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Evidence for a low level of genomic specificity of sequence-tagged-sites in *Stylosanthes*

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Abstract Genome-specific DNA markers are of great value in many applications. Recent work on different plants and animal species indicated that PCR- (polymerase chain reaction) based genetic marker systems using specific primers are highly genome-specific. To test the genome specificity of sequence-tagged-sites (STSs) as genetic markers in *Stylosanthes*, 20 pairs of primers were generated. Fifteen were from randomly selected single-copy *Pst*I genomic clones, and the other five were from two known gene sequences. These primer pairs were analysed against a set of 24 genotypes representing 12 different *Stylosanthes* species. Thirteen of these primer pairs amplified successfully. Overall, there was a low level of genome specificity, suggesting a low degree of genomic divergence within this group of *Stylosanthes* species. Of the 312 entries (24 genotypes by 13 primer pairs), PCR amplifications were unsuccessful (little or no products) in only 16 cases. The number of banding patterns detected by each of these primer pairs varied from 2 to 12 with an average pair-wise polymorphism of 44.3%. The level of intraspecific variation detected on normal agarose gels was only 3.8%. Further evidence that diploid *S. hamata* and diploid *S. humilis* are progenitors of tetraploid *S. hamata* and that *S. viscosa* is a progenitor of *S. scabra,* was obtained.

Key words *Stylosanthes* · DNA marker · Sequence-tagged-sites

Introduction

The level of genomic specificity of a genetic marker system can have a significant impact on its application. Highly genome-specific markers are of great value in applications such as species identification and genome relationship studies, but they are not very useful in applications such as interspecific introgression studies and comparative mapping experiments (Devos et al. 1995). While a percentage of markers in any given marker system can be genome-specific, the ratio of specific/non-specific markers can vary dramatically between different marker systems.

Recent work on different plant species indicates that PCR-based genetic marker systems with specific primers are highly genome-specific. For example, the SSR (simple sequence repeats or microsatellite) primer pairs designed using the gene sequences of storage proteins in hexaploid wheat only generated PCR products from one of the three genomes (Devos et al. 1995), while the STS primers generated in chrysanthemum often amplified a single fragment from each of the polyploid genotypes (Wolff et al. 1994). It has also been observed that some STS primers derived from barley clones failed to amplify wheat sequences (Tragoonrung et al. 1992).

The genus *Stylosanthes* includes some of the most important tropical pasture legume species. It consists of 40 species based on morphological characteristics (Kirkbride and de Kirkbride 1985). These species are diploids or polyploids with a basic chromosome number of x=10 (Cameron 1967). A set of genome-specific markers would be very helpful in elucidating genome relationships and identifying diploid progenitors of polyploid species. In this paper we report a lack of genome specificity and the levels of inter- and intra-specific polymorphism detected by STSs among 12 *Stylosanthes* species.

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Materials and methods

Genetic stocks

Twenty four genotypes were used in this study. They were chosen to represent 12 *Stylosanthes* species (diploid and tetraploid *S. hamata* are treated as two different entities in this paper) (see Table 1). Three of these species, *S. guianensis*, *S. scabra* and tetraploid *S. hamata*, were selected because of their importance in tropical pastures. An-

other three species, *S. humilis*, diploid *S. hamata* and *S. viscosa*, were selected because they are putative progenitors of *S. scabra* and tetraploid *S. hamata* (Stace and Cameron 1984; Curtis et al. 1995). Seeds for these accessions were obtained from the Australian Tropical Forages Genetic Resource Centre, Division of Tropical Crops and Pastures, CSIRO Cunningham Laboratory.

DNA isolation

Leaves from two to four mature plants of each genotype were harvested. Freeze-dried leaf tissue was used for DNA extraction following the method described by Liu and Musial (1995).

DNA sequencing and primer synthesis

Twenty single-copy clones were selected from a *Pst*I genomic library reported previously (Liu and Musial 1995). Both ends of all of these clones were sequenced using an Applied Biosystems 373A instrument and fluorescent chain-termination dideoxynucleotides, as directed in the Applied Biosystems Prism sequencing kit. The computer program OLIGO (version 3.3) was used to select optimal oligonucleotides for STS primer pairs. Sequences for another five primer pairs were derived from two gene sequences, *shst2* and *shst3* (Smith et al. 1995). An oligonucleotide was selected only when its Tm was greater than 50°C and when its 3′-terminus was not complementary to itself or to the other primer with which they form a pair. Primers were systhesized using a Beckman Oligo 1000 instrument

PCR amplification

PCR amplifications were performed in 24-μl reaction mixtures containing 78.2 mM Tris-HCl (pH 8.8), 19.4 mM $(NH_4)_2SO_4$, 0.53% (v/v) Triton X-100, 233 μg/ml gelatin, 5.8 mM $MgCl_2$, 130 mM dNTPs, 0.3 μM primers, 1.1 U *Taq* DNA polymerase (Biotech), and 10 ng of genomic DNA templates. The reactions were overlaid with mineral oil and subjected to 32 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 90 s. Amplification products were separated on 1.5% agarose gels in TBE buffer and visualized by staining with ethidium bromide.

Results

STS production

Of the 20 single *Pst*I clones sequenced, oligonucleotides suitable as STS primer pairs (suitability determined according to criteria described above) could not be selected for five of them. Oligonucleotide primer pairs for the other 15 clones were selected and synthesized. Together with the five sets of primer pairs selected from the sequences of the *shst2* and *shst3* genes, they were assayed by PCR amplification of two *Stylosanthes* genotypes, *S. scabra* cv Fitzroy and *S. hamata* cv Verano, the two cultivars from which all of the primer sequences used in this study were derived.

Using the PCR conditions described, 13 of the 20 primer pairs successfully amplified DNA products from these two genotypes. The sequences of these 13 primer pairs are listed in Table 2.

Genome specificity

The 13 primer pairs which successfully amplified DNA of *S. scabra* cv Fitzroy and *S. hamata* cv Verano were further assayed against two accessions of each of the 12 different *Stylosanthes* species. These results showed that the level of genome specificity of STSs among *Stylosanthes* species was low. Among 312 entries (24 genotypes × 13 primer pairs), PCR products were not detectable in only 16 cases which, interestingly, were all diploid species (Tables 1 and 2). When a primer pair failed to amplify one of the two genotypes of a species, the other would also generate little, if any, product (i.e., *S. grandifolia*; Fig. 1).

Intra- and inter-specific variation

Only two accessions were selected to represent each of the 12 species. Thus the level of polymorphism detectable within species could not be precisely estimated. However, the present study suggests that STSs are unlikely to reveal a high level of intraspecific variation on normal agarose gels. Of all the 156 pair-wise comparisons (12 pairs of genotypes with 13 primer pairs), only six displayed differences. Two of these were between the two *S. viscosa* accessions (one with primer pair S131 and the other with primer pair 3F3), two were between the two *S. leiocarpa* accessions (one with primer pair S68 and the other with 3F3), one was between the two *S. grandifolia* accessions with primer pair S131, and the other one was between the two diploid *S. hamata* accessions with primer pair 3F1. It is of note that all the variation was detected among diploid genotypes; none of the tetraploid genotype pairs showed polymorphism (Table 1).

As expected, the interspecific variation was much higher. The number of banding patterns revealed by the 13 primer pairs varied between 2 and 12 (Tables 1 and 2), with an average of 4.5 patterns detected per primer pair. The average pair-wise polymorphism between all the primer-genotype combinations was 44.3%.

Genome relationships

The genomic relationships between the 12 species could not be properly analysed in this study due to the small number of primer pairs used and the low level of polymorphism detected. However, the data indicates, as demonstrated previously (Stace and Cameron 1984; Curtis et al. 1995), that diploid *S. hamata* and diploid *S. humilis* are progenitors of tetraploid *S. hamata*. Seven of the thirteen primer pairs (S68, S200, S259, S268, 3F1, 3F3 and 2F3) amplified fragments which were different between the two diploid species. With only one exception, all the fragments of the two diploid species had counterparts in tetraploid *S. hamata*, and the tetraploid displayed no fragments which were not present in either of the diploid species. The exception was with the two diploid *S. hamata* accessions. Intraspecific variation was detected between the two accessions by primer pair 3F1. The fragments of the tetraploid *S. hamata* genotypes corresponded to that of CPI 55823 but not that of CPI 65369 (Table 1).

Table 1 The 24 genotypes used for STS analysis and their banding patterns revealed by each of the 13 primer pairs

CPI no[a]	Species	Ploidy level[b]	Banding pattern[c]												
			S18	S68	S71	S131	S200	S247	S259	S268	S284	S320	3F1	3F3	2F3
33437	*S. guianensis*	Diploid	a	–	a	a	a	a	a	a	a	a	afi	a	de
33706A	*S. guianensis*	Diploid	a	–	a	b	a	a	a	a	a	a	afi	a	de
34925	*S. scabra*	Tetraploid	a	b	b	b	a	ab	b	a	a	a	deh	be	cd
105533	*S. scabra*	Tetraploid	a	b	b	b	a	ab	b	a	a	a	deh	be	cd
33941	*S. viscosa*	Diploid	–	b	b	ab	a	a	b	–	ab	a	d	c	c
34904	*S. viscosa*	Diploid	–	b	b	b	a	a	b	–	ab	a	d	b	c
55823	*S. hamata*	Diploid	a	b	b	b	–	a	b	a	a	a	gh	e	c
65369	*S. hamata*	Diploid	a	b	b	b	–	a	b	a	a	a	egh	e	c
37037	*S. hamata*	Tetraploid	a	ab	b	b	a	a	bc	a	a	a	bgh	de	bcd
109345	*S. hamata*	Tetraploid	a	ab	b	b	a	a	bc	a	a	a	bgh	de	bcd
68841	*S. humilis*	Diploid	a	ab	b	b	a	a	c	–	a	a	b	d	bd
40272A	*S. humilis*	Diploid	a	ab	b	b	a	a	c	–	a	a	b	d	bh
92898	*S. capitata*	Tetraploid	a	b	abc	b	a	a	b	a	a	ab	hj	fi	abc
93123	*S. capitata*	Tetraploid	a	b	abc	b	a	a	b	a	a	ab	hj	fi	bc
92483	*S. debilis*	Diploid	a	b	b	b	a	a	b	a	a	a	b	g	bd
93046	*S. debilis*	Diploid	a	b	b	b	a	a	b	a	a	a	b	g	bd
78192	*S. leiocarpa*	Diploid	a	b	a	b	a	a	b	a	a	a	b	h	bd
92857	*S. leocarpa*	Diploid	a	c	a	b	a	a	b	a	a	a	b	g	bd
54835	*S. macrocephala*	Diploid	a	b	c	b	b	a	b	a	a	a	b	h	ac
105462	*S. macrocephala*	Diploid	a	b	c	b	b	a	b	a	a	a	b	h	ac
52132	*S. grandifolia*	Diploid	a	–	ab	bc	a	a	b	a	a	a	fi	–	de
93067	*S. grandifolia*	Diploid	a	–	ab	b	a	a	b	a	a	a	fi	–	de
33433	*S. angustifolia*	Diploid	a	–	b	b	a	a	b	a	a	a	c	i	bd
40326	*S. angustifolia*	Diploid	a	–	b	b	a	a	b	a	a	a	c	i	bd

[a] Commonwealth plant Introduction number
[b] Ploidy levels are all quoted from Stace and Cameron (1984)
[c] Banding patterns are represented by a letter or a combination of letters. Each letter represents one STS product. Fragment amplified by each primer pair were ordered according to their sizes with 'a' denoting the largest fragment; '–' indicates no detectable product

Table 2 Primer sequences, number of banding patterns, level of polymorphism detected, and species not amplified

Primer pair	Code	Primer sequences	Pattern (polymorphism)	Species not amplified
SsCS18P1 SsCS18P2	S18	5′ TCC ATT TAA ACT CAG AGG TGC C 3′ 5′ TGG CAA TTG CTC TCT CTG GC 3′	2 (16%)	*S. viscosa*
SsCS68P1 SsCS68P2	S68	5′ CTG CTG CAA TTC TGG TCC TC 3′ 5′ GGT GGC AAC AAT GGA AAG CA 3′	4 (64%)	*S. angustifolia; S. grandifolia; S. guianensis*
SsCS71P1 SsCS71P2	S71	5′ ATC TTC TCA ATG TCT TTC GGA 3′ 5′ AGA AGC GCA TGC AAG CAA ATC C 3′	5 (64%)	None
SsS131P1 SsS131P2	S131	5′ ATG AGG CGC GGT GAG GTT GC 3′ 5′ CAG AGG TTG CCA GTA TTT GC 3′	3 (16%)	None
SsCS200P1 SsCS200P2	S200	5′ ATT CGA AAG TGG CAC TGA CG 3′ 5′ AGC TAT TCC CTA CTT GTC TCA CG 3′	3 (30%)	Diploid *S. hamata*
SsCS247P1 SsCS247P2	S247	5′ CCA GAT TGG GTT CGG ATT CG 3′ 5′ GAG AAA CAG ATG GCA TCA GA 3′	2 (16%)	None
SsCS259P1 SsCS259P2	S259	5′ TCT CTG GCT CAA ATG AAA TG 3′ 5′ GCA AGA GAA CCA AAG TAA CAA AAA G 3′	4 (43%)	None
SsCS268P1 SsCS268P2	S268	5′ CAG CGG GTG GAG AAA AGA AG 3′ 5′ AGG AAC AAG TGC TGA AGA ATA ATG 3′	2 (29%)	*S. humilis*; *S. viscosa*
SsCS284P1 SsCS284P2	S284	5′ TCC GAA AAA CCA GAC ACA GG 3′ 5′ AAG GTC TGC CAT GGT ATT GT 3′	2 (16%)	None
SsCS320P1 SsCS320P2	S320	5′ GGT CCA GTA GAG AAG CAG AGA TG 3′ 5′ TCA CTG TCG TCA TAG CCT TTA CC 3′	2 (16%)	None
SHST3F1 SHST3R18	3F1	5′ TAA CTC TTG CCA GCC TCA 3′ 5′ CTG CAC CTG CCA TGA ATC CCA C 3′	10 (87%)	None
SHST3F3 SHST3R3	3F3	5′ GGT TAA CAT AAT AAA GCA TG 3′ 5′ GTC TTG TAA ACA ATT CCA AGC 3′	12 (95%)	*S. grandifolia*
SHST2F3 SHST2R16	2F3	5′ AAG AAC AAG AAA CTC TTC TGG 3′ 5′ CCA TGT AGT TCA CTG CTG ACC GAG 3′	7 (84%)	None

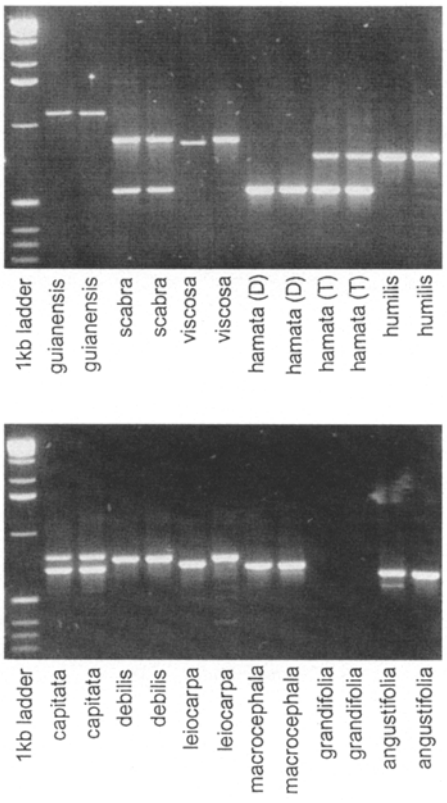

Fig. 1 Variation in the length of PCR products obtained with primer 3F3. A 1-kb ladder was used as the DNA fragment-size marker, and the order of genotypes is the same as that listed in Table 1

A similar analysis was conducted for *S. scabra* and *S. viscosa*. The results support the hypothesis that *S. viscosa* is one of the progenitors of *S. scabra* (Stace and Cameron 1984). However, their relationship is less obvious compared to that of tetraploid *S. hamata* with its two progenitors. Of the 13 primer pairs, two (S18 and S268) failed to amplify DNA from *S. viscosa*, and another five (S68, S71, S200, S259 and S320) amplifed monomorphic fragments from all of the four accessions of *S. scabra* and *S. viscosa*. Of the remaining primer pairs, the STS patterns of *S. scabra* and *S. viscosa* matched well for S247 and 3F1, but did not match for S284. The other two primer pairs (S131 and 3F3) detected intraspecific variation between the two *S. viscosa* accessions. In both cases the *S. scabra* fragments corresponded to that of CPI 34904 but not to that of CPI 33941 (Table 1). It is believed that CPI 33941 and CPI 34904 represent two highly different forms of *S. viscosa* (DF Cameron, personal communication).

Discussion

Genetic markers based on PCR with specific primers appear to be highly genome specific in some plant species (Tragoonrung et al. 1992; Wolff et al. 1994; Devos et al. 1995). However, the present study, employing 13 pairs of STS primers and 24 genotypes from 12 different species, indicates that this is unlikely to be the case in *Stylosanthes*. The low level of genomic specificity of STSs may simply indicate that the different *Stylosanthes* species are less divergent, even when compared to the differences between the constituent genomes of allopolyloid species such as wheat and chrysanthemum.

The low level of genomic specificity revealed by STSs reduces the value of this marker system when used for species identification in *Stylosanthes*. In many cases, STSs behaved similarly to most restriction fragment length polymorphism (RFLP) probes, i.e., they generated products from a wide range of closely related species. However, the same property also makes *Stylosanthes* STSs more attractive for work such as interspecific gene transfer and comparative mapping experiments. The conservation between *Stylosanthes* species seems to be close enough to allow most PCR primers designed for use in one species to be used in another. Further, compared to Southern-blot analyses, STSs, like other PCR-based marker systems, are much simpler and more convenient once primers become available.

Use of STSs in intraspecific work can be problematic however. This is because only a low level of intraspecific polymorphism can be detected by this marker system. Unlike SSRs, which targets highly variable sequences of a genome, STSs are non-selective and amplify random sequences with respect to the level of polymorphism. Further, compared with RFLPs, STSs generate much shorter fragments. Thus STSs would have less chance of detecting polymorphism caused by insertion/deletion events. *Stylosanthes* is not alone in this regard. Similar results were obtained in barley (Tragoonrung et al. 1992) and even in some highly polymorphic species such as pearl millet (Money et al. 1994). Methods to remedy such a problem include the use of high-resolution electrophoresis systems (Tragoonrung et al. 1992), restriction enzyme-digestion of PCR products (Tragoonrung et al. 1992), and the design of allele-specific primers (Wu et al. 1989).

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References

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

Curtis MD, Cameron DF, Manners JM (1995) Molecular evidence that diploid *Stylosanthes humilis* and diploid *Stylosanthes hamata* are progenitors of allotetraploid *Stylosanthes hamata*. Genome 38:344–348

Devos KM, Bryan GJ, Collins AJ, Stephenson P, Gale MD (1995) Application of two microsatellite sequences in wheat storage proteins as molecular markers. Theor Appl Genet 90:247–252

Kirkbride JH, de Kirkbride CG (1985) Typification of *Stylosanthes* (Leguminosae) and its sections. Taxon 36:455–458

Liu CJ, Musial JM (1995) Restriction fragment length polymorphism detected by cDNA and genomic DNA clones in *Stylosanthes*. Theor Appl Genet 91:1210–1213

Money TA, Liu CJ, Gale MD (1994) Conversion of RFLP markers for downy mildew resistance in pearl millet to sequence-tagged-sites. In: Witcombe RJ, Duncan RR (eds) Use of molecular markers in sorghum and pearl millet breeding for developing countries. Proc ODA Plant Sci Res Conf, Overseas Development Administration, London, pp 65–68

Smith FW, Ealing PM, Hawkesford MJ, Clarkson DT (1995) Plant members of a newly defined family of sulfate transporters reveal functional sub-types. Proc Natl Acad Sci USA 92:9373–9377

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

Tragoonrung S, Kanazin V, Hayes PM, Blake TK (1992) Sequence-tagged-site-facilitated PCR for barley genome mapping. Theor Appl Genet 84:1002–1008

Wolff K, Peters-Van Rijn J, Hofstra H (1994) RFLP analysis in chrysanthemum. I. Probe and primer development. Theor Appl Genet 88:472–478

Wu DY, Ugozzoli L, Pal BK, Wallace RB (1989) Allele-specific enzymatic amplification of beta globin genomic DNA for diagnosis of sickle cell anemia. Proc Natl Acad Sci USA 86:2757–2760