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Genetic diversity in natural populations of *Stylosanthes scabra* Fabaceae using ISSR markers

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ABSTRACT. Genetic diversity is the basis for genetic improvement as it can provide the basis for new cultivars. *Stylosanthes scabra* is a grasslands legume that presents potential economic importance in tropical and subtropical regions. Plants of the genus *Stylosanthes* naturally occur in semiarid native pastures in northeastern Brazil and are highly favored by grazing animals; therefore Brazil's Northeast stands out as an center of this genus. We evaluated the genetic diversity of *S. scabra* using ISSR molecular markers; naturally occurring samples were harvested from Santa Cruz do Capibaribe, Floresta, Sertânia and Petrolina in Pernambuco state, Brazil. We selected seven ISSR primers for amplification and analyzed 75 individuals, obtaining 88 bands, which amplified with 95% polymorphism at the species level. The AMOVA test revealed that 40% of the total genetic variation occurs within populations and 60% among populations. Population differentiation was 0.332 and the migrant number per generation was 0.5. Grouping analysis confirmed a high level of differentiation among populations and that the greatest variability was in Santa Cruz do Capibaribe and Petrolina regions. The ISSR markers were efficient for genetic diversity quantification in *S. scabra*, which presented greater variability among populations

than within them. Overall, the population variability found will be useful for breeding programs for this species.

Key words: Molecular markers; Legumes; Variability; Breeding programs

INTRODUCTION

Pasture quality decline in Brazilian semi-arid Northeast in dry seasons has stimulated studies of native forage plants (Santana Neto et al., 2015), and forage legumes have been used as a protein and minerals source to correct the deficiency of natural pastures (Tufarelli et al., 2010; Idowu et al., 2013). The incorporation of forage legumes into the ruminant diet as a complementary forage improves feed intake and reduces feed costs (Pen et al., 2013; Olafadehan et al., 2014).

Stylosanthes is a forage legume genus predominantly perennial with extensive adaptation and resistance to biotic and abiotic factors (Pangga et al., 2004; Costa et al., 2008; Nagaich et al., 2013). This genus can also biologically fix nitrogen by association with diazotrophic bacteria (Mendonça et al., 2017) and it is very useful for the recovery of degraded pastures (Fabrice et al., 2015).

There are 25 species of *Stylosanthes* in Brazil, of which 13 are endemic (Santos Garcia et al., 2011). *Stylosanthes scabra* is a species with widespread occurrence in Brazil's Northeast (Calles and Schultze Kraft, 2016) and can grown in low fertility soils, particularly low phosphorus content, conferring an advantage over other species (Gonzalez et al., 2000; Oliveira et al., 2016). Although *S. scabra* populations occur in various environments in Brazil, genetic studies at the DNA level have not been performed (Queiroz et al., 2001).

Stylosanthes scabra has some commercial cultivars, such as Seca, launched in partnership between the International Center for Tropical Agriculture (CIAT) and the Commonwealth Scientific and Industrial Research Organization (CSIRO). This cultivar has stood out for its adaptability and agronomic performance under dryland agriculture conditions in subtropical climates. It has over 17% crude protein, with a low level of tannin (Mpanza et al., 2013). According to Akinlade et al. (2008), *S. scabra* can reach a dry mass production of up to 1.97 $Ton.ha^{-1}$.

To explore *S. scabra*'s full potential it is essential to know its genetic variability to support plant-breeding programs (Araújo et al., 2016). Genetic diversity evaluation within and among populations of a species can generate information for genetic conservation in situ of natural populations (Gonçalves et al., 2010). The existence of variability in natural populations also allows the evolution of new genetic combinations (Medrano et al., 2014), with higher capacity for evolution and adaptation to changes in environmental conditions (Srihari et al., 2013).

Four types of markers are used for genetic diversity characterization: morphological, biochemical, molecular and cytological. Molecular markers have the advantage of use at any stage of plant development (Martuscello et al., 2015; Vieira et al., 2015). Among molecular markers, ISSRs (Inter Simple Sequence Repeat) stand out, because they do not require prior DNA sequence information and laboratory procedures show good transferability (Dias et al., 2015).

ISSR are semi-arbitrarily amplified by PCR using a complementary primer for a designated microsatellite (Nilkanta et al., 2017). In the *Stylosanthes* genus, ISSR markers

are considered more efficient than both RAPD and STR markers to detect genetic variability (Nagaich and Chandra, 2009). We evaluated the genetic diversity distribution in four populations of *S. scabra* in the semiarid region of Pernambuco state, Brazil using ISSR markers.

MATERIAL AND METHODS

Samples of 76 *S. scabra* plants were used from four natural populations in the semiarid region of the Pernambuco state, Brazil (Table 1) and 19 individuals represented each population.

Table 1. *Stylosanthes scabra* populations studied in Pernambuco state, Brazil.

Populations	Latitude	Longitude	Sample site description
Santa Cruz do Capibaribe	08º21'01" a 08º21'46"	40º20'19" a 40º21'52"	Pasture
Floresta	08º31'77" a 08º43'24"	38º28'06" a 38º29'22"	Caatinga (roadside)
Sertânia	08º00'52" a 08º32'35"	36º27'58" a 37º36'28"	Caatinga (roadside)
Petrolina	08º58'32" a 08º59'03"	40º16'12" a 40º45'16"	Banks of a irrigation canal

Young leaves DNA were extracted following the methodology adjusted for this genus by Santos Garcia et al. (2012). The DNA extracted was quantified by comparison with lambda standards (Invitrogen, Carslbad, CA, USA) with known concentrations (100, 200 and 500 $ng.\mu L^{-1}$) in 0.8% agarose gel. DNA purity was confirmed in a spectrophotometer under UV light (260/280 nm).

Seven ISSR primers (Table 2) were used. The 25 µL reaction mixtures contained 1x PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.8 µM of primer, 1 U of Platinum Taq DNA Polymerase (Invitrogen), 0.25 µM of each dNTP (Invitrogen), 25 ng of DNA template, and sterile distilled water to 25µL. DNA amplifications were performed on thermocycler under the following conditions: 94°C for 4 min (initial denaturation), followed by 30 cycles of 94°C for 30s, 50°C for 1 min, and 72°C for 90s, with a final extension step at 72°C for 7 min (Nagaichand and Chandra 2009).

Table 2. ISSR primers selected for *Stylosanthesscabra* populations, sequence, total fragment number per primer, polymorphism percentage (LP).

Primer	Sequence	Loci	Polymorphism (%)
UBC 1	ACACACAACACACACACT	10	100
UBC 2	GAGSGSGAGAGAGAGAT	12	100
UBC 808	AGAGAGAGAGAGAGAGC	9	100
UBC 810	CTTCATTTCACTTCA	16	100
UBC 813	GAGAGAGAGAGAGAGAA	15	95.5
UBC 879	CTTCATTTCACTTCA	12	100
UBC 888	BDBCACACACACACACA	14	71.4
Total		88	95.3

The amplification products were separated on a 2% agarose gel stained with Sybr Gold (Invitrogen) using the 100 bp marker (Invitrogen) and visualized under ultraviolet light and recorded on a VilberLourmat digital photo-documentator. The polymorphisms were tabulated according to the presence (1) or absence (0) of bands. Genetic diversity was estimated through effective alleles number (Ne), Shannon Index (I), heterozygosity within populations (H_s), genetic differentiation coefficient (G_{ST}) and migrant number in the population (Nm) using the GenAlex 6.5 software (Peakall et al., 2012).

Genetic identity and genetic distance among populations were computed using the model proposed by Nei (1978). Molecular variance analysis (AMOVA) was used to estimate genetic diversity distribution within (among individuals within the population) and among populations. The "structure" program (Pritchard et al., 2000) based on Bayesian statistics was used to infer the group's number (k). The correlation between the geographic distance and genetic distance among *S. scabra* populations was made by the Genes program using 9999 permutations (Cruz, 2013).

RESULTS AND DISCUSSION

The segregated amplicons numbers ranged from 9 for the UBC808 to 16 loci for UBC810 primer (Figure 1) and reached 100% polymorphism (Table 2). These values are similar to those of other studies that used dominant markers in *Mimosa caesalpinaefolia* (Araújo et al., 2016), in *Croton tetradenius* (Almeida Pereira et al., 2017) and in *Desmanthus sp*. (Costa et al., 2017).

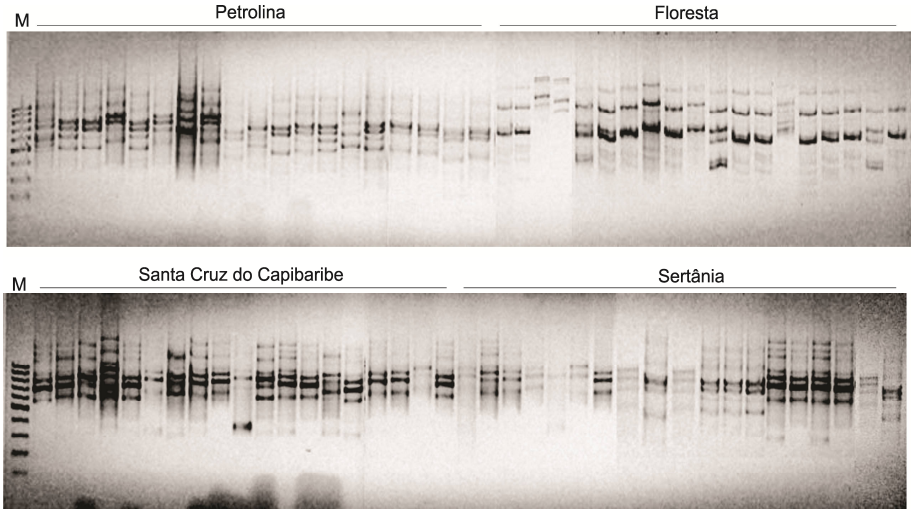

Figure 1. ISSR primer UBC810 electrophoresis profiles of four *Stylosanthes scabra* populations native to Pernambuco state, Brazil. M: molecular mass markers (Ladder 100 bp).

Santa Cruz do Capibaribe and Petrolina populations presented the highest intra-populational genetic diversity (HS) (Table 3), likely due to seed transport by animals, since the Petrolina population is located on the banks of an irrigation canal that allows animals to drink water, while Santa Cruz do Capibaribe population is located in a pasture used for grazing.

The genetic differentiation coefficient was 0.334, considering all populations (Table 3). According to Lu et al. (2005), values of 0.30 represent a high degree of differentiation among populations, suggesting low rates of gene flow among populations. In contrast, it is not possible to differentiate populations when the gene level flow is high (Ambiel et al., 2010). This confirms the proposition of Collevatti et al. (2013) that genetic differentiation coefficient is one of several genetic parameters that define a specie's relation with the environment.

Table 3. Genetic differentiation parameters in four populations of *Stylosanthes scabra* in Pernambuco state, Brazil.

Populations	Ne	I	H_S	G_{ST}	N_M
Santa Cruz do Capibaribe	1.241	0.212	0.141		
Floresta	1.235	0.206	0.138		
Sertânia	1.150	0.134	0.089		
Petrolina	1.242	0.197	0.136		
Média	1.217	0.187	0.126		
Total	1.434	0.445		0.334	0.500

Effective allele number (Ne), Shannon index (I), Heterozygosity within populations (H_s), genetic differentiation coefficient (G_{ST}), migrant number in the population (Nm).

The migrant number in the population was only 0.5 individuals per generation (Table 3) confirming the isolation of the populations. *Stylosanthes scabra* is predominantly autogamous, so the low migrant number discards the occurrence of relevant genetic migration in the process of population differentiation (Zamora et al., 2015). The genetic distance among populations varied from 0.244 between Sertânia and Santa Cruz do Capibaribe, and 0.505 between Santa Cruz do Capibaribe and Petrolina (Table 4). The closest populations were those with the smaller genetic distance, with the more geographically distance also presenting a greater genetic distance.

Table 4. Geographic distance and genetic distance for four populations of *Stylosanthes scabra* in Pernambuco state, Brazil.

	Santa Cruz do Capibaribe	Floresta	Sertânia	Petrolina
Santa Cruz do Capibaribe		345 Km	161 Km	624 Km
Floresta	0.466		183 Km	278 Km
Sertânia	0.244	0.275		464 Km
Petrolina	0.505	0.402	0.325	

The matrix correlation between genetic and geographic distances was significant according to the Mantel test, at 1% probability, which is uncommon in similar studies (Jamnadas et al., 2006; Vigna et al., 2011), since according to Zhao et al. (2012), environmental factors, mating system, population size and genetic flow are more important than geographic distance in population differentiation.

The Petrolina population had more privative bands (Figure 2) and greater variability, contributing to the appearance of DNA fragments (Szpiech and Rosenberg, 2011). According to Ellstrand (2014), the occurrence of private bands is related to the average number of exchanged migrants per generations among populations and their presence may indicate reduced gene flow.

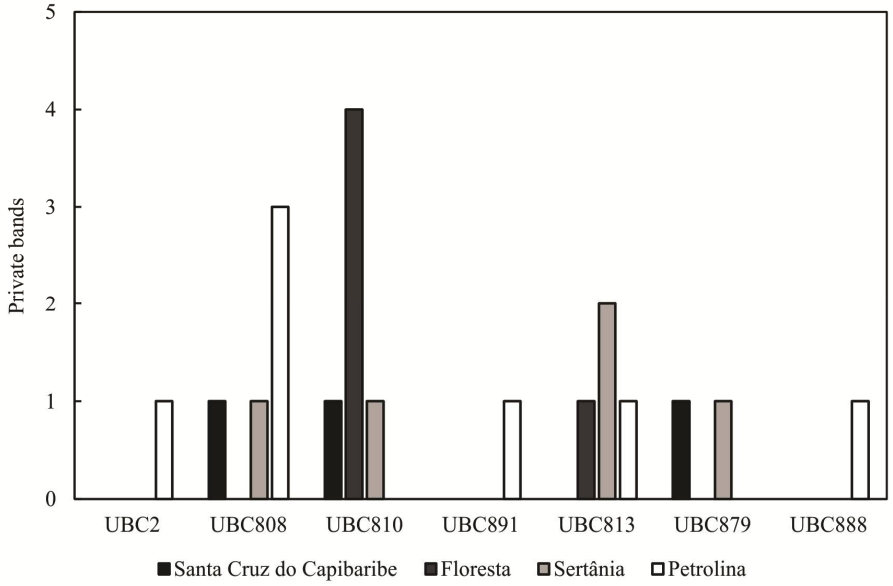

Figure 2. ISSR primer private bands in *Stylosanthes scabra* natural populations from Pernambuco state, Brazil.

Most of the genetic variation (60%) was among populations and 40% within populations, according to the AMOVA results. Species with auto fecundation exhibit greater genetic diversity among populations (Buzatti et al., 2012), which does not happen for plants with high rates of allogamy. A study by Santos Garcia et al. (2012) with microsatellite markers, *S. capitata* (which may present allogamy above 20%) did not show a correlation between the genetic distance and the collection sites, although these authors examined few samples per collection site.

The lower genetic diversity within populations reinforces the need for conservation and protection of natural populations in this region. The between-population variability represents an adaptation strategy to the different environments as a process of genetic maximization in the species evolution (Queiroz, et al., 2011). Thus, it is unnecessary to collect many individuals per *S. scabra* population, but rather to collect fewer individuals from more populations, in future collection efforts.

Considering the overall sample, four groups were formed following the collection sites (Figure 3). This grouping occurs by geographic isolation and *S. scabra*'s autogamy (Chandra et al., 2009). In these cases, superior genotypes tend to stand out in the population and have a larger offspring number, increasing within-population similarity. Barros et al. (2005) also observed a trend of watershed separation for *Stylosanthes* accesses, collected in Bahia and Minas Gerais, both states of Brazil.

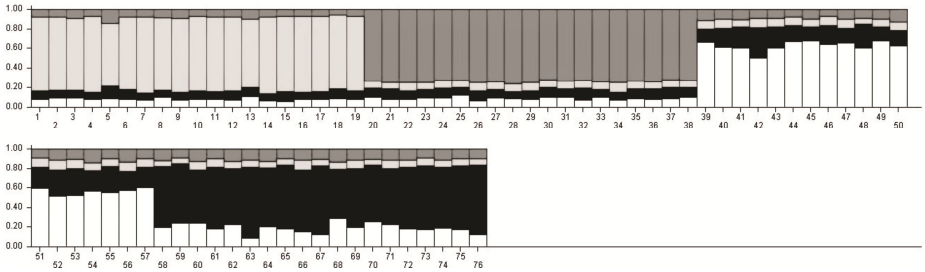

Figure 3. Representation of 76 individuals from four natural populations of *Stylosanthes scabra*, according to molecular data with ISSRs using the "Structure" program. The individuals are represented by vertical bars with coloration according to each group. Group 01 - Santa Cruz do Capibaribe; Group 02 - Floresta; Group 03- Sertânia and Group 04 - Petrolina.

Bayesian analyzes showed a maximum K value = 4, confirming the well-defined genetic structure of the four populations. These results confirm what was expected due to the low number of migrants and high genetic differentiation among the populations. According to Rossi et al. (2014), genetic structuring of populations occurs when the maximum K value coincides with the population number and the individuals of each population are grouped. Thus, there is intra-population genetic variability and the four sampling locations may be recognized as four distinct populations.

Finally, the ISSR markers used in our study were able to determine the genetic variability in *S. scabra*. There was a greater variability among populations than within populations, which showed the importance of *S. scabra* collections in different regionis for the construction of germplasm banks. We suggest future collections, emphasizing Santa Cruz do Capibaribe and Petrolina to exploring the higher variability in these places.

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