AoB PLANTS http://aobplants.oxfordjournals.org/

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Identification of *Stylosanthes guianensis* varieties using molecular genetic analysis

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Received: 2 June 2011; **Returned for revision:** 27 July 2011; **Accepted:** 5 January 2012; **Published:** 10 January 2012

Citation details: Santos-Garcia MO, Karia CT, Resende RMS, Chiari L, Vieira MLC, Zucchi MI, Souza AP. 2012. Identification of *Stylosanthes guianensis* varieties using molecular genetic analysis. *AoB PLANTS* **2012**: pls001; doi:10.1093/aobpla/pls001

Abstract

Background and aims The botanical classification of *Stylosanthes guianensis* is controversial, and few studies have used molecular markers to analyse this species. We used microsatellite markers to study the genetic diversity and population structure of *S. guianensis* and compare our results with the current infraspecific botanical classification.

Methodology A representative sample from the *S. guianensis* Brazilian germplasm collection (150 accessions) was analysed using 20 microsatellite loci. A model-based Bayesian approach implemented in the software STRUCTURE was used to assign accessions into clusters. A dendrogram was constructed based on Roger's genetic distances.

Principal results The number of alleles per locus varied from 2 to 11, with an average of 4.7. The observed (H_O) and expected (H_E) heterozygosity values varied from 0 to 0.58 (mean of 0.18) and from 0.04 to 0.83 (mean of 0.55), respectively. Nine groups were assembled in STRUCTURE, and these groups were consistent with clusters inferred from the genetic distances and taxonomic varieties described for *S. guianensis*. The G_{ST} among the nine groups was 0.46.

Conclusions The low H_O and the G_{ST} values observed are in agreement with the outcrossing rate (26 %) estimated for this species. The data indicate a high genetic diversity among and within the botanical varieties and suggest that microsatellite-based information can be combined with classical taxonomy to elucidate infraspecific levels.

Introduction

The genus *Stylosanthes* belongs to the family Fabaceae and consists of 48 species found in tropical and subtropical regions of the Americas, Africa and Southeast Asia (Costa and Ferreira 1984; Costa 2006). The genus has two centres of diversity, the most important being located in central Brazil (Ferreira and Costa 1979; Stace and Cameron 1984). It includes 45 % of all *Stylosanthes* species and exhibits the greatest degree of phenotypic variation and endemism (Costa 2006). Mexico and the

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Caribbean Islands are also major centres of *Stylosanthes* diversity (Stace and Cameron 1984).

Stylosanthes guianensis (Aubl.) Sw. ($2n = 20$) is the most widespread *Stylosanthes* species and exhibits great phenotypic variation (Williams *et al.* 1984; Vieira *et al.* 1993). This species is native to South and Central America, where it is widely distributed, although not in the equatorial zone (Williams *et al.* 1984). *Stylosanthes guianensis* is considered to be a promising forage crop in the Brazilian savannahs, and many accessions produce a large amount of dry matter and retain their leaves during the dry season (Andrade and Karia 2000; Maass and Sawkins 2004).

The taxonomic classification of *S. guianensis* is controversial, and different taxonomic groups have been proposed based on different morphological characters. Mannetje (1977, 1984) recognized seven different *S. guianensis* varieties: *S. guianensis* var. *dissitiflora, S. guianensis* var. *gracilis*, *S. guianensis* var. *guianensis*, *S. guianensis* var. *intermedia*, *S. guianensis* var. *longiseta*, *S. guianensis* var. *marginata* and *S. guianensis* var. *robusta*. Ferreira and Costa (1979) considered Mannetje's varieties to be different species. They proposed that *S. guianensis* was composed of the Mannetje *S. guianensis* var. *guianensis* but subdivided this species into four different botanical varieties. Analyses of random amplified polymorphic DNA (RAPD) markers (Kazan *et al.* 1993; Vieira *et al.* 1997), sequence-tagged site and internal transcribed spacer (ITS) sequences (Vander Stappen *et al.* 1998, 1999*b*) demonstrated a high genetic diversity among the varieties proposed by Mannetje (1984), supporting their separation into distinct species. More recently, Costa (2006) used ITS sequencing and classical taxonomy to review the genus *Stylosanthes*. The ITS data support the classification proposed by Ferreira and Costa (1979) and Brandão *et al.* (1985), according to whom there are four botanical varieties of *S. guianensis*: *S. guianensis* var. *guianensis*, *S. guianensis* var. *canescens*, *S. guianensis* var. *microcephala* and *S. guianensis* var. *pauciflora*.

Vieira *et al.* (1993) analysed the karyotypes of the botanical varieties described by Ferreira and Costa (1979) and Brandão *et al.* (1985), and four species considered to be *S. guianensis* varieties by Mannetje (1977, 1984). Their results demonstrate that the varieties proposed by Ferreira and Costa (1979) and Brandão *et al.* (1985) have similar karyotypes, suggesting that *S. g. canescens*, *S. g. microcephala* and *S. g. pauciflora* may all have evolved from *S. g. guianensis*. The four species considered to be *S. guianensis* varieties by Mannetje (1977, 1984) have distinct karyotypes, supporting the classification proposed by Ferreira and Costa (1979) and Brandão *et al.* (1985).

Microsatellites, or simple sequence repeats (SSRs), are useful markers for a variety of applications in plant genetics because they are codominant, multiallelic, easily detected by polymerase chain reaction, relatively abundant and provide good coverage of the genome (Powell *et al.* 1996*a*). Microsatellite markers are available for three species of *Stylosanthes*: *S. guianensis* (Vander Stappen *et al.* 1999*a*; Santos *et al.* 2009*a*), *S. capitata* (Santos *et al.* 2009*b*) and *S. macrocephala* (Santos *et al.* 2009*c*). Vander Stappen *et al.* (1999*a*) described 18 genomic microsatellites in *S. guianensis* and tested them in 65 genotypes of *S. guianensis* and in the related species *S. gracilis*, *S. hippocampoides*, *S. grandifolia*, *S. acuminata* and *S. longiseta*. All 18 microsatellites were polymorphic among *S. guianensis* and its related species, and 16 were polymorphic within *S. guianensis*. Karia (2008) tested the microsatellites developed by Vander Stappen *et al.* (1999*a*) to evaluate the relationships among 437 accessions of the *S. guianensis* Brazilian germplasm collection and found that only seven were polymorphic among the accessions. Because of the limited number of markers, many relationships were unclear, and few could be clarified with respect to the population structure and to the clustering of the botanical varieties based on the results of Karia (2008). Considering the small number of polymorphic microsatellites available for this species and the need to better evaluate the Brazilian germplasm collection, Santos *et al.* (2009*a*) developed 46 new genomic microsatellites, 20 of which were polymorphic when tested in 20 *S. guianensis* accessions.

Santos-Garcia *et al.* (2011) tested both sets of microsatellites (Vander Stappen *et al.* 1999*a*; Santos *et al.* 2009*a*) in 20 accessions that were used as maternal parents in a progeny array and found that only five were polymorphic and could be used to determine the mating system of *S. guianensis*. Based on the microsatellite data, these authors estimated the outcrossing rate in *S. guianensis* to be 26 %, indicating that the species presents a mixed mating system with predominance of autogamy.

Despite the importance of *S. guianensis* as forage in tropical areas and the fact that some Brazilian regions contain most of the diversity of this genus, few studies have been conducted to determine the genetic diversity in the Brazilian germplasm collection. The Brazilian *S. guianensis* collection was established in a series of collecting trips around Brazil and other South American countries, and it has not been subjected to any breeding selection. Genetic studies of this collection may provide valuable information for planning new collection trips as well as for the study of natural populations. Karia (2008) was the first to study this collection using molecular markers, but this analysis did not provide enough information to evaluate the genetic diversity of

the collection or to clarify the botanical classifications and the relationships among the accessions.

Considering the classifications proposed by Ferreira and Costa (1979), Brandão *et al.* (1985) and Costa (2006), we have selected a representative sample of the *S. guianensis* Brazilian germplasm collection consisting of 150 accessions that were either classified as one of the four botanical varieties or remained unclassified. We are dealing with botanical varieties of *S. guianensis* in this study, and their classification is still controversial. Currently, microsatellite markers have been the most widely used for inferring population structure that ultimately defines variation at the infraspecific level.

Considering that, we used microsatellite markers to analyse the *S. guianensis* accessions with the goals of (i) estimating the genetic diversity and relationships present among the accessions, (ii) determining the population structure of the samples and (iii) comparing the observed population structure to the proposed botanical varieties. The microsatellite-based population structure observed in these samples was generally in agreement with the proposed botanical varieties of *S. guianensis*.

Materials and methods

Plant material

A total of 150 accessions from the Brazilian Institution Embrapa Cerrados were selected to evaluate the genetic diversity, the population structure and the relationships among them. A list of the accessions with their sample codes, accession numbers, places of origin and botanical varieties is provided in Table 1.

DNA extraction

DNA was extracted from the bulk leaf samples of four plants per accession using the cetyltrimethyl ammonium bromide method (Doyle and Doyle 1990) as modified by Bellon *et al.* (2007).

SSR amplification and detection

Twenty microsatellite loci were amplified from the DNA of all of the sampled accessions as previously described by Santos *et al.* (2009*a*). Amplified fragments were separated on 6 % acrylamide gels and visualized by silver staining according to Creste *et al.* (2001).

Data analysis

The Genetic Data Analysis program (Lewis and Zaykin 2000) was used to estimate the observed and expected heterozygosities. Allele frequencies and Roger's genetic distance, as modified by Wright (1978), were estimated between all pairs of accessions using Tools for Population Genetic Analysis (Miller 1997).

The STRUCTURE software package (Pritchard *et al.* 2000) was used to subdivide the accessions into the appropriate number of genetic clusters independent of any prior information concerning the geographic origin of the accessions. STRUCTURE uses a Bayesian approach to identify clusters based on their fit to Hardy–Weinberg proportions and their linkage equilibria. Ten STRUCTURE runs were performed by setting the number of populations (K) from 1 to 20. For each run, the burn-in time was set to 200 000, and the replication number was set to 300 000. The most likely number of distinct clusters (K) was determined according to the procedure described by Evanno *et al.* (2005). A graphical representation of the population assignments obtained from STRUCTURE was generated using R v.2.9.1.

DARwin 5.0 (Perrier and Jacquemoud-Collet 2006) was used to define the genetic relationships among accessions based on Roger's genetic distance and the UPGMA (unweighted paired group method) clustering method. FSTAT (Goudet 2001) was used to estimate Nei's G_{ST} (Nei 1973) among the groups obtained by the STRUCTURE analysis.

Results

We tested 20 genomic microsatellite loci developed by Santos *et al.* (2009*a*) and found that all loci were polymorphic within the group of accessions studied here. These 20 microsatellites generated 94 alleles that were used to study the genetic diversity among 150 *S. guianensis* accessions. The number of alleles ranged from 2 to 11 (Table 2), with an average of 4.7 alleles per locus. The SG03G8 locus exhibited the greatest number of alleles (11), followed by the SG03E2 locus with 10 alleles.

The observed and expected heterozygosity values (H_O and H_E) ranged from 0.00 to 0.58 (mean of 0.18) and from 0.04 to 0.83 (mean of 0.55), respectively. The markers with a higher observed heterozygosity were SG01D7 and SG01B9. In the case of bulk samples, a heterozygous pattern may be observed as a result of the presence of heterozygous plants in the DNA pool or variation within the accession (Zhang *et al.* 2008).

Roger's genetic distances were estimated for each pair of accessions and varied from 0.00 (among several accessions) to 0.94 (between accessions 19 and 140), with an average of 0.66.

A model-based analysis performed by STRUCTURE was used to investigate the possible population structure within the collection sample (150 accessions). The

Table 1 The *S. guianensis* collection used to assess the genetic diversity at 20 microsatellite loci. *Shown are the sample codes, the respective accession numbers in the Embrapa Cerrados and CIAT (International Center for Tropical Agriculture) germplasm collections, the geographic collection site and the botanical variety of each accession.*

Code	Embrapa Cerrados	CIAT number	Geographic site[a]	Botanical variety[b]
1	135	1297	Distrito Federal	*pauciflora*
2	464	n.a.	Tocantins	n.a.
3	517	1705	Mato Grosso	n.a.
4	648	1950	Colombia	*guianensis*
5	662	136	Colombia	n.a.
6	1121	1507	Venezuela	*pauciflora*
7	1132	1890	Venezuela	*pauciflora*
8	1139	1975	Colombia	n.a.
9	1144	2034	Bahia	*pauciflora*
10	1153	2315	Bahia	n.a.
11	1229	n.a.	Minas Gerais	*microcephala*
12	1230	2950	Minas Gerais	*guianensis*
13	1231	2951	Minas Gerais	n.a.
14	1235	n.a.	Goiás	n.a.
15	1237	2615	Tocantins	*canescens*
16	1239	2659	Pará	n.a.
17	1354	2991	Espirito Santo	*pauciflora*
18	1360	2549	Piaui	*pauciflora*
19	1365	2812	Venezuela	*pauciflora*
20	1368	2742	Minas Gerais	*pauciflora*
21	1369	2529	Bahia	*pauciflora*
22	1371	10 107	Goiás	*microcephala*
23	1372	2439	Pernambuco	*pauciflora*
24	1619	2748	Venezuela	n.a.
25	2203	2987	Bahia	*pauciflora*
26	2464	10 993	Tocantins	*guianensis*
27	2725	2974	Bahia	*pauciflora*
28	2734	2458	Paráiba	*pauciflora*
29	2738	2542	Ceará	*pauciflora*
30	2740	2708	Goiás	*pauciflora*
31	2741	2436	Alagoas	*pauciflora*
32	2761	2727	Distrito Federal	*pauciflora*
33	2771	2992	Espírito Santo	*pauciflora*
34	4142	n.a.	Tocantins	*guianensis*
35	4144	n.a.	Tocantins	*canescens*
36	4157	n.a.	Minas Gerais	*canescens*
37	4172	n.a.	Goiás	*canescens*

Continued

Table 1 ***Continued***

Code	Embrapa Cerrados	CIAT number	Geographic site[a]	Botanical variety[b]
38	4173	n.a.	Tocantins	*canescens*
39	4174	n.a.	Maranhão	n.a.
40	4188	n.a.	Bahia	*pauciflora*
41	4193	n.a.	Bahia	*pauciflora*
42	4199	n.a.	Bahia	*pauciflora*
43	4227	n.a.	São Paulo	*canescens*
44	4233	n.a.	São Paulo	*canescens*
45	4237	n.a.	Minas Gerais	*guianensis*
46	4238	n.a.	Rio de Janeiro	*guianensis*
47	4239	n.a.	Minas Gerais	*guianensis*
48	4240	n.a.	Rio de Janeiro	*guianensis*
49	4262	n.a.	Minas Gerais	*guianensis*
50	4264	n.a.	Minas Gerais	*guianensis*
51	4286	n.a.	Goiás	n.a.
52	4292	n.a.	Goiás	*pauciflora*
53	4300	n.a.	Minas Gerais	*guianensis*
54	4302	n.a.	Rio de Janeiro	*microcephala*
55	4306	n.a.	Minas Gerais	*guianensis*
56	4308	n.a.	Minas Gerais	*canescens*
57	4314	n.a.	Pará	*guianensis*
58	4315	n.a.	Pará	*guianensis*
59	4322	n.a.	Pará	*guianensis*
60	4323	n.a.	Pará	*guianensis*
61	4324	n.a.	Pará	*guianensis*
62	4331	n.a.	Pará	*guianensis*
63	4336	n.a.	São Paulo	n.a.
64	4338	n.a.	São Paulo	n.a.
65	4364	n.a.	São Paulo	n.a.
66	4528	n.a.	Goiás	n.a.
67	4530	n.a.	n.a.	*guianensis*
68	5187	n.a.	Minas Gerais	*pauciflora*
69	5192	n.a.	Minas Gerais	*pauciflora*
70	5204	n.a.	Goiás	*canescens*
71	5206	n.a.	Minas Gerais	*canescens*
72	5221	n.a.	São Paulo	*canescens*
73	5233	n.a.	São Paulo	*guianensis*
74	5236	n.a.	Minas Gerais	*microcephala*
75	5239	n.a.	Goiás	*microcephala*
76	5248	n.a.	Goiás	*pauciflora*

Continued

Table 1 ***Continued***

Code	Embrapa Cerrados	CIAT number	Geographic site[a]	Botanical variety[b]
77	5262	n.a.	Mato Grosso do Sul	*microcephala*
78	5277	n.a.	Minas Gerais	*pauciflora*
79	5279	n.a.	Minas Gerais	*microcephala*
80	5294	n.a.	n.a.	n.a.
81	5295	n.a.	n.a.	n.a.
82	5349	n.a.	Bahia	n.a.
83	1361	2445	Pernambuco	n.a.
84	5426	n.a.	n.a.	n.a.
85	5428	10 488	Pará	n.a.
86	5429	10 416	Pará	n.a.
87	5430	2649	Maranhão	n.a.
88	5432	2700	Goiás	*microcephala*
89	5433	2677	Tocantins	*microcephala*
90	5435	101 24	Minas Gerais	n.a.
91	5436	10 126	Espírito Santo	n.a.
92	5439	10 792	Minas Gerais	*canescens*
93	5440	10 793	Minas Gerais	*canescens*
94	5441	10 794	Minas Gerais	*canescens*
95	5445	10 799	Minas Gerais	*canescens*
96	5447	10 802	Minas Gerais	*canescens*
97	5454	10 825	Minas Gerais	*canescens*
98	5456	10 849	Minas Gerais	n.a.
99	5458	10 852	Minas Gerais	*canescens*
100	5460	10 854	Minas Gerais	*canescens*
101	5462	10 876	Minas Gerais	*canescens*
102	5464	10 888	Minas Gerais	n.a.
103	5469	10 808	Minas Gerais	n.a.
104	5471	10 826	Minas Gerais	*microcephala*
105	5474	10 872	Minas Gerais	n.a.
106	5476	n.a.	Minas Gerais	*microcephala*
107	5477	10 855	n.a.	*microcephala*
108	5484	10 814	Minas Gerais	n.a.
109	5488	10 821	Minas Gerais	*pauciflora*
110	5489	10 824	Minas Gerais	*guianensis*
111	5493	10 830	Minas Gerais	*guianensis*
112	5500	10 838	Minas Gerais	*guianensis*
113	5505	10 843	Minas Gerais	*guianensis*
114	5506	10 820	Minas Gerais	*guianensis*
115	5509	10 847	Minas Gerais	n.a.

Continued

Table 1 ***Continued***

Code	Embrapa Cerrados	CIAT number	Geographic site[a]	Botanical variety[b]
116	5510	10 848	Minas Gerais	*guianensis*
117	5513	10 858	Minas Gerais	*guianensis*
118	5514	10 859	Minas Gerais	*guianensis*
119	5515	10 862	Minas Gerais	*guianensis*
120	5516	10 863	Minas Gerais	n.a.
121	5518	10 866	Minas Gerais	n.a.
122	5519	10 867	Minas Gerais	n.a.
123	5523	10 882	Minas Gerais	*guianensis*
124	5525	10 886	Minas Gerais	*guianensis*
125	5532	10 896	Minas Gerais	n.a.
126	5533	10 899	Minas Gerais	*guianensis*
127	5536	10 904	Minas Gerais	*guianensis*
128	5538	10 908	Minas Gerais	n.a.
129	EPAMIG-493[c]	n.a.	n.a.	n.a.
130	2745	n.a.	n.a.	n.a.
131	EPAMIG-1044[c]	n.a.	n.a.	n.a.
132	EPAMIG-1448[c]	n.a.	n.a.	n.a.
133	EPAMIG-443[c]	n.a.	n.a.	n.a.
134	NC 1099 A[c]	n.a.	Minas Gerais	n.a.
135	NC 2270[c]	n.a.	Pará	*guianensis*
136	n.a.	10 283	Minas Gerais	*guianensis*
137	n.a.	10 285	Minas Gerais	*pauciflora*
138	IPF 394/75[c]	n.a.	n.a.	n.a.
139	EPAMIG-1529[c]	n.a.	Minas Gerais	n.a.
140	EPAMIG-1557[c]	n.a.	Minas Gerais	*guianensis*
141	EPAMIG-1670[c]	n.a.	Minas Gerais	*guianensis*
142	EPAMIG-1787[c]	n.a.	Minas Gerais	*guianensis*
143	EPAMIG-1691[c]	n.a.	Minas Gerais	*guianensis*
144	EPAMIG-1994[c]	n.a.	Minas Gerais	*guianensis*
145	2600	n.a.	Tocantins	n.a.
146	2676	n.a.	Tocantins	*microcephala*
147	2689	n.a.	Tocantins	*microcephala*
148	2694	n.a.	Tocantins	*microcephala*
149	LC 4297[c]	n.a.	n.a.	n.a.
150	LC 4471[c]	n.a.	n.a.	n.a.

n.a., not available.
[a]Geographic sites indicate the Brazilian state or other country where the plant was collected.
[b]According to Ferreira and Costa (1979) and Brandão *et al.* (1985).
[c]For accessions without a number in the Embrapa Cerrados collection, the identification numbers in other germplasm collections or collector numbers are shown.

Table 2 The results of the analysis of 150 accessions of *S. guianensis* using 20 microsatellite loci (Santos et al. 2009a). Shown are the locus names, primer sequences, repeat motifs, number of alleles per locus (A), observed heterozygosity (H_O) and expected (H_E) heterozygosity.

Locus	Primer sequence (5′–3′)	Repeat motif	*A*	H_O	H_E
SG03A7	5′TACGGAAGTCCCATTAGTGAGG3′ 5′GGCTGCCGGAAGTTGACG3′	$(AG)_8$	6	0.16	0.66
SG03B5	5′AGGTGGATGCGAGTTCTT3′ 5′TCCCTTCTACCGAGTGTTC3′	$(TG)_7$	2	0.00	0.04
SG03D4	5′GATCGGTCGGGTTGGCTACTAT3′ 5′CCTATTATCCCCATTCCTCACA3′	$(GT)_8$	3	0.03	0.59
SG03E1	5′CCAAAGCGTAGAGAATGATGAG3′ 5′CAAATGGAGCGAAAGGACAA3′	$(GT)_8$	2	0.00	0.43
SG03D1	5′GTGGCGAAAAATCTAAAATGTC3′ 5′GGTGGAATCCCTAACTGAAGA3′	$(GT)_6$	4	0.11	0.59
SG03G2	5′GGTTGGAATATGGAGGAAGA3′ 5′GAGGAAAACTAAACAAAGCAGA3′	$(GT)_6$	7	0.11	0.78
SG03G4	5′TTGCCTTTATCCTTGTCACTCA3′ 5′ATCAAGAATCCAATACCAAATG3′	$(TG)_8$	5	0.06	0.68
SG01B9	5′TTACGCGAAAACCCGAACA3′ 5′GCACCTACAAAAGCTACACCAT3′	$(AC)_7$	5	0.54	0.58
SG03G8	5′AATTAAAGAGGAGGAGGAAAGT3′ 5′TGGAGAAGTAAAAGACAGTGAG3′	$(CT)_{11}\ldots(CA)_9$	11	0.10	0.78
SG01C2	5′TGAGAAGCACAAGGGATAAGGA3′ 5′CGAACCGGACCAAACCAT3′	$(GT)_7$	2	0.05	0.36
SG01B12	5′ATTGTTAGGGATAGGTGATTA3′ 5′TCGGAGTTGATTTGATTATG3′	$(AC)_7\ldots(CT)_4(TC)_4$	2	0.15	0.44
SG01D7	5′ACATAAACTGGACAGGGTGATT3′ 5′ATTTTTGCGAGGTGCTAAGTC3′	$(AC)_7$	4	0.58	0.51
SG01D3	5′AGATGGGCTAGATACGGAGATA3′ 5′TCGGAGTTGGTTTGATGGT3′	$(AC)_6\ldots(AC)_7$	2	0.18	0.18
SG03A9	5′AGTCCCAGTACCCAGAACA3′ 5′AACCCCTTTTTAACACAACA3′	$(CA)_7$	2	0.00	0.45
SG03E10	5′TCCCAGCTCGTATGAAGAAGTT3′ 5′GGACCCGGAGCACCTATC3′	$(GT)_7$	7	0.20	0.83
SG03B10	5′CTCTAAACGATGAAAATGAACG3′ 5′AAAGGAACAAAGGACGAGACAG3′	$(GT)_3G(GT)_4\ (TG)_3(GT)_6$	7	0.35	0.79
SG03E2	5′GTGCCCTTGAGCCCCCTTTAGT3′ 5′GAGCGGCGATCGGAGTTTGT3′	$(AC)_8$	10	0.35	0.68
SG03E7	5′AAGATGGGCCAAAAGGAACAAA3′ 5′TGGAGTGGCTTACCGTGATTAC3′	$(AC)_6\ldots(CT)_5$	4	0.03	0.73
SG01A7	5′TACCTTGAATCCGCACCTATGA3′ 5′CACCCGAACACCTAATCCTAAA3′	$(AG)_5\ldots(GT)_8$	5	0.49	0.56
SG03E9	5′GGTCAAATGGGGCAAAAGA3′ 5′ATCGAAGAGGAAAAGGCTAACT3′	$(AC)_5\ldots(CA)_5$	4	0.11	0.40

number of clusters was set to nine, following the statistics presented in Evanno *et al.* (2005). In the ancestry plot (Fig. 1B), each accession is represented by a horizontal bar, and the length of each segment of the bar is proportional to the accession's estimated ancestry fraction from each of the nine groups. The same ancestry plot is shown in more detail in Supplementary Figure S1 [see Additional Information], which includes the sample codes for the accessions. The majority of the model-based groups were in agreement with the currently proposed *S. guianensis* taxonomic classification (Ferreira and Costa 1979; Brandão *et al.* 1985). Some of the groups could also be correlated with the geographic origins of the accessions.

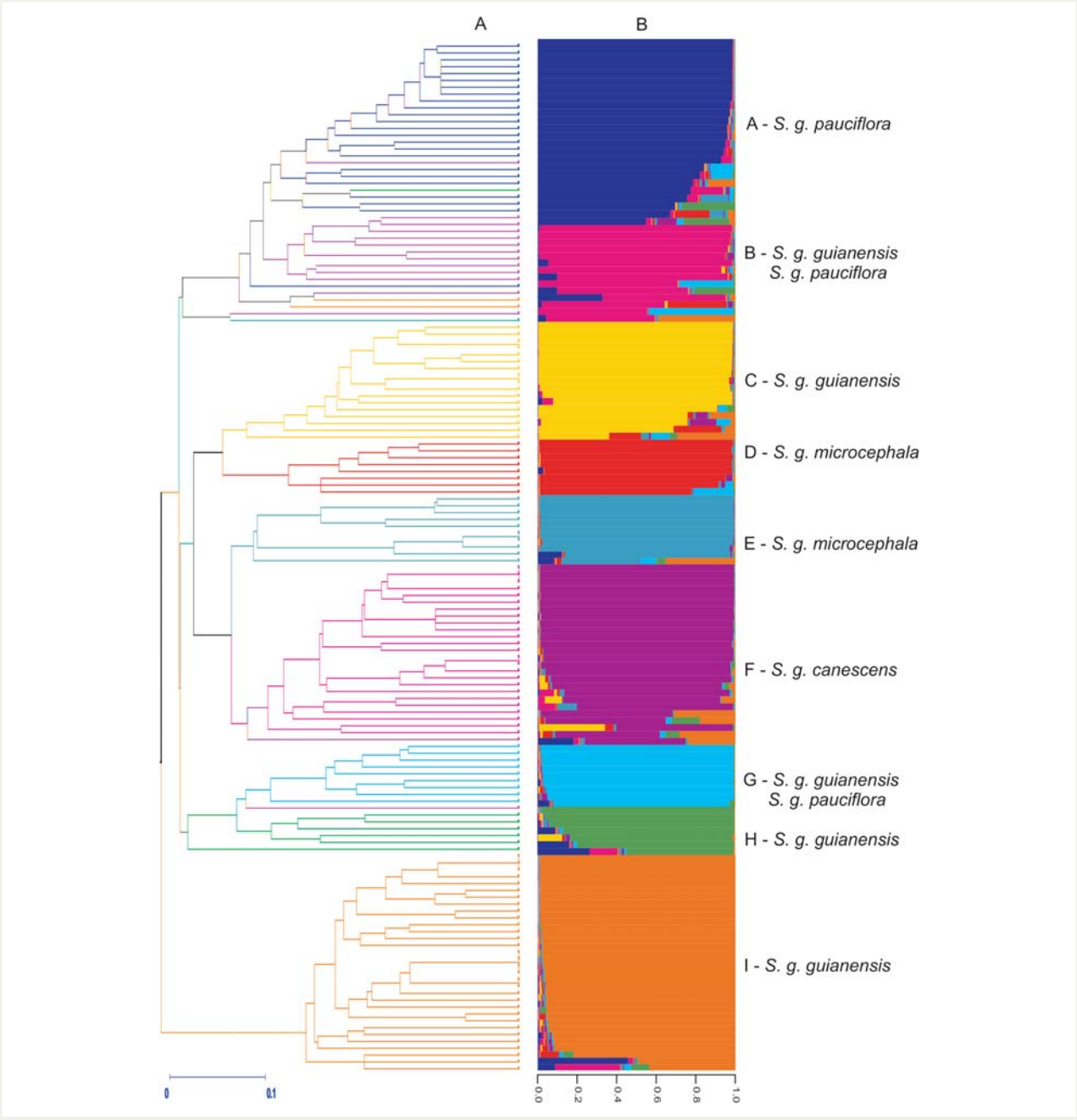

Fig. 1 (A) The Roger's genetic distance dendrogram of 150 *S. guianensis* accessions constructed using the UPGMA method implemented in DARwin. The colours in the dendrogram indicate the accession's group as assigned by STRUCTURE analysis. (B) The bar plot obtained from the model-based ancestry analysis of the same *S. guianensis* accessions implemented in the STRUCTURE software.

The number of accessions belonging to each variety that were assigned to one of the nine groups with >80 % probability is shown in Table 3. Of the 40 accessions belonging to *S. g. guianensis*, 11 were assigned to Group C, 5 to Group H and 14 to Group I. These groups are formed mostly by accessions belonging to this variety, which showed the greatest genetic diversity among all *Stylosanthes* varieties. The remaining 10 accessions from *S. g. guianensis* were distributed along with *S. g. pauciflora* accessions. Three of the accessions were assigned to Group B, two were assigned to Group G, and five were not assigned to any group with >80 % probability. Of the 28 *S. g. pauciflora* accessions, 13 were assigned to Group A (formed mostly by this variety), seven were assigned with *S. g. guianensis* in Groups B and G, two were in distinct Groups (E and I), and six were not assigned to any group with >80 % probability.

The 15 *S. g. microcephala* were mostly clustered in Groups D and E, both with six accessions. Group D contained six accessions belonging to *S. g. microcephala* and two non-classified accessions, and Group E contained six accessions belonging to *S. g. microcephala*, one accession from *S. g. pauciflora* and three non-classified accessions. Of the remaining three *S. g. microcephala* accessions, two were assigned to Group I and one to Group A.

The *S. g. canescens* accessions were clustered in a distinct Group (F) composed exclusively of this variety. Of the 20 *S. g. canescens* accessions studied, 14 were assigned to Group F.

In the dendrogram constructed using DARwin and the UPGMA method (Fig. 1A), the clusters, with few exceptions, were consistent with the groups generated using the Bayesian approach in STRUCTURE. The same dendrogram labelled with the sample code for the accessions is provided in Supplementary Figure S2 [see Additional Information].

The genetic differentiation among the *S. guianensis* groups, as clustered by the Bayesian approach, was estimated based on Nei's G_{ST} as 0.46, indicating that 46 % of differences resulted from the variation among groups. The remaining 54 % was a function of the genetic variation within groups.

Discussion

All 20 microsatellite markers from Santos *et al.* (2009*a*) were polymorphic among the 150 accessions analysed. The same set of microsatellite markers was tested previously (Santos-Garcia *et al.* 2011), but only three were found to be polymorphic in that study, probably because the previous study was based on samples from breeding material, and many of the genotypes were closely related. In contrast, we have studied a diverse germplasm collection of accessions obtained in collecting trips around Brazil and other countries in South America.

The mean number of alleles per locus observed in this study (4.7) was higher than that reported by Vander Stappen *et al.* (1999*a*) for 65 *S. guianensis* genotypes and some related species but lower than that observed by Karia (2008) among 437 *S. guianensis* accessions; those authors reported mean numbers of alleles per locus of 3.7 and 6.43, respectively. These differences can be attributed to the number of accessions studied by each of the authors.

Considering the observed and expected heterozygosities, our data revealed a deficit in heterozygosity that was consistent with the *S. guianensis* outcrossing rate (26 %) that was estimated based on microsatellite data (Santos-Garcia *et al.* 2011). The predominance of autogamy reduced the number of heterozygous samples, but some heterozygosity was maintained as a result of the 26 % outcrossing.

The genetic distances found among the studied accessions were higher than those previously reported for the species. The highest genetic distance (0.94) was observed between accessions 19 and 140. Accession 19 belongs to *S. g. pauciflora* and was collected in

Table 3 The inferred ancestry of the 150 *S. guianensis* accessions relative to the groups obtained using STRUCTURE (A-I). Shown are the percentage of individuals belonging to each botanical variety that are assigned to each of the nine groups and the number of individuals assigned to each group with >80 % membership probability (in bold).

Botanical variety	A	B	C	D	E	F	G	H	I	$P < 0.80$
S. guianensis var. *guianensis*	0.02	**3**/0.07	**11**/0.31	0.01	0.00	0.02	**2**/0.08	**5**/0.12	**14**/0.36	5
S. guianensis var. *microcephala*	**1**/0.07	0.00	0.00	**6**/0.40	**6**/0.40	0.00	0.00	0.00	**2**/0.13	0
S. guianensis var. *pauciflora*	**13**/0.59	**5**/0.21	0.00	0.01	**1**/0.05	0.00	**2**/0.07	0.01	**1**/0.06	6
S. guianensis var. *canescens*	0.01	0.01	0.02	0.00	0.01	**14**/0.79	0.00	0.00	**2**/0.13	4
Unidentified	**3**/0.11	**1**/0.09	**1**/0.07	**1**/0.05	**3**/0.09	**6**/0.14	**4**/0.11	**1**/0.04	**13**/0.30	14

Venezuela; accession 140 belongs to *S. g. guianensis* and was collected in the Brazilian state of Minas Gerais. Accession 140 was one of the most divergent, and its mean genetic distance (0.75) is greater than the overall mean genetic distance (0.66). Kazan *et al.* (1993) studied 31 *S. guianensis* accessions and observed a mean genetic distance of 0.26 using RAPD. Faleiro *et al.* (2003) also used RAPD to study 35 accessions and observed genetic distances varying from 0.04 to 0.54. Microsatellite markers are codominant and multiallelic with a high degree of polymorphism, making them more useful than dominant markers for revealing diversity (Laborda *et al.* 2005). Powell *et al.* (1996*b*) compared the expected heterozygosities and the estimated genetic similarities based on different molecular marker types for the evaluation of a set of soybean accessions. Their results show that microsatellites have higher expected heterozygosity (0.61) and lower estimated genetic similarities (0.45) among accessions relative to RAPD markers (0.31 and 0.72, respectively).

STRUCTURE analysis was performed to investigate the population structure in the germplasm collection to compare the genetic population structure with the described botanical varieties. The nine groups generated based on microsatellite analysis were mostly consistent with the botanical classification. The *S. g. microcephala* and *S. g. canescens* varieties were well differentiated and formed individual groups. Although most of the accessions belonging to *S. g. guianensis* and *S. g. pauciflora* formed distinct groups, some of them were mixed together in other groups. These mixed groups generally contained small numbers of individuals, many of which were not classified into varieties. *Stylosanthes g. pauciflora* was recognized as a new botanical variety in 1985 (Brandão *et al.* 1985); the three other varieties were recognized in 1979 (Ferreira and Costa 1979). This may have affected the classification process, and some of the accessions belonging to *S. g. pauciflora* may have been incorrectly classified as *S. g. guianensis*.

In previous studies, most *S. g. microcephala* accessions did not group together (Faleiro *et al.* 2003; Karia 2008). In the present work, these samples clustered into two diverse groups. Of the 15 *S. g. microcephala* accessions, five from the state of Minas Gerais were assigned to Group D, and six accessions (two from the state of Goiás and four from the state of Tocantins) were assigned to Group E. In both groups, the majority of the accessions were *S. g. microcephala*. The soil in Goiás and Tocantins is considered less fertile than the soil in Minas Gerais, and this difference in soil fertility may be the main driver of the observed genetic differentiation (Karia 2008). Our data reinforce the idea that the presence of *S. g. microcephala* in Minas Gerais is associated with more fertile soils, as proposed by Costa (2006). However, further studies are necessary to address this question and should include the collection and analysis of more plants from those regions. If genetic differentiation based on soil conditions is confirmed by further studies, this information could be useful for the development of new commercial varieties that are adapted to specific soil conditions.

The genetic data obtained in this study are also consistent with previously published karyological findings. All varieties have similar total chromosome lengths except for *S. g. microcephala*, which has visibly smaller chromosomes and the most asymmetrical karyotype (Fig. 2) (Vieira *et al.* 1993). This variety predominantly formed Groups D and E (Fig. 1B). *Stylosanthes g. canescens* and *S. g. microcephala* are quite close in the dendrogram (Fig. 1A); in those varieties, chromosome 10 is submetacentric (2.58 and 2.55 as arm ratios, respectively) (Table 4), which is distinct from chromosome 10 in the other varieties. However, *S. g. canescens* has a uniquely submetacentric chromosome 8 (1.76 arm ratio). As revealed from the UPGMA-based dendrogram and the model implemented in the STRUCTURE software, *S. g. guianensis* and *S. g. pauciflora* share alleles at many of the microsatellite loci under investigation. The karyotypes of these two varieties are also very similar, except for chromosome 10, which is significantly smaller in *S. g. guianensis* than in *S. g. pauciflora* (7.08 and 8.56 relative lengths, respectively). As described above, this taxon was separated from *S. g. guianensis*.

The G_{ST} observed in the present study was higher than that observed with allozymes in plants that exhibit a mixed mating system (Hamrick and Godt 1996), probably because autogamy is predominant in *S. guianensis* (26 % of outcrossing, as described above). In general, G_{ST} values in autogamous or predominantly autogamous

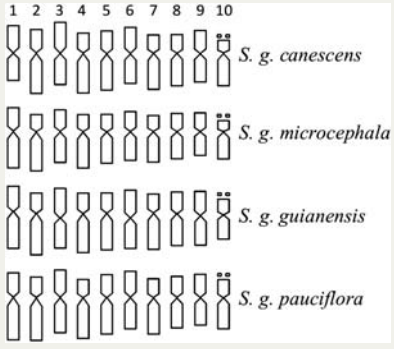

Fig. 2 Ideograms of *S. guianensis* varieties. The chromosome measurements were obtained from Vieira *et al.* (1993).

Table 4 The relative lengths and arm ratios of the chromosomes in *S. guianensis* varieties (modified from Vieira *et al.* 1993).

Variety	Chromosome pairs									
	1	2	3	4	5	6	7	8	9	10
Relative length										
S. g. canescens	11.94	11.17	10.87	10.43	10.35	9.70	9.58	9.11	9.00	7.83
S. g. guianensis	11.15	11.07	10.75	10.59	10.13	10.31	9.75	9.65	9.52	7.08
S. g. microcephala	12.16	11.36	10.66	10.80	10.09	9.94	9.40	9.19	8.72	7.70
S. g. pauciflora	11.64	10.97	10.80	10.31	10.39	9.96	9.59	8.91	8.86	8.56
Arm ratio										
S. g. canescens	1.49	1.75	1.00	1.99	1.62	1.13	2.01	1.76	1.22	2.58
S. g. guianensis	1.47	2.02	1.1	1.92	1.82	1.10	1.90	1.44	1.20	1.53
S. g. microcephala	1.55	2.30	1.48	2.14	1.79	1.47	1.96	1.48	1.28	2.55
S. g. pauciflora	1.74	2.07	1.29	2.27	1.59	1.19	1.92	1.42	1.18	1.74

species are higher than those in allogamous species (Hamrick and Godt 1996; Maki *et al.* 2003). High variation values (30 %) were observed between groups of Mexican and South American accessions of *Stylosanthes humilis* using amplified fragment length polymorphism (AFLP) (Vander Stappen *et al.* 2000). The variation was even higher in AFLP studies of *S. humilis* (59 %) and *S. viscosa* (66 %) (Sawkins *et al.* 2001).

Stylosanthes g. microcephala and *S. g. guianensis* were distributed in more than one group of the STRUCTURE analysis. The genetic differentiation (G_{ST}) between the groups formed by *S. g. microcephala* was low (18 %) and, as discussed above, may be related to the soil conditions. However, the genetic differentiation among the three clusters formed mostly by the *S. g. guianensis* variety was 49 %, which is similar to the overall G_{ST} (46 %). Because most of the accessions from this variety that were included in this study were collected in the same Brazilian state, no conclusion can be drawn about the correlation between these data and the geographic origin of the accessions. More botanical and genetic studies of this variety should be conducted to determine whether it should be subdivided into multiple varieties based on the existing variation.

The accessions that were not classified into botanical varieties were randomly distributed in the genetic groups. Although groups were generally dominated by one botanical variety under both clustering methods (STRUCTURE and UPGMA), some superposition was observed. Considering these facts, the classification of individuals in each botanical variety based exclusively on the genetic groups cannot be accomplished with confidence, showing that taxonomic information is fundamental for correct classification. The consistency between the molecular analysis and botanical classification could be increased by the analysis of a larger number of microsatellite markers.

Conclusions and forward look

This study has revealed valuable information about the relationships among a large number of *S. guianensis* accessions, showing a population structure that is generally consistent with the taxonomic classification proposed by Ferreira and Costa (1979), Brandão *et al.* (1985) and Costa (2006). These data show that molecular markers such as microsatellites can provide complementary information to address botanical questions at the infraspecific level. These data are important for existing germplasm conservation efforts and will help in planning new collecting trips and studies of natural populations of *S. guianensis* in its diversity centre. Moreover, information about the genetic diversity of germplasm collections is essential for their use in breeding programmes, mainly in guiding controlled crosses, as well as for the identification of natural and induced hybrids, and for monitoring the variability in subsequent generations.

Additional information

The following additional information is available in the online version of this article –

File 1. Fig. 1: The bar plot obtained from the model-based ancestry analysis of the *S. guianensis* accessions

implemented in the STRUCTURE software. The numbers indicate the sample code given in Table 1.

File 2. Fig. 2: The Roger's genetic distance dendrogram for 150 *S. guianensis* accessions constructed using the UPGMA method implemented in DARwin. The colours in the dendrogram indicate the accession's group as assigned by STRUCTURE analysis. The numbers indicate the sample code given in Table 1.

Sources of funding

The work was funded by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grant no. 2005/51010-0). M.O.S.-G. received a scholarship from FAPESP (2005/52211-9), and A.P.S. and M.L.C.V. received research fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Contributions by the authors

M.O.S.-G. performed experimental and statistical analyses and drafted the manuscript; M.I.Z. participated in statistical analysis; C.T.K. and L.C. participated in the germplasm selection and the design and implementation of the study; M.L.C.V. participated in the cytogenetic experiments and their interpretation; R.M.S.R. and A.P.S. conceived of the study and participated in its design and coordination. A.P.S. helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We thank the State of São Paulo Research Foundation (FAPESP) for financial support (proc 2005/51010-0) and scholarships, and the Brazilian National Council for Scientific and Technological Development (CNPq) for the research fellowships awarded to A.P.S. and M.L.C.V. We would also like to thank Dr Alexandre Franco Garcia for his help with the figures.

Conflict of interest statement

None declared.

References

Andrade RP, **Karia CT**. **2000**. O uso de Stylosanthes em pastagens no Brasil. In: *Anais do Simpósio de Forragicultura e Pastagem: Temas em Evidência, 1*. Lavras: , 273–309.

Bellon G, **Faleiro FG**, **Ferreira CF**, **Karia CT**, **Fonseca KG**, **Santos JR**, **Teixeira MA**, **Junqueira KP**. **2007**. Validação e otimização de protocolo simplificado de extração de DNA a partir de tecido foliar. In: *Anais do Congresso Brasileiro de Melhoramento de Plantas*. São Lourenço: Sociedade Brasileira de Melhoramento de Plantas, 1–5.

Brandão M, **Costa NMS**, **Schultze-Kraft R**. **1985**. Pauciflora: uma nova variedade de Stylosanthes guianensis (Aub.) Sw. Anais do 36 Congresso Nacional de Botanica, Curitiba: Sociedade Botanica do Brasil, 235–241.

Costa N. **2006**. *Revisão do género Stylosanthes*. PhD Thesis, Universidade Técnica de Lisboa, Portugal.

Costa NMS, **Ferreira MB**. **1984**. Some Brazilian species of *Stylosanthes*. In: Stace HM, Edye LA, eds. *The biology and agronomy of Stylosanthes*. Sydney: Academic Press, 53–101.

Creste S, **Tulmann Neto A**, **Figueira A**. **2001**. Detection of single sequence repeat polymorphisms in denaturing polyacrylamide sequencing gels by silver staining. *Plant Molecular Biology Reporter* **19**: 299–306.

Doyle J, **Doyle J**. **1990**. Isolation of plant DNA from fresh tissue. *Focus* **12**: 13–15.

Evanno G, **Regnaut S**, **Goudet J**. **2005**. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611–2620.

Faleiro FG, **Faleiro ASG**, **Cordeiro MCR**, **Karia CT**. **2003**. *Metodologia para operacionalizar a extração de DNA de espécies nativas do cerrado visando análises moleculares*. Planaltina: Embrapa Cerrados.

Ferreira MB, **Costa NMS**. **1979**. *O gênero Stylosanthes Sw. no Brasil*. Belo Horizonte: EPAMIG.

Goudet J. **2001**. FSTAT (Version 1.2): a computer program to calculate F-statistics. **http://www2.unil.ch/popgen/softwares/fstat.htm** (16 March 2011).

Hamrick J, **Godt M**. **1996**. Effects of life history traits on genetic diversity in plant species. *Philosophical Transactions of the Royal Society of London Series B* **351**: 1291–1298.

Karia CT. **2008**. *Caracterização genética e morfoagronômica de germoplasma de Stylosanthes guianensis (Aubl.)* Sw. PhD thesis, Universidade Federal de Goiás, Brazil.

Kazan K, **Manners JM**, **Cameron DF**. **1993**. Genetic relationships and variation in the *Stylosanthes guianensis* species complex assessed by random amplified polymorphic DNA. *Genome* **36**: 43–49.

Laborda PR, **Oliveira KM**, **Garcia AA**, **Paterniani ME**, **Souza AP**. **2005**. Tropical maize germplasm: what can we say about its genetic diversity in the light of molecular markers? *Theoretical and Applied Genetics* **111**: 1288–1299.

Lewis PO, **Zaykin D**. **2000**. Genetic Data Analysis: computer program for the analysis of allelic data. **http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php** (16 March 2011).

Maass BL, **Sawkins MC**. **2004**. History, relationships and diversity among *Stylosanthes* species of commercial significance. In: Chakraborty S, ed. *High-yielding anthracnose-resistant Stylosanthes for agricultural systems*. Sydney: CSIRO, 9–26.

Maki M, **Yamashiro T**, **Matsumura S**. **2003**. High levels of genetic diversity in island populations of the island endemic *Suzukia luchuensis* (Labiatae). *Heredity* **91**: 300–306.

Mannetje L. **1977**. A revision of varieties of *Stylosanthes guianensis* (Aubl.) Sw. *Australian Journal of Botany* **25**: 347–362.

Mannetje L. **1984**. Considerations on the taxonomy of the genus *Stylosanthes*. In: Stace H, Edye L, eds. *The biology and agronomy of Stylosanthes*. Sydney: Academic Press, 1–22.

Miller MP. **1997**. Tools for population genetic analysis (TFPGA): a Windows program for the analysis of allozyme and molecular population genetic data. **http://www.marksgeneticsoftware.net/tfpga.htm** (16 March 2011).

Nei M. **1973**. Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Sciences of the USA* **70**: 3321–3323.

Perrier X, **Jacquemoud-Collet J**. **2006**. DARwin software. **http://darwin.cirad.fr/darwin** (16 March 2011).

Powell W, **Machray GC**, **Provan J**. **1996*a***. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science* **1**: 215–222.

Powell W, **Morgante M**, **Andre C**, **Hanafey M**, **Vogel J**, **Tingey S**, **Rafalski A**. **1996*b***. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding* **2**: 225–238.

Pritchard JK, **Stephens M**, **Donnelly P**. **2000**. Inference of population structure using multilocus genotype data. *Genetics* **155**: 945–959.

Santos MO, **Karia CT**, **Resende RM**, **Chiari L**, **Jungmann L**, **Zucchi MI**, **Souza AP**. **2009*a***. Isolation and characterization of microsatellite loci in the tropical forage legume *Stylosanthes guianensis* (Aubl.) Sw. *Conservation Genetics Resources* **1**: 43–46.

Santos MO, **Sassaki RP**, **Chiari L**, **Resende RMS**, **Souza AP**. **2009*b***. Isolation and characterization of microsatellite loci in tropical forage *Stylosanthes capitata* Vogel. *Molecular Ecology Resources* **9**: 192–194.

Santos MO, **Sassaki RP**, **Ferreira THS**, **Resende RMS**, **Chiari L**, **Karia CT**, **Faleiro FG**, **Jungmann L**, **Zucchi MI**, **Souza AP**. **2009*c***. Polymorphic microsatellite loci for *Stylosanthes macrocephala* Ferr. et Costa, a tropical forage legume. *Conservation Genetics Resources* **1**: 481–485.

Santos-Garcia MO, **Resende RMS**, **Chiari L**, **Zucchi MI**, **Souza AP**. **2011**. Mating systems in tropical forages: *Stylosanthes capitata* Vog. and *Stylosanthes guianensis* (Aulbl.) Sw. *Euphytica* **178**: 185–193.

Sawkins MC, **Maass BL**, **Pengelly C**, **Newburry HJ**, **Ford-Lloyd BV**, **Maxted N**, **Smith R**. **2001**. Geographical patterns of genetic variation in two species of *Stylosanthes* Sw. using amplified fragment length polymorphism. *Molecular Ecology* **10**: 1947–1958.

Stace HM, **Cameron DF**. **1984**. Cytogenetics and the evolution of *Stylosanthes*. In: Stace H, Edye L, eds. *The biology and agronomy of Stylosanthes*. Sydney: Academic Press, 49–72.

Vander Stappen J, **Campenhout SV**, **Lopez SG**, **Volckaert G**. **1998**. Sequencing of the internal transcribed space region ITS1 as a molecular tool detecting variation in the *Stylosanthes guianensis* species complex. *Theoretical and Applied Genetics* **96**: 869–877.

Vander Stappen J, **Weltjens I**, **Volckaert G**. **1999*a***. Microsatellite markers in *Stylosanthes guianensis*. *Molecular Ecology* **8**: 514–517.

Vander Stappen J, **Weltjens I**, **Campenhout SV**, **Volckaert G**. **1999*b***. Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. *Theoretical and Applied Genetics* **98**: 1054–1062.

Vander Stappen J, **Weltjens I**, **Lopez S**, **Volckaert G**. **2000**. Genetic diversity in Mexican *Stylosanthes humilis* as revealed by AFLP, compared to the variabiliy of *S. humilis* accessions of South American origin. *Euphytica* **113**: 145–154.

Vieira MLC, **Aguiar-Perecin MLR**, **Martins PS**. **1993**. A cytotaxonomic study in twelve Brazilian taxa of *Stylosanthes* Sw., Leguminosae. *Cytologia* **58**: 305–311.

Vieira MLC, **Fungaro MH**, **Jubier MF**, **Lejeune B**. **1997**. Determination of taxonomic relationships among Brazilian taxa of *Stylosanthes* Sw., Leguminosae, using RAPD markers. *Pesquisa Agropecuária Brasileira* **32**: 305–310.

Williams RJ, **Reid R**, **Schultze-Kraft R**, **Costa NM**, **Thomas BD**. **1984**. Natural distribution of *Stylosanthes*. In: Stace H, Edye L, eds. *The biology and agronomy of Stylosanthes*. Sydney: Academic Press, 73–101.

Wright S. **1978**. *Evolution and the genetics of populations*, Vol. 4. *Variability within and among natural populations*. Chicago: University of Chicago Press.

Zhang X, **Blair MW**, **Wang S**. **2008**. Genetic diversity of Chinese common bean (*Phaseolus vulgaris* L.) landraces assessed with simple sequence repeat markers. *Theoretical and Applied Genetics* **117**: 629–640.