



## Rhizobial diversity from stem and root nodules of *Discolobium* and *Aeschynomene*

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**ABSTRACT.** Among the genera that exhibit stem nodulation, *Aeschynomene* and *Discolobium* are found in the Pantanal Mato-grossense (wetlands in the state of Mato Grosso, Brazil). Isolates obtained from the stem and root nodules of *D. pulchellum*, *D. psoraleaeifolium*, *D. leptophyllum* and *A. fluminensis* were collected from various locations in the Pantanal de Poconé sub-region and phenotypically characterized and genotyped by restriction analysis of the 16S rDNA gene. Of the 282 isolates obtained from the stem and root nodules, 84.3% alkalized YMA media and 74.1% showed slow growth. No differences in either the phenotype or the genotype among the rhizobial populations isolated from the root or stem nodules of the species *Discolobium* and *A. fluminensis* were observed. Among the isolates obtained from *D. pulchellum*, there was a group that was not similar to any of the reference strains used, and most of the isolates analyzed by PCR-RFLP were similar to the genus *Bradyrhizobium*.

**Keywords:** Flooding-tolerant legume, symbioses, Pantanal, 16S rDNA, biological nitrogen fixation, PCR-RFLP.

### Diversidade de rizóbios de caule e raiz de *Discolobium* e *Aeschynomene*

**RESUMO.** Entre os gêneros que exibem nodulação caulinar *Aeschynomene* e *Discolobium* são encontrados no Pantanal Mato-grossense (áreas inundáveis no Estado do Mato Grosso, Brasil). Isolados obtidos de nódulos de caule e raiz de *D. pulchellum*, *D. psoraleaeifolium*, *D. leptophyllum* and *A. fluminensis* foram coletados em vários pontos no Pantanal de Poconé e caracterizados fenotípica e genotipicamente, pela análise de restrição do gene 16S rDNA. Dos 282 isolados obtidos de nódulos de caule e raiz 84,3% alcalinizaram o meio YMA e 74,1% apresentaram crescimento lento. Não se observou diferença fenotípica nem genotípica entre as populações de rizóbio isoladas de nódulos radiculares ou caulinares das espécies de *Discolobium* e de *A. fluminensis*. Entre os isolados obtidos da espécie *Discolobium pulchellum* obteve-se um grupo que não agrupou similaridade com nenhuma das estirpes de referência utilizadas e a maior parte dos isolados analisados pelo PCR/RFLP apresentou similaridade com o gênero *Bradyrhizobium*.

**Palavras-chave:** leguminosas tolerantes a alagamento, simbiose, Pantanal, 16S rDNA, fixação biológica de nitrogênio, PCR-RFLP.

### Introduction

Prokaryotes that fix atmospheric nitrogen (N<sub>2</sub>) are an extremely important group of microorganisms for various ecosystems because they are responsible for the entrance of nitrogen into the ecosystem. The ability to fix atmospheric nitrogen is widely distributed among prokaryotes with a range of phylogenetic relationships. However, the ability to fix atmospheric nitrogen and induce nodulation in legumes is restricted to members of the Proteobacteria phylum (MOULIN et al., 2001; SHIRAIISHI et al., 2010).

In Brazil, studies of the diversity of the bacteria capable of inducing nodulation in leguminous plants have been conducted in cultivated áreas

(ALBERTON et al., 2006), Caatinga (scrubland) ecosystems (TEIXEIRA et al., 2010; REIS JR. et al., 2010), the Amazon Forest (JESUS et al., 2005; LIMA et al., 2009) and Cerrado (savanna) regions (REIS JR. et al., 2010; ZILLI et al., 2004). However, there are few studies of the diversity of nitrogen-fixing bacteria in the Pantanal biome. The Pantanal is the most extensive wetland region in the world. It is located in the Upper Paraguay River Basin of central South America and is composed of low-lying pluvial flatlands impacted by the rivers that drain into the basin. Its abundant flora and fauna are influenced by four great biomes, namely, the Amazon, the Cerrado, the Chaco and the Atlantic Forest biomes. The Paraguay River and its

tributaries run through the Pantanal, forming extensive flooded areas that serve as shelter for many animals. Due to the declivity of these lowlands, source water from the Paraguay River takes four months to flow through the Pantanal (ADÁMOLI, 1987).

The symbiosis between leguminous plants and nitrogen-fixing bacteria generally occurs in the roots and represents the most important nitrogen-fixing system for agriculture. Some legumes may receive up to 100% of their required nitrogen by biological nitrogen fixation (BNF). In Brazil, one of the most successful examples of this is the soybean, which contributes 10 billion dollars annually to the economy. Some legumes, however, form nodules in the stems. Hagerup reported the first example of this phenomenon in 1928 when he observed green nodules and the presence of starch grains in *Aeschynomene aspera*, suggesting the occurrence of photosynthesis.

Plant species that exhibit stem nodulation are typically tropical or subtropical hydrophytes and are found in wetlands, rivers or lake margins. They belong to the genera *Aeschynomene*, *Sesbania*, *Neptunia* and *Discolobium* (BOIVIN et al., 1997; DREYFUS; DOMMERGUES, 1981; LADHA et al., 1992; LOUREIRO et al., 1994). The only bacterial isolates reported to induce stem nodulation belong to the genera *Bradyrhizobium* and *Azorhizobium* (DREYFUS et al., 1988; MOLOUBA et al., 1999; WONG et al., 1994). Together with nodule formation, some species develop a large number of parenchymal cells, facilitating the intake of sufficient oxygen for the various metabolic functions needed in flooded ecosystems (LADHA et al., 1992).

Members of the *Discolobium* genus are found in the flooded areas of the Pantanal Mato-grossense region and contain three species that form true stem nodules: *D. pulchellum*, *D. psoraleaeifolium* and *D. leptophyllum* (JAMES et al., 2001; LOUREIRO et al., 1994). These nodules are connected to the stem vascular system rather than to the adventitious root system originating from the stem. According to Loureiro et al. (1994), the stem nodules of *A. fluminensis* only form in submersed stems; however, the root nodules form in both submersed and non-submersed roots, but this occurs to a lesser degree in the former. In *Discolobium* spp., the stem nodules must be submersed, and nodulation only occurs after the formation of the parenchymal tissue (LOUREIRO et al., 1994). Furthermore, the nodules rapidly reach senescence when exposed to the air.

Among the species that exhibit stem nodulation, members of *Aeschynomene* and *Discolobium* genera are the only species reported to occur in the Pantanal. The species of these genera play an important ecological role in the region by serving as excellent food sources for animals due to their high protein content. The objectives of this study were to investigate the stem and root nodulation associated with these species and to perform phenotypic and molecular characterization, in the latter case by restriction analysis of the 16S rDNA gene, of bacterial isolates obtained from nodules of species of the *Discolobium* and *Aeschynomene* genera in the Pantanal.

## Material and methods

### Investigation of nodulation

The investigation of nodulation in *A. fluminensis*, *D. pulchellum*, *D. psoraleaeifolium* and *D. leptophyllum* was carried out at various sites in the Pantanal de Poconé in the northern part of the Pantanal. At each sampling location, three individuals of each species were chosen at random to determine the nodulation. The first sampling area consisted of three flooded collection points along a 50 km stretch of the Transpantaneira Highway. The next area contained two collection points within the Ypiranga Farm, which is located 10 km from the city of Poconé (Mato Grosso State). The first of these points was in a flooded area, while the second was in a pasture area that was not yet flooded at the time of the study.

The third area was in the Porto Cercado region and was located 145 km from the capital, Cuiabá, in a private natural heritage reserve owned by the Pantanal Regional Commercial Training Service (SESC-Pantanal). This reserve covers an area of 50 thousand hectares (ha) and is located between the Cuiabá and São Lourenço rivers. There were four flooded sampling points in this area. The first was within the area of Posto Biguazal, which covers an area of 6,289 ha; the second was in Baía Bonita; the third was in the area of Posto Espírito Santo, which covers an area of 6,181 hectares; and the last was located approximately 10 km from the SESC Pantanal headquarters along the Poconé-Porto Cercado Highway (Table 1).

**Table 1.** Sampling area, number of collection points in each area, point flood situation and species found in each area.

Sampling area	Number of Collection points	Flood situation	Species found
Transpantaneira Highway	Three points	All flooded	<i>D. pulchellum</i> , <i>D. psoraleaeifolium</i> and <i>D. leptophyllum</i>
Ypiranga Farm	Two points	Only one flooded	<i>D. pulchellum</i>
Porto Cercado (SESC-Pantanal)	Four points	All flooded	<i>D. pulchellum</i> and <i>A. fluminensis</i>

### Isolation of bacteria from nodules and phenotypic characterization

After collection, the root and stem nodules were dehydrated in flasks containing silica gel. To isolate the bacteria present in the nodules, the nodules were washed with alcohol (70% v/v, 1 min.) to break the surface tension and were superficially disinfected with hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>, 5 min.) before being washed five times with sterile distilled water. The surface-disinfected nodules were crushed on Petri dishes containing solid YMA medium with congo red indicator. The Petri dishes were incubated at 28°C for seven days with daily observation. The rhizobial isolates were transferred to Petri dishes containing YMA medium with bromothymol blue and were purified and stored. For phenotypic characterization, the pH change in the growth medium was determined along with the following colony parameters: growth period; size; shape; edge type; transparency; color; mucus quantity, consistency and elasticity.

### Biodiversity analysis

The results of the phenotypic characterization were used to construct binary matrices that were then used for cluster analysis by calculating Jaccard's similarity index and by the UPGMA method available in the PAST program (PAleontological STatistics) (HAMMER et al., 2001). From the phenotypic similarity dendrogram, groups with 80% similarity were chosen and used as operational taxonomic units (OTUs) to calculate the richness, Shannon's diversity index and the evenness index in addition to their use in rarefaction analysis. This analysis, carried out using the EstimateS version 8.0 program in accordance with the method described by Magurran (1987), allowed plotting curves of the variation of Shannon's index as a function of the number of isolates from each plant species or nodule type. In this case, the program selected the isolates at random to compose samples that had between one and the maximum number of isolates from each plant species or nodule type, and it then calculated the Shannon index value for each sample.

### RFLP analysis of amplified 16S rDNA gene fragments

For the restriction analysis of the ribosomal 16S rDNA gene, total DNA from the isolates of each phenotype group obtained from the cluster analysis, together with DNA from the reference strains *Bradyrhizobium elkanii* (BR 29), *B. japonicum* (BR 111), *Rhizobium etli* (BR 10026), *Sinorhizobium fredii* (BR 112) and *Azorhizobium caulinodans* (BR 5410), was extracted as described by Teixeira et al. (2010).

Amplification of the 16S rDNA was carried out using the primers Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and Y3 (5'-ACCTTGTTACGACTTCACCCCAGTC-3'). Each 100 µL reaction received 2 µL of a 1:20 dilution of the total DNA as template, 1U of DNA polymerase (Invitrogen, São Paulo, Brazil), 2 mM of MgCl<sub>2</sub>, 200 µM of each dNTP and 0.5 µM of each primer. The PCR reactions were carried out in a Perkin-Elmer GeneAmp PCR System 9600 thermocycler. The amplification program consisted of an initial denaturing step at 93°C for 2 min., 35 cycles of denaturing at 93°C for 45 sec., annealing at 62°C for 45 sec., and extension at 72°C for 2 min., and a final extension at 72°C for 5 min.

The amplification products were digested separately with the endonucleases *Hinf*I, *Dde*I, *Hae*III, *Hha*I, *Msp*I, *Alu*I and *Rsa*I, and electrophoretic separation of the fragments was performed at 80 V for 210 min. in agarose gel (3% w/v) (TEIXEIRA et al., 2010).

## Results and discussion

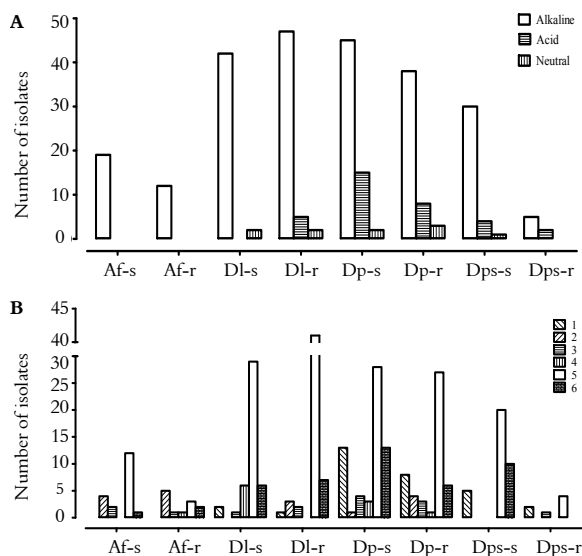
### Nodulation

The species *D. pulchellum*, *D. psoraleaeifolium* and *D. leptophyllum* were found at the sampling points along the Transpantaneira Highway. All three species exhibited stem nodules, while root nodules were only obtained from *D. psoraleaeifolium* and *D. leptophyllum*. On Ypiranga Farm, in both the flooded area and the pasture area, the only species found was *D. pulchellum*, and root and stem nodules were observed in both areas. At Posto Biguazal, one of the sampling points in the private reserve, the only species found was *D. pulchellum*, and there were no stem or root nodules on any of the plants collected even though the area was flooded. Similarly, at the other three sampling sites in the private reserve, the only species found was *D. pulchellum*; however, at these sites the plants had both stem and root nodules. The species *A. fluminensis* was found only at the collection point beside the Poconé-Porto Cercado Highway and these specimens had both root and stem nodules.

### Phenotypic characterization

Two hundred and eighty-two isolates were obtained and phenotypically characterized (19 from *Aeschynomene fluminensis* stem, 12 from *Aeschynomene fluminensis* root, 62 from *Discolobium pulchellum* stem, 49 from *Discolobium pulchellum* root, 54 from *Discolobium leptophyllum* stem, 44 from *Discolobium leptophyllum* root, 35 from *Discolobium psoraleaeifolium* stem and 7 from *Discolobium psoraleaeifolium* root).

It was observed that 84.3% of the isolates alkalinized the YMA culture medium and 74.1% exhibited slow growth, taking from five to six days to grow (Figure 1). The characteristics with the greatest variation among the isolates, thus being the most important for discrimination, were colony size, growth time, type of pH change and mucus transparency and elasticity. Fifty percent of the isolates showed medium or high mucus production, and the other 50% showed low production. Conversely, the majority of isolates produced butyric mucus, which does not coalesce in a dish and permits the formation of well-defined colonies. This parameter is usually important in characterizing rhizobial strains, but in this study it was not useful due to the low degree of variation. Mucus production is a marked characteristic among bacteria of this group mainly when using a culture medium rich in carbon. Fast-growing strains generally produce more mucus than slower growing ones (SPRENT, 1994). Strains that do not produce mucus are called dry and are more common among slow-growing rhizobia.



**Figure 1.** The number of isolates obtained with respect to the type of pH change in the culture medium (A) and with respect to growth time in days (B) according to the species and the organ from which the nodule was obtained. Af-s - *Aeschynomene fluminensis* stem, Af-r - *Aeschynomene fluminensis* root, DI-s - *Discolobium leptophyllum* stem, DI-r - *Discolobium leptophyllum* root, Dp-s - *Discolobium pulchellum* stem, Dp-r - *Discolobium pulchellum* root, Dps-s - *Discolobium psoraleaeifolium* stem, and Dps-r - *Discolobium psoraleaeifolium* root.

All the isolates obtained from *A. fluminensis* alkalinized the medium, and the majority exhibited a growth time of five days, while none displayed a growth time of one day (Figure 1). The isolates

from *D. leptophyllum* and *D. psoraleaeifolium* nodules exhibited greater variation with respect to the growth time and the type of pH alteration than those from *A. fluminensis*. In these two species, there was a predominance of isolates that alkalinized the medium and displayed a growth time of five days (Figure 1). These results corroborate those of Martins et al. (1997), who reported that 90% of the isolates from the root nodules of *Discolobium* spp. exhibited slow growth and alkalinized the culture medium.

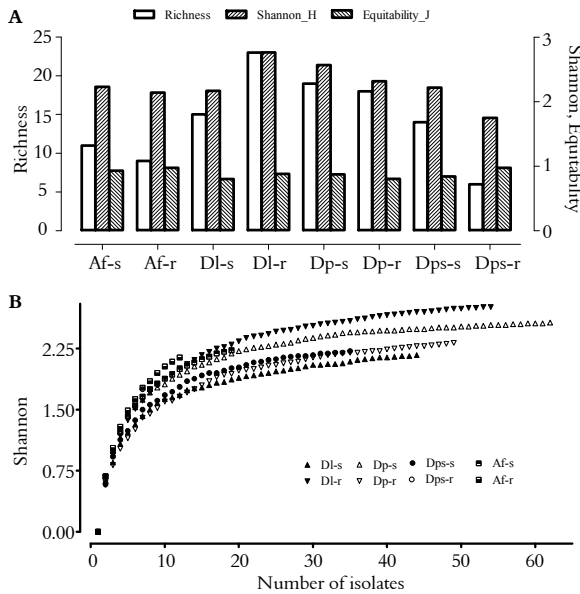
The results of the present study indicate that nodulation in *D. pulchellum* and *D. leptophyllum* can be induced by a group of rhizobia that is more diverse than the groups that induced nodulation in the other species studied here. Isolates obtained from these two species of *Discolobium* had growth times ranging from one to six days and included bacteria that caused acidic or alkaline pH changes in addition to those causing no pH change (Figure 1).

#### Biodiversity analysis

The greater rhizobial diversity obtained from *D. pulchellum* and *D. leptophyllum* nodules was confirmed both by calculating the diversity indices and by rarefaction analysis (Figure 2). Based on the phenotypic characterization, the isolates were grouped into one dendrogram (data not shown) that indicated 48 phenotypic groups at 80% similarity. For these two *Discolobium* species, the group richness varied from 15 (*D. leptophyllum* stem) to 23 (*D. leptophyllum* root), and Shannon's index ranged from 2.167 (*D. leptophyllum* stem) to 2.761 (*D. leptophyllum* root) (Figure 2A). Less diversity was observed for *D. psoraleaeifolium* and *A. fluminensis*, in which the richness varied between 6 (*D. psoraleaeifolium* root) and 14 (*D. psoraleaeifolium* stem), and Shannon's index ranged from 1.748 (*D. psoraleaeifolium* root) to 2.229 (*A. fluminensis* stem). Finally, the species presenting the greatest diversity also had the highest degree of evenness (Figure 2A).

Some authors have suggested rarefaction analysis as a tool to assess the effect of sample size on the estimate of the diversity of nodule isolates (JESUS et al., 2005). In the present study, this type of analysis showed that the number of isolates obtained from *D. pulchellum* (stem and root), *D. pulchellum* (stem and root) and *D. psoraleaeifolium* (root only) were sufficient to obtain an accurate diversity estimate because the values of Shannon's index stabilized. However, for *D. psoraleaeifolium* (root) and *A. fluminensis* (stem and root), a greater number of

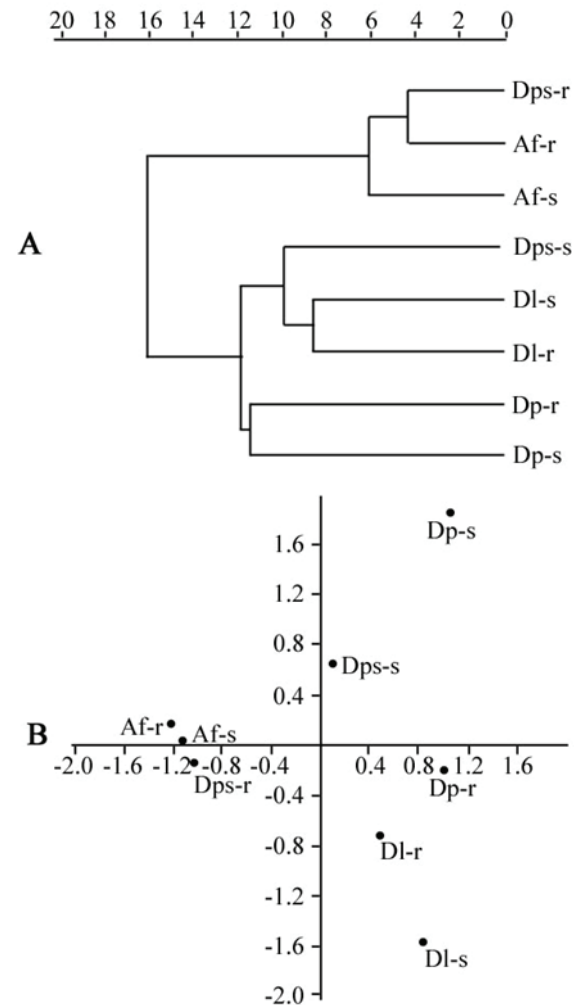
isolates would be necessary to provide a sufficiently good diversity estimate because complete stabilization of the Shannon's index values was not observed (Figure 2B). The species *Aeschynomene fluminensis* was found at only one sampling spot, and for *D. psoraleaeifolium* (root) and *A. fluminensis* (root), only 7 and 12 isolates were obtained, respectively. These limitations in the geographical origin of the samples and in the number of isolates obtained can partly explain the lower diversity observed.



**Figure 2.** The richness, Shannon and evenness indices of the community of rhizobium isolates (A) and the variation of the Shannon indices, as estimated by the rarefaction analysis (B), based on the phenotypic characterization and with reference to the species and organ from which the isolate was obtained. Af-s - *Aeschynomene fluminensis* stem, Af-r - *Aeschynomene fluminensis* root, DI-s - *Discolobium leptophyllum* stem, DI-r - *Discolobium leptophyllum* root, Dp-s - *Discolobium pulchellum* stem, Dp-r - *Discolobium pulchellum* root, Dps-s - *Discolobium psoraleaeifolium* stem, and Dps-r - *Discolobium psoraleaeifolium* root.

At the community level for each plant species, there were smaller distances between the species with similar diversity levels. Based on the distance dendrogram and the ordination diagram, the species *D. psoraleaeifolium* (root), *A. fluminensis* (stem) and *A. fluminensis* (root), which had a lower diversity of isolates, formed a distinct group from the other species that displayed greater diversity, namely, *D. pulchellum* (stem), *D. pulchellum* (root), *D. pulchellum* (stem), *D. pulchellum* (root) and *D. psoraleaeifolium* (stem) (Figure 3). This observation shows that the species that form communities with lower diversity also form communities with lower distance among species when compared to the isolates from more

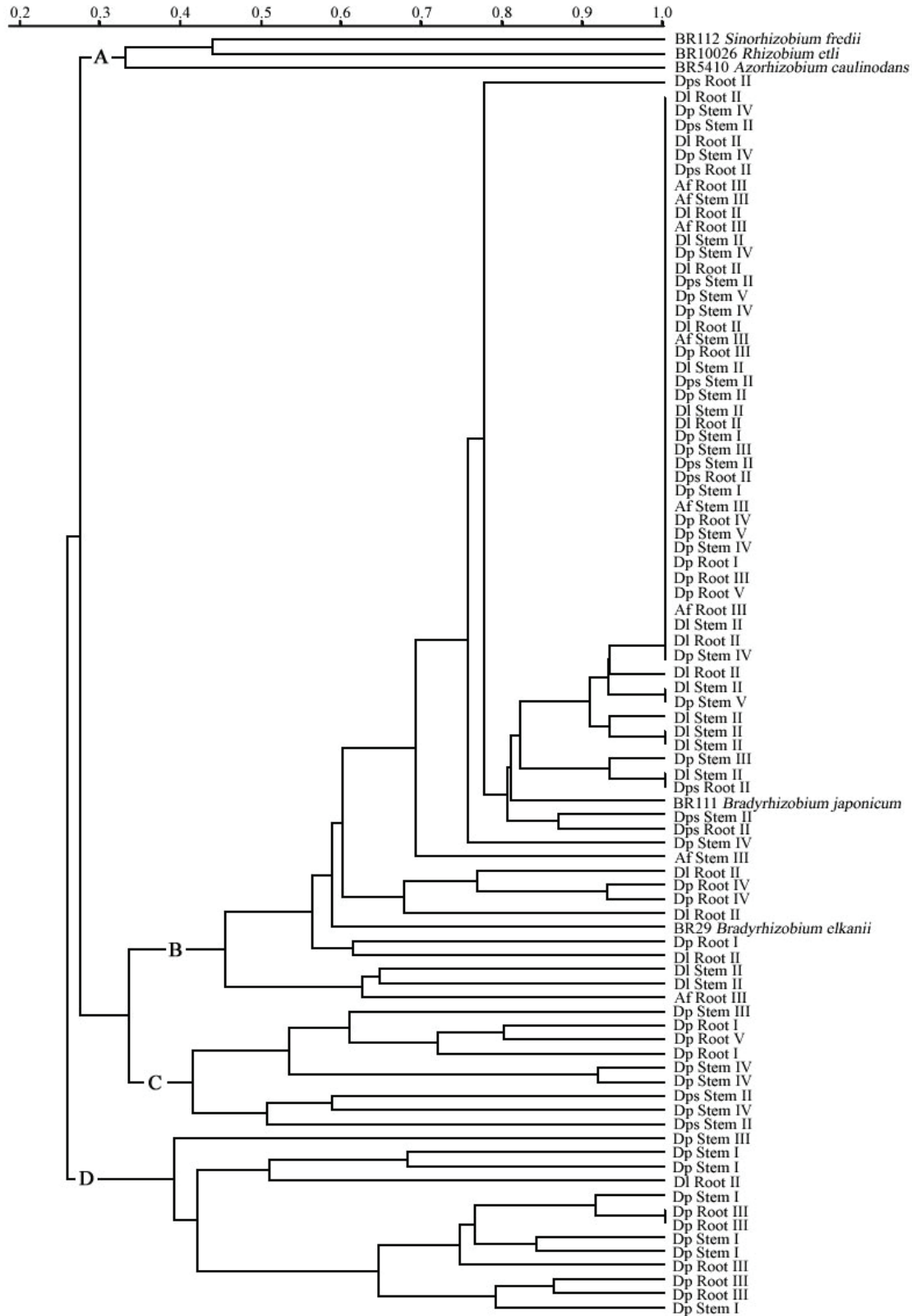
diverse communities. The most diverse communities, namely, *D. pulchellum* (stem), *D. pulchellum* (root), *D. pulchellum* (stem), *D. pulchellum* (root) and *D. psoraleaeifolium* (stem), had different compositions to those of the other species and were separated by the ordination analysis (Figure 3).



**Figure 3.** Distance dendrogram (cophenetic correlation 0.8773) (A) and ordination diagram (component 1 - 63% and component 2 - 13%) (B) of the different communities of nodule isolates from each plant species and organ. Af-s - *Aeschynomene fluminensis* stem, Af-r - *Aeschynomene fluminensis* root, DI-s - *Discolobium leptophyllum* stem, DI-r - *Discolobium leptophyllum* root, Dp-s - *Discolobium pulchellum* stem, Dp-r - *Discolobium pulchellum* root, Dps-s - *Discolobium psoraleaeifolium* stem, and Dps-r - *Discolobium psoraleaeifolium* root.

**RFLP analysis of amplified 16S rDNA gene fragments**

Among the 282 isolates obtained, 86 represented distinct phenotypic clusters and were selected for molecular characterization by restriction analysis of the 16S ribosomal gene. All of the enzymes utilized produced polymorphic bands, and the isolates were clustered into four main groups (Figure 4).



**Figure 4.** A genetic similarity dendrogram based on PCR-RFLP of the 16S rDNA gene among the reference strains *Bradyrhizobium elkanii* (BR 29), *B. japonicum* (BR 111), *Rhizobium etli* (BR 10026), *Sinorhizobium fredii* (BR 112) and *Azorhizobium caulinodans* (BR 5410) and the 86 nodule isolates obtained from *Aeschynomene fluminensis* (Af), *Discolobium leptophyllum* (Dl), *Discolobium pulchellum* (Dp), and *Discolobium psoraleaeifolium* (Dps) plants collected from SESC-Pantanal (I); the Transpantaneira highway (II); the Poconé-Porto Cercado highway (III); the Ypiranga farm (pasture) (IV) and the Ypiranga farm (flooded area) (V).

No trends were observed among the groups of rhizobia in relation to the root or stem from which the nodules were obtained. This suggests that the different isolates obtained have the ability to produce nodules from the stems and roots simultaneously with no additional factor that determines specificity in relation to nodulation in the stem or root. In this respect, Martins et al. (1997) tested the nodulation induction capacity and the biological nitrogen fixation of isolates from the stems and roots of the species *D. pulchellum* and *D. psoraleaeifolium* in *D. pulchellum* under flooded conditions. The results obtained showed that there was no specificity of nodulation in relation to the root or stem. Dreyfus et al. (1988) observed that rhizobia isolated from stem and root nodules from *Sesbania* and *Aeschynomene* had the ability to produce dual nodulation. However, specific rhizobia that induce nodulation in roots but are incapable of doing so in stems were also reported by Dreyfus et al. (1984).

Group A comprised the reference strains belonging to the species *Sinorhizobium fredii* (BR 112), *Rhizobium etli* (BR 10026) and *Azorhizobium caulinodans* (BR 5410). None of the isolates obtained in this study clustered with these strains. Group B comprised 64 isolates and the reference strains belonging to the species *B. japonicum* (BR 111) and *B. elkanii* (BR 29).

Group B contained a subgroup with 52 isolates that exhibited approximately 80% similarity to the reference strain BR 111 from *B. japonicum*. In this sub-group, there were 47 isolates that displayed 100% of the similarity in four different branches and contained 10 isolates from the nodules of *D. leptophyllum* (stem), eight from *D. leptophyllum* (root), 14 from *D. pulchellum* (stem), five from *D. pulchellum* (root), five from *D. psoraleaeifolium* (stem), four from *D. psoraleaeifolium* (root), three from *A. fluminensis* (stem) and three from *A. fluminensis* (root).

These results show that the nodule isolates that induce nodulation in the stems and roots of *Aeschynomene* and *Discolobium* growing in the Pantanal are taxonomically close to each other and also to the *Bradyrhizobium* genus. The species *A. fluminensis* was considered by Boivin et al. (1997) to be highly host-specific in relation to nodulation by rhizobia, and isolates of this species have been described as belonging to the *Bradyrhizobium* genus (WONG et al., 1994; MOLOUBA et al., 1999). The data obtained in the present study corroborate these findings because all of the isolates of *A. fluminensis*

that alkalinized the culture medium were close to each other in terms of their distribution in Group B of the dendrogram obtained from the PCR-RFLP analysis and were also similar to the two strains of *Bradyrhizobium* that were evaluated.

Groups C and D, formed by 9 and 13 isolates, respectively, did not show similarity to any of the reference strains evaluated. Other than one isolate obtained from *D. leptophyllum* (root) and two from *D. psoraleaeifolium* (stem), all of these isolates were obtained from *D. pulchellum* (stem and root). The fact that these isolates did not exhibit similarity to any of the reference strains that use mannitol as a carbon source, together with the fact that they were obtained from plant species for which there is no extensive knowledge of the associated symbionts, suggests that these isolates may comprise a new rhizobial group.

## Conclusion

No difference was observed among the rhizobia from *Discolobium* spp. and *A. fluminensis*.

In addition, no difference was observed among the rhizobia from the stem and root nodules.

It was observed that the stem and root nodule isolates from *Discolobium* belong to the *Bradyrhizobium* genus.

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