

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

---

Insecta Mundi

Center for Systematic Entomology, Gainesville,  
Florida

---

9-25-2020

## 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

Andrei Sourakov

University of Florida, asourakov@flmnh.ufl.edu

Cassandra Doll

Washington State University, cassandra.doll@wsu.edu

Alyssa M. Quinn

University of Florida

Lei Xiao

University of Florida, lxiao@ufl.edu

Eric Anderson

University of Florida

Follow this and additional works at: <https://digitalcommons.unl.edu/insectamundi>



Part of the [Ecology and Evolutionary Biology Commons](#), and the [Entomology Commons](#)

---

Sourakov, Andrei; Doll, Cassandra; Quinn, Alyssa M.; Xiao, Lei; and Anderson, Eric, "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" (2020). *Insecta Mundi*. 1287.

<https://digitalcommons.unl.edu/insectamundi/1287>

This Article is brought to you for free and open access by the Center for Systematic Entomology, Gainesville, Florida at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Insecta Mundi by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

A journal of world insect systematics

# INSECTA MUNDI

---

---

0797

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

Andrei Sourakov

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

Cassandra F. Doll

School of Biological Sciences,  
Washington State University 14204 NE Salmon Creek Ave, Vancouver, WA 98686, USA

Alyssa M. Quinn

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

Lei Xiao

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

Eric Anderson

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

Date of issue: September 25, 2020

Center for Systematic Entomology, Inc., Gainesville, FL

**Sourakov A, Doll CF, Quinn AM, Xiao L, Anderson E. 2020.** 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. *Insecta Mundi* 0797: 1–16.

Published on September 25, 2020 by  
**Center for Systematic Entomology, Inc.**  
P.O. Box 141874  
Gainesville, FL 32614-1874 USA  
<http://centerforsystematicentomology.org/>

**INSECTA MUNDI** is a journal primarily devoted to insect systematics, but articles can be published on any non-marine arthropod. Topics considered for publication include systematics, taxonomy, nomenclature, checklists, faunal works, and natural history. *Insecta Mundi* will not consider works in the applied sciences (i.e. medical entomology, pest control research, etc.), and no longer publishes book reviews or editorials. *Insecta Mundi* publishes original research or discoveries in an inexpensive and timely manner, distributing them free via open access on the internet on the date of publication.

*Insecta Mundi* is referenced or abstracted by several sources, including the Zoological Record and CAB Abstracts. *Insecta Mundi* is published irregularly throughout the year, with completed manuscripts assigned an individual number. Manuscripts must be peer reviewed prior to submission, after which they are reviewed by the editorial board to ensure quality. One author of each submitted manuscript must be a current member of the Center for Systematic Entomology.

Guidelines and requirements for the preparation of manuscripts are available on the *Insecta Mundi* website at <http://centerforsystematicentomology.org/insectamundi/>

**Chief Editor:** David Plotkin, [insectamundi@gmail.com](mailto:insectamundi@gmail.com)  
**Assistant Editor:** Paul E. Skelley, [insectamundi@gmail.com](mailto:insectamundi@gmail.com)  
**Layout Editor:** Robert G. Forsyth  
**Editorial Board:** Davide Dal Pos, Oliver Keller, M. J. Paulsen  
**Founding Editors:** Ross H. Arnett, Jr., J. H. Frank, Virendra Gupta, John B. Heppner, Lionel A. Stange, Michael C. Thomas, Robert E. Woodruff  
**Review Editors:** Listed on the *Insecta Mundi* webpage

**Printed copies (ISSN 0749-6737) annually deposited in libraries:**

CSIRO, Canberra, ACT, Australia  
Museu de Zoologia, São Paulo, Brazil  
Agriculture and Agrifood Canada, Ottawa, ON, Canada  
The Natural History Museum, London, UK  
Muzeum i Instytut Zoologii PAN, Warsaw, Poland  
National Taiwan University, Taipei, Taiwan  
California Academy of Sciences, San Francisco, CA, USA

Florida Department of Agriculture and Consumer Services,  
Gainesville, FL, USA  
Field Museum of Natural History, Chicago, IL, USA  
National Museum of Natural History, Smithsonian Institution,  
Washington, DC, USA  
Zoological Institute of Russian Academy of Sciences, Saint-  
Petersburg, Russia

**Electronic copies (online ISSN 1942-1354, CDROM ISSN 1942-1362) in PDF format.**

Printed CD or DVD mailed to all members at end of year. Archived digitally by Portico.  
Florida Virtual Campus: <http://purl.fcla.edu/fcla/insectamundi>  
University of Nebraska-Lincoln, Digital Commons: <http://digitalcommons.unl.edu/insectamundi/>  
Goethe-Universität, Frankfurt am Main: <http://nbn-resolving.de/urn/resolver.pl?urn:nbn:de:hebis:30:3-135240>

**Copyright held by the author(s).** This is an open access article distributed under the terms of the Creative Commons, Attribution Non-Commercial License, which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited. <http://creativecommons.org/licenses/by-nc/3.0/>

# 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

Andrei Sourakov

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA  
asourakov@flmnh.ufl.edu

Cassandra F. Doll

School of Biological Sciences,  
Washington State University 14204 NE Salmon Creek Ave, Vancouver, WA 98686, USA

Alyssa M. Quinn

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

Lei Xiao

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

Eric Anderson

McGuire Center for Lepidoptera and Biodiversity,  
Florida Museum of Natural History, University of Florida, Gainesville, FL 32611, USA

**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.

**ZooBank registration.** urn:lsid:zoobank.org:pub:352F472B-9F53-4B68-9F4A-15A2A6DC88DE

## 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 20<sup>th</sup> 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).

| <i>Automeris io</i>   | <i>Automeris louisiana</i>  |
|---|---|
| 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 <i>A. louisiana</i> (Fig. 2A2)  | Eggs twice as large as in <i>A. io</i> (Fig. 2A1)   |
| 4 <sup>th</sup> instar monomorphic, orange-brown uniformly striped (Fig. 2B2)   | 4 <sup>th</sup> 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 <i>A. louisiana</i> , golden-brown (Fig. 2C2, C3)   | Cocoons larger than in <i>A. io</i> , 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

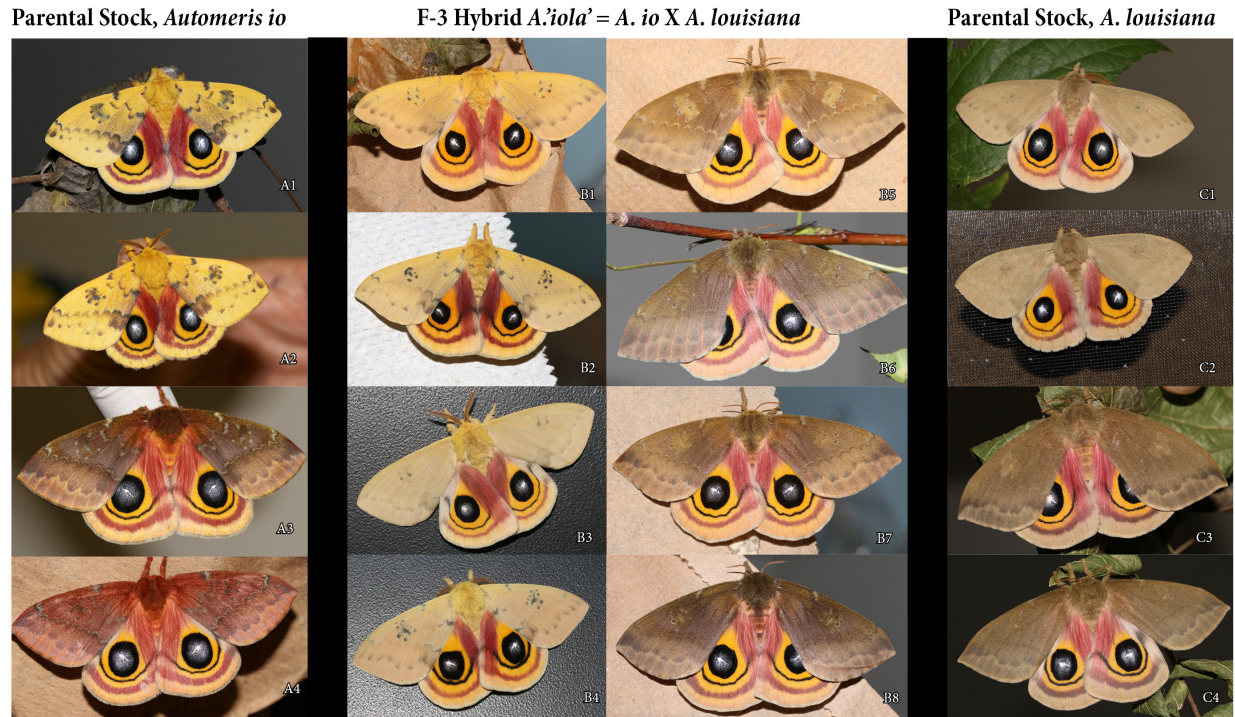
In the present study, not only were we able to produce fertile hybrids (F-1) of *Automeris louisiana* and *A. io*, but we also successfully bred these hybrids and reared second (F-2) and third (F-3) hybrid generations, at which point we terminated our experiments. While most of the successful pairings 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 1<sup>st</sup> 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)** 4<sup>th</sup> 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 2<sup>nd</sup> instar. **D)** *A. io*, F-1 Texas X Florida cross. **E)** *A. io*, late 1<sup>st</sup> 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 school-book 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.

|                     |      |      |      |      |
|---------------------|------|------|------|------|
| <i>A. ‘iola’</i>    | 0.00 | 0.16 | 0.31 | 0.47 |
| <i>A. io</i> hybrid | 0.16 | 0.00 | 0.47 | 0.31 |
| <i>A. louisiana</i> | 0.31 | 0.47 | 0.00 | 0.47 |
| <i>A. io</i> FL     | 0.47 | 0.31 | 0.47 | 0.00 |

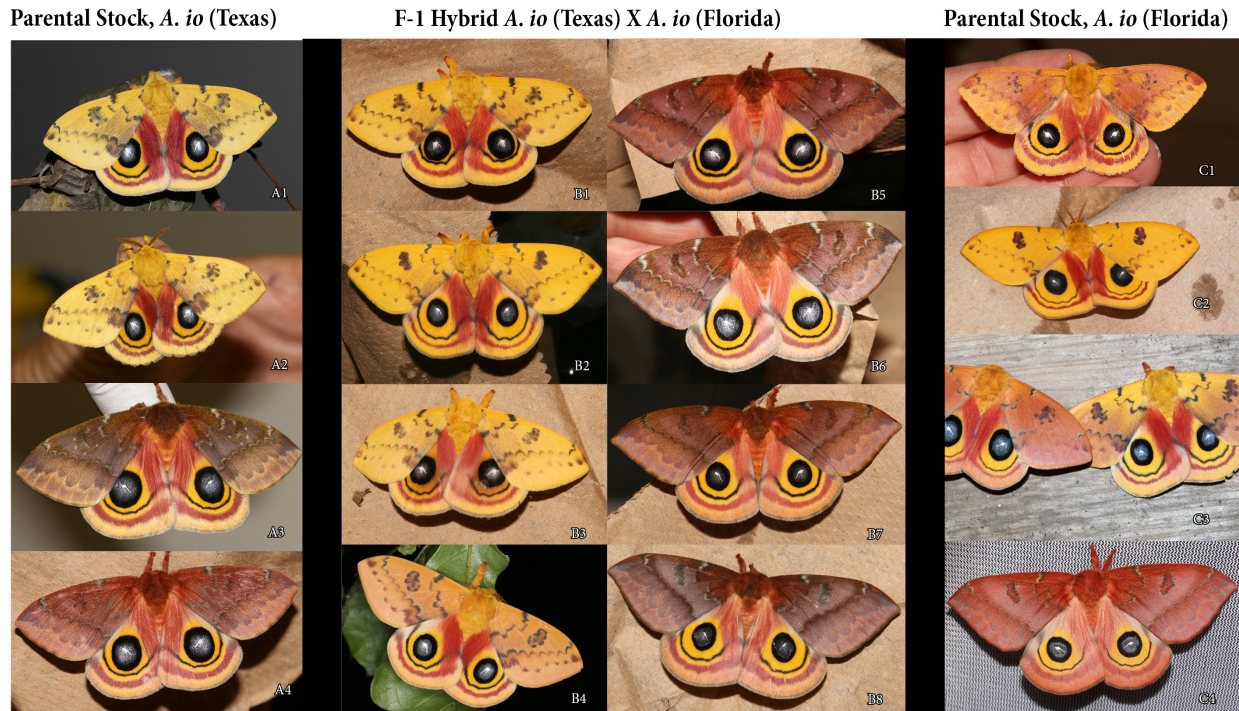




**Figure 4.** Life history of the hybrid *Automeris 'iola'* (*A. louisiana* X *A. io*). **A)** Fertile eggs. **B)** neonate larvae. **C)** late 1st instar. **D)** 2<sup>nd</sup> instar. **E, F)** 3<sup>rd</sup> instar. **G, H)** 4<sup>th</sup> instar. **I)** 4<sup>th</sup> and 5<sup>th</sup> instar.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

## Acknowledgments

We thank Keith R. Willmott and André V. L. Freitas for their thoughtful reviews of the manuscript, which was greatly improved by their comments. We also thank James Mallet and Tianzhu Xiong for discussion of the species concept in context of our hybridization experiments.

## Literature Cited

- Adamski D, Marques D, Mayo KG, Mayo R. 2019.** Description of larval and pupal stages of a bilateral gynandromorph reared from a cross between *Citheronia regalis* (Fabr.) and *C. splendens* (Druce) (Lepidoptera: Saturniidae). *The Journal of the Lepidopterists' Society* 73: 135–144.
- Adès D, Cocault R, Lemaître R, Vuattoux R. 2005.** Hybridation entre *Graellsia isabelae* (Graells) male et *Actias sinensis* (Walker) femelle: Obtention des femelles. *Lambillionea* 105(2): 321–325.
- Brou VA. 2005.** Spotlight on rearing: *Automeris louisiana* Ferguson & Brou 2005. *Southern Lepidopterist News* 27(4): plate 2.
- Collins MM, Rawlins JE. 2013.** A transect for reproductive compatibility and evidence for a “hybrid sink” in a hybrid zone of *Hyalophora* (Insecta: Lepidoptera: Saturniidae). *Annals of Carnegie Museum* 82(2): 193–223.
- Collins MM, Weast RD. 1961.** Wild silk moths of the United States: Saturniinae; experimental studies and observations of natural living habits and relationships. Collins Radio Company; Cedar Rapids, Iowa. 138 p.
- Deshmukh R, Baral S, Gandhimathi A, Kuwalekar M, Kunte K. 2018.** Mimicry in butterflies: co-option and a bag of magnificent developmental genetic tricks. *Wiley Interdisciplinary Reviews: Developmental Biology* 7(1): e291.
- Dietrich MR. 2003.** Richard Goldschmidt: hopeful monsters and other ‘heresies’. *Nature Reviews Genetics* 4(1): 68–74.
- Dittrich-Reed DR, Fitzpatrick BM. 2013.** Transgressive hybrids as hopeful monsters. *Evolutionary Biology* 40(2): 310–315.
- Ferguson DC, Brou VA. 1981.** A new species of *Automeris* Hübner (Saturniidae) from the Mississippi River delta. *The Journal of the Lepidopterists' Society* 35(2): 101–105.
- Goldschmidt R. 1931.** Analysis of intersexuality in the gypsy moth. *The Quarterly Review of Biology* 6(2): 125–142.
- Hajibabaei M, Janzen DH, Burns JM, Hallwachs W, Hebert PDN. 2006.** DNA barcodes distinguish species of tropical Lepidoptera. *Proceedings of the National Academy of Sciences* 103: 968–971.
- Hall DW. 2014.** Io Moth. Featured Creatures, # EENY-608. Available at [http://entnemdept.ufl.edu/creatures/misc/io\\_moth.htm](http://entnemdept.ufl.edu/creatures/misc/io_moth.htm). (Last accessed May 21, 2020.)
- Hayakawa M, Hattori K, Sugiyama S, Ozawa T. 1992.** Age-associated oxygen damage and mutations in mitochondrial DNA in human hearts. *Biochemical and biophysical research communications* 189(2): 979–985.
- Janzen DH. 2003.** How polyphagous are Costa Rican dry forest saturniid caterpillars? p. 369–379. *In*: Basset Y, Novotny V, Miller SE, Kitching RL (eds.). *Arthropods of tropical forests. Spatio-temporal dynamics and resource use in the canopy*. Cambridge University Press; Cambridge, United Kingdom. 490 p.
- Janzen DH, Hajibabaei M, Burns JM, Hallwachs W, Remigio E, Hebert PD. 2005.** Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. *Philosophical Transactions of the Royal Society B: Biological Sciences* 360(1462): 1835–1845.
- Jiggins CD, Salazar C, Linares M, Mavarez J. 2008.** Hybrid trait speciation and *Heliconius* butterflies. *Philosophical Transactions of the Royal Society B: Biological Sciences* 363(1506): 3047–3054.
- Jolly MS, Sen SK, Prasad GK, Sengupta AK. 1979.** Some cytological observations on interspecific hybrid of *A. pernyi* GM and *A. roylei* Mr. (Saturniidae). *Cytologia* 44(1): 259–263.
- Kryukov AP. 2019.** Phylogeography and hybridization of corvid birds in the Palearctic Region. *Zhurnal Genetiki i Selektii = Vavilov Journal of Genetics and Breeding* 23(2): 232–238.
- Lemaire C. 1971.** Revision du genre *Automeris* Hubner et des genres voisins. *Biogéographie, Ethologie, Morphologie, Taxonomie. (Lep. Attacidae)*. *Mémoires du Museum National d'Histoire Naturelle* 68: 1–232.
- Lemaire C. 1973.** Revision du genre *Automeris* Hubner et des genres voisins. *Biogéographie, Ethologie, Morphologie, Taxonomie. (Lep. Attacidae)*. *Mémoires du Museum National d'Histoire Naturelle* 79: 233–422.
- Lemaire C. 1974.** Revision du genre *Automeris* Hubner et des genres voisins. *Biogéographie, Ethologie, Morphologie, Taxonomie. (Lep. Attacidae)*. *Mémoires du Museum National d'Histoire Naturelle* 92: 423–576.
- Lemaire C, Wolfe KL. 1993.** Two new *Automeris* from western Mexico (Lepidoptera: Saturniidae: Hemileucinae). *Tropical Lepidoptera* 4(1): 39–44.
- Lukhtanov VA, Sourakov A, Zakharov EV. 2016.** DNA barcodes as a tool in biodiversity research: testing pre-existing taxonomic hypotheses in Delphic Apollo butterflies (Lepidoptera, Papilionidae). *Systematics and Biodiversity* 14(6): 599–613.
- Manley TR. 1978.** Genetics of conspicuous markings of the Io moth. *Journal of Heredity* 69(1): 11–18.
- Manley TR. 1990 (1991).** Heritable color variants in *Automeris io* (Saturniidae). *Journal of Research on the Lepidoptera* 29(1–2): 37–53.
- Manley TR. 1993.** Diapause, voltinism, and foodplants of *Automeris io* (Saturniidae) in the southeastern United States. *The Journal of the Lepidopterists' Society* 47(4): 303–321.
- Mavárez J, Salazar CA, Bermingham E, Salcedo C, Jiggins CD, Linares M. 2006.** Speciation by hybridization in *Heliconius* butterflies. *Nature* 441(7095): 868–871.
- Mayr E. 1963.** *Animal species and evolution*. Harvard University Press; Cambridge, Massachusetts. 797 p.

- Meier JI, Salazar PA, Kučka M, Davies RW, Dréau A, Aldás I, Power OB, Nadeau NJ, Bridle JR, Rolian CP, Barton NH. 2020.** Haplotype tagging reveals parallel formation of hybrid races in two butterfly species. *bioRxiv*. Available at <https://doi.org/10.1101/2020.05.25.113688> (Last accessed May 27, 2020.)
- Nuelle RJ Jr., Aicezs KK, Nuelle RJ III, Whitbeck M. 2018.** *Automeris louisiana* (Lepidoptera: Saturniidae) populations in the chenier plain habitat of coastal Texas, with new distributional and larval host plant records. *Journal of Entomology and Zoology Studies* 6(2): 1182–1188.
- Peigler RS. 2012.** Diverse evidence that *Antheraea pernyi* (Lepidoptera: Saturniidae) is entirely of sericultural origin. *Tropical Lepidoptera Research* 1: 93–99.
- Peigler RS, Williams BD. 1984.** Two interesting artificial hybrid crosses in the genera *Hemileuca* and *Anisota* (Saturniidae). *The Journal of the Lepidopterist's Society* 38: 51–56.
- Platt AP. 1975.** Monomorphic mimicry in Nearctic *Limenitis* butterflies: experimental hybridization of the *L. arthemis-astyanax* complex with *L. archippus*. *Evolution* 29(1): 120–141.
- Salazar CA, Jiggins CD, Arias CF, Tobler A, Bermingham E, Linares M. 2005.** Hybrid incompatibility is consistent with a hybrid origin of *Heliconius heurippa* Hewitson from its close relatives, *Heliconius cydno* Doubleday and *Heliconius melpomene* Linnaeus. *Journal of Evolutionary Biology* 18(2): 247–256.
- Scriber JM. 1978.** The effects of larval feeding specialization and plant growth form on the consumption and utilization of plant biomass and nitrogen: an ecological consideration. *Entomologia Experimentalis et Applicata* 24(3): 694–710.
- Scriber JM, Ording GJ. 2005.** Ecological speciation without host plant specialization; possible origins of a recently described cryptic *Papilio* species. *Entomologia Experimentalis et Applicata* 115(1): 247–263.
- Sipko T, Trepet S, Gogan PJ, Mizin I. 2010.** Bringing wisents back to the Caucasus mountains: 70 years of a grand mission. *European Bison Conservation Newsletter* 3: 33–44.
- Sourakov A. 2013.** Larvae of io moth, *Automeris io*, on the coral bean, *Erythrina herbacea*, in Florida—the limitations of polyphagy. *The Journal of the Lepidopterists' Society* 67(4): 291–298.
- Sourakov A. 2015.** Gynandromorphism in *Automeris io* (Lepidoptera: Saturniidae). *News of the Lepidopterists' Society* 57: 118–129.
- Sourakov A. 2018.** Size, spines and crochets: defences of luna moth caterpillars against predation by brown anoles. *Journal of Natural History* 52(7–8): 483–490.
- Sourakov A, Plotkin D, Kawahara AY, Xiao L, Hallwachs W, Janzen D. 2015.** On the taxonomy of the erythrina moths *Agathodes* and *Terastia* (Crambidae: Spilomelinae): Two different patterns of haplotype divergence and a new species of *Terastia*. *Tropical Lepidoptera Research* 25(2): 80–97.
- Sourakov A, Shirai LT. 2020.** Pharmacological and surgical experiments on wing pattern development of Lepidoptera, with a focus on the eyespots of saturniid moths. *Tropical Lepidoptera Research* 30(1): 4–19.
- Sourakov A, St Laurent, RA, Dexter K, Doll C. 2017.** Experimental evidence for polyphenism in *Automeris io* (Lepidoptera: Saturniidae) in north Florida. *Tropical Lepidoptera Research* 27(2): 117–119.
- Tuskes PM, Tuttle JP, Collins MM. 1996.** *The wild silk moths of North America*. Cornell University Press; Ithaca, New York. 250 p.
- Wilson J, Romfh P. 2017.** Citizen Science Project Report: LA Eyed Moth. Available at <http://tmn-cot.org/Presentations/AT-Day-A-louisiana-Presentation-01202019.pdf> (Last accessed May 21, 2020.)
- Zhang W, Kunte K, Kronforst MR. 2013.** Genome-wide characterization of adaptation and speciation in tiger swallowtail butterflies using de novo transcriptome assemblies. *Genome Biology and Evolution* 5(6): 1233–1245.
- Zakharov EV, Caterino MS, Sperling FA. 2004.** Molecular phylogeny, historical biogeography, and divergence time estimates for swallowtail butterflies of the genus *Papilio* (Lepidoptera: Papilionidae). *Systematic Biology* 1: 193–215.
- Zong E, Fan G. 1989.** The variety of sterility and gradual progression to fertility in hybrids of the horse and donkey. *Heredity* 62(3): 393–406.

Received June 24, 2020; accepted August 15, 2020.

Review editor David Plotkin.



## 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.

```

      10      20      30      40      50      60      70      80      90     100
AS-9 TL7 651 LepF1 E12 LepF1  GGATAGTAGGAACCTCATTAAAGATTGCTAAATCGAGCCGAATTAGGAACCCCTGGATCTTTAAATTGGAGATGACCAAAATTTATAACTATTGTAAACAGC
AS-10 TF 798 LepF1 F12 LepF1  GAATAGTAGGAACCTCATTAAAGATTGCTAAATCGAGCCGAATTAGGAACCCCTGGATCTTTAAATTGGAGATGACCAAAATTTATAACTATTGTAAACAGC
AS-6 LL 784 LepF1 B12 LepF1   GGATAGTAGGAACCTCATTAAAGATTGCTAAATCGAGCCGAATTAGGAACCCCTGGATCTTTAAATTGGAGATGACCAAAATTTATAACTATTGTAAACAGC
AS-4 FL 2016-5_920 LepF1_H11_L GAATAGTAGGAACCTCATTAAAGATTGCTAAATCGAGCCGAATTAGGAACCCCTGGATCTTTAAATTGGAGATGACCAAAATTTATAACTATTGTAAACAGC

      110     120     130     140     150     160     170     180     190     200
AS-9 TL7 651 LepF1 E12 LepF1  TCATGCTTTTATTATAAATTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTCCCTTAATATTAGGAGCTCCTGATATAGCT
AS-10 TF 798 LepF1 F12 LepF1  TCATGCTTTTATTATAAATTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTCCCTTAATATTAGGAGCTCCTGATATAGCT
AS-6 LL 784 LepF1 B12 LepF1   TCATGCTTTTATTATAAATTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTCCCTTAATATTAGGAGCTCCTGATATAGCT
AS-4 FL 2016-5_920 LepF1_H11_L TCATGCTTTTATTATAAATTTTTTTATAGTAATACCTATCATAATTGGAGGATTTGGTAATTGACTAGTCCCTTAATATTAGGGGCTCCTGATATAGCT

      210     220     230     240     250     260     270     280     290     300
AS-9 TL7 651 LepF1 E12 LepF1  TTCCTCGAATAAATAAATAAGATTTTGACTTCCTCCCCCATCCTTAACACTTTTAAATTTCAAGAAGAATTTAGAAAAATGGAGCTGGTACTGGATGAA
AS-10 TF 798 LepF1 F12 LepF1  TTCCTCGAATAAATAAATAAGATTTTGACTTCCTCCCCCATCCTTAACACTTTTAAATTTCAAGAAGAATTTAGAAAAATGGAGCTGGTACTGGATGAA
AS-6 LL 784 LepF1 B12 LepF1   TTCCTCGAATAAATAAATAAGATTTTGACTTCCTCCCCCATCCTTAACACTTTTAAATTTCAAGAAGAATTTAGAAAAATGGAGCTGGTACTGGATGAA
AS-4 FL 2016-5_920 LepF1_H11_L TTCCTCGAATAAATAAATAAGATTTTGACTTCCTCCCCCATCCTTAACACTTTTAAATTTCAAGAAGAATTTAGAAAAATGGAGCTGGTACTGGATGAA

      310     320     330     340     350     360     370     380     390     400
AS-9 TL7 651 LepF1 E12 LepF1  CAGTATATCCCCCTCTTCTCTCAATATTGCTCACAGAGTTCTTCTGTTGATTTAGCTATTTTTCTCTCATTTAGCTGGTATTTCTCAATTTTAGG
AS-10 TF 798 LepF1 F12 LepF1  CAGTATATCCCCCTCTTCTCTCAATATTGCTCACAGAGTTCTTCTGTTGATTTAGCTATTTTTCTCTCATTTAGCTGGTATTTCTCAATTTTAGG
AS-6 LL 784 LepF1 B12 LepF1   CAGTATATCCCCCTCTTCTCTCAATATTGCTCACAGAGTTCTTCTGTTGATTTAGCTATTTTTCTCTCATTTAGCTGGTATTTCTCAATTTTAGG
AS-4 FL 2016-5_920 LepF1_H11_L CAGTATATCCCCCTCTTCTCTCAATATTGCTCACAGAGTTCTTCTGTTGATTTAGCTATTTTTCTCTCATTTAGCTGGTATTTCTCAATTTTAGG

      410     420     430     440     450     460     470     480     490     500
AS-9 TL7 651 LepF1 E12 LepF1  AGCTATAATTTTATTACTACAATCATTAAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTAACAGCTTTC
AS-10 TF 798 LepF1 F12 LepF1  AGCTATAATTTTATTACTACAATCATTAAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTAACAGCTTTC
AS-6 LL 784 LepF1 B12 LepF1   AGCTATAATTTTATTACTACAATCATTAAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTAACAGCTTTC
AS-4 FL 2016-5_920 LepF1_H11_L AGCTATAATTTTATTACTACAATCATTAAATATACGTTTAAATAATATATCTTTTGATCAAATACCTTTATTTGTATGAGCTGTTGGAATTAACAGCTTTC

      510     520     530     540     550     560     570     580     590     600
AS-9 TL7 651 LepF1 E12 LepF1  CTTTACTCCTTTCTACCTGTTTGTAGCTGGAGCCATTACTATAATTATAACAGATCGTAATCTTAATACTCTTTTTTTGACCCCTGCTGGAGGAGGAG
AS-10 TF 798 LepF1 F12 LepF1  CTTTACTCCTTTCTACCTGTTTGTAGCTGGAGCCATTACTATAATTATAACAGATCGTAATCTTAATACTCTTTTTTTGACCCCTGCTGGAGGAGGAG
AS-6 LL 784 LepF1 B12 LepF1   CTTTACTCCTTTCTACCTGTTTGTAGCTGGAGCCATTACTATAATTATAACAGATCGTAATCTTAATACTCTTTTTTTGACCCCTGCTGGAGGAGGAG
AS-4 FL 2016-5_920 LepF1_H11_L CTTTACTCCTTTCTACCTGTTTGTAGCTGGAGCCATTACTATAATTATAACAGATCGTAATCTTAATACTCTTTTTTTGACCCCTGCTGGAGGAGGAG

      610     620     630     640     650     660
AS-9 TL7 651 LepF1 E12 LepF1  ATCCCTATTTTATATCAACATTTATTTGATTTTTGGACATCAAGAAGTTTAA-
AS-10 TF 798 LepF1 F12 LepF1  ATCCCTATTTTATATCAACATTTATTTGATTTTTGGACATCANAAAG-TTT A
AS-6 LL 784 LepF1 B12 LepF1   ATCCCTATTTTATATCAACATTTATTTGATTTTTGGACATCAGGAAGTTTAAA-----
AS-4 FL 2016-5_920 LepF1_H11_L ATCCCTATTTTATATCAACATTTATTTGATTTTTGGACATCCNNAAGTTTAA
    
```

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 |