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Identification of selected cultivars and accessions of *Stylosanthes* by isozyme analysis

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Abstract

This paper establishes standard isozyme patterns for cultivars and selected accessions of *Stylosanthes scabra*, *S. guianensis* and *S. hamata* using seeds from single plants. A total of 9 isozyme systems with 13 isozyme loci was investigated. The banding patterns were used to discriminate among all cultivars of *S. scabra* and *S. guianensis* and the cv. Verano from the other cultivars and accessions of *S. hamata*. All accessions and cultivars of *S. scabra* and *S. guianensis* showed differences at a number of loci ranging from 1 to 6. The isozyme patterns obtained for the leucine amino peptidase-2 (LAP-2) and isocitrate dehydrogenase (IDH) were sufficient to place the cultivars into species groups. Markers developed on single seeds from single plants were used to detect differences, both within and between cultivars or accessions. These differences were restricted to either one or two of the three isozyme systems, acid phosphatase (ACP), phospho-gluco isomerase (PGI-2) and LAP-2. In *S. hamata,* one seed lot of cv. Amiga and two seed lots of cv. Verano showed intra-seed lot differences in ACP. In *S. scabra*, one seed lot of accession CPI 55857 showed differences in ACP and PGI-2. In *S. guianensis*, one seed lot of cv. Cook showed differences in PGI-2 isozyme. This genetic variability is thought to have been caused by outcrossing.

Resumen

En el presente artículo se establecen los patrones standard de isoenzimas de los cultivares y accesiones seleccionadas de Stylosanthes scabra, S. guianensis *y* S. hamata, *mediante el uso de semillas obtenidas de plantas individuales. Se investigó un total de 9 sistemas de isoenzimas con 13 loci isoenzimas. Los patrones de banda fueron utilizados para discriminar todas las accesiones de* S. scabra *y el cv. Verano de los otros cultivares y accesiones de* S. hamata. *Todas las accesiones y cultivares de* S. scabra *y* S. guianensis *mostraron diferencias en el número de locis en el rango de 1 a 6. Los patrones de leucine amino peptidasa-2 (LAP-2) e isocitrato dehidrogenasa (IDH) fueron suficientes para ubicar a los cultivares en grupos específicos. Se utilizaron marcadores, desarrollados en semillas individuales obtenidas de plantas individuales, para detectar diferencias dentro y entre cultivares o accesiones. Estas diferencias fueron restringidas a una o dos de los tres sistemas de isoenzimas, fosfatasa ácida (ACP), fosfo-gluco isomerasa (PGI-2) y LAP-2. En* S. hamata, *un lote de semillas del cv. Amiga y dos lotes de semillas del cv. Verano mostraron diferencias dentro de lote de semillas con el sistema ACP. Un lote de semillas de* S. guianensis *cv. Cook mostró diferencias con los sistemas ACP y PGI-2. Se considera que esta variabilidad genética pudo haberse debido al cruzamiento.*

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Introduction

The production, persistence and expansion of *Stylosanthes*-based pastures in Australia have been seriously limited by the fungal disease anthracnose caused by *Colletotrichum gloeosporioides*. There are two distinct pathogen types (Irwin and Cameron 1978) and four different races have been identified within each type in Australia (Irwin *et al.* 1986). Since the early 1960s, no fewer than 11 cultivars of *Stylosanthes* have been released in Australia (Oram 1990); however, large-scale commercial seed production has ceased

for all except *S. guianensis* cv. Oxley, *S. hamata* cv. Verano and cv. Amiga, and *S. scabra* cv. Seca because new pathogenic races have arisen to cause widespread damage to the other cultivars.

One simple strategy to combat such a variable pathogen is to use genetic diversity by physically mixing different genotypes of the host, each carrying resistance to a different race (Wolfe 1985). This strategy has been successfully used to restrict diseases in annual crops including wheat and barley, and is currently being tested in the perennial *Stylosanthes*-based pastures. While the benefits of using mixtures were not apparent in the short term (Chakraborty *et al.* 1991), there is evidence of low levels of anthracnose in a 13-year old pasture sown to an inter-specific mixture of *Stylosanthes* (R.D. Davis personal communication). Many new plant phenotypes were identified in the plant progeny, suggesting considerable recombination of the gene pool through natural outcrossing.

In an effort to study how mixtures can restrict anthracnose progress, field plots of mixtures of *Stylosanthes* genotypes have been established at various locations in Queensland. One aim of this study is to monitor changes in host and pathogen populations so as to predict the long-term viability of these mixtures. It is necessary to genetically characterise and distinguish components of a mixture in order to identify definite genetic changes in their progeny. Seed protein patterns have been used in the past (Robinson and Megarrity 1975) to identify *Stylosanthes* introductions at the species level. However, isozymes are recognized as better biochemical markers for advancing genetic studies, as they are single gene characters, commonly co-dominant in effect and are relatively unaffected by the environment. The usefulness of isozymes in genetically characterising cultivars is well established for many plants including pastures (Collins *et al.* 1984). This paper reports on the use of isozymes for identifying the various components in these genotype mixtures. Selected accessions and cultivars of *S. hamata* and the other commercially used species, *S. guianensis* were also included in this study for comparison. Initially, isozyme markers were identified using seeds collected from a single plant. These markers were then used to detect variation within authentic early generation breeder's seeds of cultivars or accessions from one or more sources.

Materials and methods

Seed source

Initial analysis. Seeds for the initial analysis were obtained from the Australian Tropical Forages Genetic Resource Centre and grown in pots in a glasshouse at St. Lucia, Brisbane, Australia. All analyses were carried out on single seed extracts; initially, seeds from a single plant were used to establish standard isozyme patterns of *S. scabra*, *S. hamata*, *S. guianensis* and *S. humilis*, and then commercial and other seed lots were used to verify these patterns. The relative mobility of the isozyme bands expressed as the R_f values was the mean of 10 seeds.

Variations in cultivars or accessions

Seeds of cvv. Verano and Amiga were obtained from a range of sources (Table 4) and 25 seeds per seed lot were analysed for each cultivar. For all other accessions and cultivars, the range of variability in authentic early generation seed was determined from 10 seeds of each lot (Table 4). These seeds were either from commercial sources or from field plots of early generation breeder's seed grown for seed multiplication by the Australian Tropical Forages Genetic Resource Centre. Lot 13 of *S. scabra* cv. Seca was bulked from seeds of 6 plants grown in a glasshouse by Dr D.F. Cameron of CSIRO.

Sample preparation and electrophoresis

Samples of single seeds were lightly scarified with emery paper and placed on moist filter paper in a petri dish at 32 °C overnight. The following morning, each seed was crushed in a drop of chilled crushing buffer (Table 1) and absorbed onto 3 x 7 mm wicks of filter paper (Whatman 3M) through a single layer of Miracloth (Calbiochem). The wicks were arranged in a microtitre tray kept chilled over crushed ice until loaded in the gels.

Crushing buffer A consisted of 0.01 M phosphate buffer, pH 7.5 containing 12% soluble polyvinyl pyrrolidone (PVP-40), 0.3% bovine serum albumin (BSA), 0.03% ethylene diamine tetraacetic acid (di sodium EDTA) and 0.01M 2-mercaptoethanol. Crushing buffer B consisted of the same phosphate buffer as above with 5% PVP, 20% sucrose, 0.01M 2-mercaptoethanol

Table 1. Electrophoretic conditions used to resolve *Stylosanthes* isozymes.

Tray buffer	Gel buffer	Crushing buffer[1]	Running conditions	Isozyme resolved
pH 7.9 0.028M LiOH 0.192M borate	pH 7.9 0.002M LiOH 0.019M borate 0.04M tris 0.04M citrate	A A B	250V, 45mA, 2.5h 200V, 40mA, 3h 200V, 50mA, 2.5h	PGI (EC 5.3.1.9) ADH (EC 1.1.1.1) EST (EC 3.2.1)
pH 8.7 0.1M NaOH 0.3M borate	pH 7.9 0.015M tris 0.001M citrate	D D	200V, 35mA, 1.5h 200V, 35mA, 1.5h	ACP (EC 3.1.3.2) LAP (EC 3.4.11.1)
pH 8.7 0.1M NaOH 0.3M borate	pH 7.6 0.016M tris 0.003M citrate	A	200V, 50mA, 3h	TPI (EC 5.3.1.1)
pH 6.8 0.036M tris 0.01M citrate	tray buffer diluted 1:3 with water	D,A D D	250V, 35mA, 3.5h 250V, 35mA, 4.5h 250V, 35mA, 3.5h	MDH (EC 1.1.1.37) IDH (EC 1.1.1.42) ME (EC 1.1.1.40)

[1] See text for details.

and 0.01% non-ionic detergent, Triton X-100. Crushing buffer C consisted of 0.05M phosphate buffer, pH 7.5, containing 9.6% PVP, 0.8% BSA and 0.01M 2-mercaptoethanol.

Isozymes were separated using horizontal starch gel electrophoresis on gels containing 15.2% starch (Sigma) using different buffer systems, depending on the isozyme system to be analysed (Table 1). Electrophoresis was carried out at 4 °C. Then the gels were sliced and incubated in staining media at 37 °C in the dark until the bands appeared.

The acid phosphatase (ACP) staining medium consisted of 50 mg α-naphthyl acid-phosphate dissolved in 50% (v/v) acetone, 75 mg fast garnet GBC, 2.0 ml 0.1M $MgCl_2$ in 100 ml 0.2M acetate buffer pH 5.0. The alcohol dehydrogenase (ADH) staining medium consisted of 4.0 ml 0.01M nicotinamide adenine dinucleotide (NAD), 4.0 ml 0.01M nitro blue tetrazolium (NBT) and 2.0 ml 0.1M $MgCl_2$, in 50 ml of 0.05M tris-HCl pH 8.0 to which was added 2.0 ml each of ethanol and propanol just prior to incubation. The esterase (EST) staining medium consisted of 50 mg of fast blue RR, 7.0 ml of 1.0M phosphate buffer pH 6.0 and 55 ml of distilled water. To this was added 0.5 ml each of 5.0% α-naphthyl acetate and 5.0% β-naphthyl butyrate made up in 50% (v/v) acetone. Iso-citrate dehydrogenase (IDH), malate dehydrogenase (MDH), malic enzyme (ME), and phospho-gluco isomerase (PGI) staining were carried out according to the methods of Torres *et al.* (1978). Leucine amino peptidase (LAP) and triose-phospho isomerase (TPI) staining were carried out according to the methods of Vallejos (1983). All gels were incubated in the staining medium at 37 °C until the bands appeared.

After staining, the gels were rinsed in distilled water and fixed in 50% ethanol for one hour before recording the results. The putative alleles were designated by letters, depending on their mobility as described by Torres *et al.* (1978). The fastest was called F, followed by I, M, S and V in the descending order of mobility. When more than one locus for a given enzyme existed, the locus encoding the most anodal (fastest migrating) form was designated 1 and the next 2 and so on.

Results

Standard isozyme patterns

ACP Two bands, corresponding to two alleles were observed for this enzyme. At this locus, four different phenotypes were observed. The single banded phenotypes were labelled FF or SS and the two banded phenotype was labelled FS. This pattern is usually indicative of a gene/enzyme system with a monomeric structure. A few accessions analysed showed a complete absence of bands at the corresponding sites.

ADH Both single banded and three banded phenotypes were observed for this enzyme. The single banded phenotypes were either FF or MM and the three banded phenotypes were FM or MS. The three banded phenotype is usually indicative

of a dimeric structure of the enzyme system where two bands correspond to the two alleles and the third intermediate band corresponds to the intragenic heterodimer.

EST Single banded and two banded phenotypes were observed for this enzyme, indicating a monomeric structure. The single banded phenotypes were either FF or SS while the two banded phenotype was FS.

IDH It showed a dimeric structure with a fast and a slow band corresponding to two alleles, together with an intermediate heterodimeric band. A total of five alleles was observed for this enzyme in all the accessions tested. They were labelled F, I, M, S and V in the descending order of mobility. In the heterozygotic genotypes that showed FV, the intermediate heterodimeric band corresponded to the M allele. Similarly, the intermediate heterodimeric band of the genotype IS overlapped with the band corresponding to the M allele.

LAP The activity of this enzyme was resolved into two zones corresponding to two gene systems, *Lap-1* and *Lap-2*. The faster of the two, *Lap-1*, showed either one banded (FF or SS) or two banded (FS) genotypes implying a monomeric structure. All accessions of *S. guianensis* showed a complete absence of bands under the same assay conditions. *Lap-2* showed three distinct alleles, F, I and S. The single banded genotypes were labelled as II or SS while the two banded genotypes were labelled FS or IS. None of the cultivars analysed exhibited the FF genotype in them. All accessions of *S. guianensis* showed the II genotype and those of *S. hamata* showed the FS pattern. All accessions of *S. scabra* showed the IS genotype except CPI 93116 which had an SS genotype.

MDH The activity of this enzyme system was resolved into three zones of isozymes with similar mobility which corresponded to three gene systems. They were designated *Mdh-1*, *Mdh-2* and *Mdh-3*. *Mdh-1* had four allelic forms labelled F, I, M and S. The genotype, FM, was shown by all accessions of *S. scabra* and *S. hamata*, whereas accessions of *S. guianensis* showed a different genotype, IS. The heterodimeric bands of genotypes FM and IS overlapped with those corresponding to the F and I alleles of *Mdh-2*. *Mdh-2* showed three alleles designated F, I and S. Accessions of *S. guianensis* showed the IS genotype while those of *S. hamata* showed FS. All accessions of *S. scabra* also showed the genotype FS except Q10042, which had an IS genotype. The heterodimeric band of IS overlapped with the band corresponding to the S allele in *Mdh-1*. *Mdh-3* showed two alleles, F and S and the one banded genotype was labelled SS while the three banded genotypes were labelled FS. None of the accessions tested showed the FF pattern.

ME The two alleles found in this enzyme had very similar mobilities. All the accessions tested had single bands and were either FF or SS. A heterozygous genotype, FS, was not detected in any of the accessions tested.

PGI The activity of this enzyme resolved into two zones corresponding to two gene systems. However, the faster zone, corresponding to the gene *Pgi-1* did not show any polymorphism and was restricted to a single band. *Pgi-2* on the other hand had useful variations and showed two types of one banded genotypes which were labelled either FF or SS. A heterozygous genotype was not detected in the accessions analysed.

TPI The activity of TPI was resolved into two zones, corresponding to 2 gene systems labelled *Tpi-1* and *Tpi-2*. *Tpi-1* had three alleles, F, M and S and showed either one banded (MM or FF) or three banded (FM or MS) genotypes thus indicating the dimeric nature of the enzyme. *Tpi-2* also showed three alleles F, M and S. All accessions tested in the three species showed three banded genotypes which corresponded either to FM or MS.

The patterns obtained could be used to discriminate among all cultivars of *S. scabra* and *S. guianensis*, but those of *S. hamata* could only distinguish between cv. Verano and the other *S. hamata* cultivars or accessions. The putative genotypes are summarised in Table 2. The detected alleles are compared schematically in Figure 1. There was no indication of gene duplication in any of the isozymes tested, despite accessions of *S. scabra* and *S. hamata* used in this study being tetraploids. Of all the isozymes tested, LAP-2 proved to be the best to discriminate between the first 3 species, *S. scabra*, *S. guianensis* and *S. hamata*, as the cultivars and accessions of these 3 species showed unique profiles (Table 2). A matrix (Table 3) was compiled with the data shown in Table 2 to reveal how any one accession differed from another. This was restricted

Table 2. Putative isozyme genotypes of accessions of *Stylosanthes* spp. Absence of bands is denoted by a dash.

Species or accession cultivar	ACP	ADH	EST	IDH	LAP 1	LAP 2	MDH 1	MDH 2	MDH 3	ME	PGI 2	TPI 1	TPI 2
S. scabra													
40205 Fitzroy	SS	FM	FF	FV	FF	IS	FM	FS	FS	FF	FF	FM	MS
40292 Seca	SS	FM	FF	FV	SS	IS	FM	FS	FS	FF	SS	FM	MS
Q10042	SS	FM	FF	FV	SS	IS	FM	IS	FS	FF	SS	FM	MS
36260	SS	FM	FF	IS	SS	IS	FM	FS	SS	FF	SS	FM	MS
55857	SS	FM	FF	FV	FS	IS	FM	FS	FS	FF	FF	FM	MS
55860	SS	FM	FF	FS	FS	IS	FM	FS	FS	FF	SS	FM	MS
93116	FS	MM	FF	FV	FS	SS	FM	FS	FS	FF	SS	MM	MS
S. guianensis													
Q8558 Endeavour	—	MM	FS	MM	—	II	IS	IS	SS	SS	FF	FM	FM
38754 Cook	—	FF	FS	MM	—	II	IS	IS	SS	SS	FF	FM	FM
11491 Oxley	—	FF	SS	MM	—	II	IS	IS	SS	FF	SS	FF	FM
40255 Graham	SS	FF	FS	MM	—	II	IS	IS	SS	SS	SS	FM	FM
18750	SS	FF	FS	MM	—	II	IS	IS	SS	SS	FF	FM	FM
34911	SS	FF	SS	MM	—	II	IS	IS	SS	SS	SS	FM	FM
S. hamata													
38842 Verano	—	MS	FF	IS	FF	FS	FM	FS	FS	SS	SS	MS	MS
55822 Amiga	FF	MS	FF	IS	FF	FS	FM	FS	FS	SS	SS	MS	MS
92412	FF	MS	FF	IS	FF	FS	FM	FS	FS	SS	SS	MS	MS
55830	FF	MS	FF	IS	FF	FS	FM	FS	FS	SS	SS	MS	MS
S. humilis Paterson	SS	FM	FF	MM	SS	IS	FM	FF	SS	SS	FF	FM	MS

Table 3. Genetic relationship between different accessions based on the number of isozyme loci at which they differ. The matrix is compiled from data in Table 2.

	Fitzroy	Seca	Q10042	36260	55857	55860	93116
S. scabra							
Fitzroy	0	2	3	4	1	3	6
Seca		0	1	2	2	2	5
Q10042			0	3	3	3	5
36260				0	4	3	7
55857					0	2	5
55860						0	5
93116							0

	Endeavour	Cook	Oxley	Graham	18750	34911
S. guianensis						
Endeavour	0	1	4	3	2	4
Cook		0	4	2	1	3
Oxley			0	4	5	3
Graham				0	1	1
18750					0	2
34911						0

to the accessions of only 2 species, *S. scabra* and *S. guianensis*. It was based on the number of loci at which the isozyme patterns differed. For example, it revealed that cvv. Fitzroy and Seca differed at two loci whereas the differences between cv. Fitzroy and CPI 93116 were evident at 6 isozyme loci. A similar matrix was not prepared for *S. hamata* because of the similarity of the accessions. The only difference that could be detected in this species was in ACP isozyme where all accessions except Verano showed a homozygous FF band (Table 2).

Variation within and between cultivars or accessions of commercial and other seed lots

The putative isozyme genotypes obtained from different seed lots, both from breeders and commercial sources, are summarised in Table 4. Only

Table 4. Putative isozyme genotypes of ACP, PG12 and LAP2 of the different seed lots analysed (25 or 10 seeds analysed per seed lot)

Lot no.	Species	Accession Cultivar	Year	Source	Putative genotypes		
					ACP	PGI2	LAP2
1	*S. hamata*	55822 Amiga	1986	Breeders seed	25FF	25SS	25FS
2			1989	Basic seed 2nd generation	23FF, 2FS	25SS	25FS
3			1989	Commercial 2nd generation	25FF	25SS	25FS
4			1990	Commercial 3rd generation	25FF	25SS	25FS
5		38842 Verano	1971	Breeders seed	25-[2]	25SS	25FS
6			1985	Commercial	25-	25SS	25FS
7			1986	Commercial	21-, 4FS	25SS	25FS
8			1989	Commercial	19-, 6FF	25SS	25FS
9			1990	Commercial	25-	25SS	25FS
10	*S. scabra*	36260	1982	CSIRO[1]	10FS	10SS	10IS
11		40205 Fitzroy	1974	CSIRO	10FS	10FF	10IS
12		40292 Seca	1983	CSIRO	10SS	10SS	10IS
13		40292 Seca	1983	Bulk seeds (from 6 plants)	10SS	10SS	10IS
14		55857	1980	CSIRO	8SS, 2FS	8FF, 2SS	10IS
15		55860	1977	CSIRO	10SS	10SS	10IS
16		93116	1983	CSIRO	10FS	10SS	10SS
17		Q10042	1978	CSIRO	10FS	8FF, 2SS	10IS
18	*S. guianensis*	11491 Oxley	1973	CSIRO	10-	10SS	10IS
19		11493 Oxley	1974	CSIRO	10-	10SS	10II
20		18750	1973	CSIRO	10-	10SS	10II
21		34911	1974	CSIRO	10-	10SS	10II
22		38754 Cook	1973	Commercial	10-	7SS, 2FF, 1FS	10II
23		40255 Graham	1973	Commercial	10-	10SS	10IS
24		Q8558 Endeavour	1972	Commercial	10SS	10SS	10II

[1] High purity, early generation seed from CSIRO germplasm collection.
[2] No ACP Bands

3 out of 13 isozyme loci, ACP, PGI-2 and LAP-2, showed differences both within and between certain seed lots. For example, in *S. hamata*, zymograms for most seeds examined in the commercial and other samples of the two cultivars were identical to those described above for these 2 cultivars for 12 of the 13 isozymes. Seed lot 2 of cv. Amiga showed an ACP pattern corresponding to FS in 2 out of 25 seeds. In the commercial seed lot 7 of cv. Verano, 4 out of 25 seeds showed an ACP pattern corresponding to FS while in lot 8, 6 out of 25 seeds showed an ACP pattern corresponding to FF. In *S. scabra*, seed lot 14 of CPI 55857 showed differences in both ACP and PGI-2 genotypes; 2 out of 10 seeds had FS for ACP and 2 out of 10 seeds had SS for PGI-2. The PGI-2 genotype of the accession Q10042 also showed a difference with 2 of the 10 seeds showing the SS genotype. Neither of the two seed sources of Seca showed any variation in the 13 isozyme loci studied. In *S. guianensis* cv. Cook, lot 22 showed differences in PGI-2, with 1 out of 10 seeds showing an FS and 2 out of 10 seeds showing an FF genotype. In the case of Oxley, the 2 introductions, CPI 11491 and CPI 11493, which make up the cultivar, could be readily distinguished using their LAP-2 banding patterns. There were no differences within each of the 2 accessions and the isozyme pattern of the accession CPI 11493 was also identical to that of the single-plant seeds of this accession used in the preliminary studies (Table 2).

Discussion

Results of this investigation demonstrate the usefulness of isozyme analysis of seeds in identifying the different species of *Stylosanthes*. They also show that differences in the isozyme patterns of 9 isozyme systems discriminate between all cultivars of *S. scabra* and *S. guianensis* and some selected accessions and cultivars of *S. hamata*, thus establishing the usefulness of isozyme

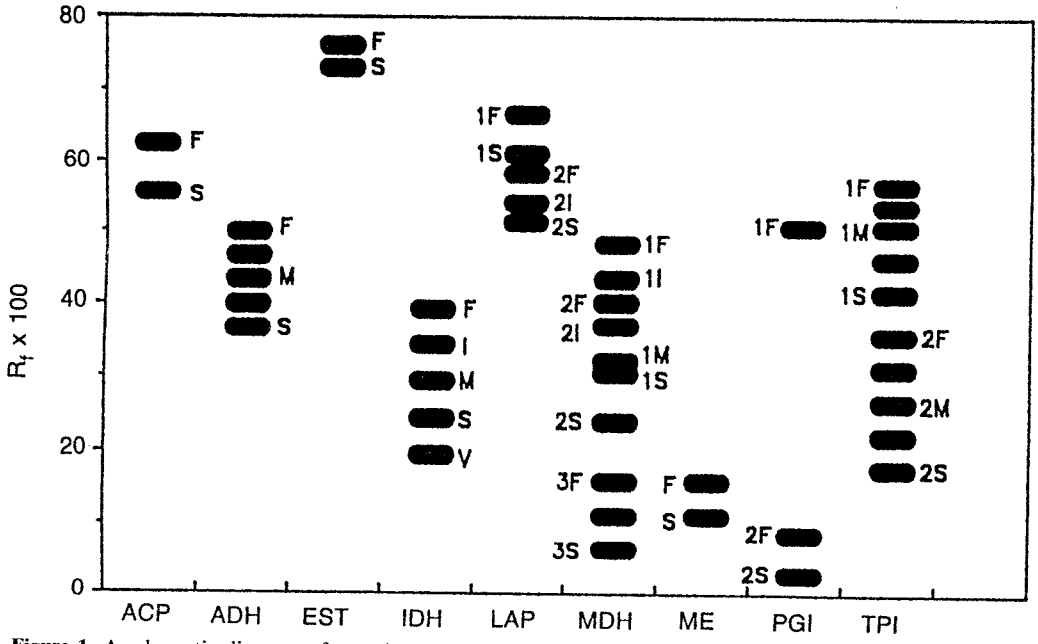

Figure 1. A schematic diagram of putative genotypes with allelic designations for the isozymes acid phosphatase (ACP), alcohol dehydrogenase (ADH), esterase (EST), iso-citrate dehydrogenase (IDH), leucine amino peptidase (LAP), malate dehydrogenase (MDH), malic enzyme (ME), phospho-gluco isomerase (PGI) and triose-phospho isomerase (TPI) of *Stylosanthes* spp.

analysis for identifying the various components of the genotype mixtures. Although only 9 isozyme systems were investigated, the presence of more than one controlling gene for some isozymes such as LAP, TPI and MDH increased the total number of loci that could be investigated to 13. Having established the experimental conditions and the standard isozyme patterns with seeds from single plants, one or more seed sources of all accessions were analysed to detect both inter- and intra-accession variability. For this purpose, both commercial and high purity, early generation seeds from the CSIRO germplasm collection have been used. These results show that, with the exception of a few cultivars or accessions where some variability is evident, the differences in the isozyme patterns can be used to discriminate the cultivars of all 3 species studied here.

Cultivars of *S. scabra* and *S. guianensis* were easily discriminated by these methods as they had more than one difference in each case as shown in Table 3. The two cultivars of *S. hamata* showed a difference in only one isozyme, ACP (Table 2).

The use of isozyme analysis in distinguishing cultivars is common in crop plants including pasture species such as subterranean clover (Collins *et al.* 1984). For species of *Stylosanthes*, isozyme analysis has been used to study species inter-relationships and ploidy levels (Stace and Cameron 1984) and to detect outcrossing in the *S. scabra* cultivar Fitzroy (Stace 1982).

In this study, LAP-2 turned out to be the best marker for distinguishing the 3 species; II genotype was unique to *S. guianensis*, FS was unique to *S. hamata* and IS and SS were seen in *S. scabra*. There were other isozymes that were useful in identifying a particular species. For example, the MM genotype of IDH was unique to *S. guianensis*. The IS genotype of both MDH-1 and MDH-2, SS genotype of MDH-3 and FM genotypes of TPI-2 were also specific for *S. guianensis*. Similarly, the MS genotype of ADH was unique to *S. hamata*. No isozyme genotype was specific for *S. scabra*, at least among the 13 loci investigated. However, some of the cultivars of *S. scabra* showed unique alleles, F and V in their IDH genotypes. From a practical viewpoint, it is very useful to have one single isozyme such as LAP-2 in order to make a broad separation at the species level. Depending on the number of cultivars in the mixture, one or more other systems can then be used for further discrimination.

All accessions of *S. scabra* and *S. hamata* used in this study were tetraploids while those of *S. guianensis* were diploid. The results suggest that the tetraploid accessions of *S. hamata* and *S. scabra* are genetically close to each other as they share similar alleles. The diploid *S. guianensis* accessions, on the other hand, have a number of isozyme genotypes with unique alleles and this may have important implications in the phylogenetic relationships of the species. An interesting feature that emerged from this study was the absence of the LAP-1 isozyme from *S. guianensis*.

In the initial study, the absence of ACP was evident in all seeds of cultivars Endeavour, Cook and Oxley. However, in the commercial seed lot of Endeavour (seed lot 24, Table 4) all 10 seeds showed an SS genotype. In the light of this, the presence of null alleles in this species cannot be taken for granted and should be further investigated. ACP was also absent in Verano which was the only feature that distinguished Verano from Amiga, CPI 92412 and CPI 55830.

Isozyme patterns of some of the commercial and other seed lots differed from those established with seeds from single plants. These differences were specifically restricted to three isozymes, ACP, PGI-2 and LAP-2 (Table 4). The intrusion of an S allele in seed lots of Amiga and Verano may have been due to cross pollination with contaminants such as *S. scabra* and *S. humilis* (Townsville Stylo). Crosses between Verano and *S. scabra* are observed quite commonly in areas where these species are grown together and this would be a potential source of the S allele of ACP. Townsville stylo is naturalised in areas around Mareeba in North Queensland, where seed crops of Amiga are grown. As shown in our analysis, *S. humilis* cv. Paterson shows an SS genotype for ACP (Table 2). In the mainly self-pollinating *S. scabra*, 1–2% outcrossing has been estimated using isozyme analysis (Stace 1982). Outcrossing rates vary from 1.6 to 22% in selected accessions of *S. guianensis* (Cameron and Irwin 1986). In populations of *S. capitata* up to 20% outcrossing has been reported (Miles 1983).

Similar variations in isozyme patterns were also detected in some other cultivars of the other 2 species (Table 4). The highly successful *S. scabra* cv. Seca now differs from the original accession CPI 40292 in stem coloration and time of flowering, and shows a range of different morphological and phenological characteristics in the various environments where it is grown. It is interesting to note that, in the early commercial seed source of Seca used in the present study, no variability in the 13 isozyme loci was found. Seed lot 22 of *S. guianensis* cv. Cook was the only case where three different allelic combinations of PGI-2 were detected; 2 out of 10 had FF, one had FS while the remainder showed the standard SS pattern.

To detect further variations in each of the nine Verano and Amiga seed lots, over 100 seeds from each lot were planted in a glasshouse and 10–12 week-old seedlings were visually examined. This failed to detect any *S. humilis*, *S. scabra* × *S. hamata* crosses or any other obvious off-types in the seed lots. This may mean that the isozyme variation we observed in these cultivars represents the natural variation within populations of each cultivar. In common with many other commercial seed production systems (Arus 1983), other factors such as contamination of seed harvesting and cleaning equipment may also account for some of this variability.

Considering the fact that variation in any given accession or cultivar occurred in only one or two of the 13 isozyme loci that were investigated, it would be reasonable to assume that authentication of most accessions or cultivars of interest in the mixtures could be achieved using the isozyme analysis reported in this paper.

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