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RESEARCH ARTICLE

MOLECULAR FINGERPRINTING OF RECENTLY NOTIFIED SUGARCANE (*SACCHARUM* L.) VARIETIES USING STMS MARKERS

G. Hemaprabha* P.J. Priji and T.S. Sarath Padmanabhan

Abstract

DNA fingerprints of 13 recently notified sugarcane varieties for commercial cultivation for Peninsular zone (Co 99004, Co 2001-13, Co 2001-15, Co 0218, Co 0403 and Co 06027), North Western zone (Co 0118, Co 0237, Co 0238, Co 0239, Co 05009 and Co 05011) and East coast zone (Co 06030) have been developed to aid their unambiguous identification using a set of sugarcane specific sequence tagged microsatellite markers. Among the primers, NKS 23 produced more number of unique bands in the investigated material. Based on the bands detected on silver stained 7.5% non-denaturing polyacrylamide gels that resolved the PCR products, unique bands specific to each variety were identified. This information along with the overall banding profile would serve as fingerprints of each variety for proper varietal identification in the context of plant variety protection and varietal registration. As sugarcane is vegetatively propagated, these fingerprints remain uniform and stable making fingerprinting a reliable method of varietal identification in the crop. Moreover, genetic diversity among the clones was quantified through graphical genotyping using GGT software that revealed a moderate genetic similarity of 0.773 among the varieties which are gene pools for high yield, juice quality and resistance to various abiotic and biotic stresses. Three genetically more diverse clones (Co 06027, Co 06030 and Co 05011) and 22 diverse combinations have also been identified for their judicious utilization in breeding for better genetic gains.

Key words: Sugarcane, notified varieties, fingerprinting, STMS markers, GGT, genetic diversity

Introduction

Sugarcane varieties are usually identified based on morphological characters. Distinctiveness, Uniformity and Stability (DUS) guidelines have been formed based on a set of morphological descriptors of the crop for their clear identification (Amalraj et al. 2011). This characterization based on gross morphology is easy and economical, but is hampered by the influence of weather and physiographic factors that alter cane morphology and invite constant attention in assuring varietal purity. DNA fingerprinting is a proven technology that relies on the application of molecular marker techniques to identify cultivars. Though several molecular marker systems are available for the identification of plant breeders' materials, more useful and widely acknowledged are the microsatellite markers. Results of microsatellite analysis in sugarcane have indicated the power of this marker system in characterizing and identifying cane varieties in several countries (Cordeiro et al. 2000; Piperidis 2003, Jannoo et al. 2001, Selvi et al. 2003) Hemaprabha et al. 2011). This exercise of molecular characterization eases identification of cultivars for variety registration and dispute settlement. Though

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DNA profiling has not been adopted by the International Union for the Protection of New Varieties of Plants (UPOV) as an essential character, the revised UPOV (1991) convention has included DNA profiling technique as an additional requirement in extending the Plant Breeders' Rights to essentially derived varieties. Sequence tagged microsatellite sites (STMS) is found to be a useful molecular marker system for plant variety characterization by the UPOV - Working Group on Biochemical and Molecular Techniques and DNA-Profiling (UPOV-BMT, 2002**).** STMS is a PCR based approach of utilizing microsatellites, in which unique microsatellite loci are amplified using specific primers designed from the unique flanking DNA sequences and have the ability to detect a large number of discrete alleles repeatedly and accurately (Smith and Helentjaris1996). In pioneering work on these lines in Australia, Cordeiro et al. (2000) used over 100 microsatellite sequences on a set of five lines. Fingerprinting sugarcane through use of random decamer microsatellite and telomere sequences identified specific sequences for differentiating cane varieties (Harvey and Botha 1995). Due to its high specificity, STMS markers can distinguish even closely related germplasm lines. Already a set of STMS markers with high discriminatory power has been identified for generating distinct DNA profiles (Hemaprabha et al. 2006, Leena and Hemaprabha 2010). The present study is aimed at generating stable and unique fingerprints of a set of notified sugarcane varieties with a set of proven STMS primers. Also, the polymorphism information was utilized to identify genetically diverse combinations and clones to breed better varieties.

Materials and methods

The plant material included 13 recently released sugarcane varieties from Sugarcane Breeding Institute. The name of the variety, parentage and zone of release are given in Table 1. STMS primer pairs were selected from the markers developed from the microsatellite repeats of the sequences present in the Genbank databases (www.nrcpb.org/STMS.html) as given in Table 2.

Table 1. Sugarcane varieties fingerprinted, their parentages and specific agroclimatic zone of release

Sl.No.	Variety	Parentage	Agroclimatic zone of release
1	Co 0118	Co 8347 x Co 86011	North Western zone
2	Co 0237	Co 93016 GC	North Western zone
3	Co 0238	CoLk 8102 x Co 775	North Western zone
4	Co 0239	Co 93016 GC	North Western zone
5	Co 05009	Co 8353 x Co 62198	North Western zone
6	Co 05011	CoS 8436 x Co 89003	North Western zone
7	Co 99004	Co 62175 x Co 86250	Peninsular zone
8	Co 2001-13	Co 7806 PC	Peninsular zone
9	Co 2001-15	Co 85002 x Co 775	Peninsular zone
10	Co 0218	Co 8353 x Co 86011	Peninsular zone
11	Co 0403	Co 8353 x Co 86011	Peninsular zone
12	Co 06027	CoC 671 x IG 91-1100	Peninsular zone
13	Co 06030	CoC 671 x IG 91-1100	East coast zone

Table 2. STMS primers used in fingerprinting elite genotypes of sugarcane

STMS Primer	Primer sequence		Fragment size (bp)	Total no. of bands	No. of Polymorphic bands	Polymorphism %
	Forward (F)	Reverse (R)				
NKS 1	tggcatgtgtcatagccaat	ccccaactgggacttttaca	241-447	4	3	75
NKS 2	gctgtcccgttccaagttac	gcgaccggattatgatgatt	187 - 567	13	11	84.61
NKS 3	cgtgttcctcttcaacaacg	tgcttcgctatatatgggttca	180 - 398	10	3	30.00
NKS 7	ttacagcctggagctcgttt	cgaagcctctcctctcctc	208-431	4	0	0
NKS 8	gtgacagcggcttgttcag	ttaaacacgcagccattcag	180- 390	8	6	75.00
NKS 9	ctttcagtggccatctccat	gaatgcgcagggataggata	170 - 440	11	8	72.72
NKS 11	caccactcacatccacttgc	tatggagagatgctgctgct	130 - 330	9	4	44.44
NKS 12	cagccacgtgatgctttct	ccgatccatcagtttcaggt	233-410	2	0	0
NKS 14	ttccaccagtgacattcagc	ccaacagcagcttcttcctt	183-330	5	2	40
NKS 16	gacagaatatgccatggataacaa	cgttctctggtcctattgagc	188 - 680	15	6	40.00
NKS 23	taaacccccgaaaaagaacc	tccggaggtagatccatttg	142-1260	10	9	90.00
NKS 25	tccatgcatgcgtgtagttt	agtgcacaacgttcttgctg	219-365	6	2	33
NKS 27	tggatttgggtaaggatgga	taatgcctctgggctcaaat	197-427	12	8	67
NKS 28	gtgctgggattctgagcttc	gcaagttcttggcctttgtt	210-326	4	1	25
NKS 30	ctccttctccttcgcatcct	caccttctggagcacgtta	132-292	10	3	30
NKS 31	aaccaccactcatcgtcctc	caccgagttcccattgttct	233-306	4	3	75
NKS 34	cgtcttgtggattggattgg	tggattgctcaggtgtttca	120 - 950	14	12	85.71
NKS 42	accgattgttcagtgggaag	acctagcaatttacaagagaattaga	170 - 580	14	12	85.71
NKS 46	acaataaccccgcagacatc	taatgcgtcatttggagcag	138-204	5	2	40
NKS 49	ctcacgtcctgttggtgcta	tacatgggacacatgcttgc	150 - 800	13	11	84.61
NKS 52	ggcctatggaacgaagttca	cagccttttcttcgcaaaac	162-210	4	2	50
NKS 54	ctatacggcaaacgcaacct	tatacgtcgcatgcaccatc	198-450	10	5	50
NKS 57	cgagcctccctccatagatt	accaccaccaacctcatctc	110-190	9	8	88.88

PCR amplification and electrophoresis: DNA from all the clones was isolated using CTAB method (Murray and Thompson, 1980) and quantified using Nanodrop DNA/RNA quantifier. Twenty three sugarcane specific STMS primers with high polymorphism information content were used to screen these clones (Table 2). PCR reactions were performed in MJ Thermal cycler PTC 100 with a total reaction volume of 10 µl containing 25 ng of template DNA, 1pMol of Forward and Reverse Primers, 2mM of dNTPs, 1.75 mM $MgCl_2$ and 0.5 U Taq. Cycling conditions were: one cycle of 5 minutes at 94°C, 30 seconds at appropriate annealing temperature (ranging from 51° C to 59° C depending on the primer) and 50 seconds at 72° C, with a final extension of 5 minutes at 72° C.

PCR products were resolved on a 7.5% non-denaturing polyacrylamide gel using 1X TBE buffer and silver stained. The gel was observed in a Gel Documentation System (Alpha Innotech) and unique bands specific to each clone were identified. Wherever there was ambiguity, the STMS analysis was repeated to confirm the presence or absence of bands.

Graphical genotyping

The presence / absence of each marker was scored as 1 / 0 respectively and analyzed using the software GGT 2.0: graphical genotypes developed by Dr. HJ Finkers, Wageningen University (GGT 2.0: Versatile Software for Visualization and Analysis of Genetic Data, 2010) in order to generate the graphical representation of molecular marker data for each variety. The analysis was also done to estimate similarity matrices among the possible combinations and to generate dendrogram to understand the clustering of varieties based on genetic distance.

Results and discussion

Fingerprinting commercial varieties

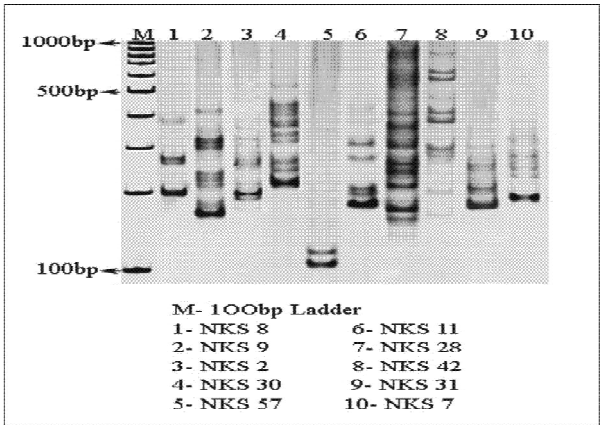
Sugarcane is traditionally described by a set of morphological descriptors, many of which are generally influenced by the environment. In India, DUS testing guidelines have been finalized with a set of morphological characters. However fingerprinting of varieties has become more relevant for protection of own breeders against appropriation of their varieties by genetic engineers and in securing position in WTO through the National Plant Variety protection system (Chowdhury 2005). Sugarcane specific STMS markers have been developed from Genbank databases (www.nrcpb.org/STMS.html), enriched sugarcane libraries (Parida et al. 2009) and EST sequences. From these, a set of STMS markers with high discriminatory power that distinguished somaclones, mutants and other morphologically more similar clones developed from a popular cultivar CoC 671 has been identified (Hemaprabha *et al*. 2011). The present study made use of these markers to generate molecular profiles of 13 promising clones and to identify a set of unique markers to discriminate a variety from others.

The primers (Table 2) enabled development of DNA fingerprints of recent varieties. The profiles generated for each variety are given in Fig. 1. The unique bands identified for each variety are listed in Table 3. It could be seen that the band sizes ranged from 120 bp to 1000 bp and majority were within 180 bp to 520 bp. While the primer NKS 11 produced smaller fragments ranging from 180 bp to 390 bp, NKS 23 produced the widest range of product sizes from 142 bp to 1260 bp. Sizes of discriminating bands ranged from 151 bp to 900 bp in Co 0118 and Co 0239 with NKS 23 and NKS 33 respectively. The primer NKS 23 showed the maximum polymorphism (90 %) and produced more number of unique bands, while NKS 7 and 12 failed to produce any unique band in the investigated material. Among the varieties, Co 0118 and Co 06027 gave more number of unique bands. This uniqueness could be attributed to their genetic nature, in that Co 06027 is evolved from a cross involving an intergeneric hybrid derived from *Erianthus arundinaceus* as a parent, whereas Co 0118 is a selection from the cross

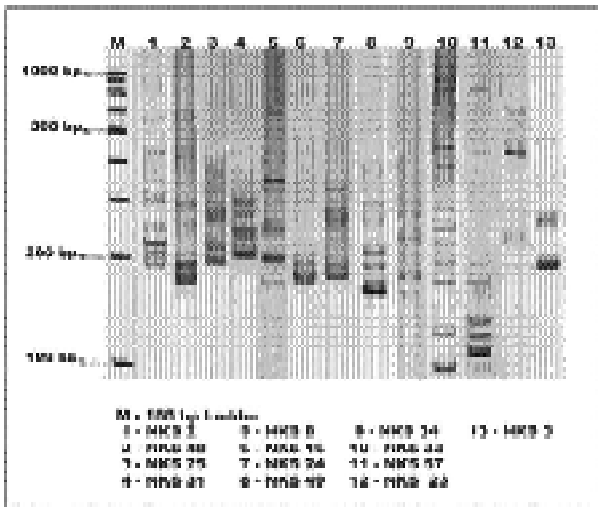
involving a subtropical parent Co 8347 with a tropical one Co 86011 with different pedigrees. The unique bands identified for each variety along with the molecular profiles generated with a set of primers (as shown in Fig. 1) would serve as signatures or fingerprints of the 13 varieties to aid their identification in case of ambiguity in varietal purity/ identity.

STMS technology holds good in detecting finer differences at molecular level. Such primers with high discriminatory power are also useful in genetic

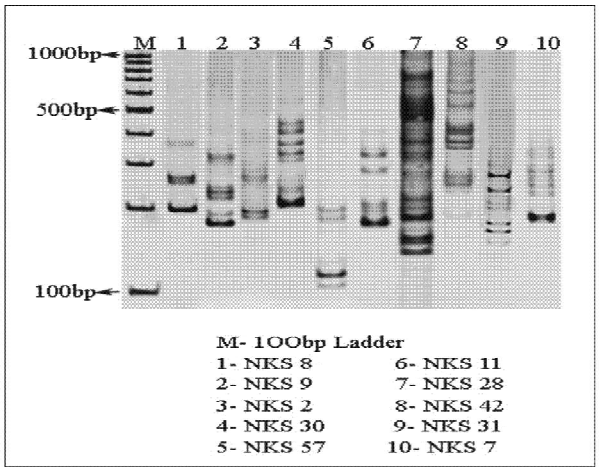
a. Co 0118



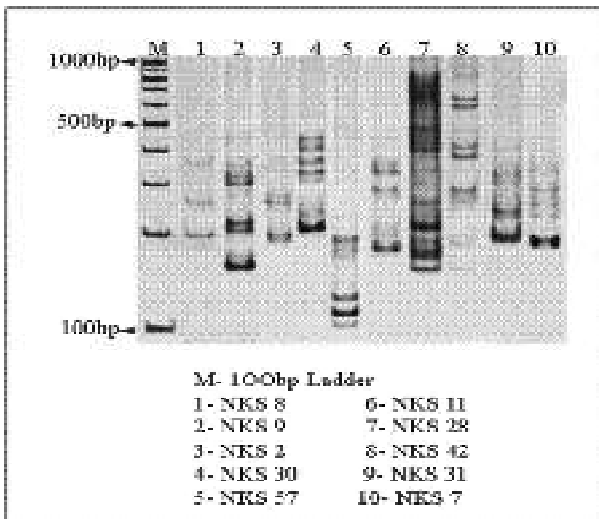
b. Co 0237



c. Co 0238



d. Co 0239



e. Co 05009

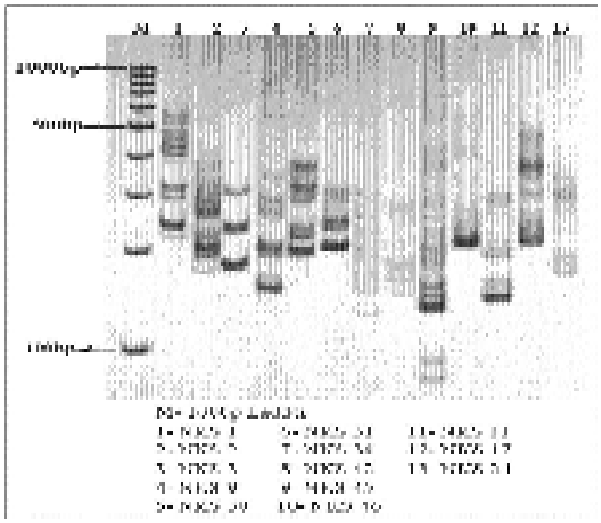
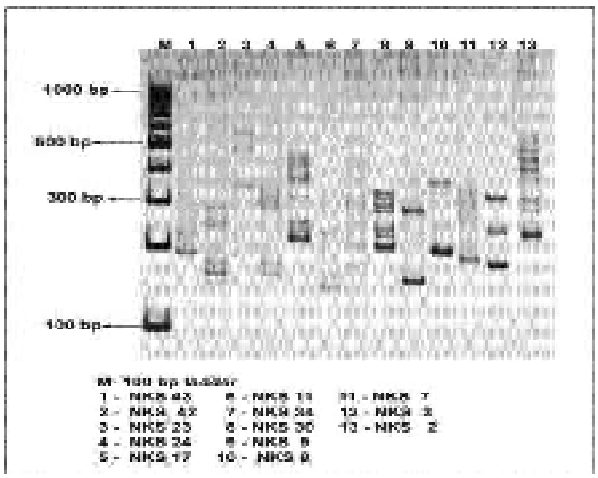
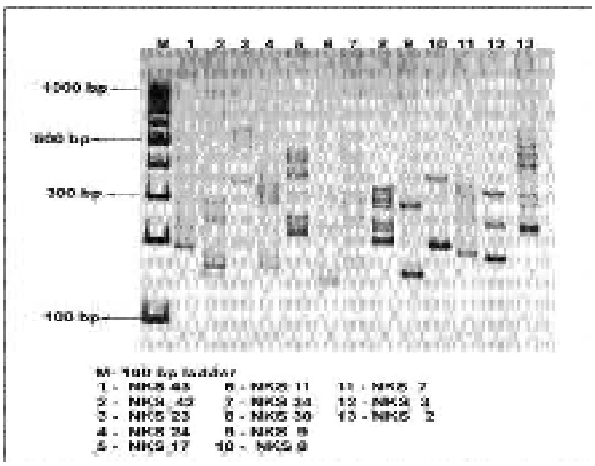


Fig. 1. Molecular fingerprints of 13 notified sugarcane varieties

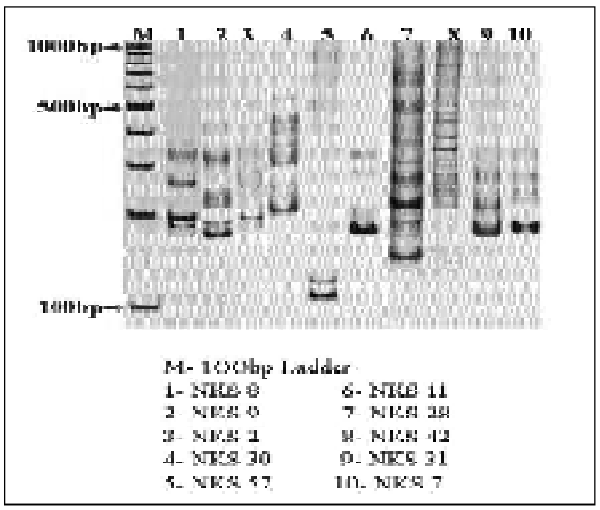
f. Co 05011



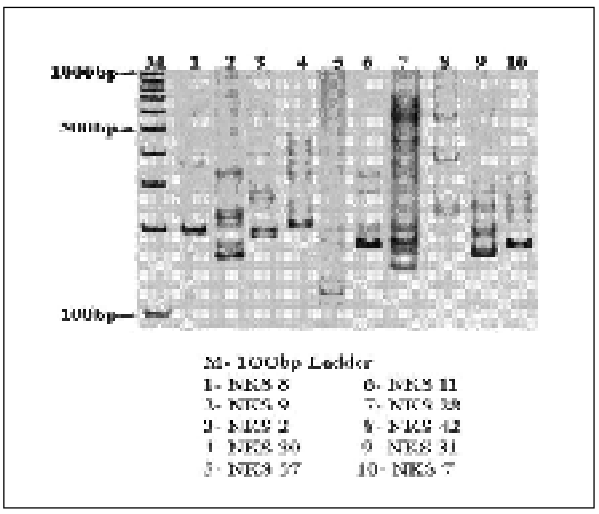
g. Co 99004



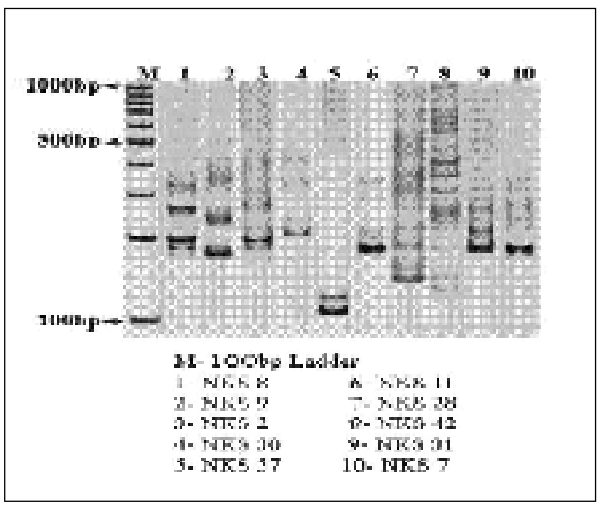
h. Co 2001-13



i. Co 2001-15



j. Co 0218



k. Co 0403

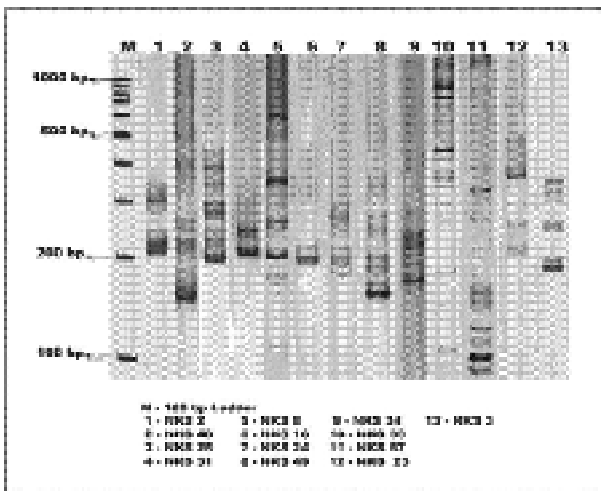
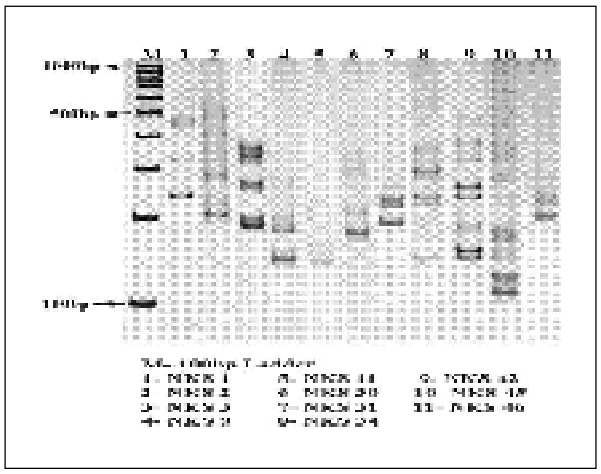


Fig. 1. Molecular fingerprints of 13 notified sugarcane varieties

l. Co 06027



m. Co 06030

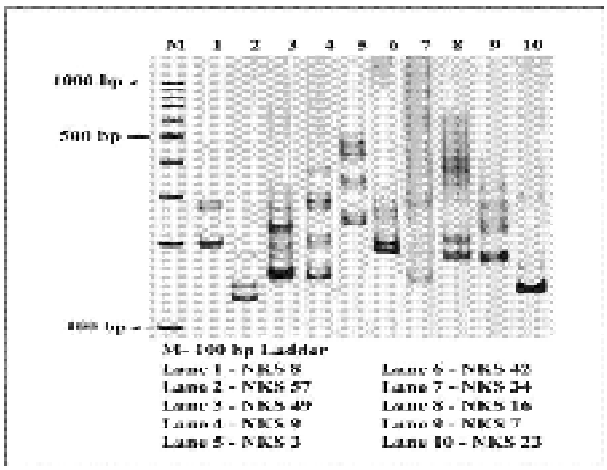


Fig. 1. Contd.

fidelity testing. Through STMS profiling the investigating group could detect a few mislabeled clones and clones with gross morphological similarity (data not included).

Varietal distinction using molecular markers could be made more feasible with tests that are straight forward, inexpensive, reliable, reproducible and capable of unambiguous analysis. STMS primers viz. NKS 2, 3, 8, 11, 14, 23, 24, 30, 31, 46, 52 and 57 were the best primers with regard to their efficiency in generating unique bands in the present set of materials. Sugarcane being vegetatively propagated through stem cuttings, once generated, the fingerprints remain stable and reliable for varietal distinction even after several crop cycles, making DNA fingerprinting an efficient and reliable technique in the crop.

Comparative molecular profiling of 13 varieties

Comparative profiling with markers on a set of genotypes would benefit identifying genetically more similar/ diverse types. Suitable software developed for plant breeders, i.e. GGT 2.0: graphical genotyping was made use of to generate graphical representation of molecular marker data. The

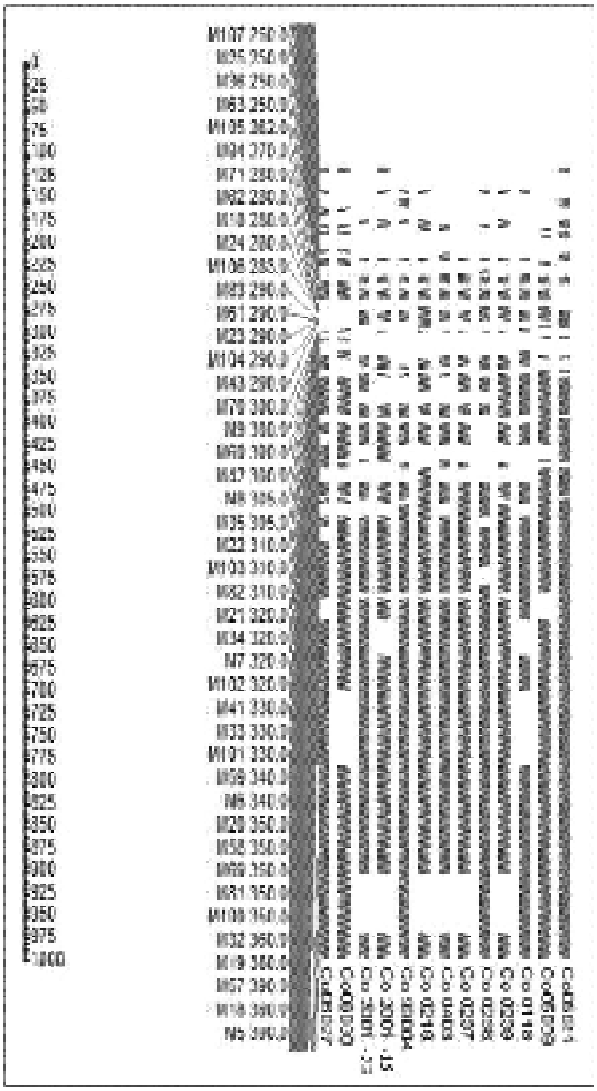


Fig. 2. Graphical genotyping of 13 sugarcane varieties based on 23 STMS primers

Table 3. Unique STMS markers detected in 13 notified sugarcane varieties

Variety/ pairs of varieties	STMS primer	Presence of band (bp)	Absence of band
Co 0118	NKS 11	-	307,335
	NKS 16	-	530
	NKS 23	151	
	NKS 25	228	
	NKS 57	805	
	NKS 3	340	
Co 0218	NKS 2	562	
	NKS 16	255	
	NKS 23	451	
Co 0237	NKS 2	320, 567	
	NKS 17	381	
	NKS 57	-	133
Co 0238	NKS 3	-	387
	NKS 34	318	-
	NKS 42	508	426,524,581
	NKS 23	330	-
Co 0239	NKS 11	197	
	NKS 48	306	
	NKS 34	318	
	NKS 33	900	
	NKS 43	492	
Co 2001-13	NKS 42	330	
	NKS 49	135, 370, 433	
	NKS 7	205	
Co 2001-15	NKS 17	-	353
	NKS 49	500	-
	NKS 38	754	
	NKS 23	465	
Co 0403	NKS 24	420	
	NKS 9	220	
Co 99004	NKS 43	167, 484, 575	
	NKS 7	207	
Co 05009	NKS 3	220	250
Co 05011	NKS 34		160
Co 06027	NKS 8	179,376,412	
	NKS 17	520	
	NKS 42	420	250
	NKS 30	-	
	NKS 49	468	
Co 06030	NKS 9	247	
	NKS 11	250	
	NKS 22	202	164
	NKS 23	247	

present analysis is based on the assumption that the primers chosen are independently assorted in the absence of perfect linkage maps in sugarcane. Fig. 2 shows the graphical genotyping pattern of 13 varieties based on 23 STMS primers. This information would be an important tool in the process of selection and evaluation of sugarcane clones. Besides, DNA profiles could be used to quantify genetic diversity among the varieties to further use the diverse clones / combinations to harness better genetic gain in sugarcane improvement. GGT generated similarity among the 13 varieties to assess their usefulness as parents in varietal improvement. Similarity indices between the 66 possible combinations based on Jaccard's coefficient (Table 4) revealed a mean similarity index of 0.773 and 22 combinations were significantly more diverse, in contrast to 13 genetically similar combinations. Co 06027 and Co 06030 were more diverse than the rest. Co 06027 was significantly dissimilar (SI< 0.651) from 10 varieties viz. Co 2001-13, Co 2001-15, Co 99004, Co 0118, Co 0218, Co 0403, Co 0237, Co 0239, Co 05009 and Co 05011, while Co 06030 was dissimilar from eight others viz. Co 2001-13, Co 2001-15, Co 99004, Co 0218, Co 0403, Co 0237, Co 0239 and Co 05009 as a reflection of their genetic constitution. Both the varieties are intergeneric hybrids made up of sugarcane and *E. arundinaceus* genomes, in contrast to the rest developed through hybridization within *Saccharum* genus. The third more diverse genotype Co 05009 was distinctly diverse from the subtropical varieties Co 0237, Co 0238, Co 0239 and Co 0118. The above mentioned 22 diverse combinations may be utilized in genetic improvement of sugarcane. The rest of the genotypes fell within genetically similar category. NJ dendrogram (Fig. 3) depicting genetic diversity clearly showed the clustering of Co 06027 with Co 06030 and Co 05011 with Co 05009 and fell distant from the rest of the genotypes.

Table 4. Similarity index among all possible combinations involving 13 commercial varieties

	Co 06027	Co 06030	Co 2001 -13	Co 2001 -15	Co 99004	Co 0218	Co 0403	Co 0237	Co 0238	Co 0239	Co 0118	Co 05009	Co 05011
Co 06027	1.00												
Co 06030	0.83	1.00											
Co 2001 - 13	0.62	0.65	1.00										
Co 2001 - 15	0.63	0.64	0.85	1.00									
Co 99004	0.61	0.62	0.83	0.80	1.00								
Co 0218	0.62	0.63	0.80	0.79	0.79	1.00							
Co 0403	0.64	0.63	0.84	0.83	0.77	0.78	1.00						
Co 0237	0.65	0.65	0.83	0.82	0.80	0.77	0.85	1.00					
Co 0238	0.66	0.67	0.77	0.76	0.76	0.79	0.72	0.78	1.00				
Co 0239	0.65	0.64	0.85	0.82	0.84	0.81	0.79	0.84	0.72	1.00			
Co 0118	0.61	0.71	0.79	0.80	0.76	0.77	0.79	0.80	0.78	0.80	1.00		
Co 05009	0.56	0.61	0.75	0.70	0.72	0.75	0.69	0.72	0.66	0.72	0.70	1.00	
Co 05011	0.62	0.68	0.67	0.68	0.65	0.69	0.67	0.65	0.63	0.65	0.65	0.73	1.00

Thus STMS based fingerprinting technology yielded more loci per primer to generate unique fragments with greater proportion of polymorphism and high discrimination power. A practical application of this technology lies in varietal distinction of the recent

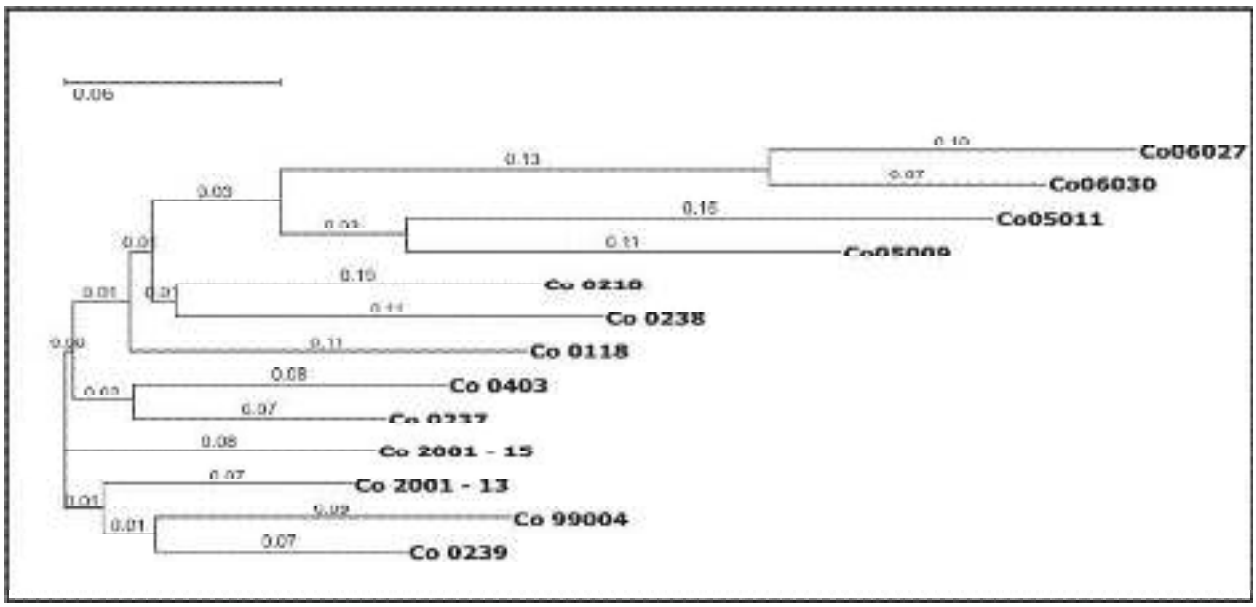


Fig. 3. NJ dendrogram depicting genetic distance among 13 sugarcane varieties

sugarcane varieties developed at Sugarcane Breeding Institute and in identifying genetically more diverse combinations to benefit sugarcane development through breeding.

References

Amalraj VA, Premachandran MN, Bakshi Ram, Karuppaiyan R, Sumitradevi, Raffee Viola, Remadevi AK (2011) DUS characteristics of reference varieties of sugarcane (Saccharum L.), Sugarcane Breeding Institute, Coimbatore.128p.

Choudhury RK (2005) Development of DUS system in India and future needs. In ICAR-IPA National Conference on IPR and Management of Agricultural Research held at New Delhi from August 27-29.

Cordeiro GM, Taylor GO, Henry RJ (2000) Characterization of microsatellite markers from sugarcane (*Saccharum* sp) a highly polyploid species. Plant Sci. 155: 161-168.

Harvey M, Botha FC (1996) Use of PCR-based methodologies for determination of DNA diversity between *Saccharum* varieties. Euphytica. 89: 257-265.

Hemaprabha G, Leena Lavanya, Priji. PJ, Arun Krishna, Vincy J, Swapna Simon, Govindaraj P (2011) Molecular fingerprinting of commercial varieties and elite clones of sugarcane (*Saccharum* sp.) using STMS markers. Proc 4th IAPSIT Intern Sugar Conference IS -2011, Nov, 21-25. pp: 624-629.

Hemaprabha G, Govindaraj P, Singh NK (2006) STMS markers for fingerprinting of varieties and genotypes of sugarcane (*Saccharum* spp.). Indian J Genet 66 (2): 95-99.

Jannoo N, Forget L, Dookun A (2001) Contribution of microsatellites in sugarcane breeding in Mauritius. Proc. Int. Soc. Sugarcane Technol. 24: 637-639

Leena Lavanya D, Hemaprabha G (2010) Analysis of genetic diversity among high sucrose genotypes of sugarcane (*Saccharum* spp.) derived from CoC 671 using sugarcane specific microsatellite markers. Presented in the third National Congress on Plant breeding and Genomics held at Coimbatore 7-9, 2010 pp. 15-16.

Murray MG, Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acid Research 8: 4321-4325.

Parida S, Kalia S, Kaul S, Dalal V, Hemaprabha G, Selvi A, Pandit A, Singh A, Gaikwad K, Sharma T, Srivastava P, Singh N, Mohapatra T (2009) Informative genomic microsatellite markers for efficient genotyping applications in sugarcane. TAG. Theoretical and Applied Genetics 118 (2), 327-38

Piperidis G (2003) Progress towards evaluation of SSR as a tool for sugarcane variety identification. ISSCT IV Mol. Biol. Workshop, Montpellier, France.

Selvi A, Nair NV, Balasundaram N and Mohapatra T (2003) Evaluation of maize microsatellite markers for genetic diversity analysis and fingerprinting in sugarcane. Genome 46: 394 - 403.

Smith S, Helentjaris T (1996) DNA fingerprinting and plant variety protection. In: A.H. Paterson (Ed.). Genome Mapping in Plants. R.G. Landes Company. pp. 95-110.

UPOV—BMT (2002) BMT/36/10. Progress report of the 36th session of the technical committee, the technical working parties and working group on biochemical and molecular techniques and DNA profiling in particular. Geneva.