



## Using genetic diversity information to establish core collections of *Stylosanthes capitata* and *Stylosanthes macrocephala*

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### Abstract

*Stylosanthes* species are important forage legumes in tropical and subtropical areas. *S. macrocephala* and *S. capitata* germplasm collections that consist of 134 and 192 accessions, respectively, are maintained at the Brazilian Agricultural Research Corporation Cerrados (Embrapa-Cerrados). Polymorphic microsatellite markers were used to assess genetic diversity and population structure with the aim to assemble a core collection. The mean values of  $H_o$  and  $H_e$  for *S. macrocephala* were 0.08 and 0.36, respectively, whereas the means for *S. capitata* were 0.48 and 0.50, respectively. Roger's genetic distance varied from 0 to 0.83 for *S. macrocephala* and from 0 to 0.85 for *S. capitata*. Analysis with STRUCTURE software distinguished five groups among the *S. macrocephala* accessions and four groups among those of *S. capitata*. Nei's genetic diversity was 27% in *S. macrocephala* and 11% in *S. capitata*. Core collections were assembled for both species. For *S. macrocephala*, all of the allelic diversity was represented by 23 accessions, whereas only 13 accessions were necessary to represent all allelic diversity for *S. capitata*. The data presented herein evidence the population structure present in the Embrapa-Cerrados germplasm collections of *S. macrocephala* and *S. capitata*, which may be useful for breeding programs and germplasm conservation.

**Keywords:** *Stylosanthes*, tropical forage, microsatellites, genetic diversity, core collection.

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### Introduction

The genus *Stylosanthes* Sw. (Fabaceae) consists of approximately 48 species distributed throughout the tropical regions of the Americas, Africa and Asia (Costa and Ferreira, 1984; Mannelje, 1984; Kumar and Sane, 2003). Brazil is considered the major center of *Stylosanthes* diversity comprising 45% of all the species within this genus (Ferreira and Costa, 1979; Stace and Cameron, 1984). The central region of Brazil is recognized as having the highest phenotypic variation and endemism for this genus (Costa N, 2006, PhD thesis, Universidade Técnica de Lisboa, Lisboa, Lisbon, Portugal).

Some *Stylosanthes* species are used as pasture legumes and thus have economic importance in tropical and subtropical regions (Edye and Cameron, 1984). Some of these species can also be used for soil improvement through nitrogen fixation, regeneration of degraded wastelands, and for promoting water and soil conservation (Chakraborty, 2004).

*Stylosanthes macrocephala* M.B. Ferr. et Sousa Costa belongs to the section Styposanthes (Mannelje, 1984). It is a diploid species with  $2n = 20$ . This species occurs on the sandy soils of the Brazilian Cerrado and Caatinga (Costa N, 2006, PhD thesis, Universidade Técnica de Lisboa, Lisbon, Portugal), and several of its ecotypes are tolerant to anthracnose (*Colletotrichum gloeosporioides*), which is the most important disease that affects this genus (Costa and Ferreira, 1984). The perennial subshrub *S. capitata* Vog. ( $2n = 40$ ) occurs in Brazil and Venezuela. It has both prostrate and erect forms. The plant produces a large amount of

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seeds and dry matter, and its inflorescences have a high nutrition value (Williams *et al.*, 1984; Costa N, 2006, PhD thesis, Universidade Técnica de Lisboa, Lisbon, Portugal).

Major collections of important crop plants are held in gene banks around the world. These collections serve as repositories of the biodiversity available for each species and thus are a valuable resource for genes useful to plant breeders. The efficient maintenance and use of germplasm are commonly restricted due to the lack of genetic information and/or by the large numbers of accessions in these collections (Virik *et al.*, 1995). Molecular markers, along with morpho-agronomic data and ecological descriptions of sampling sites have proven to be relevant for evaluating germplasm (Westman and Kresovich, 1997; Zong *et al.*, 2009). The use of molecular markers can also help to select material for establishing a core collection, *i.e.*, a group of accessions from an existing germplasm collection that is chosen to represent the genetic spectrum of the entire collection (Hao *et al.*, 2006). Microsatellites or simple sequence repeats (SSRs) have proven to be among the most suitable markers for such purposes (Huang *et al.*, 2002; Hao *et al.*, 2006; Landjeva *et al.*, 2006; Wang *et al.*, 2006; Ebana *et al.*, 2008; Blair *et al.*, 2009; Cipriani *et al.*, 2010).

In this study, we evaluated the genetic diversity and population structure in accessions of the Embrapa-Cer-

rados germplasm collections of *S. macrocephala* and *S. capitata* using polymorphic SSRs. Based on this diversity information, we determined the minimum sample size acceptable for a core collection of each species.

## Materials and Methods

### DNA extraction and PCR

A total of 326 accessions from the Embrapa-Cerrados germplasm collections were used in this study: 134 accessions of *S. macrocephala* and 192 of *S. capitata* (Tables 1 and 2). The SSR markers developed by Santos *et al.* (2009a) (13 SSR *S. macrocephala* loci) and Santos *et al.* (2009b) (15 SSR *S. capitata* loci) were used to assess the genetic diversity of these accessions.

Total DNA was extracted from leaves of three plants from each accession according to the cetyltrimethylammonium bromide method described by Faleiro *et al.* (2003). PCR amplifications were performed using a PTC-200 (MJ Research) thermocycler in a 20- $\mu$ L final reaction volume consisting of 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 0.25 mM of each dNTP (Invitrogen), 0.8  $\mu$ M of each primer, 1U *Taq* DNA polymerase (Invitrogen) and 20 ng genomic DNA. The amplification protocol consisted of an initial denaturation step at 94 °C for 1 min, followed by

**Table 1** - List of 134 accessions of *S. macrocephala* from the Embrapa-Cerrados germplasm collection that were analyzed for 13 microsatellite markers. The sample codes, the respective accession numbers and BRA or CIAT numbers in the germplasm collection of Embrapa-Cerrados (CPAC) and the place of origin are shown.

Code	CPAC	BRA/CIAT	Place of origin	Code	CPAC	BRA/CIAT	Place of origin
1	139	BRA-003697	Distrito Federal	40	1341	BRA-023264	Bahia
2	1030	CIAT 1942	Unknow	41	1345	BRA-022583	Bahia
3	1031	BRA-007773	Goiás	42	1346	BRA-023523	Bahia
4	1032	BRA-007820	Goiás	43	1347	BRA-023191	Bahia
5	1033	BRA-009032	Bahia	44	1367	BRA-022284	Goiás
6	1036	BRA-003008	Bahia	45	1370	BRA-023019	Bahia
7	1037	BRA-008052	Bahia	46	1373	BRA-022781	Bahia
8	1039	CIAT 2079	Bahia	47	1376	BRA-022985	Bahia
9	1040	BRA-008184	Bahia	48	1378	BRA-023329	Bahia
10	1043	BRA-008958	Bahia	49	1382	BRA-022586	Bahia
11	1190	CIAT 2270	Minas Gerais	50	1383	BRA-022616	Bahia
12	1191	CIAT 2271	Minas Gerais	51	1636	CIAT 1413	Unknow
13	1192	BRA-012297	Minas Gerais	52	1639	BRA-012866	Bahia
14	1193	CIAT 2273	Minas Gerais	53	1640	BRA-012947	Bahia
15	1194	CIAT 2274	Minas Gerais	54	2227	BRA-013030	Bahia
16	1196	CIAT 2276	Minas Gerais	55	2229	BRA-007226	Unknow
17	1197	CIAT 2277	Minas Gerais	56	2230	BRA-028487	Goiás
18	1198	CIAT 2278	Minas Gerais	57	2231	BRA-28495	Bahia
19	1200	CIAT 2280	Minas Gerais	58	2239	BRA-022828	Bahia
20	1201	CIAT 2281	Minas Gerais	59	2254	BRA-032883	Minas Gerais
21	1202	CIAT 2282	Minas Gerais	60	2255	BRA-032891	Minas Gerais
22	1204	CIAT 2284	Minas Gerais	61	2256	BRA-032905	Minas Gerais

Table 1 (cont.)

Code	CPAC	BRA/CIAT	Place of origin	Code	CPAC	BRA/CIAT	Place of origin
23	1205	CIAT 2285	Minas Gerais	62	2257	BRA-032913	Minas Gerais
24	1206	CIAT 2286	Minas Gerais	63	2258	BRA-032921	Minas Gerais
25	1303	-	Unknow	64	2259	BRA-032930	Minas Gerais
26	1304	-	Unknow	65	2260	BRA-032948	Minas Gerais
27	1305	-	Unknow	66	2261	BRA-032956	Minas Gerais
28	1306	-	Unknow	67	2262	BRA-032364	Minas Gerais
29	1307	BRA-017124	Distrito Federal	68	2263	BRA-032972	Minas Gerais
30	1308	BRA-017639	Goiás	69	2265	BRA-032999	Minas Gerais
31	1309	BRA-017281	Distrito Federal	70	2266	BRA-033006	Minas Gerais
32	1310	BRA-017663	Distrito Federal	71	2267	BRA-033014	Minas Gerais
33	1311	BRA-017442	Goiás	72	2710	BRA-028673	Goiás
34	1332	BRA-023202	Bahia	73	2711	BRA-028720	Bahia
35	1333	BRA-023124	Bahia	74	2712	BRA-028789	Piauí
36	1335	BRA-022837	Bahia	75	2713	BRA-028878	Bahia
37	1337	BRA-023345	Bahia	76	2714	BRA-028886	Bahia
38	1339	BRA-023493	Bahia	77	2715	BRA-028908	Bahia
39	1340	BRA-022829	Bahia	78	2716	BRA-028967	Bahia
79	2719	BRA-029025	Bahia	107	2709	-	Unknow
80	2720	BRA-029041	Bahia	108	2264	BRA-032981	Minas Gerais
81	2777	BRA-006245	Distrito Federal	109	2717	BRA-028771	Bahia
82	2778	BRA-011088	Minas Gerais	110	2790	BRA-008826	Bahia
83	2782	BRA-009008	Bahia	111	2792	BRA-008257	Bahia
84	2783	BRA-008061	Bahia	112	4135	BRA-036901	Unknow
85	1035	BRA-008010	Bahia	113	4137	BRA-036889	Unknow
86	2795	BRA-034142	Minas Gerais	114	1041	BRA-008222	Bahia
87	4136	BRA-036871	Unknow	115	1189	CIAT 2231	Rio de Janeiro
88	4138	BRA-036927	Unknow	116	1045	BRA-008362	Bahia
89	4139	BRA-036919	Unknow	117	1199	CIAT 2279	Minas Gerais
90	4140	BRA-036935	Unknow	118	1187	BRA-008168	Bahia
91	4166	BRA-036862	Unknow	119	1302	-	Minas Gerais
92	4167	BRA-036820	Unknow	120	1380	BRA-022721	Bahia
93	4168	BRA-036838	Unknow	121	1641	BRA-015261	Goiás
94	4200	BRA-036897	Unknow	122	1642	BRA-015253	Goiás
95	4271	BRA-037541	Unknow	123	1646	BRA-050173	Minas Gerais
96	4378	BRA-036854	Unknow	124	2232	BRA-028509	Bahia
97	5184	BRA-042731	Minas Gerais	125	2235	BRA-022516	Bahia
98	5296	-	Unknow	126	2268	BRA-033022	Minas Gerais
99	1362	BRA-23361	Bahia	127	2721	BRA-029076	Bahia
100	208		Goiás	128	2779	BRA-007773	Goiás
101	1336	BRA-024350	Bahia	129	2780	BRA-007820	Goiás
102	1363	BRA-022641	Bahia	130	2784	BRA-011126	Bahia
103	1361	BRA-022411	Bahia	131	2789	BRA-006301	Unknow
104	1358	BRA-022501	Bahia	132	2794	BRA-034215	Minas Gerais
105	2251	BRA-023566	Minas Gerais	133	4377	BRA-036846	Unknow
106	2252	BRA-024252	Bahia	134	4971	BRA-041441	Goiás

\*The geographic sites indicate the Brazilian states in which the plants were collected.

**Table 2** - List of 192 accessions of *S. capitata* from the Embrapa-Cerrados germplasm collection that were analyzed for 13 microsatellite markers. The sample codes, the respective accession numbers and BRA or CIAT numbers in the germplasm collection of Embrapa-Cerrados (CPAC) and the place of origin\* are shown.

Code	CPAC	BRA/CIAT	Place of origin	Code	CPAC	BRA/CIAT	Place of origin
1	515	BRA-006751	Mato Grosso do Sul	40	1160	BRA-001830	Maranhão
2	650	CIAT 1405	Mato Grosso do Sul	41	1161	BRA-001856	Maranhão
3	704	BRA-007251	Minas Gerais	42	1162	BRA-001899	Piauí
4	705	CIAT 1078	Bahia	43	1163	BRA-001902	Piauí
5	706	BRA-005886	Bahia	44	1164	BRA-001911	Piauí
6	707	BRA-001791	Maranhão	45	1165	BRA-000400	Piauí
7	901	CIAT 2249	Minas Gerais	46	1166	BRA-001929	Piauí
8	906	CIAT 1419	Goiás	47	1167	BRA-001937	Piauí
9	908	CIAT 1440	Ceará	48	1168	CIAT 2220	Bahia
10	909	CIAT 1441	São Paulo	49	1169	BRA-006190	Goiás
11	913	BRA-006742	Mato Grosso	50	1170	BRA-009181	Pernambuco
12	915	CIAT 1892	Venezuela	51	1171	BRA-007544	Ceará
13	916	CIAT 1899	Venezuela	52	1172	BRA-007522	Ceará
14	918	CIAT 1924	Venezuela	53	1174	BRA-007595	Ceará
15	922	BRA-007625	Distrito Federal	54	1177	CIAT 2259	Minas Gerais
16	924	BRA-007749	Goiás	55	1178	CIAT 2260	Minas Gerais
17	925	BRA-007871	Goiás	56	1182	CIAT 2265	Minas Gerais
18	926	BRA-007846	Goiás	57	1183	CIAT 2266	Minas Gerais
19	928	BRA-007803	Goiás	58	1185	CIAT 2268	Minas Gerais
20	929	BRA-007838	Goiás	59	1186	CIAT 2269	Minas Gerais
21	931	BRA-009059	Bahia	60	1278	BRA-017787	Minas Gerais
22	934	BRA-007960	Bahia	61	1279	BRA-017795	Minas Gerais
23	935	BRA-007994	Bahia	62	1281	-	Minas Gerais
24	936	BRA-008001	Bahia	63	1282	BRA-017881	Goiás
25	938	BRA-008087	Bahia	64	1283	BRA-017043	Goiás
26	939	BRA-008176	Bahia	65	1284	BRA-017094	Goiás
27	940	BRA-008231	Bahia	66	1285	BRA-016659	Goiás
28	943	BRA-008907	Bahia	67	1286	BRA-016675	Goiás
29	944	BRA-008869	Bahia	68	1287	BRA-016713	Goiás
30	945	BRA-008818	Bahia	69	1288	BRA-016519	Goiás
31	947	BRA-008401	Bahia	70	1289	BRA-016586	Goiás
32	949	BRA-008532	Bahia	71	1290	BRA-016403	Bahia
33	950	BRA-008583	Bahia	72	1291	BRA-016186	Goiás
34	951	BRA-008621	Bahia	73	1292	BRA-016268	Goiás
35	952	BRA-008681	Bahia	74	1293	BRA-016144	Goiás
36	953	BRA-008761	Bahia	75	1294	BRA-015962	Goiás
37	956	CIAT 2218	Bahia	76	1295	BRA-022136	Goiás
38	957	CIAT 2219	Bahia	77	1296	BRA-016039	Goiás
39	959	CIAT 2228	São Paulo	78	1297	BRA-016098	Goiás
79	1298	BRA-017507	Goiás	121	2226	BRA-032859	Minas Gerais
80	1299	BRA-017566	Goiás	122	2681	BRA-029084	Bahia
81	1300	BRA-017396	Goiás	123	2682	BRA-029068	Bahia
82	1328	BRA-013517	Goiás	124	2685	BRA-028860	Bahia
83	1350	BRA-011749	Maranhão	125	2686	BRA-028851	Piauí
84	1357	BRA-13371	Maranhão	126	2687	BRA-028843	Piauí
85	1384	BRA-022314	Maranhão	127	2689	BRA-028827	Piauí
86	1386	BRA-023485	Goiás	128	2691	BRA-028738	Bahia
87	1387	BRA-024317	Bahia	129	2692	BRA-028746	Bahia

Table 2 (cont.)

Code	CPAC	BRA/CIAT	Place of origin	Code	CPAC	BRA/CIAT	Place of origin
88	1388	BRA-023299	Bahia	130	2694	BRA-028681	Goiás
89	1389	BRA-22446	Bahia	131	2695	BRA-028762	Piauí
90	1392	BRA-022772	Bahia	132	2696	BRA-028657	Goiás
91	1394	BRA-024261	Bahia	133	2835	BRA-014346	Ceará
92	1395	BRA-022373	Bahia	134	2837	BRA-014362	Ceará
93	1588	BRA-012874	Bahia	135	2839	BRA-014443	Piauí
94	1590	BRA-012955	Bahia	136	2840	BRA-035190	Piauí
95	1591	BRA-003671	Bolivia	137	2841	BRA-014397	Piauí
96	1592	BRA-013021	Sergipe	138	2842	BRA-001848	Maranhão
97	1594	BRA-013935	Sergipe	139	2844	BRA-031160	Mato Grosso
98	1596	BRA-014036	Bahia	140	4123	BRA-036137	Ceará
99	1597	BRA-014117	Bahia	141	2821	BRA-035173	Venezuela
100	1600	BRA-015202	Goiás	142	2822	BRA-012840	Bahia
101	1598	BRA-015229	Goiás	143	2823	BRA-001881	Maranhão
102	1601	BRA-015199	Goiás	144	2826	BRA-014401	Piauí
103	1608	CIAT 2829	Venezuela	145	2828	BRA-035181	Mato Grosso
104	1609	BRA-011720	Maranhão	146	2829	BRA-014532	Maranhão
105	1611	BRA-050173	Minas Gerais	147	2830	BRA-014508	Maranhão
106	1612	BRA-033219	Distrito Federal	148	2831	BRA-014281	Ceará
107	1616	BRA-028177	Goiás	149	2833	BRA-035157	Distrito Federal
108	1617	CIAT 2946	Goiás	150	2834	BRA-000850	Piauí
109	1618	BRA-040738	Colômbia	151	2798	BRA-014311	Ceará
110	2207	BRA-027961	Bahia	152	2807	BRA-005924	Minas Gerais
111	2208	BRA-028002	Goiás	153	2809	BRA-005908	Venezuela
112	2209	BRA-028053	Venezuela	154	2811	BRA-021491	Distrito Federal
113	2211	BRA-028185	Minas Gerais	155	2817	BRA-005975	Venezuela
114	2212	BRA-014265	Ceará	156	2813	BRA-001864	Maranhão
115	2213	BRA-014320	Ceará	157	2819	BRA-013455	Paraíba
116	2214	BRA-014427	Piauí	158	4125	BRA-036081	Ceará
117	2215	BRA-007579	Ceará	159	4129	BRA-036153	Piauí
118	2216	BRA-008240	Bahia	160	4130	BRA-036161	Maranhão
119	2217	BRA-028258	Bahia	161	4131	BRA-036188	Maranhão
120	2224	BRA-032832	Minas Gerais	162	4155	BRA-035971	Bahia
163	4156	BRA-035963	Bahia	178	4354	BRA-036129	Ceará
164	4158	BRA-035955	Goiás	179	4355	BRA-036145	Ceará
165	4159	BRA-036048	Bahia	180	4356	BRA-036170	Maranhão
166	4341	BRA-035939	Distrito Federal	181	4357	BRA-036196	Maranhão
167	4343	BRA-035980	Bahia	182	4359	BRA-036218	Goiás
168	4344	BRA-035998	Bahia	183	4360	BRA-036226	Goiás
169	4345	BRA-036005	Bahia	184	4362	BRA-037583	Goiás
170	4346	BRA-036013	Bahia	185	4363	BRA-037605	Goiás
171	4347	BRA-036021	Bahia	186	4364	BRA-037770	São Paulo
172	4348	BRA-036030	Bahia	187	4973	BRA-041467	Minas Gerais
173	4349	BRA-036056	Ceará	188	4974	BRA-041475	Minas Gerais
174	4350	BRA-036064	Ceará	189	4977	BRA-041505	Minas Gerais
175	4351	BRA-036072	Ceará	190	4981	BRA-041543	Bahia
176	4352	BRA-036099	Ceará	191	4982	BRA-041556	Bahia
177	4353	BRA-036111	Ceará	192	4984	BRA-041572	Bahia

\*The geographic sites indicate the country or Brazilian states in which the plants were collected.



30 cycles of 94 °C for 1 min, 60 °C for 1 min and at 72 °C for 1 min, with a final extension step at 72 °C for 5 min. PCR-amplified DNA fragments were separated by electrophoresis on 6% denaturing polyacrylamide gels at 75 W for approximately 2 h and then stained with silver nitrate according to Creste *et al.* (2001). Allele scoring was done by comparison to a 10-bp DNA ladder (10-330 bp range) (Invitrogen).

### Data analysis

Allele frequencies, observed and expected heterozygosities ( $H_O$  and  $H_E$ ) and Roger's genetic distance modified by Wright (1978) were calculated using the Tools for Population Genetic Analysis (TFPGA) software (Miller, 1997). Population structure was inferred using STRUCTURE 2.0 software (Pritchard *et al.*, 2000), and the accessions were assigned to groups based on their genotypes. STRUCTURE uses model-based clustering in which a Bayesian approach identifies clusters based on their fit to Hardy-Weinberg and linkage equilibria.

The optimum number of populations (K) was selected after ten independent runs with a burn-in period of 300,000 and 400,000 replications using a model that does not allow for admixture or correlated allele frequencies. The procedure described by Evanno *et al.* (2005) was used to estimate the most probable number of distinct genetic groups (K) in each germplasm collection. Nei's  $G_{ST}$  among the groups defined by the STRUCTURE analysis was calculated using the software FSTAT (Goudet, 2001).

Genetic relationships among the accessions based on the genotypic data and Roger's genetic distance were estimated using a Neighbor-Joining method in DARwin 5.0 software (Perrier and Jacquemoud-Collet, 2006).

Finally, by using the software COREFINDER (Cipriani *et al.*, 2010) we assembled a core collection that should represent 100% of the genetic diversity present within the entire collection.

### Results

We used SSR markers developed for *S. macrocephala* and *S. capitata* to genotype all of the accessions in germplasm collections of both species. In *S. macrocephala*, 61 alleles were identified at 13 microsatellite loci, and 51 alleles were identified at 15 loci in *S. capitata*. In *S. macrocephala* the range was 2 to 11 alleles per locus (4.7 average) (Table 3), with  $H_E$  values ranging from 0.02 to 0.85 (0.36 on average) and  $H_O$  values varying from 0.01 to 0.17 (0.08 on average), thus representing a low level of genetic diversity. With regard to the *S. capitata* descriptive data, the numbers of alleles ranged from 2 to 9 for all of the loci analyzed (3.4 on average) (Table 4); the  $H_E$  values ranged from 0.27 to 0.74 (0.50 on average), and the  $H_O$  values from 0.04 to 0.87 (0.48 on average). Roger's genetic distance values among the *S. macrocephala* accessions ranged

**Table 3** - The 13 microsatellite loci used for the analysis of the Embrapa germplasm collection of *S. macrocephala*. The number of alleles (N) and observed and expected heterozygosities ( $H_O$  and  $H_E$ , respectively) are indicated for each locus.

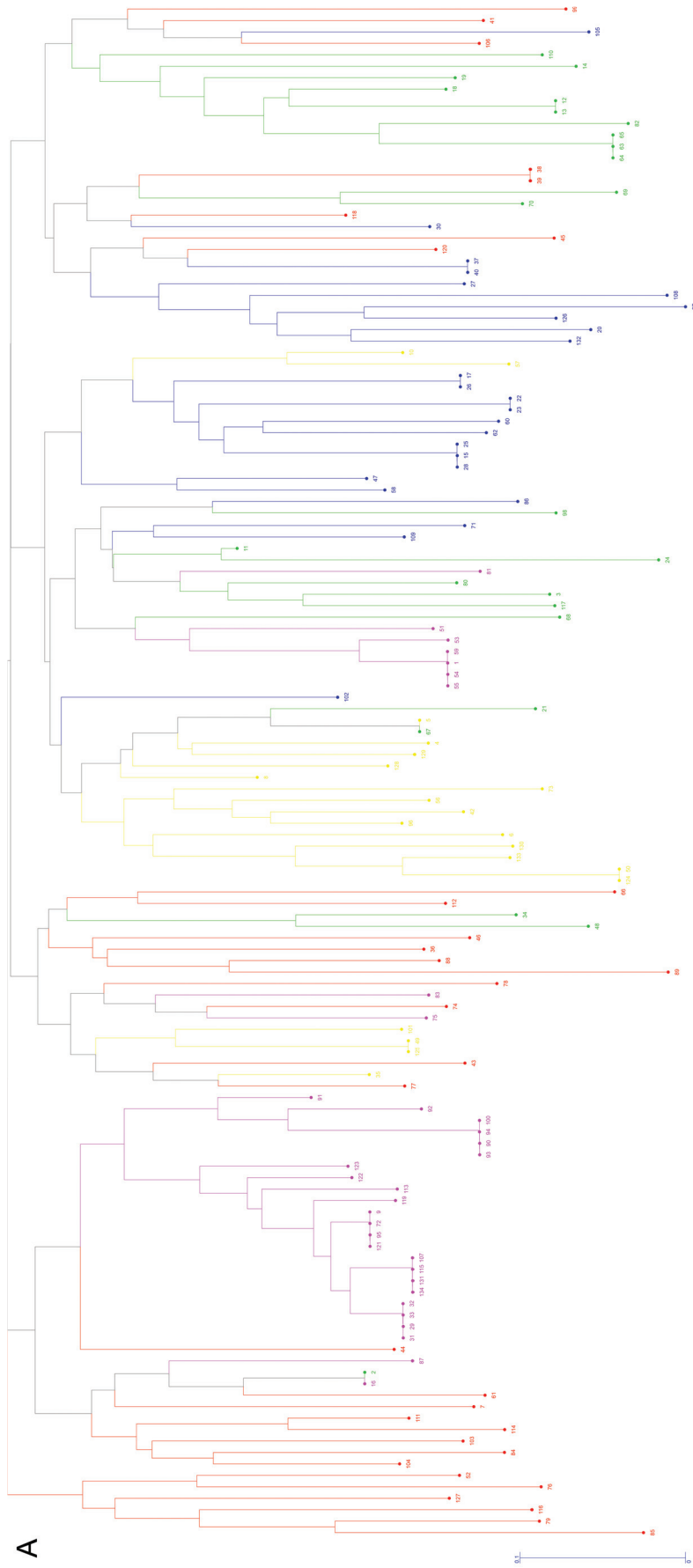
Locus	N	$H_O$	$H_E$
SM02 A5	2	0.01	0.07
SM02 A10	3	0.01	0.02
SM01 D3	2	0.02	0.04
SM02 A2	11	0.19	0.85
SM01 B11	7	0.05	0.70
SM02 C9	3	0.04	0.07
SM02 G2	2	0.07	0.26
SM02 G5	8	0.14	0.80
SM01 B5	2	0.02	0.04
SM01 B6	3	0.07	0.36
SM02 A8	3	0.16	0.54
SM02 A9	4	0.08	0.14
SM02 G3B	11	0.17	0.75
Average	4.7	0.08	0.36

**Table 4** - The 15 microsatellite loci used for the analysis of the Embrapa germplasm collection of *S. capitata*. The number of alleles (N) and observed and expected heterozygosities ( $H_O$  and  $H_E$ , respectively) are indicated for each locus.

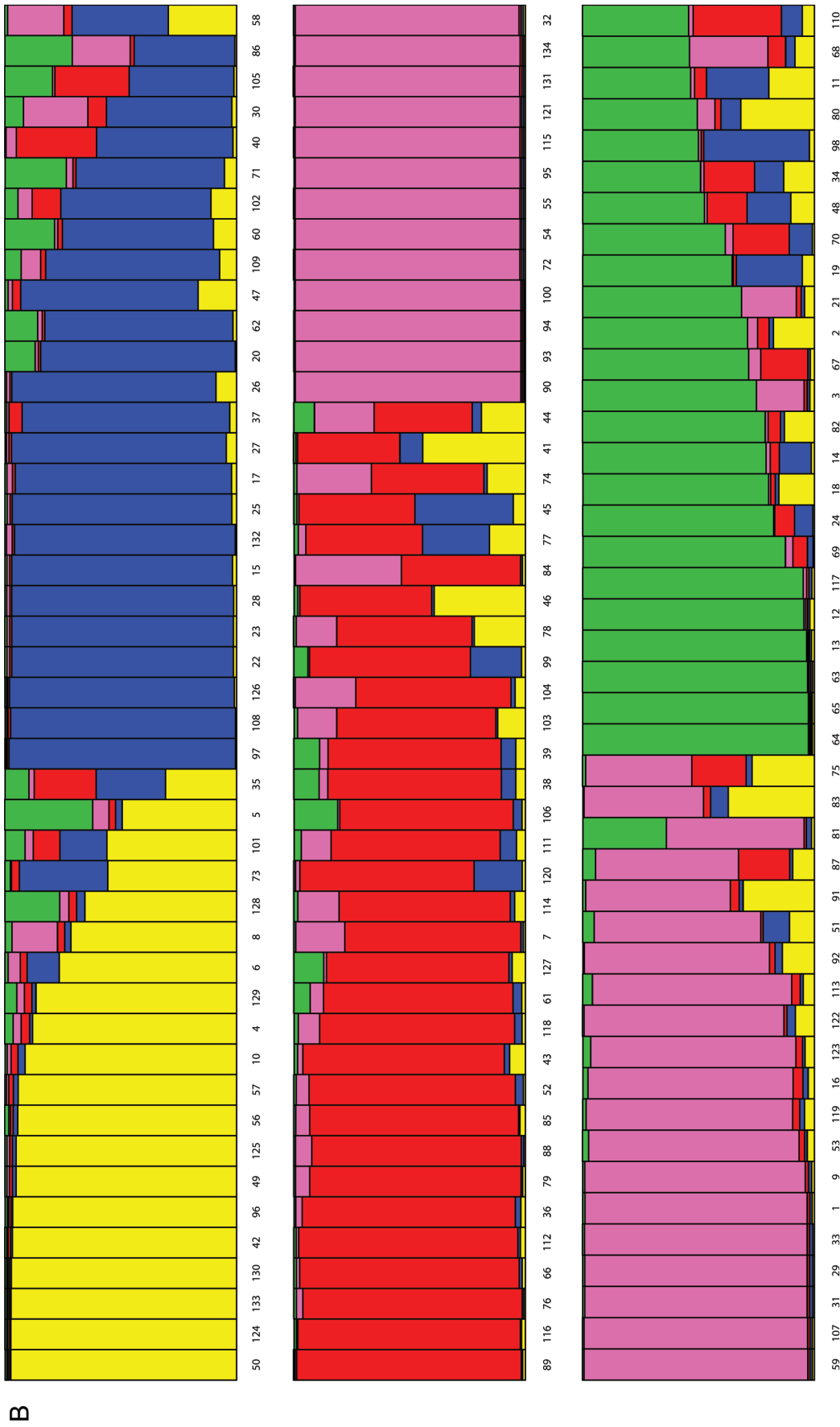
Locus	N	$H_O$	$H_E$
SC 01 TF6A	3	0.52	0.40
SC 01 C7B	5	0.87	0.59
SC 01 E10A	3	0.68	0.45
SC 01 E4	3	0.52	0.40
SC 01 E11	3	0.64	0.44
SC 01 A5	8	0.40	0.66
SC 01 E10B	3	0.72	0.46
SC 01 TG9	3	0.48	0.44
SC 01 B3	3	0.41	0.41
SC 01 TF11A	3	0.45	0.46
SC 01 TG12A	5	0.39	0.61
SC 01 A2A	3	0.28	0.27
SC 02 E12	9	0.41	0.71
SC 01 H5	5	0.04	0.54
SC 01 H6A	6	0.45	0.71
Average	4.3	0.48	0.50

from 0 to 0.83, with an average of 0.54, whereas these values ranged from 0 to 0.85 (0.50 average) for *S. capitata*.

The method of Evanno *et al.* (2005) was used to define the maximal  $\Delta K$ , which was at  $K = 5$  in the *S. macrocephala* germplasm collection, based on the STRUCTURE analysis (Figure 1). Cluster analysis revealed that 75 of the accessions (57%) were assigned to a single group with



**Figure 1** - Genetic diversity among *S. macrocephala* accessions. (A) As constructed from the Roger's dissimilarity matrix using the NJ method. (B) Bar plot representation of the percentage of the gene pool in each *S. macrocephala* accession.



**Figure 1 (cont.)** - Genetic diversity among *S. macrocephala* accessions. (A) As constructed from the Roger's dissimilarity matrix using the NJ method. (B) Bar plot representation of the percentage of the gene pool in each *S. macrocephala* accession.



more than 80% probability, whereas the other 59 accessions represented a mixture of different groups. Group D comprised the largest number of non-mixed accessions, with 79% of the individuals in this cluster showing more than 80% probability of membership. In contrast, most accessions in groups C and E had less than 80% probability of membership (59% and 62%, respectively). The descriptive data calculated for the individual clusters revealed that  $H_O$  ranged from 0.03 in group D to 0.14 in group C, and that  $H_E$  values varied from 0.14 in group D to 0.38 in group C.

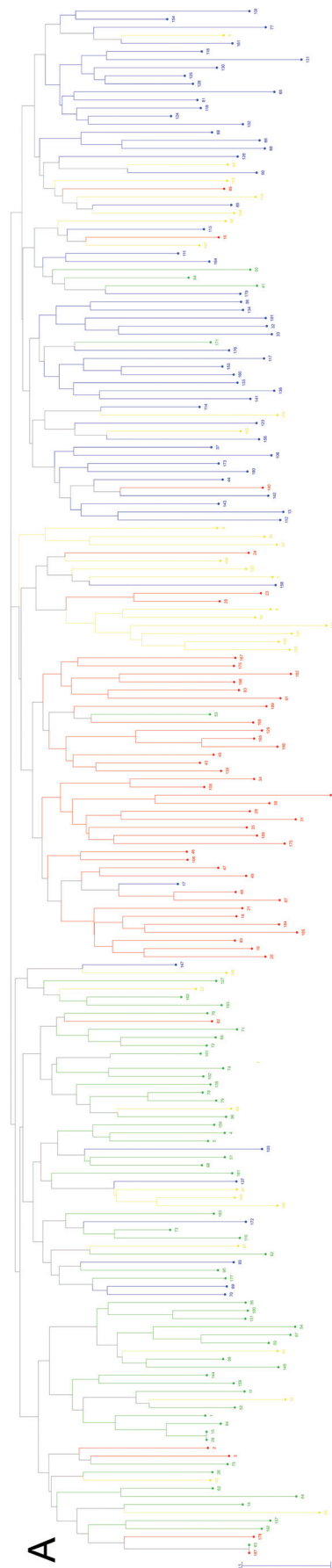
The STRUCTURE procedure clustered the *S. capitata* germplasm accessions into four groups (Figure 2), wherein 131 accessions (68%) were assigned to a single group with more than 80% probability of membership, and the remaining 61 accessions were so to different groups. Group D contained the largest number of accessions assigned with more than 80% membership probability (97%), whereas group A contained the highest percentage of mixed accessions (61%).  $H_O$  values ranged from 0.40 in group A to 0.56 in group C, and  $H_E$  values varied from 0.40 in group A to 0.49 in groups C and D. The Nei's genetic diversity among the groups ( $G_{ST}$ ) was calculated to infer the proportion of genetic diversity due to differences among the groups clustered by STRUCTURE in both species.  $G_{ST}$  values were 27% and 11% for *S. macrocephala* and *S. capitata*, respectively.

We used DARwin software to arrive at a Neighbor-Joining (NJ) tree derived from the Roger's genetic distance results (Figures 1 and 2). In this analysis, the clusters formed by STRUCTURE with high levels of mixed accessions (less than 80% probability) became dispersed along the NJ tree.

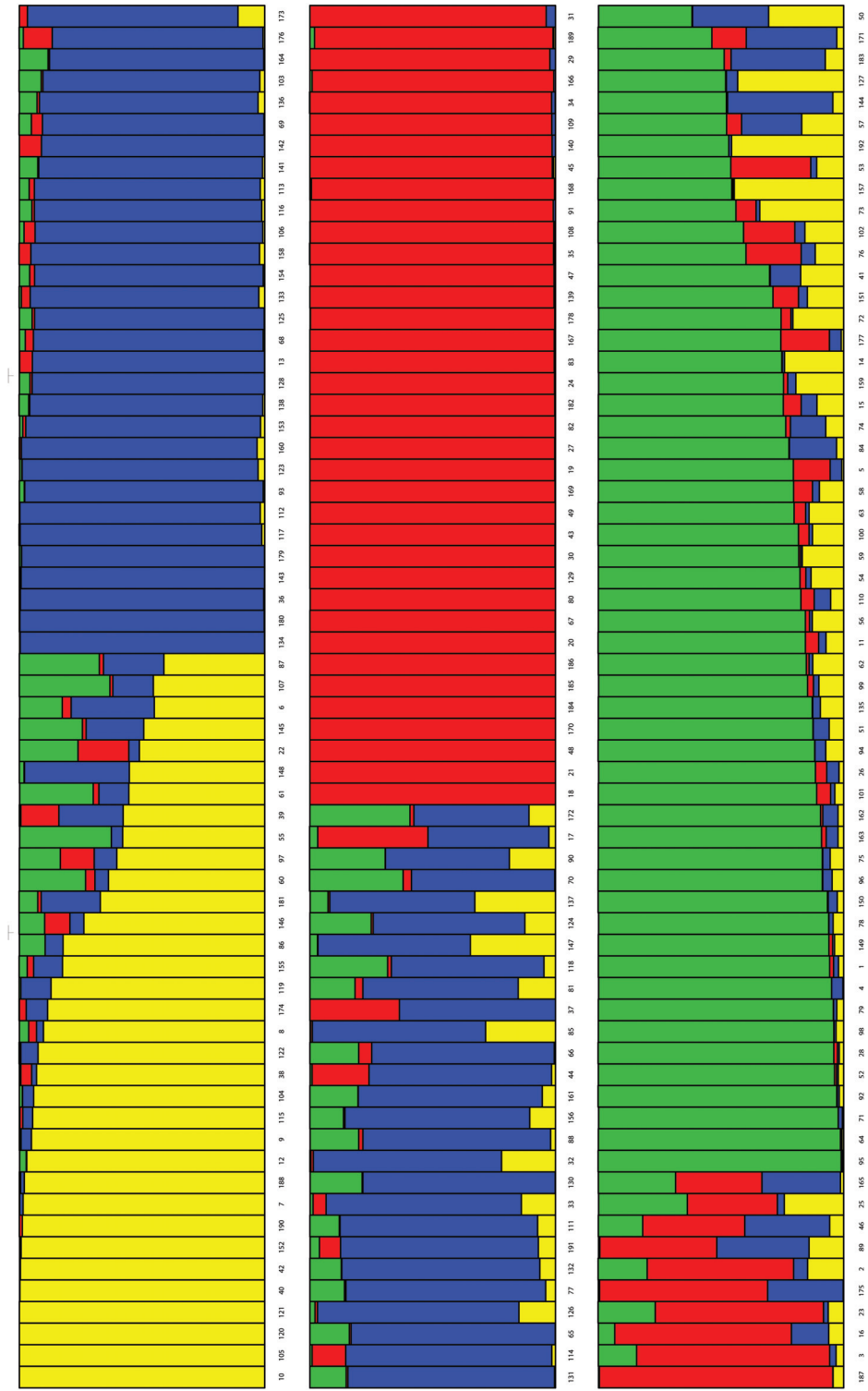
We assembled representative core collections for both species (Figure 3), aiming to obtain 100% of the genetic diversity observed in this study. This goal was accomplished with 23 accessions of *S. macrocephala* and 13 accessions of *S. capitata*. The alleles identified in this study were fully represented in these core collections.

## Discussion

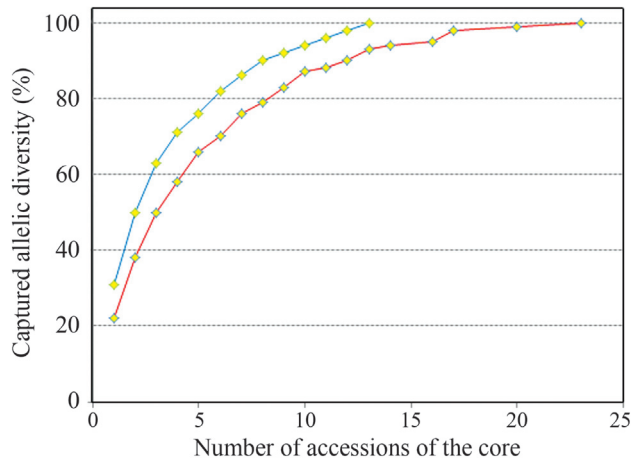
The SSR markers analyzed in this work were suitable for evaluating the genetic information in the accessions of *S. macrocephala* and *S. capitata*. Santos *et al.* (2009c) observed the same range of alleles per locus (2 to 11) in 20 accessions of this same *S. macrocephala* germplasm collection, but with a smaller average of four alleles per locus. In *S. capitata*, another study observed a range of alleles per locus that varied from 2 to 7 alleles per locus and averaged 3.3 in 20 accessions of the same germplasm collection analyzed using eight microsatellites (Santos *et al.*, 2009b). In *S. guianensis* (Aubl.) Sw., the analysis of 20 loci in 20 accessions revealed allele numbers between two and seven, with an average of four (Santos *et al.*, 2009c). However, when the number of *S. guianensis* accessions was increased to 150, the number of alleles per locus was equal to the vari-



**Figure 2** - Genetic diversity among *S. capitata* accessions. (A) As constructed from the Roger's dissimilarity matrix using the NJ method. (B) Bar plot representation of the percentage of the gene pool in each accession of *S. capitata*.



**Figure 2 (cont.)** - Genetic diversity among *S. capitata* accessions. (A) As constructed from the Roger's dissimilarity matrix using the NJ method. (B) Bar plot representation of the percentage of the gene pool in each accession of *S. capitata*.



**Figure 3** - Genetic diversity as a function of the number of accessions included in the *S. macrocephala* (red) and *S. capitata* (blue) core collections.

ation (2 to 11) and average (4.7) observed here for *S. macrocephala* (Santos-Garcia MO, 2009, PhD thesis, Universidade Estadual de Campinas, Campinas, Brazil). The allele sizes of *S. macrocephala* were consistent with the expected sizes reported in Santos *et al.* (2009a,c), with the exception of a few differences that occurred when higher numbers of alleles were observed for the same loci. The *S. capitata* accessions exhibited high levels of heterozygosity. Vander Stappen *et al.* (2002) showed that allotetraploid *Stylosanthes* species have high levels of fixed heterozygosity, which may explain the observed heterozygosity rates identified in the germplasm collection described for this study. As we used bulk samples, the observed heterozygosity could be explained by outcrossing and the inclusion of heterozygous individuals, or by heterogeneity in the GenBank accessions (Zhang *et al.*, 2008).

The genetic distances denoted in this study were higher than those previously reported for other species of the genus *Stylosanthes*. One possible explanation is that a larger number of accessions were analyzed here than in other studies. Furthermore, the types of molecular markers used in the previous studies were generally less polymorphic than our SSR markers. Barros *et al.* (2005) studied a subset of 86 accessions from the same *S. macrocephala* germplasm collection studied here using 15 RAPD primers and reported genetic distances ranging from 0.02 to 0.42. Hence, the microsatellite markers used herein revealed more genetic variation than the RAPD markers, similar to what has been shown in studies on other species (Powell *et al.*, 1996; Sun *et al.*, 1999; Laborda *et al.*, 2005). When evaluated using RAPD markers, the genetic dissimilarity in *S. scabra* J. Vogel was 0.06 among the accessions from Brazil, Colombia and Venezuela, and for *S. guianensis*, it averaged 0.26 among 31 accessions (Kazan *et al.*, 1993). The genetic distances among 42 *S. guianensis* accessions varied from 0.05 to 0.69 when measured using AFLP analysis (Chang-Shun *et al.*, 2004), and a recent analysis of 150

*S. guianensis* accessions using 20 microsatellite markers also resulted in high genetic distance values (Santos-Garcia *et al.*, 2012).

The population structure in the accessions of *S. macrocephala* and *S. capitata* was examined using STRUCTURE 2.0, which uses a Bayesian clustering approach to probabilistically assign individuals to populations based on their genotypes. The analysis of population structure using the model-based approach of Pritchard *et al.* (2000) provided support for the existence of genetic structure in these germplasm collections. Accordingly, five groups were formed among the *S. macrocephala* accessions, and four groups were formed among the *S. capitata* accessions.

The observed and expected heterozygosities were calculated considering the clusters as independent populations. Within the *S. macrocephala* groups we found that group C had the highest level of genetic diversity, whereas group D was the most homogeneous, with a low rate of heterozygosity. For *S. capitata*, the results showed no differences among groups. Such homogeneity was not unexpected because most of the accessions of the *S. capitata* collection were sampled in two locations only. When calculating the Nei's  $G_{ST}$  value among the groups formed by the STRUCTURE analysis approach, the *S. macrocephala* values were similar to other studies on species belonging to the Fabaceae family (Hamrick and Godt, 1996). In the *S. capitata* groups, the  $G_{ST}$  values were lower than those found for other *Stylosanthes* species. AFLP studies estimated a 30% variation between *S. humilis* accessions from Mexico and South America (Vander Stappen *et al.*, 2000), and another analysis on *S. humilis* H. B. K., based on AFLP, estimated 59% variation among groups. In contrast, the estimated variation among groups of *S. viscosa* (L.) Sw. was 66%, which is a higher degree of genetic difference than that observed for either of the species in our study (Sawkins *et al.*, 2001).

The sampling locations of the accessions of the *S. macrocephala* germplasm collection are listed in Table 1. The samples were collected in the Brazilian States of Bahia, Goiás, Minas Gerais, Piauí and the Distrito Federal, though information regarding the exact site of collection is lacking for several accessions.

Group A (Figure 1) consisted of accessions from Bahia and Goiás, and groups B and E included accessions from Bahia and Minas Gerais. Group C consisted mostly of accessions from Bahia, whereas group D included accessions from Bahia, Goiás, and the Distrito Federal. Barros *et al.* (2005) described 10 groups of *S. macrocephala* inferred from RAPD markers; 75% of all of the accessions were clustered into only one group, whereas seven of the remaining groups contained no more than two accessions. This clustering of 75% of the accessions into the same group limited the analysis of the genetic diversity and population structure in the *S. macrocephala* collection. Furthermore,

the grouping created difficulties for comparing the RAPD-derived clusters with those inferred from microsatellites. In this work, the Bayesian approach made it possible to identify patterns of genetic variation among five *S. macrocephala* clusters and clarified the relationships among accessions within the same RAPD cluster previously described by Barros *et al.* (2005). Our results showed that the accessions collected in Bahia were distributed throughout all five of the groups obtained with STRUCTURE and that the group consisting mostly of accessions collected in this state exhibited the highest levels of genetic diversity. Based on these results, we hypothesize that the state of Bahia might be the location of the origin of *S. macrocephala*. However, data from natural populations are necessary to confirm this hypothesis.

The sampling locations of the accessions of the *S. capitata* germplasm collection are listed in Table 2. The plants were collected in several Brazilian states, along with the Distrito Federal, and samples were also obtained from Colombia and Venezuela. The Colombia accession (CPAC 1618) is a mixture of several Brazilian accessions developed by Instituto Colombiano Agropecuario (ICA) as “Capita” variety and is considered a reference to *S. capitata*. The Capita variety was used as standard to check the phenotypic characterization of the *S. capitata* germplasm. Notwithstanding, most of the accessions were collected in Goiás and Bahia (54 and 39, respectively), representing 49% of the total collection. Groups A, B and C contained higher numbers of Bahia and Goiás accessions, whereas group B contained more samples from Bahia than from Goiás. Group D also contained several Bahia and Goiás accessions, but the majority of the accessions were from Minas Gerais. The only accession from Colombia was allocated to group B. The eight accessions from Venezuela were distributed among groups A, B, C and D, with five accessions from Venezuela clustering in group C, whereas each of the other groups contained only one accession each from this country. Group A comprised a great heterogeneity of localities, with accessions collected from all of the Brazilian states and South American countries, except for São Paulo and Colombia. Groups B and C contained the majority of the accessions from the northeastern states of Brazil and Goiás (central western region), whereas group D had more accessions from the southeastern states.

Due to sampling issues, many of the Brazilian states were poorly represented, and the genetic groups defined by STRUCTURE could not be correlated with geographic regions. Thus, for a more complete study of the genetic diversity of *S. capitata* in Brazil, new samples must be acquired, especially so from natural populations.

Using DARwin software, we constructed an NJ tree based on the Roger’s genetic distances for *S. macrocephala* (Figure 1) and *S. capitata* (Figure 2). For *S. macrocephala*, groups B and D, which contained the highest number of accessions assigned with more than 80% probability in the

STRUCTURE analysis, mostly remained clustered together in the tree. In contrast, other groups with more mixed individuals were randomly distributed along the NJ tree. Similar results were obtained for *S. capitata*, in which group A, with more mixed accessions, was also dispersed over the NJ tree. For the remaining groups, the majority of accessions clustered together in the NJ tree.

When directly compared, the results of the STRUCTURE and the NJ tree analyses revealed certain differences related to the number of groups and their genetic structure, but such differences are expected because these methods are based on distinct assumptions (Wang *et al.*, 2009). Model-based approaches, such as STRUCTURE, are more efficient than distance-based methods in discriminating genetic groups, as cluster identification is not affected by the genetic distance or graphical representation chosen (Pritchard *et al.*, 2000). Nevertheless, a combined analysis using different approaches may provide a better definition of the genetic diversity and structure in both of the *Stylosanthes* collections. Genetic diversity is the basis for genetic improvement, and consequently, knowledge about germplasm diversity has a significant impact on plant breeding (Huang *et al.*, 2002).

Costa and Schultze-Kraft (1993) performed a clustering analysis for *S. capitata* based on geographical regions and morpho-agronomic characteristics. As we used SSR markers obtained from genomic DNA, it is not possible to infer an association between the genetic markers and the phenotypic characters of the accessions. The groups obtained through molecular marker analysis are thus different from the ones obtained by Costa and Schultze-Kraft (1993), and both should be of importance to *Stylosanthes* breeders. In classical plant breeding programs, selection is done based on phenotypic evaluation, and improved progenies are obtained through crossing individuals of superior phenotypes and which, in general, are also genetically distant. Studies using molecular markers are complementary to phenotypic evaluation (Costa and Schultze-Kraft, 1993), and both are fundamental to genetic breeding programs.

Core collections were herein assembled for both *Stylosanthes* species, aiming to represent the entire genetic diversity identified in this study. The COREFINDER analysis showed that for *S. macrocephala*, 100% of the alleles found in this study could be represented by a core collection of 23 accessions. For *S. capitata*, only 13 accessions were necessary to represent 100% of the observed genetic diversity. Thus, we found that only a relatively small number of accessions were indeed necessary to represent the molecular diversity revealed in this study.

Certain factors may have contributed to the low number of accessions in the core collections suggested here. First, in terms of numbers of individuals collected in each region, the germplasm collection does not equally represent all of the distribution regions. As stated before, the germplasm collection includes some regions, such as the



state of Goiás, with 54 different accessions, while others have only few representatives. We think this unequal representation may to some extent compromise the genetic diversity present in the collection and is likely reflected in the reduced number of individuals necessary to fully represent allelic diversity. In addition, *S. capitata* is an allotetraploid species that exhibits high levels of heterozygosity, which may contribute to reducing the size of the core collection (Cipriani *et al.*, 2010). Sampling proportion and representation of base collection variation are the two most important characteristics to be observed when establishing a core collection (Hao *et al.*, 2006). Brown *et al.* (1987) suggested that the number of accessions in the core should account for 5 - 10% of the base collection, representing at least 70% of its genetic diversity. Van Hintum (1999) recommended that the sampling proportion should vary between 5% and 20% of the base collection, depending on the main objective. Both of the core collections proposed here represent 100% of the molecular diversity found in this study, with the number of accessions accounting for 17% and 7% of the base collection for *S. macrocephala* and *S. capitata*, respectively.

Our results demonstrate the great potential of using molecular data to construct a core collection and thus improve the management and utilization of the *Stylosanthes* germplasm collection of Embrapa-Cerrados. Nevertheless, because we used a relatively small number of genomic markers for the genetic analysis, the data presented here should not be used alone when deciding on which accessions from the germplasm collection should be discarded or maintained. Additional molecular markers, including more SSRs and single nucleotide polymorphisms (SNPs), should be used to provide better coverage of the genome. This information should be coupled with phenotypic data for traits of interest, such as phenology and disease resistance traits, to make a final decision on the accessions to be maintained. To initiate this effort, more genotyping and phenotyping should be initiated with the core collection proposed here and expanded to other accessions as necessary. In addition, the core collection can also be used in the selection of parents for future crosses, based both on genetic distance and phenotypic traits of the accessions.

Another issue that requires consideration is the genetic purity of the accessions used in this work. It was previously shown by our group that *S. capitata* and *S. guianensis* can cross-pollinate (Santos-Garcia *et al.*, 2010), but breeders have not accounted for cross-pollination during *Stylosanthes* seed multiplication. Here, we demonstrated a high level of heterozygosity in *S. capitata* in some undefined genetic groups obtained with STRUCTURE and the Neighbor-Joining based tree. These results might have been influenced by contaminations of the different accessions by seed multiplication plots established close to each other in the field.

In this work, we used polymorphic microsatellite markers to evaluate the genetic diversity of two *Stylosanthes* germplasm collections, and the results revealed a population structure among the accessions of both species. Our work indicates that even a small number of microsatellite markers is informative for genetic diversity studies in *Stylosanthes* species, providing a rapid and low-cost procedure for screening *Stylosanthes* germplasm collections. The results for *S. macrocephala* suggest some correlation between the region of collection and distribution among the groups based on the SSR markers. The same conclusion could not be reached for *S. capitata* because the collection does not equally represent the regions of distribution of this species in terms of quantity of accessions from each region, thereby indicating a need to improve sampling for this collection. The data from this study will certainly provide valuable information to geneticists and breeders for future improvement and conservation of *Stylosanthes* species.

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