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ISSR molecular markers for the study of the genetic diversity of *Mimosa caesalpiniaefolia* Benth.

Marcadores moleculares ISSR para el estudio de la diversidad genética de Mimosa caesalpiniaefolia *Benth.*

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ABSTRACT

Knowledge of the genetic diversity of the threatened tree species in the Brazilian semiarid region may contribute to the creation of effective strategies for their preservation and future use. The aim of this study was to select ISSR (Inter-Simple Sequence Repeat) primers capable of detecting genetic polymorphism among *Mimosa caesalpiniaefolia* Benth. individuals for a study of the genetic diversity of this species. A total of 27 ISSR primers were tested for their ability to amplify genomic DNA of three individuals (bulk); all of them amplified regions between the simple sequence repeats of the genome, however seven ISSR primers showed a good standard of amplification compared to the others. The seven primers selected were used to amplify the genomic DNA of nine individuals and generated a total of 78 loci, of which 52.7% were polymorphic. The content of polymorphic information of the primers ranged from 0.261 to 0.489, showing that selected primers are moderately informative; bootstrap analysis determined that 51 loci were enough to estimate the genetic diversity of the samples of individuals. We concluded that the markers generated by the primers UBC 807, UBC 824, UBC 827, UBC 840, UBC 851, UBC 873 and UBC 881 allow detection of the genetic polymorphism among individuals of *M. caesalpiniaefolia*, being useful to determine the genetic diversity of this species.

Key words: semiarid, forest species, primers, polymorphism.

RESUMEN

Conocer la diversidad genética de las especies de árboles forestales en la región semiárida de Brasil puede contribuir al desarrollo de estrategias eficaces para su conservación y uso futuro. El objetivo de este estudio fue seleccionar iniciadores de ISSR (Secuencias Intergénicas Repetidas Simples) capaces de detectar polimorfismo genético entre individuos de Mimosa caesalpiniaefolia *Benth. para estudiar la diversidad genética de la especie. Un total de 27 iniciadores ISSR fueron probados por su capacidad para amplificar el ADN genómico de tres individuos. Todos los iniciadores amplificaron secuencias intergénicas repetidas en el genoma, pero siete fueron seleccionados porque presentaron un mejor patrón de amplificación. Los siete iniciadores seleccionados fueron utilizados para amplificar el ADN genómico a partir de nueve individuos y generaron un total de 78 loci, 52,7% de los cuales fueron polimórficas. El valor del contenido de la información de los iniciadores polimórficos varió de 0,261 a 0,489, demostrando que son moderadamente informativo y el análisis del impulso se determinó que 51 loci fueron suficientes para estimar la diversidad genética de la muestra de los individuos analizados. Los resultados indican que los marcadores generados por los iniciadores UBC 807, UBC 824, UBC 827, UBC 840, UBC 851, UBC 873 y UBC 881 permiten detectar polimorfismo genético entre individuos de* M. caesalpiniaefolia *y pueden ser utilizados para estimar la diversidad genética para esta especie.*

Palabras clave: *semiárido, especies forestales, iniciadores, polimorfismo.*

Introduction

The scarceness of forest products in the Brazilian semiarid region caused by deforestation has created the need for preservation, requiring the sustainable use of the economically important native forest species, and in order to do so, the Brazilian government created a unified list of priority species in 2004, believing that cultivation and harnessing of these species could be an alternative to reduce

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the predatory exploitation in natural areas (Pareyn, 2010). Only four of the listed species are cultivated for commerce; among these is *Mimosa caesalpiniaefolia* Benth., a small-sized tree that occurs naturally in the northeastern semiarid region of Brazil in the states of Ceará and Piauí (Maia, 2004). This is an important species for forest exploitation in the northeastern semiarid region which has advanced studies of harnessing and cultivation, mainly on the exploitation of the wood used as stakes and wooden posts for fences and as an energy source (Pareyn, 2010).

Given this importance the development of studies that can support programs with the objective of maintaining and improving this species genetically are necessary. For both purposes it is relevant to characterize its genetic diversity, because according to Srihari *et al.* (2013) the existence of genetic diversity in the population to be preserved allows the evolution of new genetic combinations, thus presenting greater ability for evolution and adaptation to changes in environmental conditions, which are important characteristics not only for preservation, but also for genetic improvement.

Currently there are several techniques available that allow the study of genetic diversity in plants, including morphological, biochemical and molecular markers (Vashishtha *et al.*, 2013). The most successful of those that have been developed and put to use in the last two decades are molecular markers based on the polymerase chain reaction technique (PCR), such as Simple Sequence Repeat (SSR), Random Amplified Polymorphic DNA (RAPD) and Inter-Simple Sequence Repeat (ISSR) (Semagn *et al.*, 2006).

In the ISSR technique primers composed of di-, tri-, tetra- or pentanucleotide repetitions are used, with or without a one-to-three nucleotide anchorage system that targets the genomic microsatellite region (Zietjiewicz *et al.*, 1994), not making it necessary to perform previous genomic sequencing. In contrast to other molecular markers, the target sequences of the ISSR primers are abundant throughout the eukaryotic genome and evolve quickly, which consequently helps reveal a much larger number of polymorphic loci than other dominant markers such as RAPD (Ansari *et al.,* 2012).

These characteristics make ISSR markers attractive, especially when the discussion is about species that have not yet been studied from the genetic point of view using molecular markers, such as *M. caesalpiniaefolia*. Thus the aim of this study was to select ISSR primers capable of detecting genetic polymorphisms in *M. caesalpiniifolia* individuals to study their genetic diversity.

Material and Methods

Collection of plant material and extraction of DNA

We collected samples of leaves of nine individuals of *M. caesalpiniaefolia* derived from the Área de Experimentação Florestal (5º 53' 52.5" S, 35º 21' 31.6" W) that belongs to the Unidade Acadêmica Especializada em Ciências Agrárias, Universidade Federal do Rio Grande do Norte (UAECIA/UFRN) in the city of Macaíba, Rio Grande do Norte, Brazil. The samples were packed in a thermal container filled with ice and taken to the Laboratório de Genética e Melhoramento Florestal (LABGEM) at the UAECIA/UFRN, where they were transferred to tubes containing 2% CTAB buffered solution and stored at -20 ºC. Genomic DNA was extracted using a modification of the protocol proposed by Doyle and Doyle (1987) and quantified in a microplate spectrophotometer (Epoch™), according to the manufacturer's instructions.

Amplification of DNA with ISSR primers

The reactions for the amplification of the DNA were carried out in a thermocycler (Veriti® 96-Well) under the following conditions: initial denaturation at 94 ºC for 2 minutes; 37 cycles of 15 seconds at 94 ºC for denaturation, 30 seconds at 47 ºC for primer annealing and 1 minute at 72 ºC for extension, with a final extension for 7 minutes at 72 ºC. The PCR amplifications were carried out in 12 µL reaction containing 2.0 µL diluted DNA (1:50) added to 10.0 µL of mixed reaction [0.33 µM primers; 1.2 µL PCR buffer (Buffer IC Phoneutria®); 0.25 $mg.mL^{-1}$ BSA; 2.0 mM $MgCl_2$; 0.25 mM dNTPs; 0.5 U Taq DNA polymerase]; the final volume was completed with ultra-pure water.

PCR products were stained with bromophenol associated with GelRed™ and separated by horizontal electrophoresis in agarose gels (1.5% w/v) immersed in TAE 1X buffer (Tris-Acetate-EDTA) at constant voltage (100 V) for approximately three hours. A negative control containing only the PCR mix and

a standard molecular weight with 1000 base pairs (bp) (K181 DNA Ladder Invitrogen®) were used in each electrophoresis. The gels were photographed under ultraviolet light in a photo-documentation system (E-Box VX2).

We tested 27 ISSR primers (University of British Columbia - UBC) using genomic DNA in bulk of three *M. caesalpiniaefolia* individuals sampled randomly. We selected the primers that presented the best amplification standards (unmistakable, reproducible loci with good resolution quality and in large number), which were used to amplify the genomic DNA of the nine sampled individuals.

Each primer was evaluated with regard to the total number amplified loci, total number of polymorphic loci, rate of polymorphism and the value of the content of polymorphic information according to equation (1), proposed by Anderson *et al.* (1993):

$$PIC_i = 1 - \sum_{j=1}^{n} P_{ij}^2 \qquad (1)$$

Where: P_{ij} is the frequency of the allele "*j*" in primer "*i*".

Allele frequencies were obtained using presence (1) and absence (0) data of the amplified loci for each primer in the nine individuals using the Popgene version 1.3 software (Yeh *et al.,* 1997).

Number of loci sufficient for the study of genetic diversity

The determination of the sufficient number of loci to obtain precise estimates for genetic diversity of the sampled individuals was performed by bootstrap implemented in the Genes software (Cruz, 2001). Using a binary matrix of presence (1) and absence (0) of the amplified loci the genetic distance (Nei, 1978) for each pair of individuals was estimated. We then estimated for each pair of individuals values of simulated genetic distance after re-sampling using different numbers of loci (1; 6; 11; …; 78) with 10,000 permutations. With the values of simulated genetic distance we generated the matrices that were correlated (*r*) with the original genetic distance matrix. The adjustment between the two matrices was verified by the Kruskal stress value (Kruskal, 1964). The number of loci was considered sufficient to carry out the estimates of genetic diversity when the stress reached a value below 0.05 and the correlation was close to the maximum value ($r = 1.0$).

Table 1. ISSR primers with their respective sequences of nucleotides and total number of amplified loci.

ISSR Primer	Sequence of nucleotides (5'- 3')	Total number of loci
UBC 807	AGAGAGAGAGAGAGAGT	9
UBC 808	AGAGAGAGAGAGAGAGC	5
UBC 809	AGAGAGAGAGAGAGAGG	5
UBC 810	GAGAGAGAGAGAGAGAT	9
UBC 813	CTCTCTCTCTCTCTCTT	9
UBC 818	CACACACACACACACAG	10
UBC 821	GTGTGTGTGTGTGTGTT	2
UBC 822	TCTCTC TCTCTCTCTCA	7
UBC 824	TCTCTCTCTCTCTCTCG	8
UBC 825	ACACACACACACACACT	7
UBC 826	ACACACACACACACACC	4
UBC 827	ACACACACACACACACG	12
UBC 829	TGTGTGTGTGTGTGTGC	6
UBC 830	TGTGTGTGTGTGTGTGG	8
UBC 840	GAGAGAGAGAGAGAGAYT	11
UBC 841	GAGAGAGAGAGAGAGAYC	10
UBC 842	GAGAGAGAGAGAGAGAYG	4
UBC 843	CTCTCTCTCTCTCTCTRA	5
UBC 844	CTCTCTCTCTCTCTCTRC	7
UBC 851	GTGTGTGTGTGTGTGTYG	9
UBC 857	ACACACACACACACACYG	6
UBC 859	TGTGTGTGTGTGTGTGRC	10
UBC 860	TGTGTGTGTGTGTGTGRA	7
UBC 862	AGCAGCAGCAGCAGCAGC	8
UBC 873	GACAGACAGACAGACA	11
UBC 880	GGAGAGGAGAGGAGA	6
UBC 881	GGGTGGGGTGGGGTG	9

R, purine (A or G), Y, pyrimidine (C or T).

Results and Discussion

All 27 primers tested amplified sequences between simple repeated regions (microsatellites) in the genome of *M. caesalpiniaefolia* (Table 1). The number of loci amplified per primer varied from 2 to 12; most of them amplified regions with 5 to 10 loci.

The primers selected because they had good amplification compared to the others were UBC 807, UBC 824, UBC 827, UBC 840, UBC 851, UBC 873 and UBC 881. By evaluating the amplification standard of these primers using genomic DNA of nine individuals (Table 2) we found that only UBC 807, UBC 881 and UBC 840 amplified the same number of loci observed during the pre-selection, while UBC 851 amplified a smaller number and UBC 824, UBC 827 and UBC 873 amplified a larger number of loci. This difference in the profile of amplification of the primers is related to the kind of DNA sampled, because according to Yanaka *et al.* (2005), using DNA samples in bulk, alleles with low frequency in the population cannot be amplified.

Table 2. Total number of loci, number of polymorphic loci, rate of polymorphism and value of the polymorphic information content (PIC) of seven ISSR primers in a set of nine *M. caesalpiniaefolia* individuals.

Primer	Total number of loci	Total number of polymorphic loci	Rate of polymorphism	PIC value
UBC 807	9	5	55.6	0.451
UBC 824	16	9	56.3	0.450
UBC 827	14	4	28.6	0.261
UBC 840	11	7	63.6	0.334
UBC 851	7	3	42.9	0.381
UBC 873	12	8	66.7	0.489
UBC 881	9	5	55.6	0.416
Average	11	6	52.7	0.397
Total	78	41	–	–

The analysis of nine individuals with seven primers showed a total of 78 loci, with an average of 11 loci per primer (Table 2). The average number of loci per primer obtained in this study approached the numbers observed in *Pongamia pinnata* (L.) Pierre (10.75 loci) (Kesari *et al.,* 2010), *Praecox chimonanthus* (L.) Link (10.45 loci) (ZHAO *et al.*, 2007) and *Spondias sp.* (10 loci) (Santana *et al.,* 2011), suggesting that the average number of amplified loci by the primers selected for *M. caesalpiniaefolia* may be considered satisfactory.

The seven primers revealed a total of 41 polymorphic loci, that is, 52.7% of the total of amplified loci. However, the ability to detect polymorphism was quite variable among the primers, which varied from 28.6% (UBC 827) to 66.7% (UBC 873) with an average of 52.7% polymorphism per primer. This rate of polymorphism is low compared to values obtained with other sets of ISSR primers in *Hagenia abyssinica* (Bruce) J. F. Gmel. (81.0%) (Feyissa *et al.,* 2007), *Spondias* sp. (80.0%) (Santana *et al.,* 2011), *Thuja sutchuenensis* Franch. (76.1%) (Liu *et al.,* 2013), *Larix gmelinii* (Rupr.) (98.8%) (Zhang *et al.*, 2013) and *Erythrina velutina* Willd (98.0%) (Gonçalves *et al.,* 2014).

However, this does not necessarily mean that the primers evaluated for *M. caesalpiniaefolia* in this study amplify regions with low polymorphism. The rate of primer polymorphism may vary as a function of the population or group of individuals being evaluated, as shown by Oliveira *et al.* (2010) in populations of *Carapichea ipecacuanha* (Brot.) L. derived from different geographic regions, Yiing *et al.* (2014) in planted and natural forests of *Neolamarckia cadamba* (Roxb.) Bosser, Qian *et al.* (2013) between populations of *Calanthe tsoongiana* Tang & F. T. Wangand and by Dai *et al.* (2013) between populations of *Madhuca hainanensis* Chun & F. C. How.

The content of polymorphic information showed values from 0.261 (UBC 827) to 0.489 (UBC 873); the average rate was 0.397 for the combination of the seven primers, which according to Botstein *et al.* (1980) can be classified as moderately informative (Table 2). This evaluation confirms that the selected primers can be used to estimate the genetic diversity of *M. caesalpiniaefolia*, since ISSR primers classified as moderately informative have been used successfully in other species such as *Pongamia pinnata* (L.) Pierre (Kesari *et al.,* 2010), *Dioscorea* spp. (Velasco-Ramírez *et al.*, 2014), *Quercus brantii* Lindl. (Alikhani *et al.*, 2014) and *Trifolium* ssp. (Aryanegad *et al.,* 2013).

Through bootstrap analysis we were able to estimate the number of *loci* sufficient for the study of genetic diversity of *M. caesalpiniaefolia* (Figure 1). We have verified that, with the increase of loci analyzed in re-sampling there was an increase

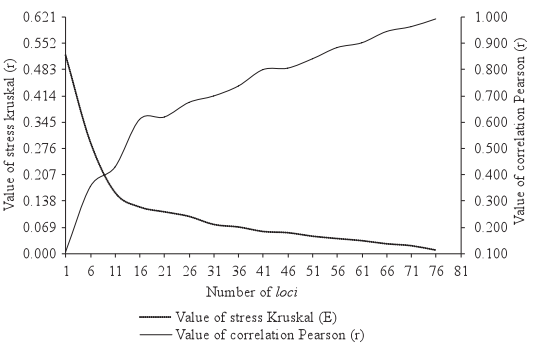

Figure 1. Values of the Pearson correlation (r) and Kruskal stress (E) as a function of the number of ISSR loci used to estimate the genetic diversity of nine *M. caesalpiniaefolia* individuals.

of the values of correlation and a reduction of the Kruskal stress values. When re-sampling was done with 51 loci, the stress was 0.046 and the correlation coefficient was 0.840.

Stress values below 0.05 indicate high precision estimates (Kruskal, 1964) and *r* values close to 1.0 indicate high positive correlation between the original genetic distance matrix and the simulated genetic distance matrix. Thus we can presume that the number of loci used (78) to carry out the genetic diversity estimates for the sampled individuals was sufficient.

The definition of the minimum number of ISSR loci for the study of genetic diversity through bootstrap analysis has been carried out for several taxa, such as *Spondias* sp. (Santana *et al.*, 2011), *Erythrina velutina* Willd. (Gonçalves *et al.*, 2014) and orchids like *Cattleya* and *Brassavola* (Fajardo *et al.*, 2014). Its use has contributed to optimize the use of resources and time, translated into a smaller number of genomic sample representatives to characterize genetic diversity (Gonçalves *et al.,* 2014).

Conclusion

We conclude that the markers generated by the primers UBC 807, UBC 824, UBC 827, UBC 840, UBC 851, UBC 873 and UBC 881 allow us to detect the genetic polymorphism among individuals of *M. caesalpiniaefolia*, being useful to determine the genetic diversity of this species.

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