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Selfing rate estimation in sugarcane under unfavorable natural conditions of crossing by using microsatellite markers

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ABSTRACT. The self-fertilization or selfing rate estimation using microsatellite markers and its impact on survival and selection rate were evaluated in families derived from polycrosses that involved parents that were widely used in sugarcane breeding in Brazil. These factors were evaluated under unfavorable natural conditions of flowering and crossing. After the germination test, the viable progeny were taken to the field for survival rate evaluation (4, 6, and 10 months) and phenotypic selection at plant cane. The selfing rate estimate based on microsatellite markers present in the progeny and absent in their female parent was 98.5 and 0% for the polycross families derived from IACSP95-5000 and SP89-1115, respectively. The survival and selection rates in the last 2 evaluations were higher for the SP89-1115 outcrossed family than the IACSP95-5000 selfed family. The IACSP95-5000 cultivar excelled either as pollen donor with fertilization capability or viable seed production even under unfavorable natural conditions of crossing. The

environment influence (temperature and humidity) had an important role during the polycross.

Key words: *Saccharum* spp; Flowering; Pollen viability; Molecular markers

INTRODUCTION

Sugarcane (*Saccharum* spp) breeding is initiated by hybridization that is usually conducted through biparental crosses, which involve only 2 parents. Besides this, polycrosses, which involve a group of parents, are also used in sugarcane (Tew and Pan, 2010). In polycrosses, many parents are arranged so that each parent has an equal probability of being pollinated by any other one (Olesen and Olesen, 1973; Bravo et al., 1981). In sugarcane, the polycross method, also known as melting pot crosses, was applied in Hawaii as a way to quickly evaluate a large number of parental combinations at a low cost.

Sugarcane is an allogamous plant with hermaphroditic inflorescence that allows outcrossing and self-fertilization or selfing. For each clone or variety involved in a cross, it is important to know its pollen fertility or viability to determine if the clone will perform as a male (high pollen fertility/viability) or as a female (low pollen fertility/viability) (Cox et al., 2000). According to Heslop-Harrison (1971), pollen that has the ability to deliver two male gametes to the embryo sac is assumed to be viable pollen. Traditionally, pollen-staining methods are adopted by sugarcane breeding programs to define pollen viability and, consequently, determine the sex of the sugarcane inflorescence, i.e., if the inflorescence will perform as a pollen donor (male) or pollen receptor (female). Because the number of crosses during the hybridization campaign is intense, the staining methods allow a rapid result. In sugarcane breeding programs, the most used pollen-staining method is the iodine-staining test that is based on staining the starch of the pollen grain (Machado Jr., 1987).

The main factors interfering with sugarcane pollen viability are the relative humidity, which should be above 85%, and the temperature range, which must not exceed 31°C or fall below 18°C (Moore, 1987; Berding and Moore, 2001). Moreover, pollen viability and the degree of sugarcane anther dehiscence can vary between different genotypes (McIntyre and Jackson, 2001).

Microsatellite markers have been used successfully for paternity testing in animals and plants (Riday and Krohn, 2010; Moroni et al., 2011; Sahli and Conner, 2011) and to estimate the outcrossing rate (Kittelson and Maron, 2000; Muluvi et al., 2004; Soengas et al., 2011). The outcrossing and/or selfing rate estimated by molecular markers can be used to verify the effective participation of all parents as pollen donors (male parents) in a polycross.

In sugarcane, there are few studies that use molecular markers to estimate the outcrossing and/or selfing rate in biparental- or polycross-derived families (McIntyre and Jackson, 2001; Pan et al., 2006; Tew and Pan, 2010). Random amplified polymorphic DNA markers were used to estimate the selfing rate of 8 families from an Australian sugarcane breeding program (McIntyre and Jackson, 2001). Tew and Pan (2010) applied microsatellite markers to identify the male parent of a family that was derived from a polycross, supplementing the pedigree information for future crosses. The knowledge of the selfing rate, whether from biparental or polycross families, is critical in breeding programs to correctly evaluate the fam-

ily's performance and thus determine the correct breeding value of the parents involved in the respective cross (McIntyre and Jackson, 2001). All of these studies were conducted with favorable environmental conditions of sugarcane crossing, i.e., high air humidity (above 85%) and temperature ranging from 18 to 31°C with an optimal temperature of 27°C (Melloni et al., 2013), which naturally occurs at the northeast region of Brazil, where the sugarcane breeding cross stations are located.

The objective of this study was to estimate the selfing rate of polycross families involving parents that are widely used in sugarcane breeding in Brazil under unfavorable natural conditions (inadequate temperature and humidity) of crossing and to assess the impact of the selfing rate on the survival and selection rate of the obtained families.

MATERIAL AND METHODS

Material

The study was conducted using 4 sugarcane cultivars (IACSP95-5000, SP89-1115, RB86-7515, and IAC91-1099), which flowered under natural conditions (without artificial photoperiod induction) 10 months after planting in June 2010 at the Sugarcane Center (Ribeirão Preto, São Paulo State, Brazil). Plant stalks with inflorescences were collected at plant cane 10 months after planting.

Polycross

The stalks with inflorescence were collected at the field and labeled with the respective cultivar name before the elimination of leaves and open florets. The pollen viability was determined by the iodine-staining test in which mature anthers are crushed on a slide using 0.1 N iodine solution, and the percentage of blue-stained pollen grains is determined using a microscope (Machado Jr., 1987). If the pollen grains are blue in the presence of iodine solution, the pollen can germinate and lead to true seed production. The greater the amount of blue-stained pollen in relation to the amount that remains yellow (not stained), the greater is the probability that the sugarcane inflorescence performs as a male parent.

The scores 1 to 9 (Table 1) were given according to the percentage of blue-stained pollen, in which 1 (100%) indicated that the cultivar was used as a male (high pollen viability) and 9 (0%) indicated that the cultivar was used as a female (low pollen viability). The cultivars IACSP95-5000, IAC91-1099, SP89-1115, and RB86-7515 presented scores of 1 (male), 3 (male), 8 (female), and 4 (male), respectively.

Table 1. Pollen viability scores according to the percentage of blue-stained pollen (%) through 0.1 N iodine.

Blue pollen (%)	Score
0	9
1 to 9	8
10 to 19	7
20 to 40	6
41 to 60	4
61 to 80	3
81 to 99	2
100	1

The stalks were taken to an isolated site and placed in a plastic bucket that was filled with an acid solution (150 ppm SO_2, 75 ppm H_3PO_4, 37 ppm H_2SO_4, and 37 ppm HNO_3). SO_2 at 10% of the total volume of the bucket solution was added daily, and the entire solution was renewed every 72 h (Liu, 1965; Mangelsdorf, 1966). On the 14th day, the inflorescences were encapsulated in porous cloth bags, and after 21 days, the spikelets were removed from the raphis and placed in bags with silica for 7 days. After 1 week, the seeds were placed in an oven at 32° ± 1°C to complete drying.

Germination test and progeny planting

A sample of 0.5 g seed was placed on germination boxes with water-based agar medium containing activated charcoal. The boxes were kept at 30° ± 1°C in a germination chamber for 7 days. The remaining seeds (progeny) from each cultivar that showed viability by the germination test were sown in plastic boxes (50 x 30 cm) with substratum (Plantmax) for 30 days. The seedlings were individualized in plastic tubes for further field planting with 1 m between rows and 0.5 m between plants.

Survival rate and percentage of selection

The survival rate was obtained 3 times (4, 6, and 10 months) after field planting. At each time, the percentage of live plants relative to the total number of progeny from each family was considered. The percentage of selection was estimated 10 months after planting based on the mass selection (phenotype selection) of healthy plants with good tillering (approximately 15-18 stalks per stool). The percentage of selected plants relative to the total number of progeny (offspring) in each family resulted in the percentage of selection.

Selfing rate estimation by molecular markers

DNA extraction and polymerase chain reaction (*PCR*)

DNA was extracted from leaf tissue using the method according to Al-Janabi et al. (1999). PCRs were performed in a final reaction volume of 15 μL (40 ng template DNA, 0.2 μM of each primer, 100 μM of each dNTP, 2.0 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KCl, and 0.5 U Taq DNA polymerase). The amplification conditions were the following: initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 s, annealing at a temperature that was specific for each primer pair (forward and reverse) for 30 s, and extension at 72°C for 30 s; and a final extension at 72°C for 3 min. The amplification products were mixed with formamide buffer (98% formamide, 10 mM ethylenediaminetetraacetic acid, 0.025% bromophenol blue, and 0.025% xylene cyanol) in a buffer-to-sample proportion of 2:1 and denatured at 95°C for 5 min. The samples were separated by electrophoresis on 6% denaturing polyacrylamide gels with a 10-bp ladder as a molecular weight marker. The bands were revealed by silver staining, according to Creste et al. (2001).

The simple sequence repeat (SSR) primer pairs SCB312, SCB436, SCB381, and SCC01 (Pinto et al., 2004; Oliveira et al., 2009) were screened in the family derived from IACSP95-5000, while SMC31CUQ, SMC2017FL, SMC1047HA (Liu et al., 2011), and

SCC01 SCA48 (Pinto et al., 2004) were used for the 24 individuals that were derived from SP89-1115. The best resolution and polymorphic primer pairs were selected for each family. Therefore, the primer pairs that were used to screen each family were not the same.

Molecular data analysis

Markers were genotyped based on their presence (1) and absence (0) for each of the microsatellite primer pairs that were evaluated. The selfing rate estimation for each polycross family was determined using the paternity exclusion test considering only the markers that were present in the progeny and absent in the female parent and thus inherited from the male parent (Buteler et al., 1997). The genetic similarity within families was estimated according to the Jaccard coefficient using the program NTSYS-PC, version 2.0 (Exeter Software, NY, USA; Rohlf, 1993).

Phenotypic data analysis

The selection and survival rate and the germination data were analyzed using the confidence interval at 95% probability.

RESULTS

Germination test

Only SP89-1115- and IACSP95-5000-derived seeds were viable, with 7 seedlings/0.5 g and 18 seedlings/0.5 g, respectively, showing a small intercept of the confidence interval at 95% probability (Table 2). After planting the remaining seeds, SP89-1115 and IACSP95-5000 (2.08 and 4.88 g) gave rise to 24 and 133 progenies, respectively, which were used to estimate the selfing rate by molecular markers.

Table 2. Weight in grams (g) and germination test (individuals/0.5 g seeds) of the polycross-derived seeds.

Cultivars	Seed weight (g)	G.T. (ind./0.5 g)	95%CI	No. of remaining seedlings
SP89-1115	2.58	7	1.10-10.89	24
IACSP95-5000	5.38	18	9.93-21.00	133
IAC91-1099	3.62	0	-	
RB86-7515	2.36	0	-	

G.T. = germination test; C.I = confidence interval.

Survival rate and percentage of selection

The SP89-1115 progeny showed a survival rate of 100% at the first 2 evaluations at 4 and 6 months and 95.83% at the last evaluation; these values did not differ significantly. For IACSP95-5000 progeny, the survival rate was 98.48, 86.36, and 82.57% at 4, 6, and 10 months, respectively. From these values, the last evaluation was significant diferent from de first and second evaluations (Figure 1). Among the families from SP89-1115 and IACSP95-5000, the survival rates from the second and third evaluations were significantly different at the 5% probability level. The percentage of plants that were phenotypically selected (healthy plants

with good tillering) and derived from SP89-1115 was significantly higher (78.26%) than that obtained for IACSP95-5000 (28.18%) (Figure 1).

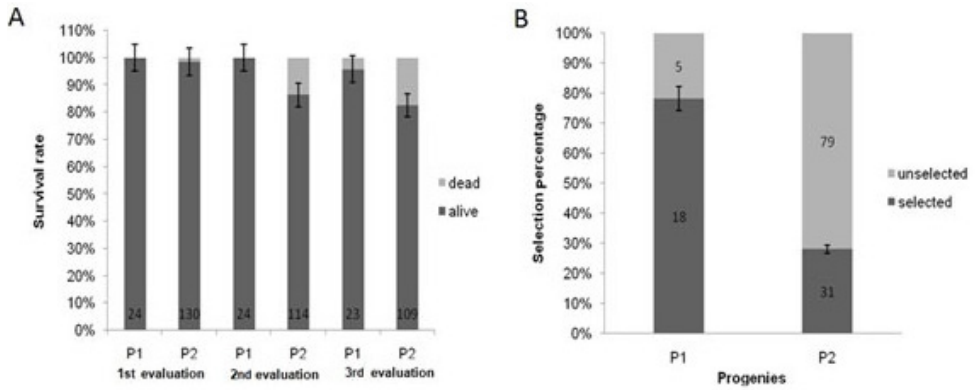

Figure 1. Evaluation of progeny at field condition. **A.** Survival rate (4, 6 and 10 months). **B.** Percentage of selection at 10 months. P1 = progeny from SP89-1115; P2 = progeny from IACSP95-5000.

Selfing rate estimation

The selfing rate estimation of the SP89-1115-derived family was performed using 6 SSR loci, which generated a total of 64 markers among the progenies and the cultivars involved in the polycross. Considering only the markers that were present in the progeny and absent in the female parent, SP89-1115 (mother), which included 28 markers (present exclusively among the parents IACSP95-5000, IAC91-1099, and RB86-7515), it was possible to identify 22 outcrossing progenies (91.6%) and 2 contaminants (individuals 23 and 24) that were identified because markers SCC01.8 and SCC01.9 were not present in any of the parents (IACSP95-5000, IAC91-1099, and RB86-7515) involved in the polycross (Table 3). Based on this result, the selfing rate for SP89-1115-derived family was estimated to be 0% since, all 24 of the individuals from this family showed at least 1 marker that was absent in the female parent (SP89-1115) and was thus inherited from the male parent. In addition, except for individuals 23 and 24, all of the progeny had IACSP95-5000 as the male parent. This can be proven by the presence of markers SMC31CUQ2, SMC31CUQ3, SCA48.4, SCB312.9, SCB312.13, and SCC01.7, which were exclusive to IACSP95-5000 and were observed in the remaining individuals that were derived from the SP89-1115 family (Table 3).

The 4 SSR loci that were screened in the IACSP95-5000-derived family and the parents involved in the polycross generated a total of 44 markers. From the selection of 23 markers that were present only among the parents (SP89-1115, IAC91-1099, and RB86-7515) and absent in the female parent, IACSP95-5000 (mother), it was possible to assume that individuals 131 and 133 resulted from outcrossing because they were the only individuals to have markers (SCB436.8, SCB436.9) that were absent in IACSP95-5000 (mother) and were inherited from a male parent. Moreover, these individuals were contaminants because the SCB436.8 and SCB436.9 markers were not present in any of the parents that were involved in the polycross (Table 4). All of the other individuals showed markers that were shared with IACSP95-5000. Thus, the selfing rate that was observed for the IACSP95-5000-derived family was 98.5%, and its complement, the outcrossing rate, was 1.5%.

Table 3. Microsatellite markers absent in the female parent SP89-1115 (mother) but observed in their progenies (24 individuals) and parents involved in the polycross.

	Parents				Individuals derived from SP89-1115																							
Markers	P1	P2	P3	P4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
SMC31CUQ1	0	1	1	1	1	0	0	1	1	0	0	1	1	0	0	0	1	1	1	0	1	0	1	1	0	0	0	1
SMC31CUQ2	0	***1***	0	0	0	***1***	***1***	***1***	***1***	***1***	***1***	0	0	0	***1***	0	***1***	0	***1***	0	***1***	***1***	***1***	***1***	0	***1***	***1***	0
SMC31CUQ3	0	***1***	0	0	***1***	***1***	0	***1***	***1***	***1***	***1***	***1***	0	***1***	***1***	***1***	***1***	***1***	***1***	0	***1***	***1***	***1***	***1***	***1***	***1***	***1***	***1***
SMC31CUQ5	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SMC2017FL1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SMC2017FL2	0	1	1	1	1	0	0	0	1	1	0	0	1	0	0	1	1	1	0	1	0	1	0	0	1	0	1	0
SMC2017FL3	0	1	0	1	1	0	0	0	1	1	0	0	0	0	0	1	1	1	0	1	0	1	0	0	1	0	1	0
SMC2017FL4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SMC2017FL9	0	1	1	0	1	1	1	1	0	0	1	1	1	1	1	1	1	0	1	1	1	1	1	0	0	1	1	1
SMC2017FL11	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCA48.2	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCA48.4	0	***1***	0	0	0	***1***	***1***	***1***	***1***	***1***	0	***1***	0	***1***	0	0	0	***1***	***1***	0	***1***	***1***	0	***1***	***1***	0	***1***	0
SCB312.4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB312.9	0	***1***	0	0	***1***	***1***	0	***1***	0	***1***	0	***1***	0	***1***	0	***1***	***1***	0	0	0	0	***1***	0	0	0	0	0	***1***
SCB312.11	0	1	1	1	1	1	1	1	1	0	1	1	0	0	0	0	0	0	1	1	1	1	0	0	1	0	1	1
SCB312.13	0	***1***	0	0	0	0	***1***	***1***	***1***	***1***	***1***	0	***1***	0	***1***	0	***1***	0	0	0	***1***	0	***1***	***1***	***1***	0	***1***	0
SCB312.14	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.6	0	1	0	1	1	0	0	0	1	1	0	0	0	1	1	0	0	1	0	1	0	1	0	1	0	0	0	0
SCB436.8	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.10	0	1	0	1	1	0	1	0	0	0	0	0	0	0	1	0	1	0	1	0	1	1	0	0	0	1	0	1
SCC01.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
SCC01.3	0	1	1	0	0	1	0	0	0	1	0	1	1	0	1	0	1	0	1	1	1	0	0	0	1	1	0	1
SCC01.4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.5	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.6	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.7	0	***1***	0	0	0	***1***	0	0	0	0	***1***	***1***	***1***	0	0	***1***	0	***1***	***1***	***1***	***1***	0	***1***	***1***	***1***	***1***	0	0
SCC01.8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1***	0
SCC01.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1***
Total	**0**	16	13	11	9	8	6	8	9	9	6	8	6	5	7	6	10	7	9	7	10	10	6	7	8	6	10	9

P1 = SP89-1115 (mother); P2 = IACSP95-5000; P3 = IAC91-1099; P4 = RB86-7515. 1 = marker presence; 0 = marker absence; ***1*** = markers exclusive to IACSP95-5000; **1*** = markers absent either in the female parent (mother) or parents involved in the polycross (contaminant individuals).

Table 4. Microsatellite markers absent in the female parent IACSP95-5000 (mother) but observed in the parents involved in the polycross [P2: IACSP95-5000 (mother); P1: SP89-1115; P3: IAC91-1099; P4: RB86-7515] and some individuals of the IACSP95-5000 progeny.

Markers	Parents				Individuals derived from IACSP95-5000																					
	P2	P1	P3	P4	1	2	3	4	5	6	7	8	…	61	62	63	64	65	66	…	128	129	130	131	132	133
SCC01.1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.3	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.4	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.5	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.7	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.8	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCC01.11	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.7	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.8	0	0	0	0	0	0	0	0	0	0	0	**1***	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB436.9	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**1***	0	0	0	0	0	0	0
SCB312.4	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB312.6	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB312.8	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB312.12	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB312.14	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB318.3	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB318.4	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB318.5	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB318.6	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
SCB318.8	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	13	10	11	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0

… = individuals between 9 and 60, and between 67 and 127; 1 = marker presence; 0 = marker absence; **1*** = contaminant individuals (individuals showing markers absent either in the female parent or parents involved in the polycross).

The genetic similarity values among the individuals (progenies) that were derived from SP89-1115 ranged from 41.5 to 73%. The average genetic similarity among the progenies was 53%, and that between the progenies and the female parent (SP89-1115) was 55.7%. On the other hand, the genetic similarity values among the IACSP95-5000 progenies ranged from 52.9 to 100%. The average similarity among the progenies was 80.70%, and that between the progenies and the female parent (IACSP95-5000) was 80.9% (Table 5).

Table 5. Similarity genetic values for SP89-1115- and IACSP95-5000-derived families.

Family	GS (min)	GS (max)	GSp	GSpg
SP89-1115	0.415	0.73	0.529	0.557
IACSP95-5000	0.529	1.00	0.807	0.809

GS (min) = minimum genetic similarity; GS (max) = maximum genetic similarity; GSp = genetic similarity among progenies; GSpg = average genetic similarity between progenies and the female parent.

According to the progeny frequency distribution relative to the genetic similarity (Figure 2), SP89-1115-derived individuals (progenies) were clustered as a class with 60% genetic similarity, while most of the IACSP95-5000-derived individuals had 90% genetic similarity; these values agreed with the type of family that was obtained: outcrossing and selfing.

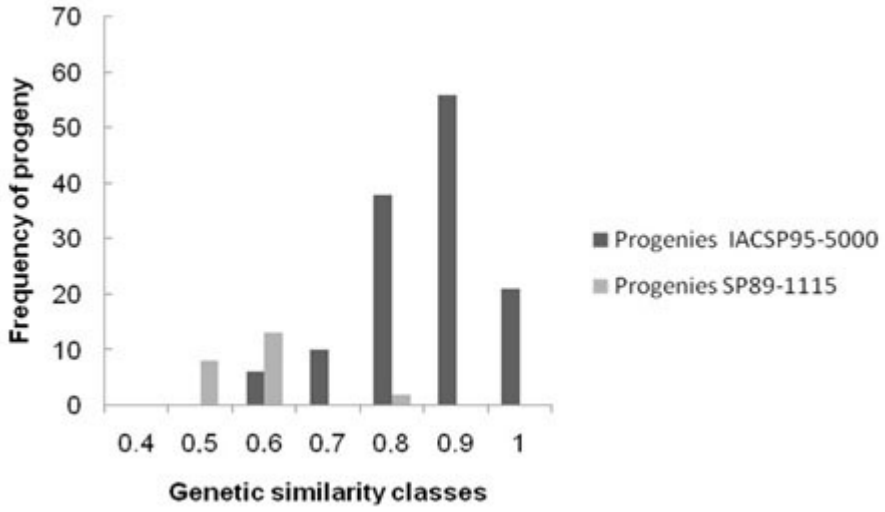

Figure 2. Frequency distribution of the IACSP95-5000 and SP89-1115 progeny in relation to genetic similarity.

DISCUSSION

Although sugarcane is an allogamous plant with hermaphroditic inflorescence, the occurrence of selfed progeny can be identified by molecular markers. In this study, microsatellite markers successfully allowed the identification of selfed and outcrossed progeny that were derived from a polycross involving 4 parents at Ribeirão Preto, São Paulo State, a region that is considered to be unfavorable for sugarcane crossing. In this region, high crossing rates require that the humidity is above 85% and the temperature around 27°-31°C, which normally

does not occur in the period (May-July) when the inflorescences are naturally observed. However, there were no previous reports that quantified the selfing/outcrossing rate of sugarcane cultivars and evaluated the performance of the derived progenies in such conditions. Consequently, the selfing rate and its complement, the outcrossing rate, were estimated under unfavorable natural conditions of crossing for the first time using Brazilian commercial sugarcane cultivars. Because no information exists for such conditions, our results also contribute to the establishment of regulatory policies for transgenic sugarcane in Brazil.

Moreover, obtaining correct scores for the pollen fertility test are important to correctly classify the parents as a male or female; in addition, male parents with similar scores should be used when planning a polycross. There was an effective participation of the pollen grains from IACSP95-5000, and almost all (98.5%) of their progeny were derived from self-fertilization. In addition, IACSP95-5000 was the pollen donor for most of the progeny (91.6%) that were derived from SP89-1115. This result was supported by the high average genetic similarity that was observed among IACSP95-5000 progeny or by the high average genetic similarity of the progeny with their female parent (IACSP95-5000). On the other hand, the same was not observed for the SP89-1115 family, where most progeny were derived from outcrossing.

The sex classification of sugarcane inflorescence as female or male through the iodine pollen-staining method, even though it is the most widely used in sugarcane breeding programs (McIntyre and Jackson, 2001), perhaps should not be considered the best way to evaluate pollen viability. The iodine-staining method relies on pollen reserves and does not guarantee effective germination; therefore, the iodine-staining method might overestimate pollen viability (Rodriguez-Riano and Dafni, 2000). In addition, the correlation between pollen viability assessed through staining methods and germination on culture medium was low when it was evaluated in different sugarcane cultivars (Melloni et al., 2013). Therefore, the pollen viability scores that were assigned here to the inflorescences that were involved in the polycross may not be correct, especially the scores of 3 and 4 that were assigned to cultivars IAC91-1099 and RB86-7515, respectively, both of which were considered to be a pollen donor (male). Besides the pollen viability, which was observed through the iodine-staining method, the temperature and humidity must be appropriate because the cultivars may have specific responses in relation to pollen viability under different conditions. In fact, IACSP95-5000 was less sensitive to these conditions and showed 100% pollen viability at Ribeirão Preto, where the humidity is low and the night temperatures are below 18°C during the sugarcane flowering period. These conditions usually prevent the pollen grain germination of most cultivars (Berding, 1981; Moore, 1987). Moreover, the monitoring of weather conditions during the polycross execution indicated minimum temperatures of 17°C for a few days and humidity below 85% during almost the entire period. Even so, this cultivar outstands the other cultivars as a pollen donor, indicating its lower sensitivity to adverse conditions.

The IACSP95-5000 cultivar, which had a score of 1 (high pollen fertility), produced a noticeably large amount of pollen compared to the other cultivars (data not shown) with less viable pollen (higher scores). The score that was given to the inflorescence that was based on the ratio of blue-stained pollen to total pollen in a microscope slide did not consider the amount of pollen that was produced by the inflorescence. This implies that, even in a polycross involving parents with different scores, parents may not have the same probability of fertilization. Thus, RB86-7515 and IAC91-1099 could be at a disadvantage in terms of pollen amount relative to IACSP95-5000. It would be interesting to evaluate the inflorescence not only based

on the pollen viability (scores), as in this study, but also by the amount of pollen that is produced to increase the number of tassels of those that produce less pollen.

The low production of viable seeds for both cultivars (RB86-7515 and IAC91-1099) can be attributed to several factors including the low amount of viable pollen that was involved in the polycross (because only 1 cultivar contributed to fertilization), temperatures that were not optimal for germination, and humidity below 85% (Berding, 1981; Moore, 1987). On the other hand, the lack of seed viability of both IAC91-1099 and RB86-7515 can be attributed to the sensitivity of the female part to unfavorable conditions of temperature and humidity.

Regarding the survival rate and comparing the IACSP95-5000 selfed family with the SP89-1115 outcrossed family, it was noted that in the last 2 evaluations (6 and 10 months) the survival rate was higher with outcrossing than with selfing. In fact, in allogamous plants, selfing can lead to inbreeding depression, i.e., the loss of vigor in the offspring that is caused by the appearance of lethal and deleterious genes in the homozygous condition, which may cause the death of some individuals (Falconer and Mackay, 1997).

Healthy plants with good growth and tillering (which reflects vigor) were considered in the percentage of selection, and the production traits due to the age of the plants (10 months) were not considered. The SP89-1115 outcrossing family presented the higher percentage of selection relative to the selfing family (the IACSP95-5000 family), which were less developed and displayed signs of illness. A similar result was reported by Silva and Gonçalves (2011), who studied segregation in the first generation of selfed sugarcane and observed a strong inbreeding depression for the plant development traits (height and stalk weight).

In general, compared to the other cultivars that were studied here, IACSP95-5000 was characterized as a good pollen donor in the environmental conditions of Ribeirão Preto, while SP89-1115 was characterized as a good pollen receiver even in bad conditions of temperature and humidity. Certainly, the natural conditions of Ribeirão Preto were a limiting factor for the polycross because of the low number of viable seeds that were produced (low number of progeny) and the almost exclusive participation of IACSP95-5000 as the pollen donor in the cross. This implies that germination may be compromised under unfavorable conditions during the crossing even when pollen is produced in natural conditions. These factors can be minimized with a photoperiod facility, which was newly built at Ribeirão Preto. This facility will allow the control of the environmental factors that drive flowering induction, panicle formation, and pollen viability.

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