Reproductive isolation between wild and domesticated chaya
(Cnidoscolus aconitifolius) in sympatry.

Miguel A. Munguía-Rosas¹ & Miguel E. Jácome-Flores²,³

¹ Laboratorio de Ecología Terrestre, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional (Cinvestav), Merida, Mexico.

² Centro del Cambio Global y la Sustentabilidad A.C., Villahermosa, Mexico.

³ CONACyT

Correspondence

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Keywords

Cladogenesis, chaya, *Cnidoscolus aconitifolius*; domestication; reproductive barriers, reproductive isolation, sympatric speciation, wild-crop plant gene flow.

**ABSTRACT**

- Reproductive isolation is a necessary condition for plant domestication in their domestication centre where crops co-occur with their wild progenitors. However, the identification of reproductive barriers and their relative contribution to reproductive isolation have been overlooked in plants under domestication.
- We assessed pre- and post-pollination reproductive barriers and their relative contribution to reproductive isolation between wild and domesticated chaya (*Cnidoscolus aconitifolius*) in its domestication centre.
- We found that wild and domesticated chaya both exhibit a high degree of reproductive isolation. However, the reproductive isolation barriers exhibited some asymmetry: while pre-pollination barriers (differential pollen production and pollinator specificity) were only detected in wild plants, post-pollination barriers (pollen-pistil incompatibility and/or failure to set fruit) were observed in both wild and domesticated plants.
- We concluded that complete reproductive isolation has evolved in sympatry in co-occurring domesticated and wild chaya.
INTRODUCTION

Reproductive isolation is a process where mating opportunities among individuals are constrained by reproductive isolation barriers (hereafter: RIBs) and plays a major role in population divergence (Coyne & Orr 1989; Baack et al. 2015). Four groups of RIBs can be recognized in plants: (1) pre-pollination barriers, which include geographic and phenological isolation as well as pollinator differentiation. (2) Post-pollination, pre-zygotic barriers such as pollen-pistil incompatibility. (3) Post-pollination, intrinsic post-zygotic barriers such as seed and hybrid sterility and, finally, (4) post-pollination, extrinsic post-zygotic barriers, in which the survival and fitness of hybrids are reduced by environmental factors (Coyne & Orr 1989; Baack et al. 2015). While it is widely recognized that the reproductive isolation observed in allopatry is primarily the result of the presence of pre-pollination geographic barriers (Bolnik & Fitzpatrick 2007; Gravilets 2003; Lowry et al. 2008), the manner in which reproductive isolation is achieved in sympatry remains poorly understood (Bolnik & Fitzpatrick 2007; Foote 2018). The key problem is that mating and recombination can break up the linkage between alleles beneficially associated with environmental variation and those associated with incompatibilities and reproductive isolation (Jiggings 2006; Foote 2018).

Plant domestication is perhaps the world’s largest and best replicated selection experiment in which, by comparing domesticated plants to their wild relatives, we can address some major questions in Evolutionary Biology (Gepts et al. 2004). However, the potential of plants under domestication as a model with which to understand how reproductive isolation arises in sympatric individuals has been overlooked (Van Raamsdonk 1995; Dempewolf et al. 2012). Most crop domestication events have occurred in specific areas of the world, known as domestication centres, where crops typically co-occur with their wild ancestors (Vavilov 1951; Harlan 1975). Despite their co-occurrence, some crops have been successfully domesticated (Ross-Ibarra & Molina-Cruz 2002; Kuriakose et al. 2009; Parra et al. 2012), suggesting the occurrence of reproductive isolation and subsequent divergence in
sympatry (Van Raamsdonk 1995). However, there is no adequate assessment of RIBs between crops and sympatric wild relatives, or of the extent of reproductive isolation, in the literature. The only study on this topic is that of Dempewolf et al. (2012) who reported that 38% of crops included in the review (n=30 crops) exhibit some (unquantified) degree of reproductive isolation from their wild progenitors, due mainly to post-zygotic barriers. However, the results of that review are biased since the studies incorporated did not consider pre- and post-pollination, pre-zygotic barriers such as phenological isolation, pollinator specificity and pollen-pistil incompatibilities. Moreover, the reviewed studies were not designed to assess reproductive isolation between crops and their wild relatives in sympathy.

Here, we assessed pre- and post-pollination RIBs in chaya (*Cnidoscolus aconitifolius*; Euphorbiaceae) in its domestication centre in Yucatan Peninsula (Ross-Ibarra & Molina-Cruz 2002). In this area, wild and domesticated plants can grow together in homegardens (Munguía-Rosas et al. 2019). Wild plants may also grow in disturbed vegetation, sometimes only a few meters away from domesticated plants (Munguía-Rosas et al. 2019). The main pollinators of chaya are bees and butterflies (Arceo-Gómez et al. 2009), which can travel the distance between wild and domesticated plants. Chaya is therefore an excellent model with which to assess how reproductive isolation arises in sympathy. In addition, chaya is a perennial clonally-propagated plant, which is relevant because perennials are often obligate out-crossers (Miller & Gross 2011) and clonal propagation may play an important role as a RIB in these plants (via clonal-sexual reproduction trade-offs). However, this assertion has little empirical support (McKey et al. 2010). Our objectives were to: (i) assess the extent of reproductive isolation between wild and domesticated chaya, and (ii) identify the reproductive barriers and their relative contribution to reproductive isolation. Since chaya has been successfully domesticated and these plants show evident morphological divergence relative to their wild relatives (Munguía-Rosas et al. 2019), we predict the incidence of high levels of reproductive isolation between domesticated and wild chaya.

**MATERIAL AND METHODS**
Study species

Chaya (*Cnidoscolus aconitifolius*) is a long-lived perennial shrub (Standley & Steyermark 1949). It is a monoecious plant species (*i.e.* the same plant produces male and female flowers) that produces short-lived (24h), self-incompatible, generalist insect-pollinated flowers (Arceo-Gómez *et al.* 2009). The fruits are dry capsules containing up to three seeds (Standley & Steyermark 1949). The leaves are deciduous and the leaf area is greater in wild individuals than in cultivated plants (Solís-Montero 2019). Wild chaya have far more urticant trichomes on the stems, leaves and fruits than the domesticated plants (Parra-Tabla *et al.* 2004; Munguía-Rosas *et al.* 2019). Cultivation is usually achieved from stem cuttings in home gardens (Munguía-Rosas *et al.* 2019). Wild plants only reproduce sexually in nature; however, asexual reproduction with stems is possible with human assistance (Munguía-Rosas *et al.* 2019). Although wild chaya has an extensive geographic distribution (from southern Texas to northern South America), the distribution of its cultivation is far more limited, being most popular in the Yucatan Peninsula, where it was domesticated (Ross-Ibarra 2003).

Study site

The study was conducted in a common garden located in the municipality of Merida on the Yucatan Peninsula (20°49’15”- 21°01’18” N; 89°41’30”- 89°33’18”W; 10-30 m a.s.l.), an area where chaya occurs both naturally and in cultivated form. We chose a common garden approach to control for plant and environmental variability and because our main interest was to assess reproductive isolation when wild and domesticated plants occur in the same site. The common garden was established in the study area as part of a bigger project in early 2017. In the garden, wild and cultivated plants collected from 20 different locations distributed across the Yucatan Peninsula were planted from stems. In each location, one stem from 1-3 cultivated plants and the same number from the closest wild plant(s) were collected and planted randomly in a plot of ca. 2000 m², at a distance of 1-1.5 m apart. Mother plants were selected to be as similar as possible. We considered “cultivated” to be a synonym for domesticated plants since cultivated plants typically exhibit an obvious domestication syndrome (*i.e.* less thorny and more branched than wild plants; Munguía-Rosas *et al.* 2019). In addition to being far thornier, the wild plants presented no evidence of having been planted (Munguía-Rosas *et al.* 2019). When this study started, in early May 2018, the garden had 33 wild and 34 cultivated plants; all of
which were of similar size and had reached sexual maturity. The common garden was surrounded by a mixture of native and exotic plants, with *Pseudia piscipula* (Fabaceae), *Ceiba pentandra* (Malvaceae), *Bursera Simaruba* (Burseraceae), *Cassia fistula* (Fabaceae), *Delonix regia* (Fabaceae), lemon (*Citrus limon*; Rutaceae) and orange (*C. aurantium*) the dominant plant species.

**Pre-pollination barriers**

**Phenology and reproductive output**

From early May 2018 to early November 2019, we counted male and female flowers weekly in a randomly selected inflorescence, as well as the inflorescences themselves, in each of 67 plants (33 wild and 34 cultivated) in the common garden. To estimate the number of flowers produced per plant, we multiplied this number by the number of inflorescences per plant. We also randomly collected 64 male flowers (31 from wild plants, 33 from domesticated plants) and 27 female flowers (13 from wild plants, 14 from cultivated plants) early in the morning (6:00-7:30 am), quantified the pollen grains present in the anthers of male flowers and measured the size (largest diameter) of the ovaries of the female flowers. In addition, to examine the microscopic anatomy of the reproductive organs, we observed six male and female flowers in a scanning electron microscope (Jeol-7600-F) with a magnification range of 45-150X.

**Pollinators and pollinator visiting rate**

During the flowering seasons of the years 2018 and 2019, we observed pollinators (*i.e.* flower visitors that made contact with the reproductive organs) in 51 flowers of wild plants (24 female, 27 male) and 29 flowers of cultivated plants (20 female, 9 male). Observations focused on 1 - 4 flowers in one accessible inflorescence per plant, 1 - 3 different plants were observed per day between 8:30 to 10:30 am. Observations lasted 20 minutes per flower. For each flower, we counted the number of visits and recorded pollinator identity to the lowest taxonomic category possible. The unbalanced sample size was due to a far lower production of male flowers in the cultivated plants (see results).

**Post-pollination barriers**

**Pollen tube growth and fruit set**
To assess pollen-pistil incompatibility, we performed inter-variety hand pollinations where pollen from wild plants was used to pollinate female flowers of cultivated plants. A reciprocal pollination treatment (i.e. wild plants pollinated with the pollen of cultivated plants) was not feasible because most male flowers of the cultivated plants produced no pollen (see results). One day prior to pollination, the buds of the female flowers were bagged and, once these buds had opened (8:00 - 9:00 am), pollen from 2-3 male flowers was deposited on the stigma to saturation. The pollinated flowers were then re-bagged and the flowers collected 30 h later, fixed in FAA (formalin-acetic acid-alcohol) and taken to the laboratory where the conspecific pollen grains on the stigma were counted. The styles were then softened in 1N KOH at 65°C for 20 min, rinsed with distilled water and stained for 20 min at 65 °C in decolorized aniline blue. The number of pollen tubes at the base of the style was then quantified under a Nikon ECLIPSE E200 fluorescence microscope (Melville, NY, USA). In total, 30 and 17 female flowers of wild and cultivated plants were processed. Again, the differences in sample size were due to differential flower production between varieties. Another 38 flowers that were treated with the inter-variety hand pollination treatment were not collected, but monitored weekly until flower abortion or fruit set. Moreover, to assess natural fruit set, 43 female flowers of the cultivated plants and 93 female flowers of the wild plants were labelled until the flowers aborted or set fruit.

Data analyses

Phenology and reproductive output

The estimated production of male and female flowers per plant was compared between wild and cultivated plants using generalized linear models with Poisson error distribution and log link function. The number of pollen grains per male flower was compared between wild and cultivated plants using a generalized linear mixed-effects (GLMM) model with Poisson error distribution and a log link function. The diameter of the ovary was also compared between wild and cultivated plants using a linear mixed-effect model with Gaussian error. In the two mixed-models, the plant was included as a random factor.

Pollinator visiting rate

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Pollinator visiting rate per flower was calculated and compared between wild and cultivated plants using a GLMM with negative binomial error distribution and log link function and including the plant as the random factor.

**Pollen tube growth and fruit set**

The proportion of pollen grains on the stigma that germinated and developed pollen tubes per pollinated flower was fitted to a GLMM with binomial error distribution. Fruit set (expressed as a binary variable) was compared among pollination treatments (open-pollinated wild, open-pollinated cultivated and inter-variety cross-pollination) using a GLMM with binomial error distribution. In both models, we used a logit link function and including the plant as a random factor.

**Relative contribution of reproductive barriers**

We estimated a barrier-specific reproductive isolation index ($RI$), as outlined by Sobel & Chen (2014), for two pre-pollination (pollinator differentiation and pollen production) and two post-pollination barriers (pollen-pistil incompatibility and failure to set fruit) in wild and cultivated plants. The $RI$ varies from -1 to 1, with the barrier increasing in strength as the index value approaches one. Negative values indicate potential facilitation of heterospecific mating and/or hybridization; however, this may not be the case and, thus, negative values should be interpreted with caution (Sobel & Chen 2014). According to these authors, $RI = 1 - 2 \times \left( \frac{H}{H + C} \right)$ (Eqn. 1), where $H$ and $C$ refers to heterospecific (in our case, inter-variety) and conspecific (in our case, intra-variety) mating/fitness, respectively. For the $RI$ due to pollinator differentiation, $H$ = exclusive pollinators and $C$ = shared pollinators. Following Martin and Willis (2007), we considered differences in pollen production at plant level (mean pollen produced by flowers x mean number of male flowers per plant) as a reproductive barrier, in this case, $C$ = pollen production in the focal plant variety and $H$ = pollen production in its counterpart. For the reproductive barrier due to pollen-pistil incompatibilities, $H$ = proportion of pollen tubes that reach the base of the style in the inter-variety hand pollinations and $C$ = proportion of pollen tubes that reach the base of the style in the intra-variety pollinations. Since intra-variety pollinations were not feasible in cultivated plants, the $RI$ due to pollen-pistil incompatibility was not calculated for this plant variety. For the reproductive barrier due to the failure
to set fruit, H = fruit set from inter-variety hand pollinations and C = fruit set from intra-variety hand pollinations.

To combine individual reproductive barriers and thus obtain the total reproductive isolation (TRI), we used the equation proposed by Sobel & Chen (2014) for sympatric populations:

\[
TRI = 1 - 2 \times \left[ \frac{\prod_{i=1}^{n} P(H)_i}{\prod_{i=1}^{n} P(H)_i + \prod_{i=1}^{n} P(C)_i} \right]
\]

(Eqn. 2)

Where \( n \) is the number of RIBs, while \( P(H)_i \) and \( P(C)_i \) are the probabilities of inter- and intra-variety gene flow.

Following the same authors, we calculated the absolute contribution (\( AC_i \)) of each reproductive barrier (\( RI_i \)) as:

\[
AC_i = RI_{[1,i]} - RI_{[1,i-1]} \quad (\text{Eqn. 3})
\]

Where \( RI_{[1,i]} \) is the combined isolation calculated by equation 2, including all barriers from the first (1) through the focal barrier (i) and \( RI_{[1,i-1]} \) is the same calculation as for \( RI_{[1,i]} \) but omitting the focal (i) barrier. Finally, the relative contribution (\( RC_n \)) of each barrier at stage n is defined as \( RC_n = \frac{AC_i}{TRI} \) (Eqn. 4) (Sobel & Chen 2014) since, in our specific case, TRI = 1, \( AC_i = RC_n \).

All of the analyses were conducted in R 3.5.1 (R Core Team 2018). The raw data are available in Appendix S1.

**RESULTS**

**Pre-pollination barriers**

**Phenology and reproductive output**

Wild and cultivated plants started flowering in early May and finished in late October with a flowering peak presented between August and September (Fig. 1). The temporal production of female flowers overlapped extensively between the wild and cultivated plants (Fig. 1A). In contrast to the
wild plants, which produced male flowers over the entire season, cultivated plants produced male flowers in discontinuous pulses. However, when both varieties produced male flowers, these overlapped (Fig. 1B). Phenology therefore plays no important role as a RIB between wild and cultivated chaya. Male and female flower production, number of pollen grains per flower and ovary size all differed significantly between wild and cultivated plants (Table 1). Cultivated plants produced 68.54% and 98.09% less female and male flowers, respectively, than wild plants (Table 1). Male flowers of wild plants produced 32749% more pollen than those of the cultivated plants (Table 1). While 100% of examined flowers in wild plants produced pollen, only 18% of the flowers of cultivated plants produced just a few (< 7) pollen grains. The size of the ovary in wild plants was 28% smaller than that of the cultivated plants (Table 1).

The morphology of the reproductive organs of male and female flowers of wild and cultivated plants was normal (Fig. 2). The ovaries in both wild and cultivated plants have three well-developed ovules (Fig.2 A, B). While all male flowers of wild plants had some pollen (Fig. 2 C), the anthers of flowers of the cultivated plants examined were completely devoid of pollen (Fig. 2D).

**Pollinators and pollinator visiting rate**

In total, 18 different species of pollinators, of three orders (Diptera, Hymenoptera & Lepidoptera), visited the flowers of the experimental plants; all except two (*Camponotus* sp; Hymenoptera: formicidae and an unidentified dipteran) visited male flowers of the wild plants and five species visited female flowers (intra-variety shared species=3 [species that visited male & female flowers of the same variety]) (Fig. 3). Only two pollinator species visited the flowers of the cultivated plants: *Camponotus* sp and an unidentified species of Halictidae (Hymenoptera). These two species visited female flowers, but only *Camponotus* sp visited male flowers (Intra-variety shared species = 1) (Fig. 3). Only two pollinator species visited male and female flowers of different varieties: an unidentified Halictidae and *Camponotus* sp (inter-variety shared species = 2) (Fig. 3).

Regarding pollinator visit rate, the flowers of wild plants (5.06 ± 1.31 visits / flower / h) received 892% more visits than flowers of cultivated plants (0.51 ± 0.49) ($\chi^2 = 5.31, P = 0.02$). Male flowers (6.44 ± 1.81) received 450% more visits than female flowers (1.17 ± 0.49) ($\chi^2 = 19.51, P <$
0.01), regardless of the variety. The interaction plant variety x flower sex was not statistically significant ($\chi^2_1 = 1.79, P = 0.18$) (Fig. 4).

**Post-pollination barriers**

**Pollen tube growth and fruit set**

Of all of the conspecific pollen grains deposited on the stigmas, $13.36 \pm 3.48\%$ developed pollen tubes in the female flowers of wild plants, but only $0.41 \pm 0.17\%$ developed pollen tubes in the cultivated plants; these differences were statistically significant ($\chi^2_1 = 14.82, P < 0.01$). Fruit set in open-pollinated flowers was 710 $\%$ higher in wild than in cultivated plants (Table 2). In contrast to wild plants, where all of the individuals except one produced fruit, only one cultivated plant set fruit. Flowers treated with inter-variety hand pollinations did not set any fruit (Table 2). The differences among pollination treatments were statistically significant ($\chi^2_1 = 13.84, P < 0.01$). *Post hoc* multiple comparisons indicated that the differences between open-pollinated wild and cultivated, as well as between open-pollinated flowers of wild plants and inter-variety hand pollinated flowers, were statistically significant. However, the differences between inter-variety hand pollinated and open-pollinated flowers of cultivated plants were not (Table 2).

**Relative contribution of reproductive barriers**

Regarding pre-pollination barriers, in wild plants, the highest $RI$ was found for pollen production ($0.99$), followed by pollinator differentiation ($0.77$). In contrast, for cultivated plants, all pre-pollination barriers had a value of $RI < 0$. Regarding post-pollination barriers, pollen-pistil incompatibility ($RI = 0.94$) and failure to set fruit ($RI = 1$) had high values in wild and cultivated plants. The relative contributions of pre-pollination barriers were also stronger in wild plants, particularly that of pollinator differentiation ($RC = 0.77$). In cultivated plants, only the failure to set fruit made a strong relative contribution to reproductive isolation ($RC = 1.00$). Despite the high $RI$ values, post-pollination barriers made little relative contribution to reproductive isolation in wild plants ($RC = 0$). Total reproductive isolation reached its highest value ($TRI=1.00$) in both wild and cultivated plants (Table 3).
DISCUSSION

In this study we assessed the reproductive barriers and their relative contribution to reproductive isolation between domesticated *Cnidoscolus aconitifolius* and co-occurring wild ancestors in their domestication centre (Ross-Ibarra 2003; Munguía-Rosas *et al.* 2019). Our results clearly show that, despite their co-occurrence, the wild and domesticated chaya exhibit a high degree of reproductive isolation.

Among the pre-zygotic barriers, pre-pollination barriers such as reduced differential pollen production and pollinator differentiation made the greatest relative contribution to reproductive isolation in wild chaya. This was mainly due to the dramatic reduction (0.6 grains per flower on average) in pollen production observed in the male flowers of cultivated plants. This almost complete lack of pollen could be considered a “magic trait” (Servedio *et al.* 2011) that prevents the opportunity for mating and thus rapidly confers reproductive isolation in wild chaya. A lack of pollen occurs relatively often in nature as a result of a spontaneous mutation. Since the reproductive organs are anatomically normal, this mutation probably affects pollen production during meiosis (*e.g.* Saumitou-Laprade *et al.* 1994). The lack of pollen is not consciously selected; rather, it is probably genetically correlated with other plant traits that are being artificially selected (Munguía-Rosas *et al.* 2019). It is likely that the lack of pollen was rapidly fixed as a character in the population of cultivated chaya as a result of clonal propagation (Ross-Ibarra 2003; McKey *et al.* 2010). Clonal propagation is common in perennial crops because it allows propagation of plants during the long juvenile phase (Miller & Gross 2011). As a result of clonal propagation, some perennial crops present a reduced investment in sexual reproduction owing to trade-offs between vegetative vs. sexual biomass/resource allocation (McKey *et al.* 2010). This could be the case in chaya, since cultivated plants produce more leaves and branches (Munguía-Rosas *et al.* 2019), but far fewer flowers, than wild plants (Table 1). In some sense, clonality contributes to reproductive isolation in chaya because it reduces the chance of gene flow via reduced reproductive investment.

Since pollen is also an important reward for pollinators of chaya (*Arceo-Gómez et al.* 2009), the lack of pollen also contributed to reproductive isolation by promoting pollinator differentiation. This notion is supported by the fact that the male flowers received far more visits than their female...
counterparts. A previous study also found pollinator differentiation between wild and domesticated cardamom (*Elettaria cardamomum*; Zingiberaceae) (Kuriakose *et al.* 2009), suggesting that pre-pollination RIBs may be more common in crops than was previously thought. In contrast to wild plants, we found negative $RI$ values for pre-pollination barriers in cultivated plants. This was because the two pollinators that visited the flowers of these plants are shared with wild plants, which may act to facilitate heterospecific pollen transfer. However, the fact that one of two shared pollinators was an ant (which are often nectar thieves e.g. Rostás *et al.* 2018) and also that the flowers of the cultivated plants were visited far less frequently, leads us to conclude that pollen transfer from wild to cultivated plants is low.

Pollen-pistil incompatibility and failure to set fruit had high $RI$ values (0.94 -1.00); however, their relative contribution to reproductive isolation was negligible in wild plants since complete reproductive isolation had already been achieved before the pollen tubes fertilized the ovary. Failure to set fruit was the only reproductive barrier detected in the cultivated plants, but it was strong enough to achieve complete reproductive isolation in this variety. Similarly, a previous study reported that inter-variety hand pollinations (domesticated x wild plants) did not produce fruit in the columnar cactus *Stenocereus stellatus* under sympatry (Casas *et al.* 1999), however, more studies are required in order to fully assess the generality of this result. Despite the almost complete absence of pollen, some open-pollinated flowers set fruit in one cultivated plant only (not considered in the inter-variety hand pollinations). It is likely that there is some intra-variety variation in the strength of barriers associated with failure to set fruit in chaya. Indeed, Martin & Willis (2007) have suggested that RIBs may not act uniformly on all individuals within a population. Chaya has only recently been domesticated (Ross-Ibarra 2003; Munguía-Rosas *et al.* 2009) and individuals with weaker pollen incompatibility probably have not been entirely eliminated from the population as a result of recent artificial selection. Evidently, more extensive investigation is required in order to fully test this hypothesis.

Reproductive isolation barriers in chaya are highly asymmetrical. While pre-zygotic barriers were sufficient to achieve complete reproductive isolation between wild and cultivated plants in the direction of cultivated to wild plants, only the post-zygotic barrier (fruit set failure) contributed to
reproductive isolation in opposite direction. In contrast to our results, Lowry et al. (2018) found that asymmetry in post-mating RIBs was greater than pre-mating RIBs in co-occurring wild sister species. However, the same authors found that pre-zygotic barriers are more common and stronger than post-zygotic barriers, which does agree with our findings in the direction of cultivated to wild. Regarding gene flow from wild to cultivated plants, our results support Dempewolf et al. (2012), who found that post-zygotic barriers were most commonly reported between crops and their wild relatives. However, it is unclear in that study whether RIBs are symmetrical or not. In fact, the strength of RIBs and their relative contribution to reproductive isolation were not formally calculated, and we therefore have no suitable reference values with which to contrast our results. More studies comparing several pre- and post-pollination barriers between crops and their wild relatives are clearly required in order to assess the generality of the results shown in this study.

In conclusion, almost complete reproductive isolation exists between co-occurring wild and domesticated chaya, and this reproductive isolation has evolved in sympatry. Moreover, the RIBs exhibited some degree of asymmetry. The lack of pollen was a key trait in attaining the observed isolation level, particularly in the direction of domesticated to wild plants. In the opposite direction, while failure to set fruit was the only barrier, it was strong enough to achieve complete reproductive isolation. The lack of pollen also prevented the domesticated plants from fertilizing their own ovules. Reproduction of domesticated chaya therefore relies on clonal propagation. Asexual reproduction can itself be considered a reproductive barrier, and this is common in cultivated and wild plants (McKey et al. 2010). Reproductive isolation would have played a major role during the early stages of domestication of several crops because most domestication events took place in domestication centres where the crops co-occur with their wild relatives. Since, even today, plants with a variable degree of domestication co-occur with their progenitors in domestication centres, these may represent an ideal study system with which to understand the mechanism that underlies sympatric speciation.

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REFERENCES


Table 1. Traits related to reproductive effort measured in wild and cultivated plants of *Cnidoscolus aconitifolius* in a common garden. Numbers of male and female flowers were estimated at plant level, while the number of pollen grains and ovary size (ovary diameter) were determined at flower level. For female and male flowers, \( n = \) the number of plants and, for pollen grains and ovary diameter, \( n = \) the number of flowers measured. The statistics associated with the variety factor (two levels: wild vs. cultivated) for each variable are shown in the final column. The numbers of flowers (male & female) were fitted to a generalized linear model with Poisson error distribution, and the values for pollen grains and ovary diameter were fitted to generalized mixed-effects models, with Poisson and Gaussian error, respectively. In both models, the plant was included as a random factor.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Wild</th>
<th>Cultivated</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>( n )</td>
<td></td>
</tr>
<tr>
<td>Female flowers (count)</td>
<td>44.79 ± 8.52</td>
<td>14.09 ± 3.34</td>
<td>( \chi^2_1 = 562** )</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>(33)</td>
<td></td>
</tr>
<tr>
<td>Male flowers (count)</td>
<td>241.41 ± 35.51</td>
<td>4.61 ± 1.74</td>
<td>( \chi^2_1 = 9831** )</td>
</tr>
<tr>
<td></td>
<td>(34)</td>
<td>(33)</td>
<td></td>
</tr>
<tr>
<td>Fruit (count)</td>
<td>152.91 ± 92.01</td>
<td>4.27 ± 4.24</td>
<td>( \chi^2_1 = 5941** )</td>
</tr>
</tbody>
</table>
Table 2. Fruit set (%) of open-pollinated flowers of wild and cultivated plants, as well as inter-variety hand pollinated flowers of Cnidoscolus aconitifolius. For the inter-variety hand pollination treatment, pollen from a male flower of a wild plant was placed on the stigma of a female flower of a cultivated plant. Sample size (Number of flowers and plants) and fruit production (Fruit set) are also shown. Different letters in the last row denote statistically significant ($P < 0.05$) differences in terms of fruit set.

<table>
<thead>
<tr>
<th>Treatment (Variety)</th>
<th>Open (Wild)</th>
<th>Open (Cultivated)</th>
<th>Cross (Wild x Cultivated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (Flowers, plants)</td>
<td>93, 10</td>
<td>43, 18</td>
<td>38, 11</td>
</tr>
<tr>
<td>Fruit</td>
<td>70</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Fruit set (%)</td>
<td>75.26$^a$</td>
<td>9.29$^b$</td>
<td>0$^b$</td>
</tr>
</tbody>
</table>

**$P < 0.01$**

$\chi^2_{1} = 418^{**}$

$F_{1,45} = 23^{**}$
Table 3. Reproductive isolation barrier indices for some pre- and post-pollination reproductive barriers assessed in sympatric wild (Wild) and cultivated (Cultivated) individuals of *Cnidosculus aconitifolius* in the domestication centre of the species. The relative contributions of each barrier for wild and cultivated plants are also shown. Total reproductive isolation (last row) presented a value of 1 for both wild and cultivated plants, meaning that full reproductive isolation was reached with these reproductive barriers in both wild and cultivated plants.

<table>
<thead>
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Fig. 1. Reproductive phenology of wild and cultivated C. aconitifolius plants in the centre of domestication of the species (Yucatan, Mexico). Mean weekly production values for female (A) and male (B) flowers per plant are presented. Dotted and continuous error bars correspond to the production of female and male flowers, respectively.

Fig. 2. Scanning electron microphotographs of the feminine (A, B) and masculine (C, D) reproductive organs of wild (A, C) and cultivated (BC) plants of *Cnidoscolus aconitifolius*. The label shows the scale bar, voltage (acc.v) and magnification (magn.). A= Cross section of the ovary of a female flower of a wild plant, B = Cross section of the ovary of a female flower of a cultivated plant, C= View of an anther of a male flower of a wild plant with pollen grains attached, D= View of an anther of a male flower of a cultivated plant; note the absence of pollen grains.

Fig. 3. Venn diagram of flower visitors observed in male and female flowers of wild and cultivated plants of *Cnidoscolus aconitifolius*. Species in the intersections are shared pollinators. S = visitor species richness. Bom sp 1= unidentified species of Bombyliidae (Diptera), UI-D= unidentified dipteran, Apis m = *Apis mellifera* (Hymenoptera: Apidae), Apidae sp 1 = unidentified species of Apidae (Hymenoptera), Cam sp1= *Camponotus* sp (Hymenoptera: Formicidae), Hal sp1- sp5 = unidentified species (five) of Halictidae  (Hymenoptera), Hel ch = *Heliconius charithonia* (Lepidoptera: Nymphalidae), Hes sp1 & sp2 = unidentified species (two) of Hesperiidae (Hymenoptera), Pho a = *Phoebis agarithe* (Lepidoptera: Pieridae), Lyc sp1 & sp2 = unidentified species (two) of Lycaenidae (Hymenoptera), UI-L= unidentified lepidopteran.

Fig. 4. Visiting rate of pollinators to flowers of *Cnidosculus aconitifolius*. Data were stratified and compared between plant varieties (wild vs. cultivated) and flower sexes (female vs. male). Asterisks indicate statistically significant differences ($P < 0.05$) between pairs of means.

Appendix S1. Raw data.
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