

TRAP, A NEW TOOL FOR SUGARCANE BREEDING: COMPARISON WITH AFLP AND COEFFICIENT OF PARENTAGE

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ABSTRACT

The choice of parents to cross is the most crucial step in any crop improvement program. A better understanding of genetic diversity among the available parental genotypes would help the breeder to make better crosses. Several molecular marker techniques have been used to assess genetic diversity in sugarcane. Target Region Amplification Polymorphism (TRAP) is fairly a new marker technique which is believed to amplify genic regions of the genome. In this study, TRAP, AFLP, and pedigree data were used to estimate genetic similarity (GS) among nine sugarcane genotypes often used as parents. The objectives were to compare TRAP with AFLP and pedigree-based coefficient of parentage (COP) for estimating GS among sugarcane genotypes. Twelve TRAP primer combinations produced a total of 444 bands, out of which 242 (55 %) were polymorphic, whereas 28 AFLP primer combinations produced a total 1325 bands out of which 686 (53 %) were polymorphic. TRAP-based GS estimates ranged from 0.67 to 0.87 with a mean of 0.75, while AFLP-based estimates ranged from 0.72 to 0.84 with a mean of 0.76. The COP-based GS estimates ranged from 0.03 to 0.36 with a mean of 0.12. The dendrograms were constructed using the unweighted pair-group method with arithmetic mean (UPGMA). Although no distinct pattern was observed in the COP dendrogram, the TRAP dendrogram was better explained by the pedigree records. The AFLP dendrogram showed some distinct cluster patterns. The associations between TRAP-COP ($r = 0.41$) and AFLP-COP ($r = 0.42$) were moderate, whereas the TRAP-AFLP ($r = 0.14$) association was low. Our results indicate that all the three methods estimate different aspects of GS. Therefore, based on the objectives of the research, some combination of TRAP, AFLP and COP would be a better choice in making decisions of which parents to cross in a crop improvement program.

Abbreviations: AFLP, Amplified Fragment Length Polymorphism; COP, Coefficient of Parentage; EST, Expressed Sequence Tag; GS, Genetic Similarity; gSSR, genome-derived Simple Sequence Repeats; RAPD, Randomly Amplified Polymorphic DNA; RFLP, Restriction Fragment Length Polymorphism; TRAP, Target Region Amplification Polymorphism.

INTRODUCTION

Sugarcane breeding efforts have contributed substantially to sugar industries worldwide. In central Queensland, Australia, for example, the cultivar Q50 was nicknamed 'mortgage buster' soon after its release because of the wealth it brought to that sugar industry. In Louisiana, the popular cultivar, LCP85-384, increased cane yield by 20-25 % and contributed to unprecedented boosts in sugar production. LCP85-384, like most dominant cultivars, has enjoyed widespread adoption in the Louisiana sugar industry, albeit to the exclusion of other cultivars. It is well known that the over reliance on a single cultivar can result in severe consequences especially in a clonally propagated crop, such as sugarcane. Therefore, tremendous effort is being made to release new cultivars that equal or surpass the performance of LCP85-384 to Louisiana growers.

The long duration of a sugarcane selection cycle is one factor limiting the rapid development of improved sugarcane cultivars. It takes about 12 to 15 years after crossing to complete a selection cycle. Because sugarcane is clonally propagated, during this 12- to 15-year period, no new opportunities are exploited for sexual recombination or the creation of new genetic variation that the breeder can capitalize on. The breeder has to rely on the initial variation created during hybridization, and no amount of selection can produce a good cultivar out of a poor cross. The choice of parents to use in crossing is, therefore, one of the most crucial decisions the breeder has to make.

The complicated genome of cultivated sugarcane (high (aneu) ploidy levels, and multiple alleles at a locus) is another factor limiting progress in sugarcane breeding programs. Cultivated sugarcane was derived by mainly crossing between two species, namely *S. officinarum* ($2n = 80$) and *S. spontaneum* ($2n = 40$ to 128), followed by backcrosses to *S. officinarum*. *Saccharum officinarum* was reported to transmit the somatic chromosome number to its F_1 progeny (Bhat and Gill, 1985; Bremer, 1961). Consequently, cultivated sugarcane harbors two genomes with about 80 % *S. officinarum* and 20 % *S. spontaneum* composition (D'Hont et al., 1996). Furthermore, chromosome numbers within cultivated sugarcane can vary (generally from about 100-130) even among full sib progenies.

Molecular markers are valuable tools that can be used to help understand and manipulate a genome as complicated as that of sugarcane. Molecular markers can be used to tag genes for traits of economic importance such that selection for these traits (via marker-assisted selection) could occur earlier in the breeding program. Molecular markers also can be used to facilitate decisions made during crossing, as using these markers can help gain a better understanding of the genetic diversity in the parental clones. That information could then play a vital role in the utilization and management of the genotypes and indeed genes in the breeding gene pool. For example, crosses could be planned between parents from divergent backgrounds to maximize heterosis while increasing genetic diversity in the cultivated gene pool.

In sugarcane breeding programs, experimental clones are often nominated as parents based upon performance in advanced stage selection trials. Ultimately, most crosses are made among parents with phenotypic superiority in one or more key attributes with the goal of combining all key attributes in the hybrid. It is believed that the probability of recovering superior progeny is higher when both parents are themselves superior. Therefore, one would like to detect genetic diversity among phenotypically superior parents. This can be a very difficult task when relying solely on pedigree records because superior phenotypic characteristics might have been obtained at the expense of genetic diversity at specific loci that have undergone selection. Pedigree-based estimates of genetic diversity may not account for allele frequency changes resulting from selection and genetic drift. By relying on pedigree records to estimate genetic diversity, one assumes that all genotypes are unrelated, which may be misleading in cultivated sugarcane where only a handful of clones were used in the original synthesis. Molecular markers on the other hand offer a direct comparison of genetic diversity at the DNA level without the simplifying assumptions inherent with the pedigree-based method.

When used for genetic diversity studies, molecular marker techniques, such as RFLP, RAPD, AFLP, and gSSR, customarily amplify random portions of the genome leading to competent estimates of genetic diversity. However, breeders may be more interested in results from genetic diversity studies when markers that co-segregate with traits of interest are used. However, even after quantitative trait loci (QTLs) for traits of interest have been identified, it has been argued that the underlying QTL-trait association is based on relatively large linkage blocks and could easily be lost with recombination. In addition, transferability of QTLs between populations remains a question in the minds of many plant breeders. The results from genetic diversity studies may, therefore, be more useful if the segments of the genome sampled or measured correspond to segments bearing the genes of interest to the breeder. This may be more important in sugarcane with its large genome size (estimated to be about 6 pg, approximately six times larger than that of rice) most of which may be duplicated and redundant (Ma et al., 2004).

Access to increasing numbers of sugarcane gene and expressed sequence tag (EST) sequences obtained from diverse cDNA libraries coupled with freely available bioinformatics tools offer new opportunities for achieving a candidate gene approach to molecular markers in sugarcane. Target region amplification polymorphism (TRAP) is a relatively new marker technique which uses gene/EST sequence information to generate polymorphic bands around targeted/putative candidate gene regions. We previously sequenced TRAP amplicons from sugarcane and showed, using Blastx analysis, that the TRAP primers successfully amplified the anticipated candidate gene regions (Alwala et al., 2006).

Our objectives were to compare TRAP, AFLP, and pedigree-based coefficient of parentage (COP) in their ability to elucidate genetic diversity and relationships among nine sugarcane genotypes frequently used as parents in the Louisiana breeding program.

MATERIALS AND METHODS

Plant material and DNA extraction

The nine sugarcane parents used in the study are described in Table 1. These genotypes are experimental clones and cultivars adapted to Louisiana's unique subtropical climate. TucCP77-042, a major cultivar in northern Argentina, was bred also in Houma, LA using Louisiana adapted clones as the recurrent parents. This group of genotypes serves as an important parental pool for sugarcane crossing in Louisiana.

Table 1. Description of the nine sugarcane genotypes used in the genetic similarity study.

Genotype	Female parent	Male parent	Comments/Agronomic characteristics
L99-238	CP79-318	LCP85-384	High sucrose content
HoCP91-552	LCP81-10	CP72-356	High tonnage; high fiber
LCP86-454	CP77-310	CP69-380	Commercial; early high sucrose content
Ho95-988	CP86-941	US89-12	Commercial; high sugar yields and good ratooning ability
LCP85-384	CP77-310	CP77-407	Commercial; leading commercial cultivar in Louisiana from 1998 to present; high sugar yield, good ratooning ability and recently showing susceptibility to rust disease
HoCP96-540	LCP86-454	LCP85-384	Commercial; released in 2003; high sugar and cane yields; good disease resistance
HoCP95-951	CP85-866	CP85-830	BC ₅ of US60-8-3; high cane yield and fiber content
TucCP77-042	CP71-321	US72-19	Commercial cultivar in Argentina; high cane yield and average sucrose content
HoCP92-624	CP81-325	CP71-1038	High sugar and cane yield; dropped due to excessive lodging; used extensively in crossing programs

Young leaves were collected from each genotype, frozen immediately and stored at -80 °C. Later, the leaves were ground in liquid nitrogen. Genomic DNA was extracted using the Plant DNeasy Mini Kit (Qiagen, Valencia, CA). DNA concentrations were estimated by known concentration of lambda DNA in 1 % agarose gel.

TRAP markers

TRAP is a simple, 2-primer polymerase chain reaction (PCR) technique (Hu and Vick, 2003). The forward (fixed) primer was designed from genes or EST sequences and the accompanied reverse (arbitrary) primer was designed to target introns or exons. Both primers are usually about 18 bp long.

In this study, the fixed primers were designed from four genes associated with sucrose metabolism, namely sucrose synthase (SuSy), sucrose phosphate synthase (SuPS), pyruvate orthophosphate dikinase (PODK), and soluble acid invertase (SAI). The primers were designed using the Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Rozen and Skaletsky, 2000). The primer optimum size, maximum size and minimum size were set to 18 nt. The optimum T_m , maximum T_m and minimum T_m were set to 53°C, 55°C, and 50°C respectively. The GenBank accession number and designed primer sequence for each gene is given in Table 2.

Table 2. Sequences of fixed and arbitrary primers used for TRAP markers.

	Gene	Sequence (5' → 3')	T_m (°C)
Fixed (forward) primers	Sucrose synthase (SuSy)	GGAGGAGCTGAGTGTTC	53
	Sucrose phosphate synthase (SuPS)	CGACAACCTGGATCAACAG	53
	Pyruvate orthophosphate dikinase (PODK)	CGTAAAGATTGCTGTGGA	53
	Soluble acid invertase (SAI)	AGGACGAGACCACACTCT	53
Arbitrary (reverse) primers	Arbi 1	GACTGCGTACGAATTAAT	53
	Arbi 2	GACTGCGTACGAATTGAC	53
	Arbi 3	GACTGCGTACGAATTTGA	53

Arbitrary reverse primer sequences were obtained from Li and Quiros (2001). The basic structure of this primer included three selective nucleotides at the 3' end, four nucleotides of AT- or GC-rich content in the core region, and 11 nucleotides as filler sequences at the 5' end. The AT and GC sequences are believed to target introns and exons, respectively. In addition, the basic rules of primer design, such as self-complementarity and maintenance of 40-60 % GC content were upheld in designing both primers (Table 2). The TRAP protocol was performed on an *i-cycler* (BioRad Labs, Hercules, CA) as described in Alwala et al. (2006). After PCR, the amplified products were run on a 7 % polyacrylamide denaturing gel for 1.5 hrs at 110 W. The gel was developed and visualized using the silver staining technique. A total of 12 TRAP primer combinations were used to screen the nine parents.

AFLP markers

The AFLP technique was chosen for comparison because it has been widely used for genetic diversity studies in sugarcane (Besse et al., 1998; Lima et al., 2002) and other crops, such as beans (Bhat et al., 2005), wheat (Tian et al., 2005) and squash (Ferriol et al., 2004). Also, the power of AFLP supposedly lies in its ability to simultaneously amplify large numbers of marker loci throughout the genome (Vuylsteke et al., 2000). Thus, it was of interest to compare it to TRAP which tends to target specified regions of the genome. AFLP analysis was performed based on the protocol described by Vos et al. (1995). Two hundred nanograms of DNA were double digested with *EcoRI* and *MseI* and linked to specific adaptors. Primers carrying one selective nucleotide were designed, based on adaptor sequence, for pre-selective amplification. *EcoRI* and *MseI* primers with three selective nucleotides were used for selective amplifications. All the PCR amplifications were carried out on an *i-cycler* (BioRad Labs, Hercules, CA). The amplified products were mixed with equal amount of dye and 5 µl of each sample was separated by electrophoresis on a 6 % polyacrylamide denaturing gel for 2 hr at 110 W. The gels were documented using the silver staining technique. A total of 28 *EcoRI* /*MseI* AFLP primer combinations were used to screen the nine parents.

Estimation of TRAP- and AFLP-derived genetic diversity and polymorphic information content

The bands from TRAP- and AFLP-derived gels were scored as '1' for presence and '0' for absence. Jaccard-similarity coefficient (1908) was used to estimate genetic diversity (GS) between pairs of genotypes as follows: $GS_{ij} = a/(a+b+c)$, where GS_{ij} is the genetic similarity measurement between individuals i and j , a represents the number of polymorphic bands present in both individuals whereas b and c are the number of bands present in individual i and j , respectively, but not in their counterparts. The bands absent in pairs of individuals were excluded from the calculation.

Allelic diversity at a given locus can be measured by polymorphism information content (PIC) wherein a marker can distinguish two alleles taken at random from a population and it was calculated as follows:

$$PIC = 1 - \sum f_i^2$$

where, f_i is the frequency of the i^{th} allele (Weir 1990). Considering the number of alleles at a locus along with their relative frequencies in a given population, an estimate of the discriminatory power of a marker can be obtained by calculating the PIC value (Vuylsteke et al., 2000).

Coefficient of parentage

The coefficient of parentage (COP), which corresponds to the probability that alleles at a locus in two individuals are identical by descent, was calculated to represent the pedigree-based measure of genetic diversity. The COP was calculated based on

Kempthorne (1957) using the PROC INBREEDING procedure in SAS (SAS Inc., 2002). The COP value between remotely related parents was assumed to be 0, and each genotype was assumed to receive half of their genome from each of its parent. All of the ancestors were assumed to be heterozygous, since sugarcane is a highly heterozygous crop, and in addition, the COP of a genotype with itself was assumed to be 0.5 rather 1.0 as for homozygous inbreds like rice (Kempthorne, 1957; Chiang and Lo, 1993; Deren, 1995).

Cluster and Principal Coordinate Analyses

For ease of interpretation, the GS values for TRAP, AFLP, and COP between pairs of genotypes were subjected to both cluster (CA) and principal coordinate (PCoA) analyses to obtain graphical representations of the relationships between the nine genotypes. The goodness of fit of the dendrograms formed from the GS matrix was evaluated by means of the cophenetic coefficient of correlation. A minimum-length spanning tree (MST) was superimposed on the PCoA plot to help detect local distortion because pairs of points which look close together in a plot may actually be far apart if other dimensions are taken into account. These analyses were performed using the NTSYS-PC ver 2.2 (CA; Rohlf, 2000) and PAST (PCoA; Hammer et al., 2001) software packages. Bootstrap analysis with 1000 replications with a 50 % consensus rule was performed using the PAUP ver 4.0 software (Sinauer Associates, Inc., MA), and the bootstrap values were superimposed on the CA dendrogram as a measure of the robustness of branches on the dendrogram.

Correlation between COP, TRAP and AFLP-derived GS

The correlation among pairs of the three genetic diversity measures was compared using two methods. The first method employed the MAXCOMP routine of NTSYS-PC software, in which two GS matrices are compared by estimating the normalized Mantel Z-statistic (Mantel, 1967). The second method estimated the simple or Pearson's correlation coefficient (r) between the measures.

Bootstrap analysis

Bootstrap analysis (Efron, 1982) was carried out to investigate if the number of markers used to generate GS were sufficient to provide precise estimates among the genotypes. Subsamples, consisting of different number of polymorphic bands, were generated by re-sampling 1000 times, with replacement, to estimate GS between every two pairs of genotypes for each subsample. The average coefficient of variation was estimated across subsamples for a given number of polymorphic bands. The analysis was performed using the Dboot software, kindly provided by Dr. A. S. G. Coelho (Universidade Federal de Goias/Goiana-GO).

RESULTS

TRAP Markers

Percent polymorphism and PIC Values

All 12 TRAP primer combinations produced multiple PCR fragments (bands) in each of the nine cultivars which ranged in size from 300 to 700 bp (Fig. 1). A total of 444 unambiguous bands were scored, of which 242 (55 %) were polymorphic (Table 3). The total number of bands amplified by individual primer combinations ranged from 19 (SuPS + Arbi 3) to 69 (SuSy + Arbi 1) with an average of 37 bands per primer combination. These two primer combinations were also responsible for the least (10) and most (41) number of polymorphic bands produced with an average of 20 polymorphic bands per primer combination. The PIC values, averaged over all polymorphic loci for individual primer combinations, varied from 0.32 to 0.40 with an overall mean of 0.36 ± 0.12 . From the polymorphism produced, it was possible to distinguish each of the nine genotypes. Sometimes only one of the primer combinations was needed to distinguish all nine genotypes. Bands were found to be uniquely present or absent in some genotypes. TRAP fragments were found to be highly reproducible (Fig. 1).

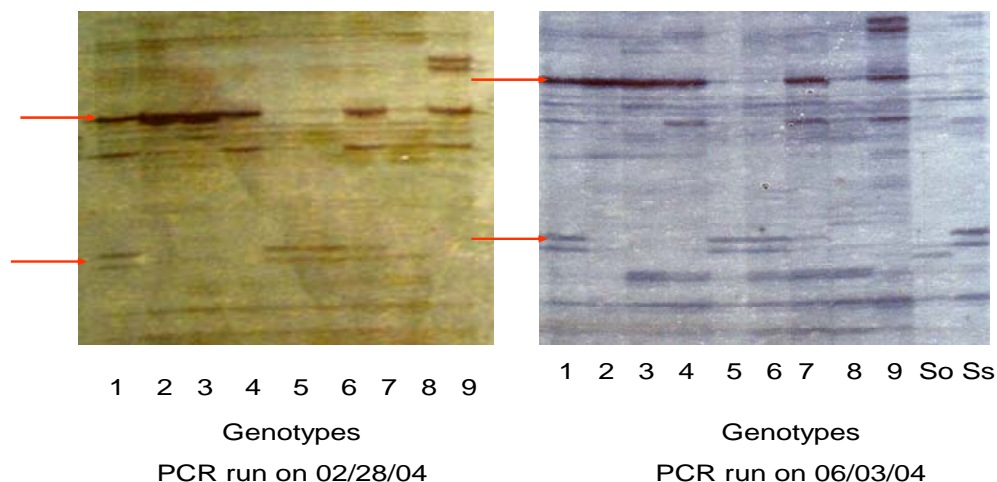


Figure 1. Reproducibility of TRAP markers depicted here by segments from two silver-stained polyacrylamide gels with polymorphic TRAP fragments generated using SuSy + Arbi 3 (see Table 1). Genotypes 1 to 9 are similar on the two gels with the reactions and gels run on different dates. Genotypes: 1 = L99-238; 2 = HoCP91-552; 3 = LCP86-454; 4 = Ho95-988; 5 = LCP85-384; 6 = HoCP96-540; 7 = HoCP95-951; 8 = TucCP77-042; 9 = HoCP92-624; So = La Stripe; Ss = SES 147 B. Arrows show identical banding patterns between the two gels.

Table 3. Percent polymorphism and PIC values of TRAP primer combinations used in genotyping nine sugarcane parents from the Louisiana breeding program.

	Primer combination	Total bands observed	Polymorphic bands observed	Percent polymorphism	PIC [†]
1	SuSy + Arbi 1	69	41	59.42	0.33
2	SuSy + Arbi 2	60	19	31.66	0.38
3	SuSy + Arbi 3	54	38	70.37	0.35
4	SuPS + Arbi 1	25	15	60.00	0.39
5	SuPS + Arbi 2	45	24	53.33	0.38
6	SuPS + Arbi 3	19	10	52.63	0.40
7	PODK + Arbi 1	31	14	45.16	0.32
8	PODK + Arbi 2	28	18	64.28	0.40
9	PODK + Arbi 3	34	15	44.11	0.35
10	SAI + Arbi 1	35	21	60.00	0.36
11	SAI + Arbi 2	20	13	65.00	0.32
12	SAI + Arbi 3	24	14	58.33	0.34
	Total	444	242		
	Average	37	20	55	0.36

[†] Polymorphism information content

Genetic diversity and relationship among genotypes

The TRAP-GS mean estimate, averaged across all pair-wise combinations of genotypes, was generally high (0.75 ± 0.04) as expected based on the shared ancestry among these genotypes (Table 1, Fig. 2). The GS values ranged from 0.67 (between HoCP91-552 and HoCP92-624) to 0.87 (between LCP85-384 and HoCP96-540) (Table 4, Fig. 3A). Data from the GS matrix were visualized using two methods, CA and PCoA.

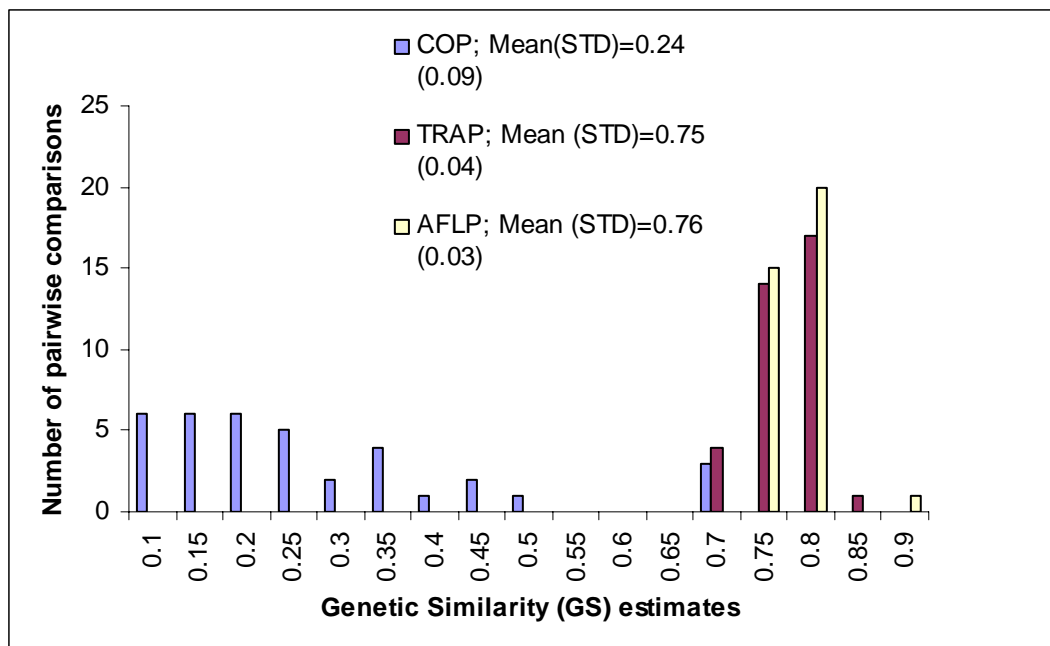
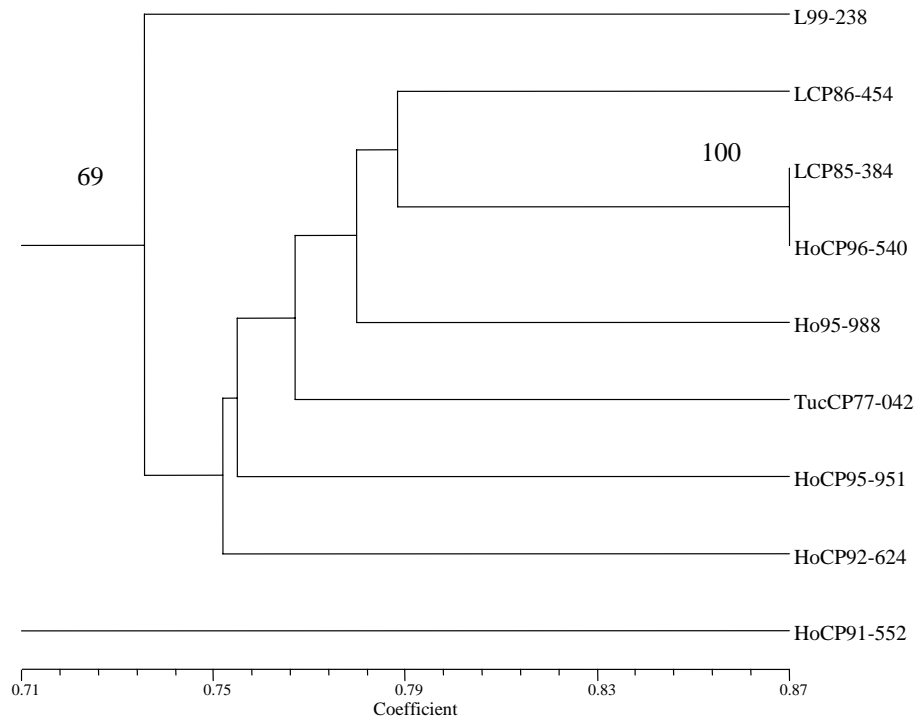


Figure 2. Frequency distribution of genetic similarity estimates based on pedigree (COP), TRAP, and AFLP data. Note: The theoretical range of genetic similarity (GS) values for molecular markers is 0 to 1, whereas COP values in a heterozygous crops, e.g. sugarcane, range from 0 to 0.5. To facilitate comparison among the different GS estimates, the COP-GS values were multiplied by 2.

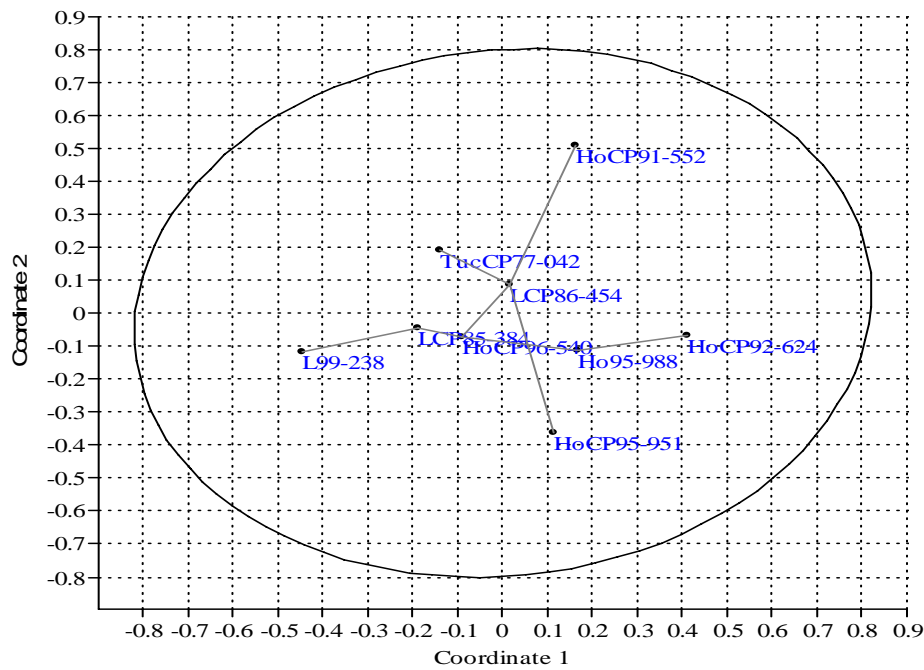
The CA yielded a dendrogram (Fig. 3A) with a cophenetic coefficient of correlation value of 0.81, which is above the 0.80 generally regarded as a good fit (Rohlf and Sokal, 1981). No distinct clusters were found. However, a subgroup was apparent between LCP85-384, HoCP96-540, and LCP86-454 (Fig. 3A), which is in agreement with the close relationship known among these genotypes (Table 4). Missing from this subgroup, however, was L99-238, a progeny of LCP85-384. The average GS between members of the group containing LCP85-384, HoCP96-540 and LCP86-454 vs. Ho95-988 is 0.78 while for L99-238 it is 0.77. This might explain why using the clustering technique placed Ho95-988 closer to the group compared with L99-238.

The bi-plot from PCoA superimposed with the MST portrayed a slightly different sub-grouping which was composed of LCP85-384 and its two progeny, L99-238 and HoCP96-540 (Fig. 3B, Table 4). Considering the first principal coordinate, LCP86-454, a sibling of LCP85-384, was placed outside of the subgroup (Fig. 3B) although the MST clearly illustrates the relationship between the two genotypes. Both the CA and PCoA portrayed HoCP91-552 as the genotype most distant from the rest of the group (Figs. 3A and 3B). In the dendrogram (Fig. 3A), the split of HoCP91-552 from the rest of the genotypes was only one of two branches supported by a bootstrap value greater than 50%.

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A



B

Figure 3. Genetic diversity pattern among nine sugarcane genotypes based on TRAP polymorphism and depicted by cluster analysis (A) and principal coordinate analysis (B). The numbers on the dendrogram (A) represent 50 % majority rule bootstrapped values.

Table 4. Pairwise genetic similarity (GS) estimates using TRAP and AFLP molecular markers compared with coefficient of parentage (COP) on nine sugarcane genotypes.

TRAP	L99-238	HoCP91-552	LCP86-454	Ho95-988	LCP85-384	HoCP96-540	HoCP95-951	TucCP77-042	HoCP92-624
L99-238	1.00								
HoCP91-552	0.68	1.00							
LCP86-454	0.76	0.78	1.00						
Ho95-988	0.72	0.71	0.78	1.00					
LCP85-384	0.79	0.73	0.79	0.78	1.00				
HoCP96-540	0.78	0.71	0.79	0.79	0.87	1.00			
HoCP95-951	0.69	0.71	0.77	0.75	0.75	0.76	1.00		
TucCP77-042	0.72	0.72	0.79	0.74	0.78	0.77	0.76	1.00	
HoCP92-624	0.70	0.67	0.76	0.77	0.75	0.75	0.76	0.75	1.00
AFLP									
L99-238	1.00								
HoCP91-552	0.78	1.00							
LCP86-454	0.79	0.8	1.00						
Ho95-988	0.73	0.78	0.76	1.00					
LCP85-384	0.80	0.77	0.75	0.76	1.00				
HoCP96-540	0.77	0.75	0.79	0.73	0.77	1.00			
HoCP95-951	0.76	0.75	0.75	0.76	0.74	0.77	1.00		
TucCP77-042	0.75	0.74	0.76	0.72	0.75	0.74	0.75	1.00	
HoCP92-624	0.77	0.74	0.73	0.72	0.84	0.79	0.72	0.74	1.00
COP									
L99-238	0.50								
HoCP91-552	0.10	0.50							
LCP86-454	0.15	0.08	0.50						
Ho95-988	0.05	0.04	0.03	0.50					
LCP85-384	0.35	0.08	0.18	0.04	0.50				
HoCP96-540	0.25	0.08	0.35	0.04	0.35	0.50			
HoCP95-951	0.20	0.11	0.11	0.06	0.20	0.15	0.50		
TucCP77-042	0.07	0.08	0.06	0.03	0.07	0.07	0.08	0.50	
HoCP92-624	0.15	0.10	0.11	0.04	0.14	0.13	0.16	0.09	0.50

Bootstrap analysis

As expected, the precision (CV %) with which one can distinguish among the nine genotypes increased with increasing numbers of polymorphic TRAP bands (Fig. 4). Using all 242 polymorphic TRAP bands, the CV for distinguishing among the nine genotypes in the study was 8.1 % (Fig. 4), which seems to be reliable considering some authors recommend a CV of 10 % (Lima et al., 2002). About 160 polymorphic TRAP bands would be necessary to measure GS among the nine genotypes with 10 % precision.

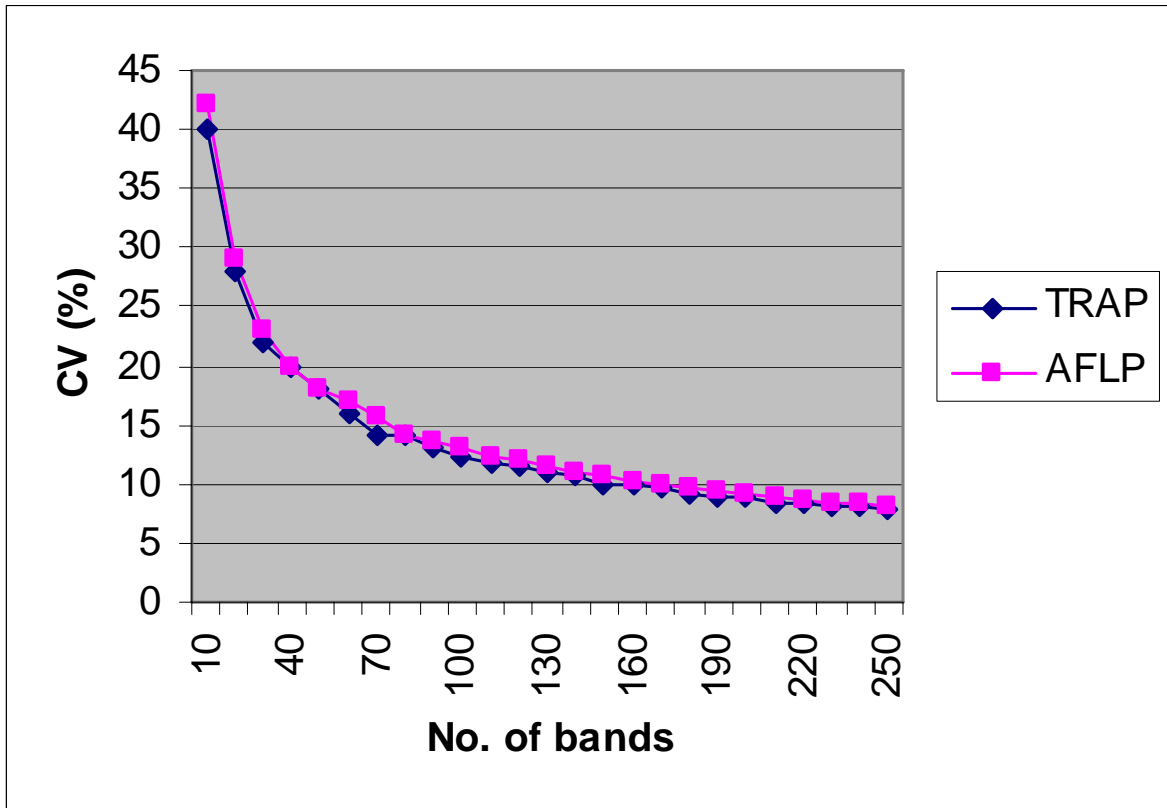


Figure 4. Number of polymorphic TRAP and AFLP bands necessary to estimate genetic similarity among nine sugarcane genotypes with a given level of precision (CV %) as estimated using bootstrap analysis.

AFLP Markers

Percent polymorphism and PIC Values

A total of 40 AFLP primer combinations were tested of which 28 primer combinations were adequate to study genetic relationships within this set of sugarcane genotypes. The 28 primer combinations produced a total of 1325 bands of which 686 (53%) were polymorphic (Table 5). The unambiguous bands ranged in size from 250 to 600bp. The total number of bands per primer combination ranged from 23 (E-AAC+M-CTC) to 81 (E-ACA+M-CTC) with an average of 47 bands per primer combination. These two primer combinations were also responsible for the least (8; E-AAC+M-CTC) and the most (44; E-ACA+M-CTC) number of polymorphic bands produced, with an average of 24 polymorphic bands per primer combination. The PIC value, averaged over all polymorphic loci for individual primer combinations, varied from 0.27 (E-ACA+M-CAC) to 0.45 (E-ACA+M-CAG) with an overall mean 0.35 ± 0.12 . As with TRAP markers, it was possible to distinguish each one of the nine genotypes. Sometimes just one AFLP primer combination was sufficient to distinguish all the genotypes. Also, bands were found to be uniquely present or absent in some genotypes.

Table 5. Percent polymorphism and PIC values of AFLP primer combinations used in genotyping nine sugarcane parents from the Louisiana breeding program.

	Primer combination	Total bands observed	Polymorphic bands observed	Percent polymorphism	PIC [†]
1	E-ACT+M-CAT	64	28	43.75	0.33
2	E-ACT+M-CAA	37	15	40.54	0.33
3	E-ACT+M-CTC	36	35	97.22	0.38
4	E-ACT+M-CTG	53	29	54.71	0.38
5	E-AAC+M-CAA	64	21	32.81	0.35
6	E-AAC+M-CTA	44	26	59.09	0.37
7	E-AAC+M-CTC	23	8	34.78	0.36
8	E-AAC+M-CTG	66	30	45.45	0.39
9	E-ACC+M-CAA	53	17	32.07	0.37
10	E-ACC+M-CTA	55	23	41.81	0.32
11	E-ACC+M-CTC	50	19	38.00	0.33
12	E-ACC+M-CTG	53	22	41.51	0.33
13	E-ACA+M-CAA	39	22	56.41	0.37
14	E-ACA+M-CTA	40	31	77.50	0.29
15	E-ACA+M-CTC	81	44	54.32	0.35
16	E-ACA+M-CTG	68	23	33.82	0.35
17	E-AGC+M-CAT	25	20	80.00	0.35
18	E-AGC+M-CAA	53	18	33.96	0.35
19	E-AGC+M-CTG	46	22	47.82	0.32
20	E-ACG+M-CAT	40	24	60.00	0.39
21	E-ACG+M-CAA	44	27	61.36	0.35
22	E-AAC+M-CAC	45	25	55.55	0.35
23	E-ACC+M-CAC	49	26	53.06	0.39
24	E-ACA+M-CAC	37	26	70.27	0.27
25	E-AGC+M-CAC	40	23	57.50	0.37
26	E-ACC+M-CAG	44	29	65.90	0.36
27	E-ACA+M-CAG	35	21	60.00	0.45
28	E-AGC+M-CAG	41	32	78.04	0.37
Total		1325	686		
Average		47	24	53	0.35

† Polymorphism information content

Genetic diversity and relationship among genotypes

The AFLP-GS estimates between pairs of genotypes ranged from 0.72 (between HoCP92-624 and Ho95-988) to 0.84 (between HoCP92-624 and LCP85-384) with a mean value of 0.76 ± 0.03 (Table 4, Fig. 2). Cluster analysis produced a dendrogram with a cophenetic coefficient of correlation value of 0.75 (Fig. 5A). The dendrogram had two distinct clusters although the bifurcation had only marginal (45 %) bootstrap support.

LCP85-384 was placed in a cluster with both of its progenies, L99-238 and HoCP96-540, but not with its half sibling LCP86-454 (Fig. 5A; Table 1). Surprisingly, the closest and most robust (100 % bootstrap support) relationship was found between LCP85-384 and HoCP92-624, a genotype with which it seemingly does not share a recent lineage (Table 1). Similar results were depicted by the PCoA with the same two groups of genotypes apparent in the first principal coordinate (Fig. 5B).

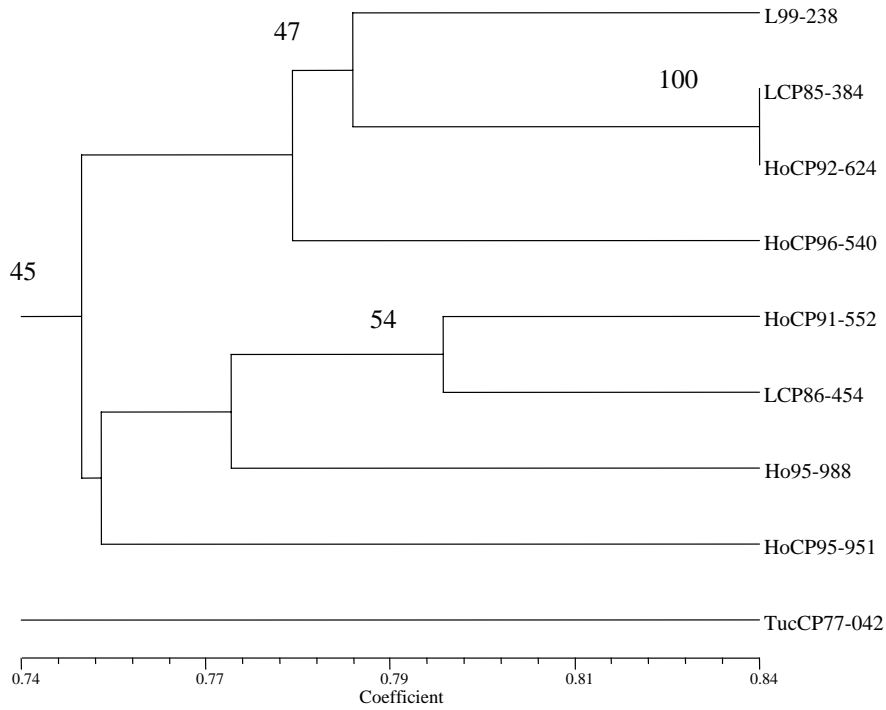
Bootstrap analysis

A total of 1325 polymorphic bands were revealed by AFLP markers. However, for comparison with the 242 polymorphic bands revealed by TRAP markers, each of 10 subsamples (with replacement) of 250 polymorphic AFLP bands was subjected to bootstrap analysis. Based on the 250 polymorphic AFLP bands, GS was measured among the nine genotypes with 8.3 % precision (Fig. 4). About 160 polymorphic AFLP bands would be necessary to measure GS among the nine genotypes with 10 % precision.

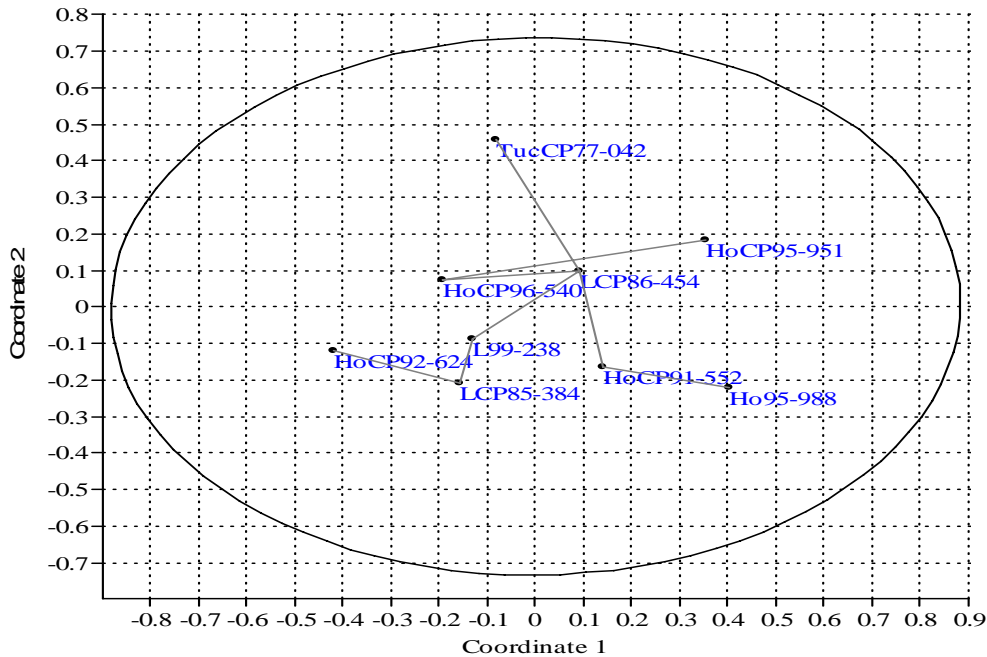
COP

Genetic diversity and relationship among genotypes

Since sugarcane is highly heterozygous, the COP-GS estimate of a genotype with itself was assumed to be 0.5 and 0 between remotely related parents. Therefore, unlike in rice for example, the COP estimates in sugarcane range from 0 to 0.5 rather than from 0 to 1. The COP-GS estimates among the nine genotypes varied from 0.03 (between TucCP77-042 and Ho95-988 and Ho95-988 and LCP86-454) to 0.36 (between HoCP96-540 and LCP85-384) with a mean of 0.12 ± 0.09 (Table 4; Fig. 2). Cluster analysis of the pair-wise COP matrix resulted in a dendrogram with a cophenetic coefficient of correlation value of 0.92 (Fig. 6A). The COP-derived dendrogram revealed no distinct pattern of diversity. For example, although one could still trace the relationship among genotypes, such as LCP85-384, HoCP96-540, L99-238 and LCP86-454, which are known to share a common lineage (Table 1), CA displayed no dichotomy between this group (related) and the non-related genotypes in the study. This dichotomy was clearly revealed by the PCoA-derived bi-plot (Fig. 6B)

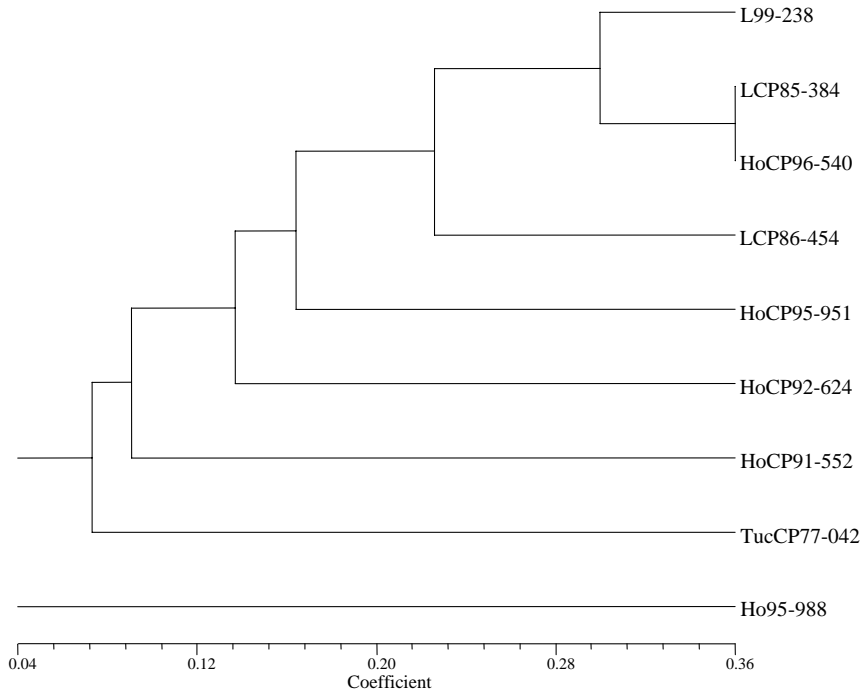


A

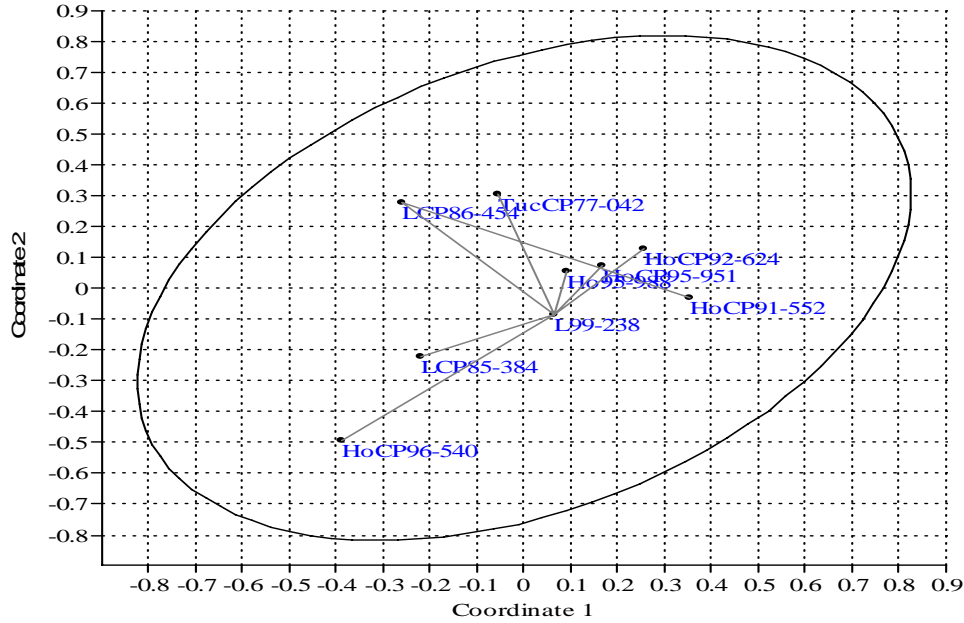


B

Figure 5. Genetic diversity pattern among nine sugarcane genotypes based on AFLP polymorphism and depicted by cluster analysis (A) and principal coordinate analysis (B). The numbers on the dendrogram (A) represent 50 % majority rule bootstrapped values.



A



B

Figure 6. Genetic diversity pattern among nine sugarcane genotypes based on the coefficient of parentage and depicted by cluster analysis (A) and principal coordinate analysis (B).

Associations between Pedigree, TRAP, and AFLP-derived GS estimates

Similar levels of association, as estimated with both the normalized Mantel Z-statistic and Pearson correlation coefficient (Table 6), were found between COP-GS with TRAP-GS and between COP-GS with AFLP-GS. Although the correlation values were significant ($P < 0.05$; $N = 36$) they were moderate (0.40). Both methods (Mantel and Pearson) also calculated a similar level of correlation between the two (TRAP and AFLP) molecular marker-derived GS estimates. The correlation between TRAP-GS and AFLP-GS estimates was much lower (0.14) compared with their respective correlations with the COP-GS (0.40).

Table 6. The Mantel's Z-statistic and Pearson correlation coefficient for pair-wise comparisons among the COP-, TRAP- and AFLP-GS estimates.

	Normalized Mantel Z statistic (R)	Pearson correlation coefficient (r)
AFLP vs COP	0.41	0.42
TRAP vs COP	0.40	0.41
AFLP vs TRAP	0.14	0.14

DISCUSSION

Comparing characteristics of TRAP and AFLP markers

The values of percent polymorphism and PIC reported in this study are typical for sugarcane. Using 21 AFLP primer combinations, Lima et al. (2002) detected an average of 50 % polymorphism among 79 Brazilian sugarcane cultivars, whereas Selvi et al. (2005) reported an average of 52 % among 28 cultivars from India using 12 primer combinations. Selvi et al. (2005) reported PIC values for AFLP that ranged from 0.31 to 0.41. TRAP analysis based upon 24 primer combinations among 61 sugarcane genotypes from Canal Point, Florida detected 58 % polymorphism and a PIC value of 0.32 (Edme, personal communication). The complex polymorphism profile displayed in sugarcane for AFLP (Besse et al., 1998; Lima et al., 2002; Selvi et al., 2005; this study) and TRAP (Arro, 2004; Edme, personal communication; this study) markers can be attributed to its large genome size, high levels of heterozygosity and aneuploidy, which is perpetuated via vegetative propagation. No two of the nine genotypes presented an identical profile, indicating that TRAP markers, besides AFLP, can be useful for sugarcane fingerprinting.

The utility of a DNA marker technique can be defined by its multiplex ratio (number of markers that can be generated in one single reaction) and the PIC (effective number of alleles that can be detected per marker in a set of individuals, i.e., the discriminatory power of the marker) (Powell et al., 1996; Vuylsteke et al., 2000). The ability of the AFLP technique to simultaneously amplify a large number of marker loci throughout the genome has been cited as a major advantage of AFLPs over other marker systems (Vuylsteke et al., 2000). In this study, AFLP was only marginally superior to

TRAP with regards to the total number of bands amplified per primer combination. However, similar PIC values were found between the two marker systems. In addition, similar numbers of polymorphic bands were necessary to distinguish among the nine genotypes with 10 % precision. The overall percent polymorphism was somewhat higher for TRAP (55 %) than for AFLP (53 %). Thus, on the basis of these data, a similar level of polymorphism detection efficiency is to be expected from these two dominant markers.

Experience in our lab has shown, however, that the relative polymorphism detection efficiency between AFLP and TRAP may be dependent upon the genetic structure of the population under study. For example, different results were obtained when the same set of TRAP and AFLP markers were used to genotype 100 individuals from an interspecific (*Saccharum officinarum* 'La Stripe' x *S. spontaneum* 'SES 147B') mapping population. The total number of bands amplified and percent polymorphism revealed by AFLP surpassed that of TRAP by about three to four fold (unpublished data) in the La Stripe x SES 147B population. Because TRAP primers are designed to target only a small and specified portion of the genome (Hu and Vick, 2003; Alwala et al., 2006), AFLP markers may be more robust for detecting polymorphism among closely related genotypes, as they are more likely to sample different segments throughout the genome. In soybean, Powell et al. (1996) found good correlations between AFLP and other markers (RFLP, RAPD and SSR) at the interspecies level, which disappeared at the intraspecies level, with AFLP giving the best resolution among genotypes. It is best to allow research objectives to guide the decision of choosing the appropriate DNA marker technique(s).

Comparing GS estimates

The mean, range, and distribution values for TRAP-GS and AFLP-GS were similar, but both were distinct from COP-GS (Table 4; Fig. 2). The mean values from TRAP-GS and AFLP-GS highlight the narrow genetic base reported for cultivated sugarcane (Mangelsdorf, 1983; Deren, 1995; Lima et al., 2002; Arro, 2004). However, judging from the COP-GS, it would appear that substantial amounts of genetic diversity exist in sugarcane. Up to 55 % (20/36) of the COP-GS were below 0.1, suggesting that only about 45 % of the genetic material segregating in the ancestral population was identical by descent between any two genotypes in this study (Table 4). In calculating TRAP-GS and AFLP-GS, only polymorphic bands were taken into consideration, yet lower levels of genetic diversity (high GS) were detected by these methods compared with the COP method. Moreover, of the total bands amplified by TRAP and AFLP markers, 45 % and 47 %, respectively, were monomorphic and therefore identical in state. This tendency of the COP method to overestimate genetic diversity compared to DNA-based methods has been reported by other authors (Cox et al., 1985; Barbosa-Neto et al., 1996; Kim and Ward, 1997).

As with several previous studies (Cox et al., 1985; Graner, 1994; Barbosa-Neto et al., 1996; Kim and Ward, 1997; Sun et al., 2003) this study found moderate levels of association between the DNA- and COP-based estimates of GS. In wheat, a low r value

of 0.27 was observed by Cox et al. (1985) between isozyme-based GS and COP. RFLP-based GS with COP in barley generated a low correlation value of 0.27 for winter type and a moderate value of 0.42 for spring type (Graner et al., 1994). Evaluating the correlation between RAPD-based GS with COP resulted in a low r value of 0.10 in potatoes (Sun et al., 2003). This disparity stems from the fact that the assumptions inherent in calculating COP are unrealistic for most cultivated species, and sugarcane is no exception (Deren, 1995). For example, the COP method assumes that both parents contribute equally (half of their alleles) to the offspring, essentially ignoring the effect of selection and genetic drift during cultivar development. As evident from Table 4, the relationship between LCP85-384 and its two progenies HoCP96540 and L99238 was not equal for TRAP-GS and AFLP-GS. Furthermore, it is well known that chromosome numbers within cultivated sugarcane can vary (generally from about 100-130) even among fullsib progenies. This can substantially affect DNA-based measurements of GS, but is yet unaccounted for by currently available models for estimating COP.

Considering that only a handful of clones were used in the original nobilization event to derive modern sugarcane, the assumption that two clones in this study are unrelated ($COP = 0$) relative to the original ancestors would be unrealistic. Thus, as opposed to the DNA-based methods, the COP method cannot account for alleles that are alike in state but not identical by descent resulting in a disproportionate downward bias of GS estimates. Incomplete pedigree records or errors in annotating parents would help to over emphasize the downward bias of COP estimates. Moreover, selfing could distort pedigree records and crossing with stray pollen could bias COP. To minimize this bias, we recalculated the correlation coefficients after eliminating COP values < 0.1 . The correlation between COP-GS and TRAP-GS increased ($r = 0.69$; $N = 16$), that for COP-GS with AFLP-GS decreased ($r = 0.31$; $N = 16$), while that between TRAP-GS and AFLP-GS remained unchanged ($r = 0.16$; $N = 16$). However, when values for the three closest known relatives were removed (i.e. $COP = 0.35$) the correlations decreased to 0.06 (COP-TRAP), 0.22 (COP-AFLP), and 0.11 (TRAP-AFLP). The lack of congruence and consistency among TRAP-GS, AFLP-GS, and COP-GS, throughout the range of diversity detected among the genotypes in this study, suggests that the three measures detect different aspects of relatedness.

Several authors (Graner, 1994; Barbosa-Neto et al., 1996; Kim and Ward, 1997; Sun et al., 2003) have recommended molecular marker-based estimates of GS to be more reliable than COP. This is largely because molecular markers such as TRAP and AFLP directly measure DNA sequence variations. However, a drawback of markers, such as TRAP and AFLP, is that the utility of bands produced by these markers can be confounded by lack of locus specificity. Without sequencing, it would be difficult to state unequivocally that bands or alleles that are identical in state (i.e. migrating to the same position on a gel) are not co-migrating non-homologous bands. Lack of adequate genome coverage is another factor that can limit the utility of DNA-based estimates of GS. This can be resolved by using markers for which the genome location is known such that markers that span the entire genome are chosen.

Comparing genetic diversity patterns among genotypes

Following CA, the least distinct pattern was obtained from the COP dendrogram while AFLP gave the most distinct pattern. However, it was easier to explain the TRAP dendrogram based on pedigree records. The genetic resolution and interpretation of the data was enhanced by including the PCoA bi-plots. In general, when the dendrogram and bi-plot were considered together, the three measures seemed to depict a somewhat similar pattern of relationship among the genotypes, the major exception being the tight relationship (100 % bootstrap support) between LCP85-384 and HoCP92-624. A closer examination of the pedigree tree revealed that the maternal grandparents of LCP85-384 (CP52-068 x L65-69) were indeed the great grandparents of HoCP92-624 (CP52-068 x CP62-258) x (CP65-357 x L65-69). One could speculate that AFLP markers may be detecting favorable alleles or blocks of genes from these ancestral parents that were preserved through independent selection for the same trait(s) in the two cultivars. As for the TRAP markers, only sucrose related primers were exemplified in this study which may not be identical to the ones detected here by AFLP markers.

CONCLUSIONS

The results showed that TRAP markers have utility for sugarcane genetic diversity studies. TRAP markers produced percent polymorphism and PIC values similar to that of AFLP markers and measured GS with the same level of precision as AFLP markers. A similarly moderate level of association was found between TRAP-GS and COP-GS estimates and between AFLP-GS and COP-GS estimates. The association between TRAP-GS and AFLP-GS was much lower. Violations of the assumptions used in calculating COP was partly responsible for the moderate level of association between COP and the two DNA-based estimates, as the COP method tends to underestimate GS. However, exclusion of subsets of data along the range of COP-GS estimates led to different levels of association between COP and TRAP, COP and AFLP and TRAP and AFLP suggesting that the three measures could be detecting different aspects of GS. Notwithstanding, with few exceptions, the dendrograms and bi-plots produced using the three measures depicted a somewhat similar pattern of diversity among the genotypes. Therefore, some combination of TRAP, AFLP and COP would likely be more useful in estimating GS, as this would compensate for the inaccuracies inherent within each of the methods.

Estimates of GS could be incorporated as a tool to assist sugarcane breeders with selecting the most divergent parents to maximize heterosis and transgressive segregation in the progeny population. The inexpensive COP could be used as a first step to assemble a large diverse group of potential parents. Molecular markers, such as TRAP and AFLP, could then be used to confirm the pedigrees. Moreover, molecular markers provide a more direct and precise estimate of allele frequency differences among the parents. Decisions could be made to decide the best crosses based on the GS values among the parents, thus allowing the breeder to focus attention and resources on the most promising crosses. Only loci for which the parents carry different alleles are expected to

contribute to genetic variance in the progeny population. If such loci co-locate with genes governing the traits being measured, then it may be possible to predict hybrid performance based on GS among parents.

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