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The application of chloroplast DNA clones in identifying maternal donors for polyploid species of *Stylosanthes*

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Abstract Organelle inheritance is strictly maternal for most plant species. This property makes organelle DNAs ideal material for identifying the maternal parents of polyploid species. A chloroplast DNA (cpDNA) clone from *Stylosanthes* was identified. Together with rice cpDNA clones, it was used in identifying putative maternal donors for polyploid *Stylosanthes* species. Of 15 taxa for which 2 or more accessions each were analysed, intra-taxon cpDNA variation was only identified within the diploid species *S. viscosa*. Of the nine basal diploid genomes identified, results from the cpDNA probes strongly suggested that Genome A1 is the maternal donor to *S.* aff. *hamata*, *S. scabra*, *S.* aff. *scabra*, *S. sericeiceps* and *S. tuberculata* and that it may also be the maternal donor to the hexaploid *S. erecta*; Genome C is the maternal donor to *S. sp*. A, *S. mexicana*, *S. subsericea* and *S. sundaica*; Genome E is the maternal donor to *S. capitata*. The maternal donor to *S. fruticosa* is likely to be Genome B3, and that to *S. ingrata* is likely to be Genome A1. The maternal donor to *S. sympodialis*, although similar to those of *S. sp*. genotypes, may not be included amongst the diploid taxa analysed in this study. The fact that none of the polyploid genotypes produced cpDNA fragments from more than one of their respective progenitors indicated that cpDNA in *Stylosanthes* is strictly maternally inherited.

Keywords *Stylosanthes* · Chloroplast DNA variation · Genetic relationships

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Introduction

The genus *Stylosanthes* (Fabaceae) is naturally distributed in tropical, subtropical and temperate regions of the Americas, tropical Africa and south-eastern Asia (Williams et al. 1984). Several of these species, including *S. scabra*, *S. hamata*, *S. viscosa*, *S. guianensis* and *S. humilis*, are widely used as forages in tropical regions (Gardener 1984; Edye 1987).

Species identification based on morphological characters in *Stylosanthes* is difficult (Mannetje 1984; Sousa Costa and Ferreira 1984). As a result, there are a substantial number of unnamed taxa in the current *Stylosanthes* seed collections (Williams et al. 1984; Schultze-Kraft et al. 1984). As with all currently described species, these unnamed taxa share a basic chromosome number of x=10. Putative genome structures for most of these taxa have been derived using molecular marker techniques (Curtis et al. 1995; Liu and Musial 1997; Liu et al. 1999).

Organelle inheritance is strictly maternal for most plant species, although biparental inheritance has also been reported on several occasions (Corriveau and Coleman 1988; Reboud and Zeyl 1994; Vivek et al. 1999). This property of uniparental inheritance makes organelle DNAs ideal markers for identifying the maternal parents of polyploid species. Such an attempt in *Stylosanthes* was made by Gillies and Abbott (1996) in their phylogenetic work using chloroplast (cp)DNA variations. However, as genome structures for most polyploid species were unknown at that time, their effort met with only limited success. In this paper we report on cpDNA variation in 18 diploid taxa and the putative maternal donors identified for 16 tetraploid and hexaploid taxa.

Materials and methods

Plant material

Genetic stocks

Fifty-one genotypes, representing 26 of the 32 described *Stylosanthes* species and six unnamed taxa that are held at the Australian

Table 1 The *Stylosanthes* accessions used, their genome structures and cpDNA types

CPI	Species	Genome[a]	cpDNA type
92463	*seabrana*	A	A1
110361	*seabrana*	A	A1
49833	aff. *hamata*	AB	A1
110116	aff. *hamata*	AB	A1
115939B	*scabra*	AB	A1
115945	*scabra*	AB	A1
115952	*scabra*	AB	A1
55816	aff. *scabra*	AB	A1
55871	aff. *scabra*	AB	A1
93099	aff. *scabra*	AB	A1
110175	*sericeiceps*	AB	A1
100452	*tuberculata*	AB	A1
76259	*ingrata*	(T)?	A1
35015	*erecta*	ABX	A1
69200	*erecta*	ABX	A1
57247	*hamata*	A	A2
110107	*hamata*	A	A2
33831	*viscosa*	B	B1
40300	*viscosa*	B	B2
33941	aff. *viscosa*	B	B3
91317	aff. *viscosa*	B	B3
25368	*fruticosa*	(T)?	B3
55797	*sp.* B	C	C1
33502A	*humilis*	C	C1
33979	*humilis*	C	C1
34148	*sp.* A	AC	C1
76255	*mexicana*	AC	C1
86137	*mexicana*	AC	C1
87484	*mexicana*	AC	C1
33943	*subsericea*	AC	C1
37206	*subsericea*	AC	C1
38604	*subsericea*	AC	C1
47477	*sundaica*	AC	C1
96907	*sundaica*	AC	C1
78478	*bracteata*	D	D1
106884	*macrocephala*	D	D1
93045	*pilosa*	E	E1
55840	*capitata*	DE	E1
78192	*leiocarpa*	F	F1
94409	*sp.*	F	F2
94410	*sp.*	F	F2
65958	*sympodialis*	AF	F3
67705	*sympodialis*	AF	F3
60692	*grandifolia*	G	G1
92840	*campestris*	G	G2
34751	*guianensis*	G	G2
78191	*hippocampoides*	G	G2
11494	*montevidensis*	G	G2
92843	*tomentosa*	H	H1
73525	*calcicola*	I	I1
91492	*calcicola*	I	I1

[a] Data from Liu and Musial (1997); (T)? indicates tetraploids with unknown genome structures

Tropical Forages Genetic Resource Centre (ATFGRC), were used in this study (Table 1).

DNA extraction and restriction fragment length polymorphism (RFLP) procedures

Leaves from two or three mature plants of each genotype were harvested. Freeze-dried leaf tissue was used for DNA extraction. Total DNA was digested with restriction enzymes *Dra*I or *Hind*III. Procedures for DNA extraction and RFLP analysis were those described by Liu and Musial (1995).

Four clones were used in this study. These include one *Stylosanthes Pst*I clone (SsCS39) and three rice chloroplast clones (pRE10, pRP10 and pRP11). The rice cpDNA clones were kindly provided by Prof. Sugiura, Nagoya University, Japan.

Clone purification and sequencing

SsCS39, which is a *Pst*I clone in pBluescript II SK$^+$ (Liu and Musial 1995), was purified using a QIAGEN plasmid mini kit (QIAGEN Pty, Clifton Hill, Australia). Sequencing reactions were set up using T3 and T7 promoter sequencing primers and analysed by the Australian Genome Research Facility, St Lucia, Queensland. Amplification products were sequenced using an Applied Biosystems 373A instrument via fluorescent chain-termination dideoxynucleotides, as described in the manual (ABI Prism Terminator Cycle Sequencing Ready Reaction kit, Perkin Elmer).

Results and discussion

Identification of a chloroplast clone from *Styosanthes* and determination of its sequence

Generally, the higher the ploidy level of a given taxon, the more DNA fragments expected to be produced by nuclear probes (Liu et al. 1999). However, such a relationship should not arise when organelle DNAs are used as DNA probes. SsCS39 behaved like an organelle DNA fragment. When used to probe total DNA digested with the restriction enzyme *Dra*I, this clone detected a single DNA fragment from each of the polyploid genotypes used in this study (see below).

The speculation that SsCS39 might be a fragment of organelle DNA was confirmed by its sequence. The clone is 499 bp in length. Its sequence is highly homoeologous to a section of the soybean chloroplast genome that has an unknown product (Fig. 1).

cpDNA types in diploid taxa

Among the 25 diploid genotypes used, SsCS39 detected six RFLP patterns when *Dra*I was used to restrict total plant DNA (Fig. 2). Each of the three rice cpDNA probes (pRE10, pRP10 and pRP11) detected three RFLP patterns when the same restriction enzyme, *Dra*I, was used (not shown). None of these cpDNA clones detected RFLP among these genotypes when total plant DNA was digested with *Hind*III.

The 25 diploid genotypes represent 18 taxa, 11 having a single genotype each and the other 7 containing 2 genotypes each (Table 1). Of the 7 taxa containing 2 genotypes, only the pair of *S. viscosa* genotypes produced different cpDNA patterns. This is not surprising because *S. viscosa* is known to have a wide geographical distribution and includes a diversity of morphological forms (Ferreira and Costa 1979; Mannetje 1984; Williams et al. 1984).

The 25 diploid genotypes were classified into nine basal groups based on their nuclear DNA similarity (Liu et al. 1999). Accessions in Genome A (Table 1) showed

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1    ATTTTTCGTC TTTCTTTATT TAATGTAAGG TAACTCAGCT TCCGTTTTAT
51   CTCATTCGTC AACTATATAA GATGATAATA ATGTTTCTAT CAAGCATAAG
101  TTCTATTACA AGTCATAGGA AAATCTATAA TCTATTCCCG CATTTTTTTA
151  TTTTCGATTC GGGAATTAGT TCTTGAATAA TTAGTTCTTG AATTTAGAAA
201  CAATAAAGAA ATAGACTAAA AATCGAAAGA ATCCACTGCC AATTTTGGCA
251  TGACGAAAAA AGATATTCTT TCATCAAAGG ACATCAATTA CTCAGATTCG
301  AAGCAATTAT ACCTATTAAT GAATAGACGG AGGAACACTT CGCGTAATGA
351  ATATTAGTCG GAATTTGAAA TCCAAAGAGG GCCCCTTCTA TCCAAAACAA
401  AATCTCTTTT TCCGAAGGAA AGGCTTTCAT TTTTTCCGTT TCATTTTCCT
451  TTTTTCCAAG TTCCTCGAAT GGGTACCCGC CCAGAAAATA GGCCAATTC
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Fig. 1 The sequence of the *Stylosanthes Pst*I clone SsCS39. The *underlined* section showed 91% identity with a section of the soybean chloroplast genome (EMB no. CHGE26948)

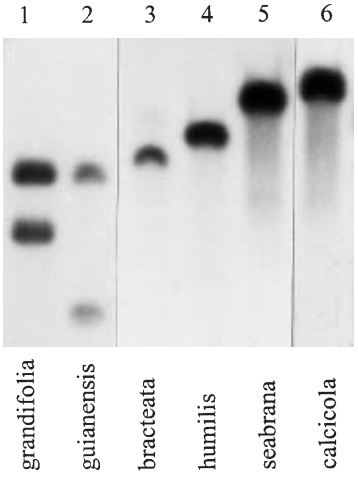

Fig. 2 Six RFLP patterns detected by probing the *Stylosanthes* cpDNA clone SsCS39 to *Dra*I restricted total DNA

2 cpDNA patterns, A1 (both accessions of *S. seabrana*) and A2 (both accessions of *S. hamata*); accessions in Genome B showed 3 cpDNA patterns, B1 (a single *S. viscosa* accession CPI 33831), B2 (a single *S. viscosa* accession CPI 40300) and B3 (both accessions of *S.* aff. *viscosa*); accessions in Genome G showed 2 cpDNA patterns, G1 (the single *S. grandifolia* accession) and G2 (all accessions of *S. campestris*, *S. guianensis*, *S. hippocampoides* and S. *montevidensis*); and accessions in Genome F showed 2 cpDNA patterns, F1 (the single *S. leiocarpa* accession CPI 78192) and F2 (both *S. sp*. accessions). Accessions in the other five genome groups showed a single cpDNA pattern each (Table 1).

The different cpDNA patterns found between *S. seabrana* and diploid *S. hamata* provided further evidence indicating that these 2 species are significantly different (Liu et al. 1999). The same situation also exists for *S. leiocarpa* and *S. sp*. Genotypes in these 2 taxa do not only have different cpDNA patterns (Table 1) but also have significantly different nuclear DNA complements (Liu et al. 1999). Similarly, the different cpDNA patterns of *S.* aff. *viscosa* and *S. viscosa* support the argument that they represent 2 different taxa.

Consistent with results from Gilles and Abbott (1996), cpDNA patterns of the 5 members of the *S. guianensis* complex (including *S. grandifolia*, *S. campestris*, *S. guianensis*, *S. hippocampoides* and *S. montevidensis*) are clearly separated from those of other species (Fig. 1). The same cpDNA pattern derived from *S. guianensis* and *S. montevidensis* (Table 1) supports the argument that these two species are closely related (Mannetje 1977). Mannetje (1984) also considered *S. grandifolia* to be the same species as *S. guianensis*, although Ferreira and Costa (1979) suggested that they should remain separate. Results from this study showed that these 2 species have different cpDNA patterns.

Identification of maternal donors for polyploid species

Of the 26 polyploid accessions used, 24 are tetraploids and the other two hexaploids. These 26 accessions represent 16 taxa. Apart from the 2 accessions of *S. fruticosa* and *S. ingrata*, putative genome structures for all other accessions are known (Table 1; Liu et al. 1999).

Surprisingly, cpDNA variation was detected between progenitors for all of the tetraploid taxa used in this study by the single *Stylosanthes* cpDNA clone SsCS39 (Table 1; Fig. 2). This allowed the identification of putative maternal donors for all these taxa. Furthermore, no contradictory results were obtained from any of the three rice cpDNA clones: they either failed to detect variation between the progenitors for a given polyploid taxon or provided results indicating the same maternal progenitor as inferred by SsCS39. In other words, without exception, identification of the maternal donor for any of the polyploid taxa used in this study can be based on a single cpDNA probe if the probe can reveal differences between the progenitors. The fact that no intraspecific cpDNA variation was detected by any of these probes for any of the polyploid taxa suggested that little cpDNA change had occurred since polyploidization.

Tetraploids with a putative AABB genome structure

Three of the four cpDNA clones (SsCS39, pRP10 and pRP11) detected RFLPs between Genomes A and B. Results from these probes indicated that Genome A1 is the maternal donor to all tetraploid accessions with a known AABB genome structure (including *S.* aff. *hamata*, *S. scabra*, *S.* aff. *scabra*, *S. sericeiceps* and *S. tuberculata*). The same cpDNA patterns produced by these five taxa confirm the results obtained from nuclear DNA probes (Liu et al. 1999) that there is only limited varia-

tion between these taxa. These results raise the question about their separate species status.

Tetraploids with a putative AACC genome structure

Of the four cpDNA probes used, two (SsCS39 and pRP10) detected cpDNA variation between Genomes A and C. Results from these two probes indicated that, without exception, Genome C is the maternal donor to all tetraploid genotypes with a known AACC genome structure (including all accessions belonging to *S. sp.* A, *S. mexicana*, *S. subsericea* and *S. sundaica*). The fact that genotypes of these four taxa produced an identical cpDNA pattern with each of the four cpDNA clones strengthens the argument that these four taxa may not be sufficiently divergent to be treated as different species (Liu et al. 1999).

S. erecta *(AABBXX)*

S. erecta is the only hexaploid species identified in *Stylosanthes* so far (Stace and Cameron 1984), and it is 1 of the only 3 species endemic to Africa (Williams et al. 1984). Results from these cpDNA clones indicated that the maternal donor to this hexaploid species is either Genome A1 or the unidentified Genome X. The third component genome, Genome B, is unlikely to be the maternal donor (Table 1).

S. capitata *(DDEE)*

Of the four probes, only one (SsCS39) detected a difference between Genomes D and E. Results from this single probe indicated that Genome E is the likely maternal donor to *S. capitata*, which has a DDEE genome structure (Liu et al. 1999).

Based on their cpDNA similarity, Gillies and Abbott (1996) proposed *S. macrocephala* to be the maternal donor to *S. capitata*. However, the relationship between *S. pilosa* and *S. capitata* was not known at the time, and this second donor species was not included in their analysis.

S. sympodialis *(AAFF)*

Results from three of the four cpDNA probes suggested that Genome F2 is the maternal donor to *S. sympodialis*. However, the fourth probe (pRE10) generated an RFLP pattern from the 2 *S. sympodialis* accessions that was different from that of any other genotype used in this study. This suggested that the maternal donor (designated as F3) was not included in this study, although this taxon seems to have similar nuclear complement to those of the 2 *S. sp.* accessions (Genome F2). These results provided additional evidence to support the argument that *S. sympodialis* is different from *S. scabra* (Williams et al 1984; Liu et al. 1999).

S. fruticosa

Based on key morphological characteristics used for species identification in *Stylosanthes*, Mannetje (1984) found it difficult to differentiate *S. fruticosa* from *S. scabra*. However, these 2 species have distinct distributions. They have also adapted to different environments (Williams et al. 1984). RAPD analysis showed that genotypes belonging to these 2 species could easily be distinguished, although the RAPD results could not determine whether the difference was at the specific or subspecific level (Glover et al. 1994).

Only a single accession was included for this tetraploid species which has an unknown genome structure. Results from the four cpDNA clones indicated that, in contrast to *S. scabra*, the maternal donor for *S. fruticosa* is likely to be Genome B3. Their different cpDNA donors could have resulted from either different directions of hybridization between the same progenitors (i.e. sharing the same nuclear complement), or different progenitors were involved in their polyploidizations (having different nuclear structures).

Mannetje (1984) also noted the existence of *Stylosanthes* specimens that morphologically appear to be intermediate between *S. sundaica* and *S. fruticosa*. He thus proposed that *S. sundaica* evolved from *S. fruticosa*. Results from these cpDNA clones do not support this hypothesis. The very different cpDNA patterns between these 2 species indicated that they most likely resulted from different polyploidization events.

S. ingrata

S. ingrata is a tetraploid with an unknown genome structure. A small number of *S. ingrata* accessions were held at ATFGRC, and they were all collected from Belize where they co-exist with a wide range of other *Stylosanthes* species (Williams et al. 1984). Based on results from the four cpDNA clones, the maternal donor for *S. ingrata* is likely to be Genome A1, which is the same as that for the tetraploids with a known AABB genome structure (Table 1).

Inheritance of cpDNA in *Stylosanthes*

Organelle inheritance in higher plant species has been investigated using phenotypic, protein or DNA markers (Smith 1989; Reboud and Zeyl 1994). A rapid method of detecting species with the potential to pass plastid DNA via pollen was also developed based on pollen grain staining (Corriveau and Coleman 1988). However, the presence of plastids in pollen is not considered to be conclusive evidence of paternal contribution because

such organelles can be excluded before or in the process of fertilization (Smith 1989).

Strict paternal plastid inheritance is common in gymnosperms but has not been reported for angiosperm species (Smith 1989). Organelle inheritance is strictly maternal for the majority of higher plants, although biparental inheritance of plastid has been reported for some outcrossing species (Reboud and Zeyl 1994).

There has been no reported work on organelle inheritance in *Stylosanthes*. In this study, all the 26 polyploid genotypes expressed cpDNAs from only 1 of their respective progenitor species. As 2–3 individual plants were used for extracting DNA for each of the 51 genotypes and four different cpDNA probes were screened (see Materials and methods), these results suggested that cpDNA in *Stylosanthes* is strictly maternally inherited.

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