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Molecular characterization of accessions of *Cratylia argentea* (Camaratuba) using ISSR markers

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ABSTRACT. *Cratylia argentea* (Desv.) Kuntze (Fabaceae) is a drought-tolerant, perennial legume found primarily in Brazil, Bolivia, and Peru. The shrub is well adapted to acid soils and exhibits high productivity and nutritional value, characteristics that would favor its use as a dry season animal forage supplement in semiarid regions. In plant improvement programs, the production of elite hybrids with superior traits is generally achieved by crossing parents that exhibit the highest level of genetic divergence. Therefore, the aim of the present study was to assess genetic diversity among 13 accessions of *C. argentea* from the same population maintained in the active germplasm bank of Embrapa Meio-Norte using inter-simple sequence repeat (ISSR) markers. Genetic similarities between *C. argentea* accessions were estimated from Jaccard coefficients, and a dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA). The set of 15 primers selected for ISSR analysis generated a total of 313 loci of which 79.23% were polymorphic. The mean number of bands per primer was 20.87, and the amplicons

ranged from 280 to 3000 bp in size. Primers UBC834 and UBC827 generated the largest number of polymorphic loci and exhibited 90.91 and 100% polymorphism, respectively. The coefficients of genetic similarity among accessions varied between 0.49 and 0.73. UPGMA cluster analysis allowed the identification of four genotypic groups and demonstrated the existence of considerable variability within the collection. Potential progenitors were selected that would offer good possibilities of obtaining unusual and favorable combinations of genes in a plant breeding program.

Key words: Molecular characterization; Forage crop; Genetic diversity; ISSR markers

INTRODUCTION

In the semiarid north-eastern region of Brazil, the low amount and irregular distribution of rainfall lead to significant levels of soil water deficits in the dry season when temperatures rise and evapotranspiration increases. During this period, the availability of quality forage is severely limited and supplementation of fodder is crucial in animal farming to obtain acceptable productivity (Andrade et al., 2010). Increasing the resistance of forage crops to climatic and abiotic stress would help to alleviate the shortage in supply, a goal that could be achieved by evaluating the intra- and inter-specific variability of native forages and crossing the most divergent genotypes to obtain elite hybrids with superior traits.

Cratylia argentea (Desv.) Kuntze, commonly known as Camaratuba, is a deep-rooting perennial legume found exclusively in South America, particularly in Brazil, Bolivia, and Peru (Argel and Lascano, 1998). This plant is well adapted to acid soils, and shows high productivity and nutritional value. Moreover, the shrub is drought-tolerant, has the capacity to retain green leaves, and exhibits regrowth under arid conditions, characteristics that would favor its use as a dry season forage supplement for cattle and sheep (Lascano et al., 2002).

Forage legumes can improve animal performance because their nutritional properties are superior to those of grasses and they improve the nitrogen content of the soil through symbiosis with nitrogen-fixing bacteria. Such legumes can contribute to the supply of forage over protracted periods and, thereby, reduce the effects of feed discontinuity for livestock associated with the exclusive use of grass pastures (Barcellos et al., 2008).

The genetic improvement of forage crops gives rise to cultivars with improved productivity, enhanced tolerance *to biotic and abiotic* stresses, and better feed conversion efficiency (Jank et al., 2011). The use of this technology has resulted in the improvement of tropical forages such as BRS Campo Grande (*Stylosanthes* spp.) and BRS Mandarim (*Cajanus cajan*), but many other genera and species could also be genetically manipulated (Euclides et al., 2010).

An important step during plant improvement is the molecular characterization of the species, since knowledge of genetic similarities facilitates the identification of heterotic groups and allows the selection of the most divergent parents for crossing (Bonato et al., 2006). Information on genetic variability among specimens within a collection is of critical importance when dealing with a species such as *C. argentea* that has yet to be domesticated (Kamada, 2006). In this context, molecular markers are of particular value because they reveal differences in plant DNA that are independent of environmental factors. The inter-simple sequence repeat (ISSR) technique is straightforward, rapid, efficient, and reproducible, and involves amplification of DNA segments

between two identical microsatellite regions oriented using the polymerase chain reaction (PCR) (Reddy et al., 2002). In the present study, ISSR markers were employed within a plant improvement and conservation program to determine the genetic diversity among accessions of *C. argentea* maintained at the active germplasm bank of Embrapa Meio-Norte (Teresina, PI, Brazil).

MATERIAL AND METHODS

Plant material

Young leaves were harvested from 13 *C. argentea* accessions (C1, C2, C3, C5, C6, C7, C8, C9, C10, C11, C12, C13 and C14), all of which originated from Codó, MA, Brazil, and had been maintained at the Germplasm Bank of Native Forages of Embrapa Meio-Norte. Samples, stored in labeled plastic bags and cooled over ice, were transported to the Laboratory of Biotechnology and Molecular Biology, Embrapa Meio-Norte, and kept at -20°C until analysis.

DNA extraction and PCR amplification

Genomic DNA was extracted from the leaves (0.1 g) of each accession using Qiagen (Venlo, Netherlands) kits following the manufacturer protocol. Aliquots of extracted DNA were subjected to electrophoresis on 0.8% agarose gel in Tris-borate-EDTA (0.5X TBE) buffer and subsequently stained with GelRed™ (1:1000; Biotium, Hayward, CA, USA). The quantity and quality of extracted DNA were determined by comparing with λ DNA standards (100 ng) and using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

DNA samples were amplified using 37 primers developed by the University of British Columbia, Vancouver, Canada. The PCR mixture contained 1.0X buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$; New England Biolabs, Ipswich, MA, USA), 0.5 µL DNA template (7.0 ng/µL), 1.0 mM dNTPs, 0.3 µM primer, 0.5 U Taq DNA polymerase (New England Biolabs) and ultrapure distilled water to a final volume of 10 µL. Amplification reactions were carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation for 90 s at 94°C, 40 cycles each comprising denaturation for 40 s at 94°C, annealing for 45 s at the temperature specified in Table 1, extension for 2 min at 72°C, and final extension for 7 min at 72°C. The resulting amplicons were separated by electrophoresis on 1.5% agarose gel in 0.5X TBE buffer for 6 h at 110 V, stained with GelRed (1:1000), visualized under a UV transilluminator, and subsequently photographed. The sizes of amplicons were estimated by comparison with a 1-kb DNA ladder (Invitrogen, Life Technologies do Brasil, São Paulo, SP, Brazil).

Statistical analysis

Analyses were performed with the aid of the PAST program version 1.34 (Hammer et al., 2001). Fifteen of the 37 primers tested were selected based on greater resolution of amplicons and higher levels of polymorphism, and these were employed in subsequent analyses. On the basis that each amplicon represented a single character, a binary matrix was created in which 1 indicated the presence and 0 indicated the absence of a specific band. From this matrix, genetic similarities between accessions of *C. argentea* were estimated using Jaccard coefficients, and a dendrogram was constructed using the unweighted pair group method with arithmetic average

(UPGMA) clustering technique. The cophenetic correlation coefficient (*r*) and the bootstrap confidence index were calculated from the binary matrix of amplified fragments and the dendrogram after 1000 permutations.

RESULTS

The 15 primers selected for ISSR analysis of *C. argentea* accessions generated 313 loci of which 248 (79.23%) were polymorphic (Table 1). The sizes of the amplicons ranged from 280 to 3000 bp. The mean number of bands per primer was 20.87, with primers UBC834 and UBC848 generating the highest (33) and UBC808 the lowest (7) numbers. Primers UBC834 and UBC827 generated the largest number of polymorphic loci, and exhibited 90.91 and 100% polymorphism (Figure 1), respectively.

Table 1. ISSR markers used to amplify genomic DNA from 13 accessions of *Cratylia argentea* (Fabaceae) in the active germplasm bank of Embrapa Meio-Norte.

Primer	Sequence 5'-3'[a]	Ta (°C)[b]	Number of amplified bands	
			Total	Polymorphic
UBC 808	AGA GAG AGA GAG AGA GC	50	7	6
UBC 810	GAG AGA GAG AGA GAG AT	52	19	14
UBC 811	GAG AGA GAG AGA GAG AC	50	27	22
UBC 812	GAG AGA GAG AGA GAG AA	50	17	8
UBC 818	CAC ACA CAC ACA CAC AG	49	8	5
UBC 827	ACA CAC ACA CAC ACA CG	60	30	30
UBC 830	TGT GTC TGT GTC TGT GG	50	8	6
UBC 834	AGA GAG AGA GAG AGA G**Y**T	54	33	30
UBC 835	AGA GAG AGA GAG AGA G**Y**C	50	30	25
UBC 836	AGA GAG AGA GAG AGA G**Y**A	54	25	23
UBC 840	GAG AGA GAG AGA GAG A**Y**T	48	22	20
UBC 842	GAG AGA GAG AGA GAG A**Y**G	52	14	10
UBC 848	CAC ACA CAC ACA CAC A**R**G	50	33	25
UBC 878	GGA TGG ATG GAT GGA T	48	18	12
UBC 886	**VDV** CTC TCT CTC TCT CT	50	22	12
Total			313	248

[a]Y = C, T; D = A, G, T; V = A, C, G and R = A, G. [b]Ta = annealing temperature.

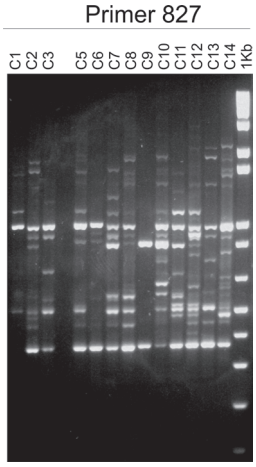

Figure 1. Electrophoretic profiles of ISSR amplifications generated by primer 827 with DNA samples derived from the 13 accessions of *Cratylia argentea* (Fabaceae) in the active germplasm bank of Embrapa Meio-Norte.

The genetic similarity matrix revealed variability among the *C. argentea* accessions with Jaccard coefficients ranging from 0.49 to 0.73 (Table 2). Pairwise comparison of individual accessions revealed that C12 and C13 showed the highest coefficient of genetic similarity (0.73), while the lowest was observed between C6 and C9 (0.49). The calculated value of *r* was 0.87, signifying that UPGMA clustering had introduced no significant distortion and that the dendrogram was a true representation of the original data.

Table 2. Jaccard coefficients showing the genetic similarities between the 13 accessions of *Cratylia argentea* (Fabaceae) in the active germplasm bank of Embrapa Meio-Norte.

	C1	C2	C3	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14
C1	1												
C2	0.57	1											
C3	0.70	0.57	1										
C5	0.65	0.62	0.60	1									
C6	0.50	0.55	0.52	0.58	1								
C7	0.57	0.59	0.59	0.63	0.53	1							
C8	0.60	0.58	0.59	0.68	0.56	0.72	1						
C9	0.55	0.53	0.57	0.57	0.49	0.58	0.58	1					
C10	0.58	0.54	0.60	0.63	0.52	0.57	0.60	0.59	1				
C11	0.61	0.53	0.62	0.65	0.56	0.58	0.64	0.57	0.66	1			
C12	0.59	0.53	0.59	0.61	0.53	0.58	0.57	0.56	0.65	0.61	1		
C13	0.57	0.53	0.57	0.61	0.53	0.57	0.57	0.54	0.65	0.63	0.73	1	
C14	0.58	0.60	0.59	0.64	0.57	0.63	0.62	0.54	0.65	0.68	0.66	0.62	1

The average coefficient of similarity considering all of the studied loci was 0.59, which was taken as the cut-off point in the dendrogram displayed in Figure 2. In this manner, the data obtained from ISSR analysis allowed the identification of four genotype groups. Groups 1, 3, and 4 comprised one accession each, namely C9, C2, and C6 with coefficients of 0.56, 0.56, and 0.54, respectively. Group 2, containing the accessions C1, C3, C5, C7, C8, C10, C11, C12, C13, and C14 with an average similarity coefficient of 0.62, could be subdivided into three subgroups as follows: subgroup 1 (C1 and C3), subgroup 2 (C5, C7, and C8), and subgroup 3 (C10, C11, C12, C13, and C14). Bootstrap confidence indices confirmed the consistency of the nodes and the reliability of the data.

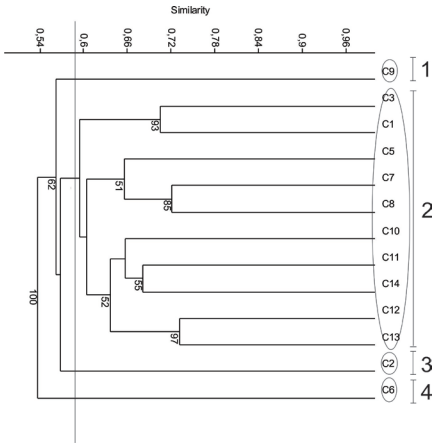

Figure 2. UPGMA dendrogram based on the 15 selected ISSR polymorphic markers showing similarity relationships between 13 accessions of *Cratylia argentea* (Fabaceae) in the active germplasm bank of Embrapa Meio-Norte. (Bootstrap values >50% are shown).

DISCUSSION

The efficiency of primers used to estimate the genetic diversity of a population is assessed by the number of polymorphic loci amplified. The ISSR primers employed in the present study afforded 79.23% polymorphism and, therefore, could be considered very efficient at determining the variability in *C. argentea.* The level of polymorphism reported herein is similar to values obtained for other leguminous species such as *Vigna mungo* (Kanimozhi et al., 2009; Karuppanapandian et al., 2010), *Arachis hypogaea* (Mondal et al., 2009), and *Caragana microphylla* (Huang et al., 2013), but higher than those reported by Souframanien and Gopalakrishna (2004) for *V. mungo* (54.5%) and by Muthusamy et al. (2008) for *V. umbellata* (61.78%).

Following ISSR analysis of the genetic relationships between *V. unguiculata* subspecies, *V. radiate*, and *V. mungo*, Tantasawat et al. (2010) reported that the mean number of bands per primer was 19.5 and that the sizes of amplified fragments varied from 200 to 3000 bp, which are similar to the values reported herein. On the other hand, the mean number of bands per primer attained in the present study was higher than those generated with *V. unguiculata* (8.6 bands/primer; Ghalmi et al., 2010) and *V. amoena* (8.1 bands/primer; Liu et al., 2013), while the amplicons derived from *C. argentea* were larger than those obtained from *Astragalus nitidiflorus* (300-1500 bp; Vicente et al., 2011) and *Derris trifoliata* (250-2000 bp; Wu et al., 2012).

Although all of the *C. argentea* accessions in the active germplasm bank at Embrapa Meio-Norte originated from the same location, three of the identified clusters contained single accessions indicating the existence of considerable diversity, which could be used in a plant improvement program. Additionally, the coefficients of genetic similarity obtained for the accessions of *C. argentea* were within the limits observed for *Medicago* sp. (0.26-0.76; Xavier et al., 2011) and *Cyamopsis tetragonoloba* (0.20-0.88; Sharma et al., 2014).

Bystricky et al. (2010) determined that the gametes of *C. argentea* flowers were mainly self-incompatible and that successful pollination was limited in the absence of pollen from other plants. This type of reproductive mechanism explains why variability exists within the accessions, since cross-pollination permits the recombination of genes from different plants and the emergence of new combinations that do not appear in the progenitors.

Crossing highly divergent accessions is a strategy usually employed in plant improvement programs, which aims to obtain a greater value hybrid. Identification of individuals with the highest level of genetic divergence facilitates the selection of appropriate parents for such crosses, since the larger the divergence between progenitors, the greater the segregation of genes in the hybrid lineages (Franco et al., 2001). From the results presented herein, it would appear that crosses between C6 and C9, C1, and C6, C3 and C6, C6 and C10, C6 and C7, and others, would offer good possibilities of obtaining unusual and favorable combinations of genes.

The present study has highlighted the importance of maintaining and amplifying accessions of *C. argentea* in the active germplasm bank of Embrapa Meio-Norte, since the genetic diversity among accessions could be exploited in future programs aimed at breeding genotypes and forage hybrids with greater productivity, nutritional value, and resistance to drought conditions.

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