

Relations between several traits linked to sexual plant reproduction in *Theobroma cacao* L.: number of ovules per ovary, number of seeds per pod, and seed weight

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Abstract Tropical perennial plants cultivated outside their place of origin call for major germplasm characterization and prebreeding work. For cacao breeding, the selection process has to optimize the weight of cacao beans produced per tree by increasing the number of pods produced, the number of seeds per pod (NoSP), and the mean weight of seeds produced (SW). NoSP and SW were studied over nine consecutive years in a collection of more than 200 clones. The number of ovules per ovary (NoOV) was also examined in a subsample of this collection in order to evaluate the ability to predict NoSP. NoSP and SW showed a relatively normal distribution with heritabilities of 0.29 and 0.51, respectively. The composite trait “mean seed weight per pod” showed a generalized extreme value distribution of intermediate heritability (0.43). NoSP and SW were found to be genetically independent. NoOV was highly heritable ($h^2=0.810$), but it did not provide good prediction of NoSP. The largest seeds were observed in genotypes derived from crosses between cultivated cacao trees, which had been formerly domesticated. Domestication therefore favored seed size, while for the

other traits examined, similar variation was found between wild and domesticated populations.

Keywords Domestication · Heritability · Fruit traits · Seed traits · Trait evolution

Introduction

Perennial plant species from the humid tropical regions can often reproduce asexually (Kinsman 1990), but the production of viable seeds still remains a necessity for the majority of perennial species. The production of viable seeds and their conservation and germination ability are, therefore, “vital” traits that are regulated through natural selection (Dalling et al. 1998). The quantity of seeds produced can, therefore, be a trait that has a selective advantage in a natural environment (Howe and Smallwood 1982). However, depending on how they are dispersed, seed size is not necessarily a trait under selective pressure, especially when animal species facilitate dispersal (Wehnecke et al. 2003). In such cases, successful seed dispersal is dependent on the choice made by the animal carrier (Gautier-Hion et al. 1985). For decades, several tropical perennial plants from the humid forests have been removed from their natural environment for agricultural use. Their targeted traits for human exploitation vary from those that enable the best survival of those species in their natural environment. Most agricultural practices and plant breeding are geared towards adaptation to human needs. Some such plants are cultivated for the production of their seeds, also called beans, such as cacao (*Theobroma cacao* L.). Genetic improvement of traits involved in sexual reproduction is, therefore, important. In this case, some of the target traits provide a selective advantage in a natural environment.

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However, knowledge of the variability of those traits in the original populations as well as their links with the species dispersal process are, therefore, necessary in order to adapt strategies for characterisation germplasm and for genetically improving the species.

Plant species cultivated for their seeds are often selected for a combined increase in both the number of seeds produced and their mean weight (Leroy et al. 1994; Hay 1995). Studying floral biology, and pollination conditions in general, contribute to a better understanding of these traits (De Reffye et al. 1978; Lescourret et al. 1997; Hayter and Cresswell 2006). For certain fruit-bearing plants, the number of fruits produced and the number of seeds per fruit can be directly related to pollination conditions (Cane and Schiffhauer 2003; Falque et al. 1995) to compatibility factors between pollen and ovule (Lanaud et al. 1987; Reinartz and Donald 1994) or to ovule fertility, which can vary between species (Gömöry et al. 2003).

The cacao plant (*T. cacao*) is a neotropical, small, evergreen tree native to South America (Motamayor et al. 2002). This plant of the Malvaceae (formerly Sterculiaceae) family is grown for its fruits, known as cacao pods, though botanically, its fruits are actually berries (von Balthazar et al. 2004). The cacao tree is a perennial plant with undefined growth and fruit production that can vary from 20 to more than 60 years, with fruits growing on both the trunk and the branches. The fruits arise from the pollination of flowers grouped in flower cushions (Swanson et al. 2008). The pods contain seeds are fermented with surrounding mucilage then dried to produce fermented dried cacao, the raw material used to make chocolate. The production of commercial cacao beans from one tree over a given period depends on the number of pods produced, the number of seeds per pod, and the mean seed weight (Lachenaud 1995). The production of a tree i between date t and date $t+s$ ($Y_i[t, t+s]$) can be written as:

$$Y_i[t, t+s] = \text{NoPods}_i[t, t+s] \times \text{NoSP}_i[t, t+s] \times \text{SW}_i[t, t+s]$$

where:

$\text{NoPods}_i[t, t+s]$	number of pods produced by tree i between date t and date $t+s$
$\text{NoSP}_i[t, t+s]$	mean number of seeds per pod for tree i between date t and date $t+s$
$\text{SW}_i[t, t+s]$	mean seed weight for tree i between date t and date $t+s$

The number of pods produced often depends on numerous environmental factors, and the heritability of this trait, estimated in various situations (Cilas 1991; Cilas et al. 1999), is relatively weak (<0.4). In fact, the trait depends on many parameters, including flowering, pollination, fruit-setting, cherville wilt, diseases, and insect attacks (Nyassé et al. 2007; Sounigo et al. 2003). Increasing the number of pods

produced remains an objective that can be achieved taking different approaches: improved agronomic practices, plant breeding, better pest, and disease control. The genetic value of a given genotype for the number of pods produced will be in interaction with other traits, such as disease and pest resistance, the ability to bear flowers, or susceptibility to cherville wilt.

The number of seeds per pod and the mean seed weight are also traits that can be improved to increase production per tree. The number of seeds per pod depends on various factors, including the number of ovules per ovary, the fertility of the ovules, which notably varies depending on the reproductive self-compatibility or self-incompatibility of the plant material and natural pollination conditions. Mean seed weight is an important trait with good heritability (Cilas et al. 1989), although the regularity of seed size is the main trait of interest for the chocolate industry.

To gain a clearer understanding of variability in the “number of seeds per pod” and “mean seed weight” traits, as well as their interdependence, they were assessed in a germplasm collection located in the State of Bahia in Brazil. The traits were evaluated for more than 200 clones over a period of 9 years. Importantly, the large number of clones and their extensive period of observation enabled a reliable estimation of the combined variability for these two traits in *T. cacao*. Furthermore, for more than half of the clones, the number of ovules per ovary was estimated in order to evaluate the impact of that trait on the number of seeds per pod. This study provides data for defining strategies to characterize the germplasm and for genetic improvement of *T. cacao* and other similar species with identical mating systems.

Material and methods

Plant material

Observations took place over nine consecutive years, from 1998 to 2006, in a collection of cacao trees at the MARS Center for Cocoa Science (Mars Inc.). The collection contains 221 clones, not only from Brazil but also from various other American countries and the Caribbean. The clones could be considered as representative of the species *T. cacao*, some being wild, i.e., coming directly from the Amazon forest considered as the center of diversification for this species (Cheesman 1944), and others selected for their desired agronomic traits. Each clone was planted in a unit plot of 16 trees, but the number of trees per plot could be smaller due to mortalities over the observation period.

Observations

Over the 9 years of observations, monthly harvests were carried out in the unit plots from the moment the trees started

bearing ripe pods. The mean NoSP and the SW were evaluated for each of the 221 clones and for each harvest. A third trait was derived from observations of the first two: the mean weight of seeds per pod: $WoSP = NoSP \times SW$. Seed weight was estimated after fermentation and drying, which are the conventional processes used to produce commercial cacao beans. Data were collected sequentially over time through successive harvests. Furthermore, for 114 of the clones, the number of ovules per ovary (NoOV) was estimated by counting the number of ovules for 10 flowers per clone.

Data analysis

The REML method (Corbeil and Searle 1976) was used to estimate the different variances (“clone” and “error” variances) for the four traits. Broad-sense heritability values were estimated for the traits, along with the associated confidence intervals, estimated by the Wald method (Agresti and Coull 1998). Estimations of heritabilities were given by the ratios of genetic variances—i.e., “clone” variances—and phenotypic variances (Cilas 1991):

$$h_b^2 = \frac{\sigma_G^2}{\sigma_P^2} = \frac{\sigma_c^2}{\sigma_c^2 + \sigma_e^2}$$

Where:

h_b^2 broad sense heritability
 σ_G^2 and σ_P^2 are, respectively, genetic and phenotypic variances
 σ_c^2 and σ_e^2 are, respectively, clone and error variances.

Genetic and phenotypic correlations were then estimated between NoSP and SW. The random model was applied for multivariate analysis, allowing for an estimation of genetic covariances and correlations between the traits (Hill 1971). The genetic correlation between the NoOV and the NoSP was also estimated. Various selection indexes were constructed graphically on the graph (x, y) of the genetic values for the two elementary traits (SW and NoSP). This method enabled interaction with the dataset of genetic values and choice of the regression line that optimized the relative genetic gains for each of the two traits. Data analyses were performed with SAS 9.1.

Results

Variability and heritability of the traits studied

The distribution of the NoSP for all the 5752 samples harvested approximated a normal distribution (Fig. 1), with the number of seeds per pod varying from three to 63 and with a mean of 29.8 seeds per pod. The mean number per

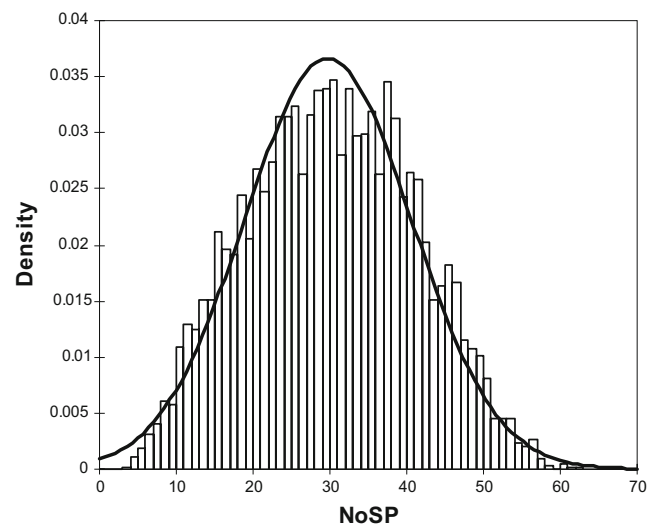


Fig. 1 Distribution of the number of seeds per pod (NoSP)

genotype varied from 12 for clone VB 265 to 46 for clone IMC 47; the clone classification (not shown) was not structured by the origin of the clones (clone series name). This widespread distribution of means did not distinguish wild clones from domesticated clones. The distribution of the SW could also be approximated to a normal distribution (Fig. 2). The seed weight varied from 0.3 to 2.8 g with a mean of 1.2 g. The mean per clone varied from 0.71 g, for clone Ucayali 6, to 2.50 g, for clone CCN 16. Most of the genotypes with large seeds (>1.70 g) corresponded to cultivated genotypes, having formerly domesticated Trinitario or Criollo ancestors (Motamayor et al. 2002). In the wild genotypes, large variations could exist within populations from the same origin: in particular, the wild Guianese population had a mean weight of one seed ranging from 0.90 g for GU 151 to 1.45 g for GU 239H. However, certain origins were characterized by having uniformly small seeds, such as the genotypes derived from the Scavina or Ucayali populations, both collected in the Upper Amazon region of Peru. The distribution of the mean seed weight per pod, corresponding to the product of the former two traits, did not follow a normal distribution. With the exception of a very large sample, it is known that the product of two normal distributions does not follow a normal distribution but a Log-normal distribution or a related distribution (Lomnicki 1967). The distribution of the mean seed weight per pod fitted well to a generalized extreme value (GEV) distribution (Fig. 3). The GEV distribution is commonly used for dissymmetric distributions with extreme values (Hosking et al. 1985). The mean of this trait ranged from 16 g for clone Ucayali 54 to 73 g for clone CCN 16.

SW and NoSP broad-sense heritabilities for these traits were estimated. The SW trait displayed the highest heritability, close to 0.5 (Table 1), while the NoSP trait

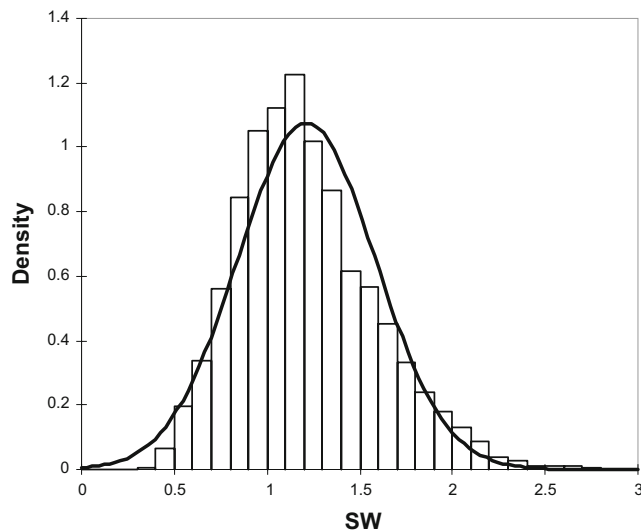


Fig. 2 Distribution of the seed weight (*SW*)

showed the lowest heritability, close to 0.3. weight of seeds per pod (*WoSP*), a composite trait ($SW \times NoSP$), had intermediate heritability, close to 0.4 (Table 1). The confidence intervals for these estimations were narrow, mainly due to the large number of pods and clones observed.

The number of ovules per ovary estimated in a subsample of 114 clones showed a normal distribution. The mean number of ovules per ovary ranged from 38 for clone Pa 8 to 65 for clone Amaz 15/15. It was not possible to structure the population according to clone origin (clone series name) using the “number of seeds per pod” trait. The estimated heritability was very high, 0.8 (Table 1); this trait had the highest level of heritability of all the traits studied. Moreover, the number of ovules per ovary is considered as a clonal descriptor (Bekele et al. 1994).

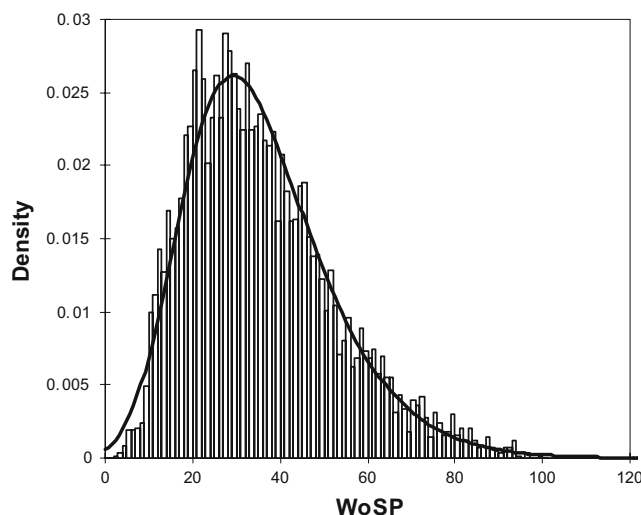


Fig. 3 Distribution of the weight of seeds per pod (*WoSP*)

Table 1 Heritabilities of the four traits studied with a confidence interval at 5%

Trait	NoSP	SW	WoSP	NoOV
h^2	0.290	0.507	0.427	0.812
Confidence interval	[0.248; 0.331]	[0.458; 0.556]	[0.379; 0.474]	[0.770; 0.856]

NoSP number of seeds per pod; *SW* seed weight; *WoSP* weight of seeds per pod; *NoOV* number of ovules per ovary

Correlations between the number of seeds per pod and seed weight

The phenotypic correlation between the number of seeds per pod and the mean seed weight was negative and significant (Table 2). Partitioning of this correlation into a genetic correlation and an environmental correlation revealed that the environmental correlation was negative and significant and that the genetic correlation was close to zero and, therefore, not significant (Table 2). This result indicated that the *NoSP* and *SW* traits were not genetically correlated and that it was, therefore, possible to select material with a large number of seeds per pod and a high mean seed weight. Conversely, the negative and significant environmental correlation indicated that for a given clone, the mean seed weight would tend to be lower for pods containing more seeds. Partitioning of the phenotypic correlation, therefore, inferred that these two traits were not genetically correlated but that there might exist an environmental or physiological dependence in the expression of the two traits.

Selection index versus the multiplicative trait

Two strategies can be used to select plant material with a higher seed weight per pod: either selecting directly for the composite trait: $WoSP = NoSP \times SW$ or constructing a selection index by assigning different weights to each of the two elementary traits. Direct selection for the composite trait entails several problems: this trait does not have a normal distribution, an environmental correlation exists between the two elementary traits that compose it, and no preference can be made for each of the two elementary traits. Using a selection index, several weighting systems can be employed. Since only two traits are involved in the

Table 2 Phenotypic, genetic, and environmental correlations between the number of seeds per pod (*NoSP*) and the seed weight (*SW*)

Correlation	Phenotypic	Genetic	Environmental
Values	-0.1143	0.0014	-0.2272
<i>P</i> values	<0.001	0.7623	<0.001

construction of this index, the different weights were tested graphically, in such a way as to interact easily at the relative selection intensities. The first weighting enabled selection of exactly the same clones as the direct selection method for the composite trait; with a selection level of around 7.5%, 17 of the 221 clones were selected (Fig. 4). Finally, the second weighting favoring SW (Fig. 5) seemed preferable because SW had a higher level of heritability and would, therefore, have a better response to selection. Moreover, a larger seed size is often considered a desirable trait by cacao farmers.

Influence of the number of ovules per ovary on the number of seeds per pod

Theoretically, with standard pollination conditions and identical ovule fertility for each clone, there should be a perfect linear relationship between the number of ovules per ovary and the number of seeds per pod. However, the correlation between the means of NoOV and NoSP per clone was only 0.48. The number of ovules per ovary only explained about 23% of the linear variation in the number of seeds per pod (Fig. 6). The number of seeds per pod was, therefore, strongly dependent on other factors affecting ovule fertilization: ovule fertility, reproductive self-compatibility or self-incompatibility of the clone, and the external pollination conditions, which could vary from one clone to another in a given location.

Discussion

Combined variability of the number of seeds per pod and seed weight

The mean seed weight showed a normal distribution and a high level of heritability (≥ 0.5), confirming previous studies

with similar results (Enriquez and Soria 1968; Engel 1983; Cilas et al. 1989). It is, therefore, relatively easy to increase seed size in the species *T. cacao* by conventional selection methods. The number of seeds per pod also showed a normal distribution. This distribution was due to the large number of pods observed (more than 5,000 pods). In fact, the number of seeds per pod for a given clone often shows a dissymmetric distribution, because of uneven, often insufficient pollination (Parvais et al. 1977; De Reffye et al. 1978; Falque et al. 1995; Lachenaud 1994, 1995). The heritability of the trait had an average value of 0.3. Selection to increase the number of seeds per pod can also be achieved by conventional selection methods, even though the selection response will be less efficient than for seed weight. The mean WoSP, which was defined as the product of the previous two traits, did not show a normal distribution. This variable fitted to a generalized extreme value distribution. The heritability of the trait was intermediate to the level of heritabilities for the two composing traits, in the order of 0.4.

The phenotypic correlation between the mean SW and the NoSP was negative and significant. However, this correlation could be divided into two correlations depending on the bivariate linear model used: a genetic correlation and an environmental correlation. The genetic correlation was close to zero, meaning the two traits were genetically independent. On the other hand, there was a negative and significant environmental correlation between these two traits, meaning that among clones an increase in seed number would lead to a decrease in seed size. This correlation was not genetic but physiological. It is, therefore, possible to increase seed weight and the number of seeds per pod by breeding, as these two traits are independent. On the other hand, agronomic practices increasing the number of seeds per pod will tend to result in a small decrease in seed weight.

Fig. 4 Selection index (first weighting: same selection as direct selection for WoSP)

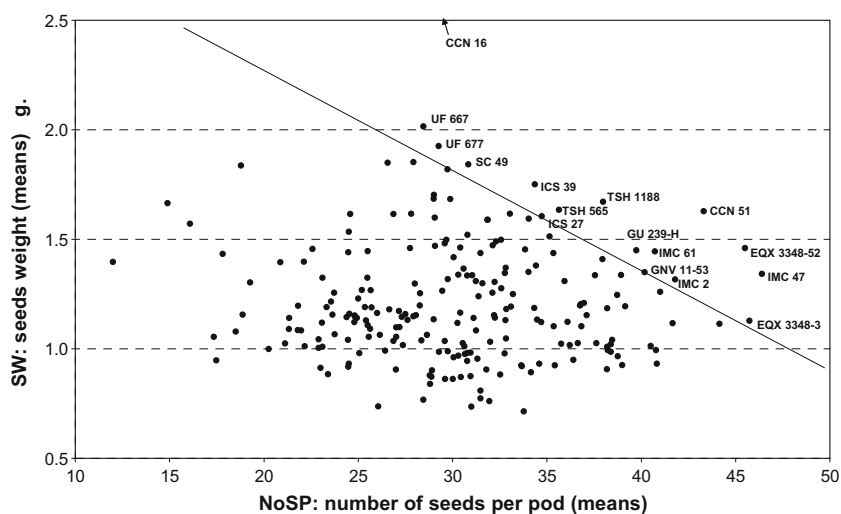
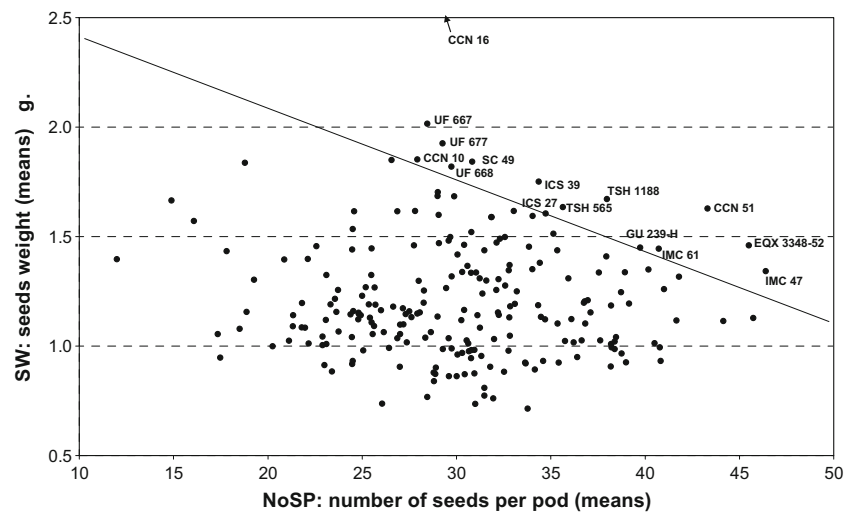


Fig. 5 Selection index (second weighting)



Implications for *T. cacao* genetic improvement strategies

To increase WoSP through breeding, it is, therefore, possible either to select directly for this composite trait or to construct a selection index with the two single traits, SW and NoSP. The latter solution seems preferable because (1) the two single traits follow a normal distribution—whereas the WoSP trait does not; (2) this method gives much more flexibility and allows selection of the desired trait by varying the “weighting system” (Ndoumbé et al. 2001).

Cacao is not the only species for which seed production can be expressed as the product or result of various single traits. In fact, in the oil palm (*Elaeis guineensis*), the selection scheme rapidly turned to reciprocal recurrent selection between a group of oil palms with a small number of large fruit bunches and a group with a large number of

small fruit bunches (Baudouin et al. 1997). In the case of cacao, of the single traits mentioned (number of pods produced, number of seeds per pod, and mean seed weight), the mean seed weight was the trait with the highest level of heritability. The other two traits would require greater control of environmental conditions and further knowledge to master their improvement more effectively.

Number of seeds per pod

The number of seeds per pod was dependent on the number of ovules per ovary. The latter trait has very high heritability in *T. cacao*, but it only weakly explains the variation in the number of seeds per pod. The number of seeds per pod also depends on the fertility of the ovaries and the ability of clones to be effectively pollinated. Large differences in ovary fertility are possible, and fertility is dependent on compatibility factors between the pollen and the ovules. Some clones are self-compatible while others are self-incompatible, but self-incompatibility is not strict and a small quantity of allopollen can sometimes overcome incompatibility (Lanaud et al. 1987; Warren et al. 1995b). Furthermore, it is possible that some clones are better pollinated than others because they are more attractive to insects (De Reffye et al. 1980). A greater understanding of this trait is necessary, especially for making better use of available genetic resources for selection.

Domestication of the species *T. cacao* and evolution of the traits studied

Recent collections from the species' zone of origin, notably French Guiana (Lachenaud and Zhang 2008) and in cultivated areas (Efombagn et al. 2008), indicate that the natural genetic resources are far from being completely exploited. Recently, several wild genotypes have been

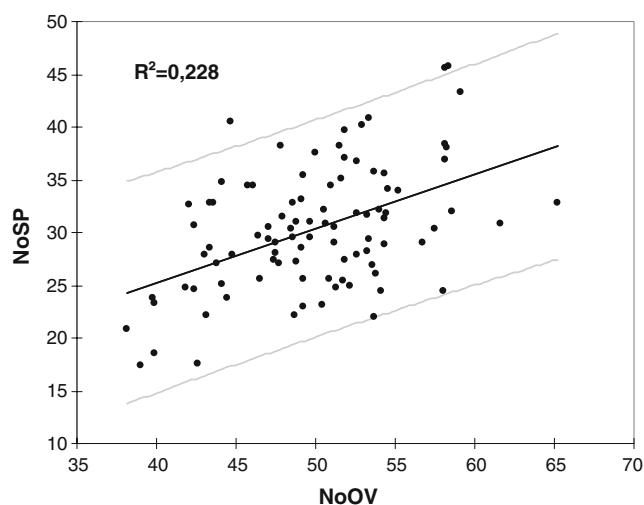


Fig. 6 Regression between number of seeds per pod and number of ovules per ovary: $\text{NoSP} = 0.512 \times \text{NoOV} + 4.80$; with a 95% confidence interval

identified as sources of resistance to certain diseases (Paulin et al. 2008), and new collections need to be made in the species' area of origin in the coming years. The traits related to sexual reproduction appear very variable in wild populations, such as the Guianese population, thus providing the possibility of finding wild genotypes displaying worthwhile traits for productivity. The domestication of the species has clearly had an impact on the evolution of the “mean weight of one seed” trait; genotypes derived from crosses with a parent formerly domesticated by the Maya have larger seeds. However, this trait is only one of the components of yield efficiency and future surveys should identify genotypes with a large number of seeds per pod to guide selection of the seed weight per pod towards significant yield gains. It was found that self-compatibility was another trait for which selection was practiced because it favors the number of pods produced (Warren et al. 1995a). However, Warren et al. (1995a) worked in a commercial plantation environment, where constraints other than those existing in a natural environment also occurred. The high variability within cacao wild populations indicates that no strong selection pressure exists on the traits related to reproductive biology, due to the coexistence of different reproduction strategies (sexual and asexual).

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