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Evaluation of genetic similarity between accessions of *Pityrocarpa moniliformis* (angico-de-bezerro) using RAPD markers

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ABSTRACT. *Pityrocarpa moniliformis* (Benth.) Luckow and Jobson, commonly known as angico-de-bezerro, is a forage legume that occurs naturally in the Caatinga of northeastern Brazil. This fast growing, vigorous, melliferous tree is well adapted to arid terrains and its branches and leaves possess high nutritional value. However, the scarcity of information regarding genetic variability within the species limits its possible exploitation as an animal forage. The aim of the study was to evaluate the genetic similarities of ten accessions of *P. moniliformis* available in the active germplasm collection of Embrapa Meio-Norte, using the RAPD markers to select those most suitable for cultivation and/or plant breeding. Polymerase chain reaction using ten selected RAPD primers generated 110 amplified loci, 106 (96.4%) of which were polymorphic. Primers A10 and M06 produced the largest number of polymorphic loci (18 and 13 bands, respectively),

while primers B18 and K15 generated the smallest number (7 bands each). The dendrogram, constructed using the Jaccard coefficients and considering a cut-off point of 0.41 allowed the separation of the ten accessions into four genotypic groups. The highest genetic similarity coefficient (0.56) was observed between group I accessions BGFAB6 and BGFAB9 and BGFAB 7 and BGFAB 8, while the lowest coefficient (0.11) was observed between accessions BGFAB3 (group IV) and BGFAB10 (group III). The results revealed that genetic variability is present in the accessions of *P. moniliformis*.

Key words: Forage legume; DNA markers; Genetic variability

INTRODUCTION

Leguminous forage crops exhibit productivities and nutritional qualities that are generally superior to those of grasses and maintain their nutritional characteristics for longer periods (Pereira et al., 2001). A survey of the uses of local native plants in three places in the Brazilian State of Piauí (Nascimento et al., 2007) revealed that *Pityrocarpa moniliformis* (Benth.) Luckow and Jobson (Fabaceae: Mimosoideae) was one of the species most commonly used by farmers as a source of animal feed. This fast-growing, vigorous, melliferous plant is well adapted to arid terrains and is frequently found in the Caatinga of northeastern Brazil, especially in the states of Maranhão, Piauí, Ceará, and Bahia, where it is known as angico-de-bezerro. It is an arboreal species ranging from 4 to 9 m, and the slender branches contain relatively high levels of protein (19.14%), P (0.16%) and Ca (0.10%). The branches and leaves of *P. moniliformis* remain green even during the dry season, thus providing an important source of fodder for cattle and goats in the Caatinga (Lorenzi, 2002). However, exploitation of this plant species as a forage crop is limited due to the lack of knowledge of the relevant characteristics and genetic variability within the species, information that is essential for the success of conservation and breeding programs.

Molecular markers have been extensively used in plant breeding programs, since they provide an unlimited number of DNA polymorphisms that are independent of environmental effects and of the physiological status of the plants, thereby allowing the early identification of individuals with desirable traits (Lanza et al., 2000). Among the molecular techniques based on the polymerase chain reaction (PCR), the method employing random amplified polymorphic DNA (RAPD) markers appears to be most advantageous because it is highly sensitive and can detect polymorphisms very fast without prior knowledge of genomic DNA sequences. Furthermore, the RAPD technique is low cost and can be readily implemented in the non-specialized laboratory (Bueno et al., 2001).

RAPD markers have been used in studies with forage legumes due to attributes with respect to cost, ease of use and polymorphism. Ulloa et al. (2003) used RAPD markers to evaluate the genetic diversity in populations of *Trifolium pretense*, and Bortolini et al. (2006) analyzed the genetic variability of 78 accessions of *Trifolium repens* with 24 RAPD primers, while Malviya and Yadav (2010) evaluated the diversity of 17 cultivars of *Cajanus cajan* using 17 RAPD primers.

The active germplasm bank of native forage species maintained by Embrapa Meio-Norte contains various accessions of *P. moniliformis* that need to be fully characterized. The aim of the present study was to evaluate the genetic similarity of ten accessions of angico-de-bezerro using the RAPD technique.

MATERIAL AND METHODS

Plant material

Ten accessions of angico-de-bezerro from the active germplasm collection of Embrapa Meio-Norte originating from the counties of Teresina (BGFAB3 and BGFAB7) and São João (BGFAB1, BGFAB2, BGFAB4, BGFAB5, BGFAB6, BGFAB8, BGFAB9, BGFAB10) in the State of Piauí, Brazil, were evaluated.

DNA extraction

Young healthy leaves were collected from each accession and immediately subjected to DNA extraction with the aid of DNeasy Plant Mini kits (Qiagen, Valencia, CA, USA), employed according to the recommendations of the manufacturer (Qiagen, 2006). Samples of leaves (100 mg) were separately macerated in 2-mL tubes, containing five 3-mm glass beads and buffers from the extraction kit, with the aid of a Precellys® 24 tissue homogenizer/grinder (Bertin, Montigny-le-Bretonneux, France). Aliquots of DNA extracted were subjected to electrophoresis on a 0.8% agarose gel in Tris-borate-EDTA (0.5X TBE) buffer and stained with SYBR® Safe DNA Gel Stain (10,000X; Invitrogen, Carlsbad, CA, USA). Genomic DNA was quantified by comparison with λ DNA standards (150 ng), and the quality estimated by spectrophotometric analysis of 2-µL aliquots using a NanoDrop (Wilmington, DE, USA) model 2000 spectrophotometer. DNA samples were diluted in Tris-EDTA (TE) buffer to a final concentration of 15 ng/µL and stored at -20°C until required for RAPD reactions.

DNA amplification

PCR amplifications were carried out in 0.2-mL microtubes using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA, USA) using a reaction mixture comprising 1.2X Invitrogen buffer (20 mM Tris-HCl, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol; pH 8.0), 3.0 mM $MgCl_2$ (Invitrogen), 0.75 mM dNTPs (Invitrogen), 1 U Taq DNA polymerase (Sigma, St. Louis, MO, USA), 0.2 µM primer, 1 µL DNA template (~15 ng), and ultrapure distilled water to a final volume of 20 µL. The PCR conditions were: initial denaturation at 92°C for 1 min, 45 cycles comprising denaturation at 92°C for 40 s, annealing at 34°C for 1 min and extension at 72°C for 2 min, and final extension at 72°C for 5 min.

Genomic DNA samples were amplified using 68 primers obtained from Operon Technologies (Alameda, CA, USA) to select the primers for RAPD reactions. Ten primers (Table 1) were chosen on the basis of resolution and high levels of polymorphism, and these were subsequently employed in the amplification of DNA samples from each of the ten *P. moniliformis* accessions. The resulting amplicons were separated by electrophoresis on a 1.5% agarose gel in 0.5X TBE at 110 V for approximately 3 h, stained with GelRed™ (Biotium, Hayward, CA,

USA), visualized under a UV transilluminator and photographed. Invitrogen 50-bp and 1-kb DNA ladders were used as molecular weight markers.

Table 1. Numbers of amplified and polymorphic bands obtained following amplification of genomic DNA from Pityrocarpa moniliformis (angico-de-bezerro) in the presence of the primers selected.

Primer	Nucleotide sequence	No. of fragments		% Polymorphism
		Amplified	Polymorphic	
A07	5' GAA ACG GGT G 3'	11	11	100
A10	5' GTG ATC GCA G 3'	18	18	100
B04	5' GGA CTG GAG T 3'	12	11	91.6
B10	5' CTG CTG GGA C 3'	8	8	100
B18	5' CCA CAG CAG T 3'	7	7	100
K15	5' CTC CTG CCA A 3'	10	7	70
M05	5' GGG AAC GTG T 3'	12	12	100
M06	5' CTG GGC AAC T 3'	13	13	100
M11	5' GTC CAC TGT G 3'	10	10	100
N06	5' GAG ACG CAC A 3'	9	9	100
Total		110	106	96

Statistical analysis

The number of polymorphic bands generated by each primer was determined by visual inspection, taking into account only those bands showing medium or strong intensities. Each band was considered to represent a single character, and a binary matrix was created, in which 1 indicated the presence of the band and 0 its absence. Genetic similarities between accessions of *P. moniliformis* were estimated from Jaccard coefficients and the corresponding similarity matrix. A dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) clustering technique. The cophenetic correlation coefficient (r) was calculated from the similarity matrix and the dendrogram. The bootstrap confidence index was also evaluated from the binary matrix of amplified fragments generating a dendrogram from 1000 permutations. The cut-off point was based on the mean genetic similarity (sg_m) calculated according to the equation: $sg_m = \Sigma\, sg_{ij} / N$, in which sg_{ij} is the genetic similarity between pairs of individuals and N is the number of pairs obtained from 1000 permutations. Analyses were performed with the aid of the PAST version 1.34 software (Hammer et al., 2001).

RESULTS AND DISCUSSION

The ten primers selected for the RAPD analysis of the accessions of *P. moniliformis* produced 110 fragments ranging in size from 375 to 3054 bp (Table 1), values that were similar to those obtained in the RAPD analysis of white clover (*T. repens*; Bortolini et al., 2006) and hyacinth bean (*Lablab purpureus*; Rai et al., 2010). The proportion of amplified loci that were polymorphic (96.4%; 106/110) was substantially higher than values previously reported for the forage legumes *Pueraria montana* var. *lobata* (55.3%; Heider et al., 2007) and *C. cajan* (74.7%; Malviya and Yadav, 2010) in RAPD reactions involving 12 and 17 primers, respectively, but slightly lower than that obtained for *Arachis pintoi* (98%) in RAPD reactions with 18 primers (Carvalho et al., 2010). The high level of polymorphic loci observed in *P. moniliformis* with a small number of primers complies with the view that non-domesticated species normally exhibit greater polymorphism than do domesticated species (Innan and Kim, 2004).

Primers A10 and M06 (Figure 1) generated the largest numbers of polymorphic loci (18 and 13 bands, respectively) while primers B18 and K15 produced the smallest numbers (7 bands each). The average number of polymorphic bands produced per primer was 10.6, a value similar to that reported for 69 accessions of *Flemingia macrophylla* (11 bands per primer; Andersson et al., 2006) and greater than that reported for 30 cultivars of *L. purpureus* (5.7 bands per primer; Rai et al., 2010).

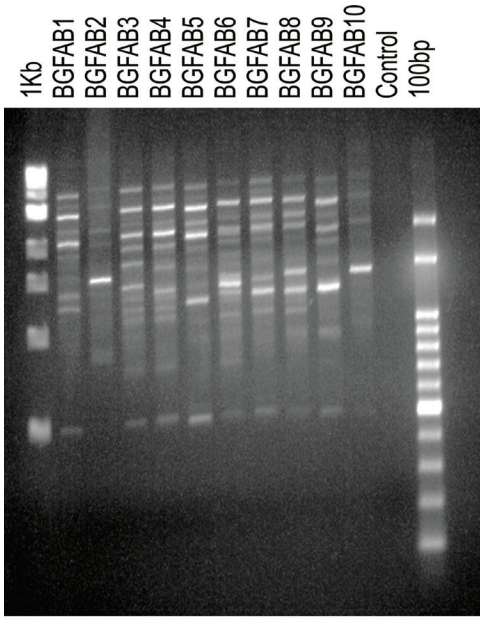

Figure 1. Electrophoretic profiles of RAPD amplifications of DNA samples from ten accessions of *Pityrocarpa moniliformis* (angico-de-bezerro) generated by primer M06.

The coefficients of genetic similarity between accessions of *P. moniliformis* ranged from 0.11 to 0.56 (Table 2). This variation was greater than that observed with domesticated and autogamous forage legumes such as *Stylosanthes macrocephala* (Barros et al., 2005) and *Vigna radiata* (Lakhanpaul et al., 2000).

Table 2. Genetic similarity matrix for ten accessions of *Pityrocarpa moniliformis* (angico-de-bezerro) generated from Jaccard's coefficients calculated on the basis of RAPD markers.

	BGFAB1	BGFAB2	BGFAB3	BGFAB4	BGFAB5	BGFAB6	BGFAB7	BGFAB8	BGFAB9	BGFAB10
BGFAB1	1									
BGFAB2	0.46	1								
BGFAB3	0.28	0.37	1							
BGFAB4	0.55	0.52	0.41	1						
BGFAB5	0.39	0.43	0.30	0.37	1					
BGFAB6	0.43	0.45	0.36	0.50	0.43	1				
BGFAB7	0.42	0.42	0.38	0.50	0.39	0.49	1			
BGFAB8	0.40	0.42	0.31	0.47	0.38	0.52	0.56	1		
BGFAB9	0.51	0.42	0.32	0.47	0.42	0.56	0.43	0.44	1	
BGFAB10	0.38	0.40	0.11	0.28	0.31	0.38	0.33	0.39	0.45	1

The large variation in genetic similarity coefficients observed in *P. moniliformis* was probably due to the non-domesticated nature of the species, since such conditions favor heterogeneity (Vieira et al., 2003). Furthermore, members of the genus *Pityrocarpa* are exclusively allogamous, a characteristic that contributes to increased genetic variability in comparison with autogamous plants (Ferreira, 2009).

The cophenetic correlation of 80% demonstrated the good agreement between the Jaccard similarity matrix and the dendrogram constructed using the UPGMA method. Delimitation of the dendrogram with a cut-off point of 0.41 revealed the genetic interrelationships between the ten *P. moniliformis* accessions and allowed the separation of the accessions into four genotypic groups (Figure 2). Group I included the accessions BGFAB1, BGFAB2, BGFAB4, BGFAB6, BGFAB9, and BGFAB7, collected in São João, PI, and accession BGFAB8, collected in Teresina, PI, suggesting that the last did not originate from the area in which it was collected. Groups II, III and IV comprised one accession each (BGFAB5, BGFAB10 and BGFAB3, respectively), indicating that the diversity of the species could be better exploited if more accessions were collected, confirming the conclusion of Barros et al. (2005) based on the identification of groups of *S. macrocephala* containing few accessions.

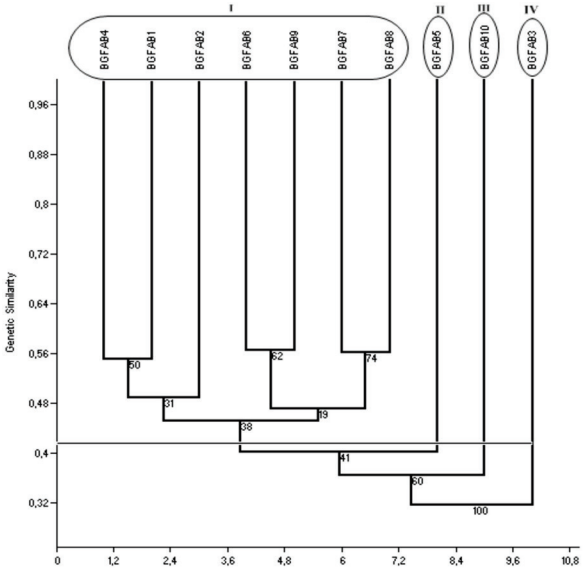

Figure 2. UPGMA dendrogram based on ten RAPD polymorphic markers showing similarity relationships between ten accessions of *Pityrocarpa moniliformis* (angico-de-bezerro).

When individual accessions were pairwise compared, the highest genetic similarity coefficients were observed between group I accessions BGFAB7 from Teresina and BGFAB8 from São João and accessions BGFAB6 and BGFAB9 from São João, while the lowest coefficient was observed between accessions BGFAB10 (group III) and BGFAB3 (group IV). The results presented herein revealed that genetic variability is present in the accessions of *P. moniliformis* available in the active germplasm collection of Embrapa Meio-Norte, which can be exploited in future breeding programs.

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