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Molecular genetic diversity and mating system in sacha inchi progenies[1]

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ABSTRACT

Sacha inchi (*Plukenetia volubilis* L.) is a species with nutraceutical benefits traditionally consumed by Amazonic indigenous and urban communities. Studies on the genetic diversity and mating system are required to preserve and make the best use of the genetic resources for this species. This study aimed to estimate the genetic diversity and mating system parameters of sacha inchi progenies using Amplified Fragment Length Polymorphism (AFLP) markers. A total of 360 progenies from 30 accessions of sacha inchi were analyzed using three AFLP primers combinations. The percentage variation between and within families and the crossing rate, among other parameters, were estimated. The AFLP markers were efficient in genetically differentiating progenies, revealing 251 (98.82 %) polymorphic fragments. The analysis of molecular variance showed that the strongest variation occurs between progenies (57.16 %). However, the genetic differentiation within progenies was considerable (42.84 %), and could be exploited in breeding programs. The estimated population outcrossing rate was high (0.957), indicating it is a predominantly allogamous species. On the other hand, 33.7 % of the crosses occurred between related individuals. The estimate for biparental crosses revealed that the progenies consisted mainly of half-siblings (66.9 %) and full-siblings (28.8 %). For purposes of breeding and *ex situ* genetic conservation, sacha inchi seeds should be collected from a large number of parent plants.

KEYWORDS: *Plukenetia volubilis* L.; plant breeding; AFLP marker.

RESUMO

Diversidade genética molecular e sistema reprodutivo em progênies de sacha inchi

Sacha inchi (*Plukenetia volubilis* L.) é uma espécie com benefícios nutracêuticos tradicionalmente consumida por populações indígenas e urbanas amazônicas. Estudos de diversidade genética e do sistema reprodutivo são necessários para a conservação e uso dos recursos genéticos da espécie. Objetivou-se estimar parâmetros de diversidade genética e do sistema reprodutivo de sacha inchi, utilizando-se marcadores Amplified Fragment Length Polymorphism (AFLP). Um total de 360 progênies de 30 subamostras da espécie foram analisadas, com três combinações de *primers* AFL. Foram estimadas, dentre outros parâmetros, a porcentagem de variação entre e dentro de famílias e a taxa de cruzamento. Os marcadores AFLP foram eficientes na diferenciação genética das progênies, revelando 251 (98,82 %) fragmentos polimórficos. A análise de variância molecular mostrou que a maior parte da variação ocorre entre famílias (57,16 %). No entanto, a diferenciação genética dentro destas foi considerável (42,84 %), podendo ser explorada em programas de melhoramento. A estimativa da taxa de cruzamento multiloco foi alta (0,957), indicando se tratar de uma espécie predominantemente alógama. Por outro lado, 33,7 % dos cruzamentos ocorreram entre indivíduos aparentados. A estimativa para cruzamentos biparentais revelou que as progênies são constituídas principalmente por meios-irmãos (66,9 %) e irmãos-completos (28,8 %). Para fins de melhoramento e conservação genética *ex situ*, sementes de sacha inchi devem ser coletadas de um grande número de plantas matrizes.

PALAVRAS-CHAVE: *Plukenetia volubilis* L.; melhoramento vegetal; marcador AFLP.

INTRODUCTION

Sacha inchi (*Plukenetia volubilis* L.) is a species of the Euphorbiaceae family native to the Amazon rainforest and originated in the Peruvian, Colombian, Venezuelan and Brazilian Amazon (Bordignon et al. 2012). It is a semi-woody perennial climber, and has been known to bear fruit for up to 10 years, when grown in Peru (Céspedes 2006). It is monoecious, with racemose inflorescences. The staminate flowers are small, white and feature a globular receptacle, while pistillate flowers are located at the base of the inflorescence and can produce seeds throughout the year, especially in

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tropical regions. They are pollinated mainly by anemophilia and entomofilia, and the most important pollinators are Formicidae (Anteparra et al. 2014). According to Cai et al. (2013), sacha inchi is a polyploid species, with chromosomes numbers ranging from 2n = 50 to 2n = 86.

Sacha inchi is a new crop that has gained prominence due to its nutritional and nutraceutical benefits. The oil extracted from the seeds contains higher levels of protein, vitamin E and essential fatty acids than those obtained from other oilseeds such as peanut, soybean and sunflower (Hamaker et al. 1992, Follegatti-Romero et al. 2009, Valente et al. 2017a). Sacha inchi is still being studied by the pharmaceutical industry and, due to its rapid growth and good soil cover, it has characteristics favorable to reforestation and protection of slopes (Bordignon et al. 2012).

In Brazil, despite the increasing number of studies carried out on sacha inchi in recent years (Rodrigues et al. 2013, Cardoso et al. 2015, Valente et al. 2017a and 2017b), the plant is still in the semi-wild condition, although it has a great potential for development in the Amazon region. Research in several areas are needed in order improve the crop.

Identifying genetically superior progenies that may be recommended for use in seed production would be an important step for improving the crop in Brazil (Valente et al. 2017a). However, previous studies are necessary to provide a basis for strategies to make the best of the available genetic resources and to maintain a broad genetic base within breeding programs (Ferreira et al. 2012). Genetic markers are a highly efficient tool for investigating the variability between and within progenies. The data obtained may help making important decisions, regarding the management and conservation of plant resources. For instance, in reciprocal recurrent selection programs, the knowledge of genetic diversity between and within progenies may speed up the development of new varieties (Ferreira et al. 2012).

Another important aspect of *P. volubilis* breeding relates to the crossing rate. Traditional methods for evaluating the reproductive system of the species, based on the observation of hybridizations, pollinator behavior, examination of floral morphology and results of controlled pollination experiments, have been used by Cachique (2006). According to the author, cross-pollination (allogamy) proved to be the most efficient breeding system. However, the methods used only provided indications regarding the sacha inchi mating system and did not provide a basis for accurately assessing the crossing rate.

This information will be useful in assessing the human action on sacha inchi populations, so that optimized strategies can be determined for sampling genetic variability (Alves et al. 2015) and appropriate genetic and statistical models selected for estimating genetic parameters (Valente et al. 2017a). It will also help in defining genetic conservation and breeding strategies (Alves et al. 2015, Sharma et al. 2017).

Amplified Fragment Length Polymorphism (AFLP) markers have been used to estimate crossing rates in open-pollinated progenies of several species (Santos & Lima Neto 2011, Hornemann et al. 2012, Santos & Gama 2013, Sharma et al. 2017). Despite the limitations of these markers, due to their dominant nature, the high degree of polymorphism that they usually reveal offsets this limitation in the analysis for determining the characteristics of the reproductive system. Studies such as those by Santos & Lima Neto (2011) and Sharma et al. (2017) show how the crossing rates obtained by AFLP markers and SSR markers are very similar in mango varieties (*Mangifera indica*) and Indian beech (*Pongamia pinnata*), respectively.

Since there is no literature on the reproductive system of *P. volubilis* based on molecular markers, and little is known about how genetic variation in individual plants is distributed, studies of this kind are necessary to speed up domestication and breeding. Thus, this study aimed to evaluate the genetic diversity between and within sacha inchi progenies and to determine the cross-breeding rate using AFLP molecular markers, in order to provide information for conservation and genetic breeding programs.

MATERIAL AND METHODS

This study evaluated the genetic diversity of the *P. volubilis* active germplasm bank in the medicinal plants section of the Embrapa Amazônia Ocidental, in Manaus, Amazonas state, Brazil. A total of 30 undomesticated accessions of the species were examined in this study, including 25 accessions (numbered 1 to 25) collected in the interior of the Amazonas state and further 5 accessions (numbered 26 to 30) collected at the Nova Jerusalém site, in Careiro Castanho, Amazonas state (3º31'45.0''S,

59º49'07.9''W). All accessions are mother families (progeny from a single tree) and were collected in their natural habitats in the Amazon rainforest.

The *in vivo* germplasm collection was set up in the field by sowing in January 2013. A randomized block experimental design was used, with five replications and two plants per plot, spaced at 3 m x 3 m and supported by a system of vertical stakes.

For purposes of genetic characterization, 12 pollinated seeds from one plant from each accession were randomly selected, duly identified and stored in a cold room for subsequent planting in styrofoam trays containing Tropstrato HT commercial substrate. At 21 days after planting, samples of plant material were collected from younger leaves of the progenies for subsequent DNA extraction. A total of 360 plants were evaluated (twelve plants per accession).

For DNA extraction, the CTAB protocol (Ferreira & Grattapaglia 1998) was used, with some modifications. Extractions from fresh leaves provided cleaner pellets that did not require cleaning with NaCl. Approximately 800 mg of fresh leaf tissue from each sample were macerated with liquid nitrogen, excluding the main vein. The quality of the extracted DNA was examined by electrophoresis in 0.7 % agarose gel, and the DNA quantified by absorbance measurements under UV light (260 nm and 280 nm), in a spectrophotometer.

The AFLP marker analysis was performed according to Vos et al. (1995), with modifications introduced by Lopes et al. (2003). For the DNA digestion reactions, a combination of EcoRI/MseI restriction enzyme was used in 200 ng of genomic DNA. Primers were used in pre-amplification reactions, complementing the restriction enzyme site sequences with a selective nucleotide. A total of twenty primers combinations were tested on a sample of sixteen individuals from the collection. The three primers with the best amplification results, both in terms of amount of polymorphism and reading quality, were used on all individuals. In the selective amplification reactions, each sample was applied to the vertical electrophoresis cell in 6 % polyacrylamide gel [acrylamide/bis-acrylamide (19:1 v/v)], under 50 W constant power, for 4 h. To develop the gel, the silver nitrate staining method was used, according to the protocol proposed by Creste et al. (2001).

From a careful reading of the polyacrylamide gels, a binary matrix was constructed, in which zero and one indicated the absence or presence of fragments. The data obtained were used to construct a genetic distance matrix between accessions (mother family) based on the Nei method (Nei 1978). To visualize the genetic differentiation between the mother families, a dendrogram was constructed by UPGMA analysis, using the R statistical software (R Development Core Team 2017). The compatibility of the cluster analysis with the original data was evaluated based on the cophenetic correlation coefficient. The consistence of the dendrogram nodes was evaluated based on 10,000 bootstraps performed with the pvclust R package (Suzuki & Shimodaira 2006).

Based on the assumption that the progeny set was in Hardy-Weinberg equilibrium, the Popgene version 1.32 (Yeh et al. 1999) was used to calculate the genetic variation within each of the 30 families. The estimates of genetic diversity included the expected heterozygosity (Nei 1978), Shannon & Weaver genetic diversity index (Shannon & Weaver 1949) and the percentage of polymorphic loci.

The distribution of genetic variation between and within families was determined using the analysis of molecular variance (Amova) (Excoffier et al. 1992). The significance test for the estimated parameters was run based on 10,000 bootstrap resamples.

P. volubilis crossing was assessed based on the mixed-crossing model and correlated according to the MLTR software (Ritland 2002). The mixed-crossing model assumes that the progenies result from a combination of self-fertilization and cross-fertilization.

The following parameters were estimated at the population level: fixation index ($\hat{F}_m$); multilocus crossing rate ($\hat{t}_m$); single-locus crossing rate ($\hat{t}_s$); crossing rate among related individuals ($\hat{t}_m - \hat{t}_s$); self-fertilization correlation ($\hat{r}_s$); multilocus paternity correlation [$\hat{r}_{p(m)}$]; and single-locus paternity correlation [$\hat{r}_{p(s)}$]. To obtain estimates, the Newton-Raphson method and a 100-bootstrap confidence interval were used, with progenies as the resampling unit. These parameters were also used to estimate the effective number of pollinating trees [$\hat{N}_{ep} = 1/\hat{r}_{p(m)}$] and define the proportion of different types of progenies (half-siblings, full-siblings and self-fertilization) found in the offspring generation, as described by Gusson et al. (2006).

RESULTS AND DISCUSSION

The three combinations of AFLP primers analyzed in 360 individuals of sacha inchi revealed high levels of polymorphism (Table 1). A total of 254 fragments were detected, 251 (98.82 %) of which were polymorphic. The total number of polymorphic fragments varied from 70 to 104, with an average of approximately 84 fragments per primer combination. In a study of the same species, Ocelák et al. (2015), assessing 173 sacha inchi accessions using ISSR markers, observed an average of only eleven polymorphic fragments per primer combination. Also studying sacha inchi, Rodrigues et al. (2013) obtained 191 polymorphic fragments using four AFLP primers combinations.

Genetic similarity relationships between mother families of *P. volubilis* were characterized by a dendrogram (Figure 1). The results obtained show the high variability between the families and suggest that there are six large groups. These cluster data will be important in determining future crosses, in order to capture maximum heterosis. Some of the results may be correlated with the prospecting site for these materials, since accessions 26 to 30 were collected in the same location at the Nova Jerusalém site and are within the same cluster (group VI). Valente et al. (2017a) also reported similar results by evaluating agronomic traits in the same accessions. Note that the cophenetic correlation coefficient (r = 0.8762) indicated a good match to the dendrogram, if compared to the dissimilarity matrix.

Table 1. Polymorphisms obtained in progenies of *Plukenetia volubilis* using three AFLP primers combinations.

Primer combination	Total fragments	Polymorphic fragments	Polymorphism (%)
E + ACA/M + CCA	104	104	100.00
E + AGT/M + CCA	80	77	96.25
E + ACA/M + CAC	70	70	100.00
Total	254	251	98.82

For evaluating the genetic diversity within the mother families, these plants were assumed to be in Hardy-Weinberg equilibrium. Expected heterozygosity values ranged from 0.074 (family 9) to 0.202 (family 1), with the Shannon & Weaver index ranging from 0.112 to 0.295 (Table 2). Parameter values closer to zero indicate a lower diversity. Since the breeding system is affected by genetic and environmental factors, a genetic variation among the seeds of a plant is the norm, especially in allogamous species. In our study, a considerable genetic diversity was observed within the families (especially 1 and 21), and estimated values are comparable to those found in small populations of another forest climber: *Arrabidaea bilabiata* (Souza et al. 2015).

The analysis of molecular variance indicated a significant difference between progenies (variance of 21.68; $p \leq 0.01$), accounting for 57.16 % of the total variation and showing a high level of genetic differentiation (Table 3). The analysis also showed that 42.84 % of the genetic variation was found among individuals within families. This shows that the species is preferably cross-fertilized, since allogamous species retain much of their genetic variability distributed

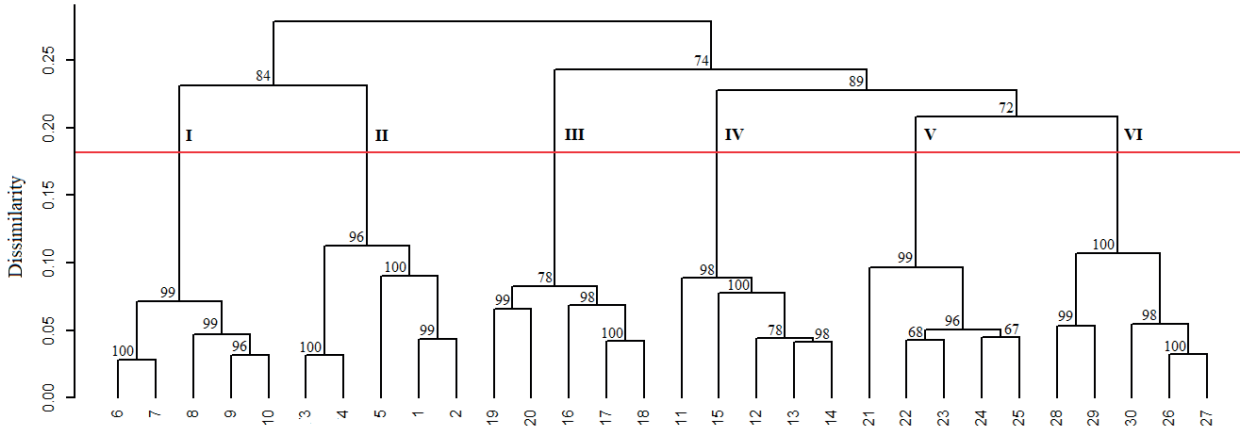

Figure 1. Grouping analysis (UPGMA) of the Nei (1978) genetic distance for 30 mother families of *Plukenetia volubilis*. Cophenetic correlation: r = 0.8762. The horizontal line in red represents the cutoff estimated by the Mojema method. The numbers above each dendrogram node correspond to the values of P for clustering based on 10,000 bootstrap samplings.

within their families, in contrast to self-pollinating species, where most of the genetic variability is between provenances (Ferreira et al. 2012).

Note that the existence of a high genetic variation within families allows them to adapt more easily to different environments, and superior individuals can be selected for breeding purposes. Moreover, if this variability within the germplasm collections is quantified, alleles can be safeguarded for conservation purposes, since some of them may be under threat of genetic erosion due to human exploitation (De Giovanni et al. 2017).

Table 2. Genetic diversity within families of *Plukenetia volubilis*.

Family	P (%)	He	I
1	50.60	0.202 (0.014)*	0.295 (0.019)
2	43.03	0.155 (0.013)	0.231 (0.018)
3	37.05	0.126 (0.012)	0.189 (0.017)
4	46.61	0.163 (0.012)	0.245 (0.018)
5	27.09	0.091 (0.011)	0.137 (0.015)
6	38.25	0.133 (0.012)	0.198 (0.017)
7	35.06	0.120 (0.012)	0.180 (0.017)
8	27.89	0.089 (0.010)	0.135 (0.015)
9	22.31	0.074 (0.010)	0.112 (0.014)
10	21.91	0.076 (0.010)	0.113 (0.014)
11	28.69	0.098 (0.011)	0.146 (0.016)
12	25.10	0.079 (0.010)	0.120 (0.014)
13	29.08	0.094 (0.011)	0.142 (0.015)
14	25.10	0.083 (0.010)	0.125 (0.015)
15	25.90	0.095 (0.011)	0.141 (0.016)
16	36.25	0.118 (0.011)	0.179 (0.016)
17	45.42	0.145 (0.012)	0.220 (0.017)
18	46.61	0.161 (0.012)	0.242 (0.018)
19	44.22	0.145 (0.012)	0.218 (0.017)
20	35.86	0.124 (0.012)	0.185 (0.017)
21	47.41	0.194 (0.014)	0.281 (0.020)
22	40.64	0.152 (0.013)	0.225 (0.018)
23	31.08	0.106 (0.011)	0.158 (0.016)
24	21.91	0.077 (0.010)	0.115 (0.015)
25	36.25	0.132 (0.012)	0.196 (0.018)
26	28.29	0.111 (0.012)	0.163 (0.017)
27	26.29	0.087 (0.010)	0.131 (0.015)
28	33.86	0.105 (0.011)	0.161 (0.016)
29	39.04	0.138 (0.012)	0.206 (0.018)
30	31.87	0.129 (0.013)	0.187 (0.018)

P = polymorphism percentage; He = expected heterozygosity, according to Nei (1978) (assuming Hardy-Weinberg equilibrium); I = genetic diversity index of Shannon & Weaver (1949). * Standard deviations in parentheses.

It is extremely important to know how a species reproduces (Turchetto et al. 2015). The information herein relating to sacha inchi is unprecedented, especially since it is based on the use of genetic markers.

The estimated multilocus crossing rate (Table 4) was high ($\hat{t}_m$ = 0.957), showing that the thirty accessions used in this study had a preference for cross-fertilization, with low levels of self-fertilization (4.3 %). On the other hand, 33.7 % ($\hat{t}_m - \hat{t}_s$ = 0.337) of crosses were between related individuals. This high frequency of crosses among relatives in the germplasm collection was not expected, since the individuals sampled in the collection came from different locations in the interior of the Amazonas state and were cultivated in a randomized block experiment. However, the cluster analysis based on AFLP markers showed that some progeny groups were genetically close, what may have contributed to this high estimated figure. In addition, the rates of crossbreeding per family ranged from 0.835 to 0.999, with no link between genetic diversity and the crossing rates obtained.

Plukenetia volubilis is a monoecious species, with hermaphrodite inflorescences of unisexual flowers. Anthesis begins early in the morning and lasts between 35 and 48 h (Cachique 2006). According to Cachique (2006), the species is also dichogamous. The low self-fertilization rate reported herein is in line with the floral characteristics of the species, which, in some ways, do not favor self-pollination. Thus, our results confirm that sacha inchi shows a preference for allogamy.

Another parameter evaluated herein was the paternity correlation [$\hat{r}_{p(m)}$], which measures the proportion of individuals generated from biparental crosses (successive crosses, even between a pair of

Table 3. Genetic variation, obtained by Analysis of Molecular Variance (Amova), between and within families of *Plukenetia volubilis*.

Source of variation	Degrees of freedom	Sum of squares	Mean squares	Component of variance	Variation (%)
Between families	29	8,015.8250	276.4078	21.6800**	57.16
Within families	330	5,361.5833	16.2472	16.2472	42.84
Total	359	13,377.4083	37.2630	37.9273	100.00
Fst = 0.5716					

** $p \leq 0.01$.

Table 4. Estimated reproductive system parameters for *Plukenetia volubilis*.

Parameter	Estimate (SD)*
Multilocus crossover rate ($\hat{t}_m$)	0.957 (0.014)
Single-locus crossing rate ($\hat{t}_s$)	0.620 (0.030)
Crossing ratio between relatives ($\hat{t}_m - \hat{t}_s$)	0.377 (0.028)
Self-fertilization correlation ($\hat{r}_s$)	0.042 (0.243)
Multilocus paternity correlation [$\hat{r}_{p(m)}$]	0.313 (0.035)
Fixation rate ($\hat{F}_m$)	0.434
Crossings involving related pollinating trees [$\hat{r}_{p(s)} - \hat{r}_{p(m)}$]	-0.027 (0.169)
Average number of donor progenies [$\hat{N}_{ep} = 1/\hat{r}_{p(m)}$]	3.195
Proportion of full-siblings [$\hat{r}_{p(m)} \ \hat{t}_m$]	0.288
Proportion of half-siblings [$\hat{t}_m \ (1 - \hat{r}_{p(m)})$]	0.669
Proportion of self-fertilized individuals ($1 - \hat{t}_m$)	0.043

* Standard deviations in parentheses.

maternal and paternal parents) (Ritland 1989). The estimated $\hat{r}_{p(m)}$ (0.313) for sacha inchi suggests that progenies do not consist exclusively of half-siblings, but of a mixture of half-siblings, full-siblings and self-fertilized siblings (Palma-Silva et al. 2015). The average number of individuals that effectively pollinated the parental families was 3.19. A low number of pollinating plants is common in tropical perennial species, as indicated by Gusson et al. (2006). Evaluating the breeding system used for jatropha (*Jatropha curcas*, Euphorbiaceae), Alves et al. (2015) reported an average of 1.5 pollen donor trees in plants of this species.

To investigate the proportions of different types of kinship in the offspring of the *P. volubilis* germplasm collection, estimated multilocus crossing rates ($\hat{t}_m$) and paternity correlation [$\hat{r}_{p(m)}$] were combined. On average, 28.8 % of offspring of the progenies analyzed were full-siblings, with the majority of individuals being half-siblings (66.9 %). In addition, it was observed that a total of 4.3 % of the offspring were self-fertilized. However, these estimates are only tentative, since the progenies in this study were sampled in a field experiment, and the spacing between plants obviously differed from that observed in natural populations.

The observed proportion of different types of kinship reveals that assuming half-sibling progenies as a basis for estimating genetic parameters in *P. volubilis* may result in overestimated additive genetic variance and other dependent parameters. Valente et al. (2017a) reported a negative estimated residual variance if sacha inchi genotypes are assumed to be half-sibling progenies. The authors also drew attention to the need of further studies on the reproductive system of this species, in order to improve the accuracy of superior genotype selection.

The estimated average inbreeding coefficient or fixation index ($\hat{F}_m$) among maternal plants was 0.434, while the expected value [$\hat{F}'_m = (1 - \hat{t}_m)/(1 + \hat{t}_m)$] (Sharma et al. 2017) was 0.022. These results indicate that there are fewer heterozygotes and more endogamy than expected in the parents. A similar result was obtained by Sharma et al. (2017), when evaluating an oleaginous species (*Pongamia pinnata*).

In general, AFLP markers revealed a high degree of polymorphism and proved to be a useful and reliable tool for characterizing genetic diversity in sacha inchi progenies, as also reported by Vašek et al. (2017). In addition, AFLP markers were highly informative in evaluating the reproductive system of sacha inchi. Our results highlight the need to apply biometric models that take into account the existence of different kinships within progenies and the need for large samples to conserve the genetic variability. *P. volubilis* exhibited a low level of self-fertilization, leading us to expect that natural populations retain a high genetic variability.

CONCLUSIONS

1. Genetic variability is lower within progenies of sacha inchi, but sufficient for use in breeding programs;
2. High rates of multilocus crossing indicate that sacha inchi is a preferentially allogamous species;
3. For breeding and *ex situ* genetic conservation purposes, seeds of the species must be collected from a large number of parent plants, in order to conserve a maximum variability.

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