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Molecular characterization and phylogenetic utility of the rDNA external transcribed spacer region in *Stylosanthes* (Fabaceae)

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Abstract Supplementary Material The nucleotide sequence of the ribosomal external transcribed spacer (ETS) region of *Stylosanthes mexicana* was determined and used to evaluate its potential for examination of intra- and inter-specific relationships in *Stylosanthes*, as compared to the use of the internal transcribed spacer (ITS) region. The entire ETS region comprises 1,145 bp and is composed of a region of non-repetitive sequences consisting of three subregions with organizational and nucleotide-sequence conservation, and a triplicated segment of about 100 bp. A primer designed in the second conserved subregion allowed us to amplify and sequence directly the 3' part (423–431 bp) of the ETS from 22 genotypes of 12 representative *Stylosanthes* species that were previously used in phylogenetic analysis of the genus. The study revealed that the right-hand part of *Stylosanthes* ETS contains approximately twice as much variable and informative characters than the ITS. Moreover, pairwise sequence-divergence values are twice as high, on average, when compared to the ITS. The ITS and ETS datasets are consistent in phylogenetic reconstruction of *Stylosanthes*, and combined parsimony analysis resulted in a strict consensus tree that is better resolved and generally better supported than trees obtained from separate analysis of the spacer regions.

Electronic Supplementary Material Supplementary material is available for this article if you access the article at http://dx.doi.org/10.1007/s00122-003-1245-9. A link in the frame on the left on that page takes you directly to the supplementary material.

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Introduction

The genus *Stylosanthes* Sw. belongs to the tribe Aeschynomeneae in the legume family and includes 30 to 45 herbaceous or suffruticose species (Costa and Ferreira 1984; 't Mannetje 1984) which are native to the tropical and subtropical regions of the Americas, tropical Africa and South-East Asia (Mohlenbrock 1958; Williams et al. 1984). The major centre of diversity for the genus is suspected to lie in the southern neotropics, particularly Brazil, with a secondary centre in the Mexico-Carribean basin (Stace and Cameron 1984). Several of the species such as *Stylosanthes guianensis, Stylosanthes hamata, Stylosanthes humilis, Stylosanthes scabra* and *Stylosanthes viscosa* have found widespread application in (sub)tropical agriculture as a pasture and forage legume (Burt and Miller 1975), as green manure (Gardener 1984), as cover crop (Thomas 1984) and, more recently, in the biological control of cattle tick (Sutherst et al. 1998). Because of its high value for improving (sub)tropical grassland, considerable effort has been invested by a number of institutions to collect, conserve and evaluate the germplasm of *Stylosanthes* (Schultze-Kraft et al. 1984).

Taxonomic and evolutionary studies are essential to managing germplasm collections and interpreting the biodiversity of *Stylosanthes* for conservation purposes. Two subgeneric sections, sect. *Stylosanthes* Vog. and sect. *Styposanthes* Vog., have been recognized by the absence or presence of an axis rudiment and the presence of one or two inner bracteoles, respectively (Kirkbride and Kirkbride 1987). While diploid species are found in both sections, polyploid species ($2n$ = 40 and 60) are restricted to sect. *Styposanthes* and they are thought to be products of intersectional hybridization between diploids, followed by polyploidization (Stace and Cameron 1984).

In this process of hybrid speciation, members of the lineages represented by *Stylosanthes calcicola* and *S. viscosa* have acted as parental genome donors for all known allopolyploids of *Stylosanthes* with the exception of *Stylosanthes capitata* (Vander Stappen et al. 1999a, 2002a, b). Because of a wide range of variation in morphological characters, the taxonomic treatment of *Stylosanthes* is very ambiguous ('t Mannetje 1984). The traditional classification is based largely on fruit morphology, in particular the shape and length of the beak, the indumentation of the pod and the width and venation of the outer bract ('t Mannetje 1984). In contrast, Ferreira and Costa (1979) emphasized the number of vascular bundles, the venation of the leaflets and the growth habit as main diagnostic features. As a result, the *Stylosanthes* germplasm collections consist of a substantial number of undetermined and inaccurately identified accessions (Schultze-Kraft et al. 1984; Sawkins et al. 2001). Given the difficulties in species delimitation and the significance of *Stylosanthes* in (sub)tropical agriculture, other techniques have been used in an attempt to complement the morphological approach (e.g. Williams et al. 1984; Stace and Cameron 1987; Vieira et al. 1993). Recently, molecular marker techniques have proven to be very useful in providing additional data for delineating species (Liu et al. 1999; Vander Stappen et al. 1999b), assessing evolutionary relationships and elucidating the genetic origin of allopolyploids (Gillies and Abbott 1996; Vander Stappen et al. 1999a; Liu and Musial 2001; Vander Stappen et al. 2002a, b), and determining genetic diversity (Vander Stappen et al. 1998, 2000; Sawkins et al. 2001) in the genus *Stylosanthes*. A large-scale phylogenetic analysis of *Stylosanthes* based on the internal transcribed spacer (ITS) region provided useful information about evolutionary relationships in the genus, especially regarding the allopolyploid species and the basic structure of the genus (Vander Stappen et al. 2002a). However, the ITS alone provided too few phylogenetically informative characters to resolve the detailed relationships within the different clades that were found, pointing to the need for additional informative characters from other loci to elucidate the relationships among closely related *Stylosanthes* species.

The external transcribed spacer (ETS) region, which is part of the intergenic spacer region of the 18S-26S nuclear ribosomal DNA repeat, may be successfully used in phylogenetic studies where ITS phylogeny gives weak results, such as in the case of recently evolved lineages, because it shares the same favorable features of the ITS, and it is generally known to evolve faster and to contain more phylogenetically informative characters than the ITS in plants (Baldwin and Markos 1998; Bena et al. 1998a). To-date, the ETS has been used in phylogenetic analysis of several plant genera belonging to the families Asteraceae (Baldwin and Markos 1998; Linder et al. 2000), Fabaceae (Bena et al. 1998b; Chandler et al. 2001) and Myrtaceae (Wright et al. 2001).

The purpose of this study was to determine the nucleotide sequence of the external transcribed spacer region of *Stylosanthes* and to evaluate its phylogenetic utility compared to the internal transcribed spacer region (ITS1 and ITS2).

Materials and methods

Plant material

Plant material of *Stylosanthes* was obtained from herbarium specimens of MEXU (Herbario Nacional de México, collector S. Gama López, Mexico City, Mexico) and GH (Gray Herbarium of Harvard University, Cambridge, USA), or from germplasm accessions of CIAT (Centro Internacional de Agricultura Tropical, Tropical Forages Collection, Cali, Colombia) and CSIRO [Commonwealth Scientific and Industrial Research Organization, Australian Tropical Forages Genetic Resource Centre, Commonwealth Plant Introduction (CPI) numbers, St. Lucia, Australia] (Table 1). The *Stylosanthes* species were selected to represent the distinct clades that were found by previous phylogenetic analysis using ITS sequence data (Vander Stappen et al. 2002a), with emphasis on the lineages represented by *S. viscosa* (clade 1b) and *S. calcicola* (clade 3). All *Stylosanthes* species under investigation are diploid, except for *Stylosanthes subsericea* which is an allotetraploid. In addition, several genotypes of *S. guianensis* and *S. viscosa* were taken in order to estimate intraspecific variation. Seeds from germplasm accessions were germinated on filter paper in Petri dishes at 25 °C. After germination, young seedlings were grown in pots. Young leaves were harvested from the plants and dried on silica gel. Total DNA was isolated from a 3-foliate leaf of either dried herbarium specimens or fresh tissue dried in silica gel, following the procedure described by Vander Stappen et al. (2000).

PCR amplification and sequencing

The complete intergenic spacer region was amplified from the species *S. calcicola, S. guianensis* (genotype A), *S. hamata, Stylosanthes mexicana, Stylosanthes pilosa* and *S. viscosa* (genotype B) by long-range PCR using the primers 26S-F and 18S-R of Bena et al. (1998a), 20 ng of total plant DNA and *TaKaRa LA Taq* according to the supplier's protocol (TaKaRa Shuzo co., Otsu, Japan). The reactions were carried out by incubation at 95 °C during 1 min, followed by 35 cycles of 10 s at 95 °C, 30 s at 58 °C, 10 min at 68 °C and a final extension step of 10 min at 72 °C on a UNOII 96 Thermocycler (Biometra, Göttingen, Germany). After electrophoresis on a 1% TAE agarose gel (GibcoBRL, Gaithersburg, USA) and visual inspection by UV illumination, the PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the specification of the supplier. The PCR products were sequenced directly using an internal 18S primer, ETS3fab-R, which is designed at the junction between 18S rDNA and the external transcribed spacer as present in several plant genera belonging to the legume family, e.g. *Glycine, Medicago, Phaseolus, Trigonella* and *Vicia*. Comparison of sequences (unambiguous readings till 500 bp on average) obtained from the above-mentioned *Stylosanthes* species revealed a conservative site suitable for the design of a second primer, ETS3sty-F, which allowed amplification and subsequent DNA sequencing of approximately 430 bp of the 3' ETS region in *Stylosanthes* in conjunction with primer ETS3fab-R. PCR reactions contained 1 × PCR buffer (Qiagen, Hilden, Germany), 200 μM of each dNTP, 1 μM of each primer, 0.625 units of HotStar*Taq* DNA polymerase (Qiagen) and approximately 20 ng total plant DNA in a total volume of 25 μl. The reactions were carried out by incubation at 95 °C during 15 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C and a final extension step of 10 min at 72 °C on a UNOII 96 Thermocycler (Biometra). The PCR products were sequenced directly in either orientation following PCR purification. Sequence data from the ETS of *S. mexicana* was used to design a species-specific internal primer, ETSi-R, to sequence

Table 1 List of *Stylosanthes* species included in this study, with genotype number, origin and EMBL/Genbank accession number

Species	Genotype	Origin	EMBL/Genbank accession number
Section *Styposanthes* Vog.			
S. calcicola Small	MEXU 252	Mexico, Yucatan	AJ489505
S. hamata (L.) Taub	CIAT 124	Colombia, Atlantico	AJ489506
S. macrocarpa S.F. Blake	MEXU 178	Mexico, Oaxaca	AJ489507
S. macrocephala M.B. Ferreira & Sousa Costa	CIAT 1643	Brazil, Brasilia	AJ489508
S. mexicana Taub.	MEXU 246	Mexico, Nuevo Leon	AJ489509 AJ489519
S. pilosa M.B. Ferreira & Sousa Costa	CIAT 2068	Brazil, Bahia	AJ489510
S. seabrana Maass & 't Mannetje	CPI 110273	Brazil, Bahia	AJ489511
S. subsericea S.F. Blake	MEXU 274	Mexico, Puebla	AJ489512
Section *Stylosanthes* Vog.			
S. guianensis (Aubl.) Sw. (A)	MEXU 281	Mexico, Chiapas	AJ489513
S. guianensis (Aubl.) Sw. (B)	MEXU 151	Mexico, Oaxaca	AJ489514
S. guianensis (Aubl.) Sw. (C)	CPI 33437	Surinam, Lelydrop	AJ512614
S. guianensis (Aubl.) Sw. (D)	MEXU 197	Mexico, Nayarit	AJ512613
S. guianensis (Aubl.) Sw. (E)	CIAT 1200	Brazil, DF	AJ512615
S. guianensis (Aubl.) Sw. (F)	CIAT 1523	Venezuela, Monagas	AJ512616
S. guianensis (Aubl.) Sw. (G)	CPI 34906	Brazil, Sao Paulo	AJ512617
S. humilis Kunth	MEXU 176	Mexico, Michoacan	AJ489515
S. leiocarpa Vog.	CPI 78192	Argentina, Corrientes	AJ489516
S. viscosa Sw. (A)	MEXU 135	Mexico, Oaxaca	AJ489517
S. viscosa Sw. (B)	CIAT 11091	Venezuela, Bolivar	AJ489518
S. viscosa Sw. (C)	CIAT 1593	Belize, Cayo	AJ512618
S. viscosa Sw. (D)	GH 5534	Cuba	AJ512619
S. viscosa Sw. (E)	CIAT 2090	Brazil, Bahia	AJ512620

Table 2 List of primers used in this study

Primer name	Primer sequence (5'–3')	Reference
26S-F	ACGTGAGCTGGGTTTAGACCGTC	Bena et al. 1998a
18S-R	CCTGCTGCCTTCCTTGGATGTGG	Bena et al. 1998a
ETS3sty-F	GACCGTGTCGGCGGATGAG	This study
ETS3fab-R	GGATCAACCAGGTAGCATCC	This study
ETSi-R	CACGGCAGGTCTTTCTGTTG	This study

the 5' part of the ETS. All primers (Table 2) were purchased from Genset (Paris, France). Purified PCR products were sequenced by the ABI PRISM DyeDeoxy terminator sequencing protocol (Applied Biosystems, Foster City, USA) and sequencing gels were run on a 373A DNA sequencer (Applied Biosystems). The DNA sequences have been deposited in the EMBL Data Library under the accession numbers shown in Table 1.

Sequence data analysis

The entire ETS sequence of *S. mexicana* was compared to data from other plant species in the EMBL DNA data library by using the FASTA program (Pearson and Lipman 1988). The subrepeat structure of the ETS of *S. mexicana* was assessed by comparing the sequence of this species to itself using the program LALIGN (Huang and Miller 1991). The same program was used to determine conserved regions between the ETS of *S. mexicana* and other plant species. Proofreading, editing and alignment of the DNA sequences were done with the program Sequencher v3.0 (Gene Codes Corporation, Ann Harbor, Mass., USA) followed by manual adjustment. The data were subsequently analyzed via Fitch parsimony (Fitch 1971) with the computer program PAUP version 4.0b10 (Swofford 2002). Heuristic searches of 100 replicates of random additions of sequences in combination with accelerated transformation (ACCTRAN) character optimization and the TBR+MULTREES branch-swapping option, were conducted. Bootstrap values (B.V.) (Felsenstein 1985) were calculated from 100 replicates of heuristic searches using random additions of sequences with TBR swapping, MULTREES and ACCTRAN options in effect. The homogeneity test of partitioned datasets was done according to Farris et al. (1995) with HomPat which is implemented in PAUP. Pairwise sequence divergence values were calculated using PAUP. The distance matrix and the NEXUS data matrices with the sequence alignments are available through the internet address http://www.agr.kuleuven.ac.be/dp/logt/Onderzoek/stylodata.htm.

Results and discussion

DNA characteristics of the entire ETS region in *S. mexicana*

The sequenced intergenic spacer (IGS) fragment of *S. mexicana* between the 5' end of the 18S rRNA gene and upstream is 1,198 bp in length and has a GC content of 63.5%. DNA sequence comparison of this fragment to data from other plant species revealed a maximum of 60% identities to the 3' ETS region from the genus *Glycine* (552 bp in length), and from the genus *Gastrolobium* and allied genera (330 bp in length). The region of similarity starts around the putative processing site and corresponds to the second region that was identified by Nickrent and

Patrick (1998) as being similar among seven legume species. The 5' ETS region of *S. mexicana* did not show any similarity to known plant sequences. The ETS is known to show a decrease of nucleotide sequence conservation upstream from the 18S gene (Kato et al. 1990; Volkov et al. 1996). The sequenced part of the intergenic spacer fragment in *S. mexicana* contains several regions similar to those of plant species in which functionality has been demonstrated previously. Based on the conserved sequence motif in the RNA polymerase I transcription initiation sites (TIS) of higher plants (Kato et al. 1990; Perry and Palukaitis 1990), the putative 5' start of *S. mexicana* rRNA transcription was assigned to position 1,145 upstream from the 5' end of the 18S rRNA gene, which means that the putative length of the ETS region is 1,145 bp in *S. mexicana*. The TIS of *S. mexicana* is identical in sequence to the ones reported in *Glycine max* and *Phaseolus coccineus*, i.e. TATTATAGGG (Fernández et al. 2000). Forty six basepairs upstream from this TIS is a conserved region (GAAAAAG) that corresponds in sequence and distance to the TIS, to one of the conserved sequences in the promotor region of legume species (Nickrent and Patrick 1998; Fernández et al. 2000). The ETS of *S. mexicana* contains one family of direct tandem repeats starting at 204 bp downstream from the TIS and including three imperfect repeated sequences of approximately 100-bp long with 78 to 59% similarity to each other. The presence of repetitive structures located downstream from the putative TIS has been reported in other plant genera and these structures might be involved in transcriptional regulation as protein binding sites (Rogers and Bendich 1987; Zentgraf and Hemleben 1992). Around 88 bp downstream from these subrepeats starts a region that contains three motifs corresponding in sequence and relative position to the conserved motifs reported previously by several authors (Perry and Palukaitis 1990; Polanco and Pérez de la Vega 1994; Fernández et al. 2000). The first motif in this region, GCG, is part of a putative primary processing site which is located at position 602 from the TIS. Approximately 100 bp downstream from this site lies the second consensus sequence, GCGNATGAGTGG, followed after 22 bp by the third motif HGKCTCCNTGC. Part of the second motif has also been reported by Bena et al. (1998a) as being highly conserved in various organisms. Polanco and Pérez de la Vega (1994) suggested that these conserved motifs may be involved in pre-rRNA metabolism as a signal for primary processing of rRNA precursors. Interestingly, the length between this motif and the 5' end of the 18S rRNA in the ETS of *S. mexicana* is 438, which is similar to what has been reported for other plant genera (Bena et al. 1998a). According to Bena et al. (1998a), the among-genera conserved length of this region may have a key function in the processing of rRNA gene transcripts.

DNA sequence characteristics and phylogenetic utility of the 3' ETS region in *Stylosanthes* as compared to the ITS (ITS1 and ITS2) region

The sequence data, derived from direct sequencing of the long-range IGS PCR product and the 3' ETS PCR product with primer ETS3fab-R, were identical. Since no ambiguous positions were found in the DNA sequence ladders, there was no evidence for the presence of multiple ETS sequence types within individuals, indicating that concerted evolution is acting as effectively on the 3' ETS as on the ITS region of *Stylosanthes* (Vander Stappen et al. 2002a). The allotetraploid species *S. subsericea* contained a single ETS sequence type with highest similarity to the ETS sequence types found in *S. viscosa*, suggesting homogenization of the ETS copies to one of its presumed parental genome donors, i.e. *S. viscosa*, as was already observed for the ITS in this species (Vander Stappen et al. 2002a). The process of homogenization of the entire parental rDNA repeat types has been reported in several allotetraploid plant species, including *Oryza* (Cordesse et al. 1990), *Gossypium* (Wendel et al. 1995) and *Nicotiana* (Volkov et al. 1999).

The main characteristics of the 3' end of the ETS region compared to the ITS (ITS1 and ITS2) region are summarized in Table 3. Length variation for the ETS region of 22 *Stylosanthes* genotypes ranged from 423 to 431 bp, which is comparable to the combined length of the ITS1 and ITS2. The G+C composition of the ETS is high and nearly equals that of the ITS. The GC balance between ITS and ETS may indicate molecular coevolu-

Table 3 Characteristics of the ITS (ITS1 and ITS2) and the 3' end of the ETS region in *Stylosanthes*, separately and combined, and phylogenetic utility

Characteristics	ITS	ETS	Combined
Size range (bp)	415–422	423–431	838–851
Mean G+C content (%)	69	65	67
Pairwise sequence divergence (range, %)			
Interspecies	0–10.58	0.23–18.14	0.12–12.34
Intraspecies	0–2.62	0.23–2.11	0.24–1.65
Number and size range (bp) of indels	6	5	11
	1–7	1–8	1–8
Variable characters (total, (%))	69 (15.9)	121 (28)	190 (22)
Informative characters (total, (%))	41 (9.4)	88 (20.4)	129 (14.9)
Number and length of most-parsimonious trees	28	10	3
	86	159	245
Consistency and retention index	0.872	0.868	0.869
	0.918	0.926	0.923

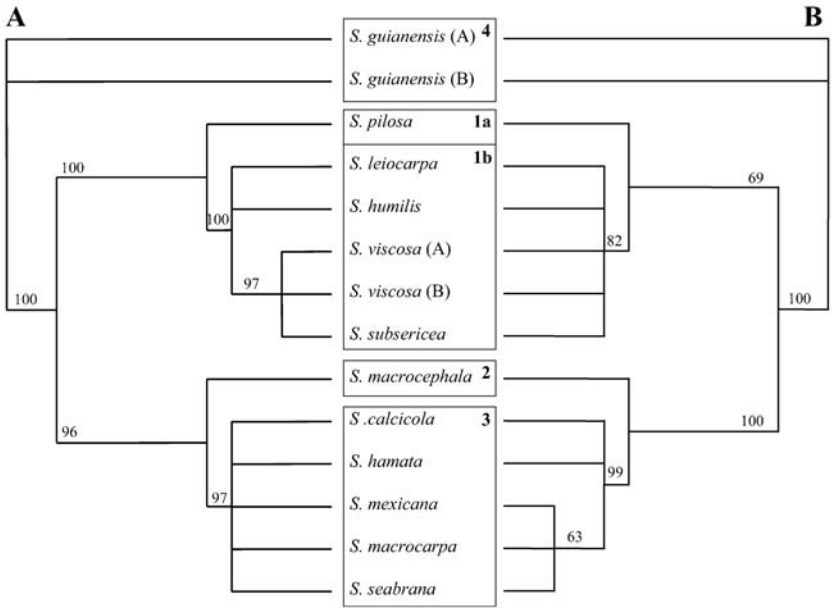

Fig. 1A, B Strict consensus trees of the most parsimonious trees obtained from the analysis of (**A**) ETS (length 159, CI 0.868, RI 0.926) and (**B**) ITS (length 86, CI 0.872, RI 0.918) sequence data from 14 genotypes representing 12 *Stylosanthes* species. Bootstrap values (%) are indicated above the branches. The numbers (*in bold*) accompanying the species correspond to the clades observed in Vander Stappen et al. (2002a)

tion at the level of base composition, as was reported by Torres et al. (1990) for the ITS1 and 2 region. Interspecific pairwise ETS sequence divergence values ranged from 0.23 to 18.14% with a mean of 12%. These values are two times higher on average, ranging from 0.96 (*Stylosanthes macrocephala – S. hamata*) to 4.37 (*Stylosanthes seabrana – S. calcicola*) when compared to values obtained from the ITS. A similar higher rate of sequence evolution by nucleotide substitution in the ETS has been observed in other plant genera (Baldwin et al. 1998; Bena et al. 1998a; Wright et al. 2001). The intraspecific divergence values in the ETS were 1% in *S. guianensis* and *S. viscosa* on average, as opposed to 1.2 and 0.4% in the ITS of the respective species. Similar to the ITS region (Vander Stappen et al. 1998), the ETS may be suitable as a molecular tool detecting variation in *S. guianensis*. In addition, the ETS may have a potential use in the molecular study of the *S. viscosa* genome, which is of importance because *S. viscosa* is regarded as an important parental genome donor for most allopolyploids in *Stylosanthes*, including the cultivated species *S. scabra* (Vander Stappen et al. 2002a).

The ETS of the 12 *Stylosanthes* species including two genotypes of *S. guianensis* (A–B) and *S. viscosa* (A–B), was readily aligned over a length of 432 bp (primers excluded). Five indels were introduced ranging in length from 1 to 8 bp, which is comparable to what has been observed in the ITS. Of these indels, three are autapomorphic while the other two are synapomorphic. There is one highly variable region between positions 321 and 335 upstream of the 5' end of 18S rRNA which is diagnostic for the major phylogenetic division in *Stylosanthes*. Of the 121 variable characters, 88 are informative. Proportionally, more phylogenetic informative characters are present in the ETS than in the ITS. Parsimony analysis of the 3' ETS region resulted in ten shortest trees of length 159, a consistency index (CI) of 0.868 and a retention index (RI) of 0.926. The strict consensus tree is shown in Fig. 1 in comparison to the strict consensus tree obtained from the ITS sequence data. The trees have similar topologies and the clades that were previously observed by ITS analysis (Vander Stappen et al. 2002a) are well supported in the ETS tree with relatively high bootstrap values. Moreover, the ETS data provides stronger support for the branching of clade 1 and the relationships within this clade, which were weakly or not supported in the ITS tree. In contrast, the inner structure of clade 3 is not resolved with the ETS data despite the fact that the ETS has accumulated more variation than the ITS. Most of this variation, however, is not informative for parsimony. Given the high proportion of autapomorphic characters and the inability of the ETS to resolve relationships among species of clade 3, these species may have experienced rapid evolution from a common ancestor. The lower pairwise ETS sequence divergence values among the species in this clade suggests a relatively recent diversification of this group of species. This has also been observed by ITS DNA sequence analysis (Vander Stappen et al. 2002a). With the exception of *S. seabrana*, which is restricted to Bahia in eastern Brazil, these species occur in the Mexican-Caribbean basin and/or the mainland of northeastern South America (Mohlenbrock 1958), which corresponds in part to the secondary centre of diversity of the genus. Morphologically they are similar and they are adapted to different environments (Mohlenbrock 1958; Jansen and Edye 1996; Gama López, in preparation). According to Stace and Cameron (1984), these species were primarily distributed in the northern Neotropics, and during the Pleistocene they migrated to the southern Neotropics. At the Pleistocene/Holocene boundary, the contraction of open dryland habitats created isolated habitats that have played a fundamental role in

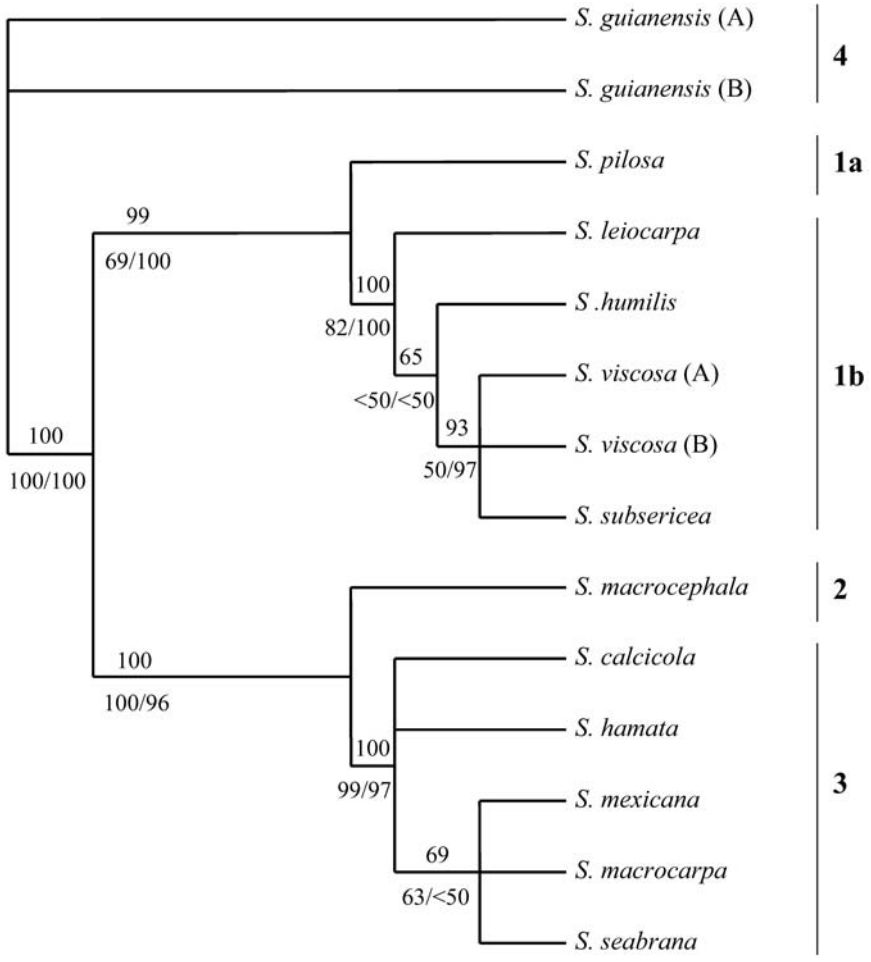

Fig. 2 Strict consensus tree of the three most parsimonious trees (length 245, CI 0.869, RI 0.923) obtained from the combined analysis of the ITS and ETS sequence data from 14 genotypes representing 12 *Stylosanthes* species. Bootstrap values (%) are indicated above the branches. Below the branches are bootstrap values from separate analysis of the ITS and ETS, respectively. The numbers (*in bold*) accompanying the species correspond to the clades observed in Vander Stappen et al. (2002a)

the speciation process of *Stylosanthes*. It is possible that these geographically or ecologically isolated species diverged within a relatively short time. When all the ITS and ETS characters are combined and analyzed using parsimony, three most-parsimonious trees with length 245, CI 0.869 and RI 0.923 are generated (Fig. 2). The partition homogeneity test shows no significant incongruence between ITS and ETS data, justifying the combination of the data. The strict consensus trees of the combined data is consistent with, and better resolved than, the consensus trees derived from the ITS and ETS separately, especially regarding the inner structure of clade 1b. The allotetraploid species *S. subsericea* is placed in the same group as *S. viscosa*. Previous studies based on ITS and STS analysis, identified *S. viscosa* as one of the possible parental progenitors of *S. subsericea* (Vander Stappen et al. 1999b, 2002a). Our results give additional support to this finding. Based on the diagnostic nucleotide positions and the congruence between ITS and 3' ETS data in the phylogenetic reconstruction of this species, a recombination event in the ribosomal DNA between the parental genome donors such as has been observed in an allopolyploid species of *Medicago* by Bena et al. (1998b), is unlikely to have occurred in *S. subsericea*.

In conclusion, combined analysis of the ITS and ETS regions contributes to a better understanding of evolutionary relationships in the genus *Stylosanthes*, which provides essential information for the collection, conservation and use of wild species related to cultivated types as genetic resources. However, further refinement of the phylogenetic estimate for closely related species is needed, perhaps by using more rapidly evolving genes.

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