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Sequencing of the internal transcribed spacer region ITS1 as a molecular tool detecting variation in the *Stylosanthes guianensis* species complex

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Abstract The internal transcribed spacer (ITS) regions 1 and 2 of the ribosomal DNA from *Stylosanthes guianensis* CIAT 1283 and cv 'Schofield' were amplified by polymerase chain reaction using conserved ITS primers from the 18S, 5.8S and 26S ribosomal genes flanking those regions. The entire region of 683 bp long was cloned, and seven clones were sequenced. Comparison of the ITS spacer regions with published DNA sequences of other plant species revealed limited homology only; this was in contrast to their comparison with the 5.8S rDNA sequences. The ITS1 region of 45 *S. guianensis* accessions was amplified by PCR and sequenced on both strands using the conserved primers ITS2-ITS5. These sequences, ranging from 201 to 204 bp, were aligned to each other to assess intra-specific polymorphism. Within the *S. guianensis* (Aubl.) Sw. species complex, 11 DNA sequence types could be distinguished based on an insertion/deletion (indel) event and 15 single base-pair substitutions. In 1 of the *S. guianensis* types, two kinds of ITS1 sequence were observed in each individual, reminiscent of an incomplete homogenization of the repeat structure in this type. Polymorphisms in the sequence of the ITS1 region were used to define molecular markers for *S. guianensis* on the basis of PCR-restriction fragment length polymorphism and selective PCR.

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Introduction

The genus *Stylosanthes* belongs to the tribe Aeschynomeneae of the family of Fabaceae and includes some 40 species and a variable number of subspecies and varieties of neotropical origin (Williams et al. 1984). Most of these are distributed throughout tropical America. The taxonomy of this genus is notoriously difficult, and it is likely that additional species will be recognized as a result of further detailed plant collecting and biosystematic investigations (Stace and Cameron 1984). The diploid *Stylosanthes guianensis* (Aubl.) Sw. originates from Central and South America and has been widely used as a pasture legume. Taxonomic groups within this species have been treated differently by different authors ('t Mannetje 1977, 1984; Ferreira and Costa 1979).

Only a few reports are available concerning genetic diversity among *Stylosanthes* species, and most of these reports are based on morphological-agronomical characters (Burt et al. 1971; Edye et al. 1974), isozyme loci (Stace 1982) and seed protein patterns (Robinson and Megarrity 1975). Variation at the DNA level as measured by molecular techniques offers an additional tool for the examination of relationships between species and populations of a genus (Doyle 1993). Kazan et al. (1993a, b) studied genetic relationships and variation in species of *Stylosanthes* at and below the species level by means of random amplified polymorphic DNA (RAPD) analysis. They concluded that the phenogram acquired by analysis of RAPD data corroborated the previously reported taxonomy of the species complex and suggested that the RAPD technique may be a useful tool for systematic analysis. Gillies and Abbott (1996) reproduced phylogenetic relationships between

a range of *Stylosanthes* taxa based on chloroplast DNA restriction fragment data. Liu et al. (1996) tested the genome specificity of sequence-tagged sites (STS) as genetic markers in *Stylosanthes*.

The 18S-26S nuclear ribosomal DNA (nrDNA) is present in tandemly repeated arrays at the nucleolus organizer region (NOR) of specific chromosomes. Each repeating unit is transcribed as a precursor which is processed into the mature 18S, 5.8S and 26S RNAs. Each rDNA unit contains genes coding for the 18S, 5.8S and 26S rDNA separated by two internal transcribed spacer regions, ITS1 and 2 (Hamby and Zimmer 1992). Because the rDNA genes are well-conserved but flank the more variable internal transcribed spacers (ITS), universal primers can be used which amplify those spacer regions (White et al. 1990). The high-copy number, rapid concerted evolution, small size and length conservation of the ITS spacer regions make them useful for amplification, sequencing and alignment to detect variation within genera (Baldwin et al. 1995). ITS sequence data have already been used successfully in Compositae (Baldwin 1993), Winteraceae (Suh et al. 1993), Fabaceae (Wojciechowski et al. 1993) and more recently in other plant families (Baldwin et al. 1995). The present paper reports the nucleotide sequence of the rDNA region between the 3′ end of the 18S rDNA and the 5′ end of the 26S rDNA gene from *Stylosanthes guianensis* genotypes and the analysis of the ITS1 spacer region in the *S. guianensis* species complex.

Our objectives were to assess the usefulness of the ribosomal DNA spacer regions to detect variation within the *Stylosanthes guianensis* species complex and hence to establish molecular tools which can be used for a large-scale screening of the genetic diversity of natural *S. guianensis* populations of Mexico.

Material and methods

Plant material

Plant material (Table 1) was obtained from existing germplasm accessions of CIAT (Centro Internacional de Agricultura Tropical, Colombia, CIAT accessions) and CSIRO (Commonwealth Scientific and Industrial Research Organization, Australia, CPI accessions).

DNA isolation and polymerase chain reaction (PCR)

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). The ITS spacer regions were amplified with primers ITS2, ITS5, ITS3 and ITS4 as described by White et al. (1990). Primers were purchased from 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, 2 U AmpliTaq DNA polymerase (Perkin Elmer Cetus, Norwall, Conn., USA) 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 1 min at 94°C, 1 min at 55°C, 1 min at 72°C and a final extension step of 5 min at 72°C on a TRIO-thermoblock (Biometra, Göttingen, Germany). PCR products were electrophoretically fractionated on a 3% TAE agarose gel (Metaphor, FMC Bio Products, Rockland, Me., USA) containing ethidium bromide and visualized under UV light. They were purified for DNA sequence analysis with the Qiaquick PCR purification kit (Qiagen, Germany).

Cloning of PCR products

The PCR product was amplified with the proofreading enzyme Pfu DNA polymerase (Stratagene, USA) because it has a high fidelity and generates blunt-end fragments (Flaman et al. 1994). Reactions were performed as described above. The PCR products were selectively precipitated by adding an equal volume of polyethylene glycol mix as described by Rosenthal et al. (1993), subsequently phosporylated according to Kanungo et al. (1993) and ligated into dephosphorylated pUC18/*Sma*I vector purchased from Pharmacia Biotech (Roosendaal, The Netherlands). This ligation mixture was used to transform competent *E. coli* strain DH5α as described by Inoue et al. (1990). Transformed bacterial cells were incubated on Ap100/IPTG/X-gal LB plates which allowed blue-white selection of the colonies. White colonies were picked out with a toothpick and directly analyzed by specific PCR.

DNA sequence analysis

Purified PCR products and cloned fragments were sequenced with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, USA) using the ITS amplification primers and vector-specific primers, respectively. Sequencing gels were run on an ABI 373A instrument (Applied Biosystems). Sequences of the ITS1 region of *Stylosanthes guianensis* genotypes were aligned with the Clustal V multiple sequence alignment program (Higgins et al. 1992), followed by manual re-alignment to correct for insertion/deletion events (indels). A matrix was produced with DNADIST, containing distance data based on Kimura's two-parameter model (Kimura 1980) The distance data was clustered by the UPGMA method. In addition, DNA sequences were analyzed by maximum likelihood (DNAML). Indels were scored as missing data. All computer analyses were performed with the software package PHYLIP (Felsenstein 1991).

Restriction fragment length polymorphism of the ITS1 region

Single digests of the amplified ITS1 products were performed with the following restriction enzymes *Alu*I, *Taq*I, *Nae*I, *Dpn*I, *Mae*II, *Bsm*I, *Sau*96I (Boehringer Mannheim, Germany), *Bsr*FI, and *Bsp*MI (New England Biolabs, USA) according to the manufacturers' instructions. Restriction fragments were fractionated on a 4% TAE agarose gel (Metaphor, FMC) at 7 V/cm for 4 h, stained with ethidium bromide and visualized under UV light.

Table 1 *Stylosanthes guianensis* accessons used in this study based on data published by Kazan et al. (1993a)

Accession	Species	Place of origin	Group Kazan	Group Edye	ITS Type
CPI accessions	S.g. complex				
39112	*S. gracilis*	Bauru, Brazil	1-B	11B	H
33434	*S. gracilis*	Sinnamary, French Guiana	1-B	11A	C
33501B	*S. gracilis*	Jusepin, Venezuela	1-B	11A	C
33501C	*S. gracilis*	Jusepin, Venezuela	1-B	11A	C
34908	*S.g.g. vulg.*	Nova Campinas, Brazil	1-B	11A	C
37512	*S.g.g. vulg.*	Rio Ceballos, Argentina	1-A-2b	7B	B
33437	*S.g.g. vulg.*	Lelydrop, Surinam	1-A-2b	7A	B
33034	*S.g.g.*	CRI Research Station	1-A-2b	7A	B
cv Schofield	*S.g.g. vulg.*	Deodora, Brazil	1-A-2b	7A	B
cv Endeavour	*S.g.g.*	Quezaltenanago, Guatemala	1-A-2b	8A	A
38222	*S.g.g. vulg.*	Quillabamba, Peru	1-A-2b	8A	A
46585	*S.g.g. vulg*	Moyuta, Guatemala	1-A-2a	8A	A
46590	*S.g.g. vulg.*	Salama Finca, Guatemala	1-A-2a	8B	A
46589A	*S.g.g. vulg.*	Salama Finca, Venezuela	1-A-2a	8B	A
38606	*S.g.g.*	Mexico	1-A-2a	8A	A
cv Cook	*S.g.g. vulg.*	Bogota, Colombia	1-A-2a	8A	A
34911	*S.g.g. vulg.*	Rio Mogi Guacu, Sao Paulo, Brazil	1-A-2a	8A	A
33706A	*S.g.g.*	Oaxaca, Loma Bonita, Mexico	1-A-2a	10A.2	A
Q8442	*S.g.g.*	Oaxaca, Salinaz Cruz, Mexico	1-A-2a	10A.1	A
86145	*S.g.g.*	Nayarit, Tepic, Mexico	1-A-1	–	A
18750	*S.g.g. mic*	Primavera, Paraguay	1-A-1	10B.2	1
38385	*S.g.g.*	Merida, Venezuela	1-A-1	10B.1	A
40567A	*S.g.g.*	Bogota, Colombia	1-A-1	10A.2	A
40255	*S.g.g.*	Santa Cruz, Bolivia	1-A-1	10B.3	A
40263	*S.g.g.*	Cera, Crato, Brazil	1-A-1	10B.3	A
40294	*S.g.g.*	Campo Grande, Brazil	1-A-1	10B.3	A
34906	*S.g.g. can*	Nova Campinas, Brazil	2-B	14A	E
43206	*S.g.g. can*	Belo Horizonte, Brazil	2-B	14A	E
34912	*S. acuminata*	Matao, Brazil	2-B	13	B
34912	*S. acuminata*	Matao, Brazil	2-B	13	B
cv Oxley	*S. hippocampoides*	Itauga, Paraguay	2-A	6A	D
38640	*S. hippocampoides*	Cardoba, Argentina	2-A	6B	D
40257	*S. grandiflora*	Montero, Bolivia	2-A	4	F
37688	*S. grandiflora*	Montero, Bolivia	2-A	4	G
40293	*S. grandiflora*	Sao Jose de Rio Preto, Brazil	2-A	16	F
92975	*S. grandiflora*	Ponta Grossa, Brazil	2-A	16	G
34918	*S. grandiflora*	ex Matao, Brazil	2-A	16	F
CIAT accessions					
10136	*S.g.g.*	Goias, Brazil			J
1283	*S.g.g.*	Meta, Brazil			J
136	*S.g.g.*	Meta, Colombia			A
184	*S.g.g.*	Valle de Cauca, Colommbia			K
2031	*S.g.g.*	Bahia, Brazil			J
Others					
82009	*S.g.g.*				J
81145	*S.g.g.*				J
cv Graham	*S.g.g.*				A

Results

Characteristics of the rDNA spacer sequence of *Stylosanthes guianensis* genotypes

The ITS1 and -2 regions of *Stylosanthes guianensis* were amplified by means of the conserved primer pairs ITS4-5 and ITS2-5 as described by White et al. (1990) (Fig. 1a). Primer pair ITS2-5, which amplifies the ITS1 spacer region, generated a PCR product of about 300 bp. The PCR products were resolved as a single sharp DNA band on a 4% metaphor agarose gel. Primer pair ITS4-5 produced bands of low intensity, while primer pair ITS3-4 did not amplify the ITS2

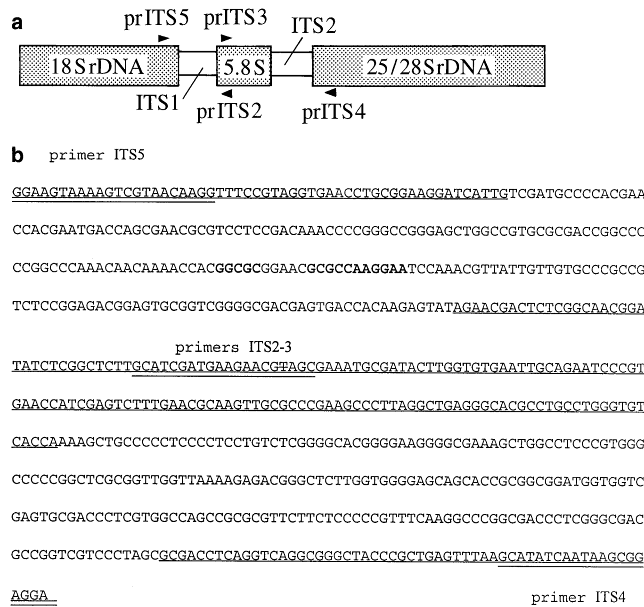

Fig. 1a,b **a** Schematic representation of the entire ITS1-5.8S-ITS2 rDNA region with the location of the conserved primers ITS2-3-4-5 as described by White et al. (1990). **b** Nucleotide sequence of the entire ITS1-5.8S-ITS2 rDNA region of *Stylosanthes guianensis guianensis* CIAT 1283. *Underlined* sequences indicate the flanking rDNA coding genes 18S, 5.8S and 26S, respectively. *Boldface print* indicates the conserved region as described by Liu and Schardl (1994). The primer binding sites of primer ITS5, ITS2-3 and ITS4 are *double underlined* with the difference in ITS2-3 to the sequence of *Stylosanthes* indicated by a *horizontal slash*. The nucleotide sequence is deposited in the EMBL DNA data library under accession number Y13481

spacer region. The PCR product from the ITS spacer region of *S. guianensis* CIAT 1283 was sequenced either directly or after cloning (Fig. 1b). The boundaries of the ITS1 and -2 regions were determined by comparing them to conserved regions of the rDNA coding sequence, i.e. the CATTG = 3′ end of 18S rDNA (Nickrent et al. 1994), AGAAC = 5′ end of 5.8S rDNA from *Gossypium*, the CACRY = 3′ end of 5.8S rDNA (Nickrent et al. 1994) and GCGA = 5′ end of 26S rDNA (Torres et al. 1990; Nickrent et al. 1994). The length of ITS1, 5.8S rDNA and ITS2 of *S. guianensis* CIAT 1283 was found to be 201, 264 and 218 bp, respectively, and the G + C content of both spacer regions was around 70%. The ITS1 region contains the conserved region unique to the central region of plant species (GGCRY . . . 4 to 7 nt . . . GYGYCAAGGAA) (Liu and Schardl 1994). The ITS2 spacer region does not contain such a conserved region. There was no indication of the existence of multiple ITS copies based on the sharp resolution of PCR products and the sequence derived from pooled PCR products. Nevertheless, this was investigated further by cloning and sequencing PCR products from the ITS1 region of *S. guianensis* CIAT 1283. All clones contained the same sequence, which may suggest complete homogenization of the ITS copies. The same conclusion was reached by the cloning and analysis of the PCR product from the entire ITS1-5.8S-ITS2 region of *S. guianensis* CIAT 1283. Hence, no evidence of intra- and inter-individual ITS length or sequence variation was found. The entire spacer region of *S. guianensis* CIAT 1283 was compared to data from other plant species in the EMBL DNA data library using the FASTA program. The ITS spacer regions are less conserved than the 5.8S rDNA. Still, the 5.8S rDNA differs in at least seven basepairs from other plant species, its closiest resemblance being to *Gossypium*, *Lupinus luteus* and mung bean. The position where primer ITS3 should bind differs at one nucleotide position from the *Stylosanthes* 5.8S rDNA sequence. This explains why primer pair ITS3-4 failed to produce a PCR product. ITS1 is 51% homologous to the ITS1 of *Clianthus puniceus*, showing a high resemblance in the central conserved region and downstream region. ITS2 is 53% and 47% homologous to the ITS2 region from *Astralagus* species and *C. puniceus*, respectively. The best homology was found in the first part. With the exception of *Gossypium*, these plant species belong to the Fabaceae.

Genetic diversity in the *Stylosanthes guianensis* (Aubl.) Sw. species complex based on the ITS DNA sequence

Several *Stylosanthes guianensis* accessions from Colombia (CIAT numbers) and Australia (CPI numbers) (Table 1) were examined to determine their genetic diversity based on the DNA sequence of the ITS1 region. Eleven different types (type A to type K) could be distinguished based on a total of 15 single base-pair substitutions and one three-base-pair indel with a transition/transversion ratio of 1.5 (Fig. 2). They were grouped according to Kimura distance followed by UPGMA and to maximum likelihood (Fig. 3), which produced the same group assignment of the different types. These results were compared to the classification of Kazan et al. (1993a), which is based on RAPD analysis (Table 1). As expected, less variation was apparent with ITS sequence analysis than with RAPD analysis. However, the main groups remained the same, except for some samples of 2-B (*S. acuminata* CPI 34912-CPI 34915). No data is available for type J and K in the RAPD analysis. *S. grandiflora* (type G, F), *S. hippocampoides* (type D) and *S.g.g.* var '*canescens*' (type E) each contain distinct ITS types. *S. acuminata* contains the same type (type B) as some S. *guianensis guianensis* accessions and therefore could not be distinguished from this group. Type J is very different from the others as a consequence of an indel and several base-pair substitutions. It is not similar to the rest of the *S. guianensis guianensis* group. *S. gracilis* (type H, C) contains 2 types of which 1 is also present in *S.g.g.* var 'vulgaris' (CPI 34908). *Stylosanthes guianensis*

Fig. 2 Multiple DNA sequence alignment of the ITS1 region from *Stylosanthes guianensis* genotypes indicated by type. *Underlined* sequences indicate restriction sites used for PCR-RFLP. Nucleotide sequences are deposited in the EMBL DNA data library under accession numbers Y13480 to Y13491

```
          TaqI       TaqI          BspMI       MaeII
type A    TCGATGCCCC ACGAACCACG AATGACCAGC GAACGCGTCC TCTGACAAAC CCCGGGCTGG
type B    .......... .......... ......A... .......... .......... ..........
type C    .......... .......... .......... .......... ..C....... ..........
type D    .......... .......... .......... .......... ..C....... ..........
type E    .......... .......... .......... .......... ..C....... ..........
type F    .......... .......... .......... .......... ..C....... ..........
type G    .......... .......... .......T.. .......... ..C....... ..........
type H    .......... .......... .......... .......... ..C....... ..........
type I    .......... .......... ......A... .....T.... .......... ..........
type J    .......... .......... .......... .......... ..C....... .......C..
type K    .......... T......... .......... .......... .......... ..........
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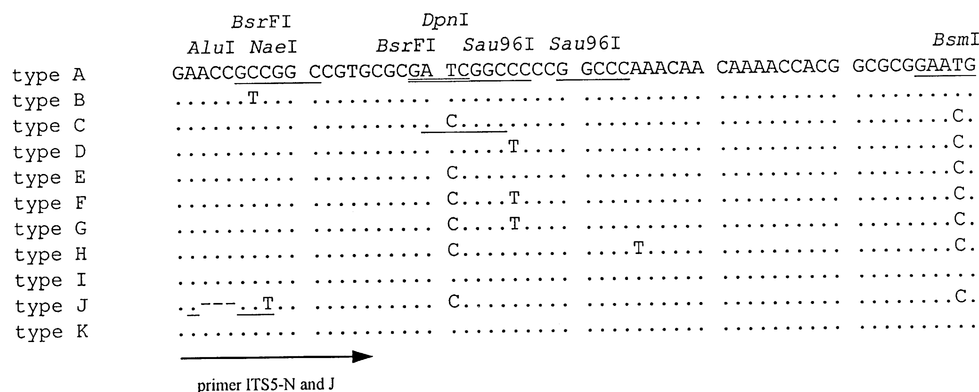


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                          MaeII
type A    CGCCAAGGAA TCCAAACGTT ATTGTTCTCC CCGCCGTCTC CGGAGACGGA GTGCGGTCGG
type B    .......... .......... .......... .......... .......... ..........
type C    .......... .......... .......... .......... .......... ..........
type D    .......... .......... .......... .......... .......... ..........
type E    .......... ......T... .......... .......... .......... ..........
type F    .......... .......... .......... .......... .......... ..........
type G    .......... .......... .......... .......... .......... ..........
type H    .......... .......... .......... .......... .......... ..........
type I    .......... .......... .......... .......... .......... ..........
type J    .......... .......... ......G.G. .......... .......... ..........
type K    .......... .......... .......... .......... .......... ..........

type A    GGCGACGAGT GACCACAAGA GTAT
type B    .......... .......... ....
type C    .......... .......... ....
type D    .......... .......... ....
type E    .......... .......... ....
type F    .......... .......... ....
type G    .......... .......... ....
type H    .......... .......... ....
type I    .......... .......... ....
type J    .......... .......... ....
type K    .......... .......... ....
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guianensis is very diverse because it contains 7 ITS1 types (types A-B-C-E-I-J-K).

Since it was not possible to amplify the ITS2 spacer region with primer pair ITS3-4, the entire ITS1-5.8S-ITS2 region was amplified with primer pair ITS4-5 (Fig. 1). Although product yield was low, fragments from *S. guianensis guianensis* cv 'Schofield' and CIAT 1283 were cloned and the ITS2 region was sequenced. This region is less variable than the ITS1 spacer region. There is no length variation and two transversions and transitions are randomly dispersed. The ITS2 spacer region was not analyzed in further.

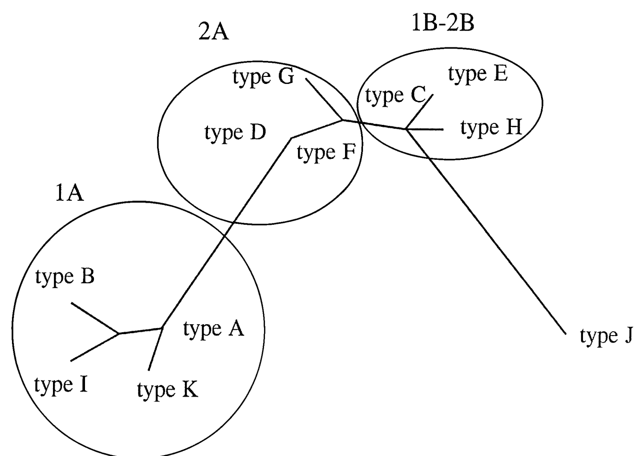

Fig. 3 Maximum likelihood tree based on the ITS1 DNA sequence, indicating relationships within the *Stylosanthes guianensis* complex and correlation with the group classification of Kazan et al. (1993a)

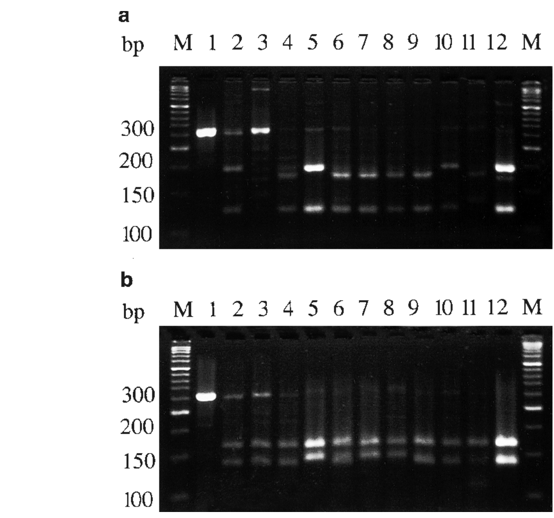

Fig. 4a, b Restriction site analysis of PCR products amplified by primer pair ITS5-ITS2 and digested by **a** *Bsr*FI and **b** *Sau*96I. *Lane 1* Control, i.e. undigested ITS1 PCR product, *lanes 2–12*: types A–K, *lane M* DNA size marker

Use of the ITS1 region as molecular tool to assess genetic diversity

Based on the ITS1 DNA sequence polymorphic sites, several sites were identified which can discriminate *Stylosanthes guianensis* genotypes by PCR-RFLP (Fig. 4). Types B-E-G-I-J-K each have a selective, unique restriction target (Table 2). The remaining types can be recognized with extra restriction digests. It is not possible to distinguish type C from H with PCR-RFLP. Most of the variation in restriction sites was found in the region preceding the highly conserved central region (Fig. 2). The usefulness of selective primers to discriminate *S. guianensis* genotypes was assessed by two selective primers containing a similar sequence except for the three-base-pair indel and 2 single base-pair substitutions (Fig. 2). Primer ITS5-J should amplify the ITS1 region of the type J, while the other primer ITS5-N should give a product in the other types in combination with primer ITS2. In contrast to what was expected, primer ITS5-N generated PCR products in all genotypes with differences in yield correlating to the types, i.e. type J gave lower yields. This may indicate the presence of multiple copies. However, this is in contrast with the results obtained from the direct sequencing approach and colony analysis. To ascertain the presence of multiple copies in type J genotypes, we sequenced directly PCR products amplified by the two selective primers in combination with primer ITS2. Primer pair ITS5-J and ITS2 produced PCR products with the expected DNA sequence, while primer pair ITS5-N and ITS2 amplified another ITS1 variant in type-J genotypes *S.g.g.* CIAT 10136 and CIAT 1283. As control, PCR products of *S.g.g.* CIAT 136 (type A) and CIAT 184 (type K) amplified by primer pair ITS5N and ITS2 were also sequenced. They contain the same DNA sequence as previously described. It seems that in type J genotypes, no complete homogenisation of the repeat has occurred in evolution. Because this product is identical to the ITS1 region of type-A genotypes and is amplified with

Table 2 List of restriction enzymes useful in discriminating *S. guianensis* genotypes with restriction targets indicated in bold

Restriction enzyme	Type										
	A	B	C	D	E	F	G	H	I	J	K
*Bsp*MI	0	0	0	0	0	**0**	**1**	0	0	0	0
*Alu*I	0	0	0	0	0	0	0	0	0	**1**	0
*Taq*I	**1**	1	1	1	1	1	1	1	1	1	**2**
*Mae*II	**1**	**1**	1	1	**0**	1	1	**1**	**2**	1	1
*Bsr*FI	**1**	**0**	**2**	1	2	**2**	2	**2**	1	1	1
*Nae*I	1	0	1	1	1	1	1	1	1	0	1
*Bsm*I	**1**	1	0	**0**	0	0	0	0	1	0	1
*Dpn*I	1	1	0	**1**	0	0	0	0	1	0	1
*Sau*96I	2	2	**2**	1	2	**1**	1	2	2	2	2

primer ITS5-N, this variant is likely to contain the indel of this type.

Discussion

The size ranges of the ITS1 and -2 spacer regions of *Stylosanthes guianensis* genotypes reported here fall within the narrow size distribution of ITS1 (194–265 bp) and ITS2 (216–224 bp) reported for other angiosperms (Baldwin 1993). The ITS1 spacer region is smaller than the ITS2 spacer region, as in other members of the Fabacea (Baldwin et al. 1995). The size of the 5.8S rDNA is one of the two reported sizes in angiosperms. The nucleotide difference in the 5.8S rDNA at the position where primer ITS3 should bind is also present in other members of the Fabaceae (Schiebel and Hemleben 1989; Yokota et al. 1989) and other plant species (D'Ovidio 1992). The G + C content is around 70% for both ITS spacer regions, which is high and not comparable to published ITS spacer regions from other members of the Fabaceae. The G + C composition of ITS1 equals that of ITS2, which indicates molecular coevolution of those regions as reported by Torres et al. (1990). On average, the 5′ end of the ITS1 region of *S. guianensis* genotypes is more variable than the 3′ end, with a particular region containing a lot of variable sites. In Cucurbitaceae, the 3′ end of the ITS1 spacer region is conserved and has a function as stem-loop structure (Torres et al. 1990). Near the center, two conserved regions described by Liu and Schardl (1994) are also present in ITS1 of *S. guianensis*. These regions show structural conservation possibly because of their role in the production of mature rRNA from primary transcripts (Venkateswarlu and Nazar 1991). This motif helps to facilitate alignment of ITS1 sequences for comparison. There is no interspersion of conserved and variable sites.

Because rDNA spacer regions are valuable for examining relationships within genera and among closely related genera (Baldwin et al. 1995), their usefulness in detecting intraspecific variation was assessed. The DNA sequence of the *Stylosanthes* 5.8S rDNA has a high homology with other plant species, which is in contrast to the spacer regions which show a low degree of homology with published sequences of other plant species. Therefore, these regions should be especially useful for quantifying relatedness among species, as was also observed by Chatterton et al. (1992) for primitive oat species. Surprisingly, intraspecific variation was detected in the ITS1 spacer region of *S. guianensis* genotypes. Ordinarily, as a result of homogenizing mechanisms associated with concerted evolution, rDNA repeats are usually very similar within individuals and species, although low intraspecific variation does exist in some plant species (Baldwin 1993; Sun et al. 1994). The most common polymorphism in the ITS spacer regions are single base-pair substitutions, which is in agreement with findings in other plant species (Baldwin et al. 1995). Transitions are more frequent than transversions. Also, most C to T transitions are preceding a G, which could be explained by deamination of methylated CpG dinucleotides (Torres et al. 1990). There are, however, no hot spots of C to T transition.

Evidence was provided for the existence of at least two copies in the individuals of type J. Apparently, concerted evolution failed to homogenize ITS1 paralogues of *S. guianensis* genotypes completely. It was not possible to detect this by the direct sequencing approach as ambiguous positions nor by the cloning strategy. It was incidently detected when specific primers were used to discriminate *S. guianensis* genotypes. The variant is probably present at a lower frequency. Two types of ITS sequences from individuals were also detected in some species in Winteraceae (Suh et al. 1993) and *Picea abies* (Karvonen et al. 1994).

The divergence values of the ITS2 sequence are slightly lower than those for ITS1 in *Stylosanthes guianensis guianensis*, which is consistent with results for Asteraceae (Baldwin 1993; Susanna et al. 1995) and Fabaceae (Wojciechowski et al. 1993). The ITS2 spacer region was not analyzed further.

The major centre of diversity of *Stylosanthes* is the southern Neotropics (particularly Brazil), with a secondary center in the Mexico-Caribbean basin and a minor development in Africa, India and Indonesia (Stace and Cameron 1984). *S. guianensis* is the most widely distributed species of the genus. Its natural distribution extends from Mexico to Argentina ('t Mannetje 1977). To obtain the greatest diversity of ITS types in the *Stylosanthes guinensis* complex, we performed ITS DNA sequence analysis on Central and South American accessions with emphasis on Brazilian accessions. The phylogenetic pattern contained within the data in Table 1 was plotted directly onto a map of the region. Figure 5 shows that most of the diversity can be found within northern/central South American accessions with the accent on Brazil.

The *Stylosanthes guianensis* species complex is treated differently by different authors. It is classified as one species with varieties according to 't Mannetje (1977, 1984), but as different species with subspecies according to Ferreira (1979) (Table 3). According to the ITS DNA sequence analysis, different species could be distinguished. *Stylosanthes guianensis guianensis* is very heterogeneous because it contains 7 ITS types (type A-B-I-J-E-C-K). This high degree of polymorphism could be the result of outcrossing (Miles 1985). These results are largely in agreement with the results of Kazan et al. (1993a), who placed types A-B-I-K in one group which is very distinct from *S.g.g.* var 'canescens' and found a close linkage between *S. hippocampoides* and *S. grandiflora.* Nevertheless, according to our analysis *S. acuminata* does not have a different ITS type than

Fig. 5 Geographical distribution of *Stylosanthes guianensis* accessions in relationship to the ITS1 types

Table 3 Taxonomic treatment of the *S. guianensis* species complex

't Mannetje (1977, 1984)	Ferreira and Costa (1979)
S. guianensis	
var robusta	*S. grandiflora*
var intermedia	*S. hippocampoides*
var gracilis	*S. gracilis*
var dissitiflora	*S. guianensis* ssp. *dissitiflora*
var longiseta	*S. longiseta*
var marginata	*S. acuminata*
var guianensis	*S. guianensis* ssp. *guianensis*
	var vulgaris
	var canescens
	var pauciflora
	var microcephala

some of the *S.g.g.* accessions and therefore cannot be regarded as a different species. This is in contrast to the hybridization results of Kazan et al. (1993a) where both species were distinguished from each other by low pollen stainability and low fertility. Also, *S. gracilis* is not distinguishable from some *S.g.g.* Based on ITS1 analysis, there is no distributional difference in *S. grandiflora*, which is in contrast to the results of Kazan et al. (1993a). Our results show that ITS DNA sequence analysis helps to elucidate taxonomic problems at the *S. guianensis* species level.

'T Mannetje (1977) recognized *S. guianensis* var 'guianensis' and *S. guianensis* var 'dissitiflora' in Mexico. This classification is based on limited collection numbers. Moreover, a recent morphological study of new Mexican *S. guianensis* collections (S. Gama, personal communication) failed to corroborate any previous taxonomic treatment of *S. guianensis* in Mexico. Genetic studies should complement the classical studies in order to clarify controversies. Because the ITS1 spacer region shows intraspecific variation, it is useful in discriminating *Stylosanthes guianensis* genotypes. Easy, quick and rather inexpensive methods are needed to study the natural variation in Mexican *Stylosanthes* populations on a molecular level. Because it is not possible to perform DNA sequence analysis on all populations, a limited number of samples need to be analyzed and the revealed variation transformed into PCR-RFLP markers. A knowledge of the genetic diversity of natural Mexican *S. guianensis* populations can contribute to efficient in situ germplasm conservation and their potential use in agronomy.

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References

Baldwin BG (1993) Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution reexamined. Am J Bot 80:222–238

Baldwin BG, Sanderson MJ, Porter JM, Wojciechowski MF, Campbell CS, Donoghue MJ (1995) The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. Ann Mo Bot Gard 82:247–277

Burt RL, Edye LA, Williams WT, Grof B, Nicholson CHL (1971) Numerical analysis of variation patterns in the genus *Stylosanthes* as an aid to plant introduction and assessment. Aust J Agric Res 22:737–757

Chatterton NJ, Hsiao C, Asay KH, Jensen KB, Wang RRC (1992) Nucleotide sequence of the internal transcribed spacer of rDNA in the primitive oat species, *Avena longiglumis* Durieu (Gramineae). Plant Mol Biol 20:163–164

D'Ovidio R (1992) Nucleotide sequence of a 5.8S rDNA gene and of the internal transcribed spacers from *Populus deltoides*. Plant Mol Biol 19:1069–1072

Doyle JJ (1993) DNA, phylogeny, and the flowering of plant systematics. BioScience 43:380–389

Edye LA,. Burt RL, Nicholson CHL, Williams RJ, Williams WT (1974) Classification of the *Stylosanthes* collection 1928–1969. CSIRO Div Trop Agron Tech Pap 15

Felsenstein J (1991) PHYLIP (phylogeny inference package), version 3.4. Computer program distributed by the author. The University of Washington, Seattle, Wash.

Ferreira MB, Costa NMS (1979) O genero *Stylosanthes* Sw. no Brasil. EPAMIG, Belo Horizonte

Flaman JM, Frebourg T, Moreau V, Charbonnier F, Martin C, Ishioka C, Friend SH, Iggo R (1994) A rapid PCR assay. Nucleic Acids Res 22:3259–3260

Gillies ACM, Abbott RJ (1996) Phylogenetic relationships in the genus *Stylosanthes* (*Leguminosae*) based upon chloroplast DNA variation. Plant Syst Evol 200:193–211

Hamby RK, Zimmer EA (1992) Ribosomal RNA as a phylogenetic tool in plant systematics. In: Soltis PS, Soltis DE, Doyle JJ (eds) Molecular systematics of plants. Chapman and Hall, New York London, pp 50–91

Higgins DG, Bleasby AJ, Fuchs R (1992) Clustal V: improved software for multiple sequence alignment. CABIOS 8 : 189–191

Inoue H, Nojima H, Okayama H (1990) High efficiency transformation of *Escherichia coli* with plasmids. Gene 96 : 23–28

Kanungo J, Pandey KN (1993) Kinasing PCR products for efficient blunt-end cloning and linker addition. Biotechniques 14: 912–913

Karvonen P, Szmidt AE, Savolainen O (1994) Length variation in the internal transcribed spacers of ribosomal DNA in *Picea abies* and related species. Theor Appl Genet 89 : 969–974

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

Kazan K, Manners JM, Cameron DF (1993b) Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. Theor Appl Genet 85 : 882–888

Kimura M (1980) A simple method for estimating evolutionary rate of base substitutions through comparitive studies of nucleotide sequences. J Mol Evol 16 : 111–120

Liu CJ, Musial JM, Smith FW (1996) Evidence for a low level of genomic specificity of sequence-tagged-sites in *Stylosanthes.* Theor Appl Genet 93 : 864–868

Liu JS, Schardl CL (1994) A conserved sequence in internal transcribed spacer 1 of plant nuclear rRNA genes. Plant Mol Biol 26 : 775–778

't Mannetje L (1977) A revision of varieties of *Stylosanthes guianensis* (Aubl.) Sw. Aust J Bot 25 : 347–362

't Mannetje L (1984) Considerations on the taxonomy of the genus *Stylosanthes.* In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes* . Academic Press, London, pp 1–21

Miles JW (1985) Evaluation of potential genetic traits and estimates of outcrossing rate in *Stylosanthes guianensis.* Aust J Agric Res 36 : 259–265

Nickrent DL, Schuette KP, Starr EM (1994) A molecular phylogeny of *Arceuthobium* (Viscaceae) based on nuclear ribosomal DNA internal transcribed spacer sequences. Am J Bot 81 : 1149–1160

Robinson PJ, Megarrity RG (1975) Characterisation of *Stylosanthes* introductions by using seed protein patterns. Aust J Agric 26 : 467–479

Rosenthal A, Coutelle O, Craxton M (1993) Large-scale production of DNA sequencing templates by microtitre format PCR. Nucleic Acids Res 21 : 173–174

Schiebel K, Hemleben V (1989) Nucleotide sequence of the 18S-25S spacer region from rDNA of mung bean. Nucleic Acids Res 17 : 2852

Stace HM (1982) Breeding systems in *Stylosanthes.* I. Observations of outcrossing in *S. scabra* at an alcohol dehydrogenase locus. Austr J Agric Res 33 : 87–96

Stace HM, Cameron DF (1984) Cytogenetics and the evolution of *Stylosanthes.* In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes.* Academic Press, London, pp 49–72

Suh Y, Thien LB, Reeve HE, Zimmer EA (1993) Molecular evolution and phylogenetic implications of internal transcribed spacer sequences of ribosomal DNA in Winteraceae. Am J Bot 80: 1042–1055

Sun Y, Skinner DZ, Liang GH, Hulbert SH (1994) Phylogenetic analysis of *Sorghum* and related taxa using internal transcribed spacers of nuclear ribosomal DNA. Theor Appl Genet 89 : 26–32

Susanna A, Jacas NG, Soltis DE, Soltis PS (1995) Phylogenetic relationships in tribe Cardueae (Asteraceae) based on ITS sequences. Am J Bot 82 : 1056–1068

Torres RA, Ganal M, Hemleben V (1990) GC balance in the internal transcribed spacers ITS1 and ITS2 of nuclear ribosomal RNA genes. J Mol Evol 30 : 170–181

Van Campenhout S, Vander Stappen J, Sagi L, Volckaert G (1995) Locus-specific primers for LMW glutenin genes on each of the group 1 chromosomes of hexaploid wheat. Theor Appl Genet 91 : 313–319

Venkateswarlu K, Nazar R (1991) A conserved core structure in the 18-25S rRNA intergenic region from tobacco, *Nicotiana rustica.* Plant Mol Biol 17 : 189–194

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols: a guide to methods and applications. Academic Press, New York, pp 315–322

Williams RJ, Reid R, Schultze-Kraft R, Costa NMS, Thomas BD (1984) Natural distribution of *Stylosanthes.* In: Stace HM, Edye LA (eds) The biology and agronomy of *Stylosanthes.* Academic Press, London, pp 73–101

Wojciechowski MF, Sanderson MJ, Baldwin BG, Donoghue MJ (1993) Monophyly of aneuploid *Astralagus* (Fabaceae): evidence from nuclear ribosomal DNA internal transcribed spacer sequences. Am J Bot 80 : 711–722

Yokota Y, Kawata T, Iida Y, Kato A, Tanifuji S (1989) Nucleotide sequences of the 5.8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. J Mol Evol 29 : 294–301