

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

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ABSTRACT. Genetic diversity is the basis for genetic improvement, providing genes for new cultivars. *Stylosanthes scabra* is a legume used in pastures, presenting economic importance in tropical and subtropical regions. Molecular markers are targeted in population studies, thus we evaluated the genetic diversity distribution of *S. scabra* in Brazilian semi-arid northeastern among and within populations, from molecular markers ISSR. Four natural populations of *S. scabra* located in the municipalities of Santa Cruz do Capibaribe, Floresta, Sertânia and Petrolina from Pernambuco state were used. We selected seven ISSR primers for amplification and analyzed 75 individuals. A total of 88 bands were amplified with a percentage of 95.27% polymorphism at the species level. AMOVA test revealed that 40.0% of the total genetic variation is within populations while 60.0% among populations. The population differentiation was 0.332 and the migrant's number per generation was 0.5. Grouping analysis confirmed a high level of differentiation among populations. The populations with the greatest variability were Santa Cruz do Capibaribe and Petrolina, and the ISSR markers were efficient for the genetic diversity quantification in *S. scabra*, which presented greater variability among populations than

within populations. In short, this variability will be very useful in future breeding programs for this species.

KEY WORDS: Molecular markers; Legumes; Variability; Breeding programs.

INTRODUCTION

The scarcity of pastures in Brazilian semi-arid north-eastern in dry seasons has stimulated the advancement in studies of native forage plants (Santana Neto et al., 2015). Forage legumes can be used as a protein source to correct the deficiency of natural pastures (Tufarelli et al., 2010), moreover contain high levels of minerals and vitamins (Idowu et al., 2013).

The incorporation of forage legumes into the ruminant diet as a complementary food improves feed intake and feed converting (Pen et al., 2013). Thus, the inclusion of these legumes in animal feed also reduce the costs with the compound (Olafadehan et al., 2014). The genus *Stylosanthes* is a forage legume, which has extensive adaptation and resistance to biotic and abiotic factors (Pangga et al., 2004, Nagaich et al., 2013), with erect and prostrate species, predominantly perennial (Costa et al., 2008). This legume obtain nitrogen by association with diazotrophic bacteria (Mendonça et al., 2017), and can be indicated for the recovery of degraded pastures (Fabrice et al., 2015). However, *Stylosanthes scabra* can develop on soils with low levels of available nutrients, particularly phosphorus, conferring an advantage over other species (Gonzalez et al., 2000).

There are twenty-five species of *Stylosanthes* in Brazil and thirteen of them are found only in national territory (Santos-Garcia et al., 2011). *S. scabra* is a species with a large occurrence in South America (Calles and Schultze-Kraft, 2016), and this species is more common in the Brazilian Northeast, occurring in different environmental conditions (Oliveira et al., 2016). In other states from Brazil, this species occurs in Goiás, Minas Gerais, São Paulo (Brandão and Costa, 1979), Mato Grosso and Mato Grosso do Sul (Costa et al., 2008).

Stylosanthes scabra has some commercial cultivars, such as the cultivar Seca, launched in partnership between the International Center for Tropical Agriculture (CIAT) and the Commonwealth Scientific and Industrial Research Organization (CSIRO), which has stood out for its adaptability and agronomic performance under rainfed conditions in the subtropical climate. The cultivar Seca contains more than 17% of crude protein with low level of tannin (Mpanza et al., 2013). According to Akinlade et al. (2008), *S. scabra* can obtain a dry mass production up to 1.97 Ton.ha⁻¹.

Exploring the full potential of *S. scabra* is essential to know the genetic variability about this species, supporting plant-breeding programs (Araújo et al., 2016). The genetic diversity evaluation of the species within and among populations can generate information for the in situ genetic conservation of the species natural populations (Gonçalves et al., 2010). In addition, the existence of variability in natural populations allows the evolution of new genetic combinations (Medrano et al., 2014), which may present greater capacity for evolution and adaptation to changes in environmental conditions (Srihari et al., 2013).

Four types of markers are used during the genetic diversity characterization: morphological, biochemical, molecular, and cytological. The molecular types present the particularity of use under analysis 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 information of the DNA sequence and present laboratory procedures with good transferability (Dias et al., 2015). ISSR are semi-arbitrarily amplified by PCR (polymerase chain reaction), using a complementary primer for a designated microsatellite (Nilkanta et al., 2017). In the *Stylosanthes* genus, ISSR markers have been considered more efficient than both RAPD and STR markers to detect genetic variability (Nagaich and Chandra, 2009). Thus, our study aimed to evaluate the genetic diversity distribution in four populations of *S. scabra* in the semiarid region of Pernambuco state from Brazil using ISSR markers.

MATERIAL AND METHODS

The samplings were carried out in September 2015. Samples of 76 *S. scabra* plants were used from 4 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	Populations characteristics
Santa Cruz do Capibaribe	08°21'01" a 08°21'46"	40°20'19" a 40°21'52"	Pasture
Floresta	08°31'77" a 08°43'24"	38°28'06" a 38°29'22"	Caatinga (road margin)
Sertânia	08°00'52" a 08°32'35"	36°27'58" a 37°36'28"	Caatinga (road margin)
Petrolina	08°58'32" a 08°59'03"	40°16'12" a 40°45'16"	Plants on the banks of the irrigation canal

For DNA extraction, young leaves were collected following the methodology adjusted for this genus by Santos-Garcia et al. (2012). The DNA extracted was quantified by comparison with lambda standards (Invitrogen, Carlsbad, CA, USA) of concentrations known in ng μ L⁻¹ (100, 200 e 500) in 0.8% agarose gel. The purity integrity of the DNA samples was confirmed in a spectrophotometer under UV light (260/280 nm).

Seven primers of ISSR (Table 2) were used. The 25 μ L reaction mixtures contained 1x PCR buffer (Invitrogen), 1.5 mM MgCl₂, 0.8 μ M of primer, 1 U of Platinum Taq DNA Polymerase (Invitrogen), 0.25 μ M of each dNTP (Invitrogen), 25 ng of DNA template, and sterile distilled water to the total volume of 25 μ L. 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 (Nagaich and Chandra 2009).

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 the digital photodocumentator Vilber Lourmat. The polymorphisms obtained using ISSR were tabulated according to the presence (1) or absence (0) of bands.

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

Primer	Sequence	Loci	Polymorphism (%)
UBC 1	ACACACAACACACACT	10	100
UBC 2	GAGSGGAGAGAGAGAT	12	100
UBC 808	AGAGAGAGAGAGAGGC	9	100
UBC 810	CTTCATTTCACTTCA	16	100
UBC 813	GAGAGAGAGAGAGAGAA	15	95.5
UBC 879	CTTCATTTCACTTCA	12	100
UBC 888	BDBCACACACACACA	14	71,42
Total		88	95.27%

Genetic diversity was estimated using the Gen Alex 6.5 software (Peakall et al., 2012), with the analysis effective alleles number (Ne), Shannon Index (I), heterozygosity within populations (Hs), genetic differentiation coefficient (GST) and migrants number in the population (Nm).

Genetic identity and genetic distance among populations were computed using the model proposed by Nei (1978). Molecular variance analysis (AMOVA) was used to reveal the distribution of genetic diversity within and among populations. In this analysis, total genetic diversity was separated into two distinct hierarchical levels: difference among populations and among individuals within the population. The "Structure" program (Pritchard et al., 2000) based on Bayesian statistics was used to infer the groups number (k). The correlation between the geographic distance and genetic distance among *S. scabra* populations was made using the Genes program using 9999 permutations (Cruz, 2013).

RESULTS AND DISCUSSION

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

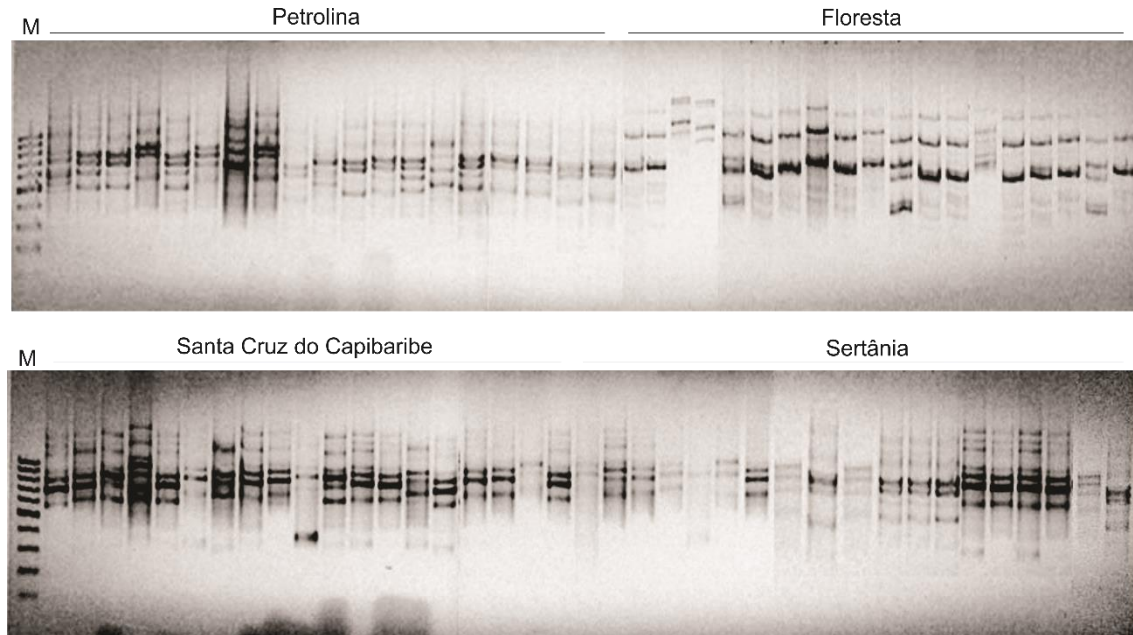


Figure 1. Electrophoresis profiles, obtained with the ISSR primer UBC810 of four *Stylosanthes scabra* populations native to the Pernambuco state, Brazil. M: molecular mass markers (Ladder 100 bp).

Santa Cruz do Capibaribe and Petrolina populations presented the highest intra-population genetic diversity (HS) among four populations (Table 3). The Petrolina population is located on the banks of an irrigation canal, allowing the animals to drink water in this place, transporting the seeds. However, the Santa Cruz do Capibaribe population is located in a pasture, allowing the animal's displacement and the transport of the seeds on this place.

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, there is no possibility of differentiating populations when the gene level flow is high (Ambiel et al., 2010). According to Collevatti et al. (2013), the genetic differentiation coefficient is one of several genetic parameters among populations, considering defining the species relation with the environment.

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

Populations	Ne	I	Hs	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 alleles number (Ne), Shannon index (I), Heterozygosity within populations (Hs), genetic differentiation coefficient (G_{ST}), migrants number in the population (N_M)

The migrants number in the population was only 0.5 individuals per generation (Table 3), reaffirming the populations isolation. *S. scabra* is predominantly autogamous, so the low migrant's number discards the

occurrence of relevant genetic migration present 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

lowest genetic distance, as well as the more distant populations geographically also presented 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 distance and geographic distance was significant according to Mantel test at 1% probability. However, the high correlation between both distances is uncommon in the literature (Jamnadas et al., 2006; Vigna et al., 2011). 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 presented the highest number of private bands (Figure 2) and greater variability, contributing to the appearance of DNA fragments (Szpiech and Rosenberg, 2011). According to Elstrand (2014), the occurrence of private bands is related to the average number of exchanged migrants per generations among populations and their presence (private bands) may indicate gene flow reduction.

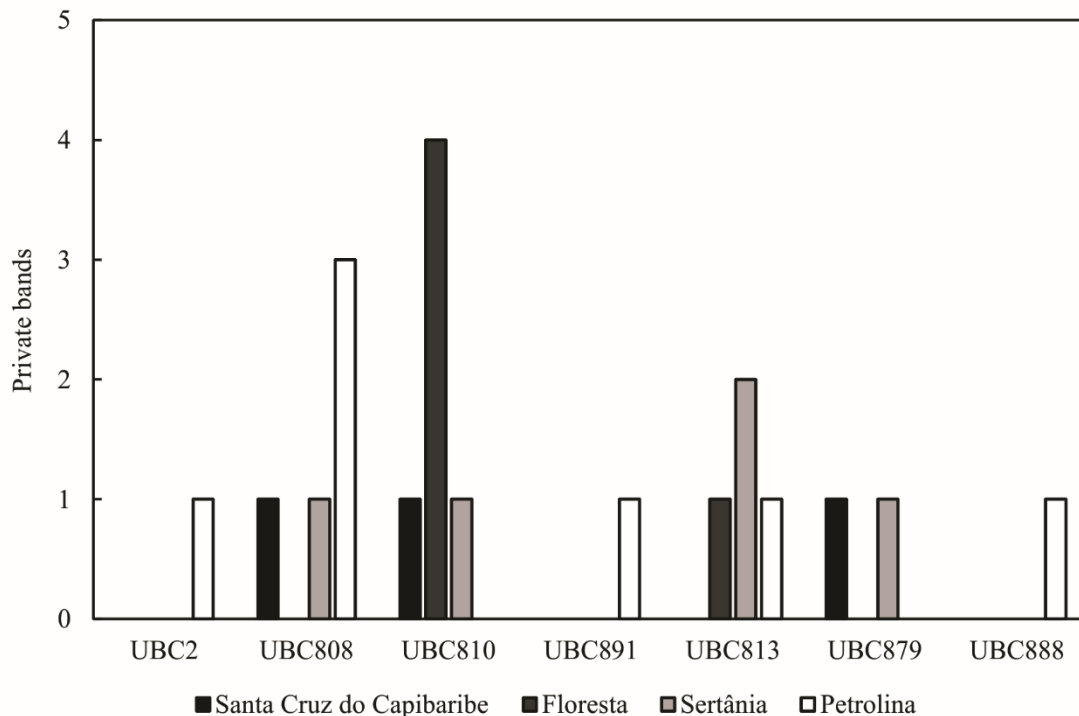


Figure 2. Private bands obtained with ISSR primers in natural populations of *Stylosanthes scabra* from Pernambuco state, Brazil.

The majority part of genetic variation (60%) was distributed among populations and 40% was distributed within populations, according to the AMOVA results. The species with autofecundation exhibit greater genetic diversity among the populations (Buzatti et al., 2012), whereas this is different for the plants with high rates of alogamy. In a study by Santos-Garcia et al. (2012) with microsatellite markers, *S. capitata* (which may present alogamia above 20%) did not present a correlation between the genetic distance and the collection sites, although few accesses by collection site were considered by these authors.

The minor genetic diversity within populations than among populations indicates the need for conservation and protection of all-natural populations in the evaluated region. Thus, it is unnecessary to collect many individuals by populations for *S. scabra*, but rather to collect few individuals in different populations, exploiting the maximum diversity in future collections.

The population disposition can be visualized by the graphical distribution of species variability (Figure 3). Thus, four groups were also formed among the samples collected and followed the divisions by collection sites, differentiating the four populations. This grouping occurs by geographic isolation and due to autogamy in *S. scabra* (Chandra et al., 2009). In these cases, superior genotypes tend to stand out and populate the population with the largest number of offspring, increasing the similarity among them. Barros et al. (2005) also observed a trend of watershed separation for *Stylosanthes* accesses, collected in Bahia and Minas Gerais, both states from Brazil.

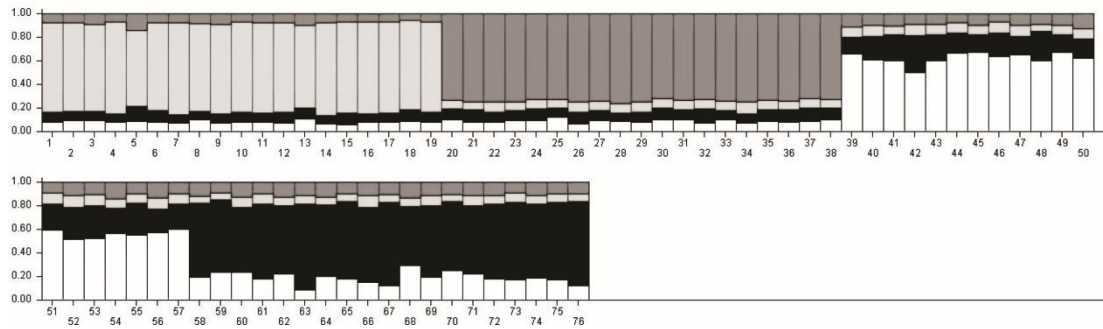


Figure 3. Representation of 76 individuals from four natural populations of *Stylosanthes scabra*, according to molecular data with ISSRs using the "Structure" program. The individuals are represented by vertical bars with coloration, according to the group belonging (four groups, $K = 4$). Group 01 - Santa Cruz do Capibaribe; Group 02 - Floresta; Group 03- Sertânia and Group 04- Petrolina.

Bayesian analyses showed a maximum K value = 4, confirming the well-defined genetic structure of the four populations. The results corroborate the expected, due to the low number of migrants and high genetic differentiation among the populations. According to Rossi et al. (2014), the genetic structuring of population occurs when the maximum K value coincides with the population's 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 cities for the construction of germplasm banks. We suggest future collections, emphasizing Santa Cruz do Capibaribe and Petrolina (both municipalities in the state of Pernambuco, Brazil), to exploring the variability of this genus in these places.

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