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Identification of *Discolobium* species indigenous to the Brazilian Pantanal ecosystem by microsatellite (SSRs) markers

Identificação de espécies de *Discolobium* do Pantanal de Mato Grosso pelo uso de marcadores microssatélites (SSRs)

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Abstract

The genus *Discolobium* comprises forage legumes indigenous to the Brazilian Pantanal. Over 80% of the Pantanal areas flood during the wet season, and stem and root nodules showing high biological N_2 fixation rates result in high protein content in *Discolobium* plants, representing a major component of the animals' diet in this area. In nature it is possible to recognize *Discolobium* species based on some phenotypic properties, as the size, shape and viscosity of the leaflets and fruit morphology. However, when cropped under controlled environmental conditions such characteristics do not express, making species discrimination difficult. In this study, the DNA of four *Discolobium* species – *D. pulchellum, D. psoraleaefolium, D. leptophyllum* and *Discolobium* sp. (non-identified species) – were amplified by PCR with eight microsatellites primers (SSRs, Simple Sequence Repeats), previously identified as effective for discriminating soybean genotypes. One of those primers, Satt 251, detected polymorphism between the *Discolobium* species, allowing their correct identification under any environmental condition. The use of molecular markers in studies with ecological and economical importance, but poorly studied tropical legume species, such as *Discolobium* spp., may considerably improve their usage and preservation.

Key words: Brazilian ecosystems, *Discolobium*, biodiversity, molecular markers, *Simple Sequence Repeats*

Resumo

O gênero *Discolobium* compreende leguminosas forrageiras nativas do Pantanal brasileiro, a maior área de terras naturalmente inundadas do mundo. Quase 80% das áreas do Pantanal são submetidas a alagamento durante a estação das chuvas, e nódulos no caule e nas raízes de *Discolobium* com altas taxas de fixação biológica do N_2 resultam em teores elevados de proteína, representando um componente importante da dieta animal. Na natureza é possível reconhecer plantas de *Discolobium*

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por alguns sinais fenotípicos, como o tamanho, forma e pegajosidade dos folíolos e a morfologia dos frutos. Contudo, quando cultivadas em ambiente controlado, tais características não se expressam, dificultando a diferenciação entre as espécies. Neste estudo, o DNA de quatro espécies de *Discolobium* – *D. pulchellum, D. psoraleaefolium, D. leptophyllum*e *Discolobium* sp. (espécie não identificada) – foi amplificado pela reação de PCR (*Polymerase Chain Reaction*) com oito *primers* do tipo microssatélite (SSRs, *Simple Sequence Repeats*), previamente identificados como efetivos para a diferenciação de cultivares de soja. Um desses *primers*, o Satt 251, detectou polimorfismo entre as espécies estudadas, permitindo a identificação correta das mesmas sob quaisquer condições ambientais. O emprego de marcadores moleculares em estudos com leguminosas tropicais de importância ecológica e ambiental, mas ainda pouco estudadas, como *Discolobium* spp., pode contribuir consideravelmente para o uso e preservação dessas espécies.
Palavras-chave: Ecossistemas brasileiros, *Discolobium*, biodiversidade, marcadores moleculares, *Simple Sequence Repeats*

Introduction

The number of morphological properties used in genetic and breeding studies is small, restricting their utilization in large-scale. Furthermore, the availability of morphological markers in genetic and breeding studies is restricted to few species used as models or with high commercial value, e.g. soybean [*Glycine max* (L.) Merr., maize (*Zea mays* L.) and tomato (*Solanum lycopersicum* L.)].

With the development and increasing use of molecular tools, the identification, genetic characterization and genome mapping of several plants have been revealed in a safer, faster and more efficient way (FERREIRA; GRATTAPAGLIA, 1998; CREGAN et al., 1999, COLLARD et al., 2005, CAIXETA et al., 2009). In this context, several DNA markers have been determined by hybridization, as well as by a variety of methods employing the polymerase chain reaction (PCR) technique, with an emphasis on studies using RFLP (Restriction Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism) methods (STEFFAN; ATLAS, 1991, CAIXETA et al., 2009).

Microsatellites, also known as Simple Sequence Repeats or SSRs, are composed by repeating units of 1-6 base pairs (bp) arranged in tandem and spread throughout the genomes. It has been broadly stated that microsatellites are advantageous over many other markers as they are highly polymorphic, abundant, typically neutral, co-dominant and relatively easy to analyze (FERREIRA; GRATTAPAGLIA, 1998, COLLARD et al., 2005; CAIXETA et al., 2009). Nowadays, a large number of SSRs markers is available and allow an extensive sampling of several important genomes without interference of the environment. In general each SSR marker is considered as a distinct and independent phenotypic character, and the interpretation of the results is quite easy – common bands represent genetic similarities among genotypes, while different bands are associated with genetic differences (COLLARD et al., 2005; CAIXETA et al., 2009).

Discolobium are forage legumes indigenous to the Brazilian Pantanal, the largest area of wetlands naturally flooded of the world and support abundant wildlife populations. Over 80% of the Pantanal flood during the wet season, and stem and root nodules showing high biological N_2 fixation (BNF) rates with *Discolobium* result in high protein contents (≈28%), representing a major component of the animal diet (LOUREIRO et al., 1994).

Wetlands are often subject to annual net losses of N from the system via leaching of the soil or gases losses, which are not balanced by inputs of N via the natural mineralization. These oligotrophic ecosystems are largely dependent on N inputs from BNF, and legume-rhizobial symbioses are some of the main contributors. Most legumes, such as *Discolobium*, that fix N_2 in flooded regions, have

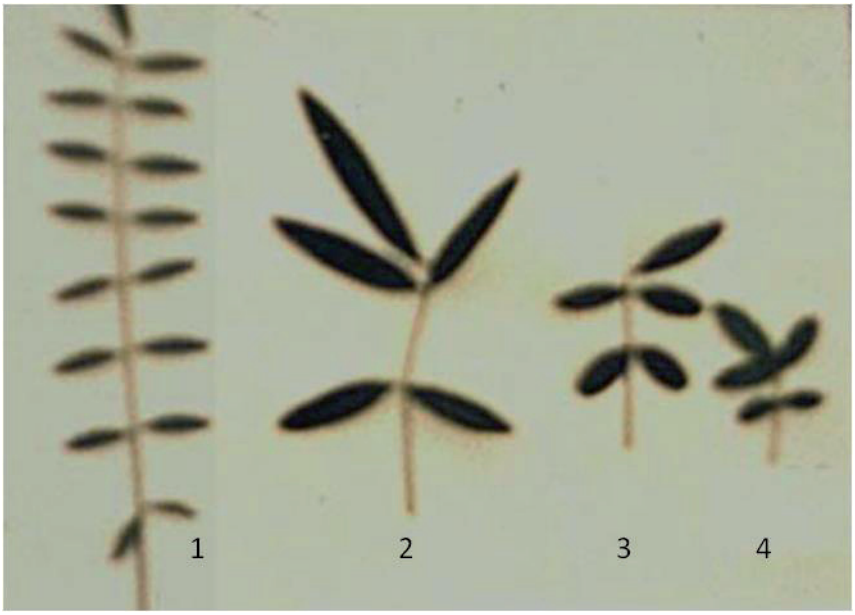
evolved additional mechanisms to optimize the O_2 supply to their flooded nodules, and are thus able to maintain BNF (LOUREIRO et al., 1994). Moreover, deforestation and conversion of native habitats to exotic pasture and crops, plus inefficient agricultural and cattle management practices, are placing great pressures on natural resources in the Pantanal and Cerrado (EATON et al., 2011). More than 40% of the Pantanal forests and savanna habitats have already been altered for cattle ranching through the introduction of exotic grasses species (PADOVANI et al., 2004). Burning – a common practice – is also increasing, and frequently leads to uncontrolled fires. The effects of introduced grasses (and herbicides) are still poorly known (HARRIS et al., 2005).

In nature it is possible to recognize *Discolobium* species based on some phenotypic properties, such as the size, shape and viscosity of the leaflets and fruit morphology. However, when cropped under controlled environmental conditions such phenotypes do not express, making it difficult to discriminate the species within the genus. Although *Discolobium* species play an important ecological role for the sustainability of the Pantanal ecosystem, characterized by low nitrogen (N) availability, there are few studies concerning the biology of this genus. The objective of this study was to search for molecular polymorphism between *Discolobium* species using SSRs markers, aiming at advancing in ecological studies and helping the preservation of the genus.

Material and Methods

Explants of four *Discolobium* species were used: three described and accepted species – *D. pulchellum* Benth., *D. psoraleaefolium* Benth., and *D. leptophyllum* Benth – and a fourth unidentified species, *Discolobium* sp. (Figure 1). In addition, *Aeschynomene fluminensis* was used as a comparison, once it is another N_2 fixing legume abundantly found in Pantanal.

Figure 1. Morphological differences observed in leaves of *Discolobium* sp. 1, *D. psoraleaefolium*; 2, *D. leptophyllum*; 3, *Discolobium* sp.; 4, *D. pulchellum*.



Source: Elaboration of the authors.

Plants were collected in the district of Poconé, an area of 17,320 km^2 localized at the latitude of 16°15', and longitude of 56°37' in the State of Mato Grosso, Brazil. Plants were maintained in a greenhouse under controlled conditions, in 10-kg pots filled with soil from the site where the plants had been collected. Plants were flooded but submitted to continuous oxygenation by constantly renewing the water, therefore simulating the original conditions of the flooded Pantanal.

For the SSR assay, leaves were harvested and kept at –80ºC. DNA extraction from leaves was obtained by using the method described by Keim, Olson and Shoemaker (1988). Two soybean cultivars were used as controls in all steps of the analysis. Eight SSR markers described for soybean by Cregan et al. (1999) were used, selected based on the study of Nicolás, Hungria and Arias (2006) by their capacity of both distinguishing different soybean cultivars, and their co-segregation with QTL (Quantitative Trait Loci) associated to some BNF traits: Satt 066, Satt 192, Satt 197, Satt 231, Satt 232, Satt 242, Satt 251 and Satt 296. Primer pairs were purchased from Research Genetics, Inc. (http://www.resgen.com) and the reactions were performed using 20 ng of genomic DNA. Reaction mixtures and amplification conditions were performed as described (NICOLÁS; HUNGRIA; ARIAS, 2006). The PCR products were separated by electrophoresis on a 1.5% (w/v) agarose + 1.5% (w/v) Synergel (Diversified Biotech, Boston, MA) gel. Gels were stained with ethidiumbromide, visualized under UV radiation and photographed.

Results and Discussion

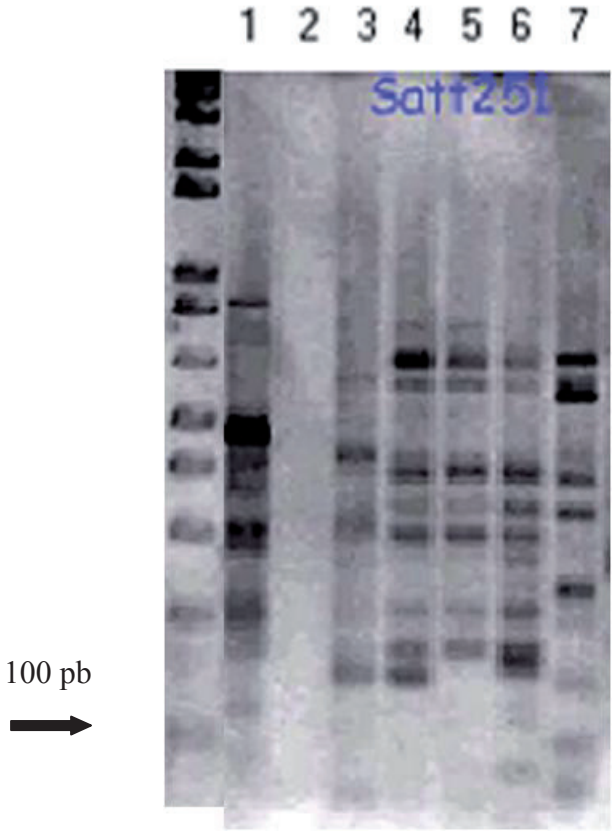
Four out of the eight primers did not detect polymorphism between the *Discolobium* species: Satt 066, Satt 192, Satt 197 and Satt 231. Polymorphism was successful achieved in all *Discolobium* species amplified with the other four primers, Satt 232, Satt 296, Satt 242, Satt 251; however, with the first three primers the PCR products had similar sizes, differing in less than 10 nucleotides (data not shown). Therefore, they might be useful for genetic studies, but not for routine analyses.

Amplification of the DNA with Satt 251 generated fragments differing in more than ten nucleotides (Fig. 2) and clearly allowed to distinguish *D. pulchellum* (line 3) and *A. fluminensis* (line 7) from *D. psoraleaefolium* (line 4), *D. leptophylum* (line 6) and *Discolobium sp.*(line 5). Both, *D. pulchellum* and *A. fluminensis* did not show a band of ~200 pb amplified with primer Satt 251. In addition, Satt 251 distinguished *D. pulchellum*, *D. psoraleaefolium* and *A. fluminensis* by a fragment of ~160 pb which was absent in *Discolobium* sp. and *D. leptophylum* (Figure 2).

Rodríguez, Alcântara Neto and Schuster (2008) used SSR assays to differentiate two varieties of soybean. One variety was susceptible and the other one resistant to soybean stem canker, the latter essentially derived from the former, in five backcross generations. Forty-two microsatellite loci distributed across the integrated genetic map of soybean, of which one locus, Satt 115, differentiated the two varieties, indicating that even essentially derived varieties can be discriminated by SSR molecular markers.

Legumes such as *Discolobium pulchellum* and *Aeschynomene* spp. are preferentially grazed by animals in the Pantanal due to their high protein content. Therefore, in addition to the stresses imposed by their seasonally flooded environments, N_2 fixing legumes are also under threat from animal and man-made pressures, and the consequences of a subsequent decline in their number/diversity may have serious and unforeseen effects on the N cycle (SPRENT; SPRENT, 1990, LOUREIRO et al., 1994).

Figure 2. Amplification of DNA with four SSR primer Satt 251. Numbers indicate: 1) Soybean cultivar Embrapa 20; 2) Soybean cultivar BRS 133; 3) *Discolobium pulchellum*; 4) *D. psoraleaefolium*; 5) *Discolobium* sp.; 6) *D. leptophyllum*; 7) *Aeschynomene fluminensis.*



Source: Elaboration of the authors.

Despite the importance of *Discolobium* to the Pantanal ecosystem, proper identification and preservation of species is limited by the lack of morphological differences under controlled conditions. Therefore, the use of the SSRs markers identified in this study with the primer Satt 251 may be useful allowing a safe characterization of the *Discolobium* species under any environmental conditions.

Conclusion

The SSR primer Satt 251 detected DNA polymorphism between *Discolobium* species and *A. fluminensis,* and thus may be recommended as a molecular marker to distinguish these species under controlled conditions or in the natural environment.

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