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Possible tobacco progenitors share long-tongued hawkmoths as pollen vectors

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Abstract. The putative ancestors of the allopolyploid hybrid *Nicotiana tabacum* have distinct flower features, apparently suited either for hawkmoth or bat pollination. This suggests that progenitors were reproductively isolated by mechanical and ethological barriers. However, the present data show that in natural populations pollen vectors could be shared by two of the possible progenitors. Pollen vectors of one of the possible male progenitors (*N. otophora*) were short- and long-tongued hawkmoths and a nectar-feeding bat, while those of the female ancestor (*N. sylvestris*) were only long-tongued hawkmoths. The latter are then the most likely vectors responsible for the presumed spontaneous hybridization. These data also suggest that interspecific pollen transfer occurred more likely in one direction.

Key words: *Nicotiana otophora*, *N. sylvestris*, tobacco, pollination, bats, hawkmoths, Solanaceae.

Introduction

Karyological and cytological evidence suggests that *Nicotiana tabacum* L. (Solanaceae) originated by a hybridization process in which an ancestor of *N. sylvestris* Spegazzini & Comes acted as maternal progenitor and a still unrecognized species of sect. *Tomentosae* as male progenitor (Goodspeed 1954, Kenton et al. 1993). *Nicotiana otophora* Grisebach, *N. tomentosiformis* Goodspeed or both, which exhibit nearly identical flower structure, are the most likely pollen donors (Lim et al. 2000, Ren et al. 2001).

At present *N. otophora* and *N. sylvestris* are the only pair of possible parents that occasionally coexist. *N. sylvestris* ranges from about 20°S in southern Bolivia down to about 30°S in NW Argentina (Fig. 1). While *N. tomentosiformis* has been found only north of this range in a restricted area around La Paz (Bolivia, about 16°32′S) (Goodspeed 1954), *N. otophora* potentially overlaps with *N. sylvestris* in southern Bolivia and NW Argentina nearly throughout its northernmost range (Fig. 1). These species are not frequently collected and to our knowledge have never been found growing next to each other at the same time. We are aware of only three sites where both species have been collected within a distance of less than 70 km: Sierra Alto del Río Seco (Salta province), Sierra de Calilegua, and Sierra de Santa Bárbara (Jujuy province) (Fig. 1) (Goodspeed 1954, Cabrera 1983).

The formation of the hybrid is apparently a rare event, and the time and place of its origin

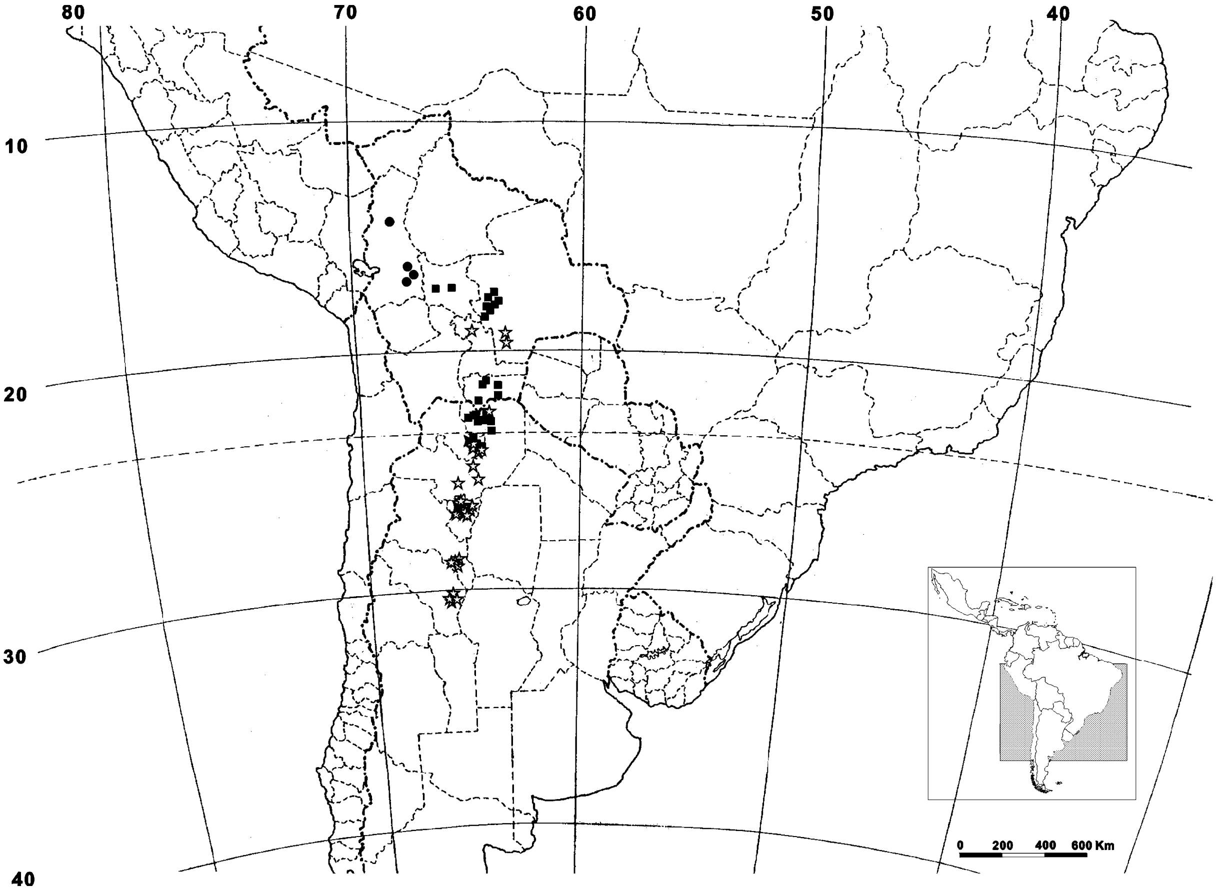

Fig. 1. Distribution of the proposed progenitors of tobacco: (■) *N. otophora*, (●) *N. tomentosiformis* and (☆) *N. sylvestris* and study sites (➤)

cannot yet be ascertained. Crossing of the progenitors could have taken place spontaneously as early as 6 m.y. BP, according to chloroplast sequence divergence (Okamuro 1985) or more recently through domestication as the lack of wild hybrids suggests. At the time when hybridization occurred the progenitors could have had a wider geographical range so the place of origin is also uncertain and it is not necessarily the present overlapping area of the progenitors. Despite these uncertainties in the identity of the male progenitors, and the place and time of origin of tobacco, the present interaction of *N. otophora* and *N. sylvestris* with their pollinators provides an instructive model of a scenario for the origin of *N. tabacum*.

Since flower structure and appearance of both parental species are very distinct, it was early suggested that they should have different pollinators. They appeared to be suited either to hawkmoth or bat pollination (Vogel 1968). *N. otophora* flowers have several features known to be adapted to bat pollination, such as nocturnal opening, broad mask-shaped corolla, copious nectar, large nectar gland, dull flower colour and inflorescences protruding from the foliage (Vogel 1968, von Helversen 1993). Conversely, *N. sylvestris* flowers have features typical of hawkmoth-pollinated plants such as long corolla tube, star-shaped corolla limb, white flower colour and intense nocturnal fragrance (Loughrin et al. 1990, Vogel 1963).

These contrasting flower features suggest the existence of mechanical and/or ethological isolation barriers between the progenitors. However, interspecific pollen transfer would not have been completely excluded. In natural populations of *N. otophora* and *N. sylvestris* we studied which animal species act as pollinators and how pollen of these *Nicotiana* species is transported.

Material and methods

Since we never found both possible progenitors growing together during the study we made samplings of two possible progenitors independently.

Hawkmoths were collected during peak flowering with light traps by the vertical sheet method (Fry and Waring 2001) in populations of *N. sylvestris* (El Laurel site, Tucuman province, 26°23′27.9″S; 65°29′9.4″W) and *N. otophora* (Sierra de Calilegua site, Jujuy province, 23°36′22.0″S; 64°57′18.6″W) distant 287 km from each other. In these populations flower visiting hawkmoths were also recorded with a photographic camera and a video recorder. In a third population, Sierra de Santa Bárbara (Jujuy province, 24°15′87.6″S; 64°26.4′27″W), where both species reportedly once coexisted, additional hawkmoths sampling was made (Fig. 1). However, neither species was flowering at this site when sampling was made. Since pollen of none of the focal species was found on the hawkmoths captured there, our data on this site are limited to the composition of the local hawkmoths fauna. The sampling was made in two nights at each site between 20:00 and 24:00 hours, with 160 W MB lamps. All arriving hawkmoths were captured during this time. To determine the plant species visited and the part of the body where the pollen was carried, a palynological analysis was performed. The pollen samples for microscopical analysis were taken in the field under a binocular microscope and mounted in glycerine jelly for later study in the laboratory (Kearns et al. 1993).

To trap bats two mist nets were laid near the *N. otophora* plants for 5 nights during peak flowering. Only one specimen of *Anoura caudifer* was caught. Pollen was removed from it by lengthwise laying and pressing a piece of clear plastic tape from the tip of the muzzle to the nape and then inspected with an epifluorescence microscope. Also pollen samples from the gut were taken after dissection. The bat is deposited in the Instituto Lillo under the accession number CML 5578.

To make metric correlations, the operative lengths and corolla depth of flowers of both species were measured. Length of flower parts (stamens, style and corolla tube) were measured considering that the proboscises of hawkmoths can be introduced deeper into the narrow parts of the corolla tube than the muzzle of a bat. Thus, in *N. sylvestris* the muzzle of a bat cannot be introduced at all while in *N. otophora* it can be introduced only in the wider distal portion. Accordingly, operative lengths and corolla depth are meant as the distance between fertile parts or corolla mouth and the depth of the corolla tube to which the mouth parts (proboscis or muzzle) can be introduced. These measurements are given as mean ± SD.

Results

Flower architectures. Flower architectures of *N. sylvestris* and *N. otophora* fit well to long-tongued hawkmoths or to *Anoura caudifer*, respectively (see Fig. 2A, B).

Position of anthers and stigmata determine that pollen is deposited onto the tongues in the former and the upper part of the head in the latter.

N. otophora flowers have relatively short tubes (32.60 ± 2.24 mm, n = 21) (Figs. 2A, 3). *N. sylvestris* flowers limit accessibility to nectar by their long (71.94 ± 6.25 mm, n = 107) and narrow corolla tubes (Figs. 2B, 4).

Pollinators. On a total of 79 hawkmoths captured in El Laurel and Sierra de Calilegua sites, microscopical analysis revealed pollen of either one or the other species of *Nicotiana* on 54 hawkmoths (Figs. 3,4). Pollen of *N. otophora* was found on an individual of *Anoura caudifer* (Figs. 2A,5), the only bat trapped. Percentage of hawkmoths carrying pollen did not differ significantly between populations (64.15% and 76.92% respectively; Williams adjusted G = 1.17, n.s.). Places of pollen deposition on hawkmoths included proboscises (94.55%), palps (13.46%), legs (5.77%), eyes (1.82%), and wings (1.82%). The bat carried *N. otophora* pollen on the muzzle, forehead, and in the gut.

Distribution of pollen-carrying individuals among the hawkmoths differed between the two populations. In Sierra de Calilegua, where only *N. otophora* occurs (site 1, Fig. 1), the

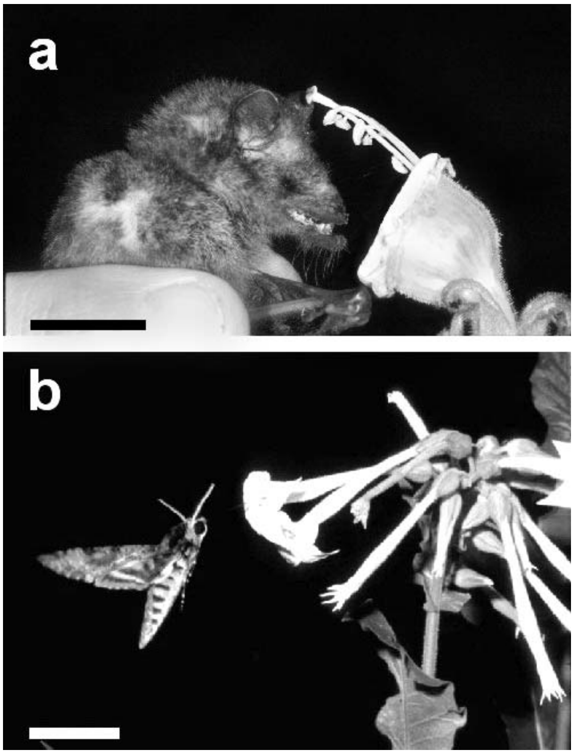

Fig. 2. **A** The bat *Anoura caudifer* posed with muzzle inside the flower of *N. otophora*. **B** The hawkmoth *Agrius cingulata* introducing its tongue into the flower of *N. sylvestris*. Bars: 30 mm

frequencies of short-tongued (60% with proboscis < 40.43 mm) and long-tongued hawkmoths (82% with proboscis > 59.62 mm) carrying pollen on the proboscis did not differ from random (Williams adjusted $G = 1.60$, n.s.) (Fig. 3). In the *N. sylvestris* population (site 3, Fig. 1) these frequencies (0% with proboscis < 44.98 mm and 73% with proboscis > 56.24 mm) significantly differed from random (Yates adjusted $G = 12.72$, $P < 0.001$) (Fig. 4).

Pictures taken of *N. sylvestris* show 16 hawkmoths; 5 are assignable to *Agrius cingulata*, 3 to *Manduca tucumana*, 1 to *Manduca diffissa* and 7 to either one of the latter two species. Of all hawkmoths 4 were approaching the flower, 2 were about to introduce the proboscis into the corolla by touching its limb with the proboscis tip and 9 had their proboscis partially (4) or completely (5) introduced in the tube.

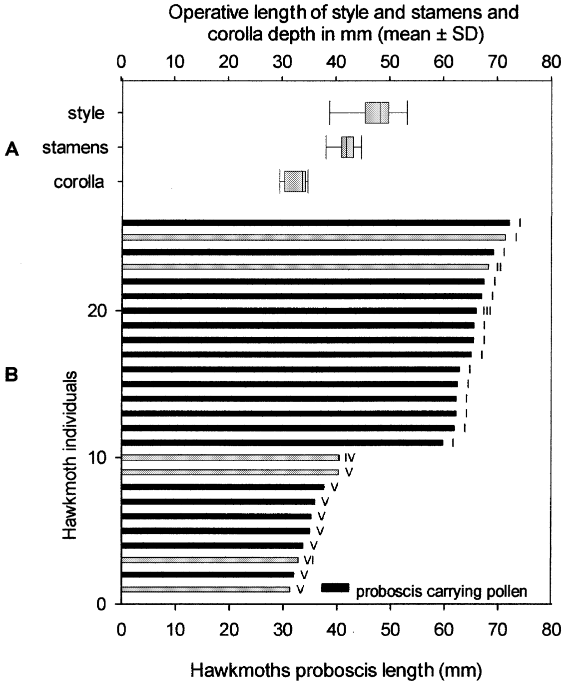

Fig. 3. Flower dimensions and pollen vectors of *N. otophora* in the Sierra de Calilegua population. **A** Total length of flowers parts. **B** Distribution of pollen among hawkmoths of all proboscis length. Hawkmoth species: I *Manduca diffissa*, II *Manduca tucumana*, III *Euryglottis aper*, IV *Eumorpha neuburgeri*, V *Erinnyis ello* and VI *Xylophanes pluto*

Those individuals of *Manduca* sp. that had their proboscis inside the tube (6) had extended legs, and when their proboscises were completely introduced into the corolla, they held to the corolla limb with the front and mid legs. Individuals of *Agrius cingulata* (5) always introduced their proboscis without extending their legs and never touched the corolla with any body part other than the proboscis.

Seven hawkmoths carried a visible amount of white pollen diffusely spread on the proximal half of two thirds of the proboscis.

The flowers the hawkmoths visited always had their corolla limb turned to the back and the flower axis was tilted downward, upward or horizontal.

The average rate of visits recorded of hawkmoths to *N. otophora* flowers was 0.37 visits/minute/flower. During 32 minutes visits

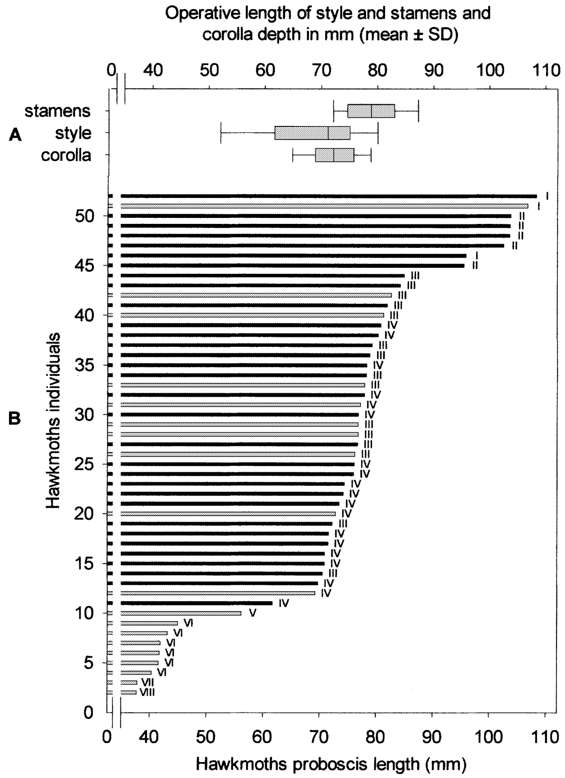

Fig. 4. Flowers dimensions and pollen vectors of *N. sylvestris* at El Laurel population. **A** Total length of flowers parts. **B** Distribution of pollen among hawkmoths of all proboscis length. Hawkmoth species: I *Agrius cingulata*, II *Manduca sexta*, III *Manduca tucumana*, IV *Manduca diffissa*, V *Manduca bergi*, VI *Manduca armatipes*, VII *Erinnyis ello* and VIII *Erinnyis oenotrus*

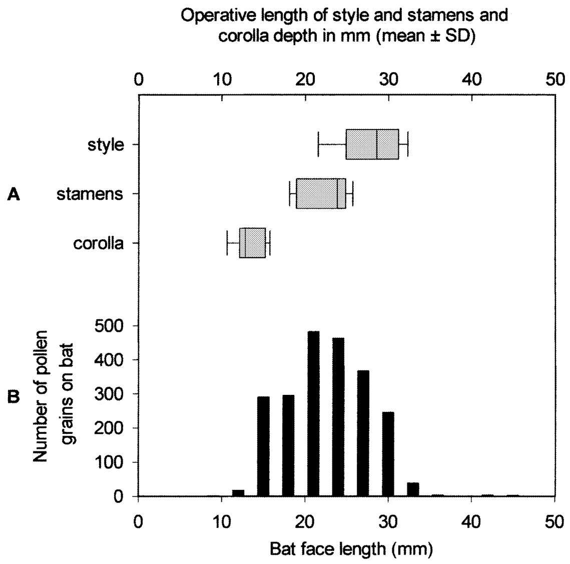

Fig. 5. Flower dimensions and pollen vectors of *N. otophora* in the Sierra de Calilegua population. **A** Operative length of flowers for bat. **B** Distribution of pollen on the face of *Anoura caudifer*

to 191 flowers were recorded. The duration of visits was in general about one and up to five seconds.

Long-tongued individuals (*Manduca* spp.) that visit flowers of *N. otophora*, usually touch the stamens and stigma with the tongue, while individuals with shorter tongues take nectar from a low position well bellow the stamens and stigma and occasionally touch the fertile parts when approaching or leaving the flowers.

We sampled 197 hawkmoths of 17 species at the Santa Bárbara site (site 2, Fig. 1) (Table 1). None of the specimens captured carried pollen of either *Nicotiana* species. The hawkmoths collected here included all but two species of the previous sampling sites. The three sampling sites share only three species, *Erinnyis ello, Manduca diffissa* and *Manduca tucumana*. The two latter hawkmoths species are the most frequent in all three of the sites and together constitute between 46 and 58% of all hawkmoths at each site.

Discussion

The present data show for the first time hawkmoth pollination of *Nicotiana sylvestris* and *N. otophora*, and also provide the first empirical evidence of bat pollination in Argentina. *N. otophora* can indeed be pollinated by bats as suggested earlier by Vogel (1968). However, long term studies are needed to determine how important these animals are in the reproductive success of this species. Flower architecture fits well with the form and dimensions of the bat's head, however, more data are needed to make a more general statement about the adaptation of *N. otophora* to bat pollination since hawkmoths are shown here to be more frequent pollinators of this species than bats. We do not know how efficient both pollinator kinds are nor whether their abun-

Table 1. Species, proboscis mean length and number of hawkmoths collected at Santa Bárbara site

Site	Species	Proboscis mean length ± SD (mm)	Number of hawkmoths collected
Santa Bárbara	*Callionima grisescens* (Rothschild, 1894)	16.33 ± 0.78	14
	Erinnyis obscura (Fabricius, 1775)	26.44 ± 1.57	3
	Xylophanes tersa (L., 1771)	32.39 ± 3.44	3
	Erinnyis ello (L., 1758)	35.15 ± 2.13	2
	Xylophanes schreiteri (Clark, 1923)	35.57 ± 1.23	6
	Xylophanes pluto (Fabricius, 1777)	38.33	1
	Erinnyis lassauxii (Boisduval, 1859)	41.74 ± 2.90	2
	Erinnyis oenotrus (Cramer, 1780)	42.49 ± 1.08	4
	Eumorpha satellita (L., 1771)	43.28	1
	Manduca armatipes (Rothschild & Jordan, 1916)	47.37 ± 2.15	4
	Eumorpha neuburgeri (Rothschild & Jordan, 1903)	49.02 ± 0.68	2
	Sphinx aurigutta (Rothschild & Jordan, 1903)	55.63 ± 14.38	4
	Manduca bergi (Rothschild & Jordan, 1903)	56.65 ± 2.83	18
	Sphinx maura (Burmeister, 1879)	57.48 ± 4.41	39
	Manduca diffissa (Butler, 1871)	65.26 ± 5.60	36
	Manduca tucumana (Rothschild & Jordan,1903)	78.84 ± 3.42	52
	Manduca sexta (L., 1763)	102.02 ± 4.52	6

dance varies between sites or from year to year. Such data would be essential to explain this apparent discordance between pollinator and flower structure. Bat pollination of *N. sylvestris* should be impossible for its long and narrow corolla tube, since *M. tucumana*, which is endemic to NW Argentina (Schreiber 1978), and *M. diffissa*, which is more widespread, are the only hawkmoth species occurring in both study sites where the progenitors reportedly coexist and since their proboscises are longer than 56 mm and thus long enough to pollinate *N. sylvestris,* they would then be the most likely pollen vectors from *N. otophora* to *N. sylvestris*.

The relationship between *N. sylvestris* and hawkmoths is of the same kind as known for other hawkmoth-pollinated plants, i.e. specialized use of available pollinators (Nilsson et al. 1985, 1987). In other hawkmoth-pollinated plant species, such a pattern results from the selective pressure, particularly on female fitness, favouring an increase in flower depths (Nilsson 1988, Maad 2000). Selection for flower depth does not appear to be acting in the same way in *N. otophora* and hence a wider range of pollen vectors are attracted.

When carried on the proboscis, pollen is more easily channelled to the stigmata in the *N. sylvestris* flowers than it is to those of *N. otophora*. This is because the styles are included in the corolla tube of the former (Fig 2B), whereas they are exerted from the short and broad corolla tube of the latter (Fig 2A). Thus visitors entering *N. otophora* flowers, particularly long-tongued ones that are known to approach flowers by hovering in a swinging fashion (Wasserthal 1993, Wasserthal 1997), have freedom of flight deflections and less chances to strike the stigma. We suggest that these contrasts in the use of pollen vectors and in flower functional morphology favoured pollen transference in one direction, i. e. from *N. otophora* to *N. sylvestris*. However, studies on interspecific pollen or pollen analogue transfer are needed to support this suggestion.

Considering the supposed antiquity of *N. tabacum* (Okamuro et al. 1985), the hybridization process must have been a very rare event. This process requires the combination of two highly fortuitous steps: first, interspecific pollen transfer between flowers of different structure and, second, formation of allotetraploid zygotes. Though the possibility of occurrence of the second step has been extensively studied in cytological and molecular fields (Kenton 1993, Okamuro 1985), empirical data were completely lacking as to how the first step could have taken place in wild populations.

Evidence presented here shows that the premating mechanical-ethological reproductive barriers between two of the possible progenitors, known to be effective in others plants (Grant 1994), can be broken down by long-tongued hawkmoths, thus facilitating the first hybridization step. However, for the hybrid to come into existence its progenitors must have coexisted closely enough and the post-mating barriers must have been overcome.

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