COMPARISON OF AFLP, TRAP AND SSRs IN THE ESTIMATION OF GENETIC RELATIONSHIPS IN SUGARCANE

By

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Abstract

THE SUGARCANE Breeding Program of the Instituto Agronômico de Campinas has been using routinely different types of molecular markers for the characterisation of their breeding clones and varieties. In the present work, we compared the genetic relationships among 82 sugarcane clones/varieties using three types of molecular markers: AFLP, TRAP and Microsatellites (SSRs). Five AFLP selective primer combinations, 10 SSRs and four TRAP fixed primers, designed from candidate genes involved in the drought tolerance response metabolism, were used in combination with three arbitrary primers. The pair-wise genetic similarity based on the Jaccard's coefficient, the dendrogram and matrix comparison were done using NTSYS Software. A total of 410 polymorphic markers were obtained: 145 AFLPs, 103 SSRs, and 160 TRAPs. Although the average genetic similarity estimates based on AFLP (0.675) and TRAP (0.655) were closer to each other than to SSRs (0.522), the correlation between TRAP and SSRs was higher (r= 0.24). The coefficient of variation was lower for AFLP and TRAP (~6.55%) than for SSRs (13%). These results indicate that the choice of molecular markers should be considered carefully, based on the purpose of the application in the breeding program, as it is not possible to select a marker system that fits all the requirements for germplasm characterisation.

Introduction

Molecular markers are essential tools in breeding programs. One of their applications is to allow the estimation of the genetic distance (GD) among elite genotypes. Genetic distance estimation revealed by molecular markers should be prioritised for crops like sugarcane, with narrow genetic base, unknown ancestors, and with no accurate or even non-existing pedigree records (Farooq and Azam, 2002). In sugarcane breeding, the choice of parents is based on agronomic traits and pedigree records, using bi-parental crosses or polycrosses among elite genotypes.

However, the lack of genealogy data, as well as errors in the identification of some genotypes has resulted in inaccurate estimation of genetic distance among sugarcane genotypes based on pedigree data. It is accepted that crosses between unrelated genotypes would maximise the number of segregating alleles resulting in a large genetic variance of the progeny (Cox *et al*., 1985; Messmer *et al*., 1993), thus increasing the probability of selecting rare and superior genotypes (Beceleare *et al*., 2005). The genetic variability within sugarcane germplasm has been estimated using different molecular markers, including random (Lima *et al*., 2002, Aitken *et al*., 2006) and functional marker systems (Pinto *et al*., 2006; Alwala *et al*., 2006).

Molecular markers are used routinely at the Instituto Agronômico de Campinas as an additional tool to assess the genetic variability among genotypes used as parents. They are also used to fingerprint commercial and pre-commercial clones and to monitor for genetic identity in germplasm collections and in commercial fields. The objectives of this study were to evaluate the usefulness of microsatellite (SSR), amplified fragment length polymorphism (AFLP) and target region amplification polymorphism (TRAP) markers in the estimation of the genetic similarities (GS) in a specific set of sugarcane genotypes and to compare the GD values among them.

Material and methods

Plant material and DNA extraction

A total of 82 genotypes, comprising commercial and pre-commercial clones, were used. These represented a set of specific genotypes issued from the 'Centro de Cana' sugarcane breeding program, that are being evaluated in the field under the Brazilian 'cerrado' (savanna) conditions for drought response. From each genotype, total DNA was extracted from a fresh meristem cylinder (Al Janabi *et al*., 1999).

Primers used

TRAP markers were obtained from four fixed primers designed from candidate genes involved in the drought tolerance response metabolism (Aquaporin – Aqua primer; late embryogenesis abundant protein – LEA primer; dehydration binding factor – DBF primer; and dehydration responsive binding elements – DREB primer) in combination with three arbitrary primers (referred to as arb1, arb2 and arb3 primers) provided by Li and Quiros (2001). TRAP PCR reactions were performed according Alwala *et al.* (2006).

AFLP analysis was carried out using five primer combinations (E-ACT/M-CAT, E-ACG/M-CTT, E-ACG/M-CTC, E-ACT/M-CAG, E-AGA/M-CTG), according Vos *et al.* (1995). A total of 10 SSRs (6 genomic and 4 derived from expressed sequence tags) were chosen based on the sugarcane functional map (Oliveira *et al*., 2007). The primer sequences and amplification conditions of genomic SSR and EST-SSRs were obtained, respectively, from Pan (2006) and Oliveira *et al.* (2009). The polymorphisms were detected in polyacrylamide gels by silver staining (Creste *et al.*, 2001).

Data analysis

Amplified fragments were scored for presence (1) or absence (0) in all 82 genotypes. The genetic similarities among all genotypes were calculated according to Jaccard´s similarity coefficient using NTSYS-PC version 2.0j (Exeter Software, Setauket, NY, USA). The polymorphic information content (PIC) for allelic diversity at a given locus was measured. As PIC for dominant markers ranges from 0 to 0.5 and for co-dominant markers from 0 to 1.0, the PIC values obtained for AFLP and TRAP were converted in PIC % to allow comparison among the different marker types.

Results and discussion

The genetic polymorphism of 82 sugarcane genotypes was assessed by TRAP, AFLP and SSR markers. The data obtained for each marker type, in terms of total number of markers analysed, number of polymorphic markers; percent of polymorphism and PIC value are summarised in Table 1.

Target region amplification polymorphisms (TRAP)

The 12 TRAP primer combinations produced a total of 225 fragments, of which 160 (71.11%) were polymorphic. The total number of bands amplified by individual primer combinations ranged from 14 (DREB/ Arb3 and LEA/Arb1) to 27 (Aquaporin + Arb3), an average of 18.75 bands per primer combination, and 13.30 polymorphic bands per primer combination. The highest number of polymorphic fragments was observed with primer combination DBF/Arb1 (18 fragments) and the lowest with DBF/Arb2 (9 fragments).

Table 1—Marker type, number of markers analysed, polymorphic markers, percentage of polymorphism, PIC values and PIC% obtained in a sample of 82 sugarcane genotypes evaluated by SSR, AFLP and TRAP markers.

Marker type	N markers	Polymorphic markers	% Polymorphism	PIC	PIC %
TRAP					
DREB +Arbi 1	15	13	86.66	0.32	0.64
DREB + Arbi 2	18	10	55.55	0.23	0.46
DREB + Arbi 3	14	10	71.42	0.23	0.46
DBF + Arbi 1	18	15	83.33	0.21	0.42
DBF + Arbi 2	16	9	56.25	0.16	0.32
DBF + Arbi 3	21	18	85.71	0.17	0.34
LEA + Arbi 1	14	10	71.42	0.38	0.76
LEA + Arbi 2	16	13	81.25	0.32	0.64
LEA + Arbi 3	20	16	80.00	0.34	0.68
ACQUA + Arbi 1	21	15	71.42	0.40	0.80
ACQUA + Arbi 2	25	15	60.00	0.35	0.70
ACQUA + Arbi 3	27	16	59.25	0.24	0.48
Total	225	160			
Average	18.75	13.30	71.09	0.28	0.56
AFLP					
E-ACT + M-CAT	31	25	80.60	0.32	0.64
E-ACG + M-CTT	32	25	78.10	0.32	0.64
E-ACG + M-CTC	34	29	85.30	0.24	0.48
E-ACT + M-CAG	40	30	75.00	0.26	0.52
E-AGA + M-CTG	45	36	80.00	0.23	0.46
Total	182	145			
Average	36.40	29.00	79.67	0.28	0.55
SSR					
Cir51	5	5	100.00	0.68	0.68
Cir56	5	5	100.00	0.71	0.71
Cir74	7	7	100.00	0.77	0.77
SMC31CUQ	6	6	100.00	0.78	0.78
SMC1047HA	15	15	100.00	0.86	0.86
SMC2017FL	13	13	100.00	0.88	0.88
SCA48	14	14	100.00	0.87	0.87
SCB100	12	12	100.00	0.90	0.90
SCB312	13	13	100.00	0.88	0.88
SCC01	13	13	100.00	0.84	0.84
Total	103	103			
Average	10.30	10.30	100.00	0.82	0.82

The average of 13.30 polymorphic bands per primer combination was lower than that (29.38 polymorphic bands) derived with 18 TRAP primer combinations on a broad set of accessions composed of sugarcane hybrids, *Erianthus*, *Miscanthus* and *Saccharum* species as reported by Alwala *et al*. (2006). Different genotypes were evaluated in our research compared to the one conducted by Alwala *et al*. (2006), and thus the different numbers of polymorphic bands observed are probably due to differences in the genetic background of the accession group analysed. In fact, the large number of different accession types evaluated by Alwala *et al*. (2006) was wider than those of our study composed exclusively of commercial and semi-commercial varieties. The DBF/Arb 2 combination gave the lowest PIC value (0.16) while ACQUA/Arb1 combination gave the highest value (0.40). The average GS obtained among the genotypes was 0.65 with the lowest similarity value observed between IACSP03-8140 and IACSP03-8189 (0.46) and highest between IACSP03-8136 and IACSP03-8125 (0.84). The GS estimated with the 160 TRAP derived markers gave a coefficient of variation (CV) of 6.50%.

Amplification fragment length polymorphism – AFLP

The five AFLP primer combinations screened produced a total of 182 bands of which 145 (79.67%) were polymorphic. The total number of bands per primer combination ranged from 31 (E-ACT + M-CAT) to 45 (E-ACG + M-CTG) with an average of 36.4 bands. The PIC values ranged from 0.23 (E-AGA + M-CTG) to 0.32 (E-ACT + M-CAT and E-ACG + M-CTT). The average GS was 0.67 with the lowest value observed between IACSP93-2094 and IACSP03-8136 (0.38) and the highest between IACSP03-8077 and IACSP03-8145 (0.88), considering a CV of 6.5%. The average GS observed among the 82 sugarcane genotypes was lower than that of 47% (CV of 4.29%) reported by Lima *et al*. (2001) on 83 sugarcane varieties from different Brazilian breeding programs.

Microsatellites (SSRs)

The 10 microsatellite primers used yielded a total of 103 alleles (markers) with an average of 10.3 alleles per locus ranging from 5 (Cir51, Cir56) to 15 (1047HA). The PIC values ranged from 0.68 (Cir 51) to 0.90 (SCB100) with an average value of 0.82 (Table 1). These values were higher than those reported for 15 sugarcane commercial varieties from Coimbatore assessed with 168 SSRs (Singh *et al*., 2008).

The average GS among the 82 sugarcane genotypes was 0.51 with the lowest pair wise genetic similarity value observed between IAC97-2028 and IACSP03-8164 (0.28 and the highest value observed between IACSP97-7077 and IACSP97-2023 (0.87) considering a CV of 13.0%. The average GS value observed here was smaller (68.3%) than that reported among 20 sugarcane varieties assessed with SSRs (Selvi *et al*., 2008).

Comparison among different marker types

As expected, the results obtained with the different molecular markers used in this research were different, since each marker system allowed us to assess the genetic variability of the 82 sugarcane genotypes at different genomic regions.

In general, the polymorphism observed in this study reflects the nature of each type of marker (AFLP, TRAP, and SSRs). The PIC value adjusted for percentage (PIC%) varied according to the respective marker type: from 0.32 (DBF/ A2) to 0.80 (ACQUA/ A1) for TRAP, 0.46 (E-AGA + M-CTG) to 0.64 (E-ACT + M-CAT and E-ACG + M-CTT) for AFLP, and from 0.684 (CIR51) to 0.896 (SCB100) for SSR.

The highest PIC% values were observed with SSR markers, probably due to the nature of this marker, which usually shows high mutation rates when compared to other types of molecular markers (Jarne and Lagoda, 1996).

The estimates of the correlation between the genetic similarity matrices and its significance (t test) were generally low: $r = 0.12$ ($t = 1.53^{ns}$, p = 0.937) for AFLP *vs* TRAP, $r = 0.011$ ($t = 0.16^{ns}$; p =0.565) for AFLP *vs* SSRs, and $r = 0.24$ ($t = 3.62^{n.s}$, p =0.999) for TRAP *vs* SSRs.

This situation was expected, since each marker system reflects the polymorphism in different portions of the genome (i.e. repetitive regions, or genome wide or target genes). The highest correlation value was observed between TRAP and SSRs and this could be partially attributed to the fact that among the 10 SSRs screened, 4 were derived from expressed sequence tags (EST-SSRs) sampling a functional region of the genome.

The range of variation of GS values was smaller for TRAP (0.45–0.84) than for AFLP (0.38 –0.88) and SSR (0.28–0.87) (Figure 1). The TRAP markers used are derived from candidates genes related to drought tolerance in sugarcane. Drought tolerance has been considered as an important trait in sugarcane breeding, and probably these genes have been under a higher selection pressure than those assessed by SSR and AFLP markers. So, there is a lower genetic variability for this trait in the pool of the genotypes sampled in comparison with those revealed by the repetitive or random markers.

The highest range in the GS distribution was generated with microsatellite data. In sugarcane, SSR markers have been considered as the most efficient marker for its characterisation, due to their reproducibility and high polymorphisms (Pinto *et al*., 2006) and also because they are ideal to establish relationships and for fingerprinting. In this study, the SSR makers used allowed the identification of several alleles including those that are unique to a variety or clone contributing to an increase in the range in the genetic diversity in the pool of genotypes assessed.

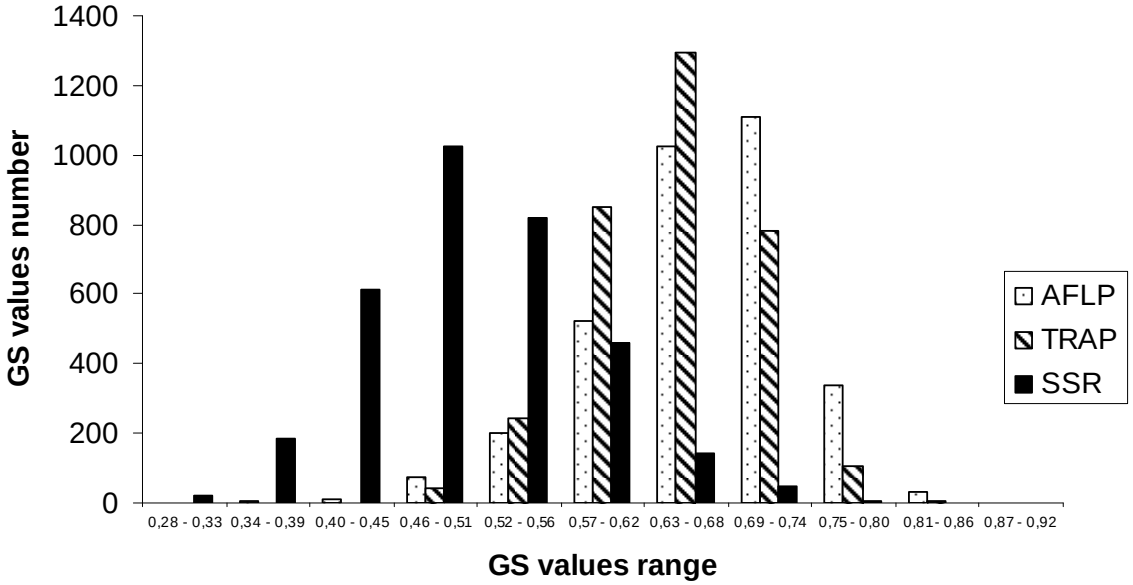

Fig.1—Frequency distribution of TRAP, AFLP and SSR-based genetic similarity values for all the 82 genotypes evaluated (3321 genotypes combinations).

According to Alwala *et al*. (2006), AFLP markers may be more robust for detecting polymorphisms among closely related genotypes, as they are more likely to sample different segments throughout the genome. However, it has been suggested that the choice of the marker (random or functional) for germplasm characterisation is dependent on the aim of the study. If the study is focused on the evolution or historical processes, random markers are suitable (Tiendiren *et al*., 2002).

For the assessment of variation in wild relatives or for establishing GS between genotypes for breeding purposes, it is more valuable to have information on the variability of specific genes that potentially affect important traits in breeding. The variation in expressed or regulatory sequences might reflect the past influences of selections, which could be different for each gene.

For instance, the characteristics that enable a sugarcane variety to show superior performance in a specific environment may depend on a limited set of genes, and the variation in genes will probably not be the same as in a group of genes involved in the expression of an independent characteristic.

Therefore the variation in these genes will most probably not be detected using random markers (Tienderen *et al*., 2002). In fact, the importance of accessing polymorphisms that account for the phenotypic variation (i.e. in target genes) in genetic breeding has contributed to the increase in the use of single nucleotide polymorphism (SNP) marker technology for sugarcane characterisation (Cordeiro *et al*., 2006).

Thus, it is important to choose a marker system related to the objective of the research. Using different markers is also an interesting approach since it provides different and complementary information on genotypes.

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COMPARAISON ENTRE TECHNIQUES AFLP, TRAP ET SSRSs POUR ESTIMER LA PARENTÉ GENOMIQUE CHEZ LA CANNE À SUCRE

Par

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MOTS-CLÉS: **Marqueur Moléculaire, Distance Génétique, Canne à Sucre.**

Résumé

LE PROGRAMME d'amélioration variétale de 'Instituto Agronômico de Campinas' fait l'usage routinier de différents types de marqueurs moléculaires pour la caractérisation des clones et variétés. Dans cette étude, nous avons comparé la parenté génomique parmi 82 clones/variétés de canne à sucre en utilisant trois types de marqueurs moléculaires: AFLP, TRAP et microsatellites (SSRs). Cinq combinaisons d'amorce sélective pour AFLP, 10 amorces SSRs et 4 marqueurs TRAP développés à partir des données des gènes candidats impliqués dans le métabolisme pour la résistance à la sécheresse ont été utilisées en combinaison avec trois amorces arbitraires. Les correspondances génétiques, basées sur la méthode du coefficient de Jaccard, du dendrogramme en classification ascendante et hiérarchique et la matrice de comparaison, ont été faites selon le logiciel NTSYS. Un total de 410 marqueurs polymorphes a été obtenu notamment 145 marqueurs AFLP, 103 SSRs et 160 marqueurs TRAP. Quoique l'estimation moyenne de la similarité génétique basée sur les marqueurs AFLP (0,675) et marqueurs TRAP (0,655) était plus près l'un de l'autre en comparaison avec les marqueurs SSRs (0.522), la corrélation entre les marqueurs TRAP et SSRs était plus élevée (r = 0.24). Le coefficient de variation était inférieur entre les marqueurs AFLP et TRAP (~6.55%) en comparaison avec les SSRs(13%). Ces résultats indiquent que le choix de marqueurs devrait être soigneusement considéré. En sus, le choix doit être basé sur l'objectif de l'application des marqueurs dans le programme d'amélioration variétale car il n'est pas possible d'adopter un système de marqueur pour tous les besoins pour la caractérisation du germoplasme.

COMPARACIÓN DE MARCADORES AFLP, TRAP Y SSRs EN LA EVALUACIÓN DE RELACIONES GENÉTICAS DE CAÑA DE AZÚCAR

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PALABRAS CLAVE: **Marcadores Moleculares, Diatancia Genética, Caña de Azúcar.**

Resumen

EL PROGRAMA de Fitomejoramiento de la Caña de Azúcar de Campinas ha utilizado de manera rutinaria diferentes tipos de marcadores moleculares para la caracterización de sus clonas mejoradas y variedades. En el presente trabajo, comparamos las relaciones genéticas entre 82 clonas/variedades de caña de azúcar usando tres tipos de marcadores moleculares: AFLP, TRAP y Microsatélites (SSR). Cinco combinaciones de iniciadores selectivos AFLP, diez SSR y cuatro iniciadores fijos TRAP fueron diseñados a partir de genes candidatos involucrados en el metabolismo de respuesta en la tolerancia a la sequía, usándoseles en combinación con tres iniciadores arbitrarios. La similitud genética basada en el coeficiente de Jaccard, el dendrograma y la comparación de matriz fueron calculados usando el programa de cómputo NTSYS. Se obtuvieron un total de 410 marcadores polimórficos: 145 AFLPs, 103 SSRs y 160 TRAP. Pese a que el promedio de los cálculos de similitud genética se sustentaron en la relación entre los marcadores AFLP (0.675) y TRAP (0.655), más cercana entre ellos que en relación a los SSRs (0.522), la correlación entre TRAP y SSR fue mayor (r = 0.24). El coeficiente de variación fue menor para AFLP y TRAP (~6.55%) que para los SSR (13%). Estos resultados indican que la elección de marcadores moleculares debe conducirse juiciosamente dependiendo del propósito para aplicarles en un programa de fitomejoramiento, ya que no es posible seleccionar un sistema de marcadores que cumpla con todos los requerimientos en la caracterización de germoplasma.