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Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers

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Abstract Nineteen sequence-tagged site (STS) primer pairs were designed on coding and non-coding regions in nine published *Stylosanthes* genes, which were mostly derived from cDNA. Direct sequencing of PCR products derived from genomic DNA allowed us to identify introns and to design specific primers flanking these introns. The use of 24 STS primer pairs for the detection of intra- and inter-specific variation in *Stylosanthes* based on size differences was tested on a core set of *Stylosanthes* species. Based on these results, 20 STS markers were selected to determine genetic relationships among 63 genotypes representing 24 *Stylosanthes* species. A total of 148 alleles were amplified and analyzed, resulting in a genetic similarity value ranging from 0.62 to 0.98 among the species. Based on cluster analysis, three main groups and three subgroups were determined, and most of the species were classified unambiguously. Alloploid species were recognized by the occurrence of more than one allele per STS marker, indicating fixed heterozygosity. Sixteen STS markers were useful for the identification of genotypes within a species. Inter-species relationships, as revealed by STS, were in general agreement with previous morphological and molecular relationship studies. These STS markers are useful as an additional tool for the identification of species, subspecies and genotypes in *Stylosanthes*, with a view to plant conservation and breeding.

Key words *Stylosanthes* sp. · Sequence-tagged site (STS) marker · Genetic variation · Polyploidy

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Introduction

The genus *Stylosanthes* (Leguminosae) includes some 40 species which have their natural distribution in the tropical, subtropical and temperate regions of the American continent, in tropical Africa and in South-east Asia. The major centre of diversity is suspected to lie in the southern neotropics, with a secondary centre in the Mexico-Caribbean Basin (Stace and Cameron 1984). Some of the species have been widely cultivated as a pasture legume. Due to the absence of stable morphological characters, the genus is notoriously difficult in its taxonomy. Differing views on just which morphological characters are useful for the classification of the genus exist, resulting in a lot of synonyms (Williams et al. 1984). Nevertheless, two sections (sect. *Stylosanthes* and sect. *Styposanthes*) have been recognized based on morphological characteristics (Kirkbride and Kirkbride 1987). The section *Stylosanthes* comprises only diploid species ($2n = 20$), whereas the section *Styposanthes* includes diploid and polyploid species ($2n = 40$ and 60) ('t Mannetje 1984). Polyploid species are thought to have been derived via hybridization and polyploidization between diploid taxa from both sections. Due to the current taxonomic confusion that has arisen from morphological analysis, other techniques have been used in an attempt to elucidate relationships in *Stylosanthes*. These include numerical pattern analysis (Burt et al. 1971; Edye et al. 1974), isozyme analysis (Stace, 1982), seed protein analysis (Robinson and Megarrity 1975), random amplified polymorphic DNA (RAPD) (Kazan et al. 1993a,b) and chloroplast (cp) DNA restriction fragment analysis (Gillies and Abbott 1996). However, most of these studies were restricted to a limited number of species.

The development of molecular markers in plant genetic diversity studies is evolving quite quickly because of the broader accessibility of techniques such as

the polymerase chain reaction (PCR) and DNA sequencing. This allows variation to be studied at the DNA level, which provides a wealth of information. Molecular studies are considered to be complementary to morphological characterization and provide genetic information of direct value in different areas of plant studies, including conservation genetics (Karp et al. 1997). The sequence-tagged site (STS) approach, whereby PCR primers are designed from low copy-number sequences, is one of the possible marker systems that can be used for molecular characterization of species. It has been applied to wheat (Chen et al. 1994; Talbert et al. 1994) and conifers (Tsumura et al. 1997) for genetic diversity studies. This approach has the disadvantage of being time-consuming and costly in development, but once primers are developed and tested, published sequences can easily be shared with other researchers and results can be reproduced. STS markers have been previously used in *Stylosanthes* to assess their genome specificity (Liu et al. 1996). However, they have not yet been used for the molecular characterization of a larger number of *Stylosanthes* species.

In the study reported here, we tested and used 20 STS markers to classify 63 genotypes representing 24 *Stylosanthes* species. In addition, their potential to identify and determine the origin of polyploid species was assessed. We were able to show that sequence-tagged site markers provide an additional tool for the assessment of relationships in the genus *Stylosanthes* and hence may improve our understanding of speciation in *Stylosanthes*.

Materials and methods

Plant material

Plant material of *Stylosanthes* was obtained from existing germplasm accessions of CIAT (Centro Internacional de Agricultura Tropical, Colombia, Cali, CIAT accessions) and CSIRO (Commonwealth Scientific and Industrial Research Organization, Australia, CPI accessions) (Table 1). Plant material of *Zornia* was obtained from S. Gama (Instituto de Biologia, UNAM, Mexico).

DNA isolation and polymerase chain reaction

Seeds were germinated in a petri dish on a water-soaked filter paper under dark conditions and grown until the seedling stage. Genomic DNA was isolated from whole seedlings on a small scale as described by Van Campenhout et al. (1995).

Primers were purchased from Eurogentec (Seraing, Belgium), Genset (Paris, France) and Pharmacia Biotech (Roosendaal, the Netherlands). PCR reactions contained 10 m*M* TRIS-HCl pH 8.3, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 µ*M* of each dNTP, 1 µ*M* of each primer, 0.2 U Super*Taq* DNA polymerase (HT Biotechnology, Cambridge, UK) and approximately 20 ng genomic DNA in a total volume of 25 µl. The reaction was overlaid with mineral oil and subjected to incubation for 4 min at 94°C, followed by 30 cycles of 30 s at 94°C, 1 min at 50°C, 1 min at 72°C and a final extension step of 5 min at 72°C. A BioOven III (BioTherm Corporation, USA) thermocycler was used to handle microtitre trays for large-scale screening. PCR products were electrophoretically fractionated on a 5% Metaphor TAE agarose gel (FMC Bio Products, Rockland, Me., USA) containing ethidium bromide and visualized under UV light.

DNA sequence analysis

PCR products were purified for DNA sequence analysis with the Qiaquick PCR purification kit (Qiagen, Germany). Purified PCR products were sequenced directly by the ABI PRISM DyeDeoxy terminator sequencing protocol (Applied Biosystems, Foster City, USA) by using either primer of the STS pairs. Sequencing gels were run on a 373A DNA sequencer (Applied Biosystems).

Data analysis

All data analysis was performed with NTSYS-PC (Rohlf 1992). Each band was scored as absent (0) or present (1). A similarity matrix based on simple matching coefficients, was produced with SIMQUAL. The similarity data was clustered by the UPGMA method using the SAHN clustering program. Additionally, principal coordinates analysis (Gower 1972) was performed with the similarity matrix by using appropriate procedures of the program NTSYS-PC. The data matrix is available upon request (a web site is in preparation).

Results

Design and characterization of STS markers in *Stylosanthes* sp.

A search in the EMBL DNA Data Library revealed the presence of 13 DNA sequences representing *Stylosanthes* genes, 11 of which were derived from cDNA. Based on 9 of these sequences, primer pairs were designed to amplify 19 regions from genomic DNA (Table 2). Six of these, which are lying in non-coding regions of cDNA, were selected by their DNA characteristics, i.e. the presence of a microsatellite motif; the other 13 were randomly selected from coding regions. In addition to exons, they may amplify introns, which are known to be more variable than exons. The primer pairs were tested first on genomic DNA of the species from which the DNA sequence was derived. Three primer pairs failed to yield amplification products. From the 13 primer pairs derived from coding regions, 7 amplified a fragment bearing at least one intron because the size of the PCR product was larger than expected. These fragments were sequenced to identify the introns and possible microsatellite motifs (manuscript in preparation). Based on these sequences, additional primer pairs (Table 3) were designed to amplify the introns specifically, thereby increasing the number of useful STS markers. The primer pairs were further tested for their ability to detect inter- and intra-species variation in a representative number of

Table 1 Characteristics of *Stylosanthes* species and genotypes used in the present study

Section[a]	Species	Genotypes used in the present study	Ploidy level according to Stace and Cameron (1984)	Ploidy level according to the present study
Stylosanthes	*S. angustifolia*	CIAT 1292	Diploid	Diploid
	S. debilis	CPI 92483	Diploid	Diploid
		CPI 93046		Diploid
	S. guianensis complex:		Diploid	Diploid
	– *S. acuminata*	CPI 34912		Diploid
	– *S. guianensis guianensis*	CPI 34906		Diploid
		CPI 46589		Diploid
		cv Schofield		Diploid
		CIAT 10136		Diploid
	– *S. gracilis*	CPI 39112		Diploid
		CPI 33501		Diploid
	– *S. grandiflora*	CPI 92975		Diploid
		CPI 40257		Diploid
	– *S. hippocampoides*	cv Oxley		Diploid
	S. humilis	CIAT 1304	Diploid	Diploid
		CPI 40266		Diploid
		CPI 33829		Diploid
		cv Paterson		Diploid
	S. ingrata	CIAT 1608	Unknown	Allotetraploid
		CIAT 76256		Allotetraploid
	S. leiocarpa	CIAT 2167	Diploid	Diploid
		CPI 78192		Diploid
	S. montevidensis	CPI 11494	Diploid	Diploid
		CPI 53962		Diploid
	S. tomentosa	CPI 92843	Diploid	Diploid
	S. viscosa	CIAT 1703	Diploid	Diploid
		CIAT 11268		Diploid
		CIAT 1817		Diploid
		CPI 84922		Diploid
		CPI 95603		Diploid
Styposanthes	*S. bracteata*	CPI 78478	Diploid	Diploid
	S. calcicola	CPI 76280	Diploid	Diploid
		CPI 91492		Diploid
	S. hamata	CPI 73523	Diploid	Diploid
		CPI 70361		Diploid
		CPI 73507		Diploid
		CIAT 1475		Diploid
	S. macrocarpa	CPI 33832	Diploid	Diploid
	S. macrocephala	CIAT 1643	Diploid	Diploid
		CPI 81086		Diploid
		CPI 81090		Diploid
	S. mexicana	CPI 67665	Diploid	Allotetraploid
		CPI 86137		Diploid
	S. pilosa	CIAT 2068	Unknown	Diploid
	S. capitata	CPI 82122	(?) Autotetraploid	Allotetraploid
		CIAT 1019		Allotetraploid
		CIAT 1078		Allotetraploid
		CIAT 1405		Allotetraploid
	S. fruticosa	CIAT 11756	Allotetraploid	Allotetraploid
		CPI 79070		Allotetraploid
	S. hamata	CPI 55822	Allotetraploid	Allotetraploid
		CPI 38842		Allotetraploid
		CIAT 177		Allotetraploid
		CPI 75171		Allotetraploid
	S. scabra	CIAT 1682	Allotetraploid	Allotetraploid
		cv Fitzroy		Allotetraploid
		cv Seca		Allotetraploid
	S. subsericea	CPI 33943	Allotetraploid	Allotetraploid
		CPI 85899		Allotetraploid
	S. sundaica	CPI 47477	Allotetraploid	Allotetraploid
	S. sympodialis	CPI 65960	Allotetraploid	Allotetraploid
		CPI 67704		Allotetraploid
	S. erecta	CPI 50979	Allohexaploid	Allotetraploid
		CPI 34118		Diploid

[a] Classification of the *Stylosanthes* species into sections according to 't Mannetje (1984)

Table 2 List of published *Stylosanthes* DNA sequences which were assessed as STS markers in this study

ID/AC[a]	Function	Reference	STS primer pair	Primer sequence 5′ → 3′	Target region[b]	PCR product	Intron[c]	Microsatellite motif
SHSHST3 X82454	Sulfate transporter cDNA	Smith et al. (1995)	SHST3F3[d]	GGTTAACATAATAAAGCATG	C	Yes	Yes (2)	(TAT), (T) (GATTTC), (A)
			SHST3R3[d]	GTCTTGTAAACAATTCCAAGC				
			SHST3F1[d]	TAACTCTTGCCAGCCTCA	C	Yes	Yes (3)	(T), (A) (TAT)
			SHST3R18[d]	CTGCACCTGCCATGAATCCCAC				
SHSHST2 X82256	Sulfate transporter cDNA	Smith et al. (1995)	SHST2F3[d]	AAGAACAAGAAACTCTTCTGG	C	Yes	Yes (2)	(ATAA) (TAT)
			SHST2R16[d]	CCATGTAGTTCACTGCTGACCGAG				
			SHST2F1	GAACCAATTCCTCTTCACGA	C	Yes	No	
			SHST2R1	GGCTTGTCAGGGAAGAAAGT				
			SHST2F4	CATATGGTCCCAATTGTATG	NC	Yes	–	(A)
			SHST2R4	TAGCATCCATTATTGGTGAG				
SHSHST1 X82255	Sulfate transporter cDNA	Smith et al. (1995)	SHST1F1	GAAGCAACTCTTCTTCACAT	C	Yes	No	(GAG)
			SHST1R1	GGCTTGTCAGGGAAGAAAGT				
			SHST1F2	CATAGCTGCTGTTGTGAACC	C	Yes	Yes (1)	
			SHST1R2	CCTATAAACACTAGTCCCAG				
			SHST1F3	GCAACTTATGGTCCCAAGAC	NC	Yes	–	(T)
			SHST1R3	TCCACCAAATCATGAAGCTG				
SHCAD1A L36823	Cinnamyl-alcohol dehydrogenase cDNA	Nourse et al. unpublished, cited in [a]	SHCAD1AF1	CCGATCTCCACACAGTCAAG	C	No	–	
			SHCAD1AR1	TGCCCGGCTCTGTCATGCCA				
			SHCAD1AF2	GCTTAGGCCATGTTGCCATC	C	Yes	Yes (2)	(TTA)
			SHCAD1AR2	ATCTCCTGAGTCTCCTTCAG				
			SHCAD1AF3	GATCCTCCTCCCATCGTCTG	NC	Yes	–	(C(T))
			SHCAD1AR3	CAAAGCTCTCAGATCAGGAG				
SHCAD3 L36456	Cinnamyl-alcohol dehydrogenase cDNA	Nourse et al. unpublished, cited in [a]	SHCAD3F1	TGTAGAATCTTGTCGCTCCT	C	Yes	No	
			SHCAD3R1	GTGAAGTACTAATGACAGTG				
SHPAL L36822	Phenylalanine ammonia-lyase cDNA	Manners et al. (1995)	SHPALF1	CATTAGGTTCTTGAATGCTG	C	Yes	No	
			SHPALR1	AGGAGTGCCATTCACCAGTG				
			SHPALF2	TTCACGGCAATGTGCCAAGG	NC	Yes	–	(TTA), (A)
			SHPALR2	AGGTATTGTATCTGTGCCCA				
SHCAPEA L77080	Cationic peroxidase gDNA	Reddy et al. (1996)	SHCAPEAF1	TAATGTTGTGTCTTGTGCTG	C	Yes	Yes (**2**)	(CTAA)
			SHCAPEAR1	GCTGCTCAAAAGCTGACAAC				
SHCAFMETH L36109	Caffeic acid O-methyltrans-ferase homo-logue cDNA	McIntyre et al. (1995)	SHCAFMSF1	CAAGGCTTATGGAATGACAG	C	Yes	Yes (2)	
			SHCAFMSR1	AGTGCTCATCACTCCAGTCA				
SHCAPE L37790	Cationic peroxidase gDNA	Curtis et al. (1995)	SHCAPEF1	CCATGATTGCTTTGTTCAAG				
			SHCAPER1	TATCAGCACAAGAAACAACA	C	No	Yes (**1**)	
			SHCAPEF2	TTATTGGTGGCTTATCGCTG	NC	No	–	
			SHCAPER2	CACAGCAGAGTTCACTGCTG				
			SHCAPEF3	TCCAGTGGCCAGATTAGGAC	NC	Yes	–	(TA), (TAA), (T)
			SHCAPER3	CCACCACAGTTTATGAGAGG				

[a] Identification and accession number of the DNA sequence, as published in the EMBL DNA Data Library
[b] C, Coding; NC, non-coding
[c] The number of introns which were detected by DNA sequencing are indicated in brackets. Bold indicates the introns which were determined in previous studies (Curtis et al. 1995; Reddy et al. 1996); the others were determined in this study
[d] Primer pairs which were previously designed by Liu et al. (1996)

Table 3 List of additional primer pairs directed to intron regions

Primer pair	Primer sequence 5′ → 3′	Target region
SHST3F32 SHST3R3	ACTTCTTGCTACGTGTCAAC cf. Table 1	SHST3F3R3 – second intron
SHST3F33 SHST3R32	CTGCCATTATCGCTCTTACC TACCAACAGCTATTGCTTCC	SHST3F3R3 – first intron
SHST3F3 SHST3R32	cf. Table 1 TACCAACAGCTATTGCTTCC	SHST3F3R3 – first intron
SHST3F1 SHST3R1	cf. Table 1 TTTGCATATCCTATGCTCTG	SHST3F1R18 – first intron
SHST3F18 SHST3R18R	TGATCCTCAATATGGCTTGT TCACTTTGTTTGCCGGAATC	SHST3F1R18 – second intron
SHST3R18F SHST3R18	GATAACAGGAGGAATCACAC CTGCACCTGCCATGAATCCCAC	SHST3F1R18 – third intron
SHST2F31 SHST2R31	TACCAGAGCAGACAAGAGAG TTAGCAGATGATGGGTTCAC	SHST2F3R16 – first intron
SHST2F16 SHST2R16S	GGTGTTGTAGCTGGCTTGGT TCCCAATTGCTATAGCTTCC	SHST2F3R16 – second intron
SHCAD1AF22 SHCAD1AR2	GCCGCTTCAGCTGCCTATCT cf. Table 1	SHCAD1AF2R2 – second intron
SHCAD1AF2 SHCAD1AR22	cf. Table 1 AGCTTCAGCAGACCAAGCAG	SHCAD1AF2R2 – first intron
SHCAPEAF1 SHCAPEAR12	cf. Table 1 TAGCCCATCTCTGCGTCC	SHCAPEAF1R1 – first intron
SHCAPEAF11 SHCAPEAR1	GCCTCAACACAAAAGACCTTG cf. Table 1	SHCAPEAF1R1 – second intron

Stylosanthes species containing two genotypes of the species *S. guianensis*, *S. humilis*, *S. hamata* (diploid and tetraploid), *S. viscosa* and the related genus *Zornia* (Table 4). All the primer pairs produced an amplification product in at least 1 other *Stylosanthes* species; 16 gave a product in all samples. Five primer pairs amplified more than one locus. Six fragments were also amplified in the related genus *Zornia*. Only 2 primer pairs failed to show any length variation. Seventeen primer pairs were useful for the detection of intra-specific variation. Primer pair SHST2F4/R4 was found not to be suitable for inter-species studies because it revealed too high a level of intra-specific variation. In total, from all the designed STS primer pairs, 64% and 51% were determined to be useful for inter- and intra-species identifications, respectively. Based on their potential to reveal polymorphism, 20 primer pairs were selected and used as STS markers in the determination of genetic relationships in *Stylosanthes* sp.

Determination of genetic relationships in *Stylosanthes* sp. by means of sequence-tagged site markers

A total of 20 primer pairs were used as STS markers in the determination of genetic relationships among 63 accessions representing 24 *Stylosanthes* species (Table 4). Fourteen primer pairs produced a PCR product in all species. Three primer pairs produced a product in all species, except in some *S. guianensis* accessions, and 3 primer pairs did not amplify in several species. Polyploid species were identified by the additive nature of the STS markers. Similarity values between the 63 accessions were calculated based on 148 fragments and are presented as a phenogram in Fig. 1. All species were unambiguously classified, except for *S. debilis*, *S. erecta*, *S. fruticosa*, *S. humilis*, *S. leiocarpa*, and *S. mexicana*. Since the genotypes within *S. debilis*, *S. erecta*, *S. fruticosa* and *S. mexicana* each belong to a different group, some of the genotypes must be considered as likely being incorrectly named and hence belonging to another species. *S. mexicana* 86137 resembles *S. humilis* 33829. Small intra-species variation was found in the species *S. guianensis*, *S. humilis*, *S. leiocarpa*, *S. calcicola*, *S. hamata*, *S. ingrata*, *S. scabra*, *S. viscosa*, and *S. capitata*. Three main groups can be distinguished. Group 1 can be subdivided into the species *S. guianensis* (1.1) and *S. montevidensis* (1.2). Group 2 contains most of the *Stylosanthes* species and can be subdivided into three groups. Group 3 contains a major group (3.1) and two minor groups (3.2, 3.3). *S. pilosa* (3.2) could not be well defined due to missing data, while we were unable to identify *S. debilis* CPI 92483 (3.3) due to significant differences with the other

Table 4 List of primer pairs which were tested on a representative number of *Stylosanthes* species

Primer pair[a]	PCR product[b]	Size range (bp)	Length variation	
			Inter-species	Intra-species[c]
SHST3F3/R3	A/1	172–550	Yes	*S. leiocarpa*, *S. guianensis*
SHST3F32/R3	NA(Sg)/1	210–430	Yes	*S. guianensis*, *S. humilis*, *S. leiocarpa*
SHST3F33/R32	NA/1	140–490	Yes	No
SHST3F3/R32	A/1/Z	240–325	Yes	*S. guianensis*, *S. leiocarpa*, *S. humilis*
SHST3F1/R1	A/1/Z	115–170	Yes	*S. guianensis*
SHST3F18/R18R	A/1	125–160	Yes	*S. guianensis*, *S. hamata*
SHST3R18F/R18	A/1	185–480	Yes	No
SHST2F31/R31	A/M/Z	165–210	Yes	*S. guianensis*
SHST2F16/R16S	A/M/Z	130–147	Yes	*S. guianensis*
SHST2F1/R1	NA/1	145	No	No
SHST2F4/R4	NA/1	200–250	Yes	*S. hamata*, *S. ingrata*
SHST1F1/R1	NA/1	130–154	Yes	*S. guianensis*, *S. capitata*
SHST1F2/R2	A/M	310–340	Yes	*S. guianensis*, *S. viscosa*
SHST1F3/R3	NA(Sg)/1	245–350	Yes	*S. calcicola*, *S. hamata* *S. ingrata*, *S. viscosa*
SHCAD1AF22/R2	A/M	80–425	Yes	*S. humilis*
SHCAD1AF2/R22	A/M	335–550	Yes	*S. guianensis*, *S. leiocarpa*
SHCAD1AF3/R3	A/1	280–340	Yes	No
SHCAD3F1/R1	A/1	350	No	No
SHPALF1/R1	A/1	400	No	No
SHPALF2/R2	NA(Sg)/1	260–350	Yes	*S. guianensis*, *S. leiocarpa* *S. hamata*, *S. humilis*
SHCAPEAF1/R12	A/1/Z	180–220	Yes	*S. guianensis*, *S. scabra*
SHCAPEAF11/R1	A/1	165–570	Yes	*S. guianensis*, *S. scabra* *S. viscosa*
SHCAFMSF1/R1	NA/1/Z	1000–1500	Yes	No
SHCAPEF3/R3	A/1	250–340	Yes	*S. guianensis*, *S. leiocarpa* *S. humilis*, *S. viscosa*

[a] Bold indicates the primer pairs which were subsequently used in the STS analysis for the determination of genetic relationships among *Stylosanthes* species
[b] Amplification in: A, all species; NA, not in all species; NA(Sg), all species, except in some *S. guianensis* subspecies; 1, one locus; M, more than one locus; Z, *Zornia*
[c] Intra-species variation detected in the core set and in all other examined *Stylosanthes* species

species. Therefore, these minor groups were not taken into consideration in subsequent analyses. Within the subgroups 2.2, 2.3 and 3.1, the species were separated into groups A and B or C, representing the diploid and polyploid species, respectively. The species in groups 3, 2.2, 2.3B belong to the section *Styposanthes*, while those in groups 1, 2.1 and 2.3A belong to the section *Stylosanthes*, except for *S. debilis* CPI 92483, *S. ingrata*, *S. mexicana* CPI 86137 and *S. macrocarpa* (Table 1). The data were also analyzed by principal coordinates analysis and visualized on a three-dimensional plot (Fig. 2). This plot gives another view of the inter-species relationships, in the sense that it clearly shows the separation of the polyploid from the diploid species. The diploid species can be separated into five groups, while the polyploid species can be separated into four groups. The additive nature of STS patterns allowed us to identify the polyploid species and to speculate about their origin. It strongly suggests that a diploid species of group 2.1 (more specifically of the group of *S. humilis*) and 2.2A are the likely diploid progenitors of a polyploid species of group 2.2B and that a diploid species of group 2.3A and one of group 2.2A are the possible progenitors of a polyploid species of group 2.3B. In group 3.1, there is also relationship between the diploid and polyploid species. One of the putative progenitors of polyploid species belonging to 3.1B is a diploid species belonging to 3.1A. However, there is no indication of another group to which the second progenitor belongs. This is also the case for *S. sympodialis*, which has a likely progenitor in group 2.2A and one unknown progenitor. Primer pair SHST3F33R32 and SHST2F1R1 amplified selectively the species of group 2.2 and 2.3B with a difference in intensity between the polyploids and the diploids. Again this strongly suggests that these tetraploid species have one of those diploid species as progenitor.

Discussion

The classification based on STS analysis of the *Stylosanthes* species into three distinct groups and three

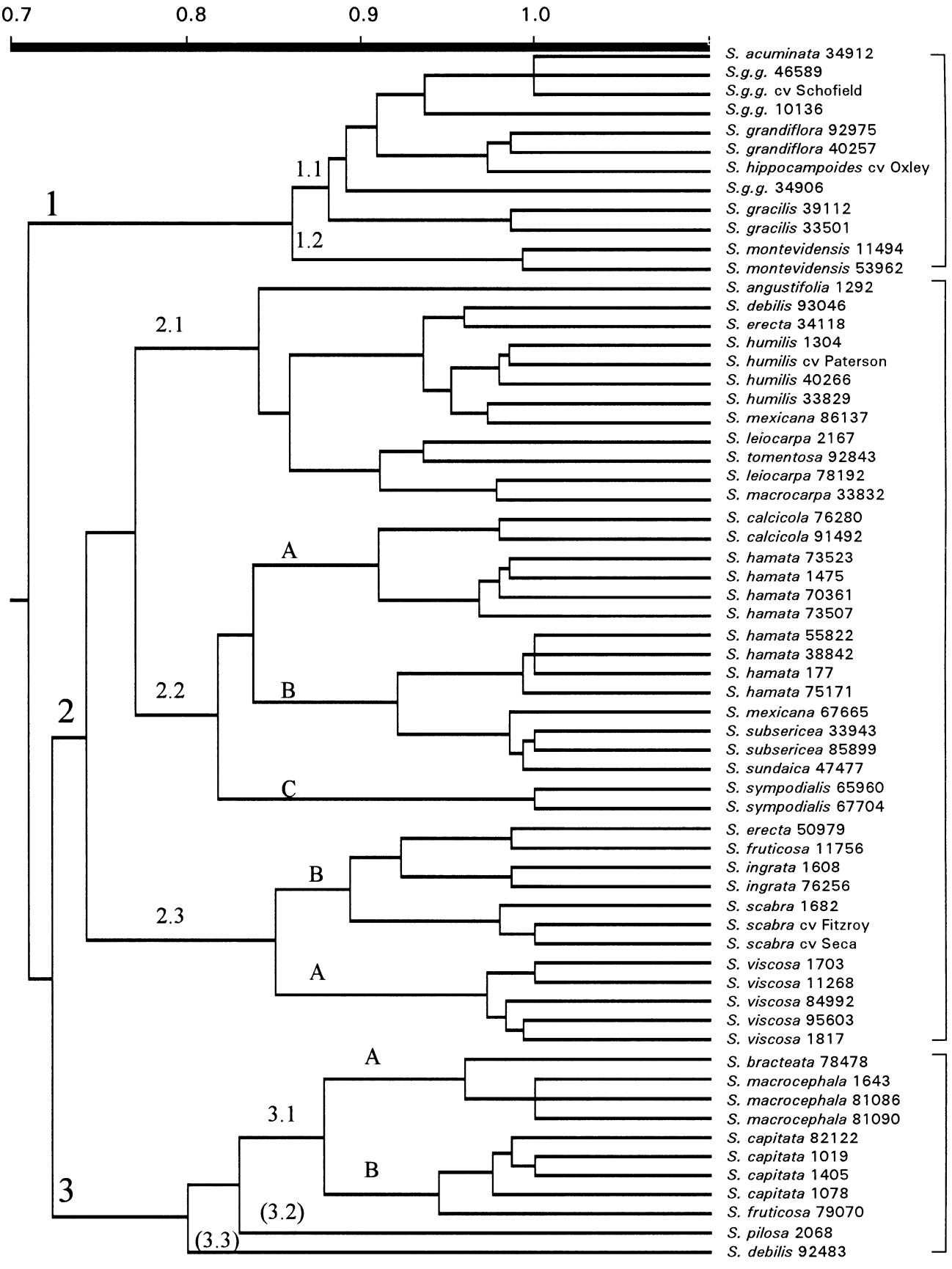

Fig. 1 Phenetic tree of 63 *Stylosanthes* genotypes, based on similarity data from 20 STSs, indicating relationships among and within the species. The *scale bar* indicates the degree of relatedness, ranging from 0.7 (70% similar) to 1 (100% similar)

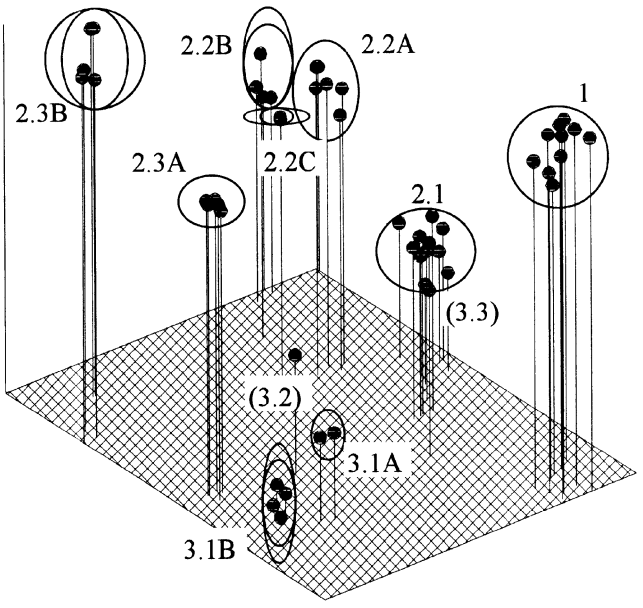

Fig. 2 Principal coordinates analysis of similarity data from STS analysis on 63 *Stylosanthes* genotypes illustrated by a three-dimensional plot. The groups as determined by the phenetic tree are *circled*. The tetraploid species are identified by *ovals*

subgroups corresponds to the classification based on ITS1 DNA sequence analysis (manuscript in preparation). The *S. guianensis* complex and *S. montevidensis* are clearly separated from the rest of the genus. This is in agreement with the morphological study ('t Mannetje 1977) and the phylogenetic study based on chloroplast DNA (Gillies and Abbott 1996). The classification based on STS markers of the subspecies and genotypes in the *S. guianensis* complex are in accordance with their classification based on ITS1 DNA sequence analysis (Vander Stappen et al. 1998) and RAPD (Kazan et al. 1993a; Vieira et al. 1997). However, *S. guianensis* CIAT 10136 is more distantly related to the other *S. guianensis guianensis* by ITS1 analysis than by STS analysis. All subspecies (*S. grandiflora*, *S. hippocampoides*, *S. gracilis*, *S. acuminata*) were unambiguously classified, except for *S. guianensis guianensis*. The genotype *S.g.g.* CPI 34906 is very different from the other *S. guianensis guianensis* accessions, which is in agreement with previous studies. Except for this genotype, three distinct groups, i.e. *S. guianensis guianensis* – *S. acuminata*, *S. grandiflora* – *S. hippocampoides*, and *S. gracilis*, can be recognized. *S. montevidensis* is classified in the same group as the *S. guianensis* complex. This has been confirmed by ITS1 DNA sequence analysis (manuscript in preparation), which showed an ITS1 distinct from those of the other species. Nevertheless, we are able to separate *S. montevidensis* from the *S. guianensis* complex, which is in contrast to cpDNA analysis (Gillies and Abbott 1996). Seed protein analysis (Robinson and Megarrity 1975) has shown a linkage between the diploid *S. hamata*, *S. calcicola* and *S. viscosa*. This is supported by our findings, except that we are able to clearly distinguish *S. viscosa* from the rest. Based on cpDNA analysis, Gillies and Abbott (1996) found relationships among the diploid species *S. humilis* – *S. leiocarpa* – *S. angustifolia*; between diploid *S. capitata* and tetraploid *S. macrocephala*; between *S. scabra* and *S. viscosa*; and between *S. fruticosa* CPI 11756 and *S. scabra*. Some of these relationships had also been determined previously by morphological-agronomical studies (Burt et al. 1971), allozyme patterns (Stace 1982) and morphology ('t Mannetje 1984). Moreover, they are well supported by our STS analysis. In contrast to Gillies and Abbott (1996), we did not find evidence that *S. sympodialis* and *S. humilis* are closely related. Instead, we found a relationship between *S. sympodialis* and group 2.2A.

STS analysis enabled us to identify incorrectly named accessions. Based on STS analysis, *S. mexicana* CPI 67665 and *S. erecta* CPI 34118 are polyploid and diploid, respectively, and therefore do not belong to the species in which they were previously classified. *S. fruticosa* CPI 11756 and 79070 are both polyploid but do not belong to the same group. Based on the similarity with previous analyses, only accession CPI 11756 is considered as *S. fruticosa*. *S. debilis* is analyzed as a diploid species, which is in agreement with cytological studies (Stace and Cameron 1984). However, one of the two accession numbers does not belong to this species because they are not related to each other based on STS markers.

We found some degree of intra-species variation in *S. viscosa*, *S. guianensis* and *S. humilis*. The widespread distribution of these species (Williams et al. 1984) may explain their polymorphic character. Nevertheless, probably more intra-specific variation may also be found by analyzing a wider number of genotypes from the other species.

Based on the additive nature of STS markers or on selective amplification, we were able to identify the polyploid species. Our identification is in agreement with that determined by cytological studies (Stace and Cameron 1984), except for *S. ingrata*, for which we did not find cytological evidence (Table 1). According to our findings, all polyploids including *S. capitata* arose from alloploidy. *S. capitata* was previously considered to be a possible autoploid (Stace and Cameron 1984). All of the polyploids studied here were identified as being tetraploid. We were not able to determine hexaploidy in *S. erecta* CPI 50979 with STS analysis. The division of the *Stylosanthes* genus into two sections is in agreement with the classification based on STS analysis. All of the recognized polyploid species in this study belong to the section *Styposanthes*. The only exception is *S. ingrata*, which belongs to the section *Stylosanthes* (Table 1). This is in contrast with previous observations that all identified species belonging to section *Stylosanthes* are diploid (Stace and Cameron 1984). This can be explained by the fact that these genotypes were

previously wrongly classified or that the present study provides evidence that polyploid species can arise from 2 species of section *Stylosanthes* and thereby remain in this section. Additional cytological and morphological study should clarify this.

STS analysis enables us to speculate about the relationship between the polyploids and their diploid progenitors. *S. viscosa* can be considered to be a possible parent of *S. scabra*, *S. ingrata*, *S. fruticosa* CPI11756 and *S. erecta* CPI 50979. Evidence for parentage between *S. viscosa* and *S. scabra* was also found by isozyme analysis (Stace and Cameron 1984), cpDNA analysis (Gillies and Abbott 1996) and previous STS analysis (Liu et al. 1996). The same conclusion can be drawn for tetraploid *S. hamata*, which is considered to have diploid *S. hamata* and *S. humilis* as progenitors, and for tetraploid *S. sundaica* which is morphologically similar to *S. humilis* (Mohlenbrock 1958). The present study indicates that it is possible to search for the genetic origin of polyploids in *Stylosanthes*, by using genetic analysis such as STS analysis and combining it with cytogenic analysis. The identification of the constituent genomes of alloploids will be useful information in plant breeding programs of existing economically important species.

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