.1 and 5.1% of the isolates from treatments 2 and 5, respectively (Table 2).

Although none of the plots had been inoculated with *B. elkanii* strains, isolates belonging to serogroup SEMIA 587 represented up to 8.0% of the isolates in treatment 4, but were absent in the plots inoculated with CPAC 15 in the second year (treatments 3, 6 and 9). Serogroup 29W was detected in all treatments except those inoculated with CPAC 7 for three consecutive years, with the highest percentages occurring in plots inoculated with CPAC 15 in the first year—24.0 and 17.9%—for treatments 4 and 5, respectively. Isolates with unknown serological reaction were detected in all treatments and represented from 13.6 (treatment 7) to 42.9% (treatment 2) of the total number of isolates. In addition, unknown serogroups occurred mainly in treatments 1, 2 and 3, inoculated with strain CPAC 7 in the first year. Double reactions occurred with very few isolates (Table 2).

Low, medium and high production of mucus as well as variability in colony size were observed in isolates from all four serogroups; however, higher mucoidy was more often verified in the CPAC 15 serotype (data not shown).

RFLP-PCR of the 16S rDNA. When the DNA of all of the isolates and reference strains were analyzed by RFLP-PCR of the 16S rDNA, two different profiles were

obtained with the *Hha*I and *Hpa*II restriction enzymes, and three were obtained with *Dde*I (Fig. 1). Both strains belonging to serogroup of CPAC 15 (parental SEMIA 566 and variant CPAC 15) showed similar profiles to *B. japonicum* USDA 6^{T}. However, serogroup of CPAC 7 (parental SEMIA 586 and CPAC 7) was different in the fingerprinting obtained with enzyme *Dde*I. The three *B. elkanii* strains (SEMIA 587, 29W and USDA 76^{T}) showed similar profiles with all three enzymes (Table 3).

The combined RFLP-PCR profiles of all isolates were compared with those of reference strains and grouped according to treatment and serotyping (Table 2). In relation to the dominant serotype CPAC 15, the great majority of the isolates showed congruence between the serological properties and the RFLP profiles. In the first three treatments—all inoculated with CPAC 7 in the first year—there were increases in the percentage of isolates showing similarity to the CPAC 15-serogroup-RFLP type from treatment 1 (non-inoculated) to 3 (inoculated with CPAC 15 in the second year), representing from 63.6 to 88.9% of all isolates belonging to this serogroup. In treatments not inoculated in the first year (7, 8 and 9), CPAC 15-serogroup-RFLP type represented the majority of the isolates, probably due to high saprophytic capacity of a few contaminants in the first year (Table 2).

Isolates of the CPAC 7-serogroup-RFLP type were present exclusively in treatments 2 and 5, the first inoculated with CPAC 7 for three consecutive years and the second inoculated with this strain in the second and third years. The CPAC 7-RFLP type represented 50% of all isolates belonging to this serogroup, the other 50% being represented by isolates of the *B. elkanii*-RFLP type (Table 2).

Although SEMIA 587 and 29W were not used as inoculants in the plots in this study, strains showing similar serogroup-RFLP types of both strains were detected in all treatments, but generally at low percentages, and probably represent contamination from other plots. In addition, the *B. elkanii*-RFLP type was detected in the CPAC 15 but not in the CPAC 7 serogroup (Table 2).

In relation to the isolates with unknown serological reaction that represented up to 42.9% of all isolates, the great majority was mainly of the CPAC 15-RFLP type and of *B. elkanii*; however, the CPAC 7 type was also detected in all but treatments 8 and 9. Unknown RFLP profiles were observed in six isolates; five others that showed cross-serological reactions were all *B. elkanii*-RFLP type. There was no indication of a relationship between treatment and the number of isolates with different RFLP types (Table 2).

rep-*PCR Fingerprinting.*. From eight to fifteen isolates were taken from each treatment and were used for the *rep*-PCR fingerprinting, resulting in one hundred and nine profiles and a dendrogram was built with those isolates as well as with the four inoculant strains. Three main clusters were observed, related to the CPAC 7, CPAC 15 and SEMIA 587-29W inoculant strains, respectively (Fig. 2).

The first cluster joined the isolates resembling CPAC 7 at a final level of similarity of 75.5% and clearly showed high variability; moreover, none of the isolates showed a profile exactly the same as the inoculant strain. Within this cluster, both the disappearance of bands and the appearance of new bands were observed in comparison to inoculant strain CPAC 7. Additionally, the first five isolates were positioned in a subcluster considerably different from the parental strain (Fig. 2).

The second and largest group included isolates identical or resembling the inoculant straing CPAC 15 at a final level of similarity of 78.8%. Within this cluster, a first group included seventeen isolates showing high similarity (91.5%) with CPAC 15, while the last twenty strains were grouped in two subclusters characterized mainly by the absence of several bands in comparison to CPAC 15. Four strains occupied an intermediate position between CPAC 15 and *B. elkanii* clusters, showing bands of both types (Fig. 2).

Inoculant strains SEMIA 587 and 29W showed identical *rep*-PCR profiles and thirty-seven isolates were grouped in a third cluster with those two strains at a final level of similarity of 66.5%. Interestingly, the variability of the isolates within this group was mainly associated with the appearance of new bands (Fig. 2).

RFLP-PCR of the DNA Region Coding for the nifH *Gene.* The DNA region containing approximately 800 bp of the *nifH* gene was amplified in twenty-nine isolates, chosen to represent all treatments, and submitted to RFLP with three restriction enzymes. Only two patterns were obtained, one related to *B. japonicum*—including strains CPAC 7 and CPAC 15—and the other related to *B. elkanii* (Fig. 3).

nodC *Partial Sequence Analysis.* Partial sequences (280 bp) of the PCR-amplified *nodC* fragments were also determined for the twenty-nine isolates used in the analysis of RFLP-PCR of the *nifH* region. The dendrogram obtained with the partial sequences of isolates and reference strains clearly showed two distinct clusters, each with a bootstrap support of 99% (Fig. 4). Again, one cluster included both RFLP-16S rDNA types of *B. japonicum* and the second included the RFLP-16S rDNA type of *B. elkanii*. Furthermore, the same cluster included isolates showing variability in the *rep*-PCR analysis. For example,

isolates from the *rep*-PCR cluster showing similarities of 69.6 [70] to 100% with CPAC 15 (Fig. 2) showed complete similarity of bases of the *nodC* sequences (Fig. 4). Additionally, for strains belonging to non-congruent RFLP and serological groups, including unknown serotypes, *nodC* group was correlated with RFLP type.

Combining the Serotyping, RFLP-PCR of the 16S rDNA and rep-*PCR Data.* Serological reaction, RFLP-PCR of the 16S rDNA and *rep*-PCR profiles of the one hundred and nine isolates were analyzed together to investigate possible mechanisms of variability occurring after the introduction of inoculant rhizobial strains and the host plant in the soil. DNA regions encoding *nodC* and *nifH* were not considered because of the full congruence with the 16S rDNA and the lower level of information, as only two types were observed, corresponding to *B. japonicum* and *B. elkanii* species.

Rhizobial variability observed seven years after the introduction in a Cerrados soils was very high, for example, isolates obtained from plots inoculated with CPAC 15 and showing high similarity in all properties to the inoculant strain (6.4%) represented as much as the isolates from plots not inoculated with CPAC 15 but with dispersion of that strain from other areas and also showing high variability in the *rep*-PCR profiles (Table 4).

Discussion

In our study, rhizobial isolates were obtained from a soil initially devoid of indigenous soybean rhizobia to which *B. japonicum* strains CPAC 15 (natural variant of SEMIA 566) and CPAC 7 (natural variant of CB 1809) were added as inoculants in different combinations for three years, with no further inoculation. SEMIA 566 was one of the first strains "selected" in Brazil; isolated from a soybean nodule in the early 1960s, it effectively nodulated the cultivars released by that time, therefore it was commercially recommended from 1966 to 1978 and greatly contributed to the successful establishment of the crop in the southern region of Brazil. More than a decade later, increased N demands of newer and more productive soybean genotypes dictated the selection of strains with higher N_2-fixation capacity. The more effective variant of SEMIA 566, CPAC 15, has been used in Brazilian inoculants since 1992 [7, 18, 19, 21, 38, 43]. Since 1986, there have been reports showing that strains related to this serogroup may occur in up to 70% of the soybean nodules growing in

the Brazilian Cerrados, even in areas that had never been inoculated before. This has been attributed to dissemination on seeds and on agricultural machinery from other soybean-growing areas in southern Brazil [7, 68], as well as by wind [7]. SEMIA 566 and CPAC 15 differ from USDA 123 in *rep*-PCR (ERIC primer), lipopolysaccharide and protein profiles [7], but they are serologically related to USDA 123 [33], considered as the most competitive of *B. japonicum* strains in the mid-western soils of the USA [*e.g.*, 15, 5]. In contrast, strain CB 1809 is recognized as very effective in fixing N_2 but poorly competitive in relation to many soybean rhizobial strains. Therefore, again in the late 1980s, a natural variant with improved competitiveness (CPAC 7) was obtained and has been commercially recommended since 1992 [7, 18, 19, 21, 38, 43]; however, competitiveness is poor when compared to CPAC 15 [33].

Differences among isolates in our study were first seen in colony size and mucus production (extracellular polysaccharides, EPSs); especially in serogroup CPAC 15, mucus production tended to increase with adaptation, similar to prior observations by Boddey and Hungria [1]. A previous study with soybean rhizobia from the Brazilian Cerrados, emplying pyrolysis mass spectrometry both of cells and their polysaccharides, showed that nodule isolates differed from the parental strains, indicating that adaptation had affected polysaccharide composition [4]. Indeed, Pellock *et al.* [43] suggested that quantitative and qualitative differences in EPSs might represent a selective advantage, allowing the strain to interact as efficiently as possible under a variety of conditions and with many cultivars or ecotypes of legumes. In addition, in *S. meliloti* three symbiotically important EPSs (succinoglycan, EPS II, and K antigen) are produced and for at least with one of them (EPS II) the production is associated with the excision of an insertion sequence [43]. As those DNA elements are often related to genetic recombination and are broadly detected in the genome of *B. japonicum* [27] they might also be related to the changes in mucoidy of adapted isolates.

Polyclonal antibodies of each strain were used in the somatic agglutination reaction. The main limitation of this methodology in ecology studies is cross-reaction with indigenous rhizobia [61]; however, that did not occur with the four inoculant strains first used as inoculants [33]. By the sixth year, serological reaction of fifty nodules collected from six plants of each replicate indicated that from 48 to 74% of the nodules were occupied by strains serologically related to CPAC 15, whereas only 2 to 9% were occupied by strains related to CPAC 7 [33]. High saprophytic capacity and competitiveness, as well as dispersion of serogroup CPAC 15 from other plots, were confirmed in the seventh year, representing from

40 to 70% of the isolates. The results showing that serogroup CPAC 15-USDA 123 become dominant in soils over time are consistent with previous reports both in the USA [56] and Brazil [33, 68] and emphasize the high saprophytic capacity of strains belonging to this serogroup. Interestingly, the studies of Freire *et al.* [8] and Mendes *et al.* [33] indicate that CPAC 15 prevailed in soybean nodules after the third year of introduction and similar reports were obtained for serogroup 123 in the USA [56]. Those observations might imply that strains belonging to serogroup CPAC 15-USDA 123 need a period of time in soils before becoming dominant. In contrast, the presence of CPAC 7 in nodules declined dramatically in the year after introduction, occupying only 2 to 9% of the nodules by the sixth year [33] and being undetectable in the seventh year.

In the USA, it has been often shown that in a soil harboring rhizobia the introduction of a new strain may fail or that the strain persists only in the first year [*e.g.*, 32, 62]. The situation is more critical if the established population belongs to a competitive serogroup such as USDA 123; inoculant rhizobia usually produce only 5 to 20% of the nodules after the first year [15, 45, 56]. Our study highlights the importance of continued inoculation with strains like CPAC 7 that are more effective in fixing N_2 but less competitive, in order to guarantee some nodule occupancy and N_2-fixation benefit while avoiding dominance of less effective but very competitive strains such as CPAC 15. Currently, massive annual inoculation using superior strains is the only strategy available to displace other established rhizobia in the tropics, where the continuous cropping year-round favors rhizobial persistence. Fortunately, in contrast to many reports from other countries, such as the USA [*e.g.*, 56, 62], soybean reinoculation in Brazil guarantees benefits in N_2 fixation and yield, probably because the inoculant rhizobia in a better physiological condition when applied to the seed [19].

In our study, by the seventh year many isolates showed unknown serological reactions. Recovery of a high percentage of unknown serogroups after some years of soybean cropping has been previously shown both in southern Brazil [8, 6] and in the Cerrados [68, 9]. Also in Australia, differences in serological patterns were found in variant strains nine years after the introduction of *B. japonicum* strain CB 1809 [12]. This may be a function of changes in cell surface, in one or more components of the outer cell membrane, including lipopolysaccharides and/or other polysaccharides. Indeed, this hypothesis is supported by the results of Johnson *et al.* [24], who showed that bacterial cell-surface markers may change over time. However, horizontal transfer of the symbiotic island from inoculant strains to

indigenous rhizobia [58, 59] might also help to explain the appearance of unknown serogroups nodulating soybean. Although Cerrados isolates belonging to unknown serogroups have mostly shown RFLP-16S rDNA types similar to CPAC 7, CPAC 15 and to *B. elkanii*, variability in the 16S-ribosomal genes may be low in *B. japonicum* and *B. elkanii* [*e.g.*, 66, 74], therefore indigenous rhizobia capable of receiving the symbiotic island might share similar profiles.

A high level of variability in *rep*-PCR profiles was observed within all three clusters, related to CPAC 7, CPAC 15 and *B. elkanii* SEMIA 587-29W. Dispersed DNA repeats, as BOX elements, differ extensively in number and location among prokaryote chromosomes and evolve at higher frequencies than insertion or deletions events; apparently they are related to a high degree of genomic flexibility, acting in specific adaptive evolution [65] and phenotypic variation [48]. Interestingly, apparently in the process of adaptation to the Cerrados soils BOX elements decreased in *B. japonicum* CPAC 15 and increased in *B. elkanii*, with both processes occurring in *B. japonicum* CPAC 7. In addition, higher variability occurred with the CPAC 7 serogroup and the lowest with most of the CPAC 15 isolates, serogroup that become dominant with time. Interestingly, an intermediate group with mixed profiles of CPAC 15 and SEMIA 587 was found, indicative of an intensive exchange of genetic material. The magnitude of the variability detected in *rep*-PCR profiles in our study supports previous reports with other isolates from the same region [9, 18, 49] and contrasts with the results obtained in less stressful conditions of soils of midwestern USA [25].

Isolates from our study fit into different RFLP-16S rDNA types. First, six isolates produced unknown patterns when compared to the reference and type strains. Second, *B. elkanii* strains SEMIA 587, 29W and USDA 76^{T} produced the same patterns with all three enzymes. Lastly, *B. japonicum* differed from *B. elkanii* with all three enzymes, however, serogroup CPAC 15 was similar to USDA 6^{T} but differed from serogroup CPAC 7 with enzyme *Dde*I. In fact, the analysis of the whole sequences of the 16S-rRNA genes of CPAC 7 and CPAC 15 [3] indicates that fragments with different sizes should be obtained with *Dde*I. As we have already mentioned, diversity in the 16S-rRNA sequences of *Bradyrhizobium* has been found to be low in most strains investigated so far [*e.g.*, 66, 74]. However, recent RFLP-PCR analysis of a collection of Brazilian *Bradyrhizobium* strains revealed differences not only in the 16S- but also in the 23S- and 16-23S-intergenic ribosomal regions, with CPAC 7 and CPAC 15 also fitting into different clusters [11]; the results were further confirmed with the complete sequencing of the 16S-rRNA gene [34]. Besides, CPAC 15 and CPAC 7 are

remarkably different in several phenotypic and symbiotic properties, as well as in protein, lipopolysaccharide, RAPD and *rep*-PCR profiles with ERIC and REP primers [1, 18, 19, 20, 21, 38, 49]. Sawada *et al.* [50] emphasized that *B. japonicum* strains are too diverse to be grouped into one species and we plan to investigate the genetic similarity of other genes in these two strains. Additionally, in studies by Germano *et al.* [11] and Menna *et al.* [34] even higher variability was confirmed within Brazilian *B. elkanii* strains; however, most of these strains were isolated from nodules of indigenous legumes species and not from soybean. Finally, it should be mentioned that in other bacterial species it has also been shown that strains showing identical 16S-rRNA gene sequences may be very different in other ribosomal regions, *rep*-PCR and even DNA-DNA hybridization, also showing differences in physiological properties. As Jaspers and Overmann [23] concluded, there is an increasing perception that natural diversity goes beyond the level of 16S rRNA.

To investigate the symbiotic genes, we chose one gene related to nodulation (*nodC*) and another one to N_2 fixation (*nifH*). These genes are relatively distant on the genome of *B. japonicum* [27], facilitating examination for possible horizontal transfer of the symbiotic island. Considering both the partial sequencing of *nodC* and the RFLP-PCR of *nifH*, CPAC 7 and CPAC 15 shared the same patterns, but differed from the profiles obtained for *B. elkanii*. Parker *et al.* [41], analyzing thirty-eight *Bradyrhizobium* strains from different continents, observed that all strains fitted either *B. japonicum* or *B. elkanii*, when the 16S-rRNA gene was considered. However, when the *nifD* gene of those same strains was analyzed, clustering occurred according to geographic region. In addition, phylogenies of both genes were statistically incongruent, suggesting that each area was initially colonized by several diverse 16S-rRNA lineages, followed by horizontal gene transfer of *nifD* within each area [41]. Likewise, analysis of ribosomal and *nod* and *nifH* genes in the *Astragalus sinicus-Sinorhizobium meliloti* [76] and *Bradyrhizobium*-several legume hosts symbioses, supported the hypothesis of horizontal transfer among diverse bacteria. [72]. The results from our study differ from those of Parker *et al.* [41] in the sense that we see full congruence between the conserved 16S-rRNA genes and symbiotic genes (*nodC* and *nifH*) of *B. japonicum* and *B. elkanii* strains. However, the variability detected in the 16S-rRNA gene of *B. japonicum* was higher than in the symbiotic genes, suggesting horizontal transfer between strains CPAC 7 and CPAC 15. Greater conservation of symbiotic genes in *B. japonicum*, in addition to the transferable nature of the symbiotic island [13, 27, 36] might be indicative of an ecological advantage related to the symbiotic effectiveness.

In conclusion, variability in several morphological, physiological, serological, genetic and symbiotic properties of isolates adapted to the Brazilian Cerrados in relation to the parental inoculant strains have been previously shown [1, 7, 49, 9]. In our study, emphasis was given to conserved and symbiotic genes and high variability was confirmed after seven years from introduction in a soil of the Brazilian Cerrados. The magnitude of rhizobial variability detected in isolates adapted to the harsh conditions of the Cerrados resembles differences obtained in RFLP and serological patterns after nine years in an Australian soil [12] and is much higher than that reported under less stressful environmental conditions in the USA [25] and France [2, 39]. The high genetic variability detected in our study is certainly related to the plasticity of the *Bradyrhizobium* genome [27]. However, without doubt, it was accelerated by the interaction with the host plant [17, 44], the adaptation to the environment [21, 44, 59, 58] and agricultural practices [6, 21, 28]. The variability in our study appears to have resulted from a variety of events including strain dispersion, genomic recombination and horizontal gene transfer. It is also reasonable to infer that the variability was associated with adaptation, saprophytic capacity and competitiveness, and not with symbiotic effectiveness, as *nodC* and *nifH* genes were more conserved. Certainly, the phenotypic and genotypic variability associated with the adaptation process has profound ecological implications that warrant further detailed study.

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Legend of figures

Figure 1. RFLP-PCR profiles of the 16S-rDNA region of reference strains of *B. japonicum* and *B. elkanii* obtained with three restriction enzymes. (M) represents the 1 kb Plus DNA Ladder (Invitrogen™) and A, B and C the profiles obtained with each restriction enzyme, as described in Table 3.

Figure 2. Cluster analysis (UPGMA with the coefficient of Jaccard) of the *rep*-PCR (primer BOX) products of soybean rhizobial isolates obtained after seven years from the introduction of inoculant strains (*B. japonicum* CPAC 15 and CPAC 7 and *B. elkanii* SEMIA 587 and 29W) in a Cerrados soil.

Figure 3. RFLP-PCR profiles of the DNA region coding for *nifH* gene of reference strains after digestion with three restriction enzymes. (M) represents the 1 kb Plus DNA Ladder (Invitrogen™), (A) the profiles with *B. japonicum* strains CPAC 7 and CPAC 15, and (B) profiles with *B. elkanii* strains SEMIA 587 and 29W.

Figure 4. Phylogenetic tree based on the *nodC* sequences of soybean rhizobial isolates and of reference strains of *B. japonicum* and *B. elkanii*. GeneBank accession numbers are given in the text. Analyses were conducted using MEGA version 3.1 and numbers in the main branches indicate bootstrap values obtained with 2,000 replicates.

Table 1. Treatments for the field experiment performed in a Brazilian Cerrados soil from which the rhizobial isolates were obtained.

Treatment	*Years*[a]							#
	1st	*2nd*	*3rd*	*4th*	*5th*	*6th*	*7th*	*Isolates*
1	CPAC 7	Non-inoc.	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	22
2		CPAC 7	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	28
3		CPAC 15	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	37
4	CPAC 15	Non-inoc.	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	25
5		CPAC 7	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	39
6		CPAC 15	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	28
7	Non-inoc.	Non-inoc.	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	22
8		CPAC 7	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	29
9		CPAC 15	CPAC 7	Non-inoc.	Non-inoc.	Non-inoc.	Non-inoc.	33

[a]Soybean was cropped every year except for the fifth year, cropped with maize.

Table 2. Percent distribution of soybean rhizobial isolates considering the serogroups and the RFLP-PCR profiles of the 16S rDNA.

Serogroup	*RFLP-PCR*	*Treatment*								
		1	*2*	*3*[a]	*4*[a]	*5*	*6*	*7*	*8*	*9*[a]
CPAC 15	CPAC 15	31.8	32.1	43.2	20.0	43.6	50.0	59.1	51.7	60.6
	CPAC 7	13.6	3.6	0.0	12.0	0.0	0.0	9.1	6.9	3.0
	B. elkanii	4.5	7.1	2.7	8.0	2.6	0.0	0.0	3.4	6.1
	unknown	0.0	0.0	2.7	0.0	0.0	3.6	0.0	0.0	0.0
Total		50.0	42.9	48.6	40.0	46.2	53.6	68.2	62.1	69.7
CPAC 7	CPAC 15	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CPAC 7	0.0	3.6	0.0	0.0	2.6	0.0	0.0	0.0	0.0
	B. elkanii	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	unknown	0.0	3.6	0.0	0.0	2.6	0.0	0.0	0.0	0.0
Total		0.0	7.1	0.0	0.0	5.1	0.0	0.0	0.0	0.0
SEMIA 587	CPAC 15	0.0	3.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	CPAC 7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.0
	B. elkanii	4.5	3.6	0.0	8.0	5.1	0.0	4.5	0.0	0.0
	unknown	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Total		4.5	7.1	0.0	8.0	5.1	0.0	4.5	3.4	0.0
29W	CPAC 15	0.0	0.0	5.4	0.0	2.6	0.0	0.0	3.4	0.0
	CPAC 7	0.0	0.0	0.0	4.0	0.0	0.0	0.0	3.4	0.0
	B. elkanii	4.5	0.0	5.4	16.0	15.4	7.1	13.6	0.0	6.1
	unknown	0.0	0.0	0.0	4.0	0.0	3.6	0.0	0.0	0.0
Total		4.5	0.0	10.8	24.0	17.9	10.7	13.6	6.9	6.1
Unknown	CPAC 15	13.6	21.4	18.9	20.0	10.3	25.0	4.5	20.7	15.2
reaction	CPAC 7	4.5	7.1	8.1	4.0	5.1	7.1	4.5	0.0	0.0
	B. elkanii	22.7	14.3	10.8	0.0	10.3	3.6	4.5	3.4	0.0
	unknown	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.4	0.0
Total		40.9	42.9	37.8	24.0	25.6	35.7	13.6	27.6	15.2

[a]Treatments 3, 4 and 9 had also one isolate with a 29W/587 double reaction, one with a 29W/CPAC 15 double reaction and three with a 29W/587 double reaction, respectively, and all with RFLP profiles of *B. elkanii*.

Table 3. Profiles[a] of RFLP-PCR of the 16S-rDNA region with three restriction enzymes obtained for each reference strain.

Strain	*Restriction enzyme*			*Serotype*
	Hha*I*	Hpa*II*	Dde*I*	
CPAC 7	b	b	b	CPAC 7
CB 1809	b	b	b	CPAC 7
CPAC 15	b	b	c	CPAC 15
SEMIA 566	b	b	c	CPAC 15
29W	a	a	a	29W
SEMIA 587	a	a	a	SEMIA 587
USDA 6^T	b	b	c	USDA 6
USDA 76^T	a	a	a	USDA 76

[a]Profiles as shown in Fig. 1.

Table 4. Percent distribution of one hundred and nine soybean rhizobial isolates based on the combined analyses of serological reaction, RFLP-PCR of the 16S rDNA and *rep*-PCR fingerprintings and on the inoculation history of the plot from which the isolates were obtained.

Characteristics of the isolates	*Isolates (%)*
Highly similar to inoculant strains[a]	
CPAC 15	6.4
CPAC 7	0.0
Showing variability[b] *in relation to the inoculant strains*	
CPAC 15	9.2
CPAC 7	0.9
Dispersion from other areas[c]	
CPAC 15	10.1
B. elkanii[d]	0.9
Dispersion[c] *and variability*[b] *in relation to the inoculant strains*	
CPAC 15	6.4
B. elkanii[d]	14.7
Mixed characteristics of the inoculant strains	
CPAC 15 and CPAC 7	
plots inoculated with both strains	2.8
plots inoculated only with CPAC 7	7.2
CPAC 15 and *B. elkanii*[d]	13.8
CPAC 7 and *B. elkanii*[d]	5.5
Mixed characteristics of the inoculant strains and unknown[e] *rhizobia*	
CPAC 15	9.2
CPAC 7	2.8
B. elkanii[d]	8.3
Mixed properties of more than one inoculant strain and unknown[e] *rhizobia*	
CPAC 15 and *B. elkanii*[d]	0.9

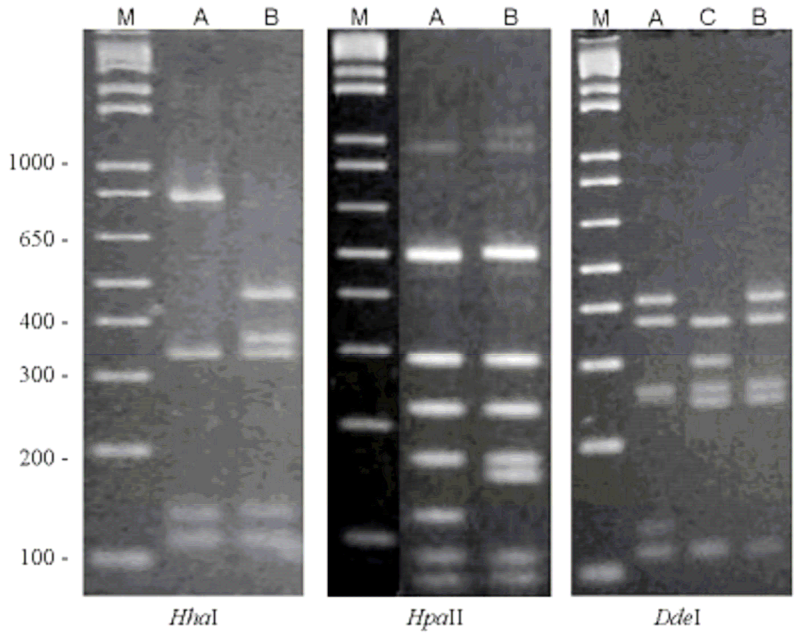
[a]To be considered similar to the inoculant strains, isolates should belong exclusively to the same serogroup, and show the same RFLP-PCR and *rep*-PCR profiles with similarity higher than 90% in comparison to the inoculant strain.

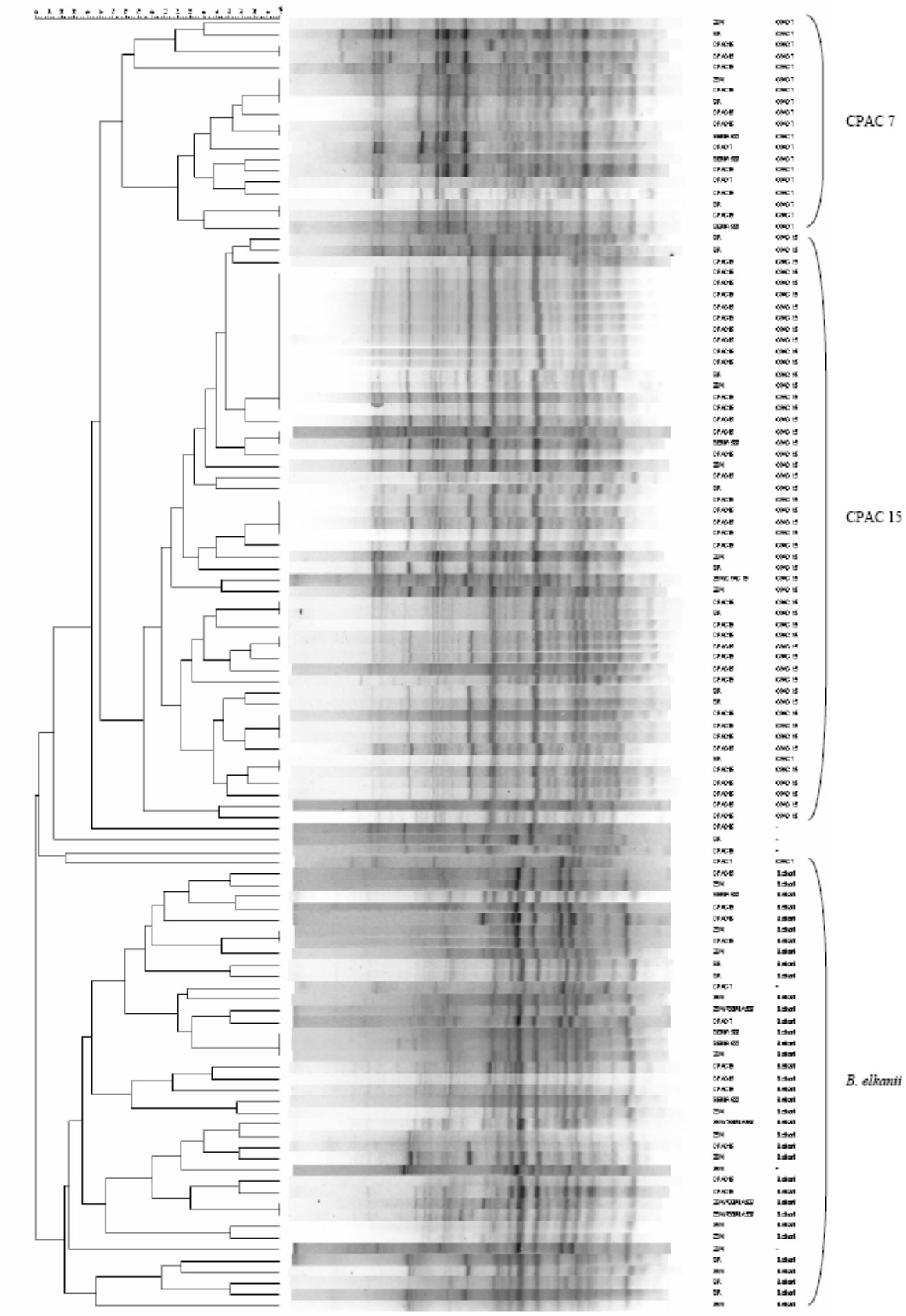
[b]Variability in *rep*-PCR profiles, with similarity lower than 90% in comparison to the inoculant strain.

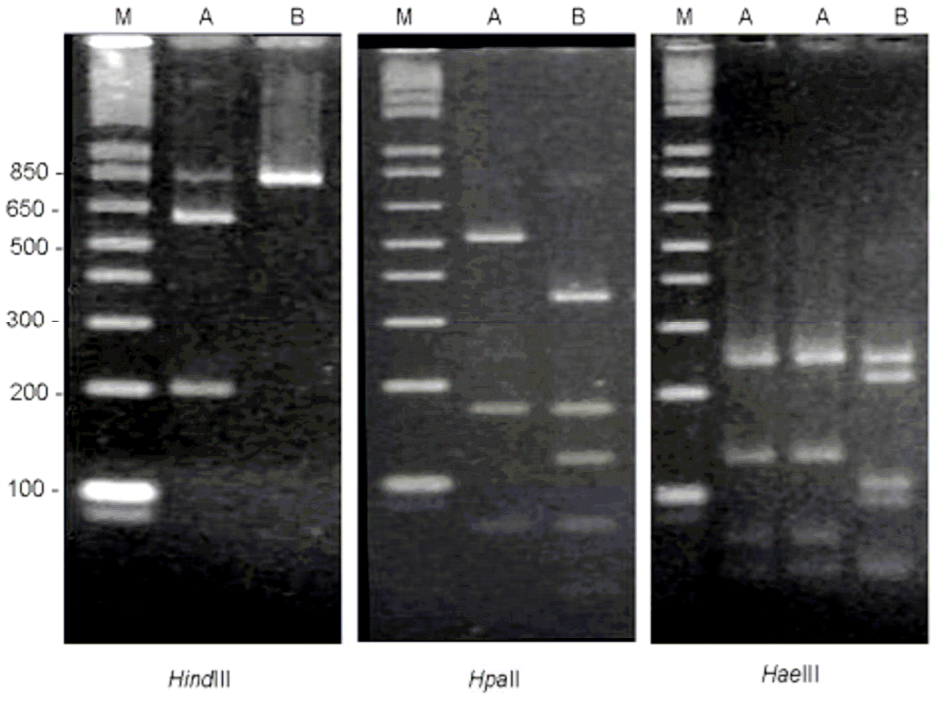
[c]Strain not used as inoculant in that plot.

[d]*B. elkanii* includes both strains SEMIA 587 and 29W as they shared similar RFLP-PCR and *rep*-PCR profiles.

[e]Showing RFLP-PCR profiles and/or serological reaction distinct from any of the inoculant strains.


M
A
B
M
A
B
M
A
C
B
1000 -
650 -
400 -
300 -
200 -
100 -
HhaI
HpaII
DdeI


CPAC 7
CPAC 15
B. elkanii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A
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500
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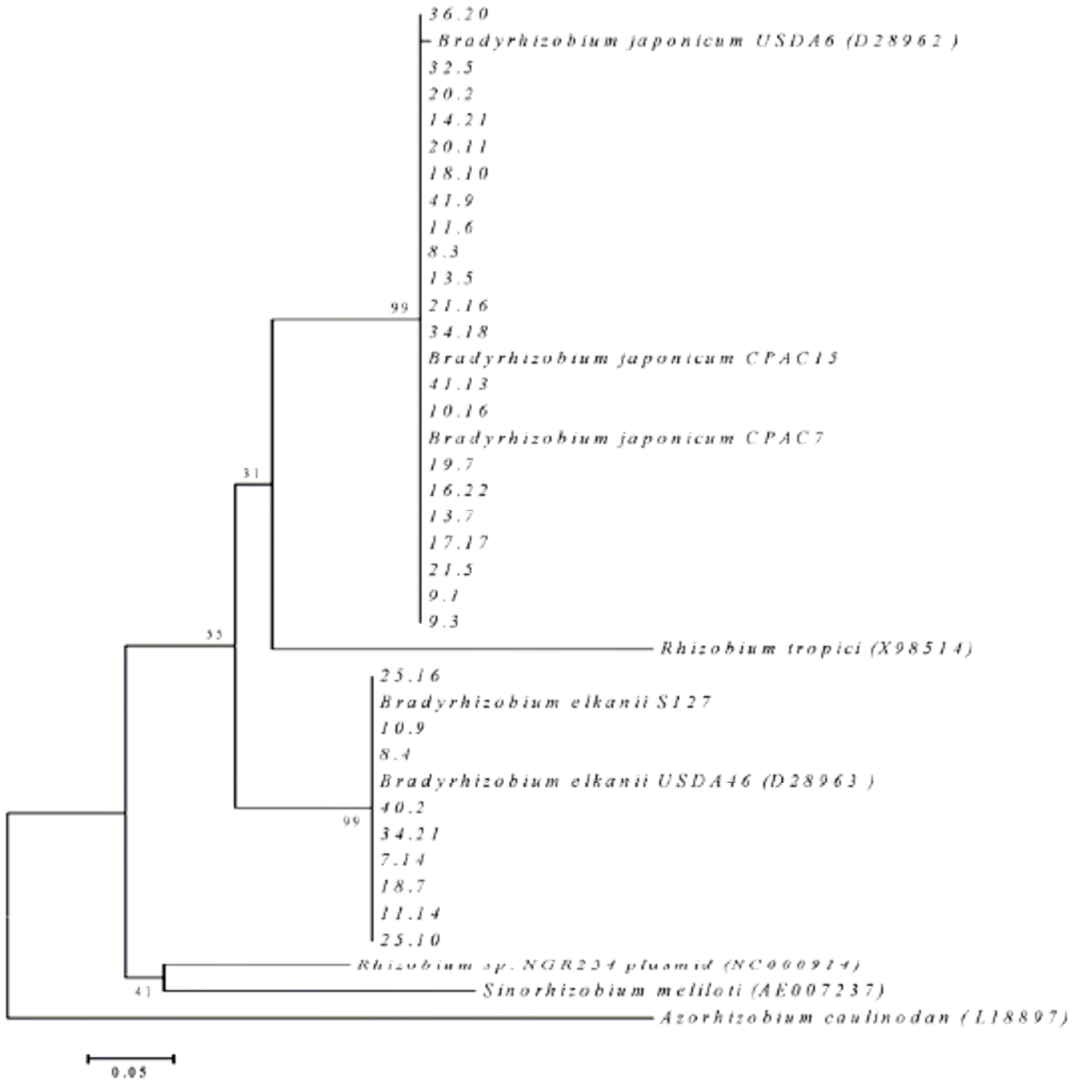
HindIII
HpaII
HaeIII


36.20
Bradyrhizobium japonicum USDA6 (D28962)
32.5
20.2
14.21
20.11
18.10
41.9
11.6
8.3
13.5
99
21.16
34.18
Bradyrhizobium japonicum CPAC15
41.13
10.16
Bradyrhizobium japonicum CPAC7
31
19.7
16.22
13.7
17.17
21.5
9.1
9.3
55
Rhizobium tropici (X98514)
25.16
Bradyrhizobium elkanii S127
10.9
8.4
Bradyrhizobium elkanii USDA46 (D28963)
99
40.2
34.21
7.14
18.7
11.14
25.10
Rhizobium sp. NGR234 plasmid (NC000914)
41
Sinorhizobium meliloti (AE007237)
Azorhizobium caulinodan (L18897)
0.05