Plant Syst. Evol. 234: 27–51 (2002)
DOI 10.1007/s00606-002-0193-1

Plant Systematics and Evolution
Printed in Austria

Phylogenetic analysis of *Stylosanthes* (Fabaceae) based on the internal transcribed spacer region (ITS) of nuclear ribosomal DNA

J. Vander Stappen[1], **J. De Laet**[2], **S. Gama-López**[3], **S. Van Campenhout**[1], and **G. Volckaert**[1]

[1]Katholieke Universiteit Leuven, Laboratory of Gene Technology, Leuven, Belgium
[2]American Museum of Natural History, Division of Invertebrate Zoology, New York, USA
[3]Universidad Nacional Autónoma de México, Laboratorio de Recursos Naturales, Municipio Tlalnepantla, Estado de México, México

Received August 14, 2001; accepted March 4, 2002
Published online: November 14, 2002

Abstract. Phylogenetic relationships in *Stylosanthes* are inferred by DNA sequence analysis of the ITS region (ITS1–5.8S–ITS2) of the nuclear ribosomal DNA in 119 specimens, representing 36 species of *Stylosanthes* and 7 species of the outgroup genera *Arachis* and *Chapmannia*. In all examined specimens of any particular diploid and (allo)polyploid species, only a single ITS sequence type was observed. This allowed us to identify a parental genome donor for some of the polyploids. In several diploid and polyploid species, different specimens contained a different ITS sequence. Some of these sequence types were present in more than one species. Parsimony analysis yielded several well-supported clades that agree largely with analyses of the chloroplast *trn*L intron and partially with the current sectional classification. Discordances between the nuclear and cpDNA analyses are explained by a process of allopolyploidization with inheritance of the cpDNA of one parent and fixation of the ITS sequences of the other. *S. viscosa* has been an important genome donor in this process of speciation by allopolyploidy.

Key words: Fabaceae, *Stylosanthes*. Internal transcribed spacer region (ITS), molecular phylogeny, allopolyploid, DNA sequence analysis.

The genus *Stylosanthes*, established by Swartz in 1788, is a member of the family Fabaceae, subfamily Papilionoideae, tribe Aeschynomeneae, subtribe Stylosanthineae (Rudd 1981). Depending on the treatment, the genus includes 30 to 45 herbaceous or suffruticose species and a variable number of subspecies and varieties of neotropical origin ('t Mannetje 1984). Most of these are native to the tropical and subtropical regions of Central and South America, while a few are distributed in the southeastern part of the United States, the Antilles, the Galapagos Islands, central and southern Africa, Madagascar, and southern India and Ceylon (Mohlenbrock 1958, Williams et al. 1984). The major centre of diversity for the genus is in the southern neotropics, particularly Brazil, with a secondary centre in the Mexico – Caribbean basin (Stace and Cameron 1984). Several species of the genus are agronomically important in tropical and subtropical agriculture as pasture and forage legumes (Burt and Miller 1975), as green manure (Gardener 1984), as cover crop (Thomas 1984), and more recently in the biological control of cattle tick (Khudrathulla and Jagannath 1998).

Stylosanthes, which is morphologically close to the genera *Arachis*, *Arthrocarpum*, *Chapmannia* and *Pachecoa*, is characterized by sessile flowers, monadelphous stamens which are often united in a closed tube, dimorphic anthers, elongate receptacles, 3-foliate leaves, and stipules that are fused with the petiole; the fruits are loments that develop aboveground (Burkart 1939, Rudd 1981). Two subgeneric sections, sect. *Stylosanthes* Vog. and sect. *Styposanthes* Vog., have been recognized by the absence or presence of an axis rudiment, which is thought to be an aborted secondary floral axis (Taubert 1891), and the presence of one or two inner bracteoles, respectively (Kirkbride and Kirkbride 1987).

The basic chromosome number of *Stylosanthes* is $x = 10$, with diploid, tetraploid and one hexaploid species identified. While diploid species are found in both sections, polyploid species are restricted to sect. *Styposanthes* and they are thought to be products of intersectional hybridization between diploids, followed by polyploidization (Stace and Cameron 1984). One exception to the intersectional origin of allopolyploids in *Stylosanthes* has been observed by Liu et al. (1999) for the species *S. capitata*, which is most probably formed by hybridization between the diploid species *S. pilosa* and *S. macrocephala*, both of sect. *Styposanthes*. With exception of *S. hamata* and *S. mexicana*, polyploids are not known to occur as subspecific races (Stace and Cameron 1984, S. Gama-López, unpubl. data). The allopolyploid origin of the polyploids has been confirmed by biochemical and molecular data (Table 1).

Apart from the division into two sections, on which all authors agree, the taxonomic treatment of *Stylosanthes* is very ambiguous. A first detailed study of the genus was done by Vogel (1838), who recognized fifteen species. Later several new species, varieties and forms were described and the classification became rather confusing (Bentham 1859, Taubert 1891, Hassler 1919, Blake 1920, Burkart 1939). This is well illustrated by the long list of synonyms in the revisions of Mohlenbrock (1958, 1960, 1963). The more recent taxonomic studies of the genus are by Ferreira and Costa (1979) and 't Mannetje (1984). Ferreira and Costa (1979) concentrated on the species of Brazilian origin and emphasized the number of vascular bundles, the venation of the leaflets and the growth habit as main diagnostic features. In contrast, 't Mannetje (1984) relied mostly on fruit morphology, stressing in particular the shape and length of the beak, the indumentation of the pod, and the width and venation of the outer bract. This differential emphasis on different characteristics may explain why several members of the *S. guianensis* complex have been classified as distinct species by Ferreira and Costa (1979) but as varieties by 't Mannetje (1977, 1984; Table 2). The occurrence of major fertility barriers between members of this complex (Hacker et al. 1988), however, supports the former taxonomic approach. Due to the lack of stable morphological characters, the identification and delimitation of several other species is still problematic ('t Mannetje 1984). For instance, *S. scabra*, *S. tuberculata* and *S. nervosa* are very difficult to distinguish because they have virtually identical pods and because the vegetative characters used to separate them are very variable, raising the question of whether the latter two species should be subsumed into *S. scabra*.

Given the difficulties in species delimitation and the agronomic importance of *Stylosanthes* in tropical agriculture, it comes as no surprise that other techniques have been used to complement the morphological approach. Most of these studies, however, were not intended to be full taxonomic surveys and/or have been restricted to widespread, well-known or agronomically important *Stylosanthes* species, while minor species have been neglected (e.g. Edye et al. 1974, Williams et al. 1984, Stace and Cameron 1987, Vieira et al. 1993). As a first step toward a phylogenetic overview of the whole genus, Gillies and Abbott (1996) performed an analysis of chloroplast DNA (cpDNA) restriction fragment length polymorphisms (RFLP) in 18 *Stylosanthes* species. A second phylogenetic study is based on sequence analysis of the

Table 1. List of polyploid *Stylosanthes* species, with their putative parental genome donors

Polyploid species	Putative parental genome donors[a]	References[b]
S. capitata (4×)	*S. macrocephala* (♀)	Gillies and Abbott (1996), Vander Stappen et al. (1999a)
	S. macrocephala × *S.* sp.	Vander Stappen et al. (1999b)
	S. macrocephala × *S. pilosa*	Liu et al. (1999)
	S. macrocephala	This study
S. erecta (6×)	*S.* sp. (clade 3) (♀)	J. Vander Stappen (unpubl.data)
	S. sp. (clade 3) × *S. viscosa*	Vander Stappen et al. (1999b)
	S. seabrana/*S. hamata* × *S.* aff. *viscosa* × *S.* sp.	Liu et al. (1999)
	S. viscosa	This study
S. fruticosa (4×)	*S.* sp. (clade 3) (♀)	Vander Stappen and Volckaert (1999)
	S. sp. (clade 3) × *S. viscosa*	Vander Stappen et al. (1999b)
	S. viscosa	This study
S. hamata s. l. (4×)	*S. humilis* × *S. hamata* s. str.	Stace and Cameron (1984, 1987), Curtis et al. (1995)
	S. humilis (♀)	Gillies and Abbott (1996), Vander Stappen et al. (1999a)
	S. humilis × *S. hamata* s. str.	Vander Stappen et al. (1999b)
	S. humilis	This study
	S. hamata s. str.	This study
	S. viscosa	This study
S. ingrata (4×)	*S.* sp. (clade 3) (♀)	Vander Stappen et al. (1999a)
	S. sp. (clade 3) × *S. viscosa*	Vander Stappen et al. (1999b)
	S. viscosa	This study
S. mexicana (4×)	*S.* sp. (clade 3) (♀) × *S. viscosa*	J. Vander Stappen (unpubl. data)
	S. viscosa	This study
S. scabra (4×)	*S. viscosa* (♀) or *S. hamata* s. str. (♀)	Gillies and Abbott (1996)
	S. seabrana × *S. viscosa*	Liu and Musial (1997)
	S. sp. (clade 3)	Vander Stappen et al. (1999a)
	S. sp. (clade 3) × *S. viscosa*	Vander Stappen et al. (1999b)
	S. viscosa	This study, Stace and Cameron (1984)
S. sericeiceps (4×)	*S.* sp. (clade 3) (♀)	J. Vander Stappen (unpubl. data)
	S. seabrana/*S. hamata* × *S.* aff. *viscosa*	Liu et al. (1999)
	S. viscosa	This study
S. subsericea (4×)	*S.* sp. (clade 3) (♀) × *S. viscosa*	Gama-López et al. (2001)
	S. viscosa	This study
S. sundaica (4×)	*S. humilis* × *S. hamata* s. str.	Stace and Cameron (1984), Liu et al. (1999)
	S. humilis (♀) × *S. hamata* s. str.	J. Vander Stappen (unpubl. data)
	S. hamata	This study
S. sympodialis (4×)	*S. humilis* (♀)	Gillies and Abbott (1996)
	S. sp. × *S. seabrana*/*S. hamata*	Liu et al. (1999)
	S. sp. (clade 1) (♀)	Vander Stappen et al. (1999a)
	S. sp. (clade 1) × *S.* sp. (clade 3)	Vander Stappen et al. (1998b)
	S. sp. (clade 1)	This study
S. tuberculata (4×)	*S. seabrana*/*S. hamata* × S. aff. *viscosa*	Liu et al. (1999)

Table 1 (continued)

Polyploid species	Putative parental genome donors[a]	References[b]
	S. sp. (clade 3) (♀)	J. Vander Stappen (unpubl. data)
	S. viscosa	This study

[a] *S.* sp. (clade 3) = *S.* aff. (*calcicola*, *hamata*, *mexicana*, *macrocarpa*, *seabrana*)
S. sp. (clade 1) = *S.* aff. (*viscosa*, *humilis*, *leiocarpa*, *angustifolia*)
[b] Biochemical and molecular data used to infer parental genome donors: cpDNA RFLP (Gillies and Abott, 1996); cpDNA *trn*L DNA sequence (Vander Stappen et al. 1999a, Vander Stappen and Volckaert 1999, J. Vander Stappen, unpubl. data); sequence-tagged site (STS) analysis (Liu and Musial 1997, Liu et al. 1999, Vander Stappen et al. 1999b, Gama-López et al. 2001); nuclear RFLP analysis (Curtis et al. 1995, Liu et al. 1999); isozymes (Stace and Cameron 1984, 1987)

Table 2. Taxonomic treatment of the *S. guianensis* species complex

't Mannetje (1977, 1984)	Ferreira and Costa (1979)
S. guianensis (Aubl.) Sw.	
var. *robusta* 't Mannetje	*S. grandifolia* M.B. Ferreira & S. Costa
	S. aurea M.B. Ferreira & S. Costa
var. *intermedia* (Vog) Hassl.	*S. hippocampoides* Mohlenbr.
	S. campestris M.B. Ferreira & S. Costa
var. *gracilis* (Kunth) Vog.	*S. gracilis* Kunth
var. *dissitiflora* (Robins. & Seat.) 't Mannetje	Not studied
var. *longiseta* (Micheli) Hassl.	*S. longiseta* Micheli
var. *marginata* Hassl.	*S. acuminata* M.B. Ferreira & S. Costa
var. *guianensis* (Aubl.) Sw.	*S. guianensis* ssp. *guianensis*
	var. *vulgaris* M.B. Ferreira & S. Costa
	var. *canescens* M.B. Ferreira & S. Costa
	var. *pauciflora* M.B. Ferreira & S. Costa
	var. *microcephala* M.B. Ferreira & S. Costa

chloroplast *trn*L intron of 35 *Stylosanthes* species (Vander Stappen et al. 1999a, J. Vander Stappen, unpubl. data). This study revealed four major clades; the clade containing the *S. guianensis* complex, *S. biflora*, and *S. montevidensis* appeared as sister group to the three other clades, which were grouped in polytomy. Because of strict maternal inheritance and low DNA sequence variation of the chloroplast DNA of *Stylosanthes*, and because of the occurrence of phenomena such as interspecific cytoplasmic gene flow, the use of nuclear DNA sequence data is highly recommendable to confirm these results and to elucidate the origin of *Stylosanthes* polyploids.

In this study, we use the internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA). The high copy number, rapid homogenization, small size and length conservation of the ITS spacer regions make them useful for amplification, sequencing and alignment to resolving relationships within genera and below the species level (Hillis and Dixon 1991, Hamby and Zimmer 1992, reviewed in Baldwin et al. 1995). In addition, due to fixation or additivity of the parental ITS repeat types in allopolyploid species, ITS sequence analysis often allows inference of maternal and/or paternal progenitors of these species (Kim and Jansen 1994, O'Kane et al. 1996, Wendel et al. 1995, Sang et al. 1995, Franzke and Mummenhoff 1999, Volkov et al. 1999). In order to better understand and to help resolve the taxonomic problems in *Stylosanthes*, it is our aim (1) to infer the phylogenetic relationships in the genus on the basis of ITS sequences; (2) to compare the

ITS results with previous taxonomic data; and (3) to provide evidence for the origin and parentage of allopolyploid species in *Stylosanthes*.

Materials and methods

Plant material. We used a total of 112 specimens of 36 different species of *Stylosanthes* (see Appendix 1; we follow Ferreira and Costa (1979) for the nomenclature). These represent a broad range of the morphological and ecogeographical diversity of the genus (Mohlenbrock 1958, Williams et al. 1984). Nine known and described species (sect. *Stylosanthes*: *S. figueroae* Mohlenbr., *S. debilis* M.B. Ferreira & S. Costa, *S. longicarpa* M. Brandão & N.M. Sousa Costa, *S. longiseta* Micheli, *S. nunoi* M. Brandão, *S. suborbiculata* Chiov.; sect. *Styposanthes*: *S. nervosa* J.F. Macbr., *S. ruellioides* Mart. ex Benth., *S. suffruticosa* Mohlenbr.) were not included because no plant material was available or because the quality of the DNA that we obtained was insufficient. Seven species of the genera *Arachis* and *Chapmannia*, each represented by a single specimen, were used as outgroups (see Rudd 1981, Beyra and Lavin 1999, and Lavin et al. 2001).

DNA isolation, polymerase chain reaction (PCR), and sequencing. Plant material for DNA analysis was obtained from existing herbarium specimens or from plants that were grown from seeds. Seeds were germinated on filter paper in Petri dishes at 25 °C. After germination, young seedlings were grown in pots. Young leaves were harvested from these plants and dried in silica gel to prevent degradation of the DNA (Chase and Hills 1991). Total DNA was isolated from a 3-foliate leaf of either dried herbarium specimens or fresh tissue dried in silica gel, following the procedure as described in Vander Stappen (1999). The entire ITS region, comprising ITS1, 5.8S gene and ITS2, was amplified by means of the universal primer pairs ITS5 and ITS4 of White et al. (1990). In cases where these primers did not amplify adequate PCR products, the ITS region was amplified using more specific primer pairs, ITS5sty (5′ CGGAAGGAT CATTGTCGATG 3′) and ITS4sty (5′ CTGACCT-GAGGTCGCGCT 3′). The 3' end of these primers bind to the first five and last three nucleotides of the ITS1 and ITS2 spacer region of *Stylosanthes*, respectively. Primers were purchased from Genset (Paris, France). PCR reactions contained 1x PCR buffer (Qiagen, Hilden, Germany), 200 μM of each dNTP, 1 μM of each primer, 0.625 units HotStarTaq 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, 1.30 min at 72 °C, and a final extension step of 5 min at 72°C on a UNOII 96 Thermocycler (Biometra, Göttingen, Germany). After visual inspection of the PCR products by electrophoresis on a 1.5% TAE agarose gel (GibcoBRL, Gaithersburg, USA) and subsequent UV illumination, the products were purified using the Qiaquick PCR purification kit (Qiagen). All steps were performed according to the specification of the supplier. Purified PCR products were sequenced directly in both directions by the ABI PRISM DyeDeoxy terminator sequencing protocol (Applied Biosystems, Foster City, USA) and by using the primer pairs ITS4–5 or ITS4–5sty. 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 Appendix 1.

Data analysis. The ITS region boundaries were determined as described in Vander Stappen et al. (1998). Proofreading and editing of the DNA sequences were done with the program Sequencher v3.0 (Gene Codes Corporation, Ann Harbor, MA, USA). Alignments were made using ClustalX (Thompson et al. 1997) with the default settings. Subsequently, the alignments were adjusted manually. Potentially informative insertion/deletion events (indels) were included in the data matrix by adding extra characters. The NEXUS data matrix with the sequence alignments of the entire ITS region is available through the internet address http://www.agr.kuleuven.ac.be/dp/logt/onderzoek/stylodata.htm.

The data were analyzed using parsimony (Farris 1970, Fitch 1971) with equal a priori character weights and unordered characters. In the alignment, gaps were treated as missing values. The basic analyses were performed with the computer program Nona 2.0 (default settings; Goloboff 1993). By default, Nona collapses all branches that have no unambiguous synapomorphies (a character provides an unambiguous synapomorphy for a branch if a state transition occurs on that branch under every possible optimization of the character on the tree). This method of

treating zero-length branches may collapse branches of a tree that cannot simultaneously be of zero-length. To remove such overcollapsed trees, it is sufficient to optimize the polytomies and keep only those trees that are still of minimal length, as pointed out by Goloboff (1993; see also Coddington and Scharff 1994). This was done using the computer program WinClada (Nixon 1999; WinClada runs Nona as a subprocess).

The most parsimonious trees were calculated with "MULT*100". This instruction carries out 100 replications of randomizing the order of the taxa, creating a tree by means of stepwise addition, and submitting it to branch-swapping by means of tree bisection and reconnection. During each replicate a maximum of 20 trees was retained ("HOLD/20" setting, the default). As descriptive measures of the fit between data and trees, we calculated consistency and retention indices (C and R; Kluge and Farris 1969, Farris 1989). As an indication of the relative lengths of the branches of the most parsimonious trees, we calculated the number of unambiguous changes for each branch. Trees are depicted with the root placed arbitrarily between the ingroup and the outgroups. To evaluate a possible influence of outgroup choice on the relationships within *Stylosanthes*, we performed alternative analyses in which either *Chapmannia* or *Arachis*, or both, were excluded.

In order to evaluate the relative support of clades, we calculated Bremer branch support, i.e., the number of extra steps needed to lose a branch in the strict consensus of near-most-parsimonious trees (Källersjö et al. 1992, Bremer 1994). Because of the high number of near-most-parsimonious trees, only trees up to one step longer than the shortest trees were calculated (command "BSU 1" in Nona). We also performed a jackknife analysis (Farris et al. 1996), using the computer program xac that was kindly provided by J. S. Farris. We ran 1000 pseudoreplicates, each with a character removal probability of 37%. Each pseudoreplicate was analyzed using ten random addition sequences and branch swapping. A pseudoreplicate was considered as supporting a clade only when that clade was present in all trees for that replicate.

Results

Characteristics of the entire ITS region in *Stylosanthes* and the outgroup species. The main characteristics of the ITS region are summarized in Table 3. Length variation for the entire ITS region of *Stylosanthes* ranged from 579 to 586 bp. The ITS1 and ITS2 regions varied in length between 196 and 204 bp and between 217 and 223 bp, respectively. When compared to *Stylosanthes*, the length of the entire ITS region in *Arachis* and *Chapmannia prismatica* was somewhat larger and smaller, respectively. The 5.8S subunit was uniformly 164 bp in all samples. The G + C content varied from 62.7 to 68 %, 53.6 to 56 % and 69.8 to 74.3 % in the ITS1, 5.8S and ITS2 region of *Stylosanthes*, respectively. Similar G + C content values were found in the ITS region of the outgroup species.

The size of the alignment of the entire ITS region over all species was 608 bp. Sixteen indels were introduced, 10 of which are variable within *Stylosanthes*. The length of the indels ranged from 1 to 9 bp; seven indels were included as additional characters in the dataset that was used for phylogenetic analysis (5 in ITS1 and 2 in ITS2). Proportionally as well as in absolute numbers ITS1 and ITS2 have about the same number of informative characters. Compared to ITS, the 5.8 S region is much more conserved, both in terms of length and in terms of variable positions.

In the diploid as well as in the polyploid species, no evidence of divergent paralogous rDNA repeat types within single individuals was found. When sequencing, no ambiguous positions were observed, indicating that only one ITS sequence type was amplified and sequenced in each individual. However, this does not necessarily mean that only a single type is present: PCR drift (Wagner et al. 1994) may result in the amplification of one repeat type, while other minor types remain undetected.

In about half of the taxa, nucleotide and/or length variation was observed when comparing different specimens. In three cases, specimens of pairs of diploid species, i.e. *S. guianensis* – *S. gracilis*, *S. cayennensis* – *S. hispida* and *S. mexicana* – *S. macrocarpa*, were found to contain the same ITS sequence type. In addition, some specimens of several polyploid

Table 3. Characteristics of the ITS region (ITS1-5.8S-ITS2) in *Stylosanthes* and outgroup species

Characteristics	ITS1	5.8S	ITS2	ITS region
Length range (bp)				
Within *Stylosanthes*	196–204	164	217–223	579–586
Within *Arachis*	206–207	164	218–220	589–592
Chapmannia prismatica	191	164	220	575
Mean G + C content (%)				
Within *Stylosanthes*	66.2	54.3	71.8	64.9
Within *Arachis*	65.5	54.2	72.4	65
Chapmannia prismatica	63.9	54.2	72.7	64.5
Variable characters (total, (%))				
Within *Stylosanthes*	59 (26.5)	5 (3)	56 (24.5)	120 (19.5)
Stylosanthes + outgroup	92 (41.2)	6 (3.6)	87 (38)	185 (30)
Informative characters (total, (%))				
Within *Stylosanthes*	41 (18.4)	4 (2.4)	41 (18)	86 (14)
Stylosanthes + outgroup	62 (27.8)	4 (2.4)	57 (25)	123 (20)

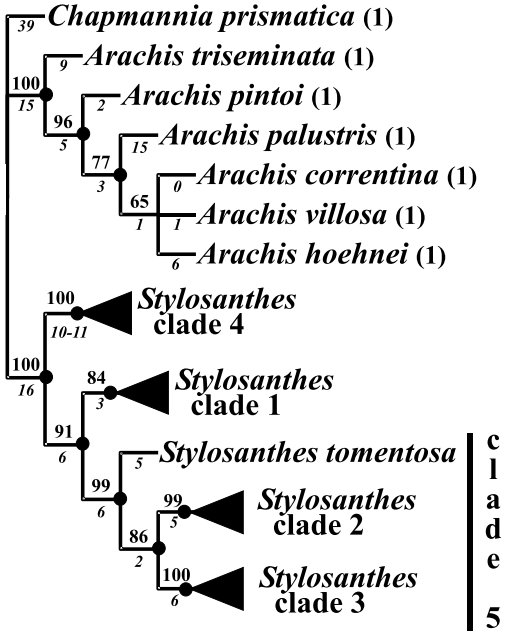
species contained an ITS region that was identical to sequences that were also observed in the diploid species (Fig. 1).

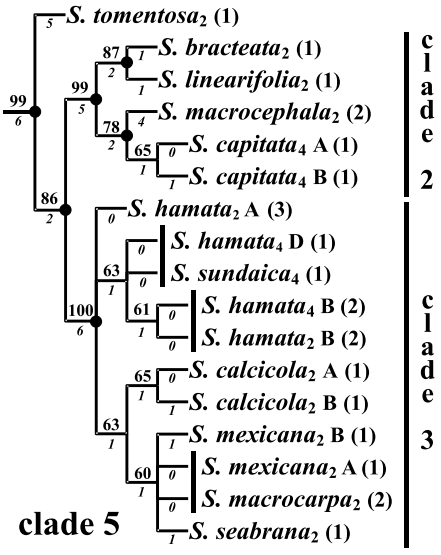
Phylogenetic analysis. A total of 57 different sequences were observed in the 112 specimens of the 36 *Stylosanthes* species under study, and an additional seven in the seven outgroup species. For clarity of presentation, identical sequences that were observed between different (sub)species, or between diploid and tetraploid specimens of *S. hamata*, were retained as separate entries in the dataset. They are depicted as horizontal bars on Fig. 1.

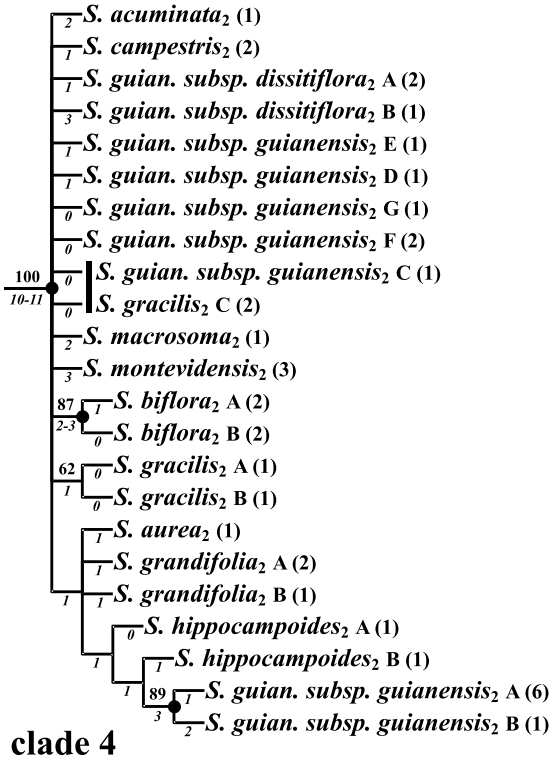
Parsimony analysis resulted in 3 most parsimonious trees, with length 321, consistency index 0.77 (excluding uninformative characters) and retention index 0.95. In each tree, unambiguous changes accounted for over 87% of the total number of steps. The strict consensus is shown in Fig. 1. The differences between the three trees are restricted to clade 4, in which the basal polytomy is partially resolved in three different ways. The relationships in *Stylosanthes* that are obtained when *Arachis* and/or *Chapmannia* are excluded are fully compatible with the consensus tree that is obtained with all outgroups included.

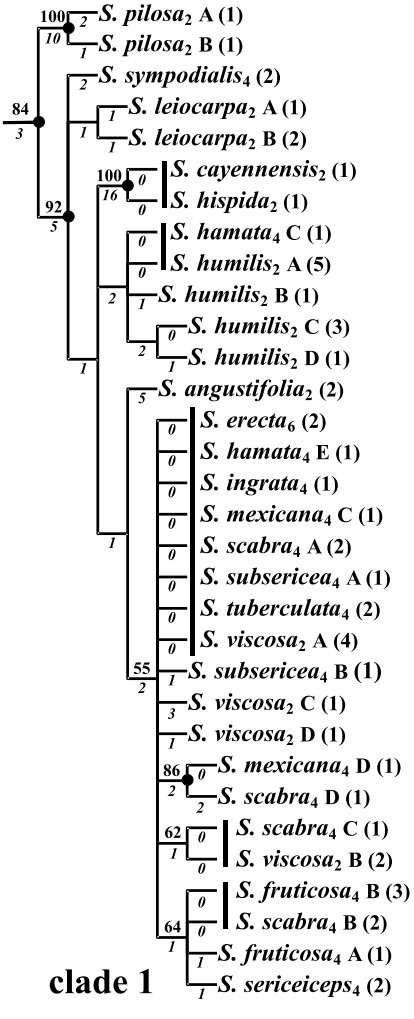
Both *Arachis* and *Stylosanthes* are highly supported monophyletic groups, each with a jackknife support (J.S.) of 100%. As we have included only one representative of *Chapmannia*, nothing can be concluded about the monophyly of this genus. Within *Stylosanthes*, several well-supported groups are present, most notably 4 clades that, apart from the polyploids, agree with clades 1 to 4 of Vander Stappen et al. (1999a). We therefore designate these with the same name. Their jackknife supports are 84%, 99%, 100%, and 100%, respectively. In addition, a sisterpair relationship between clades 2 and 3 is supported at 86%. This clade, in turn, is sister to *S. tomentosa* with a J.S. of 99%. We designate this clade as clade 5. With the exception of *S. tomentosa*, the species of section *Stylosanthes* are restricted to clades 1 and 4. Clades 2 and 3 contain all diploid species of section *Styposanthes* with the exception of *S. pilosa* (clade 1), but only 3 of the 12 polyploid taxa traditionally attributed to *Styposanthes*.

Clade 4 is sister to the rest of the genus and contains the diploid species *S. macrosoma*, *S. montevidensis*, *S. biflora,* and the diploid *S. guianensis* complex. While the clade as a whole is highly supported (J.S. 100%), its inner structure is poorly resolved. Only six nodes are present in the strict consensus, and only 3 of these have a J.S. over 50%. A clade that consists of *S. aurea*, some specimens of *S. guianensis*, *S. grandifolia* and *S. hippocampoides* is


Chapmannia prismatica (1)
Arachis triseminata (1)
Arachis pintoi (1)
Arachis palustris (1)
Arachis correntina (1)
Arachis villosa (1)
Arachis hoehnei (1)
Stylosanthes clade 4
Stylosanthes clade 1
Stylosanthes tomentosa
Stylosanthes clade 2
Stylosanthes clade 3
clade 5


S. tomentosa2 (1)
S. bracteata2 (1)
S. linearifolia2 (1)
S. macrocephala2 (2)
S. capitata4 A (1)
S. capitata4 B (1)
S. hamata2 A (3)
S. hamata4 D (1)
S. sundaica4 (1)
S. hamata4 B (2)
S. hamata2 B (2)
S. calcicola2 A (1)
S. calcicola2 B (1)
S. mexicana2 B (1)
S. mexicana2 A (1)
S. macrocarpa2 (2)
S. seabrana2 (1)
clade 2
clade 3


S. acuminata2 (1)
S. campestris2 (2)
S. guian. subsp. dissitiflora2 A (2)
S. guian. subsp. dissitiflora2 B (1)
S. guian. subsp. guianensis2 E (1)
S. guian. subsp. guianensis2 D (1)
S. guian. subsp. guianensis2 G (1)
S. guian. subsp. guianensis2 F (2)
S. guian. subsp. guianensis2 C (1)
S. gracilis2 C (2)
S. macrosoma2 (1)
S. montevidensis2 (3)
S. biflora2 A (2)
S. biflora2 B (2)
S. gracilis2 A (1)
S. gracilis2 B (1)
S. aurea2 (1)
S. grandifolia2 A (2)
S. grandifolia2 B (1)
S. hippocampoides2 A (1)
S. hippocampoides2 B (1)
S. guian. subsp. guianensis2 A (6)
S. guian. subsp. guianensis2 B (1)
clade 4


S. pilosa2 A (1)
S. pilosa2 B (1)
S. sympodialis4 (2)
S. leiocarpa2 A (1)
S. leiocarpa2 B (2)
S. cayennensis2 (1)
S. hispida2 (1)
S. hamata4 C (1)
S. humilis2 A (5)
S. humilis2 B (1)
S. humilis2 C (3)
S. humilis2 D (1)
S. angustifolia2 (2)
S. erecta6 (2)
S. hamata4 E (1)
S. ingrata4 (1)
S. mexicana4 C (1)
S. scabra4 A (2)
S. subsericea4 A (1)
S. tuberculata4 (2)
S. viscosa2 A (4)
S. subsericea4 B (1)
S. viscosa2 C (1)
S. viscosa2 D (1)
S. mexicana4 D (1)
S. scabra4 D (1)
S. scabra4 C (1)
S. viscosa2 B (2)
S. fruticosa4 B (3)
S. scabra4 B (2)
S. fruticosa4 A (1)
S. sericeiceps4 (2)
clade 1

present in the strict consensus but not supported by the jackknife analysis. Within this clade, the group of widespread *S. guianensis* ssp. *guianensis* genotypes is well-supported (J.S. 89%) and characterized by 3 unambiguous C to T transitions. A second group that is strongly supported (J.S. = 87%) consists of the two sequence types that were observed in *S. biflora*. Lastly, two of the three sequence types that were observed in *S. gracilis* form a weakly supported subclade (J.S 62%).

Clade 1 has a jackknife support of 84%. Its basal division is highly supported as well. The first branch (J.S. 100%) groups the two sequence types that were found in *S. pilosa*, the only diploid species of this clade that belongs to sect. *Styposanthes*. The other branch (92%) groups a mixture of diploid and allopolyploid species (*S. angustifolia*, *S. cayennensis*, *S. erecta*, *S. fruticosa*, *S. ingrata*, *S. hispida*, *S. hamata*, *S. humilis*, *S. leiocarpa*, *S. mexicana*, *S. scabra*, *S. sericeiceps*, *S. subsericea*, *S. sympodialis*, *S. tuberculata* and *S. viscosa*). The internal structure of this group is poorly resolved. The widespread species *S. viscosa* and most of the allopolyploid species have two unambiguous synapomorphic indels, one of a single and a second of two basepairs. Despite this, the group is barely supported by the jackknife analysis (JS 55%). Within this group, several genotypes belonging to the allopolyploid species *S. erecta*, *S. hamata*, *S. ingrata*, *S. mexicana*, *S. scabra*, *S. subsericea* and *S. tuberculata* are identical to sequence types that were found in the diploid species *S. viscosa*.

Clade 5, sister to clade 1, has a jackknife support of 99%. The basal division is between *S. tomentosa*, a species of Brazilian origin and the only species of section *Stylosanthes* in this clade, and the group that consists of clade 2 and clade 3. Clade 2 (J.S. 99%) is strictly composed of South American species, mostly of Brazilian origin. Within this clade, two monophyletic subgroups with medium to strong support are present. One is composed of the diploid species *S. bracteata* and *S. linearifolia* (J.S. 87%). The second (J.S. 78%) groups the diploid *S. macrocephala* and the tetraploid *S. capitata*. The Brazilian and Venezuelan *S. capitata* differ by one bp substitution. Clade 3 (J.S. 100%) contains two weakly supported subgroups (J.S. 63% and 63%) that are in a polytomy with a sequence of a diploid *S. hamata* specimen. The first subgroup, formed by *S. calcicola*, *S. mexicana*, *S. macrocarpa*, *S. seabrana*, is further subdivided into two weakly supported groups with the *S. calcicola* sequences sister to the rest (J.S. 65% and 60%, resp.). The second subgroup contains *S. sundaica* and some diploid and tetraploid specimens of *S. hamata*. The ITS regions of the Colombian and Venezuelan tetraploid *S. hamata* genotypes and the two genotypes of diploid *S. hamata* are identical in sequence (hamata B). However, because they have only a single unambiguous step, they are only supported at 61%. The Mexican *S. hamata* specimen and *S. sundaica* have an identical ITS sequence but lack unambiguous changes.

Fig. 1. Strict consensus of the three most parsimonious trees (length 321, C 0.77, R 0.95). Numbers above branches are jackknife support values; unmarked branches have a jackknife support below 50. Numbers below branches are the number of unambiguous steps as found in the individual three shortest trees; a range is given when the numbers differed between the trees. Full circles indicate nodes with Bremer branch support greater than one. Subscripts to taxon names indicate ploidy level (note that *S. mexicana* and *S. hamata* have both diploid and tetraploid specimens). Horizontal bars before taxon names indicate identical sequences that are found in different (sub)species, or between diploid and tetraploid specimens of *S. hamata*. Polymorphic sequences within (sub)species are indicated by a letter code following the (sub)species name (see Appendix 1). The number between parentheses is the number of specimens in which a specific sequence was observed

Discussion

ITS sequences, intraspecific variation and polyploidy. The fact that any ITS nucleotide sequence that we observed in any allopolyploid species was identical or nearly identical to a sequence that was also observed in a diploid species strongly suggests that the ITS regions of these allopolyploids can be completely attributed to only one of their two parent species. In addition, we did not find any evidence of a chimeric nrDNA array within specimens. This absence of ITS sequence polymorphism in single allopolyploid specimens suggests that their nrDNA array is fixed for the sequence type of one of the progenitors of the species to which they belong. Similar patterns have been observed in allopolyploids of other plant species, such as in *Gossypium* (Wendel et al. 1995), *Nicotiana tabacum* (Volkov et al. 1999), *Cardamine* (Franzke and Mummenhoff 1999). The presence of only a single ITS sequence type in allopolyploids is mostly attributed to the homogenizing effect of concerted evolution (Hillis and Dixon 1991), a process that removes one parental rDNA type from the hybrid genome. Alternatively, additional ITS sequence types may remain undetected by our direct sequencing strategy and cloning may be considered to rule out this possibility.

In some allopolyploid species, the intraspecific variation exceeded largely the typical interspecific variation, resulting in their polyphyletic position in the phylogenetic tree. Different phylogenetic placements of allopolyploid ITS sequences might be the result of bidirectional interlocus concerted evolution, i.e. different populations of an allopolyploid species may have become fixed for different ITS repeat types inherited from the two parental species involved in its allopolyploid origin (Wendel et al. 1995). This may be the case in the allotetraploid form of *S. hamata*. The ITS sequences that we found in this form group either with *S. hamata* in clade 3 or with *S. humilis* or *S. viscosa* and 6 polyploid species in clade 1.

Small intraspecific ITS nucleotide variation was found among different specimens of several widespread as well as geographically restricted diploid *Stylosanthes* species, which suggests that concerted evolution in these cases fails to homogenize the rDNA arrays across the entire species. This divergence could also be due to limited gene flow between populations. As discussed below, the level of intraspecific variation in these diploid species was mostly too small to cast doubt on the species delimitations. Exceptions are *S. hamata* s. str., *S. humilis*, *S. leiocarpa*, *S. mexicana*, *S. viscosa* and the species complex *S. guianensis*.

Chloroplast *trn*L versus nuclear ITS sequences. With exception of the position of most allopolyploid species, the phylogenetic relationships in *Stylosanthes* as revealed by ITS DNA sequence analysis, agree largely with results of earlier studies which were based on cpDNA sequencing (Vander Stappen et al. 1999a, J. Vander Stappen, unpubl. data). Congruence between both datasets with respect to the diploid species, suggests that reticulate evolution due to ancient hybridizations is unlikely in these species. This corresponds to the results of Stace and Cameron (1984), who observed that interspecific hybrids are completely sterile at the diploid level. Dissimilar topologies between chloroplast and nuclear trees for the allopolyploid species are due to hybridization and homogenization of the ITS copies to the paternal ITS type. Therefore, ITS data are likely to provide information about parentage in allopolyploids that is complementary to information from cpDNA data. In this study, we obtained precise information about parentage in all known allopolyploids of the genus, except of *S. capitata* and *S. sympodialis* (Table 1). The ITS phylogeny of the genus *Stylosanthes* is more resolved than previous chloroplast DNA phylogenies. The ITS regions showed an overall substitution rate 3 to 4 times that of the *trn*L intron, which is in the same range as in the genus *Gentiana* (Gielly et al. 1996). As a consequence, most relationships that were weakly supported by ITS analysis, are not

resolved by cpDNA analysis. However, in some cases, the cpDNA sequences were more informative in *Stylosanthes*. For instance, whereas no interspecific variation was detected between *S. macrocarpa* and *S. mexicana* by sequencing of the entire ITS region, the *trn*L intron showed variation between both species (Vander Stappen and Volckaert 1999).

ITS phylogeny, species relationships and taxonomic implications. The ITS region in *Stylosanthes* provides several new insights into the phylogeny of the genus, even though its limited variability often results in a poor resolution at the lower levels. This low level of sequence divergence is consistent with a recent origin of the genus, which indeed has been hypothesized to be of the late Tertiary (Raven and Polhill 1981). According to our ITS data, *Stylosanthes* is a strongly supported monophyletic genus that is in turn composed of three strongly supported clades (Fig. 1). However, even when the allopolyploid species and populations are not considered, neither of the two traditional subsections is monophyletic: the diploid species of the polyphyletic section *Styposanthes* appear as two distinct monophyletic groups within a paraphyletic section *Stylosanthes*. One of these groups consists of the single species *S. pilosa*, all other species of section *Styposanthes* form the second group. Interestingly, *S. pilosa* is one of the presumed progenitors of *S. capitata* (Liu et al. 1999; the other parent is *S. macrocephala*, see below), which is the only known intrasectional allopolyploid. In what follows, we discuss clades 1 to 5, in the order in which they appear in the cladogram.

Clade 4. Most species in this exclusively diploid clade are members of the *S. guianensis* complex. These have a wide to restricted distribution and are characterized by a loment with a minute beak ('t Mannetje 1977, 1984; Costa and Ferreira 1984). The other species in this clade are *S. montevidensis*, *S. macrosoma* and *S. biflora*. *S. montevidensis* is considered as closely related to *S. guianensis* on the basis of morphological similarities ('t Mannetje 1977), a relationship that has also been confirmed in previous molecular analysis of *Stylosanthes* (RFLP and sequence analysis of cpDNA, Gillies and Abbott (1996) and Vander Stappen et al. (1999a); RFLP and sequence-tagged site (STS) analysis, Liu et al. (1999) and Vander Stappen et al. (1999b)). *S. macrosoma* is morphologically related to *S. guianensis* (Blake 1920), and to *S. montevidensis* (Hassler 1919, Burkart 1939). Our analysis adds support to these previous morphological observations. *S. biflora* is restricted to the eastern part of the USA from 27° to 41°N (Williams et al. 1984), where it is widespread and known under different varieties and species such as *S. riparia* and *S. floridana* (Mohlenbrock 1958). To our knowledge, this is the first paper that reports a close phylogenetic relationship between *S. biflora* and *S. guianensis*. The two different sequence types that we observed in *S. biflora* form a well-supported monophyletic group (J.S. 87%).

While our ITS data confirm the close relationship between the members of the *Stylosanthes guianensis* complex, they contribute little or nothing to the question of whether they should be considered as varieties of the same species ('t Mannetje 1977) or as different species (Ferreira and Costa 1979) (Table 2).

Clade 1. The basal division in this clade sets apart *S. pilosa*, a Brazilian species with a restricted distribution in the states Minas Gerais and Bahia. The presence of *S. pilosa* in clade 1 agrees with previous results from cpDNA analysis (Vander Stappen et al. 1999a). However, as discussed above, it makes section *Styposanthes* polyphyletic. The presence of the diploid species *S. angustifolia*, *S. humilis*, *S. leiocarpa*, *S. hispida* and *S. viscosa* in clade 1, likewise, confirms results obtained previously with cpDNA analysis (Gillies and Abbott 1996, Vander Stappen et al. 1999a). In addition, clade 1 also contains the diploid species *S. cayennensis* and *S. hispida*, and the majority of the allotetraploid species and populations.

S. cayennensis, a species of French Guiana and Northern Brazil, is traditionally thought to be related to *S. guianensis*, with which it

shares glabrous loments and a short and strongly uncinate beak (Mohlenbrock 1958, Ferreira and Costa 1979). In our analysis it is present as the sister of *S. hispida*, which also is often regarded to be morphologically close to *S. guianensis*. Vogel (1838) and Mohlenbrock (1958) even considered *S. hispida* to be synonymous with *S. guianensis*. Ferreira and Costa (1979), however, re-established the species because of the loment that has two distinct fertile articulations. In our analysis, *S. hispida* and *S. cayennensis* are not part of clade 4, the clade in which the *S. guianensis* complex is present. Instead they constitute a highly divergent subclade of clade 1 (it has 16 unambiguous steps, the same number that separates *Stylosanthes* from its outgroup genera). This not only supports the view of Ferreira and Costa (1979) that *S. hispida* is distinct from *S. guianensis*, it also shows that these species are not even closely related within the genus. According to 't Mannetje (1984), *S. cayennensis* conforms exactly with the type of *S. hispida,* which is from the same area. Since we also found identical ITS types in both species, this study supports at least a close relationship between the two. Based on the sympatric distribution and both morphological and molecular similarities, *S. cayennensis* may indeed best be subsumed under *S. hispida*, as suggested by 't Mannetje (1984).

S. viscosa is a widely distributed species that ranges from Mexico and the Antilles to southern Brazil. It contains a diversity of ecogeographical and morphological forms (Mohlenbrock 1958), which may explain the occurrence of different ITS types in this species. Sawkins (1999) found seven additional ITS types in *S. viscosa*, one of which is identical to the ITS type that we found in the allotetraploid *S. fruticosa*. Similar conclusions about intraspecific variation can be drawn for *S. humilis*, which is also a widespread species with different morphological forms (Burt 1984). Within this species, sequence types C and D set apart a group of specimens from Mexico, Honduras, and Guatemala, to the exclusion of the specimens from Costa Rica, Panama, and South America; a similar pattern has been observed using the *trnLF* intergenic spacer and AFLP analysis (Vander Stappen et al. 1999a, Vander Stappen et al. 2000).

Costa and Ferreira (1984) recognized two forms of *S. leiocarpa*: the first ranges from Minas Gerais (Brazil) to Uruguay and Paraguay, while the second can be found in Bahia and the north of Brazil. Williams et al. (1984) suggested that the northern populations of *S. leiocarpa* may be taxonomically distinct: they can be separated from each other by the shape of the beak and the hairiness of the upper article of the loment. These data are consistent with the fact that both forms possess distinct ITS (this study) and cpDNA sequence types (Vander Stappen et al. 1999a).

The presence of the tetraploid species *S. sympodialis* in clade 1 suggests that one of its progenitors must belong to this clade as well. This is in agreement with previous results based on cpDNA analysis that identified *S. humilis* or a closely related species as possible maternal genome donor (Gillies and Abbott 1996, Vander Stappen et al. 1999a). Based on previous molecular data, the second genome donor has affinity with *S. seabrana* and *S. hamata* of clade 3 (Liu et al. 1999). In this study, *S. sympodialis* falls outside the subclade that contains *S. humilis*, so this latter species seems to be ruled out as a possible parent of *S. sympodialis*. It should be noted however, that support is weak in this part of the cladogram.

Two of the five allotetraploid specimens of *S. hamata* that we studied are present in clade 1. One of these groups with *S. humilis*, which indicates that this species is one of the genome donors for *S. hamata*, confirming a previous result that used RFLP analysis (Curtis et al. 1995). A second tetraploid specimen of *S. hamata* is present in the weakly supported subclade that groups the diploid species *S. viscosa* with a large group of tetraploids. This particular specimen of *S. hamata* is from Guatemala and was previously described as *S. eriocarpa* (Blake 1931) but regarded as conspecific with *S. hamata* by Mohlenbrock

(1958). Given that other evidence exists that *S. humilis* is one of the parents of tetraploid *S. hamata* (see below), and given the isolated geographical position of the specimen from Guatemala, further study seems to be justified to investigate if this specimen indeed is a representative of a separate species. Similar conclusions can be drawn for the Bolivian and Venezuelan specimens of *S. mexicana* that group with *S. viscosa* in clade 1 rather than with the diploid *S. mexicana* specimens of Mexico in clade 3 (see below for further discussion).

Except for *S. capitata* (clade 2, see below), *S. sundaica* (clade 3, see below), *S. sympodialis* and all but three specimens of *S. hamata*, all allopolyploids that we studied are in a weakly supported monophyletic group (J.S. 55%) that has *S. viscosa* as its only diploid member. In total, this clade has representatives of 9 different allopolyploid species. Even taking into account that species delimitation is far from clear in this group of allopolyploids (see below), their varied geographic distribution indicates that the widespread *S. viscosa* may have acted as a genome donor at many independent occasions.

The close relationships between the allopolyploids *S. erecta*, *S. fruticosa*, *S. ingrata*, *S. scabra*, *S. sericeiceps*, *S. tuberculata* and the diploid *S. viscosa* have been demonstrated before using isozyme, RFLP and/or STS analysis (Stace and Cameron 1984, Liu et al. 1999, Vander Stappen et al. 1999b). A relationship between *S. scabra* and *S. viscosa* is also in agreement with morphological and agronomical data (Mohlenbrock 1958, Burt 1984). 't Mannetje (1984) observed morphological resemblances between *S. scabra*, *S. tuberculata* and *S. fruticosa*, suggesting that the first two species may be merged with *S. fruticosa*. However, this ignores the fact that these entities have distinct geographic, climatic and edaphic distributions (Williams et al. 1984). Previous molecular analyses based on RAPDs have shown that there is a small degree of differentiation between *S. scabra* and *S. fruticosa* (Glover et al. 1994), but cpDNA sequence analysis failed to show differences between the two species (Vander Stappen and Volckaert 1999). ITS sequences seem even less suited to discriminate among these various species. As an example, *S. erecta*, *S. ingrata* and *S. subsericea* are generally considered as clearly distinct species with different geographic distribution patterns but our ITS data fail to discriminate these from *S. scabra*, *S. tuberculata* and/or *S. fruticosa*. The presence of different ITS types in the tetraploid species *S. fruticosa*, *S. mexicana*, *S. scabra* and *S. subsericea* may be related to independent and recurrent formation of these species by hybridization between different forms of *S. viscosa* with a second progenitor. In the case of *S. scabra*, the hypothesis of independent origins of different groups of this species is supported by the distribution of *S. viscosa*, which is very similar to that of *S. scabra*, and by the geographical structuring of genetic variation as revealed by RAPD analysis (Liu 1997).

S. subsericea has been widely collected in Honduras and sporadically in the state Oaxaca of Mexico (Mohlenbrock 1958). Because this species has some morphological affinity with *S. macrocarpa*, 't Mannetje (1984) suggested that *S. subsericea* may be reduced to *S. macrocarpa*. Our results causes doubt about the relationship between both species because they are wide apart in the cladogram. However, given that both species differ in their ploidy level (Gama-López et al. 2001), it may well be that the diploid species *S. macrocarpa* is one of the parents of a tetraploid *S. subsericea*. While this scenario argues against a reduction of *S. subsericea* to *S. macrocarpa*, it nevertheless explains the observed morphological similarities between both species. The species *S. ingrata* is known only from Belize (Mohlenbrock 1960). According to this author, the species is close to *S. montevidensis*. This contrasts with our ITS sequence data, which groups *S. ingrata* with *S. viscosa*. Based on these data and previous molecular data (Liu et al. 1999; Vander Stappen et al. 1999a, b), *S. ingrata* is considered to be an allotetraploid species of

which *S. viscosa* is one of the parents. This does not correspond to its sectional classification into sect. *Stylosanthes* ('t Mannetje 1984). This incongruity may be due to confusion in previous revisions of this species. In his first revision, Mohlenbrock (1958) listed the type specimen of *S. ingrata* under synonymy of *S. guianensis* subsp. *guianensis* with the statement that it is without fruits. In 1960, however, he re-instated the species after examination of the fruit. Although no axis rudiment was observed, the fruit contained two inner bracteoles (Mohlenbrock 1960) and two distinct fertile articulations (S. Gama-López, unpubl. data). Given these data and the knowledge that the axis rudiment is caducous in some species ('t Mannetje 1984), *S. ingrata* is best put into section *Styposanthes*, together with the other allopolyploids.

S. sericeiceps is known only from Venezuela. According to Mohlenbrock (1958), this species closely resembles *S. sympodialis*. Our data suggest that the former species is more closely related to *S. viscosa*.

Clade 5: (S. tomentosa (clade 2, clade 3)). *S. tomentosa* is found in a restricted area in the state Minas Gerais of Brazil (Ferreira and Costa 1979). It belongs to section *Stylosanthes* but, as discussed above, it is sister to a well-supported clade that consists of all diploid species of section *Styposanthes* except *S. pilosa*. A similar result was previously obtained with RFLP and STS analysis (Liu et al. 1999). A cpDNA analysis put the species in a polytomy with clades 1, 2 and 3 (J. Vander Stappen, unpubl. data).

The species of clade 2, *S. linearifolia*, *S. bracteata*, *S. macrocephala*, and the tetraploid *S. capitata*, are distinguished from the other members of section *Styposanthes* by bracts that are broad and contain 13 to 23 conspicuous nerves (Mohlenbrock 1958, Ferreira and Costa 1979). The species *S. linearifolia* and *S. bracteata*, a well-supported sister pair in our analysis, are sympatric and morphologically very close (Costa and Ferreira 1984). Previous studies have also shown close genetic relationships between *S. capitata* and *S. macrocephala* (Gillies and Abbott 1996, Liu et al. 1999, Vander Stappen et al. 1999a, b), the other fairly well-supported sister pair in clade 2. Our data, as well as these previous studies, indicate that the diploid species *S. macrocephala* is one of the genome donors that gave rise to *S. capitata*. The intraspecific variation that we found within the latter species coincides with its disjunct distribution in Venezuela and Brazil. Liu et al. (1999) suggested that *S. capitata* evolved in Brazil and then dispersed to eastern Venezuela.

Clade 3 is formed by the diploid species *S. calcicola*, *S. macrocarpa*, and *S. seabrana*, all diploid specimens of *S. hamata* and *S. mexicana*, some of the tetraploid specimens of *S. hamata* that we studied, and the tetraploid species *S. sundaica*. A close relationship between these diploid species and populations is in agreement with both morphological conclusions (Blake 1920, Mohlenbrock 1958, Jansen and Edye 1996, Gama-López et al. 2001) and results of a previous cpDNA sequence analysis (Vander Stappen et al. 1999a, Vander Stappen and Volckaert 1999). With the exception of *S. seabrana*, which is restricted to eastern Brazil, the species of clade 3 have their distribution in the northern Neotropics and/or the mainland of northeastern South America, which corresponds in part to the secondary centre of diversity of the genus.

S. hamata is widely distributed and occurs in Florida, Mexico, Central America, the Antilles, Colombia and Venezuela. The species exists in two different forms, diploid *S. hamata* s. str. and allotetraploid *S. hamata*. These two can be distinguished cytologically, genetically, geographically and morphologically, which suggests that they may represent good taxonomic species (Stace and Cameron 1987, Curtis et al. 1995, Gillies and Abbott 1996, Vander Stappen et al. 1999a, Gama-López et al. 2001). In addition, these studies show that the maternal and paternal genome donors of allotetraploid *S. hamata* are *S. humilis* and *S. hamata* s. str., respectively. Since we found the ITS types of both progenitors in allo-

tetraploid *S. hamata*, our study gives additional support to this hypothesis. The different ITS sequences that we found within the tetraploid form of *S. hamata* correspond to a gap in its distribution (Venezuela/Colombia versus Mexico) that is also reflected in differences in morphology and chloroplast type (Vander Stappen et al. 1999a, S. Gama-López, unpubl. data). All this strongly suggests that this tetraploid form may have arisen independently at least two times, involving populations of *S. humilis* and diploid *S. hamata* that were both genetically and geographically distinct.

S. sundaica is an allotetraploid species that is distributed in Indonesia and Malaysia. The species is ambiguous in its taxonomy because of its close resemblance to *S. humilis* ('t Mannetje 1984). It differs from *S. humilis* by its ploidy level and by the presence of two inner bracteoles and/or an axis rudiment (Nooteboom 1960). In contrast to cpDNA sequence analysis (J. Vander Stappen, unpubl. data), we did not find evidence that supports a close relationship with *S. humilis*. Our data do suggest, however, that one of the parent species of *S. sundaica* is the diploid form of *S. hamata*. Indeed, the tetraploid form of *S. hamata* that occurs in Mexico and *S. sundaica* have an identical ITS sequence and are in a clade that also contains diploid *S. hamata*. This relationship between *S. sundaica* and *S. hamata* has also been found with RFLP (Liu et al. 1999) and STS analysis (Liu et al. 1999, Vander Stappen et al. 1999b).

S. mexicana occurs in northeastern Mexico and in two disjunct regions in Venezuela and Bolivia, all of which have similar climatic and edaphic conditions (Mohlenbrock 1958). The Bolivian populations have been described as a separate species, *S. bangii*, but according to Mohlenbrock, *S. bangii* should be considered as a synonym of *S. mexicana* because it cannot be distinguished from the latter species. In our analysis the specimens of these three regions fall in two distinct groups: the Mexican specimens belong to clade 3, while those from Venezuela and Bolivia contain ITS types that are in clade 1, more specifically in a subclade that contains, a. o., *S. viscosa*. Interestingly, STS analysis revealed differences in ploidy level between the Mexican and the other specimens (J. Vander Stappen, unpubl. data). This provides indirect but strong evidence that the latter specimens are tetraploids that have *S. viscosa* as one of their parent species. There is, however, definitely a need to revise the disjunct populations of this species with other tools.

The two studied specimens of *S. macrocarpa*, a species from southern Mexico, contained an ITS sequence that we also found in a diploid *S. mexicana* specimen. This is in agreement with the observations of Blake (1920), who found both species to be identical except for their fruit morphology and their distribution within Mexico. The same close relationship between these species has been confirmed in a cpDNA sequence analysis (Vander Stappen and Volckaert 1999).

In conclusion, the ITS sequence data presented here provide novel information about phylogenetic relationships of the genus *Stylosanthes*, especially regarding the allopolyploid species and regarding the basic structure of the genus. The ITS data failed, however, to resolve the detailed relationships within the different well-supported clades that we found. Additional informative characters from other loci are needed to study species delimitations and to elucidate the relationships among closely related species.

We thank I. Weltjens for technical assistance. Some sequence data refer to the technical part of the work of S. Marant in partial fulfillment of her undergraduate thesis in 2000 at the K.U.Leuven. We wish to thank the curators of seed banks and herbaria cited in this paper, for gifts and use of seed and plant material. We thank J.S. Farris for providing a copy of xac. This work is part of IPGRI's research programme and was financed in part by the Belgian Administration of Development Cooperation (BADC). J. Vander Stappen acknowledges the Research Council of the K.U.Leuven for a postdoctoral fellowship. J. De Laet and S. Van Campenhout are postdoctoral fellows of the F.W.O, the Fund for Scientific Research – Flanders (Belgium).

Appendix 1. List of *Stylosanthes* and outgroup species included in this study, with ploidy level, voucher reference, location, ITS sequence type and EMBL accession number

Species (Ploidy level)[a]	Specimens[b]	Locality	ITS sequence type[c]	EMBL accession number
Stylosanthes Sw.				
Sect. *Stylosanthes* Vog.				
S. acuminata M.B. Ferreira & S. Costa (2x)*	Krapovickas 34539, NY	Brazil, Mato Grosso do Sul		AJ320282
S. angustifolia Vog. (2x)	Vander Stappen 1, BR (CPI 40236)	Brazil		AJ320283
	Vander Stappen 2, BR (CPI 33433)	French Guiana, Cayenne		AJ320284
S. aurea M.B. Ferreira & S. Costa (2x)*	Irwin 2647, NY	Brazil, Minas Gerais		AJ320285
S. biflora (L.) Britton, Sterns & Poggenb. (2x)	Kearney 674, US	USA, Tenessee	A	AJ320286
	Fernald 8321, US	USA, Virginia	A	AJ320287
	Nash 1848, US	USA, Florida	B	AJ320288
	Godfrey 4632, US	USA, North Carolina	B	AJ320289
S. campestris M.B. Ferreira & S. Costa (2x)	Irwin 22914, NY	Brazil, Minas Gerais		AJ320290
	Irwin 27551, US	Brazil, Minas Gerais		AJ320291
S. cayennensis Mohlenbr. (2x)*	Broadway 972, GH	French Guiana		AJ320292
S. gracilis Kunth (2x)	Vander Stappen 3, BR (CPI 39112)	Brazil, Bauru	A	AJ320293
	Irwin 25102, NY	Brazil, Goias	B	AJ320294
	Vander Stappen 4, BR (CIAT 2467)	Brazil, Rio Grande Do Norte	C	AJ320295
	Vander Stappen 5, BR (CPI 33501)	Venezuela, Jusepin	C	AJ320296
S. grandifolia M.B. Ferreira & S. Costa (2x)	Killeen 2347, NY	Bolivia, Santa Cruz	A	AJ320297
	Vander Stappen 6, BR (CPI 34918)	Brazil, Sao Paulo	A	AJ320298
	Vander Stappen 7, BR (CPI 92975)	Brazil, Parana	B	AJ320299

Appendix 1 (continued)

S. guianensis (Aubl.) Sw. (2x)				
subsp. *dissitiflora* (Robins. & Seat.) Mohlenbr.	Gama-López 127, IZTA	Mexico, Jalisco	A	AJ320300
	Gama-López 281, IZTA	Mexico, Chiapas	A	AJ320301
	Pringle 11433, ENCB	Mexico, Jalisco	B	AJ320302
subsp. *guianensis* (Aubl.) Sw.	Gama-López 143, IZTA	Mexico, Veracruz	A	AJ320303
	Gama-López 197, IZTA	Mexico, Nayarit	C	AJ302304
	Tenorio L. 14813, MEXU	Guatemala	A	AJ302305
	Betancur 1642, MO	Colombia	A	AJ320306
	Vander Stappen 8, BR (CPI 38385)	Venezuela, Merida	A	AJ320307
	Vander Stappen 9, BR (CPI 38222)	Peru, Quillabamba	A	AJ302308
	Vander Stappen 10, BR (CPI 33437)	Surinam, Lelydrop	B	AJ320309
	Vander Stappen 11, BR (CPI 40263)	Brazil, Ceara	A	AJ302310
	Pohl 1997, NY	Brazil, Campo do Parado	G	AJ302311
cv. canescens	Vander Stappen 12, BR (CPI 34906)	Brazil, Sao Paulo	E	AJ320312
cv. microcephala	Vander Stappen 13, BR (CIAT 1200)	Brazil, Distrito Federal	D	AJ320313
cv. pauciflora	Coradin 5675, NY	Brazil, Goias	F	AJ320315
cv. pauciflora	Vander Stappen 14, BR (CIAT 1523)	Venezuela, Monagas	F	AJ320314
S. hippocampoides Mohlenbr. (2x)	Kurtz 8303, NY	Argentina, Cordoba	A	AJ320316
	Schinini 16995, NY	Argentina, Corrientes	B	AJ320317
S. hispida Rich. (2x)*	Egler 47648, NY	Brazil, Territorio do Amapa		AJ320328
S. humilis Kunth (2x)	MacKee 11171, US	Costa Rica, Buenos Aires	A	AJ320318
	Pittier 4983, US	Panama, Cocle	A	AJ320319
	Vander Stappen 15, BR (CIAT 1363)	Venezuela, Guarico	A	AJ320320
	Vander Stappen 16, BR (CPI 61674)	Venezuela	B	AJ320321
	Vander Stappen 17, BR (CIAT 2334)	Colombia, Casanare	A	AJ320322

Appendix 1 (continued)

Species (Ploidy level)[a]	Specimens[b]	Locality	ITS sequence type[c]	EMBL accession number
	Vander Stappen 18, BR (CPI 40266)	Brazil, Pernambuco	A	AJ320323
	Gama-López 180, IZTA	Mexico, Oaxaca	D	AJ320324
	Gama-López 231, IZTA	Mexico, Nayarit	C	AJ320325
	Casco-Varela 32, MO	Honduras, Morazan	C	AJ320326
	Heyde 4162, US	Guatemala, Chupadero	C	AJ320327
S. ingrata S.F. Blake (4x)*	Record s.n., US	Belize, Vaca Falls District		AJ320329
S. leiocarpa Vog. (2x)	Vander Stappen 19, BR (CIAT 2167)	Brazil, Bahia	A	AJ320330
	Vander Stappen 20, BR (CPI 36258)	Brazil, Santa Catarina	B	AJ320331
	Vander Stappen 21, BR (CPI 78192)	Argentina, Corrientes	B	AJ320332
S. macrosoma S.F. Blake (2x)	Hassler 7606, NY	Paraguay		AJ320333
S. montevidensis Vog. (2x)	Sellow s.n., GH	Brazil, Brasil		AJ320334
	Vander Stappen 22, BR (CPI 11494)	Paraguay		AJ320335
	Petersen-Troels 3113, NY	Argentina, Corrientes		AJ320336
S. tomentosa M.B. Ferreira & S. Costa (2x)*	Vander Stappen 23, BR (CPI 92843)	Brazil, Minas Gerais		AJ320337
S. viscosa Sw. (2x)	Gama-López 135, IZTA	Mexico, Oaxaca	A	AJ320338
	Vander Stappen 24, BR (CIAT 1593)	Belize, Cayo	B	AJ320339
	Howard 5534, GH	Cuba	D	AJ320340
	CIAT 11091*	Venezuela, Bolivar	C	AJ320341
	CIAT 2773*	Venezuela, Lara	A	AJ320342
	CIAT 2341*	Colombia, Casanare	A	AJ320343
	Vander Stappen 25, BR (CIAT 10301)	Brazil, Roraima	B	AJ320344
	Vander Stappen 26, BR (CPI 34904)	Brazil, Goias	A	AJ320345
Sect. *Styposanthes* Vog.:				
S. bracteata Vog. (2x)*	Callejas 1962, NY	Brazil, Matto Grosso do Sul		AJ320346

Appendix 1 (continued)

S. calcicola Small (2x)	Gama-López 252, IZTA	Mexico, Yucatan	A	AJ320347
	Gama-López 251, IZTA	Mexico, Yucatan	B	AJ320348
S. capitata Vog. (4x)	Vander Stappen 27, BR (CIAT 10940)	Brazil, Mato Grosso	A	AJ320349
	Vander Stappen 28, BR (CIAT 1504)	Venezuela, Anzoategui	B	AJ320350
S. erecta Beauv. (6x)	Reitsma 685, NY	Gabon		AJ320351
	De Wilde 790, MO	Gabon		AJ320352
S. fruticosa (Retz.) Alston (4x)	Vander Stappen 29, BR (CPI 79070)	India	A	AJ320353
	Huntley 2069 (MO)	South Africa	B	AJ320354
	Vander Stappen 30, BR (CPI 50979)	Senegal	B	AJ320355
	Vander Stappen 31, BR (CPI 60354)	Kenya	B	AJ320356
S. hamata (L.) Taub. s. str. (2x)	Sintenis 1092, MO	Puerto Rico	A	AJ320357
	Correll 46279, NY	Bahamas	B	AJ320358
	Killip 45007, US	USA, Florida	B	AJ320359
	Standley 25126, US	Guatemala, Izabal	A	AJ320360
	Tamayo 3776, NY	Venezuela, Lara	A	AJ320361
S. hamata (L.) Taub. s. l. (4x)	Vander Stappen 32, BR (CPI 38842)	Venezuela, Maracaibo	C	AJ320365
	Vander Stappen 33, BR (CPI 55822)	Venezuela, Maracaibo	B	AJ320362
	Kirkbride 2475, US	Colombia, Magdalena	B	AJ320363
	Gama-López 136, IZTA	Mexico, Oaxaca	D	AJ320364
	Blake 7792, GH	Guatemala	E	AJ320366
S. linearifolia M.B. Ferreira & S. Costa (2x)*	Hatschbach 33374, NY	Brazil, Minas Gerais		AJ320367
S. macrocarpa S.F. Blake (2x)	Gama-López 178, IZTA	Mexico, Oaxaca		AJ320368
	Pringle 372, GH	Mexico, Oaxaca		AJ320369
S. macrocephala Ferreira & Costa (2x)	Coradin 6437, NY	Brazil, Bahia		AJ320370
	Vander Stappen 34, BR (CIAT 1643)	Brazil, Brasilia		AJ320371
S. mexicana Taub. (2x)	Gama-López 246, IZTA	Mexico, Nuevo León	A	AJ320372

Appendix 1 (continued)

Species (Ploidy level)[a]	Specimens[b]	Locality	ITS sequence type[c]	EMBL accession number
	Gama-López 248, IZTA	Mexico, Nuevo León	B	AJ320373
S. mexicana Taub. (4x)*	Bang 936, GH	Bolivia, Cochabamba	C	AJ320374
	Pittier 9679, NY	Venezuela, Caracas	D	AJ320375
S. pilosa M.B. Ferreira & S. Costa (2x)	Coradin 6280, NY	Brazil, Bahia	A	AJ320376
	Vander Stappen 35, BR (CIAT 2129)	Brazil, Bahia	B	AJ320377
S. scabra Vog. (4x)	Belém 2027, NY	Brazil, Goias	C	AJ320378
	Lima 189, NY	Brazil, Bahia	B	AJ320379
	Ehrich 290, NY	Bolivia, Tarija	A	AJ320380
	Vander Stappen 36, BR (CIAT 1368)	Venezuela, Tachira	D	AJ320381
	Vander Stappen 37, BR (CIAT 10645)	Venezuela, Nueva Esparta	B	AJ320382
	Jahn 169, NY	Venezuela, Trujillo	A	AJ320383
S. seabrana B.L. Maass & 't Mannetje (2x)	Coradin 6261, NY	Brazil, Bahia		AJ320384
S. sericeiceps S.F. Blake (4x)*	Jahn 678, GH	Venezuela, Merida		AJ320385
	Breteler 4070, NY	Venezuela, Merida		AJ320386
S. subsericea S.F. Blake (4x)	Williams 11261, MEXU	Honduras, Morazan	A	AJ320387
	Purpus 7152, F	Mexico, Oaxaca	B	AJ320388
S. sundaica Taub. (4x)	CPI 47477*	Indonesia, Bali		AJ320389
S. sympodialis Taub. (4x)*	Asplund 15976, NY	Ecuador, Manabi		AJ320390
	Vander Stappen 38, BR (CIAT 1634)	Peru, Piura		AJ320391
S. tuberculata S.F. Blake (4x)	F/Britton 3336	Bahamas		AJ320392
	Vander Stappen 39, BR (CPI 100452)	Bahamas		AJ320393
Outgroup species				
Arachis correntina (Burkart) Krapov. & W.C. Greg.	Vander Stappen 40, BR (CIAT 22249)	Argentina, Corrientes		AJ320394
A. hoehnei Krapov. & W.C. Greg.	Vander Stappen 41, BR CIAT 22244)	Brazil, Mato Grosso		AJ320395

Appendix 1 (continued)

A. palustris Krapov., W.C. Greg. & Valls	Vander Stappen 42, BR (CIAT 22245)	Brazil, Sao Paulo	AJ320396
A. pintoi Krapov. & W.C. Greg.	Vander Stappen 43, BR CIAT 22148)	Brazil, Minas Gerais	AJ320397
A. triseminata Krapov. & W.C. Greg	Vander Stappen 44, BR CIAT 22224)	Brazil	AJ320398
A. villosa Benth.	Vander Stappen 45, BR CIAT 22254)	Uruguya	AJ320399
Chapmannia prismatica (Sessé & Moc.) Thulin	CIAT 18287*	Venezuela	AJ320400

[a] Ploidy level according to Atchison (1949), Cameron (1967), Stace and Cameron (1984), Stace and Cameron (1987), Vanni (1987), Vieira (1993), Liu and Musial (1997) and Gama-López et al. (2001). The ploidy level of the species indicated by asterisks (*) was inferred from isozyme, RFLP and/or STS analysis (Stace and Cameron 1984, Liu et al. 1999, Vander Stappen et al. 1999b, J. Vander Stappen et al., unpubl. data)

[b] Sources: Commonwealth Scientific and Industrial Research Organization (CSIRO), Australian Tropical Forages Genetic Resource Centre, Cunningham Laboratory, Commonwealth Plant Introduction (CPI) numbers, St Lucia, Australia; Centro Internacional de Agricultura Tropical (CIAT), Tropical Forages Collection, Cali, Colombia; United States National Herbarium (US); New York Botanical Garden (NY); Missouri Botanical Garden (MO); Gray Herbarium of Harvard University (GH); Field Museum of Natural History (F); Herbario Nacional de México (MEXU); ENEP-Iztacala (IZTA); Herbario de la Escuela Nacional de Ciencias Biológicas (ENCB). CIAT and CSIRO accessions were grown from seed, and a herbarium specimen was prepared and deposited at the herbarium of the National Botanic Garden of Belgium (BR). No voucher is available for the specimens indicated by an asterisk

[c] Within each (sub)species in which ITS polymorphisms were observed, a unique letter code is used to identify the different sequences

References

Atchison E. (1949) Studies in the Leguminosae. IV. Chromosome numbers and geographical relationships of miscellaneous Leguminosae. J. Elisha Mitchell Sci. Soc. 65: 118–122.

Baldwin B. G., Sanderson M. J., Porter J. M., Wojciechowski M. F., Campbell C. S., Donoghue M. J. (1995) The ITS region of nuclear ribosomal DNA: A valuable source of evidence on angiosperm phylogeny. Ann. Missouri Bot. Gard. 82: 247–277.

Bentham G. (1859) Leguminosae: XXV. *Stylosanthes*. Fl. Bras. (Martius) 15: 88–93.

Beyra A.-M., Lavin M. (1999) Monograph of *Pictetia* (Leguminosae-Papilionoideae) and review of the Aeschynomeneae. Syst. Bot. Monogr. 56: 1–93.

Blake S. F. (1920) Nine new plants of the genus *Stylosanthes*. Proc. Biol. Soc. Wash. 33: 45–54.

Blake S. F. (1931) New plants from Guatemala and Honduras. Contr. U.S. Natl. Herb. 24: 4–5.

Bremer K. (1994) Branch support and tree stability. Cladistics 10: 295–304.

Burkart A. (1939) Estudios sistematicos sobre las Leguminosas – Hedisareas de la Republica Argentina y regiones adyacentes. Darwiniana 3: 232–261.

Burt R. L. (1984) Natural variation in *Stylosanthes*. In: Stace H. M., Edye L. A. (eds.) The biology and agronomy of *Stylosanthes*. Academic Press, London, pp. 103–123.

Burt R. L., Miller C. P. (1975) *Stylosanthes* – A source of pasture legumes. Trop. Grasslands 9: 117–123.

Cameron D. F. (1967) Chromosome number and morphology of some introduced *Stylosanthes* species. Austral. J. Agric. Res. 18: 375–379.

Chase M. W., Hills H. H. (1991) Silica gel: An ideal material for field preservation of leaf samples for DNA studies. Taxon 40: 215–220.

Coddington J., Scharff N. (1994) Problems with zero-length branches. Cladistics 10: 415–423.

Costa N. M. S., Ferreira M. B. (1984) Some Brazilian species of *Stylosanthes*. In: Stace H. M., Edye L. A. (eds.) The biology and agronomy of *Stylosanthes*. Academic Press, London, pp. 23–48.

Curtis M. D., Cameron D. F., Manners J. M. (1995) Molecular evidence that diploid *Stylosanthes humilis* and diploid *Stylosanthes hamata* are progenitors of allotetraploid *Stylosanthes hamata* cv. Verano. Genome 38: 344–348.

Edye L. A., Burt R. L., Nicholson C. H. L., Williams R. J., Williams W. T. (1974) Classification of the *Stylosanthes* collection 1928–1969. CSIRO Austral. Div. Trop. Agron. Tech. Pap. 15: 1–28.

Farris J. S. (1970) Methods for computing Wagner trees. Syst. Zool. 19: 83–92.

Farris J. S. (1989) The retention index and the rescaled consistency index. Cladistics 5: 417–419.

Farris J. S., Albert V. A., Källersjö M., Lipscomb D., Kluge A. G. (1996) Parsimony jackknifing outperforms neighbour-joining. Cladistics 12: 99–124.

Ferreira M. B., Costa N. M. S. (1979) O genero *Stylosanthes* Sw. no Brasil. EPAMIG, Belo Horizonte, pp. 1–107.

Fitch W. M. (1971) Toward defining the course of evolution: minimum change for a specific tree topology. Syst. Zool. 20: 406–416.

Franzke A., Mummenhoff K. (1999) Recent hybrid speciation in *Cardamine* (Brassicaceae) – conversion of nuclear ribosomal ITS sequences in statu nascendi. Theor. Appl. Genet. 98: 831–834.

Gama-López, Vander Stappen J., Dávila P., Volckaert G. (2001) Systematic study of the genus *Stylosanthes*. Abstract in The Fourth International Legume Conference, Canberra, July 2001.

Gardener C. J. (1984) The dynamics of *Stylosanthes* pastures. In: Stace H. M., Edye L. A. (eds.) The biology and agronomy of *Stylosanthes*. Academic Press, London, pp. 333–357.

Gielly L., Yuan Y. M., Kupfer P., Taberlet P. (1996) Phylogenetic use of noncoding regions in the genus *Gentiana* L.: chloroplast *trn*L (UAA) intron versus nuclear ribosomal internal transcribed spacer sequences. Molec. Phylogen. Evol. 5: 460–466.

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

Glover B. J., Gillies A. C. M., Abbott R. J. (1994) Use of the polymerase chain reaction to investigate the delimitation of two agronomically important species of *Stylosanthes* (Aubl.) Sw. Bot. J. Scotland. 47: 83–96.

Goloboff P. A. (1993) Nona version 2.0. Program and documentation distributed by the author. Tucumán, Argentina.

Hacker J. B., Clements R. J., Cameron D. F. (1988) Systematic botany and genetic improvement of tropical pastures. Acta Univ. Ups. Symb. Bot. Ups. XXVIII: 55–68.

Hamby R. K., Zimmer E. A. (1992) Ribosomal RNA as a phylogenetic tool in plant systematics. In: Soltis P. S., Soltis D. E., Doyle J. J. (eds.) Molecular Systematics of Plants. Chapman and Hall, New York London, pp. 50–91.

Hassler E. (1919) Ex herbario Hassleriano: Novitates paraguarienses. XXIII. Repert. Spec. Nov. Regni Veg. 16: 220–224.

Hillis D. M., Dixon M. T. (1991) Ribosomal DNA: Molecular evolution and phylogenetic inference. Quart. Rev. Biol. 66: 411–446.

Jansen P. I., Edye L. A. (1996) Variation within *Stylosanthes* sp. aff. *scabra* and comparison with its closest allies, *S. scabra* and *S. hamata*. Austral. J. Agric. Res. 47: 985–996.

Källersjö M., Farris J. S., Kluge A. G., Bult C. (1992) Skewness and permutation. Cladistics 8: 275–287.

Khudrathulla M. D., Jagannath M. S. (1998) Biocontrol of ixodid ticks by forage legume *Stylosanthes scabra* (Vogel). Indian J. Anim. Sci. 68: 428–430.

Kim K. J., Jansen R. K. (1994) Comparisons of phylogenetic hypotheses among different data sets in dwarf dandelions (*Krigia*): additional information from internal transcribed spacer sequences of nuclear ribosomal DNA. Plant Syst. Evol. 190: 157–185.

Kirkbride J. H., Kirkbride C. G. (1987) Typification of *Stylosanthes* (Leguminosae) and its sections. Taxon 36: 455–458.

Kluge A. G., Farris J. S. (1969) Quantitative phyletics and the evolution of the anurans. Syst. Zool. 18: 1–32.

Lavin M., Pennington R. T., Klitgaard B. B., Sprent J. I., de Lima H. C., Gasson P. E. (2001) The dalbergioid legumes (Fabaceae): delimitation of a pantropical monophyletic clade. Amer. J. Bot. 88: 503–533.

Liu C. J. (1997) Geographical distribution of genetic variation in *Stylosanthes scabra* revealed by RAPD. Euphytica 98: 21–27.

Liu C. J., Musial J. M. (1997) *Stylosanthes* sp. aff. *scabra*: a putative diploid progenitor of *Stylosanthes scabra* (Fabaceae). Plant Syst. Evol. 208: 99–105.

Liu C. J., Musial J. M., Thomas B. D. (1999) Genetic relationships among *Stylosanthes* species revealed by RFLP and STS analyses. Theor. Appl. Genet. 99: 1179–1186.

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

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

Mohlenbrock R. H. (1958) A revision of the genus *Stylosanthes*. Ann. Missouri Bot. Gard. 44: 299–351.

Mohlenbrock R. H. (1960) Recent studies in the leguminous genus *Stylosanthes*. Rhodora 62: 340–343.

Mohlenbrock R. H. (1963) Further considerations in *Stylosanthes* (*Leguminosae*). Rhodora 65: 245–258.

Nixon K. C. (1999) Winclada (BETA) version 0.9.9. Published by the author, Ithaca, NY.

Nooteboom P. (1960) *Stylosanthes* Swartz. Reinwardtia 5: 446–450.

O'Kane Jr. S. L., Schaal B. A., Al-Shehbaz I. A. (1996) The origins of *Arabidopsis suecica* (Brassicaceae) as indicated by nuclear rDNA sequences. Syst. Bot. 21: 559–566.

Raven P. H., Polhill R. M. (1981) Biogeography of the Leguminosae. In: Polhill R. M., Raven P. H. (eds.) Advances in legume systematics: Part 1. Royal Botanic Gardens, Kew, pp. 27–34.

Rudd V. E. (1981) Aeschynomeneae. In: Polhill R. M., Raven P. H. (eds.) Advances in Legume Systematics. Royal Botanic Gardens, Kew, pp. 347–354.

Sang T., Crawford D. J., Stuessy T. F. (1995) Documentation of reticulate evolution in peonies (*Paeonia*) using internal transcribed spacer sequences of nuclear ribosomal DNA: Implications for biogeography and concerted evolution. Proc. Natl. Acad. Sci. USA 92: 6813–6817.

Sawkins M. C. (1999) Geographical and genetic studies of *Stylosanthes* Sw. species. Ph.D. Dissertation, University of Birmingham, Birmingham.

Stace H. M., Cameron D. F. (1984) Cytogenetics and the evolution of *Stylosanthes*. In: Stace H. M., Edye L. A. (eds.) The biology and agronomy of *Stylosanthes*. Academic Press, London, pp. 49–72.

Stace H. M., Cameron D. F. (1987) Cytogenetic review of taxa in *Stylosanthes hamata* sensu lato. Trop. Grasslands 21: 182–188.

Swartz O. (1788). Prod. Veg. Ind. Occ. 108.

Taubert P. (1891) Monographie der Gattung *Stylosanthes*. Verh. Bot. Vereins Prov. Brandenburg. 32: 1–34.

Thomas D. (1984) Global ventures in *Stylosanthes* I. South America. In: Stace H. M., Edye L. A. (eds.) The biology and agronomy of *Stylosanthes*. Academic Press, London, pp. 451–466.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F., Higgins D. G. (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucl. Acids Res. 24: 4876–4882.

Vander Stappen J., Van Campenhout S., Gama-López S., Volckaert G. (1998) Sequencing of the internal transcribed spacer region ITS1 as a molecular tool detecting variation in the *Stylosanthes guianensis* species complex. Theor. Appl. Genet. 96: 869–877.

Vander Stappen J. (1999) Development of molecular markers for screening genetic diversity in the tropical legume genus *Stylosanthes*. Ph.D. Dissertation, Katholieke Universiteit Leuven, Leuven.

Vander Stappen J., Volckaert G. (1999) Molecular characterization and classification of *S. mexicana*, *S. macrocarpa*, *S. seabrana*, and *S. fruticosa* by DNA sequence analysis of two chloroplast regions. DNA Seq. 10: 199–202.

Vander Stappen J., Weltjens I., Munaut F., Volckaert G. (1999a) Inter-specific and progeny relationships in the genus *Stylosanthes* inferred from chloroplast DNA sequence variation. C. R. Acad. Sci. III. Life Sci. 322: 481–490.

Vander Stappen J., Weltjens I., Van Campenhout S., Volckaert G. (1999b) Genetic relationships among *Stylosanthes* species as revealed by sequence-tagged site markers. Theor. Appl. Genet. 98: 1054–1062.

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

Vanni R. (1987) Recuentos cromosomicos en *Zornia*, *Stylosanthes* y *Desmodium* (Leguminosae). Bonplandia 6: 39–43.

Vieira M. L. C., de Aguiar-Perecin M. L. R., Martins P. S. (1993) A cytotaxonomic study in twelve Brazilian taxa of *Stylosanthes* Sw., Leguminosae. Cytologia 58: 305–311.

Vogel J. R. T. (1838) *Stylosanthes*. Linnaea 12: 63–71.

Volkov R. A., Borisjuk N. V., Panchuk I. I., Schweizer D., Hemleben V. (1999) Elimination and rearrangement of parental rDNA in the allotetraploid *Nicotiana tabacum*. Molec. Biol. Evol. 16: 311–320.

Wagner A., Blackstone N., Cartwright P., Dick M., Misof B., Snow P., Wagner G. P., Bartel J., Murtha M., Pendleton J. (1994) Surveys of gene families using polymerase chain reaction: PCR selection and PCR drift. Syst. Biol. 43: 250–261.

Wendel J. F., Schnabel A., Seelanan T. (1995) Bidirectional interlocus concerted evolution following allopolyploid speciation in cotton (*Gossypium*). Proc. Natl. Acad. Sci. USA 92: 280–284.

White T. J., Bruns T., Lee S., Taylor J. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis M. A., Gelfand D. H., Sninsky J. J., White T. J. (eds.) PCR Protocols: A guide to methods and applications. Academic Press, New York, pp. 315–322.

Williams R. J., Reid R., Schultze-Kraft R., Costa N. M. S., Thomas B. D. (1984) Natural distribution of *Stylosanthes*. In: Stace H. M., Edye L. A. (eds.) The biology and agronomy of *Stylosanthes*. Academic Press, London, pp. 73–101.

Addresses of the authors: Jacqueline Vander Stappen, Steven Van Campenhout and Guido Volckaert (E-mail: guido.volckaert@agr.kuleuven.ac.be), Katholieke Universiteit Leuven, Laboratory of Gene Technology, Kasteelpark Arenberg 21, B-3001 Leuven, Belgium. Jan De Laet, American Museum of Natural History, Division of Invertebrate Zoology, Central Park West at 79th Street, New York 10024–5192, USA.

Susana Gama-López, Universidad Nacional Autónoma de México, Unidad de Biología, Tecnología y Protipos (UBIPRO), FES-Iztacala, Laboratorio de Recursos Naturales, Av. de Los Barrios S/N, Colonia Los Reyes Iztacala, Municipio Tlalnepantla, Estado de México, C.P. 54090, México. Present address: Apartado Postal 154, Cto. Parque No. 3, C.P. 53102, México.