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Genetic diversity of *Desmanthus* sp accessions using ISSR markers and morphological traits

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ABSTRACT. *Desmanthus* is a genus of forage legumes with high nutritional value, productive potential, and ability to obtain nitrogen in association with diazotrophic bacteria. The use of accurate techniques for genotype identification and characterization is essential for breeding programs. Morphological markers are widely used to know the genetic diversity and the molecular markers are fundamental in these studies. We investigated the genetic diversity among *Desmanthus* sp genotypes in Pernambuco (Brazilian Northeast State), using morphological traits and ISSR markers. Morphological and molecular characterizations were performed in 18 and 26 accessions, respectively, in plants belonging to the germplasm bank of forage legumes of Universidade Federal Rural de Pernambuco (UFRPE), Academic Unit of Serra Talhada, PE, Brazil. Eight ISSR primers were selected, and 95 loci were generated, with polymorphism of 95.79%. The allele number observed was 1.958, where the effective number was 1.359, and the Nei diversity genetic

index was 0.226. About the morphological markers, seed number was the variable that most contributed to the genetic divergence. A large amount of genetic diversity was observed among *Desmanthus* species, occurring spontaneously in Pernambuco, Brazil. Thus, the variability found in morphological and ISSR markers is importance for the improvement of *Desmanthus* spp. Our findings showed that 17L, 27L, 25F, 22F, 19S, 13Au, and 28G accessions could be used in breeding programs to explore the maximum genetic divergence.

Key words: Molecular markers; Morphological markers; Breeding programs

INTRODUCTION

Desmanthus is a genus of forage legumes occurring throughout America and has great potential for improved pasture and animal production (Rangel and Gardiner, 2009). These plants are mainly used as fodder because they have high palatability and large seed production (Fontenele et al., 2007, 2009).

In the Brazilian Northeast, *D. pernambucanus* L. Thell predominates (Pengelly and Liu, 2001), which is autogamous (2n = 26 chromosomes), originating in South America, probably in Northeast Brazil (Santos-Garcia et al., 2012). These species are shrub-like, drought resistant, and have a high capacity to obtain nitrogen by association with diazotrophic bacteria, estimating about 30 kg N ha/year coming from biological nitrogen fixation (Freitas et al., 2011).

Although some studies have evaluated the response of *Desmanthus* species to the cut (Trujillo et al., 1996; Gonzalez-V et al., 2005; Diniz Neto et al., 2013), the cultivation of this plant in Northeast Brazil is non-existent. In the semiarid region of Pernambuco, *Desmanthus* sp occurs in different soil types, as well as different environmental characteristics (Queiroz, 2012). Besides, genotypes collected in this region show desirable morphological and productive characteristics for forage plants (Calado et al., 2016).

To initiate a breeding program, it is essential to know the genetic variability within the population (Melo et al., 2011). The genetic diversity evaluation among germplasm accessions results in information from potential parents to be used in breeding programs, allowing the duplicate identification and the exchange of germplasm among researchers (Martins et al., 2012).

The use of accurate techniques for identifying and characterizing of genotypes is essential for breeding programs and protection of cultivars. Morphological markers are widely used; however, they may present low efficiency in the assessment of available variability (Silva et al., 2013). On the other hand, molecular markers are highly accurate because they detect differences in DNA level and exhibit sufficient polymorphism to discriminate genotypes (Vieira et al., 2015).

ISSR (inter-simple sequence repeat) molecular markers is an excellent tool for the genetic diversity analysis and characterization of accessions and cultivars of several species due to the high degree of polymorphism, reproducibility and low cost (Salazar-Laureles et al., 2015; Brito et al., 2016; Silva et al., 2016).

The ISSR molecular markers use repeated and short sequences of DNA to amplify anonymous loci and do not require prior knowledge of the genome. Because these sequences are dominant loci, it is not possible to distinguish heterozygotes from homozygotes. However,

multiple loci can be produced from each PCR (polymerase chain reaction) amplification (Goulão and Oliveira, 2001).

Thus, our study aimed to perform the morphological and molecular characterization of *Desmanthus* sp accessions. Thereby, we investigated the genetic diversity using ISSR markers, unprecedentedly for this genus on literature.

MATERIAL AND METHODS

The morphological characterization was conducted in 16 accessions belonging to the forage legume germplasm bank from the Universidade Federal Rural de Pernambuco - Academic Unit of Serra Talhada, PE (Table 1). The morphological characters related to the plant were: pod number per bunch; growth habit; leaf number per branch; branch number; plant height; stem diameter; leaflet number per leaf; seed number.

Table 1. *Desmanthus* sp accessions and their respective accession numbers, collection city, soil type, latitude, longitude, and altitude.

Identification	City	Soil type	Latitude	Longitude	Altitude
58F	Bom Jardim	Entisol	35º31'270"	7º48'187"	163 m
94F*	Bom Jardim	Vertisol	35º41'129"	7º50'151"	335 m
22F*	Bom Jardim	Alfisol	35º32'684"	7º47'466"	297 m
25F*	Bom Jardim	Alfisol	35º32'684"	7º47'466"	297 m
21F*	Bom Jardim	Aridsol	35º32'684"	7º47'466"	297 m
20F*	Bom Jardim	Aridsol	35º33'679"	7º48'632"	300 m
28G*	Santa Cruz do Capibaribe	Entisol	36º14'831"	07º53'504"	509 m
7G	Santa Cruz do Capibaribe	Aridsol	38º44'70"	7º94'58"	448 m
235C*	Sertânia	Ultisol	37º12'159"	8º05'186"	581 m
65F*	Sertânia	Ultisol	35º31'719"	7º46'314"	231 m
100C*	Sertânia	Aridsol	37º18'163"	8º04'039"	559 m
19S*	Serra Talhada	Entisol	38º26'65"	7º95'71"	444 m
17L*	Jataúba	Aridsol	36º26'884"	07º59'198"	517 m
16L*	Jataúba	Aridsol	36º27'227"	07º59'243"	519 m
92L*	Jataúba	Entisol	36º29'058"	08º07'084"	546 m
45L*	Jataúba	Aridisol	36º23'980"	07º58'083"	490 m
15L	Jataúba	Aridsol	36º27'227"	07º59'243"	519 m
27L*	Jataúba	Aridsol	36º26'884"	07º59'198"	517 m
50J	Petrolina	Ultisol	40º19'086"	09º04'875"	373 m
31D	Caetés	Ultisol	38º39'34'	7º99'14"	452 m
13AU*	Austrália				
10AU	Austrália				

All accessions were characterized by ISSR. *Accessions characterized morphologically and by ISSR.

The data were standardized using the following formula: x = (species mean - general mean) / standard deviation. Principal component analysis was used to identify the variables that most contributed to the data variation. A cluster analysis was performed to evaluate the similarity degree among the species studied, using Euclidean distance as a unit of measure. These analyses were performed through the Genes program (Cruz, 2013).

For molecular characterization, 22 accessions were used. For DNA extraction, young leaves were collected following the methodology adjusted for this species by Pengelly and Liu (2001). The extracted DNA was quantified by comparison with lambda standards (Invitrogen, Carlsbad, CA, USA) of concentrations known (300, 500, and 100 ng/µL) on 0.8% agarose gel. The purity integrity of the DNA samples was confirmed in a spectrophotometer under UV light (260/280 nm).

For molecular analyses, 15 ISSR primers were used from a set produced by the University of British Columbia, Vancouver, Canada (Table 2). The 25-µL reaction mixtures contained 1X PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.8 µM primer, 1 U Platinum Taq DNA Polymerase (Invitrogen), 0.25 µM of each dNTP (Invitrogen), 25 ng DNA template, and sterile distilled water to the total volume of 25 µL.

Table 2. ISSR primers selected for *Desmanthus* sp genotypes, sequence, total fragment number per primer, polymorphism percentage (LP), allele numbers (N_A), the effective number of alleles (N_E), Shannon index (I), and Nei genetic diversity (H_E).

Primer	Sequence	Loci	%LP	N_A	N_E	I	H_E
UBC 1	ACACACACACACACT	10	90%	1.888	1.442	0.387	0.254
UBC 2	GAGSGSGAGAGAGAGAT	12	100%	2.00	1.145	0.449	0.289
UBC 808	AGAGAGAGAGAGAGC	7	100%	2.00	0.132	0.317	0.192
UBC 810	GAGAGAGAGAGAGAT	16	100%	2.00	1.402	0.389	0.248
UBC 812	GAGAGAGAGAGAGAA	15	95.5%	1.933	1.523	0.456	0.303
UBC 834	AGAGAGAGAGAGAGYT	13	92.3%	1.923	1.304	0.316	0.196
UBC 845	CTCTCTCTCTCTCTRG	8	100%	2.00	1.225	0.309	0.179
UBC 888	BDBCACACACACACA	14	92.8%	1.928	1.366	0.357	0.255
Total		95	95.8%	1.958	1.359	0.361	0.226

DNA amplifications were performed on thermocycler MJ Reseach, Inc., PTC100 Programmable Thermal Controller (Watertown, USA) under the following conditions: 94°C for 1 min (initial denaturation), followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min with a final extension step at 72°C for 5 min (Santos-Garcia et al., 2012).

The amplification products were separated on a 2% agarose gel stained with Syber Gold (Invitrogen), using the 100-bp marker (Invitrogen) and visualized under ultraviolet light and recorded on the digital photo documentation Vilber Lourmat. The polymorphisms obtained using ISSR were tabulated according to the presence (1) or absence (0) of bands.

Genetic diversity was estimated using the GenAlex 6.5 software (Peakall and Smouse, 2012), with the analysis of the observed number of alleles (N_A), an effective number of alleles (N_E), Nei genetic diversity (H_E), and Shannon index (I). Similarity coefficients were calculated using the Jaccard index (Jaccard, 1908) and the Genes program (Cruz, 2013) was used to construct a dendrogram, using the unweighted pair group method with arithmetic mean (UPGMA).

RESULTS AND DISCUSSION

About the morphoagronomic markers, the seed number was the variable that most contributed to the genetic divergence, with 60.31%, followed by the branch number with a relative contribution of 30.34% (Table 3). Descriptors with the greatest contribution to divergence are the most important for the breeding program. These descriptors support to select the parents for the creation of segregating populations with a greater probability of success through the combination of these genotypes (Oliveira et al., 2016).

The accession clustering demonstrates eight formations of groups considering the mean dissimilarity of 62.5% (Figure 1). The same grouping was also suggested by the Tocher grouping. The first group is formed only for accessions collected in Jataúba, 16L, 92L, 45L, and 17L. The second group isolated 25F access and the third isolated 22F, both collected in Bom Jardim. The fourth group had 94F and 65F accessions from Bom Jardim in one branch, and 100C accession from Sertânia in another branch.

Table 3. Relative importance of eight descriptors for genetic divergence in *Desmanthus* spp.

Characters	S.j	S.j (%)
Pod number per bunch	223.0	0.47
Growth habit	80.0	0.16
Leaf number per branch	175.0	0.36
Branch number	14812.0	30.34
Plant height	121.92	0.25
Stem diameter	1997.08	4.09
Leaflet number per leaf	1815.0	3.72
Seed number per pod	29591.0	60.31

'S.j: contribution of variables to mean Euclidean distance between genotypes i and i'.

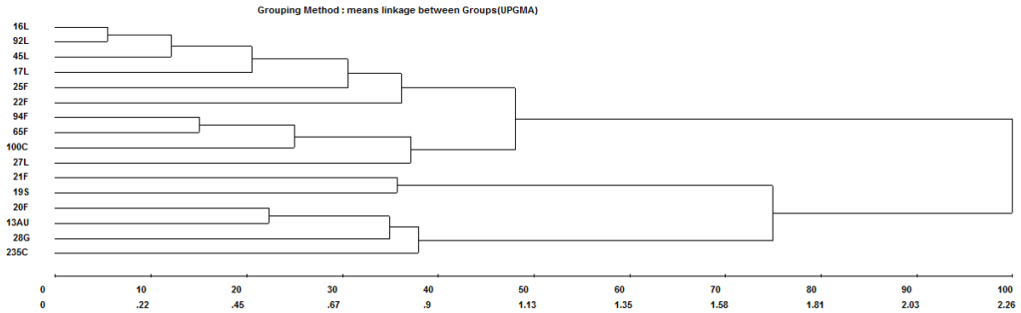

Figure 1. Morphological dissimilarity dendrogram of 16 *Desmanthus* sp accessions belonging to the germplasm bank from Universidade Federal Rural de Pernambuco, Academic Unit of Serra Talhada, PE.

The 27L accession was isolated in the fifth group, being the only accession collected in Janaúba, outside the first group. The accessions 21F from Bom Jardim and 19S from Sertânia formed the sixth group. The seventh group consisted of 20F, 13AU, and 28G accessions, from Bom Jardim, Australia, and Santa Cruz do Capibaribe, respectively. The 13AU accession is the result of genotypes collected from Brazil, despite being cultivated in Australia, The last group consists of the isolation 235C accession from Sertânia. Thus, the morphological descriptors were able to form distinct groups that can be used for selection of parents for future breeding programs.

Plant selection based on morphological characters may be efficient, allowing breeding workers to use genetic variability to aggregate desirable alleles through crosses with superior genotypes (Silva et al., 2014). According to Silva et al. (2013), genetic variability means the possibility to direct the crosses using the most divergent accessions. Thus, the most divergent accessions (235C, 42L, 22F, 25F, 28G, and 45L) can be used for future crosses to explore the variability of the *Desmanthus* sp collection.

In molecular characterization, a total of fifteen ISSR primers were tested, where eight were selected because they exhibited defined amplification patterns and high reproducibility. The primers selected to evaluate the 22 accessions amplified 95 DNA fragments (Table 2). The primer UBC808 resulted in the lowest number of amplified fragments (7) whereas the primer UBC810 generated the largest number of fragments (16) (Table 2).

The optimum number of bands estimated for *Desmanthus* sp was 84 loci (Figure 2). Thus, the total locus numbers found (94) was sufficient for the molecular characterization of

the accessions. According to Kruskal (1964), when the stress value is equal to or less than 0.05 the estimates are accurate. For *Copernicia prunifera*, 76 bands were sufficient to estimate the genetic variability (Vieira et al., 2015).

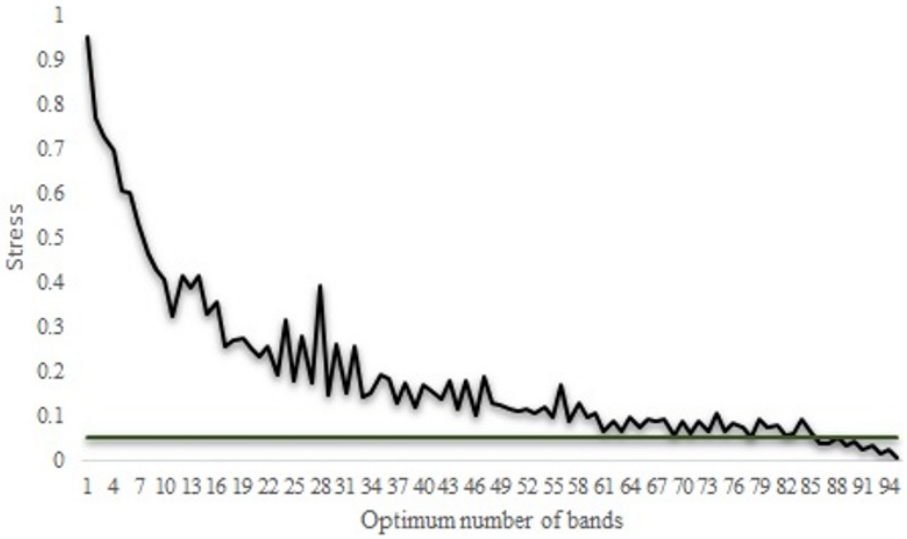

Figure 2. Estimates for increasing numbers of polymorphic ISSR markers and stress values, which indicate the ideal minimum number of bands to represent the genetic diversity in the *Desmanthus* sp accessions of Universidade Federal Rural de Pernambuco, Academic Unit of Serra Talhada, PE.

The primer UBC1 presented the lowest polymorphism (90%), whereas the primers UBC02, UBC808, UBC810, and UBC834 showed 100% polymorphism (Table 2). The mean of fragments per primer was 11.87 with polymorphism of 95.79%. The data presented here were similar to other studies found using ISSR markers in other species. Souza Neto et al. (2014) analyzed the genetic diversity of *Anthurium* and obtained 71.43% of polymorphism with the UBC845 primer, 81.82% for UBC808 and 100% with UBC10. Accessions of the biribazeiro tree when submitted to ISSR markers, presented 81.3% of polymorphism, with a mean of 9 fragments per primer (Lorenzoni et al., 2014).

The N_E in our study was 1.359 (Table 2), and I was 0.361. The H_E was 0.226, considered moderate to low. The indices varied from 0 to 1 [(0 represents the zero genetic diversity and 1 the maximum genetic diversity (Giustina et al., 2014)]. The primer UBC845 presented values of 0.179 for Nei (H) and 0.316 for I, with the lowest values in comparison to the other primers. The highest values were found when the primer UBC812 was used with values of 0.456 for H and 0.303 for I. Our values were similar to the ones reported in the literature for dominant markers (Vieira et al., 2015).

The ISSR markers enabled the differentiation among the *Desmanthus* sp accessions (Figure 3). The lowest genetic distance was observed between 58F and 94F accessions, collected from Bom Jardim, with a genetic distance of 0.37. The most divergent accessions were 235C and 19S, collected at 132 km, with a genetic distance of 0.82.

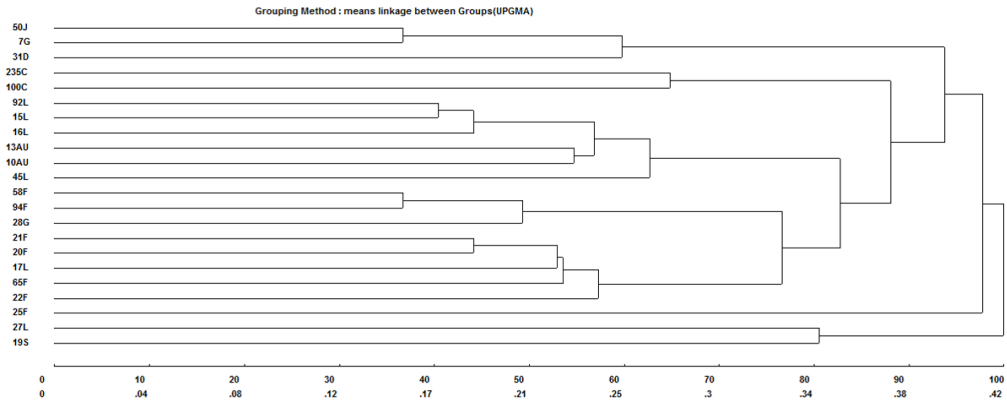

Figure 3. ISSR dendrogram dissimilarity of 22 *Desmanthus* sp accessions belonging to the germplasm bank of Universidade Federal Rural de Pernambuco, Academic Unit of Serra Talhada, PE.

The formation of six groups was observed, considering the dissimilarity of 0.75. The first group had accessions 50J from Petrolina, and 7G from Santa Cruz do Capibaribe in one branch, and the 31D accession from Caetés was found to be more isolated. The second group was formed by the 235C and 100C accessions from Sertânia. The third group consisted of three branches. One branch grouped 92L, 15L, and 16L accessions, all coming from Jataúba and with altitude above 519 m. In another branch were the two accessions coming from Australia and the last branch was formed by the 45L accession, also belonging to Jataúba, but with 490 m in altitude.

The fourth group concentrated the highest accessions number, exclusively formed by accessions from Bom Jardim, with eight accessions distributed in two branches. One branch was constituted by the 58F and 94F accessions, both from Bom Jardim and with the greater similarity among all accessions of the collection. However, in the same branch there was the 28G accession coming from Santa Cruz do Capibaribe, but collected in an Entisol similar to the soil where 58F accession was collected. The second branch was formed by 20F, 21F, 22F, 65F, and 17L accessions and only 17L was not collected in Bom Jardim.

In the fifth group, the 25F accession was isolated. This accession was the only one collected in Bom Jardim and did not group with the other accessions collected in this city. The sixth and last group was represented by 27L and 19S accessions, coming from Jataúba and Serra Talhada, respectively. These accessions have 87% of the genetic distance, although they were in the same group.

All accessions were considered distinct and did not have duplicates. The occurrence of unidentified duplicates in germplasm banks makes it expensive and difficult to maintain the material, generating problems related to the organization and access of users to the genetic resource (Gonçalves et al., 2008).

Achieving dendrograms with similar clusters between morphological and molecular characters has not always been possible. With morphological data of mangaba accession, a dendrogram similar to that produced by the RAPD technique was not obtained (Silva et al., 2013). High correlations are not always obtained due to the interaction between genotype and environment, affecting their expression (Gomes Filho et al., 2010; Samal et al., 2011).

Our selected primers are indicated for future research with genetic diversity and studies of *Desmanthus* sp populations. However, the apparent superiority of the UBC1, UBC2, UBC810, UBC812, and UBC888 primers was observed because they presented a high number of amplified fragments and Nei diversity indexes above the general average of the other primers. The grouping of some accessions was constant in both analyses, although they presented differences between the clusters of the morphological and molecular characterization. Besides, the grouping of most accessions corresponds to the geographic distribution.

Morphological characters and ISSR markers were efficient for the genetic diversity study in *Desmanthus* sp, revealing the diversity among the accessions. Thus, to exploit the maximum genetic divergence, the accessions 17L, 27L, 25F, 22F, 19F, 19A, 13Au, and 28G can be used in future breeding programs, because they differ from other accessions by both morphological and molecular markers.

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