COMPARATIVE APPLICATION OF RFLP AND AFLP TO MONITORING GENETIC DIVERSITY AMONG A GROUP OF SUGARCANE VARIETIES AND THEIR RELATIONSHIP TO FAMILY PERFORMANCE

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ABSTRACT. Five sugarcane varieties and their hybrids were evaluated for brix, plant height and stalk weight components on seedling and first clonal stages. Correlations among RFLP (DR), AFLP (DA) and genealogical (DG) parent distances, the observed family means and five predictors of family performance were calculated. High and statistically significant correlations (r=0.88-0.99*) between observed family means and midparent and high-parent heterosis were obtained for all characters and plant stages. Cluster analysis groupings on parent distances differed. The cophenetic value of UPGMA cluster analysis increased when employing molecular distance estimates calculated, considering one of each pseudoallele variant with both types of markers (RFLP, AFLP). The presence of pseudoallelism did not influence the predictive value of parental distances for family means or heterosis. These results confirmed previous reports in different crops on the limited predictive value of molecular-based parent distance estimates when markers employed are not selected for their genetic linkage to loci controlling traits studied. Present results evidenced the practical value of BLUP method to estimate brix and stalk diameter family means, considering as effects the seedling stages and the parental pairwise genetic distances. These estimates attained high correlations values (r=0.81- 0.97*) with observed family means, although slightly lower than those obtained by LS estimates and the traditional family predictors based on progeny evaluation, such as the general combining ability and midparental heterosis. Better results could be expected increasing the number of markers and selecting them for their linkage relations and magnitude of their contribution to phenotype of target characters.

Key words: RFLP, genetic markers, genetic distance, taxa, sugarcane

RESUMEN. Se evaluaron cinco variedades de caña de azúcar y sus híbridos para el brix, la altura de la planta y los componentes del peso del tallo en las etapas de postura y primer estadio clonal. Se calcularon las correlaciones de los estimados de distancia entre los padres por RFLP (DR), AFLP (DA) y las distancias genealógicas (DG), así como las medias familiares observadas y los valores de cinco predictores del comportamiento familiar. Se obtuvieron para todos los caracteres y cepas evaluadas estimados de correlación altos y estadísticamente significativos (r=0.88-0.99*) entre las medias familiares observadas y los estimados de heterosis en relación con el valor medio entre los padres y el del padre mayor. Los agrupamientos del análisis de conglomerados basados en las distancias parentales difirieron entre si. El valor cofenético del análisis de conglomerado UPGMA aumentó cuando se emplearon los estimados de distancia molecular, considerando uno de cada variante pseudoalélica presente en ambos tipos de marcadores (RFLP, AFLP). La presencia de pseudoalelismo no influyó en el valor predictivo de las distancias parentales para las medias familiares o la heterosis. Estos resultados confirman informes previos en diferentes cultivos sobre el valor predictivo limitado de los estimados de distancia parental basados en información molecular, cuando los marcadores empleados no han sido seleccionados por su ligamiento genético con los loci que controlan los caracteres estudiados. Estos resultados evidenciaron el valor práctico del método BLUP en la estimación de las medias familiares para el brix y el diámetro del tallo, cuando se consideran como efectos las cepas de postura y las distancias genéticas parentales. Estos estimados alcanzaron correlaciones altas (r=0.81- 0.97*) con las medias familiares observadas, aunque ligeramente menores que las obtenidas por los estimados LS y los predictores familiares tradicionales basados en la evaluación de las progenies, tales como la habilidad combinatoria general y la heterosis del valor medio entre los padres. Se esperan mejores resultados aumentando el número de marcadores y seleccionándolos por sus relaciones de ligamiento, así como por la magnitud de su contribución al fenotipo para los caracteres de interés.

Palabras clave: RFLP, marcadores genéticos, distancia genética, taxa, caña de azúcar

INTRODUCTION

Selection for economically important traits in animals and plants has been traditionally based on phenotypic records of progeny individuals and their relatives, preferentially, their parents.

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Breeders need accurate methods to obtain progenies with high genetic variance and mean performance. The former is partially dependent on the genetic diversity between parents, that is commonly estimated by phenotypic, genealogical or molecular marker data. Molecular markers have been successfully used in genetic and mapping studies and they are being introduced to assist phenotypic selection in introgression and other breeding programs. By employing DNA polymorphisms, it is expected to obtain a higher and faster genetic gain in relation to traditional estimation based on phenotypic data and progeny analysis.

Cultivar groupings have been determined based on molecular pairwise distance estimates in sugarcane (1-4).

Empirical and theoretical results supported the efficiency of family selection at early stage of the variety program. Some authors (5), demonstrated that the potential of a cross to produce elite progeny for a trait could be accurately predicted by its family mean at seedling stages. Thus, methods for predicting family means for brix and stalk weight components based on parent evaluation would facilitate the implementation of family selection.

RFLP markers are generally employed in mapping studies due to their reliability, allowing the integration of different maps and the application of the comparative genomic strategy between distant related species (6, 7).

More precise estimates of the genetic diversity between cultivars could be expected to be obtained by employing highly polymorphic markers such as AFLP technology (8, 9). This is a multi-locus detection system, although multiple allelles of a locus are not necessarily amplified by a single primer combination. AFLPs are generally found in genomic regions covered by RFLPs.

On the other hand, quantitative trait means are very variable across experiments limiting the efficiency of regression techniques. For this reason, the estimation of breeding values based on phenotypic data are commonly calculated by the best linear unbiased prediction (BLUP) method (3, 10, 11).

A combined approach using BLUP prediction based on trait and marker data (TM-BLUP) to avoid prior information on the mean effects associated with specific marker genotypes is being employed in animals and plants (3, 12, 16).

This study was aimed at: (1) comparing genealogical and molecular-based genetic distances (RFLP, AFLP) as genetic diversity estimates among five sugarcane cultivars commonly used as parents; (2) determining the influence of molecular pseudoallelism in the magnitude and association of these estimates; and, (3) determining the association among parent genetic distances, observed family means and traditional family performance predictors based on progeny tests.

MATERIALS AND METHODS

Plant materials. Five sugarcane cultivars, regularly employed as parents in the breeding program, were crossed in a factorial cross design (3X2) (Table I) and planted with their progenies during the spring season in a completely randomized design with two replications and 48 individuals per combination. Brix (grade), plant height (cm.), stalk diameter (mm) and the number of stalks per stool (three stalks per individual) were assessed on plant cane (PP) and first ratoon (PR) single stool seedling and clonal plant cane (CP), first ratoon (C1R) and second ratoon (C2R) stages. All plant materials were obtained from the germplasm bank of the National Institute for Sugarcane Research (INICA).

Table I. List of sugarcane parental clones and progenies studied

Female parents	Male parents	
	C 323-68 (P1)	Ja 60-5 (P2)
My 54129 (M1)	M1P1 (1)	M1P2 (2)
CP 5243 (M2)	M2P1 (3)	M2P2 (4)
Ja 64-19 (M3)	M3P1 (5)	M3P3 (6)

(-): Parents and progeny codes

RFLP analysis. Total genomic DNA from each parent was extracted from young leaves (17). DNA was digested by enzymes BamHI, EcoRI, HindIII and XhoI following supplier's recommendations and transferred to a nylon membrane (Hybond N+ Amersham). Prehybridization (5hr.) and hybridizations (overnight) were performed at 65^{0}C. Autoradiograms were then exposed to P32 (7-10 days).

Ten low copy number genomic probes of maize (BNL, UMC) and *S. spontaneum* (SSCIR) from different linkage groups were employed (18). Thirty-one probe/enzyme combinations were selected for their polymorphism and autoradiographic quality (Table II).

AFLP analysis. AFLP method was performed by using the AFLP Analysis System I Kit (Instruction manual; GIBCO BRL, Life Technologies). Genomic DNA was digested with an *Eco*R/*Mse*I enzyme combination. The preamplification step was carried out with AFLP primers having one selective nucleotide (*Eco*RI+A, *Mse*I+C). Selective amplification was performed with three selective nucleotide (*Eco*RI+ANN, *Mse*I+CNN). Thirteen primer combinations were employed (Table III). PCR samples were denaturated by adding an equal volume of formamide buffer (98% formamide, 10 mM EDTA, pH 8.0, 0.05 % bromo-phenol blue, and 0.05 % xylene cyanol), heating for 5 min at 93 ^{0}C and chilled on ice. Samples were loaded on 6.5 % polyacrilamide gel under standard sequencing conditions. AFLP fingerprints were visualized using silver nitrate staining method according to the manufacturer's instruction (Promega Cat. # TMD005). The number of polymorphic fragments with good visual resolution and intensity were scored for each primer combination. The scored fragments ranged from 200 to 700 bp size. The

size of fragments was determined by comparing sequencing ladders of control template DNA to AFLP patterns.

Table II. Parental genetic diversity revealed by RFLP

Probe-enzyme combinations (PEC)		Total number of bands	Polymorphic bands	Number of different RFLP patterns/PEC
SSCIR107	Hind III	3	2	2
(VIII)	EcoR I	5	5	4
	Xho I	4	4	4
SSCIR73	Hind III	10	7	4
(I)	BamH I	6	3	3
	EcoR I	6	4	3
	Xho I	7	3	3
BNL8.09	BamH I	6	2	1
(I, X)				
SSCIR230	Hind III	6	4	4
(VI, X)	BamH I	6	4	4
SSCIR92	BamH I	2	1	1
(VIII)	EcoR I	3	1	1
	Xho I	4	4	3
SSCIR60	Xho I	4	1	1
(III)	BamH I	5	2	2
	EcoR I	7	4	4
	Hind III	7	4	4
SSCIR76	Hind III	7	6	5
(IX)	BamH I	3	3	3
	EcoR I	5	4	4
	Xho I	4	2	2
SSCIR119	Hind III	7	5	5
(III)	BamH I	11	10	9
	EcoR I	5	1	1
	Xho I	3	2	2
SSCIR194	Hind III	3	1	1
(VII)	BamH I	11	8	7
	EcoR I	6	5	5
UMC114	Hind III	3	1	1
(IX)	EcoR I	9	7	6
	Xho I	8	6	4
		166	116 (69.8%)	103

I-X: sugarcane linkage groups (18)

Table III. Parental genetic diversity revealed by AFLP

Primer combinations	Total numbers of bands scored	Polymorphic bands	Total numbers of patterns	Non-complementary patterns
E1/M1	7	5 (71)	5	3 (60)
E2/M2	7	4 (57)	3	3 (100)
E2/M1	21	15 (71)	9	8 (88.9)
E1/M2	18	16 (88.9)	13	11 (84.6)
E3/M2	15	14 (93)	10	8 (80)
E6/M4	20	17 (85)	7	5 (71)
E4/M7	14	13 (92.9)	11	11 (100)
E5/M1	19	18 (94.7)	9	9 (100)
E7/M3	45	39 (86.7)	15	11 (73)
E6/M6	27	25 (92.6)	18	13 (72)
E7/M8	20	18 (90)	11	8 (72.5)
E8/M6	13	13 (100)	9	9 (100)
E5/M7	27	27 (100)	18	12 (66.7)
	253	219 (86.6)	138	111 (80.4)

Parental distance estimation. Genealogical distances (DG) were obtained as the complement of the coefficient of coancestry estimates, previously calculated by pedigree records (19, 20).

To estimate molecular-based distances between parents, each polymorphic RFLP and AFLP fragment was scored 1 for presence and 0 for absence. Due to the polyploid complex origin of modern sugarcane varieties as well as the presence of redundant DNA, fragments were considered as molecular phenotypic variants. The genetic similarity (S_{ij}) between each pair of genotypes was estimated by the formula proposed by Dice (21). The corresponding pairwise genetic distances were calculated as their complements ($1 - S_{ij}$).

Totally polymorphic units were considered to calculate RFLP (DR_1) and AFLP (DA_1) distance estimates, respectively. For comparative purposes to determine the influence of molecular pseudoallelism in the magnitude and association of these estimates, totally correlated variants (r=1 and r=-1) were considered once to calculate the corresponding DR_2 and DA_2 pairwise distances.

To describe AFLP polymorphism patterns of AFLP bands per primer combination were also considered.

To determine clonal diversity groups, a cluster analysis was performed on individual distance matrix using the unweigthed pair-group method with arithmetic mean (UPGMA) linkage algorithm. To determine the precision of dendrograms, individual cophenetic values (R_{co}) were calculated.

All possible pairs of the five distance matrixes were compared by Mantel test of correspondence using as criterion the normalized Mantel statistic Z and employing the matrix comparison (MXCOMP) program. For these data analysis, the package NTSYSpc, version 2.10p. (Applied Biostatistics Inc. © 2000-2001) was employed.
Family agronomic performance estimation. For each trait and plant stage, an analysis of variance was performed on individual observations according to a linear model considering as sources of variation: male and female parents, replication and their interactions. General (GCH_O) and specific (SCH_O) combining abilities, and 95 confidence intervals were calculated (22, 23), midparent (MPH_O) and highparent (HPH_O) heterosis were estimated for each trait and expressed in percentage (24). Observed family means (Y_O), the average of parent GCH_O (GCH_O) and other estimates were employed to characterize the families.

A multiple regression model considering the same factors was employed for the family mean estimation by the least-square method (LSM). Family performance indicators were computed as already described and identified by the same abbreviations with ($_E$).

Family means were also estimated by the best linear unbiased estimation method (BLUP) (10, 25), using the following models:

1) For each plant stage and trait, BLUP 1-5 estimates were calculated:

$$Y = XB + Z_1a_x + Z_2a_y + e$$

where:
y = p x 1 vector of trait observations
B = t x 1 vector of fixed effects (replication, mean)
$a_x = n_x$ x 1 vector of genetic distance effects of female parents (DG, DR_1, DR_2, DA_1 and DA_2, for BLUP 1, 2, 3, 4 and 5, respectively)
$a_y = n_y$ x 1 vector of genetic distance effects of male parents
e = vector of errors
X, Z_1, Z_2: incidence matrices of 1s and 0s relating Y to B, a_x and a_y, respectively.

2) For each trait and considering PP and PR plant stages, BLUP 6-10 estimates were calculated:

$$Y = XB + Z_1a_x + Z_2a_y + Zd + e$$

where:
y = p x 1 vector of trait observations
B = t x 1 vector of fixed effects (plant stage, mean)
$a_x = n_x$ x 1 vector of genetic distance effects of female parents (DG, DR_1, DR_2, DA_1 and DA_2, for BLUP 6,7,8,9 and 10, respectively)
$a_y = n_y$ x 1 vector of genetic distance effects of male parents
e = vector of errors
X, Z_1, Z_2: incidence matrices of 1s and 0s relating Y to B, a_x and a_y, respectively.

Association between parent and progeny estimates. Correlation analyses were performed between all parent and progeny estimates to determine their association employing Pearson' correlation coefficient (P<0.05).

RESULTS AND DISCUSSION

Association among distance estimates. Polymorphism revealed by RFLP probe-enzyme combinations is described in Table II. Fifteen polymorphic bands (12.9 %) were employed to represent the variants totally associated with them (DR_2). Similarly, polymorphism amplified by AFLP is presented in Table III. Nineteen polymorphic bands (8.7%) were employed for calculating DA_2 estimates.

Parental distance estimates were not correlated with the exception of DR_1 and DA_2 (r= 0.88*). This result could be considered as an evidence that polymorphic AFLPs selected to represent the cosegregant variants, and RFLPs most contributing to the diversity among parents were located in the same regions. In spite of this association, matrix comparisons by Z Mantel statistics showed the absence of statistically significant correlation (r= 0.80 NS) in all cases. Complete co-segregation cannot be used to prove allelism in sugarcane, as there are other possible causes that may provoke the redundancy of DNA such as: its genome size, that is considered among the larger ones in the *Poaecea*, so that it could be expected that repetitive DNA has a major contribution (26); and the interspecific hybrid origin of modern cultivars.

Parent groupings are presented in Figure 1. It can be noted that the use of one variant as representative of each pseudoalellic group improved the cophenetic values of UGMA clusters for both types of molecular markers.

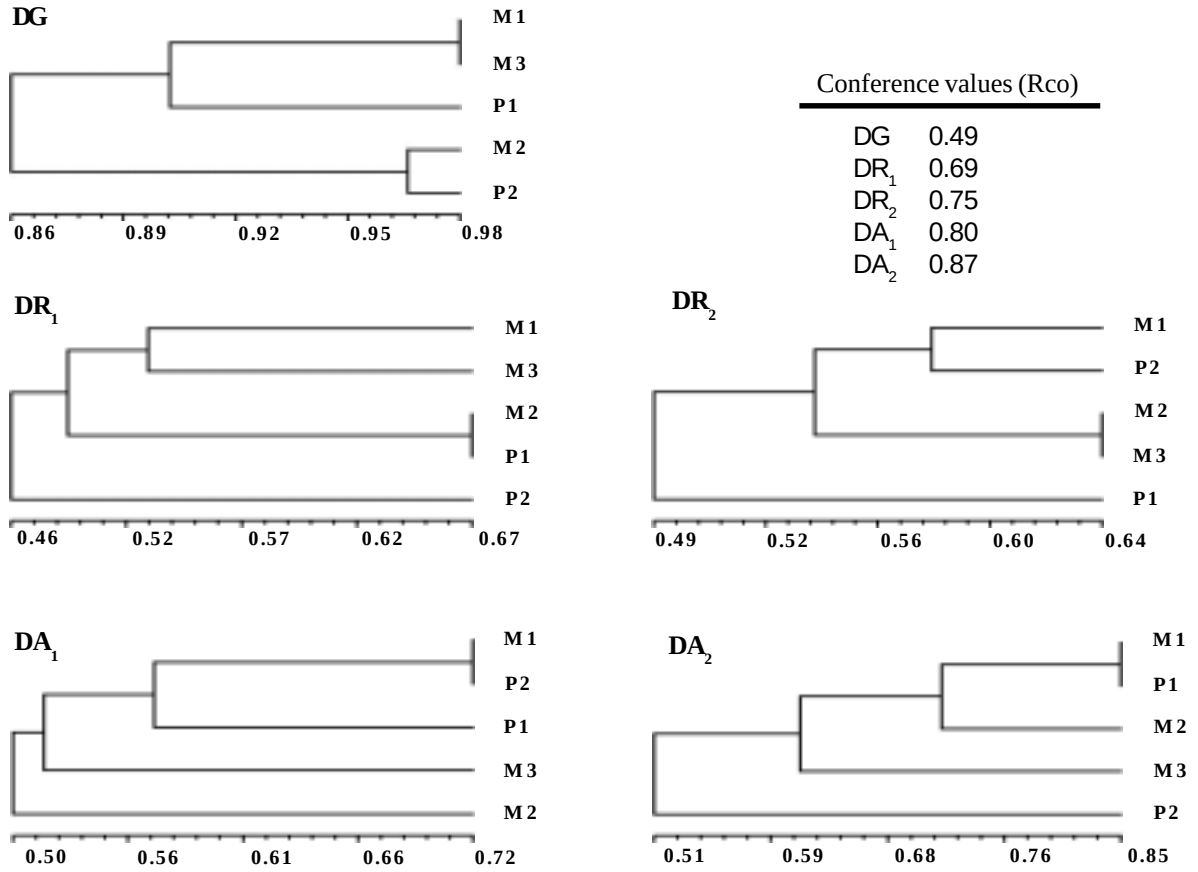

Figure 1. Parental groupings according to UPGMA cluster analysis on each matrix of pairwise distant estimates: genealogical distances (DG), RFLP-based distances with total (DR_1) and independent (DR_2) polymorphic bands; AFLP-based distances with total (DA_1) and independent (DA_2) polymorphic bands. Distances were calculated as the complements of the coefficient of coancestry and of the Dice genetic similarity estimates, respectively

Distance estimates based on RFLP data and genealogical information have been reported as highly associated and/or leading to equivalent genotype groupings (27-30). Other authors (4) reported a significant correlation (r= 0.42, P<0.001) between AFLP-based genetic similarities and the coefficient of parentage in a group of 70 cultivars regularly used as parents and *Saccharum* clones of four species. These authors concluded that AFLP based genetic distance estimates may help to determine more ccurately the degree of relationship among sugarcane cultivars.

On the other hand, the lack of association between these estimates in our study is in agreement with previous results for wheat (24), barley (31) and oat (32) as well as for a different set of sugarcane genotypes (33). Violation of genetic assumptions for the coefficient of coancestry estimation (34); distance underestimation due to the presence of common variants in non-related genotypes (35), the presence of different bands with identical electrophoretic mobility and sampling errors caused by reduced marker and genotype numbers are the most frequent sources of this bias.

Sharing highly conserved variants are probably not the cause of the lack of association between molecular and genealogical distance estimates, as it was previously demonstrated in sugarcane (33).

In sugarcane, other factors may contribute to these discrepancies, such as the major contribution of *S. officinarum* in some crosses, the somatic variation in chromosome number and the polysomic segregation (20). *Association between parent genetic distances and family performance*. In general, correlations between observed family means and MPH_O and HPH_O were highly and significant (P<0.05) (r= 0.88-0.99*) for all characters and plant stages (Table IV) . All family estimates, except SCH_O, were associated to brix in observed family means. These results are in close correspondence with the relatively high contribution of the additive genetic component to the phenotypic variance for brix in sugar cane (23).

Association among brix of observed family means in PP and the least square estimates were also high and significant with LSM, $\overline{GCH}_E$ and MPH_E (r= 0.98*), and HPH_E (r= 0.85*).

Parental distance estimates were not consistently associated to the observed family means (Table V). These results are in agreement with those reported for DG and DR_1 in another set of sugarcane cultivars (33).

Family means estimated by BLUP were highly correlated (r=0.81- 0.97*) to observed family means for brix in seedlings stages and for stalk number in PP (Table V). These results evidenced the contribution of replication and plant stages to improve the accuracy of BLUP models. These correlations were slightly lower than those obtained by LS estimates and by general combining ability and midparental heterosis, the traditional family predictors based on progeny evaluation.

Table IV. Correlations of observed family means with family performance estimates: average parental general (GCH_O) and specific (SCH_O) combining abilities and midparent (MPH_O) and highparent (HPH_O) heterosis estimates for the character studied

Trait		GCH_O	SCH_O	MPH_O	HPH_O
Brix	PP	0.98*	0.19NS	0.98*	0.88*
	PR	0.83*	0.56NS	0.95*	0.98*
	CP	0.81*	0.61NS	0.94*	0.97*
	C1R	0.82*	-0.26NS	0.80NS	0.80NS
	C2R	0.97*	0.26NS	0.97*	0.94*
Shoot diameter	PP	0.91*	0.42NS	0.95*	0.96*
	PR	0.54NS	0.84*	0.97*	0.95*
	CP	0.80NS	0.59NS	0.94*	0.94*
	C1R	0.78NS	0.62NS	0.94*	0.99*
	C2R	0.70NS	0.71NS	0.95*	0.98*
Plant height	PP	0.72NS	0.69NS	0.95*	0.91*
	PR	0.56NS	0.83NS	0.95*	0.94*
	CP	0.80NS	0.60NS	0.90*	0.89*
	C1R	0.69NS	0.72NS	0.95*	0.92*
	C2R	0.63NS	0.78NS	0.96*	0.98*
No. stalks per stool	PP	0.64NS	0.76NS	0.96*	0.98*
	PR	0.46NS	0.89*	0.98*	0.99*
	CP	0.36NS	0.93*	0.91*	0.97*
	C1R	0.55NS	0.84*	0.97*	0.99*
	C2R	0.44NS	0.90*	0.98*	0.99*

Table V. Significant correlations between observed family means and family means estimated by BLUP method

Estimates	Brix		Stalk diameter
	PP	PR	PP
BLUP1	0.81*	0.37NS	0.70NS
BLUP2	0.86*	0.29NS	0.58NS
BLUP3	0.81*	0.33NS	0.73NS
BLUP4	0.83*	0.42NS	0.55NS
BLUP5	0.86*	0.29NS	0.70NS
BLUP6	0.95*	0.82*	0.89*
BLUP7	0.97*	0.79NS	0.84*
BLUP8	0.96*	0.79NS	0.89*
BLUP9	0.94*	0.81*	0.88*
BLUP10	0.97*	0.81*	0.88*

(*), (NS): Pearson's coefficient of statistically significant (P<0.05) and non-significant correlations, respectively.
Parental distance were not associated to observed family means.
BLUP1-5: family means estimated by BLUP method considering replication and parental distance effects, DG, DR_1, DR_2, DA_1 and DA_2, respectively.
BLUP6-10: family means estimated by BLUP method considering seedling stages and parental distance effects, DG, DR_1, DR_2, DA_1 and DA_2, respectively

Although considerable research is currently conducted, correlations of molecular marker diversity with hybrid performance and heterosis have been too low (28, 36, 37, 38). In sugarcane, RFLP-based distance estimates have evidenced a limited practical value for predicting family means or heterosis among parents studied. The association between DR_1 estimates and midparental heterosis (r=059-0.63*) exhibited moderate values for brix in all stages and for other traits in clonal plant cane stage (r =0.45-0.46*), and attained also

moderate values (r= 0.40-0.52) for brix to high-parent heterosis. On the contrary, $BLUP_2$ means based on RFLP molecular distances correlated for brix of observed family means in all plant stages and for stalk weight components, in plant cane seedling stage (33).

These results demonstrated that the magnitude and significance of the association between the observed family indicators and the parental distance estimates, specially those based on molecular polymorphisms, as well as BLUP mean estimates weighed with them, depend on the number of markers, their genetic linkage and phenotypic contribution to loci controlling the characters studied, heritability of the character and the control of environmental effects and their interactions during early plant stages. However, it could be expected that information of gene mapping studies could be used to increase their predictive power.

The utility of molecular information based on genomic probes or even based on a low-dense marker map could be limited for the estimation of breeding values. It has been established that complex interactions among a high number of genes and their alleles determine useful effects and that overdominant epistatic loci are the major genetic component of inbreeding depression and heterosis in different plants (39-41). Thus, the molecular marker assisted approach is being employed to unravel the genetic basis of heterosis by identifying QTLs and genes associated with yield heterosis in plants.

On the other hand, reported results point to the need of a saturated genetic map composed by different types of markers such as RFLP, AFLP and SSR, specially for outcrossing species (42).

Results of the present work confirm previous reports on the use of molecular markers when the linkage phase between them and QTLs are not established for each family, in which they are expected to be used for parent and parent selection. If a dense marker map is available, some markers would be expected to be tightly linked to target QTLs with favorable allelles across all families and could be used without previous information of their linkage phase in each family. Selection conducted by the genetic values estimated by these markers could be substantially increased in animals and plants (43).

CONCLUSIONS

- Genealogical distance between parents were not correlated with molecular-based distance estimates, nor to the observed family mean and heterosis.
- Genotype groupings based on pairwise molecular distance estimates (RFLP, AFLP) calculated with total polymorphic bands and those calculated considering one of each pseudoallele variants differed. Latter distance estimates improved the precision of UPGMA cluster dendrograms, but do not increase their predictive value for family means or heterosis.
- Present results evidenced a limited practical value of parental estimates for predicting family means or heterosis among parents studied. Although BLUP estimated means weighed by replication and parental distance effects, improved their association to observed family means for brix in seedlings stages and for stalk number in plant cane seedling stage, these correlations were slightly lower than those obtained by the traditional family predictors based on progeny evaluation, such as the general combining ability and midparental heterosis.
- Better results can be expected to increase the number of markers and select them for their linkage relations to QTLs of interest.

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