*NOTE*

# *Passiflora cristalina* and *Passiflora miniata*: meiotic characterization of two wild species for use in breeding

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**Abstract:** *Passiflora cristalina and Passiflora miniata are two new wild species found in the southern Amazon region. This study aimed to analyze the meiotic behavior of the two species, by meiotic analysis, meiotic index and pollen grain viability, using routine methodologies of the laboratory. By the meiotic analysis, the two species were diploid with 18 chromosomes, and nine bivalents were observed in diakinesis. Laggard chromosomes and fiber spindle problems were the abnormalities observed in both species. The recombination indices were 21.6 and 18.8 for P. cristalina and P. miniata, respectively. The most common abnormal post-meiotic products were triads. The meiotic index and the pollen grain viability for P. cristalina were 90.6% and 98.9%, respectively, and 91.6% and 82.2% for P. miniata, respectively. Based on the results, both species are fertile. Thus, gene transference to sour passion fruit by interspecific hybridization is possible in breeding programs.*

**Key words**: *Chromosome, meiotic abnormalities, recombination index, meiotic index.*

## INTRODUCTION

The family Passifloraceae is composed of 18 genera, and the genus *Passiflora* is the richest in number of species within the family, comprising between 521 and 537 species (Feuillet and MacDougal 2004, Vanderplank 2007). Species of this genus are distributed in four subgenera: *Astrophea, Deidamioides*, *Decaloba* and *Passiflora* (Feuillet and MacDougal 2004). New species and mutants belonging to this genus are still being described (MacDougal 2001, Lira Júnior et al. 2014), and about 90% of them are native to the Americas (Lopes 1991). Ferreira (1994) reported the existence of more than 200 Brazilian native species, which places the country in a privileged position in relation to the genetic resources of passion fruit trees.

Two new species were described in 2011 and 2006, *Passiflora cristalina* and *Passiflora miniata*. The former belongs to the Super Section Diasthephana of the subgenus *Passiflora,* and it was named after being found in the Cristalino State Park, in the northeast of the state of Mato Grosso. The species presents red flowers, which are held erect before and during anthesis, becoming pendulous as the ovary develops (Figure 1A) (Vanderplank and Zappi 2011).

*Passiflora miniata* was described by Vanderplank (2006) as belonging to the subgenus *Passiflora*, super section Coccinea. It is originated and distributed in

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the Amazon region of Peru, Brazil, Colombia, and the Guianas (Lim 2012). The species presents red flower, and according to the author, it has three series of purple corona filaments, with small green and cream fruits (Figure 1B).

Wild species have attracted the attention of breeders due to their genetic potential, since they have genes that confer resistance to diseases or pests, besides agronomic traits of interest. Despite this, hybridization with cultivated species is not always possible, leading to underutilization (Hajjar and Hodgkin 2007). According to Nass et al. (2001), in spite of the potential as a gene repository, wild species related to the cultivars are used as last resource due to the lack of basic and useful information for breeding. McCouch et al. (2013) stated that wild species and landraces of different plant species are conserved in more than 1,700 germplasm banks; however, little genetic information on this type of germplasm is found in the literature, which means that breeders make either none, or very little use of this type of germplasm in breeding programs.

Plant breeding seeks to combine desirable alleles that are found in different genotypes in a single elite variety. The success of a breeding program depends on the ability of the breeder to transfer these desired alleles to a hybrid by constructing desired combinations of alleles in the chromosomes, and by projecting the right combination of chromosomes (Wijnker and Jong 2008). This can only be achieved by the occurrence of crossovers during meiosis. Thus, meiotic recombination plays a key role in the breeding success, since crossover occurs only during meiosis (Wijnker and Jong 2008). Therefore, meiosis is essential for breeding, since it becomes one of the sources of genetic variability, besides explaining reproductive phenomena and mechanisms of heredity (Caetano et al. 2003). In addition, basic studies of a species, such as chromosome number, ploidy level, and genome size, among others, are important for the most different fields of knowledge (Singh 2002).

The objectives of this work were to evaluate the meiotic behavior of two wild species, *Passiflora cristalina* and *Passiflora miniata*, with emphasis on meiotic analysis and on possible meiotic irregularities; and to estimate the recombination index, meiotic index and pollen viability.


***Figure 1.*** Flowers of the species *Passiflora cristalina* (A) and *Passiflora miniata* (B).

## MATERIAL AND METHODS

Flower buds were collected in five accessions of the two wild species, *Passiflora cristalina* and *Passiflora miniata*, native to the Brazilian Amazon, and naturally found in the municipality of Alta Floresta, extreme north of the state of Mato Grosso (lat 9º 53' 02" S, long 56º 14' 38" W, alt 320 m asl). The climate of the region is rainy tropical with temperatures ranging from 20 ºC to 38 ºC, with average annual temperature of 26 ºC (Köppen 1948).

For meiosis, flower buds at different stages of development were fixed in a 3:1 alcohol: acetic acid solution, and kept at 4 ºC. After 24h, the fixative solution was replaced with a 70% ethanol solution, and buds were kept in a freezer until the time of slides preparation. For slide preparation, anthers were macerated in 2% acetic carmine and, after removal of the debris and the coverslip, slides were observed using an optical microscope (Olympus BX 60). The different phases of meiosis were analyzed, and irregularities were counted.

Chiasmata were counted in 50 cells in diakinesis to estimate the recombination index (RI). The following expression was used to estimate the RI:

$$RI = n(1 + X)$$

in which n is the haploid number of chromosomes of the species, and X is the mean number of chiasmata per bivalent (Darlington1958).

The meiotic index (MI) was estimated based on five slides/buds, and each slide was prepared with four anthers, which were macerated and stained with 2% acetic carmine for the visualization and counting of post-meiotic products. Tetrads were considered as a normal post-meiotic product, and monads, dyads, triads and polyads were considered as abnormal. Based on these data, the meiotic index was calculated according to Love (1951):

$$MI = \frac{\textit{Total normal post} - \textit{meiotic products}}{\textit{Total normal and abnormal post} - \textit{meiotic products}} \times 100$$

For the estimate of pollen viability, two anthers were macerated in Alexander's triple solution, composed of Orange G, acid fuchsin, and malachite green. In this way, pollen grains were classified as viable or unviable. The viable pollen grains were red/purple, while the unviable ones were green (Alexander 1969). Five slides were prepared per species, accounting for 200 pollen grains/slide, totaling 1000 pollen grains per species. Data were transformed into viability percentage (%).

## RESULTS AND DISCUSSION

### Meiotic analysis

Both species, *P. cristalina* (Figures 2A-D, and 2F) and *P. miniata* (Figures 3A-F), presented meiosis within the normality pattern. Nine pairs of bivalents were observed in prophase I cells of both species, which confirms that the species are

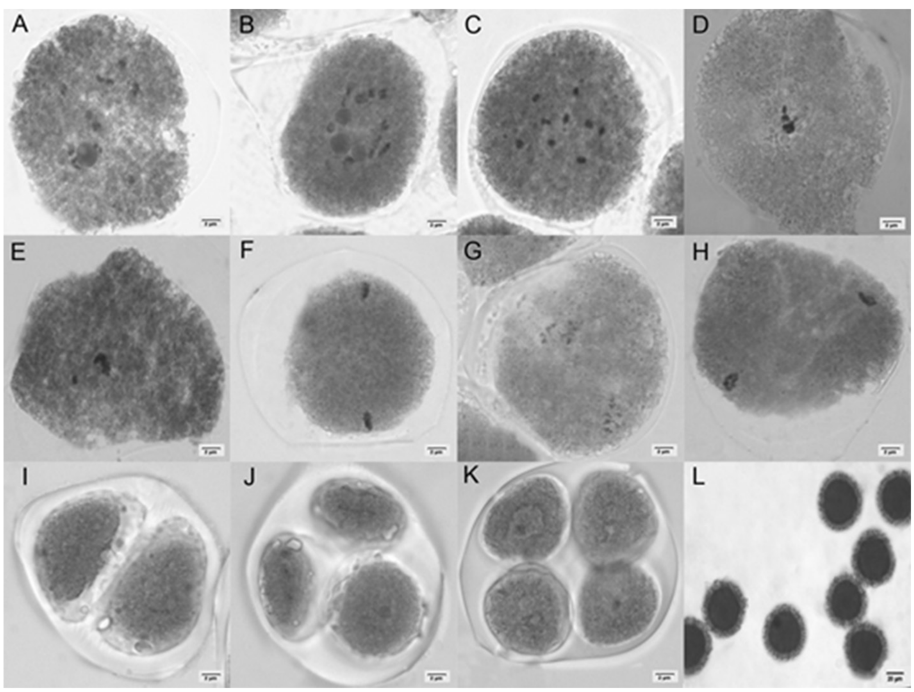


***Figure 2.*** Meiosis of *P. cristalina* (A-C) Cells in diakinesis where nine bivalents are observed; (D) Metaphase I; (E) Metaphase I with a pair of laggard chromosomes; (F) Metaphase II; (G-H) cells in anaphase II (G) and anaphase II (H) with problems in the spindle fibers: (I-K) Post-meiotic products, dyad, triad and tetrad, respectively; (L) Viable pollen grains stained with Alexander's triple solution. Bar = 2 µm (A-K) and 20 µm (L).

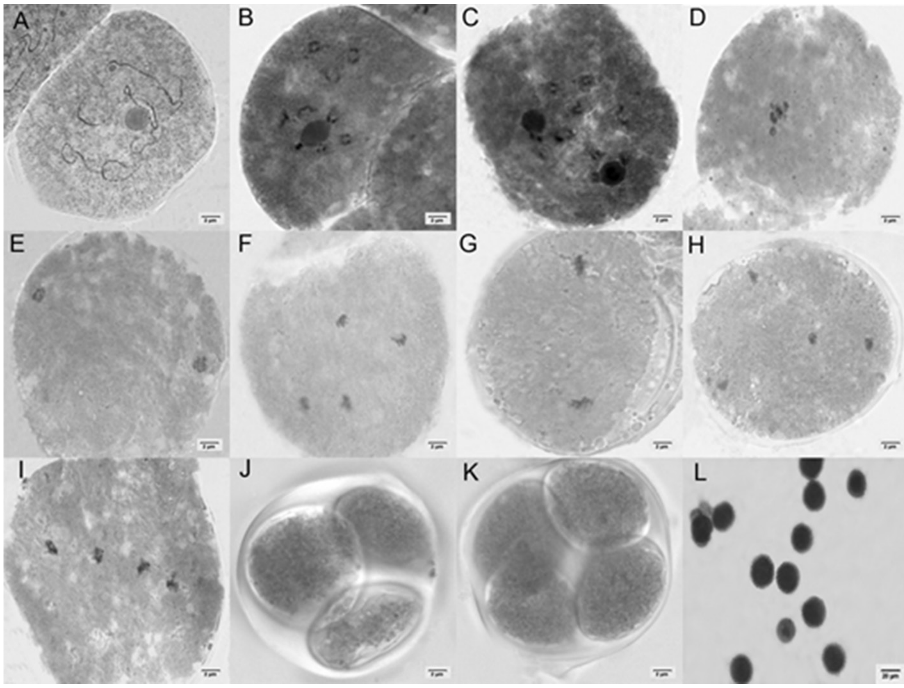


***Figure 3.*** Meiosis of *P. miniata* (A) Pachytene where nine bivalents are observed; (B-C) diakinesis where bivalents and more than one nucleolus per cell are observed; (D) Metaphase I with a pair of laggard chromosomes, outside the equatorial plate; (E) Telophase I; (F-I) Cells in anaphase II (F), metaphase II (G), and in telophase II (H-I) with spindle fiber problems; (J-K) Post-meiotic products, triad and tetrad, respectively; (L) Viable pollen grains stained with Alexander's triple solution. Bar = 2 µm (A-K) and 20 µm (L).

diploid and have 2n=18 chromosomes. According to Hansen et al. (2006), the species of the subgenus *Passiflora* have the most representative basic chromosome number (x=9 chromosomes). Although passifloras can be classified into four karyological groups, represented by n=6, n=9, n=10 and n=12 (Melo and Guerra 2003), most species present 2n=2x=18 chromosomes and are diploid. However, polyploids and aneuploidshave been reported in the family (Melo et al. 2001).

More than one pair of chromosomes associated with the nucleolus were observed in both species, indicating the presence of at least two pairs of chromosomes containing the nucleolar organizing regions (NORs) (Figures 2A and 3A-C). Cells with more than one nucleolus were also observed (Figures 2B and 3C). Barbosa and Vieira (1997) studied sour passion fruit (*Passiflora edulis*), and observed that two pairs of chromosomes were associated with the nucleolus in 33% of the cells. According to the authors, the studied species has two NORs located in the secondary constrictions of chromosomes 8 and 9. NORs are repeated DNA sequences that encode ribosomal RNA 18S, 5.8S and 26S (18S-26S), and are located in secondary constrictions. NORs are usually associated with secondary constrictions; however, not all secondary constrictions are NORs (Battistin et al. 1999). During telophase and interphase, NORs are responsible for the nucleoli formation (Besendorfer et al. 2002).

Despite their importance, studies on chiasmata frequency are rare in Passifloras (Souza et al. 2008). The number of chiasmata is an important trait for meiotic stability, since they prevent the early migration of the chromosomes, and ensure that the bivalents are oriented to opposite poles (Wijnker and Jong 2008). The chiasmatas are originated from the crossover, and are the regions that keep the homologous chromosomes together during Prophase I (Mézard 2006). In the present work, rod and ring bivalents were observed. Rod bivalents (Figure 2B) present only one chiasm in one of the chromosome arms, while ring bivalents (Figure 3C) present chiasms in the two chromosome arms (Senda et al. 2005).

For *P. cristalina,* the estimated recombination index (RI) was of 18.8, and the chiasma mean per bivalent was of 1.08 chiasmata; and for *P. miniata*, the RI was 21.6, with 1.4 chiasmata per bivalent. Forni-Martins (1996) also observed low chiasma frequency in species of Erythrina (<1.5 per bivalent). The present results corroborate those reported by Souza and Pereira (2011), who analyzed 14 wild *Passiflora* species of the n = 9 chromosome group, and obtained RI ranging from 17.6 (*P. alata*) to 23.9 (*P. malacolhyla*). Considering that the authors evaluated 50 cells in diakinesis, chiasma mean per bivalent ranged from 0.96 to 1.7, and these values are close to those reported in this study.

The maximum recombination that can be obtained in species is determined by two factors: the number of chromosomes of the species, and the number and positions of crossovers in the homologous pair. Crossovers preferentially occur in certain areas, known as recombination hotspot. Conversely, areas with very low occurrence of crossovers are known as recombination cold spots (Wijnker and Jong 2008). In passifloras, these regions (hotspot and cold spot) have not yet been defined; however, in other species, such as maize, wheat and barley, recombination increases as it distances from the centromere. In tomato and rice, the recombination tends to decrease as the crossover approaches the telomeres (Mézard 2006).

Meiosis of both species was considered as normal. However, some irregularities were recorded, such as lagging chromosomes in metaphase I (Figures 2E and 3D), and anomalies in spindle fibers, which were the most frequent (Figures 2G-H-I). Souza and Pereira (2011) analyzed the meiosis of 14 passiflora species, and reported more meiotic irregularities than those observed in this study.

In *P. cristalina*, laggard chromosomes were observed in 16.7% of the cells analyzed in metaphase I; however, they were not observed in metaphase II. This abnormality was not observed in *P. miniata*. On the other hand, they have been observed in *P. alata, P. foetida, P. cincinnata* and *P. amethystina*in both in meiosis I and meiosis II (Kiihl et al. 2010).

Laggard chromosomes are not aligned on the equatorial plate during metaphase I or anaphase I. According to Pagliarini (2000), laggard chromosome is the most frequent abnormality during nuclear division, and a possible cause is the early segregation of chiasma, or the presence of *asynaptic* or *desynaptic*genes during prophase I. Laggard chromosomes are usually lost during nuclear division, resulting in micronuclei (Risso-Pascotto et al. 2003). These micronuclei are usually observed in post-meiotic products, and may lead to the formation of unbalanced gametes, egg abortion, and the consequent formation of non-viable gametes (Battistin et al. 2006).

The most frequent abnormality observed for both species was the lack of orientation of the spindle fibers, estimated in 17.8% for *P. cristalina,* and in 35.9% for *P. miniata*. Cells with this anomaly presented the chromosomal groups aligned in the T-shaped equatorial plate (Figure 2H, Figure 3G-H), which characterizes the transversal spindle (Souza et al. 2003, Shamina 2005). Some genes affect spindle formation during meiosis I and meiosis II (Shamina et al. 1999, Shamina 2005) and may cause problems in karyokinesis and cytokinesis, consequently generating abnormal post-meiotic products, such as dyads, triads and polyads. In passifloras, convergent spindle is directly related to the formation of triads, which are formed due to asynchrony during meiosis II (Souza and Pereira 2011).

The abnormalities observed in this study did not hinder the formation of post-meiotic products of the species (Figures 2I-K and 3J-K), since *P. cristalina* and *P. miniata* presented meiotic index of 90.6 and 91.6%, respectively. Plants with meiotic index between 90 and 100% are considered as cytologically stable (Love 1951), presenting no problems in generating fertile progeny. Triads were observed in 9.3% of the *P. cristalina* cells, and in 8.4% of the *P. miniata*cells, probably due to the anomalies of the spindle fibers described above. Only one dyad was observed in *P. cristalina* (Figura 2I), and none was observed in *P. miniata*. The determination of the meiotic index assists in the verification of the meiotic regularity. Thus, the higher the value of the meiotic index, the more regular is the meiotic behavior of the species (Love 1951).

Plants fertility depends on the meiotic regularity, which may occur during the formation of pollen grains. The normal course of meiosis guarantees the viability of the gamete. However, this event is controlled by several genes, and thus mutations may occur (Pagliarini 2000). The pollen viability estimated in this study was high, since it varied, on average, from 98.9% to 82.2% in *P. cristalina* and *P. miniata*, respectively. Figures 2L and 3L show the viable pollen grains. These values are in agreement with those reported by Souza et al. (2004), who observed pollen viability greater than 90% for most of the species studied, except for *P. pentagona*, which presented 78.22% pollen viability.

Based on the results obtained in this work, the two new *P. miniata* and *P. cristalina*, both belonging to the genus and sub-genus Passiflora, present n=x=9 chromosomes, and therefore are considered as two new diploid species. Meiosis of both species was generally normal, despite the irregularities observed. Nevertheless, for being wild, they are probably still under domestication process. Both species had good meiotic index and high pollen viability, and thus they can be used in future sour passion fruit (*P. edulis* Sims) breeding programs by means of interspecific hybridization.

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