

## NUMBER OF POLLEN GRAINS, OVULES AND POLLEN-OVULE RATIO IN CACAO CLONES

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Cacao (*Theobroma cacao* L.) is a tree species, whose beans are used mainly in the chocolate manufacture. Pollination deficits have been frequently reported in cacao, impacting bean yield. In the present study, 30 cacao clones were evaluated in respect to the number of ovules and pollen grains produced. It was observed statistically significant variation among the 30 clones, for the number of pollen grains produced, ranging from 1750 to 10575 pollen grains/flower (average = 4698 pollen grains/flower). The number of ovules per flower ranged from 41.4 to 66.5 (average = 52.9 ovules/flower) and the pollen-ovule ratios from 30.7 to 186.2 (average = 88.0). There were no statistical significant differences between self-compatible and incompatible clones in respect to the pollen-ovule ratios. Considering the pollen-ovule ratios observed in this study, cacao should be classified as a facultative/obligate autogamous, according to Cruden's (1977) scale, despite the many evidences showing a high frequency of outcrossing in cacao. It can be concluded that there is genetic variance for the number of pollen grains and ovules in cacao, allowing breeding for those traits and contributing to the reduction of the yield gap associated to pollination deficit.

**Key words:** *Theobroma cacao*, pollination, mating system.

**Número de grãos de pólen, óvulos e proporção pólen-óvulo em vários clones de cacauero.** O cacauero (*Theobroma cacao* L.) é uma espécie arbórea, cujas amêndoas são usadas principalmente na manufatura do chocolate. Deficit de polinização tem sido frequentemente reportado em cacau, impactando a produção de amêndoas. No presente estudo, 30 clones de cacauero foram avaliados em relação ao número de óvulos e grãos de pólen produzidos. Foi observada variação estatisticamente significativa entre os clones, para o número de grãos de pólen produzidos, variando de 1750 a 10575 grãos de pólen/flor (média = 4698 grãos de pólen/flor). O número de óvulos por flor variou de 41,4 a 66,5 (média = 52,9 óvulos/flor) e a proporção pólen-óvulo de 30,7 a 186,2 (média = 88,0). Não houve diferenças estatisticamente significativas entre clones autocompatíveis e incompatíveis em relação às proporções pólen-óvulo. Considerando as proporções pólen-óvulo observadas neste estudo, o cacauero deveria ser classificado como autógamo facultativo/obrigatório, de acordo com a escala de Cruden (1970), apesar das muitas evidências mostrando uma elevada frequência de polinização cruzada. Conclui-se que há variância genética quanto ao número de grãos de pólen e óvulos no cacauero, permitindo melhoramento para aquelas características e contribuindo para a redução da lacuna de produção associada ao deficit de polinização.

**Palavras-chave:** *Theobroma cacao*, polinização, sistema de acasalamento.

## Introduction

Cacao (*Theobroma cacao* L.) is a fruit tree species originally from the Amazon region and planted mainly in the tropics (West Africa, Latin America, and Asia). Cocoa beans – the commercial product of the cacao tree - are mainly used for chocolate manufacture but are also used for cosmetics, pharmaceuticals, and many other products. A high bean production initiates with and requires a successful pollination; however, some authors have reported a strong pollination deficit in cacao (Soria, 1974; Parvais, Reffye and Lucas, 1977; Reffye et al., 1978; Tovar and Ortis, 1991; Lachenaud, 1994) and complementary pollination has been proposed (Soria, 1974; Nakayama, 2018) as a form of increasing yield. Cilas (1988) has shown that a high number of pollen grains per flower, reduces the number of flowers receiving no pollen in the pistil ( $r=-0.41^{**}$ ). On the other hand, cacao breeders have not considered the number of pollen grains as a trait for selection, and a few cacao germplasms have been assessed for this trait (Cilas, 1988; Godoy et al., 2009), compared with those evaluated for ovule number, for example (e.g., Lachenaud, 1991; Cilas, Machado and Motamayor, 2010). Also, in cacao, the pollen-ovule ratio has been estimated only for two clones (Godoy et al., 2009) and then a better assessment of the mating system based on this aspect, as suggested by Cruden (1977), is not possible. This paper will focus on these two aspects, the assessment of cacao germplasms for the pollen number and the estimation of the pollen-ovule ratio to infer the mating system.

Cacao flowers are hermaphrodite and present several barriers to natural pollination. Firstly, cacao has sexual incompatibility, which is quite frequent in the species and prevents incompatible plants being self-fertilized or fertilized by individuals with the same alleles of incompatibility (Knight and Rogers, 1955; Cope, 1962). Secondly, the stamens of the cacao flowers are enclosed within petal hoods, making difficult the access of the pollinator insect to the anthers (Knoke, Soria and Chapman, 1980). Thirdly, anther and stigma are in different heights in the flower (heterostyly), due to different sizes of the stamens and pistils. Fourthly, the stigma of the cacao flower often matures later than the anthers (Claus et al., 2018) and the stigma is surrounded by a barrier of

(infertile) staminodes. Fifthly, the cacao pollen grains are sticky, therefore, not easily transported by the wind, requiring a specialized pollinator insect, mainly midges of the *Forcipomyia* genus (Soria and Wirth, 1974). All these factors tend to limit the level of pollination in cacao.

On the other hand, cacao flowers are produced throughout the year, and a single tree can produce up to 125 thousand flowers per year (Lachenaud and Mossu, 1985). Nevertheless, only 1% to 5% of those flowers receive enough pollen to produce a pod (Alvim, 1984) and 50% to 79% of them receive no pollen at all in the pistil, falling off the trees without bearing pods (Parvais, Reffye and Lucas, 1977; Reffye et al., 1978; Tovar and Ortis, 1991; Lachenaud, 1994).

Pollination in cacao has been studied and modeled by different authors, including Parvais, Reffye and Lucas (1977), Reffye et al. (1978) and Tovar and Ortiz (1991). They observed that increasing the number of stigmas with sufficient pollen by natural (insect) pollination, has a significant impact on cacao yield. Also, studies with artificial pollination in cacao have shown a high increase in yield (Soria, 1974; Knoke, Soria and Chapman, 1980; Nakayama, 2018). Moreover, Falque, Lesdalons and Eskes (1996) observed that the correlation between pollination intensity and fruit setting in two clones (Sca-6 and IFC-5) was higher than  $0.92^{**}$ . Therefore, selection for high amount of pollen in the anthers could improve the pollination efficiency in cacao, improving yield, as suggested by Cilas (1988).

The first step to breed for higher number of pollen grains per flower is to assess the variability of that trait in the germplasm available for breeding. However, there are very few studies quantifying the number of pollen grains in cacao, particularly for germplasms of interest to breeding. Massaux et al. (1976), in Cameroon, observed up to 14 thousand pollen grains per flower, but did not mention the germplasm used. Cilas (1988), evaluating some hybrid trees, observed an average of 878 pollen grains per flower, ranging from 540 to 1310 pollen grains. Godoy et al. (2009) counted the number of pollen grains in two cacao clones, one self-compatible (CCN-51) and another self-incompatible (TSH-1188), and founded 2288 and 2424 pollen grains per flower, respectively.

The objective of the present study was to estimate the number of pollen grains, the number of ovules and the pollen-ovule ratio, in 30 clones of CEPEC's (Cacao Research Center) cacao breeding program, in Bahia, Brazil.

## Materials and Methods

### Germplasms

Thirty clones of the germplasm collection of CEPEC, in Ilhéus, Bahia, Brazil, were used in the present study (Table 1). Those clones are from different origins and were collected either in wild populations or selected in breeding programs; some are self-compatible, others self-incompatible.

### Pollen and Ovule Counting

For the pollen counting, floral buds were collected at random from trees randomly chosen from the 30 clones, after 15:00 h, when the buds started opening (sepal separation). After collection, buds were then immediately put in plastic cups labelled with the clone's name, covered with a young cacao leaf to keep a humid environment, and thereafter transported to the lab. The flower buds were maintained at room temperature until next day morning, to have them completely opened, with anthers dehisced and releasing pollen. Flowers were collected in two periods, Nov-Dec/2018 and Apr-Jun/2019. From each clone and period, two flowers were collected.

From the two flowers, five anthers randomly chosen among the 10 anthers of the two flowers were used for each clone and period. The method used followed the procedure suggested by Dafni (1992). An aqueous solution of approximately 500  $\mu\text{L}$  was prepared by adding three drops of methylene blue stain and four drops of neutral detergent. Then alcohol 70% was added until completing 1000  $\mu\text{L}$ . This solution was manually shaken to mix the components. After that, 100  $\mu\text{L}$  of the solution was put on a single concave microscope slide, together with the five anthers of the clone. Thereafter, five anthers were properly macerated in the solution with a glass stirring rod. After maceration, 1.0  $\mu\text{L}$  of this suspension of pollen grains was distributed in five microscope slides. Observations were then made by counting the number of pollen grains for each slide under magnification (10X) of an

optical microscope. The number of pollen grains per flower (NPGPF) was estimated by:  $\text{NPGPF} = (\text{NPGPS} \times 100 \mu\text{L}) / (1 \mu\text{L} \times 5 \text{ anthers})$ ; where, NPGPS is the average number of pollen grains per slide.

For the ovule counting, another group of flowers were used. In this case, 10 flowers were collected in Oct/2019 from the same clones used for pollen counting, and stored in small plastic tubes containing tap water, until the counting day (usually 1 day after the flower harvest). The ovaries of each of the 10 flowers were dissected and the number of ovules counted under a stereoscopic microscope Taimin TE 1L, 10X.

### Data Analysis

For all analysis, the SAS software was used (SAS Institute, 2004). For the analysis of variance of number of pollen grains (NPGPF), the effects of period and genotypes were considered, assuming as a completely randomized design, with five replicates or slides with pollen of a given clone, in each of the two periods. For the analysis of the number of ovules per flower, only the effect of clone was considered (because it was evaluated only once) and 10 replicates or flowers were used. For comparing the means, 95% confidence intervals were established for the averages of the number of pollen grains and number of ovules per flower of the clones.

The pollen-ovule ratio was computed by dividing the average number of pollen grains per flower of each clone by the average number of ovules per flower of those same clones. Since the number of pollen grains and the number of ovules were measured in different flowers of the clone (even with different number of flowers as pointed before), it was not possible to estimate the pollen-ovule ratio for each flower; therefore, no statistical analysis (ANOVA, confidence intervals) was possible to apply for this variable, as performed for the number of pollen grains and ovules. However a cluster analysis was done, using proc cluster from SAS (SAS Institute, 2004).

The averages of number of ovules, number of pollen and pollen-ovule ratios of self-compatible (23 clones, see Table 1) vs. self-incompatible clones (7 clones) were statistically compared, by the F test, from the analysis of variance, using each clone average as a replication. And a simple linear regression of number

Table 1. Origin, type and sexual incompatibility status of the 30 clones used in this study

Clone	Origin	Type	Incompatibility <sup>a</sup>
BJ-11	Brazil	Breeding Selection	SC
BN-34	Brazil	Breeding Selection	SC
CCN-10	Ecuador	Breeding Selection	SC
CCN-51	Ecuador	Breeding Selection	SC
CEPEC-2002	Brazil	Breeding Selection	SC
CEPEC-2176	Brazil	Breeding Selection	SC
CEPEC-2204	Brazil	Breeding Selection	SC
CP-49	Brazil	Breeding Selection	SC
CP-77	Brazil	Breeding Selection	SC
ICS-1	Trinidad & Tobago	Breeding Selection	SC
ICS-6	Trinidad & Tobago	Breeding Selection	SC
ICS-95	Trinidad & Tobago	Breeding Selection	SC
IMC-67	Peru	Wild	SI
Ipiran-1	Brazil	Breeding Selection	SC
LP-6	Brazil	Breeding Selection	SC
PH-9	Brazil	Breeding Selection	SC
PH-15	Brazil	Breeding Selection	SC
PH-16	Brazil	Breeding Selection	SC
PS-13.19	Brazil	Breeding Selection	SC
Salob-3	Brazil	Breeding Selection	SC
Sca-6	Peru	Wild	SI
Sca-12	Peru	Wild	SI
SIAL-169	Brazil	Breeding Selection	SC
SIC-23	Brazil	Breeding Selection	SC
SJ-2	Brazil	Breeding Selection	SC
TSH-516	Trinidad & Tobago	Breeding Selection	SI
TSH-565	Trinidad & Tobago	Breeding Selection	SI
TSH-1188	Trinidad & Tobago	Breeding Selection	SI
UF-613	Costa Rica	Breeding Selection	SI
VB-681	Brazil	Breeding Selection	SC

<sup>a</sup> SC = Self-Compatible, SI=Self-Incompatible

of pollen grains per flower was fitted on number of ovules, as another way of looking the pollen-ovule ratio but also to assess the association of these two traits.

## Results

The effect of clones was highly significant for both the number of pollen grains and the number of ovules per flower (Table 2). However, for the number of pollen grains, measured in two seasons, there was no effect

of season, but it was a significant season-by-clone interaction (F test, p-values =0.0429). In Nov-Dec/2018, the overall average of the 30 clones was 4595 pollen grains/flower (Figure 1a); while in Apr-Jun/2019 that average was 4795 pollen grains/flower (Figure 1b). While most clones had a higher number of pollen grains in Apr-Jun/2019; ICS-1, ICS-95, UF-613, Ipiran-1, among others had more pollen grains in Nov-Dec/2018 (Figure 1a,b). There was a high variability in number of pollen grains in each season and overall (Figure 1). Some clones as Sca-6, PH-18 and PH-15 had the least number of pollen grains in both seasons. On the other hand, IMC-67, ICS-1, CCN-10 had high number of pollen grains on both seasons. The overall average for all 30 clones in the two seasons was 4,698 pollen grains per flower and ranged from 1760 in Sca-6 to 10575 pollen grains per flower in IMC-67 (Figure 1c). The clones Sca-6, PH-16, PH-15 presented the smallest average; while IMC-67, ICS-1, and CCN-10 showed the highest average.

The number of ovules per ovary or flower ranged from 44.5 in ICS-95 to 66.5 in IMC-67, with an overall average of 52.9 in the 30 clones evaluated (Figure 2a). Among the clones with the smallest values were the ICS-95, UF-613, SIAL-169, and CP-49; while among those with the highest values were the IMC-67, CP-77, CCN-51 and Sca-6.

The pollen-ovule ratios were quite variable among the clones evaluated. The

Table 2. Analysis of variance for the number of pollen grains and ovules per flower measured in 30 cacao clones, in two seasons Nov-Dec/2018 and Apr-Jun/2019 (pollen grains) and one season (Nov/2019), at the Cacao Research Center (CEPEC), Bahia, Brazil

Traits	Source	DF	Mean Square	F	p-value
Pollen Grains	Season	1	19255	0.00	0.9497
	Clone	29	27676322	5.74	<.0001
	Season x Clone	28	7690637	1.60	0.0429
	Residual	127	4818066		
Ovules	Clone	29	202	33.21	<.0001
	Residual	270	6		

CV = 46.7% (Pollen Grains), 4.7% (Ovules)

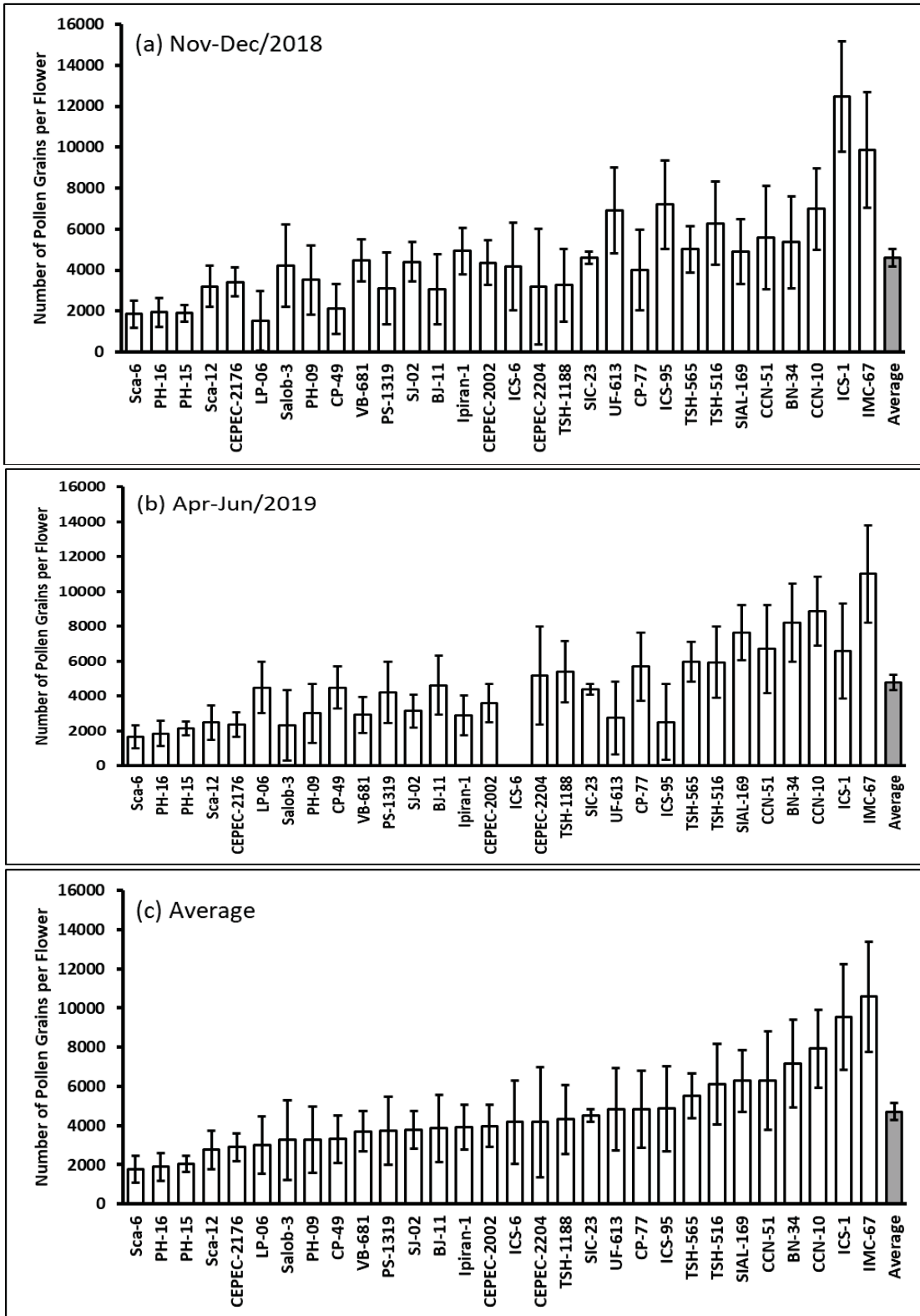


Figure 1. Average estimates and 95% confidence intervals of number of pollen grains per flower (N=6 flowers/clone/season) evaluated in 30 cacao clones in (a) Nov-Dec/2018, (b) Apr-Jun/2019, and (c) average of the two seasons.

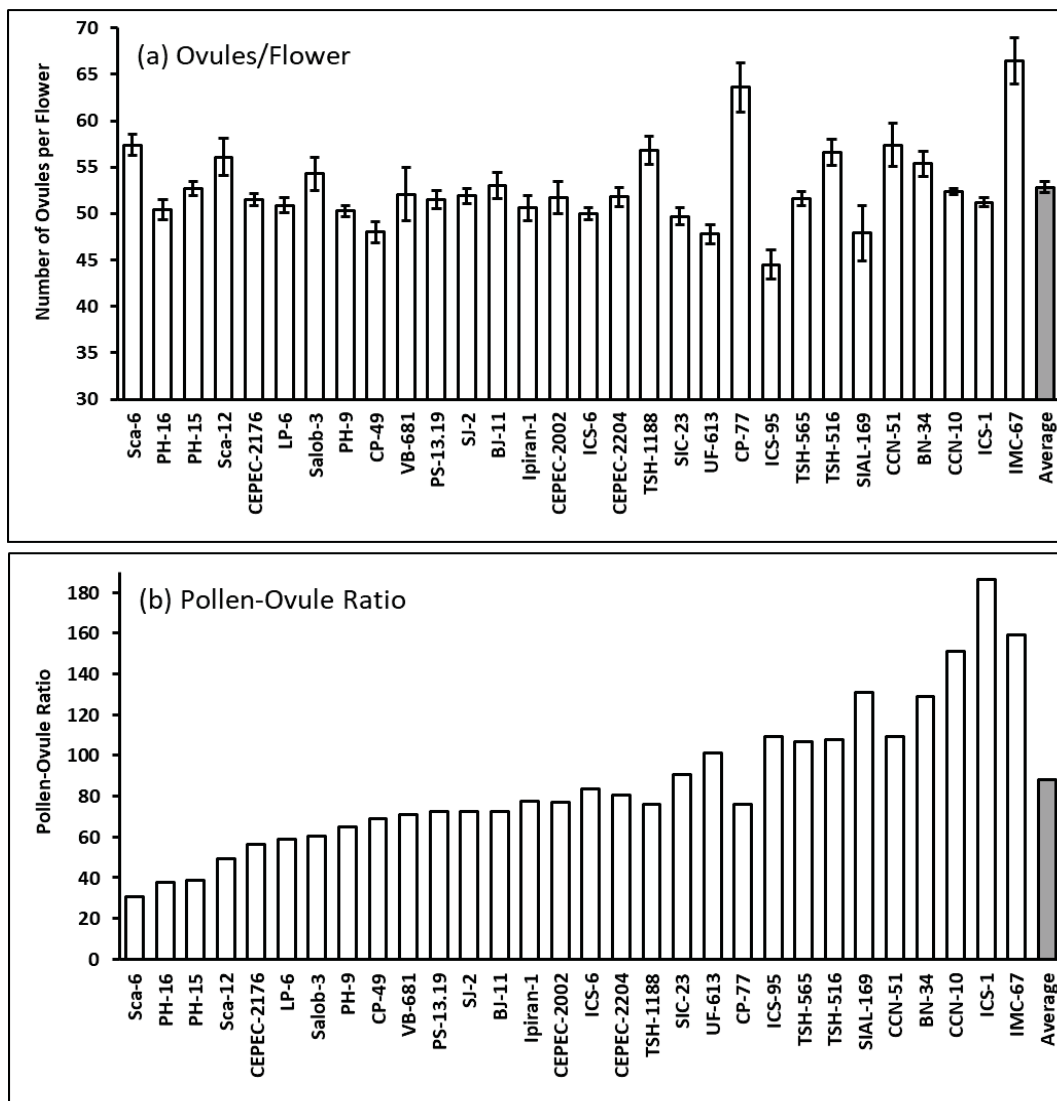


Figure 2. Average estimates of (a) number of ovules per flower (N=10 flowers/clone), and (b) pollen-ovule ratios estimated in 30 cacao clones, and the confidence intervals associated to those estimates.

ratios ranged from 30.7 in Sca-6 to 186.2 in ICS-1 (Figure 2b). Clones with low pollen-ovule ratios were Sca-6, PH-16, and PH-15; while those with high values were ICS-1, IMC-67 and CCN-10 (Figure 2b and 3). The overall pollen-ovule ratio average was 88.0 for the 30 clones.

The fit of the number of pollen grains on the number of ovules in the 30 clones evaluated for both traits was poor ( $R^2 = 0.11$ , Figure 4), indicating that the pollen-ovule ratio is not constant within the species as also shown in Figure 2b. Some clones with very similar number of ovules (e.g., 51.5 ovules/flower for CEPEC-2176 and 51.6 in TSH-565, Figure 2a) have different

number of pollen grains (2890 in CEPEC-2176 and 5500 in TSH-565), resulting in different pollen-ovule ratios (56.1 and 106.6, respectively, Figure 1c).

The averages of the seven self-incompatible clones were higher than those of 23 self-incompatible ones, for the three traits evaluated (Table 3); however, they were statistically different only for the number of ovules per flower (Table 4).

## Discussion

A good pollination is an essential step for a high production of cocoa beans. Pollination deficit has been

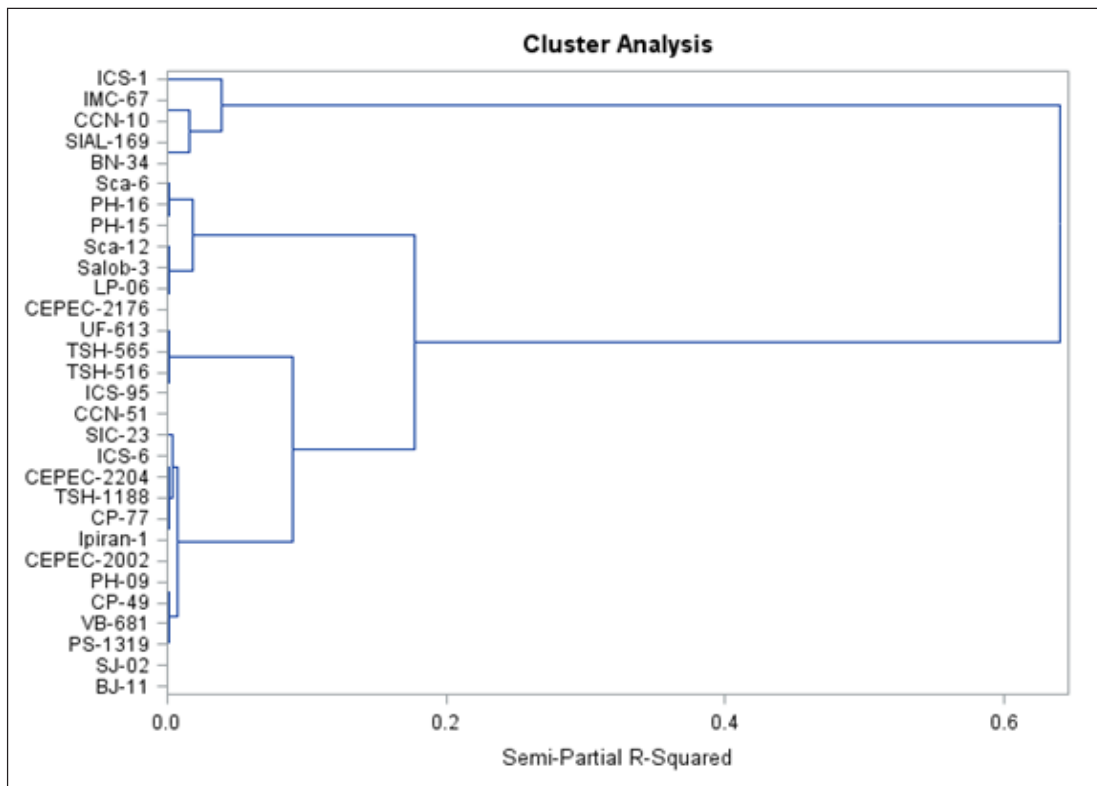


Figure 3. Clone groups based on a cluster analysis on the variable pollen-ovule ratio.

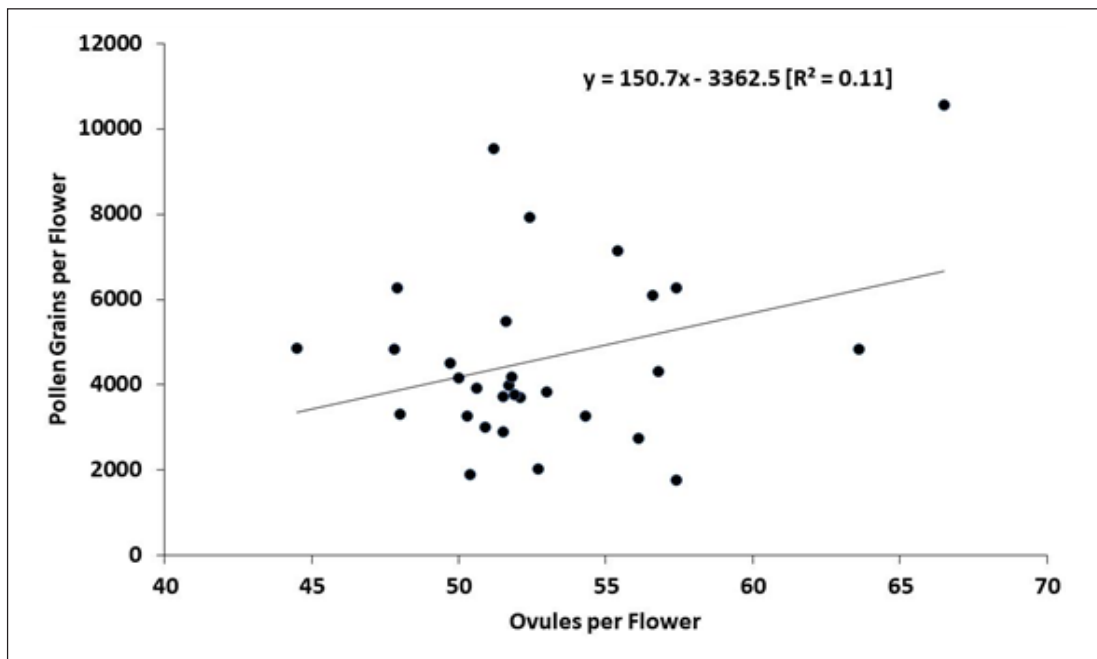


Figure 4. Average number of pollen grains per flower in 30 cacao clones as a function of number of ovules per flower in the same clones.

Table 3. Averages of number of pollen grains per flower, number of ovules per flower and pollen-ovule ratio, for 23 self-compatible and seven self-incompatible cacao clones, at the Cacao Research Center (CEPEC), Bahia, Brazil

Incompatibility	N	Pollen Grains	Ovules	Pollen-Ovule Ratio
Self-Compatible	23	4449.6	51.9	85.9
Self-Incompatible	7	5121.1	56.1	90.1

Table 4. Analysis of variance for number of pollen grains per flower, number of ovules per flower and pollen-ovule ratio, considering the two incompatibility status (Self-Compatible, Self-Incompatible) in 30 cacao clones, at the Cacao Research Center (CEPEC), Bahia, Brazil

Traits	Source	DF	Mean Square	F	p-value
Pollen Grains	Incompatibility	1	2419658	0.54	0.467
	Residual	28	4440902		
Ovules	Incompatibility	1	97	5.55	0.026
	Residual	28	17		
Pollen-Ovule Ratio	Incompatibility	1	93	0.07	0.798
	Residual	28	1388		

CV = 46.8% (Pollen Grains), 8.6% (Ovules), 40.2% (Pollen-Ovule Ratio)

claimed as one of the major gaps in cacao yield production, with yield increasing nonlinearly with the number of flowers pollinated up to 200% (Groeneveld et al., 2010). However, pollination deficit does not occur only with cacao. Burd (1994), reviewing published data from 258 plant species, from 77 families, found a pollination deficit in at least 62% of them, with a strongest impact on the frequency of fruit set, as compared with the frequency of seed set. Usually only 1% to 5% of the flowers produced by cacao trees receive enough pollen to produce pods (Alvim, 1984) and 50% to 79% of the flowers in a tree receive no pollen at all (Parvais, Reffye and Lucas, 1977; Reffye et al., 1978; Tovar and Ortis, 1991; Lachenaud, 1994). On average, seven cacao clones assessed in Ecuador for six months, received only around 16 pollen grains in the stigmas (Mena-Montoya et al., 2020), which is only around one-third of the ovules typically produced in a cacao flower.

To overcome the pollination deficit in cacao, supplementary pollination has been suggested as a way of increasing productivity based on some successful results. Falque, Lesdalons and Eskes (1996), for example, showed a high correlation between pollination intensity and fruit setting in cacao ( $r > 0.92^{**}$ ). Soria (1974) and Knoke, Soria and Chapman (1980) showed increments in yield of up to five times in plants blown

with a backpack mistblower, compared with the yield of unblown control plants. Nakayama (2018), using a tractor hauled mechanical pollination blower for 20 days, observed an increase of up to 46.3 times in fruit setting in trees of the clone CCN-51, compared with trees of the same clone naturally pollinated. Groeneveld et al. (2010) showed that in cacao yield is maximized by pollinating only 40% of the flowers available, instead 100% of them. Despite these successful results, other studies have reported unsuccessful outcomes. For example, Leite et al. (1990) have shown that the increase in yield potentially attained by supplementary pollination, can be offset by the physiological discard of small fruits (physiological cherrille wilt).

It has been suggested that increasing the number of pollen grains produced in the anthers of the flower can improve the pollination quality. For example, Cilas (1988), in Togo, monitoring the number of pollen grains produced in the anthers and the number of flowers pollinated, observed that when the number of pollen grains increased, there was a reduction of the number of flowers receiving no pollen ( $r = -0.41^*$ ). The author also studying two populations, one historically high and other low yielding, observed that the high yielding population had flowers receiving a higher number of pollen grains in their stigma (Pollen Rarity Index=0.75 vs 1.02, in high vs low yielding plants). Based on those results, the author suggested breeding for high pollen production. Also, Knoke, Soria and Chapman (1980), inducing pollination in cacao, observed that pods pollinated by the mistblower had less seeds compared with those naturally pollinated, i.e., 12.9 vs. 15.5 seeds/pod, respectively (Knoke, Soria and Chapman, 1980), suggesting a suboptimal number of pollen grains reaching the stigma. Therefore, increasing the number of pollen grains per flower increases the chances of higher pollination efficiency, allowing that in periods of low number of flowers or of pollinator insects, more pollen grains are transported to stigmas in each insect visit.

Here we quantified the number of pollen grains of 30 clones from different origins, used in CEPEC's cacao breeding program; some of them used as clones



or parents of hybrids in many other cacao producing countries (e.g., CCN-10, CCN-51, ICS-1, ICS-6, ICS-95, IMC-67, Sca-6, Sca-12, SIAL-169, SIC-23, TSH-516, TSH-565, TSH-1188, and UF-613). We observed a high variability for the number of pollen grains per flower, with some clones, like IMC-67 producing up to 10575 pollen grains per flower on average; while other clones, such as the Sca-6, producing only 1760 pollen grains per flower. Also, at least during the two seasons evaluated (Nov-Dec/2018 and Apr-Jun/2019), we did not observe a statistically significant effect of season, but we did find a barely significant season-by-clone interaction ( $p=0.0429$ ). Here we observed that high yielding clones such as CCN-10, CCN-51, BN-34, TSH-565, and BN-34 produce high number of pollen grains; by contrast other equally high yielding clones (in number of pods), such as Sca-6, PH-16, PH-15 and ICS-95 produce low number of pollen grains. Therefore, despite the association of pollen availability in the area and yield shown by some authors (Cilas, 1988), these results reinforce the fact that yield in cacao is not controlled only by the number of pollen grains produced. In other words, pollen production is only one of the many factors affecting yield. However, the high variability shown here among clones, for pollen number, suggests that breeding for high pollen number, facilitating both natural and artificial pollinations, is feasible.

Some studies show that, for each ovule fertilized, more than one pollen grain is needed in the stigma to maximize seed set. For example, Cruden (1977) observing several species verified that at least two to six pollen grains per ovule are needed for a maximum seed set. In apples, a minimum of five pollen grains are needed per ovule (Sedgley and Griffin, 1989). Falque, Lesdalons and Eskes (1996) observed that for maximum fruit set in cacao, at least 150 pollen grains are needed for each pod formed. Considering that cacao ovary has around 50 ovules, this results in a proportion of three pollen grains per ovule. This number (150 pollen grains), however, as pointed out by Falque, Lesdalons and Eskes (1996), citing some field studies in Africa and in Latin America, is lower than commonly observed in the field, under natural (insect) pollination. This was shown, for example, in the work by Mena-Montoya et al. (2020), in Ecuador, who found an average of 16 pollen grains in the stigma of seven evaluated clones. Few studies have estimated the

number of pollen grains in cacao, particularly for well identified germplasms. Massaux et al. (1976), in a preliminary work of pollen transport conducted in Cameroon, estimated 14100 pollen grains per flower. Cilas (1988), in Togo, and using a mixture of hybrid trees, observed an average of 878 pollen grains per flower, ranging over time from 540 to 1310 pollen grains. Godoy et al. (2009), in Brazil, using two cacao genotypes, one self-compatible (CCN-51) and one self-incompatible (TSH-1188), observed 2288 and 2424 pollen grains/flower, respectively. In our study, the average number of pollen grains (4698) was higher than those found in previous studies, except the 14100 pollen grains per flower found by Massaux et al. (1976). Some of the clones used in our study (e.g., IMC-67) had values not much far from the value estimated by Massaux et al. (1976). However, in the present study CCN-51 and TSH-1188 had more pollen (4320 and 6283 pollen grains, respectively) than those observed by Godoy et al. (2009). Mena-Montoya et al. (2020), instead counting the number of pollen grains in the anthers, counted those deposited in the stigma and observed they ranged from 10 to 29 in the seven clones. This of course, can be associated to a variation for insect attraction to the flower of those clones, but also to the number of pollen grains available in the anthers. In Mena-Montoya et al. (2020), unfortunately the number of pollen grains in the anthers was not counted, but CCN-51 showed the second highest number of pollen grains in the stigma (around 19) in their study. In this study it is among those with a high number of pollen grains in the anthers.

In Brazil, many farmers are using supplementary pollination, either by hand or mechanically, with successful increase in yield. Also, some studies on supplementary pollination have shown increments in fruit setting of the order of up to five times that of naturally pollinated plants (Soria, 1974; Knoke, Soria and Chapman, 1980). The use of a pollinizer clones, having high number of pollen grains per anther, can increase the efficiency of the pollination process, either by allowing to dispense more pollen grains in the stigma or by reducing the number of pollen donor flowers used to provide the proper number of pollen grains. Data from the present study showed that the clones IMC-67, ICS-1, CCN-10, ICS-9 and BN-34 produce more than 7000 pollen grains per flower and, therefore, are potential pollinizers to be utilized in manual pollination

programs in cacao. Clone CCN-10, for instance, is one of the most currently used in Brazil by farmers with that purpose. That clone, besides the high number of pollen grains produced, it also is claimed to increase the bean size by the effect of pseudo-xenia (phenotypic expression in the  $F_0$  generation, in the cotyledons, instead in the endosperm as in xenia).

Considering the results found here for the number of pollen grains, some clones can be more benefited by mechanic pollination. For example, Knoke, Soria and Chapman (1980), using a mistblower to induce pollination in cacao observed that the pods pollinated by the blower had less seeds than those naturally pollinated, despite the higher number of pods in the first, suggesting a suboptimal number of pollen grains reaching the stigmas. Using clones with a high number of pollen grains in their anthers can reduce this effect, by releasing more pollens in the blowing process. Also, it is well known in cacao (e.g., Reffye et al., 1978) that some clones do not tolerate a small number of seeds (fertilized ovules). Therefore, in principle the effect of the mechanical pollination on both the number of seeds per pod and the number of pods can be further improved by using clones with high number of pollen grains in their anthers. If that is the case, among the high yielding and widely planted cacao clones in Brazil, BN-34, CCN-10 and CCN-51 (having more 6283 pollen grains/flower), can be benefited more from blower pollinations than clones such as PH-15 and PH-16 which have a smaller number of pollen grains in their anthers (less than 2027 pollen grains/flower).

In this study it was additionally observed that the average number of pollen grains per flower in the 30 clones investigated was not correlated with the number of ovules per ovary or flower of those same clones ( $R^2=0.11$ ). Therefore, the number of pollen grains in cacao can be increased by breeding, without reducing the number of ovules. The number of ovules observed here (44.5 to 66.5, average for all clones = 53.1) was quite similar to those observed by other authors. Maharaj et al. (2011) found average values ranging from 46.0 to 66.5 ovules per ovary; Lachenaud (1991) found values ranging from 40 to 65 ovules and Cilas, Machado and Motamayor (2010), evaluating 110 clones, found values ranging from 38 to 65 ovules per ovary.

Regarding to mating system in cacao, studies have focused on quantifying the frequency of selfing/

outcrossing as reported by Harland and Flechville (1927), Voelcker (1940), Posnette (1950) and Yamada and Guries (1998), but another approach have also been suggested by Cruden (1977) and it was tested in this study. Cruden (1977), after evaluating around 100 plant species, proposed that the proportion of pollen grains to ovules (pollen-ovule ratio) was a good indicator of the mating system of that species, reflecting the pollination efficiency. In cacao, despite of the many studies on the frequency of outcrossing using phenotypic and molecular markers (Harland and Flechville, 1927; Voelcker, 1940; Posnette, 1950; Yamada and Guries, 1998), there is only one study estimating the pollen-ovule ratio and using only two germplasms (Godoy et al., 2009). The average pollen-ovule ratio observed in the present study was 88.0, ranging from 30.7 in Sca-6 to 186.2 in ICS-1. Therefore, based on Cruden's (1977) scale, cacao should be classified between a facultative and an obligate autogamous species. Godoy et al. (2009), estimating the pollen-ovule ratio in two cacao clones, one self-compatible (CCN-51) and one self-incompatible (TSH-1188), observed values of 7.2 and 7.5 for these clones, respectively, suggesting incorrectly that cacao should be classified as obligate autogamous by Cruden's scale. With these pollen-ovule values, cacao should be better classified as cleistogamous, instead obligate autogamous. In addition, Godoy et al. (2009) also did not find any statistical differences between the two clones, even though one of them was self-compatible and another self-incompatible. Similarly, we also compared the pollen-ovule ratio averages of 23 self-compatible clones with that of seven self-incompatible ones, and we did not find statistical differences between those two groups of clones, neither for the pollen-ovule ratio (87.4 vs. 90.1, respectively), nor for the number of pollen grains (4467 vs. 5121, respectively).

The results of mating system observed in the present study, as well as those found by Godoy et al. (2009), based on the pollen-ovule rate, suggesting a cleistogamic or facultative autogamous system for cacao, respectively, disagree of results based on selfing/outcrossing rates. For example, Harland and Flechville (1927), in Trinidad, using the phenotypic marker axil spot, found an outcrossing rate of 30%. Posnette (1950), in Ghana, using axil spot and albino leaves traits, found an outcrossing rate of 18% to 43%. Moreover, as pointed out before, cacao flowers have

several physiological and morphological barriers to self-pollination, including self-incompatibility (Knight and Rogers, 1955; Cope, 1962), stamens enclosed in petal hoods (Knoke, Soria and Chapman, 1980), protandry (Claus et al., 2018) and heterostyly.

In summary, many studies have pointed a pollination deficit in cacao, with many flowers receiving little or no pollen grains in the pistil (Parvais, Reffye and Lucas, 1977; Reffye et al., 1978; Tovar and Ortis, 1991; Lachenaud, 1994; Mena-Montoya et al., 2020). Complementary pollination has been successful in reducing the yield gaps from poor pollination (Soria, 1974; Groeneveld et al., 2010; Toledo-Hernández, Wanger and Tschardtke, 2017; Nakayama, 2018). In addition, some authors (Cilas, 1988) have observed that increasing the number of pollen grains in the anthers, increases the number flowers receiving enough pollen for fruit setting. Therefore, breeding for high number of pollen grains should be pursued; particularly considering that, as observed in the present study, there is a large variability for the number of pollen grains per flower among germplasm available to cacao breeders. Also, comparing the results of pollen-ovule ratios found in this study with the mating system classes proposed by Cruden (1977), cacao should be facultative or obligate autogamous. However, with high rates of crossing observed in cacao (Harland and Flechville, 1927; Voelcker, 1940; Posnette, 1950; Yamada and Guries, 1998), this apparently is not the case.

### Conclusions

There is genetic variability among clones for the number of pollen grains produced per flower, allowing breeding for this trait and eventually reducing yield gaps due to poor pollination. Despite the interaction clone x season, on average clones IMC-67, ICS-1, CCN-10, ICS-9 and BN-34 produced more than 7000 pollen grains per flower, being good candidates for breeding aiming high pollen production and also as pollenizers in supplementary pollination programs. We also observed variability for the number of ovules and for the pollen-ovule ratio. This ratio was not different in self-compatible and incompatible clones. Overall, this study contributes information to breeders interested in developing varieties with more efficient pollination mechanisms and higher number of beans, contributing to increased yield production.

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