

## *Vanilla bicolor* Lindl. (Orchidaceae) from the Peruvian Amazon: auto-fertilization in *Vanilla* and notes on floral phenology

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**Abstract** Minimal documentation exists for natural pollination in wild *Vanilla* spp., despite the economic importance of this genus, additionally commercial vanilla (*V. planifolia* Jacks.) is one of very few crops whose production depends entirely on artificial pollination. Flowering and fruiting phenology of *Vanilla bicolor* Lindl., a close relative of *V. planifolia*, was documented in a palm swamp in the Peruvian Amazon. *V. bicolor* was found to auto-fertilize via bagging experiments. This ecotype had an average fruit set per raceme of  $42.50 \pm 2.5\%$ . Pollen removal experiments suggest that stigmatic leak may be the mechanism by which auto-pollination occurs in *V. bicolor*.

**Keywords** *Aguajales* · Auto-pollination · Orchidaceae · Palm swamps · Phenology · Self-fertilization · *Vanilla*

Scant documentation exists on pollination mechanisms in wild *Vanilla* species (Lubinsky et al. 2006). With vanilla being a crop of global significance surprisingly little attention has been paid to its wild relatives for crop improvement. Recent work demonstrating historical plant breeding and crop development via hybridization opens a new window onto the possibilities of crop improvement in vanilla (Lubinsky et al. 2008a). Vanilla cultivation relies upon the use of cheap labor to carry out hand pollination in order to obtain fruits for the spice trade, and this results in vanilla being an expensive crop (Lubinsky et al. 2006). Understanding pollination in this economically valuable genus is of currency.

*Vanilla* displays considerable reproductive diversity both between and within species (Cameron and Soto Arenas 2003). Few studies have documented the natural mechanisms of pollination, phenology, and seed dispersal of *Vanilla* despite its economic importance (Lubinsky et al. 2006; Householder 2007). Recently, Euglossine bees have been shown to pollinate and disperse the seeds of *V. pompona* subsp. *grandiflora* Lindl. in the neotropics (Lubinsky et al. 2006). Furthermore, natural hybridization seems to have occurred in several neotropical *Vanilla* spp. (Nielsen 2000; Lubinsky et al. 2008a). In addition to

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out-crossing and asexual propagation, auto-pollination has also been reported in *Vanilla* (van der Pijl and Dodson 1966). Examples of species that are thought to auto-pollinate, as evidenced by their high fruit sets, atypical in outcrossing species, are *V. palmarum* (Salzm. ex Lindl.) Lindl., *V. savannarum* Britton, and *V. griffithii* Rchb. f. (Cameron and Soto Arenas 2003). The mechanisms and concomitant selective pressures for auto-pollination in *Vanilla* remain unclear at best.

There are three proposed mechanisms for auto-pollination in *Vanilla*: (1) “stigmatic leak” by which stigma lobes release a fluid, that contacts the pollen and induces germination of the pollen tubes (van der Pijl and Dodson 1966), (2) by dehydration or underdevelopment of the rostellum, which could also allow for pollen to contact the stigmatic cavity (Garay and Sweet 1974), (3) a third mechanism of self-fertilization in *Vanilla* that has remained overlooked is agamospermy. In the *Neuwiedia veratrifolia* Blume (Apostasioideae) the method of self-pollination was determined to be auto-pollination not agamospermy via emasculation experiments (Okada et al. 1996; Kocyan and Endress 2001). Thus, auto-pollination is the method of self-fertilization that occurs at the clade sister to the rest of Orchidaceae. Documentation of any of the above three mechanisms remain absent in *Vanilla*.

*Vanilla bicolor* Lindl. is a widespread locally common hemi-epiphyte endemic to the Neotropics (Christenson 1995). They are found in open swampy areas along rivers in northern South America and exposed humid thickets along mountain crests in the Caribbean (Adams 1972; Werkhoven 1986; Christenson 1995; Borhidi 1996). *V. bicolor* is a close relative of the widely cultivated *V. planifolia* Jacks (Cameron and Soto Arenas 2003; Lubinsky et al. 2008a) making it an important wild relative. Unlike the exceedingly sparse density of wild *V. planifolia* plants (Lubinsky et al. 2006, 2008a, b) *V. bicolor* occur at high densities in southern Peru and we were curious if higher density resulted in higher pollination rate due to increased reward. In this short communication we provide evidence regarding the floral phenology, pollination syndrome (i.e., outcrossing or self-fertilization) and possible mechanism of pollination in a cleistogamous ecotype of *V. bicolor* from the Southern Peruvian Amazon.

## Materials and methods

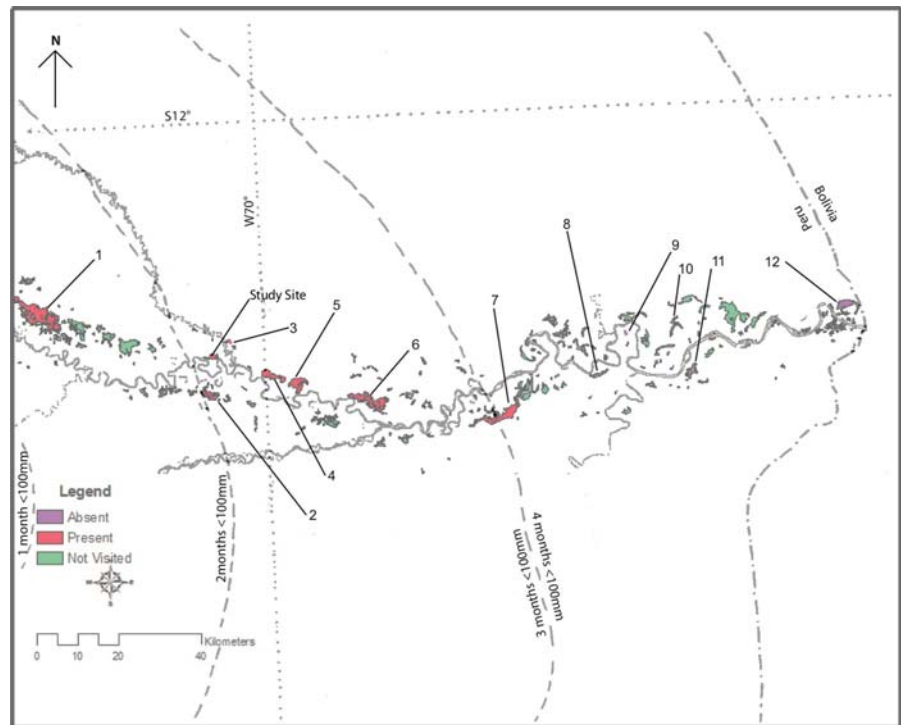
### Study site

Our study site is located in southeastern Peru in the Madre de Dios basin (Fig. 1). The Madre de Dios River is located in the headwaters of the southwest Amazon, draining a portion of the forelands of the Eastern Cordillera. The Madre de Dios River displays high rates of lateral migration and typical meander scroll morphology. Over 300 hundred wetlands occur along the current Madre de Dios River floodplain, generally hugging the terrace escarpments. Some wetlands in particular, palm swamps, are known locally as “aguajales”, named after the common name of the monodominant palm *Mauritia flexuosa* L. Carrera, or “aguaje”. *Aguajales* in this region range in size from 1 to 2,000 ha, occupying a significant portion of ecologically important floodplain habitat. Wetland soils are permanently saturated year round, fed by perennial spring inputs (personal observation, Householder 2007).

Annual average rainfall in the region where our study occurred is  $2,995 \pm 455$  mm. Rainfall is unevenly distributed throughout the year, with greater than 80% falling between October and April. Average temperature fluctuates minimally between 21 and 26°C, however southerly cold fronts rapidly progress northward from Patagonia, known as “frijajes” and are common in June through August (Amazon Conservation Association, unpublished data). These *frijajes* are known to decrease temperatures to 10°C or less in a matter of minutes. Climate data since 2001 is publicly available provided by the Amazon Conservation Association (ATRIUM 2007).

Data was collected on *V. bicolor* in the Peruvian Amazon near the Los Amigos research station in the center of an *aguajale* (palm swamp) (S12°33.453' W70°07.312' at ~330 m). We set up a 50 × 55 m plot in which phenological data (excluding the frequency of bloom and auto-fertilization tests which were recorded in the same *aguajal* but outside of the plot) for *V. bicolor* vines were recorded during August 17–18, 2005. Most plants had finished blooming during this time of the year in August during the dry season.

**Fig. 1** Map of Madre de Dios River Peru in grey with *aguajales* shown in color transposed over rainfall isohyets from Sombroek (2001)



#### Rate of fruit set in *V. bicolor*

Multiple individuals of *V. bicolor* plants are commonly encountered climbing up sides of palm trees (*Mauritia flexuosa*). We will refer to multiple *V. bicolor* vines growing up the sides of a single palm tree as a “clump”. We counted the number of vines per clump of *V. bicolor*, the number of individual racemes of *V. bicolor* per clump, the number of un-pollinated flowers per raceme, the number of pollinated flowers per raceme, and the number of buds or new flowers per raceme. An un-pollinated flower was recognized by the presence of a bract on the raceme, but lacking a flower or a fruit as abscission of flowers occurs. A pollination event was also denoted by the presence of fruit on a raceme or a flower with a drooping ovary. The height at which each raceme occurred was measured.

The average fruit set per raceme, number of racemes per clump of *V. bicolor*, average height of the racemes occurring on the *V. bicolor* vines, and percent of *V. bicolor* vines that did not produce racemes were calculated. Only racemes that had no new buds and completely finished blooming were used for calculating the fruit set rate. A total of 131

racemes from 49 clumps of *V. bicolor* growing up palm tree trunks occurred in our study plot.

Fruit set data was tested for a normal distribution by looking at a scatter plot of residuals using SYSTAT Version 9.01<sup>©</sup> SPSS Inc.1998. Residual scatter plots were made for the height of individual racemes to their fruit set rate, and fruit set rate to number of clumps of *V. bicolor* growing over individual palm trees in our study site. Scatter plots of the residuals were both normal so the next step was to perform a linear regression analysis on the data to see if any trends existed. Finally trend lines were plotted for the linear regression analyses using Microsoft Excell<sup>®</sup> 2004 edition. Linear regression analyses were conducted to see if any immediate phenological patterns stood out as well as to add to documentation of the floral phenology for comparisons between any future studies. Height of racemes and their density have been correlated with increased pollinator visitation in some plants (Pyke et al. 1977; Engel and Irwin 2003). We were also curious if these tests might point toward any possible effects of pollinators on *V. bicolor* floral phenology. Finally histograms were constructed from the data collected.

### Auto-fertilization tests

Auto-fertilization in *V. bicolor* was determined two ways (1) an intensive survey across the entire Los Amigos *aguajal* for open or partially open flowers was conducted on August 22, 2005, 17 cleistogamous flowers that showed recent evidence of fertilization (1–2 days previous) via drooping ovaries were inspected for pollen removal or pollen granules placed on the stigma (2) 10 racemes of 10 different *V. bicolor* plants were bagged with Wirlpak<sup>®</sup> plastic bags. Bags excluded any pollinators from entering the flowers. Fertilization was determined by presence of developing fruits. The following dry season in April of 2006 13 additional racemes from 13 different plants were bagged in a similar manner using 1 mm mesh net to prevent any possible temperature differences between bagged flowers and outside environment. Plants to bag were chosen based on presence of flowers and accessibility from the 50 × 55 plot.

To test for agamospermy, ten flowers from different racemes and plants had their pollen removed by hand before completion of flower development. Racemes along with removed flowers were bagged with 1 mm mesh nets and allowed to mature. They were later checked once flowers had finished blooming. Manipulated buds were periodically monitored until either fruit development began, suggesting agamospermy, or senescing flowers fell from the raceme, indicating unsuccessful pollination.

### Frequency of bloom in *V. bicolor*

Time between blooms in *V. bicolor* was calculated by recording the number of days from one flower opening to another for 10 racemes from 10 different individuals. Plants were observed daily at approximately 9:00 am for 3 weeks from August 10 to August 31, 2005. Time between blooms varied from plant to plant slightly. A bloom was counted as stereotypical closed florescence in which flowers were only slightly open (Fig. 2a). One pre-dawn and post-dusk observation was made in which five plants were watched to see if buds opened at night, but a nocturnal bloom was not observed, instead buds partially opened in morning (Fig. 2a). Flowers were also observed throughout the wet season (November–April) to look for any different trends in florescence production.



**Fig. 2** **a** Top: *Vanilla bicolor* exhibiting the typical cleistogamous state that was encountered at our study site in the Peruvian Amazon (S12°33.453' W70°07.312' at ~330 m). **b** Below: *Vanilla bicolor* exhibiting the atypical open florescence found at our study site

### Survey for *V. bicolor* in *aguajales* of Madre de Dios Peru

Thirteen different *aguajales* in the Madre de Dios Province of the Peruvian Amazon were surveyed for presence or absence of various *Vanilla* spp. The presence or absence of *V. bicolor* was noted as well as the growing condition. This was done to learn more about the preferred habitat of *V. bicolor*. This was an exhaustive survey covering 13 different *aguajales* over a 300 km stretch of the Madre de Dios drainage system in Peru. An Arc GIS map of the survey was constructed. Based on field experience it seemed that eastern *aguajales* lacked *V. bicolor*. To look for a possible explanation to this pattern the GIS map was transposed onto rainfall isohyete maps from

Sombroek (2001) to see if any rainfall and dry season intensity patterns of the region affected where *V. bicolor* grew. Upon constructing this map it was clear that *V. bicolor* was absent from *aguajales* of southern Peru that characteristically receive less than 4 months out of the year with <100 mm per month, thus a measure of dry season intensity. This was followed by a linear regression analysis to see if a relationship existed between presence and absence data and intensity of the dry season using Microsoft Excell® 2004.

## Results

### Rate of fruit set in *V. bicolor*

A summary of all results of rate of fruit set is given on Table 1. Average rate of fruit set per raceme was  $42.50 \pm 2.50\%$  ( $N = 131$ ), 0–100% fruit set per raceme (Table 1; Fig. 3a). Average number of racemes per clump of *V. bicolor* was  $5.10 \pm 0.68$  ( $N = 41$ ) with the range being 1–16 racemes per clump of *V. bicolor* (Table 1; Fig. 3b). Average height of a raceme was  $3.14 \pm 0.07$  m ( $N = 253$ ) with a range from .2 to 6.1 m in height (Table 1) (Fig. 3c). Average number of florets per raceme was  $5.25 \pm 0.18$  ( $N = 131$ ) range 1–13 (Table 1; Fig. 3d). Percentage of plants that were not in bloom during the flowering season recorded was 16.7% (8 of 48) from our study site (Table 1).

Result from the linear regression analysis between height of a raceme and its pollination rate was not significant ( $P = 0.961$ ; Fig. 4a). This indicates that

there is no relationship between the height of a raceme and its pollination rate. Linear regression analysis between fruit set rate per raceme and number of *V. bicolor* racemes growing per clump of *V. bicolor* were not significant as well ( $P = 0.112$ ; Fig. 4b). Thus there was no trend in fruit set rate and number of racemes per clump of *V. bicolor*.

### Auto-fertilization tests

After visually inspecting 17 recently fertilized flowers all 17 had no pollen removed or pollen placed on stigma area. Furthermore all of these flowers had fully developed rostellums and were not dry or shriveled in any way suggesting that auto-pollination via “stigmatic leak” or agamospermy are possible mechanisms for auto-fertilization. Results from bagged racemes from the August experiment was that 3 out of 10 bagged racemes developed fruit. These results indicate that self-fertilization is occurring in this population. Results from the April 2006 bagging experiment that included 13 racemes on different plants lend further support to this conclusion. Eighty-five percent of bagged racemes developed fruit. Seventy-one percent of all flowers developed fruit under bagged conditions in the April 2006 experiment. This again indicates that self-fertilization is occurring in this population.

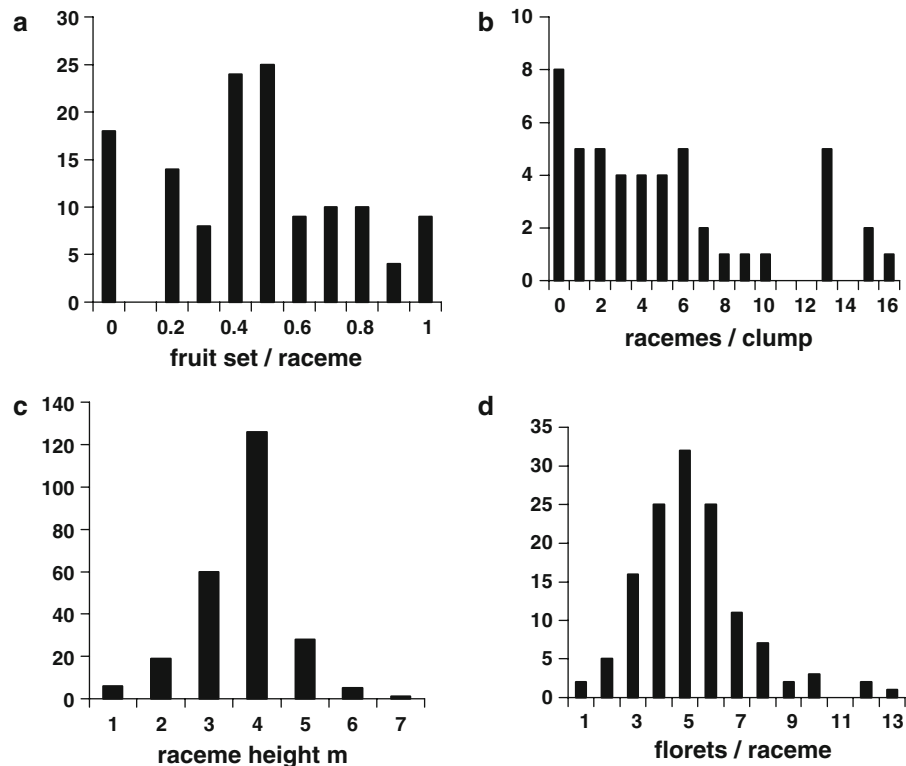
Pollen removal experiments concluded that no flowers became pollinated after pollen was removed, thus agamospermy may not be a method by which this species may self-fertilize and auto-pollination is perhaps the method for self-fertilization. This sample size (10 flowers) is rather small, but it does suggest

**Table 1** Summary of phenological data compiled on *V. bicolor* in an *aguajale* (palm swamp) in the Peruvian Amazon (S12°33.453' W70°07.312' at ~330 m) from a 50 × 55 m plot

Phenological category	$\mu \pm SE$	Range	$N$
Average fruit set/raceme	$42.50 \pm 2.5\%$	(0–100%)	$N = 131$
Average number of florets/raceme	$5.25 \pm 0.18$	(1–13)	$N = 131$
Average number of racemes/clump	$5.10 \pm 0.68$	(1–16)	$N = 41$
Average raceme height	$3.14 \pm 0.07$ m	(0.2–6.1 m)	$N = 253$
% <i>V. bicolor</i> clumps on palms not blooming	16.67%	0	$N = 8$
Days between a single bloom	5 days	0	$N = 10$

Means are followed by standard error

in which all *V. bicolor* “clumps” (multiple tangled vines of *V. bicolor*) were growing on palm trees (*Mauritia flexuosa*)



**Fig. 3** Histograms of phenological data compiled on *V. bicolor* in an *aguajale* (palm swamp) in the Peruvian Amazon (S12°33.453' W70°07.312' at ~330 m) from a 50 × 55 m

that agamospermy is not a mechanism affecting auto-pollination.

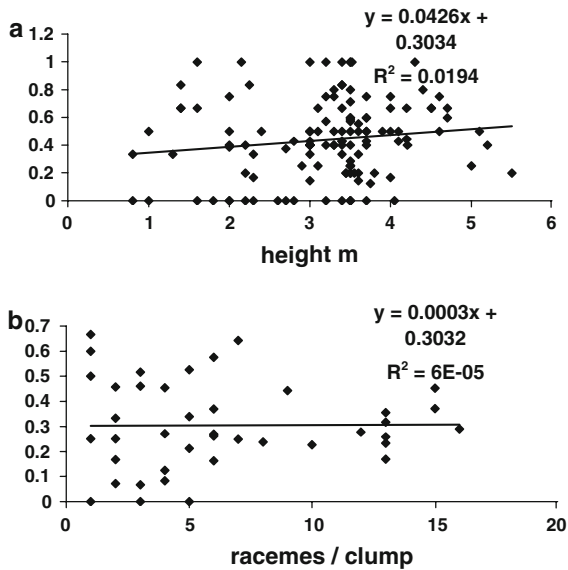
#### Frequency of bloom in *V. bicolor*

There was a single bloom for each florescence at 5-day intervals per raceme, although variation on this would be expected with a larger sample size. Each flower was only open or slightly open for a single day at a time. Over the duration of the blooming season from August to mid-September only one individual flower (Fig. 2b) was fully open out of 68 cleistogamous flowers (Fig. 2a) that were observed blooming across the *aguajale*. Thus an estimated 98.5% of flowers during the dry season exhibit a cleistogamous appearance. Cleistogamy is a common characteristic of auto-pollination in the Orchidaceae (Catling 1990). Furthermore there is no evidence of cross-pollination in cleistogamous flowers in the Orchidaceae (Catling 1990).

plot in which all *V. bicolor* “clumps” (multiple tangled vines of *V. bicolor*) were growing on palm trees (*Mauritia flexuosa*)

#### Survey for *V. bicolor* in *aguajales* of Madre de Dios Peru

Across thirteen *aguajales* sampled for *V. bicolor* a distinct geographical trend emerged. In eastern most *aguajales* *V. bicolor* was absent (Fig. 1). Size of *aguajal* did not seem to affect presence or absence of this species because relatively large and small *aguajales* both contained *V. bicolor*. Furthermore after transposing rainfall isohyete data from Sombroek (2001) onto the GIS map of *aguajales* combined with presence or absence data of *V. bicolor* distributions it was clear that intensity of the dry season prohibits this species from growing farther eastward (Fig. 1). Specifically the cut off point seemed to be in areas with <100 mm of rainfall for more than 4 months out of the year prohibiting the growth of *V. bicolor* (Fig. 1). Results from the linear regression analysis clearly indicate that the intensity of the dry season is related to where *V. bicolor* can grow in the *aguajales* of southern Peru ( $df = 12$ ;  $R^2 = 0.606$ ;  $P = 2E^{-3}$ ).



**Fig. 4** Scatter plots with linear regression trend lines. The slope of the linear regression followed by the  $R^2$  value are given in the *top right* hand corner of each linear regression analysis. **a** Top: *V. bicolor* fruit set/raceme plotted against height of raceme. **b** Below: *V. bicolor* average fruit set/clump plotted against racemes/clump

Hopefully future more detailed isohyte data will be available to test our observations as data provided here is a rough fitting of regional rainfall patterns.

## Discussion

### Auto-fertilization in *V. bicolor*

Several lines of evidence illuminate auto-fertilization as a method by which *V. bicolor* in our study site can reproduce. The combination of a high fruit set, ubiquity of closed flowers during the dry season, and fertilization of bagged flowers, all point toward auto-fertilization. Furthermore the pollination rate for this auto-fertilizing *V. bicolor* is high compared to other *Vanilla* species that are Euglossine pollinated such as *V. pompona* subsp. *grandiflora* (Lubinsky et al. 2006), which has a pollination rate of  $\sim 1\%$  (personal observation, Householder 2007). Other populations of *V. bicolor* may exhibit a non-cleistogamous phenotype (see Christenson 1995). Furthermore we provide some initial evidence that the method of auto-fertilization may be auto-pollination and not agamospermy through emasculation experiments. Also

morphological examination of *V. bicolor* flowers shows a fully developed rostellum in pollinated flowers making “stigmatic leak” the most likely mechanism of auto-pollination in this species.

Drought conditions may eliminate *V. bicolor* from *aguajales* under certain circumstances thus reducing the *Vanilla* and consequent plant diversity in those *aguajales* (Fig. 1). Meteorological evidence from the Amazon basin points to large-scale deforestation of primary forests effectively shifting sub-regional climate conditions to be significantly drier (Sombroek 1962, 2001). Implications of large-scale deforestation for conservation of plant diversity within *aguajales* would suggest a regional approach is needed. A regional approach would include maintaining substantial tracks of primary forest around *aguajales* as opposed to just fencing off *aguajales* from development.

Rapid loss of neotropical habitat where wild *Vanilla* species occur supports urgency in documenting valuable wild genetic resources in *Vanilla* (Lubinsky et al. 2008b). We provide evidence for an auto-fertilizing *Vanilla*. Discovering the underpinnings of genetic and environmental conditions that bring about this breeding system strategy in the individuals studied, could be useful in breeding programs for improving vanilla yields via auto-fertilization. This demonstrates the need to conserve wild neotropical *Vanilla* species as most life history data on these species is completely unknown.

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