MOLECULAR GENETIC DIVERSITY OF SUGARCANE GENOTYPES OF SUBTROPICAL INDIA: SSCP-PCR ANALYSIS OF SIMPLE SEQUENCE REPEATS

By

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Abstract

THE POTENTIAL of Single Strand Conformational Polymorphism (SSCP) of simple sequence repeats as a new marker system was investigated with respect to their abundance, variability and ability to detect polymorphisms and molecular genetic diversity in sugarcane genotypes. Eleven microsatellite markers (derived from genomic libraries) were used as primers to amplify genomic DNA from 42 commercial cultivars, interspecific hybrids and species of sugarcane. Amplified SSR-PCR products were subjected to SSCP to detect polymorphism to determine genetic diversity and inferring relationship of the studied genotypes. The resulting patterns ranged from few bands to highly complex multi-band ladders for most of the primers. SSCP-PCR demonstrated a high level of polymorphism between the studied genotypes. The presence or absence of individual amplified product DNA bands was scored to derive a pair-wise distance matrix between genotypes and their clustering in different groups. Thus, the SSCP-PCR analysis of simple sequence repeats is useful to differentiate even closely related taxa. The major advantage of this method is that many individual PCR products may be screened for variation simultaneously. This is the first report of the use of SSCP-PCR to differentiate between members of a highly polyploid genus.

COMMERCIAL hybrids of sugarcane are being used as parents in genetic improvement programs for subtropical India. In addition, a search is on for identification of new gene resources to break the cane yield and sucrose content plateau and to incorporate tolerance/ resistance against abiotic and biotic stresses.

Deciphering phylogenetic relationships between these genotypes could assist in understanding genome organisation in modern sugarcane and its relatives, as well as to aid breeding programs aimed at widening the genetic base of sugarcane.

Microsatellites, also known as simple sequence repeats (SSRs), are ideal genetic markers for genetic mapping (McIntyre *et al.*, 2001) and fingerprinting studies (Pan *et al.*, 2002; Piperidis *et al.*, 2001; Cordeiro *et al.*, 2000) in sugarcane because of their abundance, high polymorphism between individuals within populations or closely related genotypes, and their transferability across genera (Cordeiro *et al.*, 2001).

The assessment of genetic diversity in sugarcane genotypes of subtropical India is currently based on pedigree records, morphological and economic traits. Screening and evaluating the available genetic diversity with SSR markers will help assign unique genetic fingerprints to the varieties to facilitate molecular-based genetic relationships for exploitation of new gene resources of sugarcane to help broaden the genetic base of sugarcane in subtropical India.

Single-strand conformation polymorphisms (SSCP) rely on electrophoretic detection of differential mobilities of single stranded DNA molecules due to conformational differences resulting from small nucleotide changes in their flanking regions as well as microsatellite core region. The technique was originally developed for rapid analysis of mutations (Orita *et al.*, 1989) and has been used extensively in human genetics to detect single-point mutations (Hayashi, 1992).

In this study, we applied the SSCP technique as a novel approach to analyse SSR amplified products in order to enhance their variability, utility in diversity analysis, and ability to distinguish among

forty-two genotypes of sugarcane consisting of commercial cultivars of subtropical India, inter-specific hybrids (ISH) and species clones available at research farms of IISR, Lucknow (Table 1).

Table 1—Name of clones, interspecific hybrids and genotypes belonging to *Saccharum* species and species hybrids used in the present study.

S. officinarum	*S. spontaneum*	*S. barberi*	*S. sinense*	Inter-specific hybrids	Cultivated genotypes	
Orambo Karia Rayada Gungera	SES -69 SES -267A SES -597 SES -605 Kans Local BG-2 BG-5 Bazpur-2 Bazpur-6	Nargori Rekhra Katha-CBE Dhaur-kinara	Tukuya - No.1 Khakai Khelia Ubanaquin	ISH -135 ISH-148 ISH- 288 ISH- 309	CoLk 8102 CoLk 9617 CoLk 8901 CoJ 64 CoH 92 CoH 98 CoPt 97221 CoPt 84211 CoSe92423	BO 110 BO 128 Co 1158 Co 1148 CoLk 8001 Self -17 Self -9 CoC 671

Approximately 2 g of young leaf tissue of each genotype was used to extract and purify genomic DNA (Srivastava and Gupta, 2001). The DNA was quantified in 0.8% (w/v) agarose gels by comparison with known quantities of the lambda phage DNA and stored at –20°C. Eleven microsatellite markers (derived from genomic libraries) viz. mSSCIR 1, 5, 20, 25, 28, 36, 37, 61 and 76, (A. D' Hont and F. Paulet, pers. Comm.) and SMC477CG and SMC248CG (Cordeiro *et al.*, 2000) were used as flanking primers.

Amplification reactions were carried out using MJ Research PTC 200 thermocycler in medium containing 1 x PCR buffer, 1.25 mM d NTPs, 2 μM primer reverse and forward each, 4 mM $MgCl_2$, 1 Unit Taq polymerase and 10 ng template DNA.

Amplified products were denatured, snap chilled to convert them in single strands, separated on 10% (m/v) PAGE using TBE buffer, stained with ethidium bromide and photo-documented with AlphaImager™1220 gel documentation system. Reproducible polymorphic bands were scored for the presence or absence in each sample.

Positions of unequivocally scorable bands were transformed into a binary character matrix. Genetic similarity was estimated for each SSR primer according to Nei and Li (1979). UPGMA based cluster analysis was performed on the matrix of Nei's genetic distances and dendrograms were constructed using the computer program.

SSCP pattern

SSCP of simple sequence repeats is an efficient technique to convert all the markers, which were monomorphic in super fine resolution agarose (Ameresco brand) gel (Figure 1 a) into polymorphic ones (Figure 1b) and was able to detect high level of fragment length polymorphism among forty-two sugarcane genotypes, through distinct multiple band profiles (Figure 2) for all the primer pairs.

The resulting patterns ranged from few bands to highly complex multi-band ladders for most of the primers with product size ranging from 92–449 bp.

Each amplicon was identified by its size and number of DNA fragments. A total of 1025 bands was analysed in these genotypes, which belonged to 116 conformers of distinct molecular weight.

Among these, 98 conformers revealed more than 72% to 100% parsimony in different genotypes. 14 conformers were of unique type indicating their usefulness as specific markers whereas only four conformational variants (3.45% of all) were monomorphic in nature.

In all the genotypes, the number of conformers arising due to conformational polymorphism was more than one. The pattern of the conformers was reproducible.

Total number of conformers obtained for each primer ranged from 2 (with mSSCIR 61) to 18 (with mSSCIR 25) having an average of 93.18 fragments per primer and 24.40 conformers per genotype. Primers mSSCIR 61, SMC477CG and mSSCIR 28 showed complete parsimony and were very useful for diversity purposes.

Total polymorphism per cent obtained through SSCP analysis of SSR markers was 85%, which reinforced the utility of this approach for diversity studies in sugarcane.

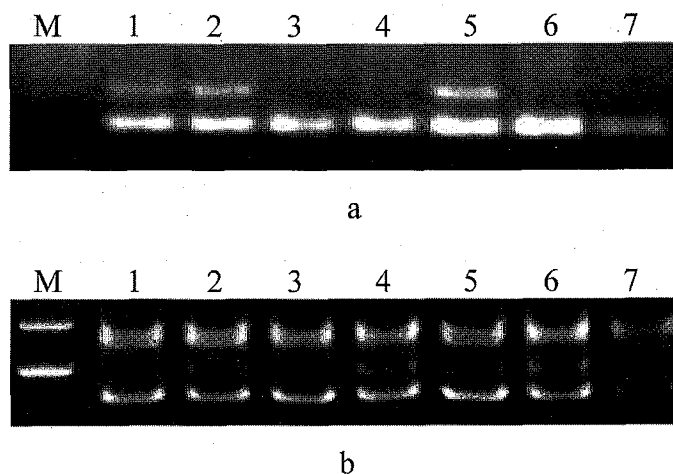

Fig. 1—SSR polymorphism in some clones of *S. officinarum* and inter-specific hybrids using primer mSSCIR36 (M=100 bp ladder, from 1 to 7 - Orambo, Karia, Rayada, Gungera, ISH-135, ISH-148 and ISH-288).
(a) in super fine resolution agarose gel
(b) SSCP profile

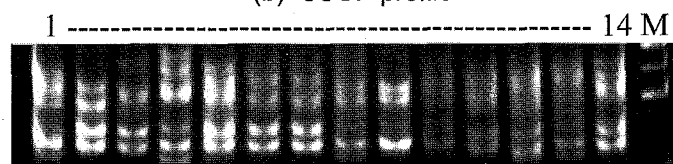

Fig. 2—SSCP Profile using primer SMC247CG (from 1 to 14 - BG-2, BG-5, Bazpur-2, Bazpur-6, Nargori, Rekhra, Katha-CBE, Dhaurkinara, Tukuya No.1, Khakai, Khelia, Ubanaquin, commercial hybrid BO 110, commercial hybrid BO 128 and M=100 bp ladder).

Genetic diversity

Mean similarity index among various groups ranged from 38 (among the clones of ISH) to 75% (among the clones of *S. officinarum*). Cultivated genotypes revealed sufficient genetic variability (S.I. = 59%). The two selfed progeny clones self-17 and self-9 were grouped in the same sub-cluster along with their parent cultivar, CoLk 8102.

The variety CoC 671 was grouped with the ISH, probably because of its role as one of the parents in derivation of two of the ISH clones. The mutant CoLk 8901 and its parent CoJ 64 were grouped separately. Between the clones belonging to *S. officinarum* and *S. spontaneum,* the mean genetic similarity was only 34%, confirming their separate evolution as envisaged by Nair *et al.* (1999) and Cordeiro *et al.* (2003).

Molecular diversity in sugarcane genotypes of subtropical India using 116 SSCP conformers generated by eleven SSR primers became apparently clearer through the dendrogram generated by UPGMA based cluster analysis performed on the matrix of Nei's matrix of genetic distances.

Two taxonomical groups were clearly resolved having different sub-clusters. The first taxonomical group consisted of clones belonging to *S. spontaneum, S. barberi, S. sinense* and interspecific hybrids but in different clusters and sub-clusters.

The other taxonomical group consisted of the clones of *S. officinarum* and commercial varieties in two sub-clusters. *S. officinarum* genotypes were quite close, whereas *S. spontaneum* clones exhibited great degree of genetic variability among themselves with respect to the presence or absence of conformers and were clustered in two different groups in one sub-cluster.

Similar response of *S. officinarum* and *S. spontaneum* clones to molecular markers has also been reported with RAPD (Harvey and Botha, 1996; Nair *et al.*, 1999), SSR (Cordeiro *et al.*, 2003), RFLP (Lu *et al.*, 1994; Burnquist *et al.*, 1995) and organellar DNA sequences (Al-janabi *et al.*, 1994).

Detection and identification methods using the PCR to amplify DNA have been used for sugarcane as well as other organisms. RAPD markers have been largely used to identify the genotypes and to determine genetic diversity (Harvey and Botha, 1996; Oropeza and Degarcia, 1997; Nair *et al.*, 1999 and

Srivastava *et al.*, 2003).

SSRs have proved more useful for germplasm characterisation and varietal identification in sugarcane as they provide higher incidence of detectable polymorphism (Cordeiro *et al.*, 2000; 2003). In the present study, application of SSCP technique to SSR primers has further helped in resolving multiple band profiles or conformers of amplicons (Figure 2). These amplicons are highly polymorphic also. Since only four conformational variants (3.45% of all) are monomorphic in nature, it is clear that SSCP is able to convert SSR amplified products into highly polymorphic banding patterns, which is extremely useful for genetic diversity studies. The major advantage of this method is that many individual PCR products may be screened for variation simultaneously and it can be extremely useful to differentiate even the closely related taxa. Besides, it is a cheap and fast technology to decipher the SSR amplified products as compared to high-resolution or metaphor agarose gels.

This technique may further be useful in sugarcane breeding programs through selection of genetically diverse parents to obtain new genetic combinations, as a marker in segregating populations for MAS, and in IPR through identification of different cultivars.

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DIVERSITÉ GÉNÉTIQUE MOLÉCULAIRE DE GÉNOTYPES DE CANNE À SUCRE DE L'INDE SUBTROPICALE: ANALYSE PAR SSCP-PCR DE RÉPÉTION DE SÉQUENCE SIMPLE

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MOTS CLÉS: Diversité Génétique, Microsatellite, SSCP-PCR, Répétition de Séquence Simple, Canne a Sucre

RÉSUMÉ

LE POTENTIEL du polymorphisme de conformation simple brin (SSCP) de répétition de séquence simple (SSR ou microsatelitte) comme un nouveau système de marqueur a été évalué par rapport à leur abondance, leur variabilité, leur capacité à détecter des polymorphismes et à révéler la diversité génétique moléculaire dans les génotypes de la canne à sucre. Onze couples d'amorce microsatellites (obtenus à partir des banques génomiques) ont été utilisés pour amplifier l'ADN génomique de 42 variétés commerciales, hybrides interspécifiques et espèces de canne à sucre. Les produits amplifiés ont été soumis au SSCP pour détecter du polymorphisme afin d'analyser la diversité génétique et la relation parmi les génotypes étudiés. Pour la plupart des amorces utilisées, les profils de bandes variaient de quelques bandes à des échelles de bandes très complexes. L'analyse SSCP-PCR a démontré un niveau élevé de polymorphisme entre les génotypes étudiés. La présence ou l'absence des bandes a été notée pour dériver une matrice de distance entre les génotypes pris 2 à 2 et pour leur regroupement dans différents groupes. Ainsi l'analyse SSCP-PCR de répétition de séquence simple apparaît efficace pour différencier les taxons même très proches. L'avantage principal de cette méthode est que beaucoup de différents produits de PCR peuvent être criblés simultanément pour la variation. Ce rapport est le première traitant l'utilisation de SSCP-PCR pour différencier les individus d'une espèce polyploïde.

DIVERSIDAD GENÉTICA MOLECULAR DE GENOTIPOS DE CAÑA DE AZÚCAR DE LA INDIA SUBTROPICAL: ANÁLISIS DE SSCP-PCR CON SECUENCIAS SIMPLES REPETIDAS

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PALABRAS CLAVE: Diversidad Genética, Microsatélites, SSCP-PCR, Simple Secuencias Simples Repetidas, Caña De Azúcar

Resumen

EL POTENCIAL del Polimorfismo Conformacional de Solo un Filamento (SSCP) de secuencias simples repetidas como un sistema nuevo de marcador fue investigado con respecto a su abundancia, variabilidad y capacidad para detectar polimorfismos y diversidad genética molecular en genotipos de caña de azúcar. Once marcadores de microsatélites (derivados de librerías genómicas) fueron empleados como iniciadores en la amplificación de ADN genómico en 42 cultivares comerciales, híbridos interespecíficos y especies de caña de azúcar. Los productos amplificados de SSR-PCR fueron sometidos a SSCP para detectar el polimorfismo y determinar así la diversidad genética y sugerir la relación entre los genotipos estudiados. Los patrones que resultaron estuvieron desde pocas bandas hasta muchas bandas altamente complejas para la mayoría de los iniciadores. El SSCP-PCR mostró la existencia de un alto nivel de polimorfismo entre los genotipos estudiados. La presencia o ausencia de bandas de ADN amplificadas fueron anotadas para realizar una matriz de distancia entre parejas en los genotipos y la conformación de diversos grupos. Por tanto, el análisis de SSCP-PCR de las secuencias simples repetidas es una herramienta muy útil para distinguir incluso individuos muy cercanos genéticamente. La ventaja principal de este método radica en el hecho de que muchos productos de PCR se pueden evaluar simultáneamente en su variación. Éste es el primer informe sobre el uso de SSCP-PCR en la separación entre los miembros de un género altamente poliploide.