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On speciation and hybridization among closely related species: establishing an experimental breeding lineage between two species of *Automeris* Hübner moths (Lepidoptera: Saturniidae) and implications for taxonomy

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Abstract. Many species of plants and a few species of animals are believed to have resulted from hybridization of parental species, and the ability of species to occasionally hybridize in captivity and in nature is even more widespread. In the present study, we describe a hybridization experiment conducted in the laboratory between the sexually dimorphic *Automeris io* (Fabricius), a widespread, variable species ranging from Canada to Costa Rica, and its congener *A. louisiana* (Ferguson and Brou), a more local, sexually monomorphic species (Lepidoptera: Saturniidae). The *A. louisiana* populations occur in a highly specialized habitat—the coastal marshland along the Gulf of Mexico in Louisiana and Texas and is nested inside the broad distribution of *A. io*, demonstrating strong differences from the latter in its ecology and morphology. No natural hybridization between the two species has been described. While the separate species status of *A. io* and *A. louisiana* is supported by morphology and ecology of their populations, we were able to create a hybrid lineage in the laboratory and maintained it for three generations. The hybrids were phenotypically intermediate between the parental species. Under a stricter reading of the biological species concept, such an ability to hybridize would be interpreted by some as a sign of conspecificity. Our experiments once again demonstrate the complexity of 'species' as a concept, which may need major redefinition in the popular interpretation of sciences.

Key words. Allopatric, biological species concept, coastal marshlands, gene flow, geographic isolation, Io moth, Louisiana eyed moth.

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Introduction

Hybrid speciation has been proposed as a mechanism for the origin of some Lepidoptera species. Perhaps the most striking example is *Heliconius heurippa* Hewitson, 1854 (Nymphalidae), which is believed to be a result of hybridization between *H. cydno* (Doubleday, 1847) and *H. melpomene* (Linnaeus, 1758) (Salazar et al. 2005). Laboratory hybridization experiments played a role in understanding this landmark system (Mavárez et al. 2006).

Less spectacular of an example, though not any less studied, is the case of hypothesized hybrid speciation in the tiger swallowtail complex in North America, where *Papilio appalachiensis* Pavulaan and Wright, 2002 (Papilionidae) is believed to be a hybrid species resulting from interbreeding of *Papilio canadensis* (Rothschild and Jordan, 1906) to the north and *Papilio glaucus* Linnaeus, 1758 to the south of its range (Scriber and Ording 2005). Like the *H. heurippa* case, this latter example attracted significant attention, including genome-level assessment of what makes a hybrid species (Zhang et al. 2013). Research on the genus *Heliconius* Kluk, species of which frequently hybridize both in nature and in captivity, continues to be a constant source of new information about how haplotypes of different species interact in hybrid zones (e.g., Jiggins et al. 2008; Meier et al. 2020).

Hybridization experiments in the laboratory are time consuming, but they can provide significant insights into species boundaries and even into the mechanisms of evolutionary developments. In Lepidoptera, an iconic example is the series of experiments with gypsy moths conducted by Richard Goldschmidt (e.g., Goldschmidt 1931) at the dawn of the 20th century that resulted in breakthroughs in biological sciences (see Dietrich 2003 and references therein). More recently, Platt (1975) explored mechanisms of wing pattern evolution in mimetic *Limenitis* Fabricius butterflies in North America by interbreeding them. Such laboratory interbreeding experiments with Lepidoptera continue to provide insights into biology, frequently yielding unusual specimens such as gynandromorphs (e.g., Adamski et al. 2019).

One of the criteria by which species are frequently evaluated as being distinct under the biological species concept (BSC; Mayr 1963), and cited mistakenly as a guiding principle, is their inability to produce the F-2 generation of hybrids. However, this abbreviated description of 'species' as a concept, familiar to almost every good high school student, does serious disservice to grasping the complexities of biodiversity and evolution. In a recent effort to revive Goldschmidt's 'hopeful monsters' theory, Dittrich-Reed and Fitzpatrick (2013) suggested that transgressive hybrids with similar recombinant phenotypes not only can establish true-breeding lineages but are also a source of evolutionary advancement. This is supported by evidence in corvid birds (e.g., Kryukov 2019 and references therewith), where wild hybrids seemed to be better adapted than the parental populations. In the world of conservation of natural species and captive breeding, another example of introgressive hybridization is that of American bison and European wisent. Efforts to restore wild wisent populations led, at some point, to hybridizing the two species from captive populations (Sipko et al. 2010 and references therewith). Among Lepidoptera in captivity, many swallowtail butterflies can be hybridized using hand-pairing techniques, and while most successful matings produce sterile hybrids, sometimes hybrids are fully fertile (Zakharov et al. 2004 and references therewith). Among other Lepidoptera, experiments by Platt (1975) mentioned above demonstrated that distinct species of *Limenitis* butterflies can be hybridized in the lab, and hybrids were successfully back-crossed with the parental stock. Such back-crosses of interspecific F-1 hybrids with the parental species in domesticated animals are known to occur among donkeys, horses, and buffalo (Zong and Fan 1989 and references therewith).

Saturniid moths are known to be hybridized by breeders in captivity, with intermediate hybrids obtained on a number of occasions, but such hybrids are rarely fertile. For instance, hybrids obtained via hybridization experiments in *Hemileuca* Hübner (Hemileucinae) and *Anisota* Hübner (Ceratocampinae) by Williams had F-1 females that were sterile (Peigler and Williams 1984). Adès et al. (2005) not only hybridized *Graellsia isabellae* (Graëlls, 1849) with *Actias sinensis* (Walker, 1855) successfully, but even obtained back-crosses from F-1 to the parental species. To our knowledge, the only case of successful laboratory introgressive hybridization in saturniids, during which a continuous multigenerational lineage of hybrids has been obtained, is known from the world of sericulture. Jolly et al. (1969) not only obtained fully fertile hybrids of *Antheraea pernyi* (Guérin-Méneville, 1855) from China and *A. roylei* (Moore, 1859) from India, despite different chromosome numbers between the two species of n=49 and n=30, respectively, but this hybrid line was successfully maintained in sericulture in India for many generations. This hybrid also showed signs of "hybrid vigor," and Peigler (2012) made the case that *A. pernyi* may be an artificially-derived line of *A. roylei* maintained in captivity for thousands of years. If he is correct, these two taxa may have been once 'conspecific' in the biological sense, and, while not undermining the importance of this system from the point of view of understanding cytology and artificial selection, such a conclusion would certainly change the significance of this system for evolutionary biology. For one, it may be an example of rapid diversification via chromosomal rearrangement, for another, it might demonstrate that chromosomal rearrangement does not lead to immediate speciation, and that even extremely different chromosome numbers by themselves may not be a reliable way of telling different species apart. In nature, largely allopatric

saturniids can hybridize in their contact zone, but don't blend and don't form hybrid species: in the genus *Hyalophora* Duncan [and Westwood] in the western US, hybrid females may show full fertility in the contact zone whereas crossing individuals of these species from widely allopatric populations can result in sterile F-1 hybrids (Collins and Rawlins 2013).

In the present study, we tested the hypothesis that two moth species in the genus *Automeris* can, in the lab, produce a true-breeding lineage with a distinctive phenotype. With over 120 described species, the genus *Automeris* presents an excellent model for studying speciation, biodiversity, phenotypic plasticity, and genetics (e.g., Lemaire 1971, 1973, 1974; Manley 1978, 1990, 1993; Lemaire and Wolfe 1993; Sourakov 2015; Sourakov et al. 2017), as well as evolutionary development (e.g., Sourakov and Shirai 2020). Here, we provide a report on our findings concerning laboratory hybridization between *Automeris io* (Fabricius, 1775) and *Automeris louisiana* Ferguson and Brou, 1981, two closely related but very distinct species, only one of which is sexually dimorphic. The details of our experiments are described below, hybrids are illustrated, and their implications for our understanding of the taxonomy and evolution of *Automeris* are discussed. In Table 1, the evidence supporting the specific status of *A. louisiana* is summarized.

Materials and Methods

While the two species are quite easily recognized, and *A. louisiana* is very specialized ecologically, both are easy to rear in the lab on a variety of hostplants. *Automeris io*, as was recently determined, undergoes six instars as males and seven instars as females and its diapause is easy to break by rearing larvae in 24-hour light (Sourakov et al. 2017). *Automeris louisiana* proved to be similar in these respects, and hence the interbreeding experiments were conducted during three consecutive non-diapausing generations.

Stocks of *Automeris louisiana* and *Automeris io* were established and reared in USDA-approved quarantine rooms at the University of Florida, at 22–24°C, 24-hour light, under USDA permit #P526P-17-03348 and in accordance with permit conditions. The young larvae were first kept in large batches (as both species are

Table 1. Morphological differences between *Automeris io* and *A. louisiana*, based on Ferguson and Brou (1981) and Sourakov (pers. obs.) (FWd and HWd – forewing and hindwing dorsal).

Automeris io	***Automeris louisiana***
Sexually dimorphic (FWd yellow/pink in male, brown in females) (Fig. 1A)	Sexually monomorphic (FWd grey in both males and females (Fig. 1C)
Discal spot on FWd very distinct	Discal spot on FWd diffused, almost indistinguishable from the rest of the wing
HWd margin yellow/pink corresponds to FWd	HWd margin olive-grey corresponds to FWd
More complex uncus of male genitalia, with 3 transverse ribs	Simpler uncus of male genitalia, with 2 transverse ribs
Eggs twice as small as in *A. louisiana* (Fig. 2A2)	Eggs twice as large as in *A. io* (Fig. 2A1)
4th instar monomorphic, orange-brown uniformly striped (Fig. 2B2)	4th instar dimorphic, dark-brown or green, with wider spiracular and subspiracular stripes (Fig. 2B1)
Mature larvae green with candy-cane stripe, with occasional yellow forms	Mature larvae green with candy-cane stripe, with occasional white-green forms
In mature larvae, the crimson red spiracular band has the same width as the white subspiracular band. It is uniformly colored, with white dots barely noticeable.	In mature larvae, the burgundy-colored spiracular band is wider than the white subspiracular band. It is darker, almost black between segments, with white dots creating spotted pattern.
Cocoons smaller than in *A. louisiana*, golden-brown (Fig. 2C2, C3)	Cocoons larger than in *A. io*, silvery-brown (Fig. 2C1)
Widespread, naturally extremely polyphagous species, ranging from Canada to Costa Rica	Local, SE US species, restricted to coastal marshland habitat of LA and TX, possibly grass-feeding only in nature

gregarious in early instars), starting in small pint-sized containers, and then in one-gallon bags. Eventually they were separated into smaller and smaller groups, with 1–2 larvae per bag in the final instars, which corresponds to their biology in the wild. Hostplant material in the form of cut branches was supplied three times per week. Emerging moths were paired in mesh 24×24×36 inch cages where they remained until eggs were laid and females died. The cages were kept in partial darkness, covered with dark cloth. Larvae of parental species were reared on sugarberry (*Celtis laevigata* Willdenow (Cannabaceae)), oak (*Quercus nigra* L. (Fagaceae)), wax myrtle (*Myrica cerifera* L. (Myricaceae)), or cherry (*Prunus serotina* Ehrh. (Rosaceae)), with *C. laevigata* also used for rearing the hybrids. In order to break the diapause, all larvae were reared and pupae were kept under a 24-hour light regime, so emergence followed 20–30 days after pupation, with a few exceptions of several diapausing *A. io* pupae. Considering 10–12 days of development as eggs, 45–70 days as larvae, and 20–30 days as pupae, each generation took approximately three months to complete.

DNA barcodes (COI, mt-DNA; Hajibabaei et al. 2006) for the analysis of genetic distance (performed with BioEdit) were obtained at the University of Florida using legs from voucher specimens via standard procedures (see Materials and Methods in Sourakov et al. 2015). Sequences can be found in table S1. Voucher specimens were deposited in the collection of the McGuire Center for Lepidoptera and Biodiversity, Florida Museum of Natural History, Gainesville,

Results and Discussion

Hybridization experiments

In the present study, not only were we able to produce fertile hybrids (F-1) of *Automeris louisiana* and *A. io*, but we also successfully bred these hybrids and reared second (F-2) and third (F-3) hybrid generations, at which point we terminated our experiments. While most of the successful pairings were not observed, and success could only be judged by fertility or lack thereof in the eggs which females laid, a single mating of a female *A. louisiana* and a male *A. io* (Texas stock) was observed around 8 AM and lasted for approximately 15 minutes. This mating, along with three others, between males of *A. louisiana* and females of *A. io* from Texas stock, resulted in fertile eggs. Larvae were reared on either sugarberry or cherry into a series of adult moths representing four broods.

Compared to the parental stock, both wing ground color and contrast of pattern in F-3 was intermediate (Fig. 1). While *A. louisiana* was characterized as a species by Ferguson and Brou (1981: 101) by "reduction or near loss of sexual dimorphism," *A. io* is highly sexually dimorphic. In this respect, the F-3 hybrids exhibit a spectrum where some pairs can be characterized as sexually dimorphic, while others are nearly monomorphic. Sexual dimorphism in Lepidoptera has been suggested to result from co-option of sex-determining genes during the formation of wing pattern (Deshmukh et al. 2018). While we do not yet have the genomic information that would allow us to determine the way in which sexual dimorphism is maintained in *A. io*, one can hypothesize that genes from both sex chromosomes and autosomes are directly or indirectly involved in wing pattern formation. It would be interesting to explore if that is still the case in *A. louisiana*, the species that appears to have lost its sexual dimorphism.

The F-1 hybrids represented three crosses of *A. io* females and *A. louisiana* males and one cross of an *A. louisiana* female with an *A. io* male. While successful initial hybridization produced more dimorphic phenotypes (Fig. S1), it was the F-2 crosses that had a wider variation in phenotypes (Fig. S2), as would be expected for inheritance with incomplete dominance in which multiple alleles are involved. For instance, some males in F-2 have dorsal forewing color with a tint of pink or yellow. In the F-3 generation, which was produced via sib-sib crosses, the variability was more limited, as would be expected giving less genetic variation, but also in accordance with the regression to the mean concept (offspring generations tend to exhibit less and less extreme variation in any given character as compared to the parents).

As a side experiment, we hybridized *Automeris io* from Houston, Texas with individuals from Gainesville, Florida and obtained morphological intermediates between these relatively distinct and geographically removed populations (Fig. 5). We also attempted several crosses of interspecific hybrids with Florida *Automeris io*, but none of them were successful.

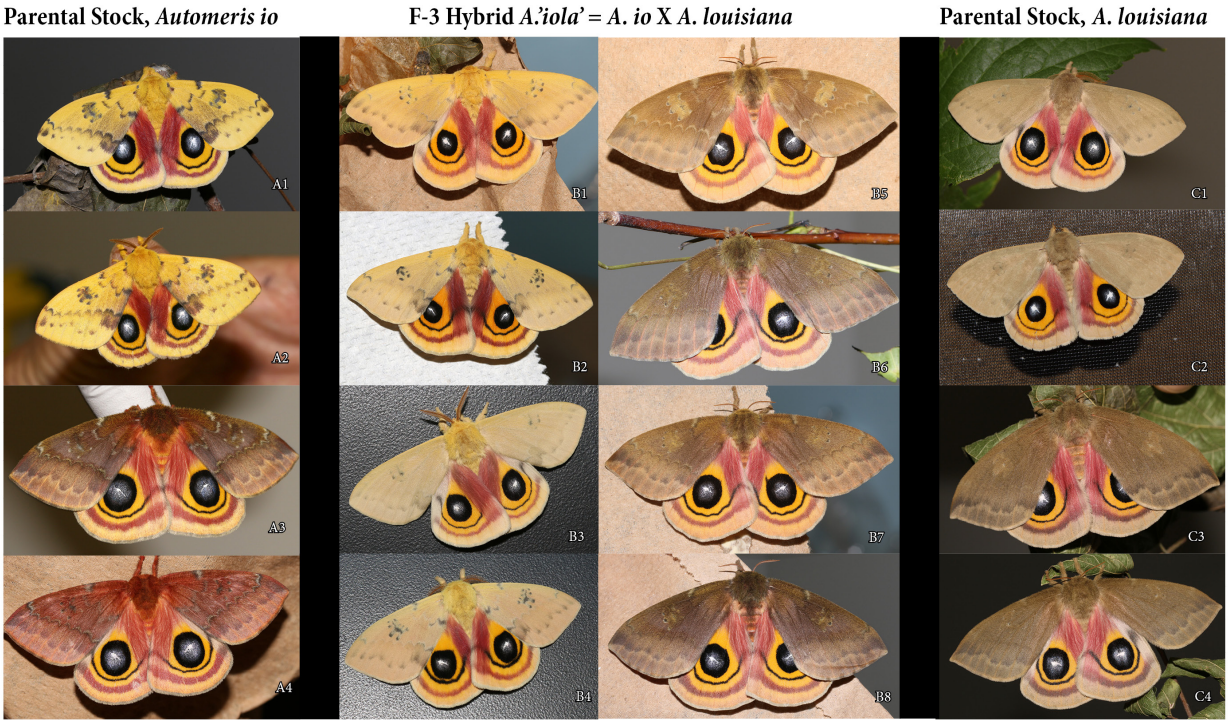

Figure 1. *Automeris* 'iola' - Hybridization between *A. io* and *Automeris louisiana*. **A, C)** Parental stock of (A) *A. io* and (C) *A. louisiana* (A1, A2, C1, C2 males, A3, A4, C3, C4 females). **B)** The F-3 hybrids (*Automeris* 'iola') between the parental stocks (B1–B4 males, B5–B8 females).

Taxonomy and evolution

Automeris louisiana was characterized in the original description of Ferguson and Brou (1981) as a sister species to *A. io* that lacks sexual dimorphism and is allopatric with the latter, narrowly distributed in the coastal marshes of Texas and Louisiana (see also Nuelle et al. 2018). In contrast, *A. io* is much more broadly distributed, from Canada to Costa Rica (e.g., Janzen 2003), is sexually dimorphic and variable, both in wing pattern as witnessed by the many formally described subspecies (Tuskes et al. 1996), but also in terms of recently described polyphenism in its southern US populations (Sourakov et al. 2017). The transition between the two species is characterized as abrupt, with no hybridization zone described to date, which led Ferguson and Brou to suppose the existence of reproductive isolation. Based on our lab experiments, overall similarity between the two species, and limited genetic evidence presented below, we can hypothesize that *A. louisiana* may have originated from *A. io* relatively recently, perhaps during the last glaciation period. For instance, it may have formed populations in refugia along the coast, which 20,000 years ago extended much further into the Gulf of Mexico than it does today. Perhaps these refugia were less affected by cold temperatures, and, as climate has warmed, the *A. io* populations may have repopulated the adjacent areas to the north but were already reproductively isolated from *A. louisiana*. This is just one scenario of how the two species may have diverged.

In Lepidoptera taxonomy, the standards for description of species and subspecies are extremely variable between individual researchers and even taxonomic groups, with practices surrounding taxonomy of "showy" species such as silk moths favoring splitting over lumping. One can therefore guess that, should a phenotype like this of our interspecific hybrid (to which we henceforth refer to as *Automeris* 'iola') be discovered as an isolated population in nature, it would most likely be named as a separate species or subspecies. In Table 1 and Figures 1 and 2, we illustrate key differences between the two parental species, demonstrating how different the two taxa are from one another, including the immature stages. The 1st instar *A.* 'iola' larvae seemed to have variable shades of head capsules, something that was not observed in the parental stock (Fig. 3). In Figure 4, the life cycle of the hybrid *A.* 'iola' is illustrated (the life cycle of *A. louisiana* is also shown in Figure S3).

Figure 2. Observed differences in immature stages between the parental stocks of *Automeris louisiana* (left) and *A. io* (right). **A)** Eggs; in *A. louisiana* (A1), eggs are significantly larger than in *A. io* (A2). **B)** 4th instar larvae; in *A. louisiana* (B1), they are dimorphic, chocolate-brown or pale-green with white stripes and with a maroon spiracular stripe, while in *A. io* (B2), they are always light brown. **C)** Cocoons; in *A. louisiana* (C1) they are lighter and appear tighter-woven than in *A. io* (C2 – Texas brood, C3 – Florida brood).

Figure 3. First instar larvae of hybrid *Automeris* 'iola' vs. *A. io*. **A)** F-1, neonate larvae. **B)** F-2 neonate larvae. **C)** F-3, molting to 2nd instar. **D)** *A. io*, F-1 Texas X Florida cross. **E)** *A. io*, late 1st instar, Florida.

While we crossed the two *Automeris* species repeatedly to determine the presence of post-zygotic isolation between them, and found that they interbreed, forming phenotypic intermediates, we do not consider this experiment significant enough for the taxonomic status of the two species to change, as there is ample evidence from their morphology and distribution that suggests they are well reproductively isolated in nature and not by the means of any geographical barrier. Since hybridization commonly occurs between different species of animals, from lions and tigers to *Heliconius* butterflies, both in the wild and in the lab, we see our experiments as another contribution to developing a more nuanced concept of species, which is far more complex than the short schoolbook definition of "populations that are unable to interbreed." We encourage researchers working in the area of *A. louisiana* distribution to keep an eye out for unusual phenotypes of *Automeris* – perhaps hybridization between the two species studied here does occasionally occur in nature.

We analyzed mitochondrial DNA barcodes of specimens resulting from the experiments described above (Table 2).

Genetic distances between mitochondrial DNA barcodes alone cannot be used to answer the question of whether individuals or populations belong to same or different species. However, they can serve as useful

Table 2. Genetic distances (%) between mitochondrial DNA COI "barcode" sequences sampled from breeding lines. Row 1, *Automeris* 'iola,' is a hybrid of female *A. io* (TX) and male *A. louisiana*. Row 2, *Automeris io* hybrid, is a hybrid of a female from Texas and a male from Florida.

A. 'iola'	0.00	0.16	0.31	0.47
A. io hybrid	0.16	0.00	0.47	0.31
A. louisiana	0.31	0.47	0.00	0.47
A. io FL	0.47	0.31	0.47	0.00

Figure 4. Life history of the hybrid *Automeris* 'iola' (*A. louisiana* X *A. io*). **A)** Fertile eggs. **B)** neonate larvae. **C)** late 1st instar. **D)** 2nd instar. **E, F)** 3rd instar. **G, H)** 4th instar. **I)** 4th and 5th instar. **J)** mature larva. **K)** cocoon.

characters for screening for potential cryptic species and help inform taxonomic decisions. For instance, a difference of at least 3% between two geographically isolated and morphologically distinct populations may be safely considered a good indication of separate species (Lukhtanov et al. 2016), though some authors use a lower threshold. However, in the present case, the genetic distance between taxa is less than 1%, corresponding to normal intraspecific variation. From the alpha-taxonomy standpoint, such mt-DNA distance in barcode region would not support a separate species status. However, there are many examples of genera where mt-DNA barcodes are not useful in understanding species boundaries, and species are instead delimited using knowledge of biology, distribution, hybridization, and whole-genome analysis.

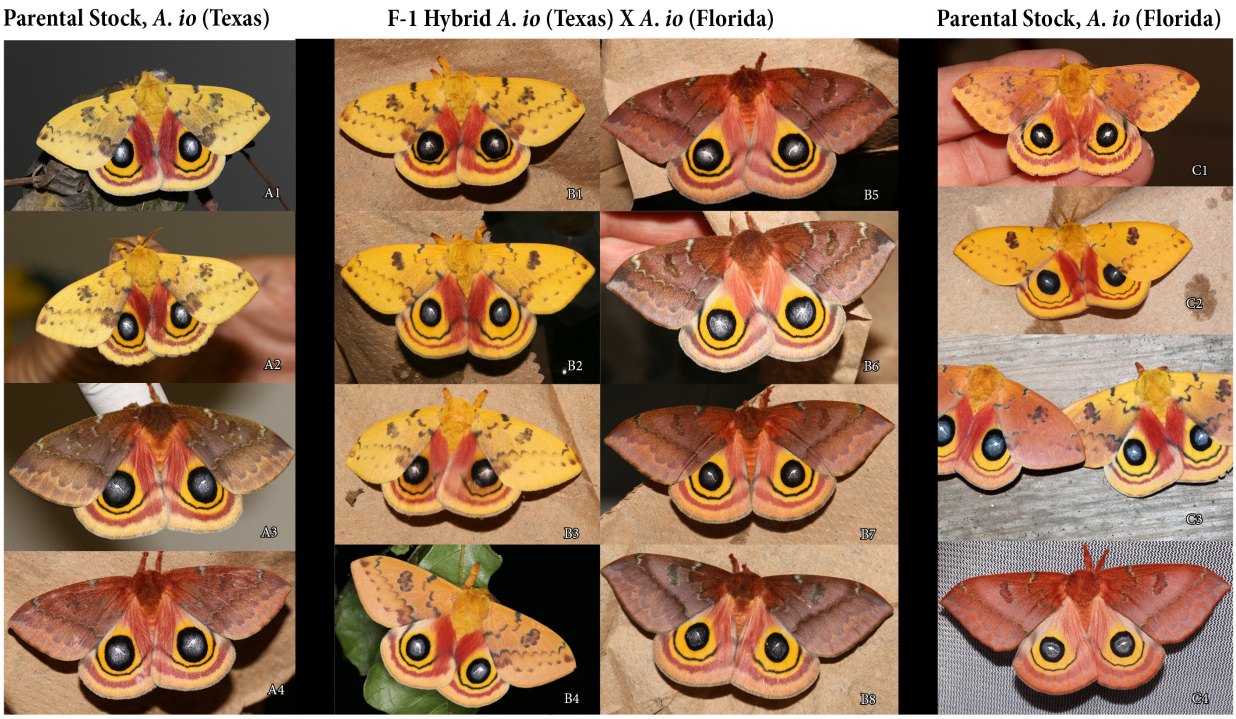

Figure 5. Hybridization between *A. io* populations from Texas and Florida. **A, C)** Parental stock of (A) *A. io* from Houston area, Texas (A1–A2 non-diapausing males, A3–A4 females) and (C) *A. io* from Gainesville area, northcentral Florida (C1–C2 non-diapausing males, C3 diapausing males, C4 female). **B)** The F-1 Texas–Florida hybrids (B1–B3 non-diapausing males, B4 diapausing male, B5–B8 females).

While we do not intend, within the scope of the present publication, to engage in detailed discussion of the *A. io* species complex, we would also like to report that we sequenced 36 DNA barcodes of captive-bred *A. io* specimens from Gainesville, Florida; 12 each from three consecutive generations. All females in this line were daughters or granddaughters of the same founding mother, and our goal was to observe if any mutations may have occurred within such a short timespan. This investigation was prompted by the fact that in humans, even within one generation, mt-DNA is known to undergo mutations (e.g., Hayakawa et al. 1992) and hence we decided to assess if this may be also happening in Lepidoptera. We found no evidence that this mt-DNA region underwent any changes within three generations - all 36 sequences were identical.

Ecology of *Automeris io* and *A. louisiana*

There are some interesting behavioral differences between the two species that we observed during rearing. *Automeris io* is well defended against predators, with caterpillars delivering toxins via syringe-like spines. We experienced their stings many times during our years of rearing caterpillars, and these stings have been shown to be an effective defense strategy against predators (e.g., Sourakov 2018). *Automeris louisiana* caterpillars share this feature with *A. io*, but, when handled, they are less likely to cause a painful sting. Their stinging action seems to be delayed, manifesting itself as pain not immediately, like in *A. io*, but a few moments later (Sourakov, Doll, pers. obs.). Whether the difference is due to mechanical, physiological, or chemical properties of the spines and associated glands and toxins remains to be investigated. One can hypothesize however, that, to avoid predation in nature, *A. louisiana* caterpillars rely less on chemical defense and more on cryptic coloration and their ability to wiggle and fall off the hostplant at the first sign of danger. Compared to *A. io*, *A. louisiana* larvae separate from the hostplant much more readily when disturbed. Such a behavioral adaptation is common among other Lepidoptera, including armyworms, *Spodoptera* Guenée, and various Arctiinae (larvae of which are known as woolly bears). It is possible that the behavioral differences between the two species result from different host

associations: *A. louisiana* caterpillars feed predominantly or exclusively on grassy vegetation which would allow them to easily crawl back up their hostplant after the danger passes.

Automeris io is a highly polyphagous species (Hall 2014 and references therewith) that lays eggs on a wide variety of plants, usually in small groups. In Florida alone, we have found *A. io* eggs and larvae on plants as diverse as *Celtis* L., *Rhododendron* L., *Prunus* L., *Cercis* L., *Erythrina* L., *Entada* Adans. and *Crotalaria* L. While the last three hostplants did not prove to be very suitable for rearing larvae because of high mortality, *A. io* demonstrates the ability to develop in the wild and in the lab on some very toxic plants (e.g., Sourakov 2013). This ability to detoxify defensive plant compounds is variable among individual broods and greatly depends on genetic factors, such as level of inbreeding (Sourakov, unpublished data), and is probably also variable geographically. In contrast, *Automeris louisiana* is known to be naturally associated with a specific habitat, the coastal marshes in Louisiana and Texas, where it has been observed to utilize hostplants dominant in that habitat – robust grasses, such as cordgrass, *Spartina alterniflora* Loisel. (Poaceae) and sturdy bulrush, *Bolboschoenus robustus* (Pursh) Soják (Cyperaceae) (Wilson and Romfh 2017; Nuelle et al. 2018). In the lab, however, *A. louisiana*, just like *A. io*, can be reared on a variety of woody hostplants, as was determined initially by Brou (2005). In our experience, *A. louisiana* larvae perform especially well on the black cherry, *Prunus serotina*, the host that on occasion can be toxic to *A. io* (Sourakov, pers. obs.).

Polyphagy is common among *Automeris*. For instance, Janzen (2003) identified *Automeris zugana* Druce, 1886 as the most polyphagous of all saturniids occurring within the Guanacaste Conservation Area, Costa Rica, feeding on 84 species of plants in 66 genera and 20 families. However, the more we learn about species boundaries, the more we might discover that hostplant associations (in addition to geographic isolation) may drive speciation. In the case of Costa Rican fauna studied by Janzen's group, a presumed single polyphagous species has, on a number of occasions, turned out to be several cryptic species with more limited host associations. This has been shown in several skipper species occurring at their study site (Guanacaste Conservation Area) and there is an indication that it may prove to be the case with *A. zugana* as well (Janzen et al. 2005).

The ability to feed on a variety of hostplants in captivity does not mean that these hostplants are utilized in nature, as can be observed, for example, with satyrine butterflies such as various Euptychiina or Pronophilina, which can be frequently reared on domesticated grasses in the lab, while in nature will only utilize a very specific, frequently highly endemic species (e.g., Freitas, pers. comm.; Sourakov, per. obs.). For the analysis of costs and benefits of feeding on different hostplants by both generalist and specialist Lepidoptera larvae, we refer the readers to Scriber (1978), who used over 800 larvae spanning 22 species, including *A. io*, to test various factors (secondary plant compounds, water contents, height above ground, etc.) that can contribute to the supposed host specialization observed in *A. louisiana*. Future research should be focused on better understanding the ecology of *A. louisiana* and ecological barriers that prevent this species from being absorbed by *A. io*. Additionally, the genetic basis of adaptations exhibited by *A. louisiana* and *A. io* populations will be very interesting to explore.

Conclusions

1) In captivity, *Automeris io* and *A. louisiana* formed a hybrid lineage which was maintained for three generations with no sign of sterility.
2) Hybrids between *Automeris io* and *A. louisiana* are morphological intermediates between parental stocks.
3) While species status of the two taxa is supported by the authors of the present study, this experiment suggests that there may occur an occasional hybridization between these two recently evolved species.

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Supplementary Materials

Figure S1. The F-1 hybrid *Automeris* 'iola' (*A. louisiana* crossed with *A. io* from Texas). **A)** Males. **B)** Females. All represent four different crosses between female *A. io* and male *A. louisiana* except B4 represents a cross of female *A. louisiana* with male *A. io*.

Figure S2. The F-2 hybrid *Automeris* 'iola' (*A. louisiana* crossed with *A. io* from Texas). **A)** Males. **B)** Females. Two different hybrid broods are represented by siblings as follows: A1–A3, B1 and A4, B2–B4.

Figure S3. Immature stages of *Automeris louisiana*. Last instar larvae (bottom) occasionally exhibited pale-green dorsally patterned coloration that was not encountered in *A. io*.

Table S1. Mitochondrial DNA COI "barcode" sequences sampled from breeding lines:
AS-9_TL7_651_LepF1_E12_LepF1 - *Automeris* '*iola*' (hybrid of female A. io (TX) and male *A. louisiana*).
AS-10_TF_798_LepF1_F12_LepF1 - *A. io* hybrid, female from TX crossed with male from FL AS-6_LL_784_LepF1_B12_LepF1 - *A. louisiana*.
AS-4_FL_2016-5_920_LepF1_H11_L - *A. io*, Gainesville, FL.

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                                10        20        30        40        50        60        70        80        90       100
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AS-9_TL7_651_LepF1_E12_LepF1    GGATAGTAGGAACTTCATTAAGATTGCTAATTCGAGCCGAATTAGGAACCCCTGGATCTTTAATTGGAGATGACCAAATTTATAATACTATTGTAACAGC
AS-10_TF_798_LepF1_F12_LepF1    GAATAGTAGGAACTTCATTAAGATTGCTAATTCGAGCCGAATTAGGAACCCCTGGATCTTTAATTGGAGATGACCAAATTTATAATACTATTGTAACAGC
AS-6_LL_784_LepF1_B12_LepF1     GGATAGTAGGAACTTCATTAAGATTGCTAATTCGAGCCGAATTAGGAACCCCTGGATCTTTAATTGGAGATGACCAAATTTATAATACTATTGTAACAGC
AS-4_FL_2016-5_920_LepF1_H11_L  GAATAGTAGGAACTTCATTAAGATTGCTAATTCGAGCCGAATTAGGAACCCCTGGATCTTTAATTGGAGATGACCAAATTTATAATACTATTGTAACAGC

                                110       120       130       140       150       160       170       180       190       200
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AS-9_TL7_651_LepF1_E12_LepF1    TCATGCTTTTATTATAATTTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTTCCCTTAATATTAGGAGCTCCTGATATAGCT
AS-10_TF_798_LepF1_F12_LepF1    TCATGCTTTTATTATAATTTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTTCCCTTAATATTAGGAGCTCCTGATATAGCT
AS-6_LL_784_LepF1_B12_LepF1     TCATGCTTTTATTATAATTTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTTCCCTTAATATTAGGGGCTCCTGATATAGCT
AS-4_FL_2016-5_920_LepF1_H11_L  TCATGCTTTTATTATAATTTTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTTCCCTTAATATTAGGGGCTCCTGATATAGCT

                                210       220       230       240       250       260       270       280       290       300
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AS-9_TL7_651_LepF1_E12_LepF1    TTCCCTCGAATAAATAATATAAGATTTTGACTTCTTCCCCCATCCTTAACACTTTTAATTTCAAGAAGAATTGTAGAAAATGGAGCTGGTACTGGATGAA
AS-10_TF_798_LepF1_F12_LepF1    TTCCCTCGAATAAATAATATAAGATTTTGACTTCTTCCCCCATCCTTAACACTTTTAATTTCAAGAAGAATTGTAGAAAATGGAGCTGGTACTGGATGAA
AS-6_LL_784_LepF1_B12_LepF1     TTCCCTCGAATAAATAATATAAGATTTTGACTTCTTCCCCCATCCTTAACACTTTTAATTTCAAGAAGAATTGTAGAAAATGGAGCTGGTACTGGATGAA
AS-4_FL_2016-5_920_LepF1_H11_L  TTCCCTCGAATAAATAATATAAGATTTTGACTTCTTCCCCCATCCTTAACACTTTTAATTTCAAGAAGAATTGTAGAAAATGGAGCTGGTACTGGATGAA

                                310       320       330       340       350       360       370       380       390       400
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AS-9_TL7_651_LepF1_E12_LepF1    CAGTATATCCCCCTCTTTCTTCTAATATTGCTCACAGAGGTTCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTAGCTGGTATTTCCTCAATTTTAGG
AS-10_TF_798_LepF1_F12_LepF1    CAGTATATCCCCCTCTTTCTTCTAATATTGCTCACAGAGGTTCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTAGCTGGTATTTCCTCAATTTTAGG
AS-6_LL_784_LepF1_B12_LepF1     CAGTATATCCCCCTCTTTCTTCTAATATTGCTCACAGAGGTTCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTAGCTGGTATTTCCTCAATTTTAGG
AS-4_FL_2016-5_920_LepF1_H11_L  CAGTATATCCCCCTCTTTCTTCTAATATTGCTCACAGAGGTTCTTCTGTTGATTTAGCTATTTTTTCTCTTCATTTAGCTGGTATTTCCTCAATTTTAGG

                                410       420       430       440       450       460       470       480       490       500
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AS-9_TL7_651_LepF1_E12_LepF1    AGCTATTAATTTTATTACTACAATCATTAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTACAGCTTTC
AS-10_TF_798_LepF1_F12_LepF1    AGCTATTAATTTTATTACTACAATCATTAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTACAGCTTTC
AS-6_LL_784_LepF1_B12_LepF1     AGCTATTAATTTTATTACTACAATCATTAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTACAGCTTTC
AS-4_FL_2016-5_920_LepF1_H11_L  AGCTATTAATTTTATTACTACAATCATTAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTACAGCTTTC

                                510       520       530       540       550       560       570       580       590       600
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AS-9_TL7_651_LepF1_E12_LepF1    CTTTTACTCCTTTCTCTACCTGTTTTAGCTGGAGCCATTACTATATTATTATTAACAGATCGTAATCTTAATACTTCTTTTTTTGACCCTGCTGGAGGAGGAG
AS-10_TF_798_LepF1_F12_LepF1    CTTTTACTCCTTTCTCTACCTGTTTTAGCTGGAGCCATTACTATATTATTATTAACAGATCGTAATCTTAATACTTCTTTTTTTGACCCTGCTGGAGGAGGAG
AS-6_LL_784_LepF1_B12_LepF1     CTTTTACTCCTTTCTTTACCTGTTTTAGCTGGAGCCATTACTATATTATTATTAACAGATCGTAATCTTAATACTTCTTTTTTTGACCCTGCTGGAGGAGGAG
AS-4_FL_2016-5_920_LepF1_H11_L  CTTTTACTCCTTTCTCTACCTGTTTTAGCTGGAGCCATTACTATATTATTATTAACAGATCGTAATCTTAATACTTCTTTTTTTGACCCTGCTGGAGGAGGAG

                                610       620       630       640       650       660
                                ....|....|....|....|....|....|....|....|....|....|..
AS-9_TL7_651_LepF1_E12_LepF1    ATCCTATTTTATATCAACATTTATTTTGATTTTTTGGACATCAAGAAGTTTTAA-
AS-10_TF_798_LepF1_F12_LepF1    ATCCTATTTTATATCAACATTTATTTTGATTTTTTGGACATCANAAG-TTT-A
AS-6_LL_784_LepF1_B12_LepF1     ATCCTATTTTATATCAACATTTATTTTGATTTTTTGGACATCAGGAAGTTTAAA--------
AS-4_FL_2016-5_920_LepF1_H11_L  ATCCTATTTTATATCAACATTTATTTTGATTTTTTGGACATCCNNAAGTTTAA
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DNA Distance Matrix based on 1-643

AS-9 TL7 6	0.0000	0.0016	0.0031	0.0047
AS-10 TF 7	0.0016	0.0000	0.0047	0.0031
AS-6 LL 78	0.0031	0.0047	0.0000	0.0047
AS-4 FL 20	0.0047	0.0031	0.0047	0.0000