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Transferability of STS markers in studying genetic relationships of marvel grass (*Dichanthium annulatum*)

Author Details

Raghvendra Saxena — Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi - 284 003, India

Amaresh Chandra (*Corresponding author*) — Crop Improvement Division, Indian Grassland and Fodder Research Institute, Jhansi - 284 003, India
e-mail: amaresh62@yahoo.com

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Abstract

Transferability of sequence-tagged-sites (STS) markers was assessed for genetic relationships study among accessions of marvel grass (*Dichanthium annulatum* Forsk.). In total, 17 STS primers of *Stylosanthes* origin were tested for their reactivity with thirty accessions of *Dichanthium annulatum*. Of these, 14 (82.4%) reacted and a total 106 (84 polymorphic) bands were scored. The number of bands generated by individual primer pairs ranged from 4 to 11 with an average of 7.57 bands, whereas polymorphic bands ranged from 4 to 9 with an average of 6.0 bands accounts to an average polymorphism of 80.1%. Polymorphic information content (PIC) ranged from 0.222 to 0.499 and marker index (MI) from 1.33 to 4.49. Utilizing Dice coefficient of genetic similarity dendrogram was generated through un-weighted pair group method with arithmetic mean (UPGMA) algorithm. Further, clustering through sequential agglomerative hierarchical and nested (SAHN) method resulted three main clusters constituted all accessions except IGBANG-D-2. Though there was intermixing of few accessions of one agro-climatic region to another, largely groupings of accessions were with their regions of collections. Bootstrap analysis at 1000 scale also showed large number of nodes (11 to 17) having strong clustering (>50). Thus, results demonstrate the utility of STS markers of *Stylosanthes* in studying the genetic relationships among accessions of *Dichanthium*.

Key words

Cluster analysis, Genetic resources, Marvel grass, Polymorphism, STS markers

Introduction

Marvel grass (*Dichanthium annulatum* Forsk.), is an important perennial grass species of tropical and subtropical regions. In India, it is a constituent of two major grass covers *i.e.*, *Dichanthium-Cenchrus-Lasiurus* and *Sehima-Dichanthium* (Dabadghao and Shankarnarayan, 1973). Though highest green biomass is obtained in monsoon season, it persists and survives under harsh and dry environmental conditions. To date around 20 species of the genus have been reported, 8 of these are reported in India in various agro-ecological zones (Arora *et al.,* 1975). Two species *viz.*, *Dichanthium annulatum* and *Dichanthium caricosum* are widely used for forage production. The basic chromosome number in *Dichanthium* is 10, however *Dichanthium annulatum* complex shows different ploidy levels with chromosome number 2n = 2X = 20, 4X = 40 and 6X= 60 with distinct morphological characters (Mehra, 1961; Fedorov, 1974). It is largely apomictic in nature, however, facultative apomict lines are also reported. Despite this, high levels of DNA polymorphism and agro-morphological variations have been reported among accessions collected from similar eco-geographical situations (Agarwal *et al.,* 1999; Chandra *et al.,* 2004, 2006). In India, a collection of more than 250 accessions (collected from different parts of the country) is maintained at Indian Grassland and Fodder Research Institute (IGFRI), Jhansi, India. Despite of discrete germplasm holding, only one variety (Marvel-8) has been so far released in India. This suggests that further collections of germplasm should be undertaken to broaden the genetic base available to breeders as the identification of lines from core germplasm is the best option for selecting suitable lines for a particular agro-climatic zone since breeding by conventional methods in apomictic grass is tedious and difficult.

DNA markers, such as random amplified polymorphic DNA (RAPD) have been used in genetic and breeding studies in many

plant species (Williams *et al.,* 1993). Of the various molecular diagnostic techniques available, RAPD is easy to perform and cost-efficient, does not require radioactive compounds and analysis can reveal a high degree of polymorphism but repeatability of such markers is always doubtful. In the absence of stringent and gene specific markers like SSR and STS in *Dichanthium*, we have tried STS primers of a range legume *i.e.*, *Stylosanthes* which have worked well in case of another apomictic tropical grass genus *Cenchrus* (Chandra and Dubey, 2007). Since the tropical fodder grasses have been less intensively studied than other members of the family Gramineae and limited DNA sequences available in the database, transferability of STS markers developed largely on the basis of functional gene sequences was attempted to genetically analyze the *Dichanthium annulatum* accessions.

Materials and Methods

DNA extraction and STS analysis: Thirty *Dichanthium* accessions were examined, representing collections from the central north (Bundelkhand) and the southern plateau and hills (Dharwad and Bangalore) of India. All accessions were tetraploids in nature (Saxena and Chandra, 2006). These accessions were collected from their natural habitats, *i.e.*, grasslands and open fields, and maintained in experimental fields of the Indian Grassland and Fodder Research Institute, Jhansi (25°27'N, 78°35'E). The fresh and young leaves from three plants of each accession were used for total genomic DNA isolation and enzyme extraction. Each accession was established in three rows each consisting of three plants for morphological evaluation. Here DNA was isolated from one row by taking fresh leaves (2-3 g) from three plants together as described earlier (Chandra *et al.*, 2004). Because of the apomictic nature of *Dichanthium* homogeneity within plots was maintained. For STS-PCR, 17 STS primer pairs used in the present investigation were primarily derived from the Pst1 clones obtained from *S. scabra* cv Fitzroy and S. hamata cv Verano (Liu and Musial, 1995) as well as different coding and non-coding regions of gene sequences (Curtis *et al.*, 1995; Liu and Musial 1995; Manners *et al.*, 1995; Smith *et al.*, 1995; Liu *et al.*, 1996; Reddy *et al.*, 1996; Vander Stappen *et al.*, 1999). The OLIGO (version 3.0) computer program was used to select the optimal oligonucleotide for STS primer pairs as described by Liu *et al.* (1996). 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 synthesized by Sigma Genosys company according to the sequences by Liu and Musial (1995) and Vander Stappen *et al.* (1999). PCR amplification for STS loci followed the procedure described by Liu *et al.* (1996) with modification in genomic DNA concentration and inclusion of primer extension step for 5 min as a last step of amplification. The total reaction volume was 25 µl, which contained 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^{-1} BSA, 5.8 mM $MgCl_2$, 130 mM dNTPs, 0.3 µM of primers, 1.2 unit of Taq DNA polymerase (Bangalore Genei, India) and 25 ng of genomic DNA templates. The reactions were overlaid with mineral oil. The PCR protocol consisted of 32 cycles of 94°C for 60 s, 52°C for 60 s, 72°C for 90 s; one cycle at 72°C for 5 min; and 4°C soak. The reaction was carried out in MJ Research (PTC-200) thermocycler.

To evaluate the discriminatory power of STS in *Dichanthium*, the polymorphic information content (PIC) and marker index (MI) were calculated across assay units, assuming that each primer with 30 *Dichanthium* genotypes was an assay unit, and each polymorphic DNA fragment within an assay unit was a single dominant marker locus as the case reported for AFLPs marker in celeriac (Muminovic *et al.*, 2004). The PIC value was calculated employing the formula of Roldan-Ruiz *et al.* (2000): $PIC_i = 2f_i (1 - f_i)$, where f_i is the frequency of the amplified allele (band present) and $(1 - f_i)$ is the frequency of the null allele (band absent) of marker i. MI was determined as the product of PIC and the number of polymorphic bands per assay unit (Powell *et al.*, 1996). The binary data generated on the basis of presence (1) and absence (0) of the bands was analyzed for genetic similarity among the accessions based on Dice's similarity coefficients (s). Amplification failure of a sample or missing data was coded as 9. Dendrogram was constructed by Sequential Agglomerative Hierarchical and Nested (SAHN) clustering using the Un-weighted Pair Group Method with Arithmetic mean (UPGMA) algorithm. A band was considered polymorphic if the band was present in some accessions and absent in others while monomorphic if the band was present among all the accessions. Boot strap analysis was carried out at 1000 scale limit to understand the level of clustering of the different nodes (Yap and Nelson, 1996).

Results and Discussion

All 17 STS primers were tested at three different annealing temperatures. *i.e.*, 52, 55 and 58°C and based on amplification patterns and consistency of bands 52°C was observed as most suitable annealing temperature for amplification of *Dichanthium annulatum* DNA (Fig. 1). Of the 17 STS primers, 14 performed well and gave discrete amplification reactions at 52°C (Table 1). Three primers namely SsCS71P1/SsCS71P2, SHST1F3/SHST1R3 and SHCAPEF3/SHCAPER3 gave no amplification reactions. The number of bands generated by individual primer pair ranged from 4 to 11, whereas polymorphic bands from 4 to 9. Among 30 accessions the level of polymorphism ranged from 66.66 to 100%. Primers SHST1F1/SHST1R1 and SHCADIAF2/HCADIAR2 produced no monomorphic bands and thus exhibited maximum (100%) polymorphism, whereas rest of 12 primers generated monomorphic bands which ranged from 1 to 4.

In total 106 bands were recorded from 14 primers based on their clarity and reproducibility. Of these, 84 bands were polymorphic and 22 were monomorphic in nature. Among 14 STS primers, the maximum (11) bands were obtained with primer pair SHPALF2/SHPALR2 and minimum (4) from SHCADIAF2/SHCADIAR2. Though in general less numbers of monomorphic bands were amplified, a maximum of 4 monomorphic bands out of 10 were obtained with primer SHCAPEAR12/SHCAPEAF1 which exhibited least polymorphism *i.e.*, 60%. To assess the suitability and discrimination ability of STS primers, polymorphic information content (PIC) and marker index (MI) were also estimated for each

Table - 1: Description of sequence tagged-sites (STS) markers used in 30 *Dichanthium* accessions

STS primer pairs	Sequences	Target region	Intron	Microsatellites motif , if any	Total bands	No. of polymorphic bands	% polymorphism	PIC	MI	Annealing temperature	References
SHCADIAF2 SHCADIAR2	5' GCTTAGGCCATGTTGCCATC 3' 5' ATCTCCTGAGTCTCCTTCAG 3'	C	Yes	(TTA)	4	4	100	0.0.420	1.68	52 ^{0}C	Vander Stappen *et al.*, 1999
SHST3F3 SHST3R3	5' GGTTAACATAATAAAGCATG 3' 5' GTCTTGTAAACAATTCCAAGC 3'	C	Yes	(TAT), (T),(GATTTC),(A)	10	9	90.0	0.499	4.49	52 ^{0}C	Smith *et al.*, 1995
SHPALF2 SHPALR2	5' TTCACGGCAATGTGCCAAGG 3' 5' AGGTATTGTATCTGTGCCCA3'	NC	Yes	(TTA), (A)	11	8	72.72	0.499	3.99	52 ^{0}C	Manners *et al.*, 1995
SHCAPEAF1 SHCAPEAR1	5' TAATGTTGTGTCTTGTGCTG3' 5' GCTGCTCAAAAGCTGACAAC 3'	C	Yes	(CTAA)	9	7	88.99	0.488	3.42	52 ^{0}C	Reddy *et al.*, 1996
SHST3F1 SHST3R18	5' TAACTCTTGCCAGCCTCA 3' 5'CTGCACCTGCCATGAATCCCAC 3'	C	Yes	(T), (A), (TAT)	8	6	75.0	0.489	2.93	52 ^{0}C	Smith *et al.*, 1995
SHST2F3 SHST2R16	5' AAGAACAAGAAACTCTTCTGG 3' 5'CCATGTAGTTCACTGCTGACCGAG 3'	C	Yes	(ATAA), (TAT)	9	6	66.66	0.482	2.89	52 ^{0}C	Smith *et al.*, 1995
SHCAPEAF1 SHCAPEAR12	5' TAATGTTGTGTCTTGTGCTG3' 5'TAGCCCATCTCTGCGTCC3'	NC	Yes	-	10	6	60.0	0.363	2.18	52 ^{0}C	Vander Stappen *et al.*, 1999
SHCAPEAF11 SHCAPEAR1	5'GCCTCAACACAAAAGACCTTG3' 5' GCTGCTCAAAAGCTGACAAC 3'	NC	Yes	-	8	7	87.50	0.233	1.63	52 ^{0}C	Vander Stappen *et al.*, 1999
SsCS247P1 SsCS247P2	5' CCAGATTGGGTTCGGATTCG 3' 5' GAGAAACAGATGGCATCAGA 3'	-	-	-	5	4	80.0	0.433	1.73	52 ^{0}C	Liu *et al.*, 1996
SsCS268P1 SsCS268P2	5' CAGCGGGTGGAGAAAAGAAG3' 5'AGGAACAAGTGCTGAAGAATAATG 3'	-	-	-	7	6	85.71	0.495	2.97	52 ^{0}C	Liu *et al.*, 1996
SsCS284P1 SsCS284P2	5' TCCGAAAAACCAGACACAGG 3' 5' AAGGTCTGCCATGGTATTGT 3'	-	-	-	6	5	83.33	0.489	2.44	52 ^{0}C	Liu *et al.*, 1996
SsCS4P1 SsCS4P2	5' ACAAGGTCCAAGAGAGCAAC 3' 5' ACATTTCTTTCCTCCACAGC 3'	-	-		6	5	80.00	0.364	1.82	52 ^{0}C	Liu *et al.*, 1996
SsCS15P1 SsCS15P2	5' GGTCCTCCAATAGAAACTGC '3 5' GCTACCTGGGCTTTTGGC 3'	-	-	-	8	6	75.00	0.222	1.33	52 ^{0}C	Liu *et al.*, 1996
SHST1F1 SHST1R1	5' GAAGCAACTCTTCTTCACAT 3' 5'GGCTTGTCAGGGAAGAAAGT 3'	C	No	(CAG)	5	5	100	0.491	2.95	52 ^{0}C	Smith *et al.*, 1995

C = Coding, NC = Non-coding, NR = No reaction

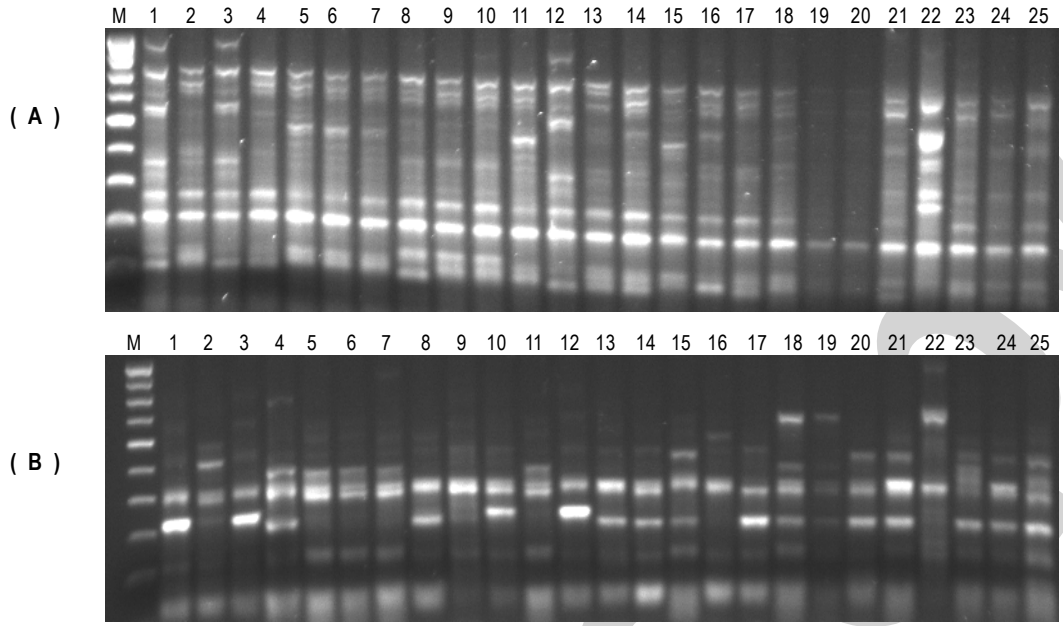

Fig. 1: Representative gel photographs of STS with primer pairs (A) SHCAPEAF1/SHCAPEAR1 (B) SHST2F3/SHST2R16 depicting clear separation and scorable bands with a set of *D. annulatum* accessions. M = 100 bp ladder as molecular weight marker

primer pairs which ranged from 0.222 to 0.499 with an average of 0.426 per primer pair. The maximum PIC (0.499) was shown by SHPALF2/SHPALR2 and SHST3F3/SHST3R3 primers whereas minimum (0.222) was obtained from SsCS15P1/ SsCS15P2 (Table 1). Marker index (MI) ranged from 1.33 to 4.49 with an average of 2.60. Maximum MI (4.49) was recorded with SsCS71P1/ SsCS71P2 and minimum 1.33 with primer SsCS15 P1/ SsCS15P2 (Table 1).

Based on 106 amplified STS bands, the generated dendrogram revealed 3 major clusters namely cluster I, II and III (Fig. 2). These clusters were separated at 75% genetic similarity level. Out of 30 accessions, 29 were grouped in various clusters. The remaining accession (IGBANG-D-2) belonging to South India separated from rest of the accessions at 70% genetic similarity and formed a distinct and separate node (Fig. 2). Cluster I was observed as largest cluster with 21 accessions. The intra-cluster similarity ranged from 79 to 100%. Cluster I joined cluster II at 75% genetic similarly, whereas cluster III joined cluster I and II at 74%. Cluster I embodied accessions collected from northern and central plateau region of India. Cluster I was further subdivided into two subclusters (sub-cluster IA and sub-cluster IB) which joined at 79% genetic similarity level. Sub-cluster IA embodied 17 accessions, which was further clustered into three small groups namely group I, II and III with 4, 8 and 5 accessions, respectively. Intra-cluster similarity in sub-cluster IA ranged from 79 to 100%. Sub-cluster IB possessed 4 accessions (IG 97-247, IG 95-25, IG 97-241 and IG-97-151) with intra-cluster genetic similarity ranging from 90-98%. Two accession namely IG 97-247 and IG 95-25 shared 98% genetic similarity and joined with accession IG 97-241 at 96% similarity level (Fig. 2).

Cluster II embodied six accessions (IGTGD-4, IGBANG-D-1, IGKMD-10, IG3108, IG 97-189 and IG 97-132) where intra-cluster similarity ranged from 77 to100%. Of these six accessions, IG 97-189 and IG 97-132 shared 100% genetic similarity and occupy the same position in dendrogram. This cluster possessed accessions mostly dominated from southern part of India (IGTGD-4, IG BANG-D-1, 1GKMD-10 and IG3108) with two accessions from northern part of India (IG97-189 and IG 97-132). Accessions from South India showed 83% genetic similarity among themselves which further joined to IG 3108 by 81%. This cluster was separated from cluster I at 75% genetic similarity level. Cluster III embodied only two accessions (IG 97-144 and IGKMFD-1). These two accessions grouped together at 80% similarity level, which was further separated from rest of cluster at 74% genetic similarity (Fig. 2). The reliability of dendrogram and clustering of accessions was evaluated with 1000 boot straps using Win Boot software (Yap and Nelson, 1996). The boot strap value ranged from 7.0 to 100 at various nodes of dendrogram. Total 12 nodes in dendrogram were identified, which exhibited more than 50% bootstrap value (Fig. 2).

The application of STS markers of a range legume provided an additional class of markers for studying molecularly unexplored crop like *Dichanthium annulatum*. Transferability rate was very high as only three primers of the total 17 did not react. Dendrogram based on genetic similarity obtained from these transferred STS markers illustrated the distinct clustering patterns of accessions. STS markers have been proved to be extremely valuable tool in the analysis of gene pool variations of crops during the process of cultivar development and classification of germplasm (Liu *et al.,* 1996). These markers are extremely sensitive and can detect allelic

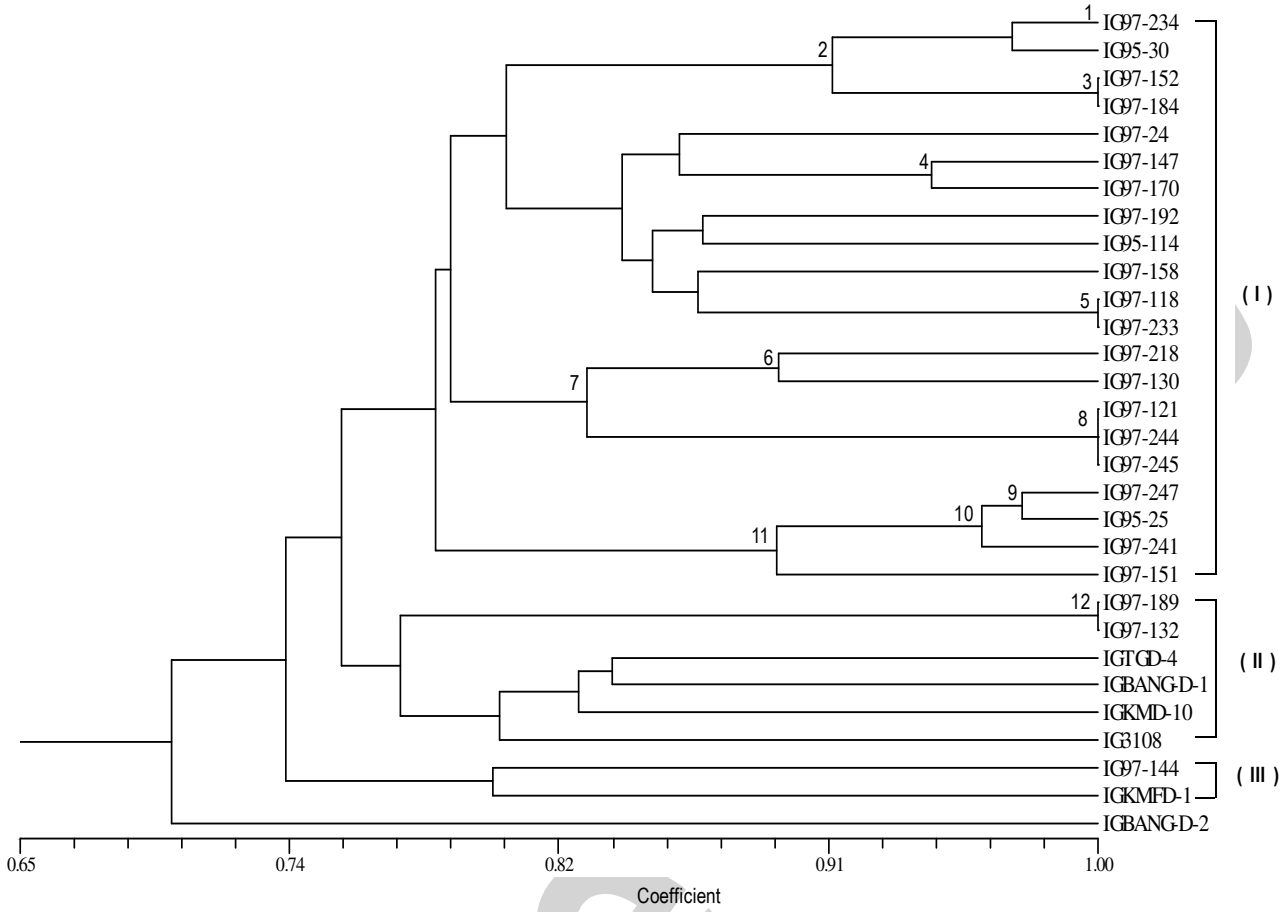

Fig. 2: Dendrogram of 30 *Dichanthium* accessions derived from Dice similarity values based on STS markers data. In total, 12 nodes, as depicted in the body of dendrogram were observed having strong clustering (>50 bootstrap values).

variations during cultivar development. The STS markers based on the sequence of rye grass and its related species have been successfully employed in 18 Gramineae species and average amplification success reported was 81% (Lem and Lallemand, 2003). Among *Dichanthium* accessions, when this marker system which have been developed from both known genes sequences and single copy *Pst1* clones, successfully detected the genetic diversity and revealed high level of polymorphism (80.1%). Using these *Stylosanthes* STS primers a high level of polymorphism have been also reported in another tropical grass *Cenchrus* (Chandra and Dubey, 2007), however low genome specificity of these STS markers have been reported in *Stylosanthes* (Liu *et al.,* 1996). Since some of the markers used were developed from coding regions of the genes the obtained polymorphism can be further increased by restriction digestion of amplified products, preferable where number of bands are less. Suitability of STS markers in estimating genetic diversity in *Dichanthium* accessions was further assessed by estimating the polymorphic information content (PIC) and marker index. A fairly high PIC values (0.222 to 0.499) and MI (1.33 to 4.99) was sufficient enough to compare the utility of such markers in genetic relationships study.

Due to low genome specificity of the *Stylosanthes* STS markers, these markers were employed in studying the DNA profiles of *D. annulatum* accessions, which are otherwise have been studied only with dominant marker like RAPD (Chandra *et al.,* 2004, 2006). While percentage of markers in any given marker system can be genome specific, the ratio of specific / non-specific marker can vary dramatically between different marker system. The STS markers generated in chrysanthemum often amplified a single fragment from each of the ploypoid genotypes (Wolff *et al.,* 1994), while some STS primers derived from barley clones failed to amplify wheat sequences (Tragoonrung *et al.,* 1992). Due to low intra-specific polymorphism as observed in *Stylosanthes*, use of this marker in intra-specific work can be problematic (Liu *et al.,* 1996), however when these markers were used in *Dichanthium* showed reasonable level of polymorphism (80.1%) indicated usefulness of such markers especially where not much information about reliable marker is available. The high level of polymorphism as shown here by STS markers could be valuable in studies that require a high number of polymorphic loci, such as population association genetics.

We are in process of sequencing some of the amplified products to visualize the nature of the fragments which may generate idea about why higher number of bands is obtained when primers were used in unrelated texta even using these STS primers at the same Tm as used in *Stylosanthes*. Though more than one alleles (2 to 9) have been observed when STS based on sequences of rice, wheat, barley, maize, oat and fescue were used in ryegrass and showed more polymorphism (67.9%) than those STS developed from sequences of ryegrass itself (50%) (Lem and Lallemand 2003). Most interestingly, only 10.7% of STS markers derived from related species of Gramineae gave no amplification, by comparison to 21.4% of the STSs derived from ryegrass itself. The resultant STS primers sets which showed 82% amplification success in unrelated texta could be employed in deciphering both inter and intra-specific polymorphism in available germplasm of different species of tropical grasses where otherwise not much information regarding molecular markers and genome analysis is available.

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