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Research Article

Diagnostic fingerprints ISSR/SSR for tropical leguminous species *Stylosanthes capitata* and *Stylosanthes macrocephala*

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ABSTRACT: *Stylosanthes capitata* Vogel and *Stylosanthes macrocephala* M.B.Ferreira & Sousa Costa are two forage leguminous species of agronomic importance for animal husbandry in tropical environments. The physical mixture of both species (80 % *S. capitata* and 20 % *S. macrocephala*) comprises the commercial cultivar "Estilosantes Campo Grande". However, proximity of fields for seed production may contaminate seed lots, compromising seeds quality. The combined use of dominant and co-dominant molecular markers is an appropriate strategy to certificate genetic purity and perform diversity studies of cultivars. In this research, a set of ISSR (Inter-Simple Sequence Repeat polymorphic DNA) and SSR (Simple Sequence Repeat polymorphic DNA) molecular markers were standardized to characterize *S. capitata* and *S. macrocephala* species and evaluate the genetic purity of commercial samples. Four ISSR markers (UBC 2, 864, 885, 886) and SSR marker SC18-01 G4B showed precise species-specific electrophoretic fingerprints for both species. Electrophoretic patterns of ISSR molecular markers should be displayed first to confirm the sample identification. The structure analysis showed that the less contaminated sample was *S. capitata* with 97 % of its genetic composition assigned to a single genetic cluster vs. 95 % for *S. macrocephala*. *S. capitata* has greater genetic diversity ($ISSR_{He}$:0.292; SSR_{He}:0.57) than *S. macrocephala* ($ISSR_{He}$:0.285; SSR_{He}:0.16); however, this difference was only significant with SSR molecular markers. As these genetic resources have considerable ecological, agronomic and economic importance, tools for accurate species identification and genetic studies are essential for further seed multiplication, as well as for improvement and conservation of cultivars.
Keywords: genetic purity, genetic diversity, seed production

Introduction

The *Stylosanthes* genus belongs to the family Leguminosae and subfamily Papilionoideae (Azani et al., 2017). Despite its high intraspecific and interspecific diversity, which has hindered its taxonomy (Mannetje, 1984; Maass and Sawkins, 2004; Stappen et al., 2002), 43 species of the genus *Stylosanthes* are recognized by the International Legume Database & Information Service - ILDIS (Roskov et al., 2018). In Brazil, 31 species occur and 12 are endemic (Costa and Valls, 2015). However, agricultural use is limited to species *S. guianensis* (Aubl.) Sw. (diploid), *Stylosanthes capitata* Vogel (tetraploid) and *Stylosanthes macrocephala* M.B.Ferreira & Sousa Costa (diploid) (Maass and Sawkins, 2004; Jank et al., 2011; Roskov et al., 2018).

In tropics, both species *S. capitata* and *S. macrocephala* grow well on acid, infertile soils in sub-humid savannah environments and exhibit tolerance to anthracnose (Grof et al., 1979; Miles and Lascano, 1997). Two cultivars were released, one in Colombia, *S. capitata* cv. Capica (CIAT 10280, CPAC 1618) and one in Brazil, *S. macrocephala* cv. Pioneiro (CIAT 1281 = BRA-003697); however, their adoption was constrained by weak agronomic performance (Miles and Lascano, 1997). Later, it was released in Brazil the cultivar named 'Estilosantes Campo Grande', formed by the physical mixture of anthracnose resistant accessions of *S. macrocephala* (20 %), with productive accessions of *S. capitata* (80 %) (Embrapa, 2000; Cook et al., 2005; Jank et al., 2011), and it is currently the most used commercial cultivar. However, because of the proximity of fields for seed production of *S. capitata* and *S. macrocephala*, and their morphological similarity, contamination of seed lots may occur, compromising seeds quality.

The combined use of dominant (as ISSR-Inter-Simple Sequence Repeat) and co-dominant (as SSR-Simple Sequence Repeat) molecular markers is an adequate strategy to certificate genetic purity and perform diversity studies on cultivars, both for scientific and commercial purposes. Thus, this study aimed to standardize a set of ISSR (Inter-Simple Sequence Repeat polymorphic DNA) and SSR (Simple Sequence Repeat polymorphic DNA) molecular markers to characterize the forage leguminous species *Stylosanthes capitata* Vogel and *Stylosanthes macrocephala* MB. Ferreira & Sousa Costa that comprise cultivar 'Estilosantes Campo Grande' and evaluate genetic purity of commercial samples.

Materials and Methods

Plant material

Seeds of *Stylosanthes capitata* Vogel and *Stylosanthes macrocephala* MB. Ferreira & Sousa Costa species used for this research were from two separated commercial seed lots, kindly provided by a Brazilian commercial seed company that uses these species to comprise the

mixed commercial cultivar named "Estilosantes Campo Grande". Additionally, seeds of *S. macrocephala* cv. Pioneiro were used for fingerprint confirmation.

Sampling of plant material

Leaf samples from 80 adult individual plants of *S. capitata* were collected in the experiment conducted in the year 2015 under the "Temperature Free-Air Controlled Enhancement and free-air carbon dioxide enrichment" facility (Trop-T-FACE), installed in field conditions, in the municipality of Ribeirão Preto, São Paulo, Brazil (21°10′30″ S, 47°48′38″ W, altitude: 546 m). For comparison, 80 samples of seeds of the commercial *S. macrocephala* and four samples of seeds of *S. macrocephala* cv. Pioneiro were germinated *in vitro* and their seedlings were sampled and stored at -20 °C for further analysis. Also, foliar samples of 117 adult plants that showed a different morphology in the field, evidencing seed lot contamination, were stored at -20 °C for further identification.

DNA extraction and ISSR/SSR amplifications

Total genomic DNA was extracted from the leaves of each plant sampled using the method described by Alzate-Marin et al. (2009), except for adult field plants contaminated with *Stylosanthes* sp. that were extracted using a commercial DNeasy Plant Mini Kit, since the previous protocol did not work for these samples.

ISSR/SSR primers characterization

Fifteen ISSR markers were tested for amplification of *S. capitata* and *S. macrocephala* according to the official list published by the University of British Columbia in Canada (Table 1). In addition, fifteen SSR primer pairs developed by Santos et al. (2009a; 2011) were tested and standardized for amplification of *S. capitata* and cross-transferability to *S. macrocephala* (Tables 1 and 2).

The SSR/ISSR PCR amplifications were similar to those reported in Moraes Filho et al. (2015). Since SSR amplifications with the annealing temperature previously described for these primers (60 °C) only worked in about 47 % of our amplifications, we tested annealing temperatures between 55 and 62 °C. The temperatures that were successful in the amplification of *S. capitata* were used to confirm cross-transferability of these markers in *S. macrocephala* (Santos et al., 2009a; 2011). The PCR products were denatured and separated in 8 % denaturing polyacrylamide gels (SSR) and 8 % non-denaturing polyacrylamide gels (ISSR) stained with silver nitrate (Sanguinetti et al., 1994). Alleles were sized by comparison to a standard 10-bp (SSR) and 50-bp (ISSR) DNA ladder.

Table 1 – Sequences of the molecular markers tested.

ISSR		SSR	
Loci	Primer[π]	Loci	Primer Forward and Reverse
UBC-1	ACACACACACACACACT	SC 18-01 B3[a]	5' GGCTAAAGAACGGCTAATG 3' 5' TCGAAAGATCCAAGAACAAA 3'
UBC-2	GAGAGAGAGAGAGAGAT	SC 18-01 C7B[a]	5' CCGACCAAGGGGGATGTC 3' 5' AAGTAGCAGCGGCGAGACC 3'
UBC-13	CTCTCTCTCTCTCTCTT	SC 18-01 E11[a]	5' TGGAGACAACACCCTTATG 3' 5' ATTCTATTACTCTTGCCTTTTCT 3'
UBC-820	GTGTGTGTGTGTGTGTC	SC 18-01T F11A[a]	5' CTTCTTTATCCCCACCTTTTT 3' 5' AGCACACTCTTTGATGATGAG 3'
UBC-834	AGAGAGAGAGAGAGAGYT	SC 18-01 G4B[a]	5' GCATAGCAGCATAGGTAGTAAA 3' 5' ATGCCAGGGCTGATAGAAG 3'
UBC-845	CTCTCTCTCTCTCTCTRG	SC 18-01 A2A[b]	5' AGCAGCATAGGGAATAAAAT 3' 5' CAAAGGCCTAATCAACTGTG 3'
UBC-851	GTGTGTGTGTGTGTGTYG	SC 18-01 B4[b]	5' GCTTAGGCCTTATCCAGAA 3' 5' TTGAATTTGTTATTGCTACTACTT 3'
UBC-858	TGTGTGTGTGTGTGTGRT	SC 18-01 E4[b]	5' CGGCAACTGGGAAAAATAA 3' 5' ATGGGTAATCACAAATCTTCAG 3'
UBC-860	TGTGTGTGTGTGTGTGRA	SC 18-02 E12[b]	5' AGGGGAAGGGCAAATGGT 3' 5' GCATAGATGGCAAACAGAGACA 3'
UBC-862	AGCAGCAGCAGCAGCAGC	SC 18-01T F2[b]	5' CTGACCCCACCTAATGAGAAA 3' 5' AGCAAAACAAAACAAACAACACTA 3'
UBC-864	ATGATGATGATGATGATG	SC 18-01T G9[b]	5' TCCAGCTAAAGGGCAACACA 3' 5' CCACCGCACACCAGAGATT 3'
UBC-866	CTCCTCCTCCTCCTCCTC	SC 18-01T G12A[b]	5' ATGCTGATTTTTGGCTCTTTT 3' 5' CCCCTTTTGAACGGATTG 3'
UBC-885	BHBGAGAGAGAGAGAGA	SC 18-02 H1[b]	5' GTCATTGTCGTCGTCACC 3' 5' ACCGCATAGCTGTCTTTATT 3'
UBC-886	VDVCTCTCTCTCTCTCT	SC 18-01T H4[b]	5' GGTATATGGGAGTTCTTGTTCT 3' 5' TTTGTTTGTTTTGCTTTTGTA 3'
UBC-897	CCGACTCGAGNNNNNNATGTGG	SC 18-01 H5[b]	5' GCATCATTTGCATTTGTTTT 3' 5' CTATCACCTCTCCATACCTTATC 3'

[π]N = (A,G,C,T), R = (A,G), Y = (C,T), B = (C,G,T), D = (A,G,T), H = (A,C,T), V = (A,C,G). [a]Santos et al. (2009a); [b]Santos et al. (2011).

Table 2 – SSR molecular markers developed for *S. capitata* (Santos et al., 2009a; 2011), standard annealing temperatures tested and transferability to *S. macrocephala* and *S. macrocephala* cv. Pioneiro. [1]Indicate the temperature of successful DNA amplification.

Loci	Size range (bp)	Tested temperatures for *S. capitata* (°C)								Transferability to *S. macrocephala*			*S. Macrocephala* cv. Pioneiro[£]
		55	56	57	58	59	60	61	62	Reported	This work[£]	Temperature Tested/amplified[1]	
SC 18-01 B3[a]	222-225	/	+	/	-	/	-	/	/	-	-	56[1],60	-
SC 18-01 C7B[a]	318-330	/	+	/	-	/	-	/	/	+[a]	+	56[1],58,60	+
SC 18-01 E11[a]	235-239	/	+	/	/	/	-	/	/	-	-	56,60	-
SC 18-01T F11A[a]	188-186	/	-	/	-	/	+	/	/	-	-	56,58,60	-
SC 18-01 G4B[a]	250-255	-	+	/	/	/	-	/	/	+[a]	+	56[1]	+
SC 18-01 A2A[b]	232-238	/	/	/	/	/	+	/	/	+	-	60[1]	-
SC 18-01 B4[b]	238-242	/	-	-	-	/	-	/	-	-	-	55,56	-
SC 18-01 E4[b]	300-310	/	/	/	/	/	+	/	/	+	+	60[1]	+
SC 18-02 E12[b]	270-305	/	-	/	+*	/	+	/	-	+	+	58,60[1]	+
SC 18-01T F2[b]	196-198	/	/	/	/	/	+	/	/	-	-	60	-
SC 18-01T G9[b]	242-245	/	/	/	/	/	+	/	/	+	+[α]	60[1]	+[α]
SC 18-01T G12A[b]	240-260	/	-	/	-	+	-	/	-	-	-	59	-
SC 18-02 H1[b]	204-218	/	/	/	+*	+*	+	-	-	+	-	58[1],59[1],60[1],61,62	-
SC 18-01T H4[b]	182-195	-	-	-	-	-	-	/	-	-	-	55,57	-
SC 18-01 H5[b]	192-196	+	/	/	/	/	-	/	/	-	-	55,60	-

+ = amplified; +* = amplified with lower efficiency; [α]some samples amplify and others do not; - = not amplify; / = not tested; [£]For transferability, four plants were tested; [a]Santos et al. (2009a); [b]Santos et al. (2011).

Statistical Analyses

The statistical analyses were similar to those fully described by Moraes Filho et al. (2015) for ISSR and SSR molecular markers. The GenAlex 6.5 software (Peakall and Smouse, 2012) was used to generate the genetic distance matrix according to Nei (1972) and calculate genetic diversity parameters and genetic differentiation between populations (AMOVA, F_{ST}) (Excoffier et al., 1992; Weir and Cockerham, 1984). The differences between means were performed with One-way ANOVA, followed by post-hoc Tukey (significance level 0.05), using Excel tools and the PAST Sofware (Hammer et al., 2001). The software MEGA 5 (Tamura et al., 2011) was used to generate a dendrogram of genetic dissimilarity based on the UPGMA algorithm. The software Structure 2.0 (Pritchard et al., 2000) was used to investigate the genetic structure of samples from a cluster analysis based on models.

Results

Standardization and selection of molecular markers

Nine ISSR markers (UBC 1, 2, 834, 851, 860, 862, 864, 885, 886) of 15 markers tested (Table 1) showed amplification products in *S. capitata* and *S. macrocephala*. Except for ISSR 1, the remaining eight markers were used to perform the genetic analysis of both species (Table 1 and Table 3).

From the fifteen SSR markers developed for *S. capitata*, annealing temperatures previously described (60°) for seven primers were confirmed and temperatures for the other six were standardized (Tables 1 and 2) (Santos et al., 2009a; Santos et al., 2011). We did not obtain amplification products with SSR SC18-01T H4 and SC18-01 B4 primers in our laboratory conditions after testing with seven and five temperatures, respectively (Table 2). Moreover, cross-transferability of five SSR markers to *S. macrocephala* was confirmed (Santos et al., 2009a; 2011), using related and new annealing temperatures (Table 2).

Table 3 – Parameters of genetic diversity for *S. capitata* and *S. macrocephala* species with molecular markers ISSR. *A* = number of alleles observed; *Ae* = effective number of alleles [1 / (p^2 + q^2)]; *He* = Nei genetic diversity [2*p*q]; PL=number of polymorphic loci; %P = percentage of polymorphic loci; SE = standard error.

	ISSR	*A*	*Ae*	*He*	PL	%P
S. capitata	UBC-2	1.71	1.40	0.284	18.00	85.70
	UBC-834	2.00	1.39	0.246	09.00	100.00
	UBC-851	2.00	1.58	0.338	13.00	100.00
	UBC-860	1.90	1.61	0.344	10.00	90.00
	UBC-862	2.00	1.80	0.428	11.00	100.00
	UBC-864	1.50	1.47	0.283	12.00	75.00
	UBC-885	1.87	1.28	0.191	14.00	87.50
	UBC-886	1.95	1.45	0.275	19.00	95.00
Mean		1.84	1.49	0.292	13.25	91.65 %
SE		0.05	0.03	0.016	-	3.14
S. macrocephala	UBC-2	2.00	1.59	0.323	21.00	100.00
	UBC-834	1.77	1.43	0.256	08.00	89.00
	UBC-851	1.85	1.52	0.301	12.00	92.31
	UBC-860	2.00	1.54	0.312	10.00	100.00
	UBC-862	1.82	1.75	0.392	10.00	90.91
	UBC-864	1.63	1.48	0.270	12.00	75.00
	UBC-885	1.94	1.32	0.211	15.00	93.75
	UBC-886	1.90	1.40	0.245	18.00	90.00
Mean		1.87	1.50	0.285	13.25	91.36 %
SE		0.04	0.03	0.017	-	2.78

Although SC18-01T G9 presents less reliable results in *S. macrocephala*, as it amplifies some samples and not others, this primer should be used with caution (Table 2). SSR molecular markers SC18-02 E12, SC18-01 E4, SC18-01 G4B, and SC18-01 C7B, which amplified both species, were selected for the genetic characterization proposed.

Genetic structure

The cluster (Figures 1A and B) and the ancestry analysis (ISSRα = 0.050/SSRα = 0.036) (Figures 1C and D), with the two type of molecular markers, suggest that populations of both species are divided into two genetic groups, agreeing with the identification of individuals for each species. According to the STRUCTURE analysis, the "purest" sample was *S. capitata*, with 97 % of its genetic composition assigned to a single genetic cluster. The *S. macrocephala* sample showed more considerable genetic mixture with 95 % attributed to its principal ancestral group.

The joint analysis of both markers showed four contaminants in both groups analyzed, three (4 %) in the species *S. macrocephala* (M2, M11, and M12) and one (1 %) in the species *S. capitata* (C10.13) (Figures 1A, B, C and D), which were removed for subsequent analyses. However, it is essential to highlight that this data was obtained after the removal of 117 contaminants in the field; therefore, it does not correspond the real *S. capitata* seed lot purity.

The analysis of molecular variance (AMOVA) showed that most genetic variability was found among species, and the ISSR analysis indicated a higher value (ΦST = 23.09) than the SSR markers (ΦST = 3.15) (Table 4). The F_{ST} values showed differentiation (SSR-F_{ST} = 0.366 ± 0.061) and a small number of historical gene flow (SSR-Nm = 0.509 ± 0.155) between *S. capitata* and *S. macrocephala* species.

Table 4 – Molecular Analysis of Variance (AMOVA) ($p < 0.05$). Df = Degrees of freedom; SS = Sum of Squares; EV = Estimated Variance; %V = Percentage of variance.

	Df	SS	EV	%V
ISSR Markers				
Between Species Φst	1	1812.23	23.09	67 %
Within Species Φct	154	1754.01	11.39	33 %
Total	155	3566.24	34.48	100 %
SSR Markers				
Between Species Φst	1	247.61	3.15	60 %
Within Species Φct	154	321.30	2.09	40 %
Total	155	568.91	5.24	100 %

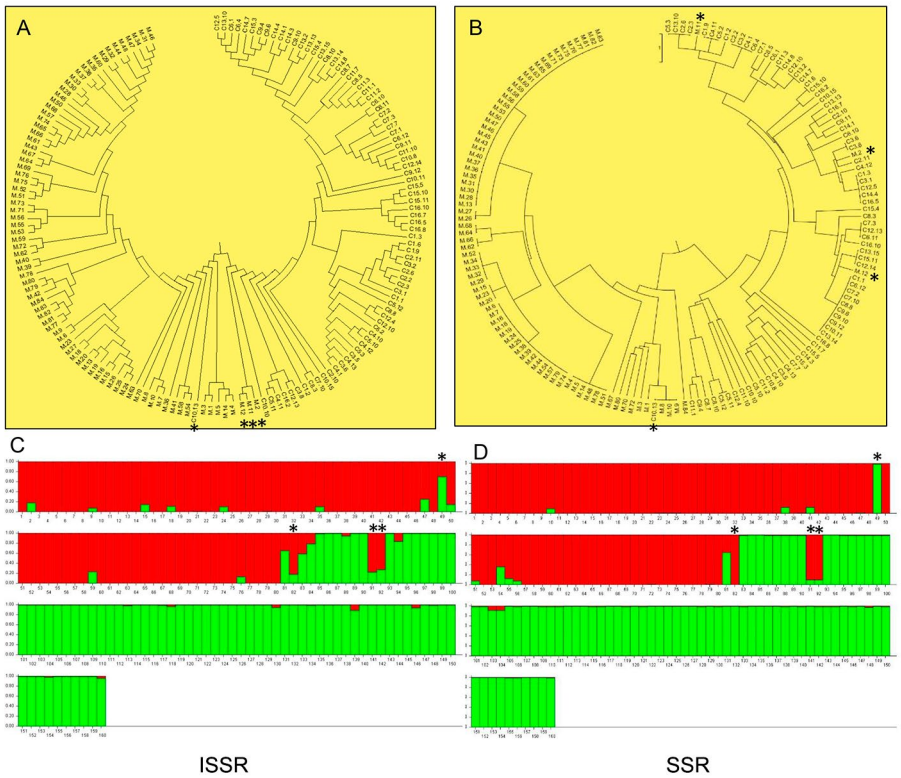

Figure 1 – Above (A, B): UPGMA dendrogram, demonstrating the genetic relationships among species *Stylosanthes capitata* and *Stylosanthes macrocephala* with ISSR/SSR markers. Below (C, D): Relationship of ancestry for the genetic cluster of individuals of the species *S. capitata* (red) and *S. macrocephala* (green), respectively. Both species samples were grouped into two major clusters. See the contaminant individuals (*) 49 (C10.13) of *S. capitata* and individuals M2 (82), M11(91) and M12 (93) of *S. macrocephala*, respectively.

Diversity genetic analysis

The eight selected ISSR primers generated 116 loci, with an average of 14.5 loci per primer, ranging from nine (UBC-834) to 21 (UBC-2). The average percentage of polymorphism of *S. capitata* (M = 92 %) was similar to *S. macrocephala* (M = 92 %) (Table 3). No differences were observed for parameters *A* (Number of different alleles), *Ae* (Number of effective alleles) and *He* (expected heterozygosity) between populations of both species (He_{Sc} = 0.292 ± 0.016; He_{Sm} = 0.285 ± 0.017). Markers UBC 2, 864, 885 and 886 formed specific haplotypes of each species, including samples from *S. macrocephala* cv. Pioneiro, although the first two amplified higher quality fingerprints (Figures 2A and B).

The four SSR markers were polymorphic and generated 19 alleles (*A*) for *S. capitata* with an average of 4.75 ± 1.11, ranging from two (SC18-01 G4B) to seven (SC18-02 E12) (Table 5). For *S. macrocephala*, ten alleles were amplified, a smaller number than for *S. capitata*, with an average of 2.50 ± 0.87, ranging from one (SC 18-01 G4B, monomorphic) to five (SC18-01 E4) (Table 5). *S. capitata* exhibited higher average values of *He* than *S. macrocephala* (He_{Sc} = 0.57 ± 0.09/He_{Sm} = 0.16 ± 0.14). Lower values of *F* were observed in *S. capitata* (-0.06 ± 0.41), while the highest values were found in *S. macrocephala* (0.42 ± 0.26), for which *F* was positive and different from zero, indicating heterozygote deficiency, possibly due to the presence of monomorphic loci. In this sense, the lower diversity of *S. macrocephala* must be viewed with caution since the SSR markers used were transferred from *S. capitata*, which can present reduced polymorphism, as discussed by Guidugli et al. (2010). Nonetheless, Santos et al. (2012) reported similar interrelationships and results in 192 *S. capitata* (*He* = 0.50) and 134 *S. macrocephala* (*He* = 0.36) accessions, considering that the SSR markers used for the analysis of *S. macrocephala* were developed for the species (Santos et al., 2009b). Therefore, our diversity data are consistent with data previously published (Santos et al., 2009a; 2009b; 2012). According to the repeatability and quality, SC18-01 G4B is the most reliable SSR species-specific marker, amplifying simultaneously monomorphic alleles of different sizes by both species (Figure 2C).

DNA analysis of contaminant plants in the field

The fingerprint analysis of molecular markers ISSR UBC-864 and SSR SC18-01 G4B showed that contaminant plants discarded in the field belong to species *S. macrocephala* (Figures 2B and C), endorsing contamination of *S. capitata* commercial seeds used in the ex-

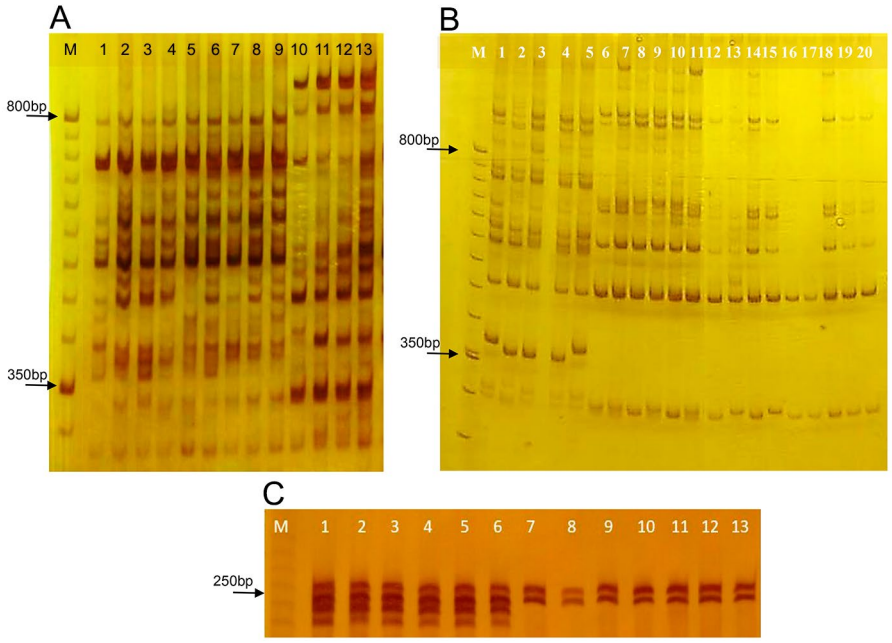

Figure 2 – Diagnostic fingerprints of *Stylosanthes capitata* and *Stylosanthes macrocephala* species generated by the amplification of ISSR markers UBC-2 (A) and UBC-864 (B), and SSR SC18-01 G4B (C) in polyacrylamide gel 8 % non-denaturing (ISSR) and denaturing (SSR). A) Channels 1-4, 5-9 and 10-13 correspond to DNA amplification of the control samples of *S. macrocephala*, *S. macrocephala* cv. Pioneiro and *S. capitata*, respectively. B) Channels 1-5, 6-11 and 12-20 correspond to DNA amplification of the control samples of *S. capitata*, *S. macrocephala*, and some contaminant individuals observed in the field, respectively. C) Channels 1-6, 7-9, 10-11 and 12-13 correspond to DNA amplification of control samples of *S. capitata*, *S. macrocephala*, two contaminant individuals observed in the field and *S. macrocephala* cv. Pioneiro, respectively. M is the molecular weight marker (50bp/ISSR, 10pb/SSR/Invitrogen). According to (B) and (C), contaminating plants in the field belong to *S. macrocephala*.

periment. Some plants of *S. macrocephala* survived in the field and some differences were observed on their morphological characteristics regarding *S. capitata* when adults, such as smaller and narrower leaflets, hairiness, more branched and thin stems, more spaced trifoliate leaves, smaller flowers with brown nectar-guides (Figures 3A and B). However, according to Cook et al. (2005), brown nectar-guides are also observed in *S. fruticosa*, *S. guianensis*, *S. hamata*, *S. humilis*, *S. scabra*, *S. seabrana* and *S. viscosa* thus this morphological character must be used carefully as a phenotypic marker.

Discussion

In species of genus *Stylosanthes*, without a list of stable descriptors available for morphological characterization (Maass and Sawkins, 2004), molecular markers such as RAPDs, RFLPs, AFLPs, ITS, SSRs, SRAPs, and ISSRs can improve knowledge about germplasm and accesses deposited in collections, aiming their use in breeding programs (Santos et al., 2009a; 2009b; 2012; Vieira et al., 1997; Liu et al., 1999; Sawkins et al., 2001; Vander Stappen et al., 2002; Huang et al., 2014; Nagaich and Chandra, 2009). The ISSR markers are advantageous because the same primer may produce, simultaneously, distinctive fingerprints of different species (Zietkiewicz et al., 1994; Godwin et al., 1997; Sepúlveda-Nieto et al., 2017), as observed in *Stylosanthes hamata* (Nagaich and Chandra, 2009) and in this work. This type of molecular markers can be used efficiently for identification of genotypes, protection of intellectual property rights, and genetic purity analyses (Zietkiewicz et al., 1994; Rakoczy-Trojanowska and Bolibok, 2004; Kumar and Sharma, 2011). This economic strategy allows to confirm the identification of samples, avoiding wasting time with poorly classified materials that may falsely increase levels of genetic diversity within species, as observed by Santos et al. (2012) in studies with Germplasm Bank accessions of *S. capitata*. However, for microevolutionary purposes, microsatellite markers stand out as the most suitable for

Table 5 – Genetic diversity across *S. capitata and S. macrocephala* samples analyzed using simple sequence repeat (SSR) markers. N = Number of individuals, *A* = Number of alleles; *Ae* = Effective number of alleles; H_0 = Observed heterozygosity; *He* = Expected heterozygosity; F = Fixation index; CI-F = Jackknife confidence intervals for the fixation index; N, *A*, *Ae*, *Ho*, *He* and F are reported as means (SE = standard error).

Species	Loci	N	*A*	*Ae*	*Ho*	*He*	*F*	CI-F
S. capitata	SC18-02 E12	77	7.00	4.14	0.21	0.76	0.73	
	SC18-01 E4	74	4.00	1.59	0.18	0.37	0.52	
	SC18-01 G4B	79	2.00	2.00	1.00	0.50	-1.00	
	SC18-01 C7B	78	6.00	2.93	0.99	0.66	-0.50	
Mean		77.00	4.75	2.66	0.59	0.57*	-0.06	-0.26 - 0.08
SE		1.08	1.11	0.57	0.23	0.09	0.41	-
S. macrocephala	SC18-02 E12	76	2.00	1.01	0.01	0.013	-0.01	
	SC18-01 E4	66	5.00	2.38	0.18	0.579	0.69	
	SC18-01 G4B	76	1.00	1.00	0.00	0.000	1.00	
	SC18-01 C7B	69	2.00	1.03	0.03	0.029	-0.02	
Mean		71.75	2.50	1.36	0.06	0.16*	0.42	0.22 - 0.56
SE		2.53	0.87	0.34	0.04	0.14	0.26	-

*$p \leq 0.05$.

Figure 3 – Leaves, flowers and inflorescences of adult plants of the species *Stylosanthes capitata* (A) and *Stylosanthes macrocephala* (B) observed in the Trop-T-FACE experiment.

crossing systems, gene flow and paternity studies, for their codominance of the alleles, easy detection via PCR, and high allelic diversity (Oliveira et al., 2006; Avise, 2004; Vieira et al., 2016). The synteny among related species allows SSR cross-transferability, and that the success depends on the phylogenetic distance (Zhu et al., 2005; Vieira et al., 2016).

In this work, we characterized the first time eight ISSR markers that can be used simultaneously in the genetic study of *S. capitata* and *S. macrocephala*, four of them amplifying fingerprints characteristics that can aid in characterization and identification of germplasm. Similarly, we finely characterized 15 previously developed SSR markers, with a gradient of annealing temperatures (Table 2) and verified their cross-transferability to *S. macrocephala* and *S. macrocephala* cv. Pioneiro. Also, for the first time, heterologous SSR markers SSR SC18-02 E12, SC18-01 E4, SC18-01 G4B, SC18-01 C7B (Santos et al., 2009a; 2011) were genetically characterized in *S. macrocephala* (Table 5) and primer SC18-01 G4B was identified as the most reliable species-specific marker, amplifying simultaneously monomorphic alleles of different sizes in each species sample and identifying directly contaminant samples. Although *S. macrocephala* was a possible diploid ancestor of *S. capitata* (Maass and Sawkins, 2004), both species display a clear separation, as shown by the cluster, ancestry and genetic structure analyses (AMOVA and F_{ST}). Regarding diversity, the analysis with SSR markers shows higher values for *S. capitata*. In general, the two sets of molecular markers grouped samples of both species similarly and highlighted their genetic similarities and differences and the contamination cases in the samples.

The species-specific bands ISSR/SSR identified between both species are useful for the analysis of genetic purity, mainly because the mixture of both species comprise the cultivar "Estilosantes Campo-grande", currently the most important Brazilian forage legume. As the Stylosanthes genus has considerable agronomic, agroforestry and silvopastoral importance, both as a protein source and for soil conservation, accurate identification of this species is essential for further improvement of cultivars and transference of desirable features from wild species to domesticated ones (Gillies and Abbott, 1998; Cameron and Chakraborty, 2004). The integration of knowledge of diversity patterns, geographic information (Costa and Shultze-Kraft, 1990; Barros et al., 2005; Maass and Sawkins, 2004) and physiology (Martinez et al., 2014) allows understanding suitable ecological conditions for conservation of this species in nature as well as its most appropriate use, aiming at genetic improvement to reduce impacts of climate change in the future.

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