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Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers

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Summary. Random amplified polymorphic DNA (RAPD) markers were generated from 20 cultivars and accessions representing four agronomically important species of *Stylosanthes*, *S. scabra*, *S. hamata*, *S. guianensis*, and *S. humilis*. Approximately 200 fragments generated by 22 primers of arbitrary sequence were used to assess the level of DNA variation. Relatively low levels of polymorphism (0–16% of total bands in pairwise comparisons) were found within each species, while polymorphisms between the species were much higher (up to 46%). Very few polymorphisms (0–2%) were detected between the individuals of the same cultivar or accession. A phenogram of relationships among the species was constructed based on band sharing. Four main clusters corresponding to each species were readily distinguished on this phenogram. The allotetraploid species *S. hamata* and its putative diploid progenitor, *S. humilis*, were more similar to each other than to *S. scabra* and *S. guianensis*. No variation in RAPD markers was found between the two commercial *S. hamata* cvs 'Verano' and 'Amiga'. Cultivar 'Oxley' in *S. guianensis* was considerably different from the other cultivars and accessions of this species. The phylogenetic distinctions obtained with RAPDs were in agreement with other studies from morphology, cytology, and enzyme electrophoresis. The low level of polymorphisms observed within each species suggested that interspecific crosses may be a better vehicle for the construction of RAPD linkage maps in *Stylosanthes*.

Key words: Polymerase chain reaction – Random amplified polymorphic DNA – *Stylosanthes* spp.

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Introduction

The genus *Stylosanthes* (*Leguminosae*) has been estimated to consist of about 40 species that are classified into two sections (*Stylosanthes* and *Stylposanthes*) on the basis of morphological characteristics (Kirkbride and de Kirkbride 1985). The section *Stylosanthes* contains all of the known diploid species (2n = 2x = 20), whereas the section *Styposanthes* includes both diploid and polyploid species (Mannetje 1984). Four species of the genus *Stylosanthes*, *namely S. scabra*, *S. hamata*, *S. guianensis* and *S. humilis*, have been widely cultivated as tropical forage legumes. Among these, *S. scabra* and *S. hamata* are allotetraploids (2n = 2x = 40) and appear to be highly diploidized with regular bivalent formation at meiosis and disomic inheritance (Stace and Cameron 1984); the other 2 species, *S. guianensis* and *S. humilis*, are diploid (2n = 2x = 20). These 4 cultivated species are predominantly self-pollinating with a low but variable degree of outcrossing (Miles 1985; Stace 1982). The limited studies that have been carried out on interspecific hybrids have reported complete sterility (Cameron 1974), although normal bivalent formation and moderate seed set have been observed in the synthetic *S. humilis* × *S. hamata* allotetraploid hybrid (Cameron 1968).

Breeding for disease resistance and other characters would benefit from the development of molecular marker technology for this genus of legumes. Variation at the DNA level as measured with molecular techniques offers a number of advantages over morphological data with respect to examination of the basic taxonomic relationships of the species. Variation detected by the molecular analysis of DNA can be quantified, provides many characters, and is not

subject to environmental effects. Although chloroplast DNA (cpDNA) (Palmer et al. 1988 for review) and nuclear RFLPs (Debener et al. 1990; Havey and Muehlbauer 1989; Kesseli et al. 1991; Kochert et al. 1991; Miller and Tanksley 1990; Song et al. 1990) have been employed in taxonomic studies, their use is limited due to the fact that they are costly, relatively slow, and technically demanding. Random amplified polymorphic DNA (RAPD) analysis, however, can circumvent some of the problems associated with RFLP analysis. In RAPD analysis single oligonucleotides of random sequence are used for amplification of DNA by the polymerase chain reaction (PCR) (Williams et al. 1990). Polymorphisms can be simply identified as the presence or absence of an amplification product on the ethidium bromide-stained agarose gels. This provides relatively easy and rapid assessment of the differences in the genetic composition of related individuals. Polymorphisms generated by RAPD assays have been used for assessments of variation (Welsh and McClelland 1990; Williams et al. 1990), fingerprinting (Caetano-Anolles et al. 1991; Klein-Lankhorst et al. 1991), cultivar (Hu and Quiros 1991; Welsh and McClelland 1991) and pathotype (Crowhurst et al. 1991; Goodwin and Annis 1991) identification, and genetic mapping (Williams et al. 1990). However, RAPDs have not been used for the extensive quantitative phylogenetic comparison of plant genotypes.

Although several reports are available concerning the genetic variability of some *Stylosanthes* species as measured by morphological and agronomic characters (Burt et al. 1971; Edye et al. 1974), isozyme loci (Stace and Cameron 1984), and seed protein patterns (Robinson and Megarrity 1975), no data is yet available on variation at the DNA level. Our objectives in the research reported here were to investigate variation in random amplified polymorphic DNA markers in the agronomically important species of *Stylosanthes* to determine genetic relationships and to estimate the feasibility of constructing RAPD linkage maps. In this investigation we compared the results obtained with RAPDs to results obtained from previously reported studies that used conventional taxonomic characters.

Materials and methods

Plant material

The frequencies of RAPDs were assessed among 20 predominantly self-pollinated cultivars and accessions belonging to four species of *Stylosanthes*: *S. scabra* (cvs 'Fitzroy', 'Seca'; CPIs Q10042, 36260, 93116, 55860), *S. hamata* (cvs 'Vernao', 'Amiga'; CPIs 55830, 92412), *S. guianensis* (cvs 'Graham', 'Endeavour', 'Oxley', 'Cook'; CPIs 18750, 34911), and *S. humilis* (cv 'Paterson'; CPIs 40270, 34752, 33830). These were obtained from the Australian Tropical Forages Genetic Resources Centre, Division of Tropical Crops and Pastures, CSIRO Cunningham Laboratory. Plants were grown in either a controlled environment room (16-h daylength; 25 °C and 28 °C night and day temperature, respectively) or in a glasshouse. Five individuals were examined for each accession or cultivar.

DNA extraction method for PCR and PCR conditions

Total plant DNA for PCR was isolated from young leaves according to the following protocol modified from Edwards et al. (1991). A single leaflet was ground in an Eppendorf tube with a disposable grinder (Eppendorf); 300 µl of DNA extraction buffer (200 m*M* TRIS HCl, pH 7.5, 250 m*M* NaCl, 25 m*M* EDTA, 0.5% SDS w/v) was added, and the tubes were vortexed for a few seconds. The extracts were centrifuged in a microfuge (13000 rpm) for 2 min, and the supernatant was transferred to a new Eppendorf tube. The DNA was precipitated with isopropanol, dried, and dissolved in 300 µl TE. After being extracted once with equal volumes of phenol and chloroform, the DNA was ethanol precipitated and redissolved in 100 µl TE. DNA concentrations were estimated with a fluorimeter (Model TKO 100, Hoefer Scientific Instruments, San Francisco). Amplifications were done in 25 µl of reaction mixture containing 67 m*M* TRIS HCl (pH 8.8), 16.6 m*M* $(NH_4)_2SO_4$, 0.45% (v/v) Triton-X-100, 200 µg/ml gelatine, 4 m*M* $MgCl_2$, 200 µ*M* dNTPs, 0.1–0.2 µ*M* primer, 25 ng of genomic DNA, and 1.5 units of Taq polymerase (Biotech International). After an initial heat denaturation at 94 °C for 5 min the reaction mix was subjected to amplification in a Perkin Elmer-Cetus thermocycler for 40 cycles consisting of 1 min at 94 °C, 1 min at 45 °C, and 2 min at 72 °C. Amplified fragments were resolved on a 1–1.5% agarose gel containing TRIS-Borate recipe buffer, visualized, and photographed after staining with ethidium bromide (EtBr). Primer sequences were obtained either from the Queensland Institute of Medical Research (Brisbane, Australia) or from Operon Technologies Inc. (Alameda, Calif.) (Table 1).

Data analysis

Polymorphisms between accessions/cultivars were calculated using the index of genetic distance (1 − F). F values were obtained by using the method of Wang and Tanksley (1989) based on the theory of Nei and Li (1979), which involved estimating the fraction of shared fragments (F) between two cultivars/accessions using the formula $F = 2m_{xy}/(m_x + m_y)$, where m_{xy} was the number of RAPD fragments shared by the two cultivars/accessions and m_x and m_y were the number of RAPD fragments in

Table 1. Primer sequences used in the study

Primer number	Sequence (5′–3′)	Primer number	Sequence (5′–3′)
3	GCATGCTTGC	69	GGAAGTCGCC
4	ACTCAGCATG	70	AGTCGTCCCC
6	GCTTCGATACG	71	ACGCATCGCA
7	CTCAATGCTGG	72	CTGCATCGTG
8	GTAACTTGCCT	73	GAAACACCCC
9	TCACGATGCA	74	TGTAGCTGGG
10	ATTGCGTCCA	76	CTTCCGCAGT
49	GGTGATCAGG	77	ACGCGCATGT
66	GGTCGGAGAA	78	GACGCCACAC
67	TCGGACGTGA	79	ACCAGGTTGG
68	AGACGTCCAC	84	CTGACCAGCC

[a] Reference number in CSIRO primer bank

each cultivar/accession. The SAS computer program (SAS, 1985) was used for cluster analysis of the 1−F values (McQuitty procedure of SAS, based on the Unweighted Pair Group Method with Arithmetic Mean, UPGMA), and the Proc Tree procedure of SAS was used to generate a phenogram.

Results

Optimization of PCR amplification of DNA from Stylosanthes spp.

The 22 oligonucleotides of random sequence (mostly 10 mers) were tested as primers to amplify the DNA from 20 cultivars/accessions of four *Stylosanthes* species. These 22 primers were selected from 90 primers because of their consistent amplification of DNA segments from *Stylosanthes* (Fig. 1). Depending on the primer/accession combination and the amplification conditions used, the number of amplification products resolved varied between 5 and 15 with a size range from 0.25 kb to 1.9 kb. All amplifications were found to be reproducible when repeated at different times under the same amplification conditions. Higher molecular weight bands (> 2.0 kb) which could not be reproduced consistently were not included in the data analysis. A sampling of five individuals for each accession/cultivar in most instances produced the same banding pattern and thus further demonstrated the reproducibility of the assays. However, changes in amplification conditions affected the number of bands produced. Among the amplification parameters examined, Mg^{2+} ion concentration was found to be very important for successful amplifications. At lower Mg^{2} ion concentrations (i.e. 2 m*M*) the number of fragments produced for a given primer was drastically reduced or no fragments at all were produced. However, higher concentrations (4 m*M*) increased the number of bands resolved, probably by stabilizing the hybridization of primer to template DNA (Fig. 2). Reproducible variation was also observed in the relative intensities of bands generated with 1 DNA sample.

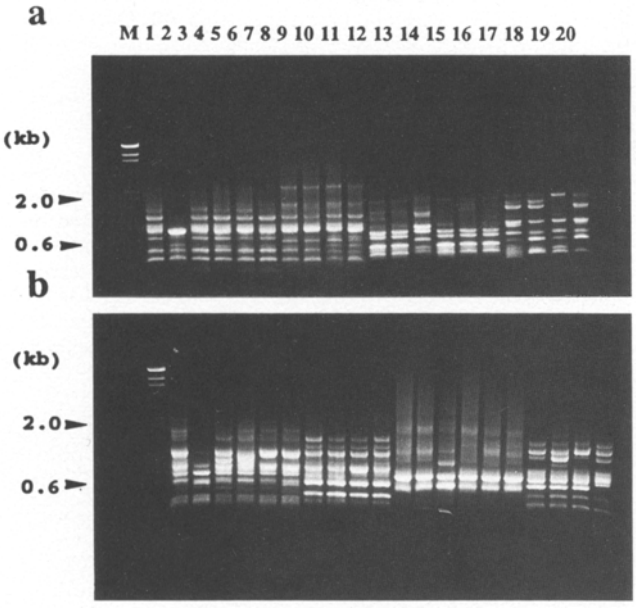

Fig. 1a, b. RAPDs revealed by amplification with **a** primer 68 and **b** primer 70 in 20 cultivars/accessions belonging to four *Stylosanthes* species. *M* DNA size marker (*Hin*dIII-cut λ DNA), *lanes 1–6 S. scabra* cultivars/accessions 'Fitzroy', 'Seca', Q10042, 36260, 93116, 55860, *lanes 7–10 S. hamata* cultivars/accessions 'Verano', 'Amiga', 55830, 92412, *lanes 11–16 S. guianensis* cultivars/accessions 'Graham', 'Endeavour', Oxley, 18750, 'Cook', 34911, *lanes 17–20 S. humilis* cultivars/accessions 'Paterson', 40270, 34752, 33830

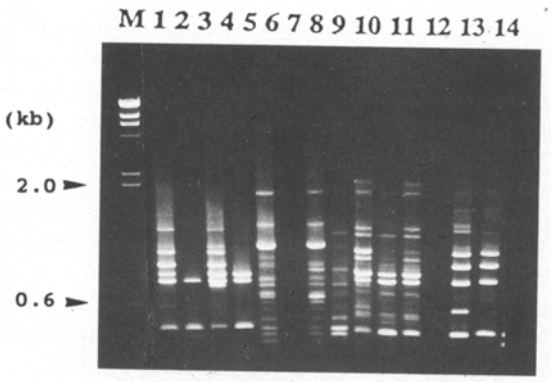

Fig. 2. Effect of Mg ion concentration on PCR amplification of DNA from *Stylosanthes* cultivars/accessions. DNA samples from the same individuals were amplified with primer 10 using either 4 or 2 m*M* Mg^{2+} and run on alternate lanes. *M* DNA size marker (*Hin*dIII-cut λ DNA), *lanes 1 and 2* 'Fitzroy', *lanes 3 and 4* 36260, *lanes 5 and 6* 'Verano', *lanes 7 and 8* 55860, *lanes 9 and 10* 'Graham', *lanes 11 and 12* 'Endeavour', *lanes 13 and 14* 'Paterson'

RAPDs in Stylosanthes spp.

Between 0 and 2% of the total bands in pairwise comparisons were polymorphic within each accession/cultivar. Such polymorphisms were detected in *S. humilis* accession 92412 (1%), in *S. guianensis* cvs 'Oxley' (2%), and 'Endeavour' (2%), and in *S. scabra* cv 'Seca' (2%) (Fig. 3). Similarly, low levels of polymorphisms varying from 0–25% were found within each species (Table 2). However, higher levels of polymorphisms were detected between species (28–46%). The total number of RAPD bands detected among the species was 145, giving an average frequency of 6.6 RAPD bands per primer.

It is important to note that the nature of the RAPD polymorphisms detected within each species of *Stylosanthes* was different from that observed between species. Most of the polymorphism within each species

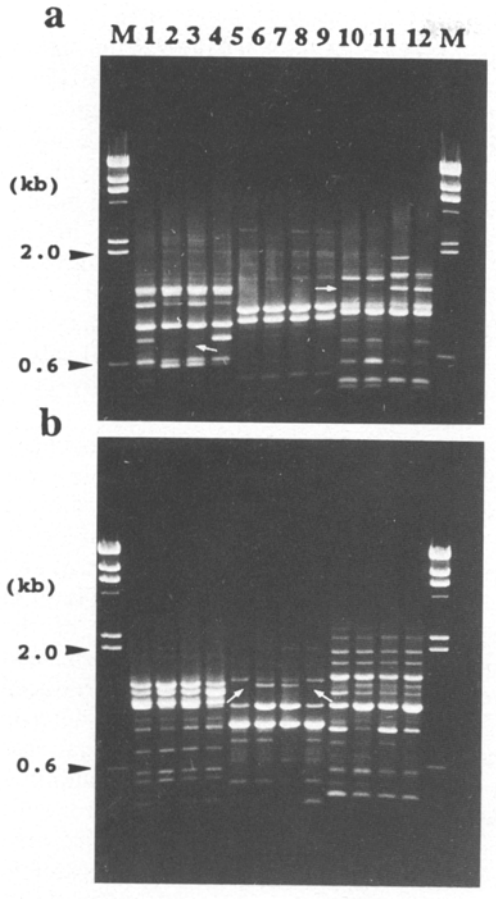

Fig. 3a, b. Intra-accessional RAPDs revealed by amplification with **a** primer 84 and **b** primer 49. **a** M DNA size marker (*Hin*dIII-cut λ DNA), *lanes 1–4* 92412, *lanes 5–8* 33830, *lanes 9–12* 'Oxley'. **b** *lanes 1–4* Seca, *lanes 5–8* Endeavour, *lanes 9–12* Paterson. *Arrows* indicate the polymorphic fragments

was due to the variation in relatively minor or weakly staining bands. Polymorphisms between species, however, consisted of variation in both major and minor amplified fragments. It is likely that intense monomorphic bands are derived from multiple copy sequences containing homomorphic core fragments.

Phenetic analysis of RAPD data

DNA fingerprints produced by each primer were used to obtain 1 − F values in pairwise comparisons of the accessions/cultivars according to the formula of Nei and Li (1979). A cluster analysis based on these values was performed to generate a phenogram. This approach has been widely used to demonstrate the genetic differences among taxonomic groups using RFLP analysis (Havey and Muehlbauer 1989; Kesseli et al. 1991; Wang and Tanksley 1989). We considered each RAPD fragment as a distinct allele, and thus fragments of equal size in two genotypes were products

Table 2. Frequency of RAPD polymorphisms (1 − F × 100) among the cultivars/accessions belonging to four *Stylosanthes* species

Accessions	*Stylosanthes scabra*				*Stylosanthes hamata*			*Stylosanthes guianensis*					*Stylosanthes humilis*			
	Seca	Q10042	36260	93116	55860	Verano	Amiga	55830	92412	Graham	Endeavour	Oxley	18750	Cook	34911	Paterson	40270	34752	33830
Fitzroy	15	9	8	9	10	34	33	32	34	40	40	46	44	46	45	40	36	36	31
Seca		11	13	12	13	35	34	31	38	39	40	43	38	43	43	38	36	39	42
Q10042			9	10	9	34	34	31	36	41	42	43	41	38	37	39	39	38	42
36260				9	11	33	34	33	33	44	45	45	44	45	44	40	41	40	39
93116					7	32	33	35	33	45	45	43	46	47	45	44	45	41	43
55860						34	35	33	40	39	42	43	40	37	39	39	43	40	42
Verano							0	4	5	43	45	45	44	43	46	28	30	30	29
Amiga								4	5	43	45	45	43	43	45	28	30	30	29
55830									6	41	40	41	40	43	43	27	29	28	33
92412										42	43	44	42	43	43	29	30	30	28
Graham											10	20	7	12	14	40	38	42	44
Endeavour												25	14	10	13	41	43	43	42
Oxley													18	18	15	41	45	47	45
18750														10	12	41	45	47	45
Cook															6	42	45	43	42
34911																43	41	44	41
Paterson																	8	8	9
40270																		9	13
34752																			16
33830																			

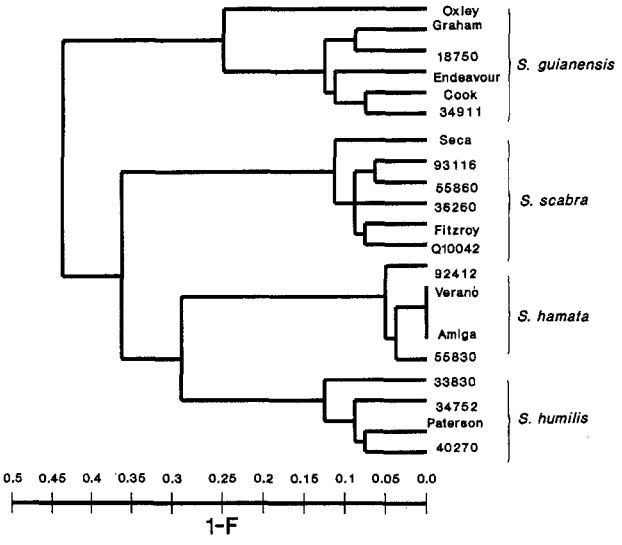

Fig. 4. Clustering of *Stylosanthes* spp. by UPGMA analysis of 1−F values from pairwise comparisons of RAPDs between cultivars/accessions

of monomorphic alleles. The phenogram obtained by this procedure readily separated the genotypes analysed into the four main species groups (Fig. 4). Allotetraploid *S. hamata* was more similar to diploid *S. humilis* than to the other two species examined (1−F = 0.27). The *humilis/hamata* group was more similar to allotetraploid *S. scabra* than to diploid *S. guianensis*.

The most homogeneous species group was *S. hamata* with a 1−F value for the species of only 0.05. The *S. hamata* cvs 'Verano' and 'Amiga' could not be distinguished on the fragment patterns obtained with the 20 primers. The screening of these 2 cultivars with a total of 90 primers and subsequent digestion of the amplification products with several restriction enzymes also failed to produce any polymorphisms (K. Kazan unpublished). Within the *S. humilis* group, accession 33830 was clearly differentiated from the other 3 accessions while in the *S. scabra* set of accessions, the cv 'Seca' was separated from the other 5 accessions. The greatest degree of intraspecific diversity was displayed by the *S. guianensis* group, with the 'Oxley' cultivar clearly differentiated from the other 5 cultivars and accessions with a 1−F value of 25%, which is close to the value distinguishing the species of *S. hamata* and *S. humilis*.

Discussion

The results demonstrate that RAPD technology can be used to identify DNA polymorphisms among cultivars and accessions of the agronomically important legume species *Stylosanthes*. RAPDs may arise from either single base changes in the primer annealing sites or insertions and deletions in the region between the priming sites (Caetano-Anolles et al. 1991; Gillet 1991; Williams et al. 1990). The degree of homology between the fragments of similar size is yet to be demonstrated, but it is likely that fragments shared by two closely related individuals are allelic. This may not be true when comparing the higher taxonomic groups (i.e. genera), which show greater differentiation, because the fragments of equal size may be derived coincidentally from non-allelic genomic regions. In our study each accession was precisely assigned to the species group determined by conventional taxonomy.

The relatively close similarity observed between tetraploid *S. hamata* and diploid *S. humilis* is consistent with the hypothesis that *S. humilis* is a diploid progenitor of tetraploid *S. hamata*. Previous evidence for this hypothesis was based on isozyme patterns and supported by the observation of an improved level of fertility (compared with complete sterility of the diploid *S. humilis* × *S. hamata* hybrid) and formation of only bivalents in the synthetic tetraploids produced between *S. humilis* and *S. hamata* (Stace and Cameron 1984). Results from analyses of seed-protein patterns (Robinson and Megarrity 1975), rhizobial affinities (Date et al. 1979), and morphological and agronomic characters (Burt and Williams 1979) are also consistent with *S. hamata* (2n = 4x = 40) being an allotetraploid between *S. humilis* (2n = 2x = 20) and a diploid *S. hamata* (2n = 2x = 20) species. A similar but independent formation of allotetraploidy between *S. viscosa* (2n = 2x = 20) and an unknown diploid species has been suggested for *S. scabra* (Stace and Cameron 1984). *S. viscosa* accessions were not included in our study, but further studies could be conducted to test the degree of similarity between *S. scabra* and *S. viscosa*. It is likely that the other diploid putative progenitor species of *S. scabra* may be found in a broader survey of diploid species of *Stylosanthes*.

The low level of polymorphisms observed among cultivars/accessions of each species may partly be due to the predominantly self-pollinated nature of the species. Stace (1982) reported only 1–2% outcrossing in *S. scabra*, but outcrossing in *S. guianensis* has been reported to vary from 1.6 to 21.9% (Cameron and Irwin 1986; Miles 1985). The slightly higher level of intraspecific variation and the existence of intra-accessional variation in *S. guianensis* that was detected in this investigation with RAPDs may be partly explained by the higher outcrossing rates of the species. This result agrees with other studies correlating increased genetic diversity with higher outcrossing rates (Gottlieb 1981; Miller and Tanksley 1990). The fact that seed of these accessions originated from a collection of a small number of wild plants followed

by multiplication of a limited number of plants in quarantine upon introduction to Australia would further restrict the within-accession variation.

Of the 20 accessions investigated in the present work, 14 were included in a classification of a set of 154 accessions based on 28 morphological and 12 agronomic (MA) attributes (Burt et al. 1971). Burt et al. (1971) recognized 28 groups, and their placement of the above 14 accessions was quite similar to that developed in our similarity matrix. Within the *S. guianensis* accessions, the similarity of 'Graham' with 18750 (MA group 10) and 'Cook' with 34911 (MA 8) was identified. In their work, 'Endeavour' also fell within MA 8, but 'Oxley' was again clearly separated (MA 6). Although the taxonomic treatment of *Stylosanthes* has been approached somewhat differently by Mannetje (1984) and Ferreira and Costa (1979), both agree in their recognition of the divergence of 'Oxley' from the other accessions of *S. guianensis*. In crosses among these accessions, the fertility of crosses with 'Oxley' is only 1–5% of the parental fertility, while crosses among the remaining accessions do not show reduced fertility (Cameron 1977 and unpublished).

Differentiation among the *S. scabra* accessions 'Seca', 'Fitzroy' and 36260, which were also included in the classification of Burt et al. (1971), was recognized by their placement in 3 different MA groups, although the 'Fitzroy' and 'Seca' MA groups were more similar to each other than to the 36260 MA group. In the case of *S. humilis*, 34752 was grouped with Australian naturalized ecotypes very similar to 'Paterson', while 33830 and 40270 were located in a separate MA group. Crosses among *S. humilis* accessions support our separation of 33830 from the other 3 accessions as crosses of 33830 and a similar accession 33829 to an Australian ecotype show reduced fertility and seed set while crosses among and between Australian naturalized ecotypes 34752 and 40270 show normal fertility and seed set (Cameron 1970 and unpublished). In the MA classification work, 'Verano' was the only *S. hamata* tetraploid and was grouped with some diploid accessions of *S. hamata*. At higher levels in the MA classification, perenniality and longevity of perennials were the major attributes differentiating species groups. Thus, the annual *S. humilis* was isolated with another annual species from the perennial species groups containing *S. guianensis*, *S. hamata*, and *S. scabra*, but the perennials with shorter longevity, including *S. guianensis*, were then grouped at a higher level with the annual *S. humilis* cluster (Burt et al. 1971). Higher order groupings from our RAPD analysis were quite different, with *S. humilis* first grouping with *S. hamata* and the resulting cluster then grouping with *S. scabra*.

The information presented here will also be useful for the selection of potential parents for RAPD mapping populations. The greatest intraspecific variation was observed between *S. guianensis* cv 'Oxley' and other accessions of the species, but sterility in this cross may be problematic for linkage analysis. It seems likely that intraspecific variation for all of the species examined is not sufficient for the easy construction of a genetic map within *Stylosanthes* spp. Hybrids of the diploid crosses *S. humilis* × *S. hamata* and *S. humilis* × *S. guianensis* showed complete sterility (Cameron 1968). This excludes their use in genetic linkage analysis. The modest fertility of hybrids between the tetraploid species *S. scabra* and *S. hamata* may permit their utilization as potential parents for linkage analysis. Agronomically important cultivars, 'Fitzory' (*S. scabra*) and 'Verano' (*S. hamata*), also differ by some morphological (Edye et al. 1974) and important agronomic characteristics, i.e. resistance to anthracnose (Irwin et al. 1986).

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