

Symbiotic nitrogen-fixing bacterial populations trapped from soils under agroforestry systems in the Western Amazon

Paula Marcela Duque Jaramillo[§], Amanda Azarias Guimarães, Ligiane Aparecida Florentino^{§§}, Karina Barroso Silva, Rafaela Simão Abrahão Nóbrega[¶], Fatima Maria de Souza Moreira^{*}

Universidade Federal de Lavras – Depto de Ciência do Solo, C.P. 3037 – 37200-000 – Lavras, MG – Brasil.

*Corresponding author <fmoreira@dcs.ufla.br>

Edited by: Cláudio Marcelo Gonçalves de Oliveira

ABSTRACT: Cowpea (*Vigna unguiculata*) is an important grain-producing legume that can forego nitrogen fertilization by establishing an efficient symbiosis with nitrogen-fixing bacteria. Although inoculating strains have already been selected for this species, little is known about the genotypic and symbiotic diversity of native rhizobia. Recently, *Bradyrhizobium* has been shown to be the genus most frequently trapped by cowpea in agricultural soils of the Amazon region. We investigated the genetic and symbiotic diversity of 148 bacterial strains with different phenotypic and cultural properties isolated from the nodules of the trap species cowpea, which was inoculated with samples from soils under agroforestry systems from the western Amazon. Sixty non-nodulating strains indicated a high frequency of endophytic strains in the nodules. The 88 authenticated strains had varying symbiotic efficiency. The SPAD (*Soil Plant Analysis Development*) index (indirect measurement of chlorophyll content) was more efficient at evaluating the contribution of symbiotic N₂-fixation than shoot dry matter under axenic conditions. Cowpea-nodulating bacteria exhibited a high level of genetic diversity, with 68 genotypes identified by BOX-PCR. Sequencing of the 16S rRNA gene showed a predominance of the genus *Bradyrhizobium*, which accounted for 70 % of all strains sequenced. Other genera identified were *Rhizobium*, *Ochrobactrum*, *Paenibacillus*, *Bosea*, *Bacillus*, *Enterobacter*, and *Stenotrophomonas*. These results support the promiscuity of cowpea and demonstrate the high genetic and symbiotic diversity of rhizobia in soils under agroforestry systems, with some strains exhibiting potential for use as inoculants. The predominance of *Bradyrhizobium* in land uses with different plant communities and soil characteristics reflects the adaptation of this genus to the Amazon region.

Keywords: legume-nodulating bacteria, biodiversity, symbiotic promiscuity, chlorophyll content

Received March 15, 2013

Accepted July 19, 2013

Introduction

Agroecosystems generally support lower plant species diversity than ecosystems without human intervention. The reduction in above-ground diversity can have a marked influence on the diversity of edaphic organisms, affecting the biochemical processes essential for the sustainability of the forest. Agroforestry systems that seek to minimize these effects have been implemented by many communities in the Amazon, including those of the upper Solimões River (<http://www.biosbrasil.ufla.br/>). Several researchers conducted studies in the Amazon biome and have demonstrated the great diversity of N₂-fixing legume-nodulating bacteria (LNB) strains isolated from nodules in the field or by using different species as trap plants under controlled conditions to capture LNB from the soil (Guimarães et al., 2012; Chagas Junior et al., 2010; Lima et al., 2009; Moreira et al., 1998; Moreira et al., 1993).

Cowpea [*Vigna unguiculata* (L.) Walp] is an important food crop in Africa and Asia, and the most widely grown grain legume in northern and northeast Brazil, where it is grown primarily by small producers and represents one of the main subsistence crops. This species is considered promiscuous and can establish symbiosis with several species and genera of LNB belonging to the alphaproteobacteria and betaproteobacteria classes (Moreira, 2006). Nevertheless, cowpea can respond to inoculation with select strains of the genus *Bradyrhizobium* with an increase in productivity (Guimarães et al., 2012). The use of cowpea in studies assessing LNB diversity in soils is relevant because it also allows for the identification of native strains that are adapted to local conditions and potentially useful as inoculants. These studies also assess the need for inoculation when efficient native strains are not available.

Recently, *Bradyrhizobium* was shown to be the predominant microsymbiont of cowpea in agricultural soils from the Amazon (Guimarães et al., 2012). Agroforestry systems in the same region have varying soil characteristics (Moreira et al., 2009) as well as varying diversity of plant species, which could affect rhizobia diversity. The objective of this study was to investigate the genetic and symbiotic diversity of nitrogen-fixing bacteria from agroforestry systems in the upper Solimões River, western Amazon. Additionally, the indirect measurement of

[§]Present address: Universidade de Brasília – Depto de Biologia Celular, Campus Universitário Darcy Ribeiro – 70910-900 – Brasília, DF – Brasil.

[¶]Present address: Universidade Federal do Recôncavo da Bahia – Centro de Ciências Agrárias, Ambientais e Biológicas – Rua Rui Barbosa 710, Centro – 44380-000 – Cruz das Almas, BA – Brasil.

^{§§}Present address: Universidade José do Rosário Vellano, Faculdade de Agronomia, Rod. MG 179 km 0 – Campus Universitário - 37130-000, Alfenas, MG – Brasil.

chlorophyll content (SPAD index) was evaluated as a measure of N₂-fixation for the first time in cowpea.

Materials and Methods

Strain origin

Strains were isolated from soil samples collected from the municipality of Benjamin Constant, Amazonas State (4°21'–4°26' S and 69°36'–70°1' W), near the border of Brazil, Colombia, and Peru. The area comprises the town of Benjamin Constant and the communities of Guanabara II and Nova Aliança.

Six windows were distributed in the area: windows 1 and 2 in Guanabara II, windows 3, 4, and 5 in Nova Aliança, and window 6 in Benjamin Constant, where many chemical, physical, and biological studies of soils have been carried out by the BiosBrasil project (<http://www.biosbrasil.ufla.br>). The windows comprise the six land use systems representative of the region: primary forest, secondary forest (late successional state), secondary forest (early regeneration state), agroforestry systems, agriculture, and pasture. The location of sampling points and, sampling procedures were described by Moreira et al. (2009) and Guimarães et al. (2012).

A total of 148 strains were obtained from the following sampling points under agroforestry systems: 24, 22, 25, 20, 17, 24A, and 17A at window 2 (Guanabara II); and 66, 67A, and 67 at window 5 (Nova Aliança) (<http://www.biosbrasil.ufla.br>). The chemical characteristics of the soil samples were in the following ranges: pH in water: 4.6–5.5; K⁺: 34–124 mg dm⁻³; P: 1.4–10 mg dm⁻³; S: 3.3–173 mg dm⁻³; Al³⁺: 5–36 mmol_c dm⁻³; Ca²⁺: 4.6–12 mg dm⁻³; and Mg²⁺: 1.0–3.6 mg dm⁻³. Average sum of bases ranged from 65 to 158 mmol_c dm⁻³. Micronutrient levels were as follows: Fe²⁺: 75.6–231.7 mg dm⁻³; Zn²⁺: 2.0–14.6 mg dm⁻³; Mn²⁺: 11.2–99.7 mg dm⁻³; B: 0.0–0.7 mg dm⁻³; and Cu²⁺: 0.8–3.7 mg dm⁻³. Average organic matter content was 18 g kg⁻¹; and base saturation ranged from 30 to 62 %. Moreira et al. (2009) provides more details of these soils as compared to other land use systems in the same area. No commercial bacterial inoculants were applied to any of these soils.

At the agroforestry sampling points the following genera known to establish symbiosis with rhizobia were found: *Acacia*, *Calopogonium*, *Centrosema*, *Dalbergia*, *Derris*, *Desmodium*, *Entada*, *Inga*, *Machaerium*, *Mimosa*, *Pueraria*, *Piptadenia*, *Pityrocarpa*, *Swartzia*, and *Tachigali*; the tree species *Inga edulis* was the most abundant, followed by the herb *Calopogonium mucunoides*.

Cultural characteristics of each strain were evaluated in 79 media (Fred and Waksman, 1928) as described previously (Guimarães et al., 2012; Moreira et al., 1993).

Strain authentication and symbiotic efficiency

Authentication as symbiotic nitrogen-fixing bacteria was verified for all 148 strains via procedures described in detail by Guimarães et al. (2012), in two successive experiments carried out in axenic conditions

with nutrient solution (Hoagland and Arnon, 1950) in the greenhouse. The experiments were conducted over a period of 35 days, and 75 and 73 strains were analyzed in the first and second experiments, respectively. Strains approved as inoculants to cowpea by the Brazilian Ministry of Agriculture were included as positive controls (Moreira, 2006; Martins et al., 2003).

Symbiotic efficiency of nitrogen-fixing bacteria was also evaluated with the SPAD (*Soil Plant Analysis Development*) index of leaves of the middle and upper thirds of the plant, determined using a Minolta SPAD-502 chlorophyll meter that was previously calibrated according to the manufacturer's instructions by averaging 30 readings per plant. The indirect measurement of chlorophyll content using Minolta SPAD-502 chlorophyll meters has been validated for crops such as maize, beans, and soybean, demonstrating a positive, linear correlation between SPAD index values and the nitrogen content of plants and other related variables (Pan and Smith, 2000; Argenta et al., 2002; Poustini et al., 2007; Remans et al., 2008; Vollmann et al., 2011).

All data were tested for normality. The results were analyzed by analysis of variance (ANOVA) with the number of nodules (NN) transformed to the square root of (x + 1) as recommended by SAS Learning Edition 2.0. Mean values were grouped by the Scott-Knott test ($p \leq 0.05$) using SISVAR (Ferreira, 2011).

Characterization of genetic diversity by BOX-PCR

The genetic diversity of 73 strains, out of a total of 88 authenticated as cowpea-nodulating strains, was evaluated by BOX-PCR as described by Guimarães et al. (2012), following previous described procedures (Pule-Meulenberg et al., 2010; Rademaker et al., 1997; Versalovic et al., 1994). The following type strains were included: *Cupriavidus taiwanensis* (LMG19424^T), *Burkholderia sabiae* (BR3405), *Sinorhizobium* sp. (BR6806), *Azorhizobium doebereineriae* (BR5401^T), *Bradyrhizobium* sp. (UFLA 03-84), *Rhizobium tropici* (CIAT899^T), and *Azorhizobium caulinodans* (ORS571^T).

The genetic diversity of the strains was analyzed by the presence or absence of polymorphic bands in the gel. The data were grouped with the Unweighted Pair Group Mean Arithmetic Method (UPGMA) algorithm and Jaccard coefficient using BioNumerics 6.5 software (Applied Maths, Sint-Martens-Latem, Belgium).

Characterization of genetic diversity by sequencing of the 16S rRNA gene

A total of 22 strains, including at least one from each cultural group and representative of genotypes determined by BOX-PCR, were selected for sequencing of the 16S rRNA gene. Bacterial growth conditions, extraction of genomic DNA (Ausubel et al., 2005), DNA quantification, and PCR conditions (Lane, 1991) are described in Guimarães et al. (2012).

The quality of sequences was verified using Phred and submitted to BLAST for comparison with other se-

quences deposited in GenBank (<http://www.ncbi.nlm.nih.gov/GenBank>). Only sequences greater than 400 bp in length were used in the phylogenetic analysis. Sequence alignment was performed with ClustalW, and the phylogenetic tree was constructed using the neighbor-joining method in the Kimura 2 model (Krasova-Wade et al., 2003; Saitou and Nei, 1987) using the parameters in MEGA version 5 (Tamura et al., 2011). A bootstrap confidence analysis was performed with 1000 replications.

Nucleotide sequence accession numbers

The sequences determined in this work have been deposited in GenBank under accession numbers KC113601 to KC113623.

Results

The 148 strains clustered into nine groups according to growth rate (GR) and pH (Figure 1). Among the fast-growing strains, 46 (31 %) acidified the medium (FA), six (4 %) did not alter the pH of the medium (FN), and 22 (15 %) alkalized the medium (FAL). Of the intermediate-growing strains, eight (5 %) acidified the medium (IA), 25 (17 %) did not alter the pH of the medium (IN), and 25 (17 %) alkalized the medium (IAL). Among the slow-growing strains, two (1 %) acidified the medium (SA), two (1 %) did not alter the pH of the medium (SN), and 12 (8 %) alkalized the medium (SAL).

Of the 148 strains evaluated in the two authentication experiments, 88 (59 %) exhibited a capacity to nodulate. Strains that failed to nodulate were predominantly fast-growing, and among these, the most frequent were those that acidified the medium (FA). Controls with

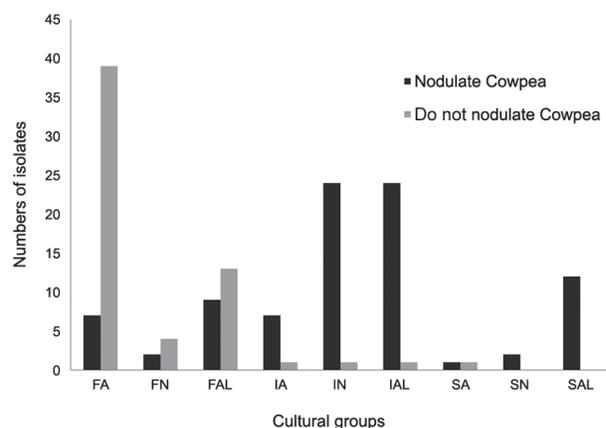


Figure 1 – Distribution in cultural groups of 148 bacterial strains isolated from soil samples under agroforestry systems according to growth rate and alteration of medium pH: fast-growing with medium acidification (FA), no alteration of pH (FN), or medium alkalization (FAL); intermediate-growing with medium acidification (IA), no alteration of pH (IN), or medium alkalization (IAL); slow-growing with medium acidification (SA), no alteration of pH (SN), or medium alkalization (SAL).

and without nitrogen supplementation (52.5 mg N L⁻¹) showed no nodulation, indicating no external contamination in the experiment, which is a requirement for this type of study.

No strain had shoot dry matter (SDM) similar to the control with high nitrogen content [corresponding to the highest relative efficiency (RE); statistical "group a"]. Strains exhibited varying REs and were grouped by the Scott-Knott test into two groups: intermediate (statistical group "b"), which comprised 12 strains, and inefficient (statistical group "c"), which comprised 80 strains and the control without nitrogen supplementation. Among the reference strains, which have been used as cowpea inoculants, the BR 3267 strain (RE = 56 %) performed best. Among the land use system strains, UFLA 03-282 (RE = 79 %), which was identified as *Rhizobium etli* with 99 % similarity (Table 1), demonstrated the highest efficiency.

According to the SPAD index, the reference strains UFLA 03-84, INPA 03-11B, and BR 3267, a group of 51 strains, and the control supplemented with nitrogen demonstrated the highest values (statistical group "a"), indicating that these strains were effective at fixing nitrogen, which was not reflected in the SDM. The other 16 strains were considered inefficient (statistical group "c"), as they were similar to the control without N supplementation. The remaining 21 strains had intermediate efficiency (statistical group "b"). Significant and positive correlation coefficients were obtained for SPAD index with both SDM ($r = 0.47, p < 0.01$) and relative efficiency ($r = 0.52, p < 0.01$).

A total of 75 genotypes, most of which were restricted to only one strain, were obtained after clustering the BOX-PCR profiles with over 70 % similarity (Figure 2). The 73 strains from the agroforestry system, which were distributed among 68 genotypes, did not exhibit a banding pattern similar to any of the seven reference/type strains evaluated, indicating a high level of diversity among them.

After genotypes were determined by BOX-PCR, 23 strains were selected for phylogenetic analysis and partial sequencing of the region 3' - 5' that encodes the 16S rRNA gene. These sequences ranged between 416 bp and 850 bp and exhibited 96-100 % identity with existing sequences in GenBank (Table 1). The phylogenetic relationships between the sequences are shown in Figure 3.

Sequencing of the 16S rRNA gene indicated that all sampling sites exhibited great diversity (Table 1). The largest number of strains (70 %) belonged to the genus *Bradyrhizobium*, and most of these were *Bradyrhizobium* sp. at varying percentages of similarity. Other genera identified were: *Rhizobium*, *Ochrobactrum*, *Paenibacillus*, *Bosea*, *Bacillus*, *Enterobacter*, and *Stenotrophomonas*.

Discussion

The fact that non-nodulating strains were predominantly fast-growing indicates that these strains were in-

Table 1 – Origin (sampling point), relative efficiency, SPAD index, cultural characteristics and identification (based on 16S rRNA sequences extant in GenBank) of strains that nodulate and fix nitrogen in symbiosis with cowpea.

| Strain | Sampling point ^a | RE % ^b | SPAD index ^c | Cultural characteristics ^d | Length (bp) of 16S rRNA sequence | Most similar sequence found in GenBank | | |
|-------------|-----------------------------|-------------------|-------------------------|---------------------------------------|----------------------------------|--|--------------|------------------|
| | | | | | | Species | % Similarity | Accession number |
| UFLA 03-225 | 17 | 72 b | 55.80 a | FA | 719 | <i>Ochrobactrum anthropi</i> | 99 | JF309272.1 |
| UFLA 03-226 | 17 | 41 c | 43.14 a | FA | 725 | <i>Paenibacillus humicus</i> | 99 | JF309272.1 |
| UFLA 03-227 | 17 | 46 c | 40.73 a | SAL | 784 | <i>Bradyrhizobium</i> sp. | 100 | JN375585 |
| UFLA 03-230 | 17 | 60 b | 60.10 a | IAL | 792 | <i>Bradyrhizobium</i> sp. | 100 | GU433457.1 |
| UFLA 03-234 | 17A | 30 c | 14.40 c | IN | 583 | <i>Bradyrhizobium</i> sp. | 99 | FJ375585 |
| UFLA 03-236 | 17A | 44 c | 49.63 a | IAL | 842 | <i>Bradyrhizobium</i> sp. | 100 | GU433457 |
| UFLA 03-237 | 17A | 42 c | 43.93 a | IAL | 651 | <i>Bradyrhizobium</i> sp. | 99 | JN375584.1 |
| UFLA 03-239 | 17A | 49 c | 39.96 a | IN | 633 | <i>Bradyrhizobium</i> sp. | 98 | FJ418695 |
| UFLA 03-241 | 17A | 41 c | 34.36 b | FAL | 780 | <i>Bradyrhizobium</i> sp. | 100 | AM748956 |
| UFLA 03-244 | 17A | 22 c | 18.30 c | IN | 571 | <i>Bosea thiooxidans</i> | 96 | AF508112 |
| UFLA 03-245 | 17A | 47 c | 65.56 a | SA | 699 | <i>Bacillus pumilus</i> | 100 | JN540803 |
| UFLA 03-251 | 17 | 46 c | 23.00 c | FA | 850 | <i>Enterobacter cloacae</i> | 99 | FN401343 |
| UFLA 03-259 | 20 | 31 c | 35.46 b | FAL | 735 | <i>Stenotrophomonas maltophilia</i> | 99 | JF747159 |
| UFLA 03-261 | 20 | 41 c | 44.33 a | FA | 687 | <i>Paenibacillus</i> sp. | 100 | EU839649 |
| UFLA 03-266 | 20 | 51 c | 59.73 a | FA | 681 | <i>Ochrobactrum anthropi</i> | 100 | HE578783.1 |
| UFLA 03-268 | 20 | 50 c | 50.53 a | IAL | 716 | <i>Bradyrhizobium</i> sp. | 99 | GQ181045 |
| UFLA 03-270 | 22 | 31 c | 52.50 a | FAL | 719 | <i>Bradyrhizobium</i> sp. | 100 | NR041785 |
| UFLA 03-273 | 22 | 40 c | 18.83 c | FN | 726 | <i>Bradyrhizobium</i> sp. | 99 | NR041785 |
| UFLA 03-280 | 66 | 38 c | 30.10 b | IN | 719 | <i>Bradyrhizobium</i> l sp. | 99 | NR041785 |
| UFLA 03-282 | 67 | 79 b | 59.90 a | IA | 698 | <i>Rhizobium etli</i> | 99 | FJ534690 |
| UFLA 03-286 | 67 | 31 c | 25.70 b | IAL | 784 | <i>Bradyrhizobium</i> sp. | 98 | GU433457 |
| UFLA 03-290 | 67 | 36 c | 34.63 b | IN | 585 | <i>Bradyrhizobium</i> sp. | 99 | JN375585 |
| UFLA 03-291 | 20 | 23 c | 23.10 c | SAL | 574 | <i>Paenibacillus daejeonensis</i> | 99 | NR02517 |

^aGPS (www.biosbrasil.ufla.br); ^bMeans of relative efficiency based on Shoot Dry Matter (SDM) of inoculated treatment compared with SDM of control with mineral N by the following formulas: RE = [SDM inoculated/SDM control mineral N(52.5 mg N L⁻¹)] x 100. ^cIndirect measurement of chlorophyll content by SPAD index. ^b and ^cRE and SPAD index of control with mineral N (52.5 mg N L⁻¹) belong to statistical group "a": RE N control = 100 %, and SPAD N control = 41.53. Absolute control (no inoculation and no N added): RE = 29 %, SPAD = 15.5. The same letters in the same column belong to the same group at a 5 % significance level (Scott-Knott test). The RE of each strain as well as of the controls was the mean for three replicates, and each replicate had one plant; ^dCultural characteristics in medium 79: (FA) fast growth, medium acidification; (FAL) fast growth, medium alkalization; (IA) intermediate growth, medium acidification; (IN) intermediate growth, no alteration of medium pH; (IAL) intermediate growth, medium alkalization; (SA) slow growth, medium acidification; (SAL) slow growth, medium alkalization.; FN- fast growth, no alteration of medium pH.

ternal or external contaminants of the nodules that grew faster than the rhizobia during the isolation process because nodules were firm, exhibited no signs of decomposition, and were harvested from fully-grown plants that were not in senescence.

The SPAD index, an indirect measurement of chlorophyll content, confirmed the N₂ efficiency of various strains that presented the highest SDM, and the SPAD index and SDM were significantly correlated. However, while a total of 51 strains (out of 88) were considered efficient by the SPAD index (because they were similar to the control with mineral nitrogen), none reached the SDM of the mineral nitrogen. This occurred probably due to the short period plants were allowed to grow (35 days), during which nitrogen uptake was not yet converted to dry matter. Thus, although the measurement of SDM *per se* is fast and less expensive, SPAD is a more effective method for evaluating symbiotic N₂ fixation under axenic conditions over shorter periods. On the other hand, the equipment to measure the SPAD index is not expensive and can provide a faster analysis than N anal-

ysis by Kjeldahl method. These results corroborate results obtained in pots with *Phaseolus vulgaris* (Poustini et al., 2007). Under field conditions significant correlations between SPAD index and yields were obtained for maize (Argenta et al., 2002), beans (Remans et al., 2008), and soybean (Pan and Smith, 2000; Volmann et al., 2011).

A high diversity of the native rhizobia populations of agroforestry was revealed by rep-PCR, a high-resolution technique used to assess bacterial diversity (Versalovic et al., 1994). Rep-PCR is considered to be a universal tool for studies of genomic variation in prokaryotes and reflects the variability of the entire genome (Igual et al., 2001). In our study, BOX-PCR, which is a specific type of rep-PCR, proved to be an efficient technique for assessing the diversity of nitrogen-fixing bacteria that nodulate cowpea from agroforestry systems. The high diversity of strains was confirmed by 16S rRNA sequence, although 16S rRNA gene sequencing does not offer good species-level resolution among members of the *Bradyrhizobium*, thus requiring further testing to identify species belonging to this genus (Guimarães et al., 2012; Willems et al., 2001).

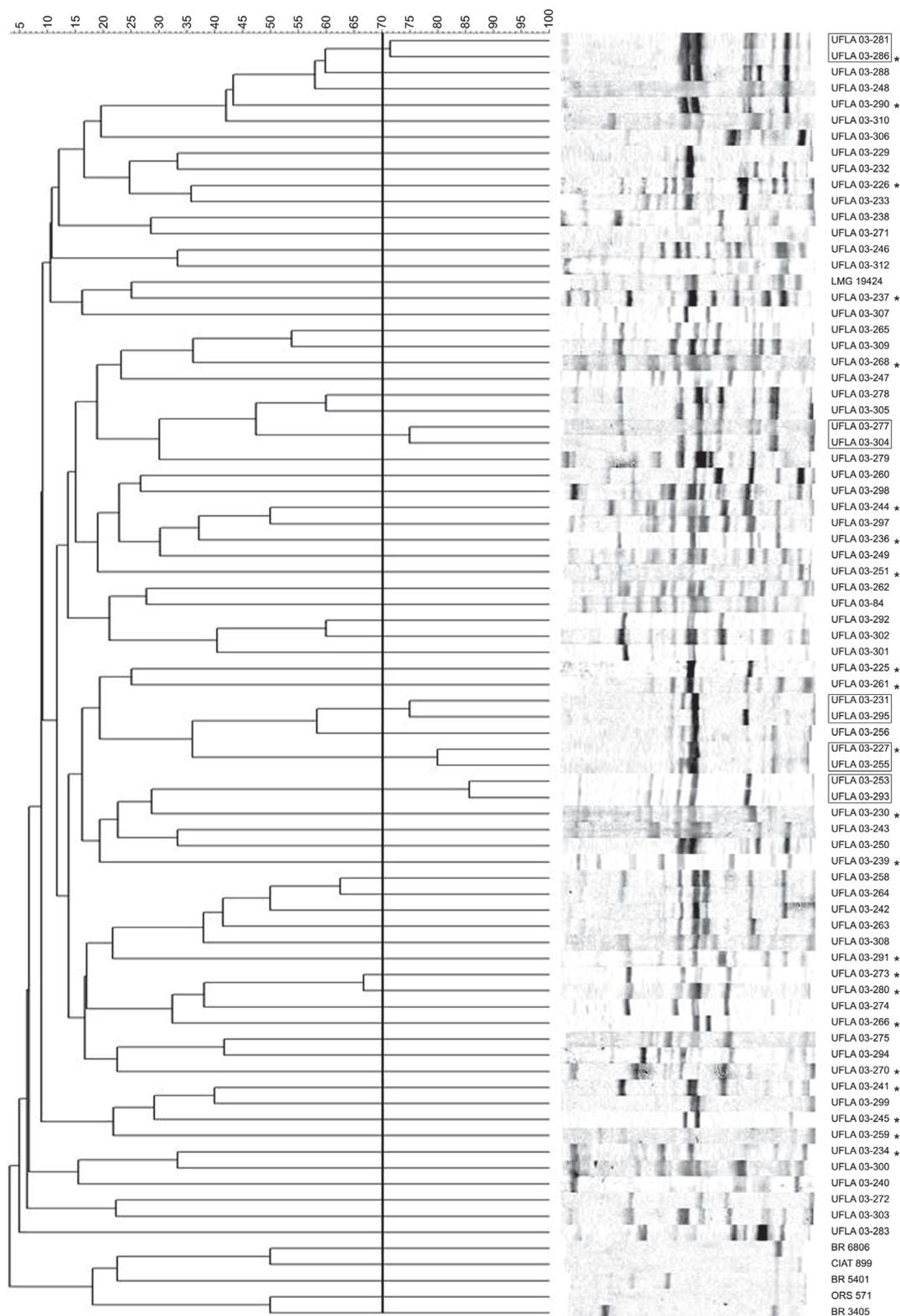


Figure 2 – Dendrogram based on profiles obtained by rep-PCR using the BOX primer of cowpea-nodulating strains isolated from soil samples under agroforestry systems in the western Amazon and of type strains. Genotypes were determined using similarity values of 70 %. *indicates the isolates for which 16S rRNA gene has been sequenced (Table 1).

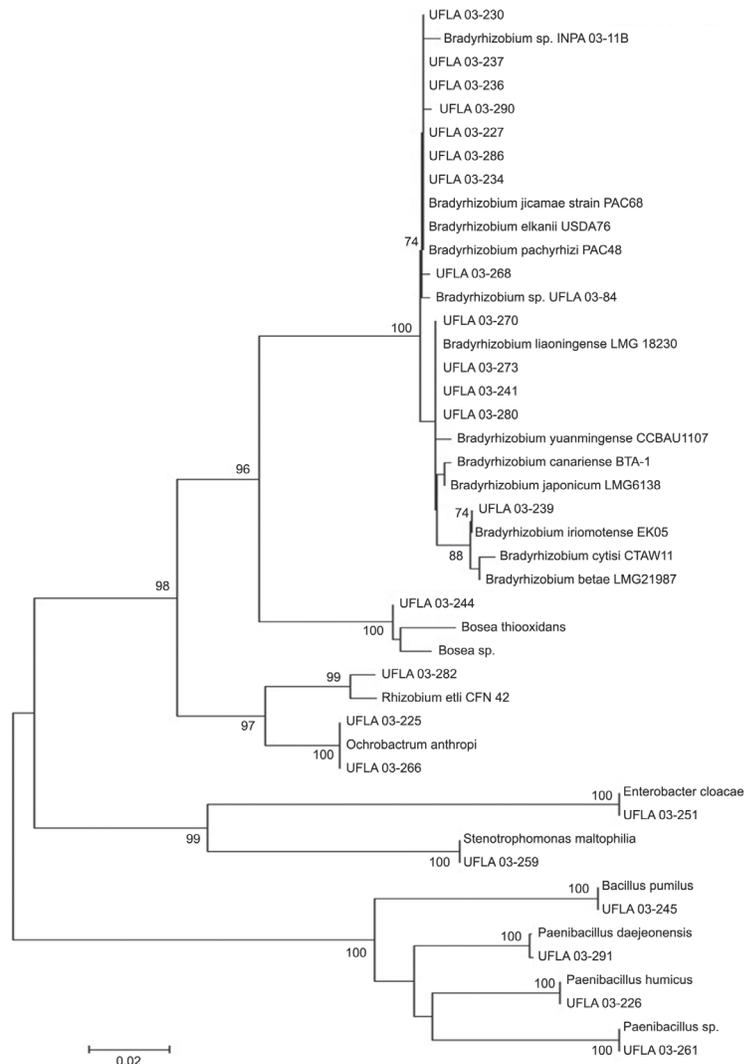


Figure 3 – Dendrogram showing the genetic relationships between partial sequences of the 16S rRNA gene from cowpea-nodulating strains compared with GenBank using MEGA version 4 (26). Bootstrap values are based on 1000 replications.

In addition to *Bradyrhizobium* spp., other species and genera were identified in this study: *Bacillus pumilus*, *Paenibacillus daejeonensis*, *Paenibacillus humicus*, *Enterobacter cloacae*, *Boresea thiooxidans*, *Stenotrophomonas maltophilia*, *Rhizobium etli*, and *Ochrobactrum anthropi*. The latter two species belong to N₂-fixing, legume-nodulating bacterial genera, although *O. anthropi* has not previously been reported as such. Lima et al. (2009) reported that the genus *Stenotrophomonas* is potentially legume-nodulating in the same study area as in the present study, but there are no reports on the nodulation capacity of the other species, and further work is needed to confirm that they are indeed LNBS and not non-symbiotic endophytes that are commonly isolated from legume nodules (Sprent, 2009; Gyaneshwar et al., 2011). Their taxonomic position and nodulation capacity should be assessed in future studies. Nevertheless, there

are reports that these species promote plant growth through other processes. Silva et al. (2007) studied the effects of different *Paenibacillus macerans*, *Paenibacillus durus*, *Paenibacillus polymyxa*, and *B. pumilus* strains on the symbiosis between *Bradyrhizobium* and cowpea, and showed that these strains stimulated nodulation and improved nitrogen fixation efficiency.

Few studies in Brazil have assessed the genotypic diversity of native populations that establish symbiotic relationships with cowpea. Zilli et al. (2006) sequenced the 16S rRNA gene from 14 strains: 11 isolated from cowpea nodules of plants grown in Cerrado soils in Piauí state (BR); one isolated from the semi-arid region of Pernambuco state (BR); one isolated from the Integrated Agroecology Production System (Sistema Integrado de Produção Agroecológica - SIPA) in Seropédica, state of Rio de Janeiro, Brazil (22°48'S; 43°41'S, 33 m

a.s.l.); and one strain (BR2001) previously approved by the Brazilian Ministry of Agriculture (MAPA) as a cowpea inoculant but later replaced by the reference strains cited in this study. Of these 14 strains, nine grouped with *Bradyrhizobium elkanii* (ATTC49852//USDA 76^T) with a similarity of 98 %, three with *Bradyrhizobium japonicum* type strain (ATCC10324^T/LMG 6138) and *B. liaoningense* (ATCC700350/LMG18230) strains, and two grouped indirectly with *B. elkanii* and with the group formed by *B. japonicum* and *B. liaoningense*. The diversity of 20 rhizobia isolates from upland and floodplain soils based on morphological, physiological, and symbiotic characteristics was reported in another study conducted in the Amazon using cowpea as a trap plant (Chagas Junior et al., 2010). However, genetic diversity and identification of rhizobia were not provided in the study.

A few strains, isolated from nodules collected from cowpea growing in the field, were identified as *Bradyrhizobium* by 16S-23S rDNA IGS sequencing, after screening of a larger number of nodules (88 and 270) by PCR-RFLP analysis of this gene in Senegal, West Africa (Krasova-Wade et al., 2004) (four strains) and South Africa, Ghana, and Botswana (Pule-Meulenberg et al., 2010) (13 strains). It may be that the small number of strains sequenced and bias in obtaining isolates, such as restriction to slow-growing alkalizing isolates, hindered the isolation of other genera.

Lima et al. (2009) identified rhizobia from different land use systems in the western Amazon, including the same agroforestry system sampling points of this study, using siratro (*Macroptilium atropurpureum*) as a trap plant. The authors identified the following species in the agroforestry system: *R. etli*, *R. tropici*, *R. galegae*, *Rhizobium* sp., *Sinorhizobium medicae*, *Burkholderia* sp., *Azorhizobium* sp., *Bradyrhizobium* sp., *B. elkanii*, *B. japonicum*, and *Mesorhizobium* sp. Some, but not all, of these species were also identified in the present study, in addition to other genera (*Paenibacillus*, *Stenotrophomonas*, *Enterobacter*, *Bosea*, and *Ochrobactrum*). These results illustrate the need to use more than one species of trap plant when assessing the diversity of nitrogen-fixing, legume-nodulating bacteria in native populations; moreover, siratro captures a higher diversity of legume-nodulating bacteria than cowpea.

Guimarães et al. (2012) found high genetic and symbiotic diversity of strains isolated from agricultural soils in the same sampling area by using cowpea as trap plant. Although they also found *Bradyrhizobium* to be a predominant genus among isolates, other genera were found (*Rhizobium*, *Burkholderia*, and *Achromobacter*). However, a lower diversity was found when compared to agroforestry systems, probably due to the higher diversity of legume genera in agroforestry systems (15) than in agricultural systems (6). Thus, the diversity of phenotypes, genotypes, and species of legume-nodulating bacteria found in this study is probably associated with the diversity of legumes in this land use system, because

they belong to genera that have already been reported to host a great diversity of legume-nodulating bacteria (Moreira, 2006). Even with the more diverse plant composition and soil attributes in agroforestry than in agriculture, the genus *Bradyrhizobium* was the most frequent trapped by cowpea, although this species is also able to nodulate with many other genera like *Burkholderia*, *Azorhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Rhizobium* (Moreira, 2006), *Achromobacter* (Guimarães et al., 2012), and *Cupriavidus* (Silva et al., 2012).

In conclusion, cowpea was found to nodulate with a high diversity of nitrogen-fixing bacteria genotypes isolated from soils under agroforestry systems. Most genotypes (70 %) were identified as belonging to different species in the genus *Bradyrhizobium*, while the remaining genotypes belonged to the genera *Rhizobium*, *Ochrobactrum*, *Stenotrophomonas*, *Paenibacillus*, *Enterobacter*, and *Bosea*. The nodulating strains exhibited varying N₂-fixing efficiencies, but most of them established an efficient symbiosis with cowpea. The SPAD index was more efficient than SDM at evaluating the contribution of symbiotic N₂-fixation under axenic conditions over shorter periods.

Acknowledgements

We thank CAPES and CNPq for student fellowships, CNPq for a research fellowship and grant, and the GEF/UNEP-GF2715-02 (CSM-BGBD) project for financial support. This work presents part of the findings of the international project "Conservation and Management of Below-Ground Biodiversity" implemented in seven tropical countries: Brazil, Cote d'Ivoire, India, Indonesia, Kenya, Mexico, and Uganda. This project is coordinated by the Tropical Soil Biology and Fertility Institute of CIAT (TSBF-CIAT), with co-financing from the Global Environmental Facility (GEF), and implementation support from the United Nations Environment Program (UNEP). The Brazilian Co-executing Institution was Universidade Federal de Lavras, and in Brazil the CSM-BGBD project was named BiosBrasil. Views expressed in this publication are those of their authors and do not necessarily reflect those of the authors' organization, the United Nations Environment Program, and the Global Environmental Facility.

References

- Argenta, G.; Silva, P.R.F.; Mielniczuk, J.; Botollini, C.G. 2002. Plant parameters as indicators of nitrogen status in maize. *Pesquisa Agropecuária Brasileira* 37: 519-527.
- Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A.; Struhl, K. 2005. *Current Protocols in Molecular Biology*. Wiley, New York, NY, USA.
- Chagas Junior, A.F.; Oliveira, L.A.; Oliveira, N.A. 2010. Phenotypic characterization of rhizobia strains isolated from Amazonian soils and symbiotic efficiency in cowpea. *Acta Scientiarum Agronomy* 32: 161-169.

- Ferreira, D.F. 2011. Sisvar: a computer statistical analysis system. *Ciência e Agrotecnologia* 35: 1039-1042.
- Fred, E.B.; Waksman, S.A. 1928. *Laboratory Manual of General Microbiology*. McGraw-Hill, New York, NY, USA.
- Guimarães, A.A.; Jaramillo, P.M.D.; Nóbrega, R.S.A.; Florentino, L.A.; Silva, K.B.; Moreira, F.M.S. 2012. Genetic and symbiotic diversity of nitrogen-fixing bacteria isolated from agricultural soils in the western Amazon by using cowpea as the trap plant. *Applied and Environmental Microbiology* 78: 6726-6733.
- Gyanešwar, P.; Hirsch, A.M.; Moulin, L.; Chen, W.-M.; Elliot, G.M.; Bontemps, C.; Santos, P.E.; Gross, E.; Reis Jr, F.B.; Sprent, J.I.; Young, J.P.W.; James, E.K. 2011. Legume-nodulating betaproteobacteria: diversity, host range and future prospects. *Molecular Plant-Microbe Interactions* 24: 1276-1288.
- Hoagland, D.; Arnon, D.I. 1950. The water culture method for growing plants without soil. University of California, Berkeley, CA, USA. (AES Circular, 347).
- Igual, J.M.; Valverde, A.; Cervantes, E.; Velásquez, E. 2001. Phosphate-solubilizing bacteria as inoculants for agriculture: use of updates molecular techniques in their study. *Agronomie* 21: 561-568.
- Krasova-Wade, T.; Ndoye, I.; Braconnier, S.; Sarr, B.; de Lajudie, P.; Neyra, M. 2003. Diversity of indigenous bradyrhizobia associated with three cowpea cultivars (*Vigna unguiculata* (L.) Walp.) grown under limited and favorable water conditions in Senegal (West Africa). *African Journal of Biotechnology* 2: 13-22.
- Lane, D.J. 1991. 16S/23S rRNA sequencing, p. 115-148. In: Stackebrandt E.; Goodfellow M., eds. *Nucleic acid techniques in bacterial systematics*. Wiley, New York, NY, USA.
- Lima, A.S.; Nóbrega, R.S.A.; Barberi, A.; Silva, K.; Ferreira, D.F.; Moreira, F.M.S. 2009. Nitrogen-fixing bacteria communities occurring in soils under different uses in the western Amazon Region as indicated by nodulation of siratro (*Macroptilium atropurpureum*). *Plant and Soil* 319: 127-145.
- Martins, L.M.V.; Xavier, G.R.; Rangel, F.W.; Ribeiro, J.R.A.; Neves, M.C.P.; Morgado, L.B.; Rumjanek, N.G. 2003. Contribution of biological nitrogen fixation to cowpea: a strategy for improving grain yield in the semi-arid region of Brazil. *Biology and Fertility of Soils* 38: 333-339.
- Moreira, F.M.S.; Nóbrega, R.S.A.; Jesus, E.C.; Ferreira, D.F.; Perez, D.V. 2009. Differentiation in the fertility of inceptisols as related to land use in the upper Solimões river region, western Amazon. *Science of the Total Environment* 408: 349-355.
- Moreira, F.M.S. 2006. Nitrogen-fixing Leguminosae nodulating bacteria, 280 p. In: Moreira, F.M.S.; Siqueira, J.O.; Brussaard, L., eds. *Soil biodiversity in Amazonian and other Brazilian ecosystems*. CAB International, Wallingford, UK.
- Moreira, F.M.S.; Gillis, M.; Pot, B.; Kersters, K.; Franco, A.A. 1993. Characterization of rhizobia isolated from different divergence groups of tropical leguminosae by comparative polyacrylamide gel electrophoresis of their total proteins. *Systematic and Applied Microbiology* 16: 135-146.
- Moreira, F.M.S.; Haukka, K.; Young, J.P.W. 1998. Biodiversity of rhizobia isolated from a wide range of forest legumes in Brazil. *Molecular Ecology* 7: 889-895.
- Pan, B.; Smith, D.L. 2000. Preincubation of *B. japonicum* cells with genistein reduces the inhibitory effects of mineral nitrogen on soybean nodulation and nitrogen fixation under field conditions. *Plant and Soil* 223: 235-242.
- Poustini, K.; Mabood, F.; Smith, D.L. 2007. Preincubation of *Rhizobium leguminosarum* bv. *phaseoli* with jasmonate and genistein signal molecules increases bean (*Phaseolus vulgaris* L.) nodulation, nitrogen fixation and biomass production. *Journal of Agricultural Science and Technology* 9: 107-117.
- Pule-Meulenberg, F.; Belane, A.K.; Krasova-Wade, T.; Dakora, F.D. 2010. Symbiotic functioning and bradyrhizobial biodiversity of cowpea (*Vigna unguiculata* L.Walp.) in Africa. *BMC Microbiology* 10: 89-100.
- Rademaker, J.L.W.; Frank, J.L.; Bruijn, F.J. 1997. Characterization of the diversity of ecologically important microbes by rep-PCR genomic fingerprinting, p. 1-26. In: Akkemanns, A.D.L.; van Elsas, J.D.; de Bruijn, F.J. *Molecular microbial ecology manual*. Kluwer Academic, Dordrecht, Netherlands.
- Remans, R.; Ramaekers, L.; Schelkens, S.; Hernandez, G.; Garcia, A.; Reyes, J.L.; Mendez, N.; Toscano, V.; Mulling, M.; Galvez, L.; Vanderleyden, J. 2008. Effect of *Rhizobium-Azospirillum* coinoculation on nitrogen fixation and yield of two contrasting *Phaseolus vulgaris* L. genotypes cultivated across different environments in Cuba. *Plant Soil* 312: 25-37.
- Saitou, N.; Nei, M. 1987. The neighbor joining 440 method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4: 406-25.
- Silva, K.; Florentino, L.A.; Silva, K.B.; Brandt, E.; Vandamme, P.; Moreira, F.M.S. 2012. *Cupriavidus necator* isolates are able to fix nitrogen in symbiosis with different legume species. *Systematic and Applied Microbiology* 35: 175-182.
- Silva, V.N.S.; Silva, L.E.S.F.; Martínez, C.R.; Seldin, L.; Burity, H.A.; Figueiredo, M.V.B. 2007. Strains of *Paenibacillus* promoters of the specific nodulation in the symbiosis *Bradyrhizobium* - caupi. *Acta Scientiarum Agronomy* 29: 331-338.
- Sprent, J.I. 2009. *Legume Nodulation: A Global Perspective*. Wiley-Blackwell, Chichester, UK.
- Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28: 2731-2739.
- Versalovic, J.; Schneider, M.; Bruijn, F.J.; Lupski, J.R. 1994. Genomic finger-printing of bacteria using repetitive sequence-based polymerase chain re-action. *Methods in Molecular and Cellular Biology* 5: 25-40.
- Vollmann, J.; Sato, T.; Walter, H.; Schweiger, P.; Wagentristsl, H. 2011. Soybean di-nitrogen fixation affecting photosynthesis and seed quality characters. *Soil, Plant and Food Interactions* 496-502.
- Willems, A.; Coopman, R.; Gillis, M. 2001. Phylogenetic and DNA-DNA hybridization analyses of *Bradyrhizobium* species. *International Journal of Systematic and Evolutionary Microbiology* 51: 111-117.
- Zilli, J.E.; Valichesi, R.R.; Rumjanek, N.G.; Simões-Araújo, J.L.; Freire, F.R.F.; Neves, M.C.P. 2006. Symbiotic efficiency of cowpea *Bradyrhizobium* strains in Cerrado soils. *Pesquisa Agropecuária Brasileira* 41: 811-818.