Phylogeny and divergence time estimation of Io moths and relatives (Lepidoptera: Saturniidae: *Automeris*)

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Abstract

The saturniid moth genus *Automeris* includes 135 described species. Their geographic distribution ranges from the eastern half of North America to as far south as Peru. *Automeri*s moths are cryptically colored and their forewings resemble dead leaves, with conspicuously colored, elaborate eyespots hidden on their hindwings. Despite their charismatic nature, the evolutionary history and relationships within *Automeris* and between closely related genera, remain poorly understood. In this study, we present the most comprehensive phylogeny of *Automeris* to date, including 80 of the 135 described species. We also incorporate two morphologically similar hemileucine genera, *Pseudautomeris* and *Leucanella*, as well as a morphologically distinct genus, *Molippa*. We obtained DNA data from both dry-pinned and ethanol-stored museum specimens and conducted Anchored Hybrid Enrichment (AHE) sequencing to reconstruct a high-quality dataset for phylogenetic analysis. The resulting phylogeny supports *Automeris* as a paraphyletic genus, with *Leucanella* and *Pseudautomeris* nested within, with the most recent common ancestor dating back to 26 mya. This study lays the foundation for future research on various aspects of *Automeris* biology, including anti-predator defense mechanisms, ecological adaptations, geographical distribution patterns, and potential drivers of speciation.

Introduction

Saturniidae, commonly known as wild silkmoths, is renowned for its remarkable morphological features, such as large body size, vibrant wing patterns, and distinctive hindwing markings. According to the most recent published checklist [1], this family comprises a staggering 3,454 validated species, divided into eight subfamilies and 180 genera. The genus *Automeris* (subfamily Hemileucinae) is the most diverse genus within the family. These species can be found across a vast geographic range, from North America to Peru. They thrive in diverse

biotopes, spanning from lush tropical rainforests to arid xerophytic habitats, and in a wide range of altitudinal levels, from the sea level up to 4000 m [2].

One distinctive feature of all *Automeris* species, as well as closely related genera, is their easily recognizable wing ornamentation on both the fore- and hindwings. Forewings typically exhibit subdued hues and a cryptic leaf-like pattern. In stark contrast, hindwings have vibrant colors and each prominently features a large eyespot. Eyespots on the hindwings of *Automeris* moths make up the conspicuous coloration component of a deimatic display– thought to deter possible predators by startling, frightening, or confusing them, causing predators to pause or abandon their pursuit [3, 4]. The presence of eyespots in most species of *Automeris* suggests they confer a selective benefit, although eyespots of a few species are minimized or vestigial, which may be a secondary evolutionary loss. There has been extensive research on molecular mechanisms of eyespot development, primarily in butterflies [5, 6, 7] and some studies have examined the evolutionary origins of eyespots in a few representative species of Lepidoptera [8, 9, 10] but research into the evolution of eyespots in *Automeris* and relatives has yet to be conducted. The evolution of eyespot size and shape is believed to be driven by adaptive pressures instead of developmental constraints, thus they are not likely to constrain adaptive radiation of size, shapes, and patterns of eyespots [11, 12].

Historically, researchers have heavily relied on wing pattern variation to establish systematic hypotheses and determine relationships within *Automeris* [13, 14], with the assumption that species most similar in appearance are most closely related. In his extensive study of the subfamily Hemileucinae, Lemaire [15] identified 135 species within *Automeris*, classified into nine species groups based on their physical appearance and genitalia morphology. Despite being a charismatic and extremely diverse genus, knowledge regarding the evolutionary relationships between *Automeris* species, closely related genera, and the timing of the group's divergence from other hemileucine lineages remains limited. While some studies have included *Automeris* and other hemileucine species in large-scale phylogenetic analyses of Lepidoptera [1, 16], a comprehensive phylogenetic analysis focused on *Automeris* is yet to be conducted.

In this study we present the most comprehensive *Automeris* phylogeny that includes 80 of the 135 described species and 6 subspecies. Additionally, we include two morphologically similar hemileucine genera - *Pseudautomeris* and *Leucanella*, and a morphologically distinct hemileucine genus, *Molippa,* to investigate relationships of *Automeris* with these genera. By constructing a well-represented phylogeny, we aim to uncover the diversification patterns within the genus and shed light on the timing of its evolutionary divergence from other lineages. Furthermore, a detailed phylogeny will provide a framework for future studies on various aspects of *Automeris* biology, including their anti-predatory defense, ecological adaptations, geographical distribution patterns, and potential drivers of speciation.

Materials and Methods

Taxon sampling and extraction

We sampled and extracted all previously unsequenced species and subspecies of *Automeris* (85), *Leucanella* (4), *Pseudautomeri*s (6), and *Molippa* (2) available in the McGuire Center for Lepidoptera and Biodiversity (MGCL), Florida Museum of Natural History (FLMNH). Specimens were obtained from two collection types: dry, pinned specimens and those stored in ethanol at -80°C, which have been wing-vouchered following Cho et al. [17] and are specifically stored for use in molecular studies.

One specimen per taxon was selected from the collections. Pinned specimens were carefully selected based on their condition, focusing on those with intact wings and body parts. Priority was given to more recently collected specimens during the selection process to enhance the likelihood of successful DNA extraction. Two legs were pulled from each sample and placed in a 1.5 mL microcentrifuge tube. Corresponding identification labels for samples were recorded on the respective tubes, linking them to those collection data present in the MGCL specimen database. Similarly, molecular collection samples were taken from their respective cold storage boxes, and partial thoracic tissue was dissected and placed in a labeled 1.5 mL microcentrifuge tube, and all relevant information was recorded in the collective database. The extracted thorax tissue was then placed in a labeled 1.5 mL microcentrifuge tube, and all relevant information was recorded in a collective database. Determination of DNA extraction from legs or thorax tissue was based on availability within the MGCL pinned and molecular collection. Due to the likelihood of higher DNA yield, specimens from the molecular collection were given priority. If specimens were available in both molecular and pinned collections, specimens were chosen from the molecular collection.

Extractions were completed following the specified protocol of the Qiagen DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). For quality control, extracted DNA was checked for concentration and fragmentation with a Qubit 2.0 fluorometer and electrophoresis gels (Fisherbrand Electrophoresis Power Supplies, FB200). After DNA extraction, if the desired concentration of DNA was not achieved, samples were re-extracted if tissue was still available. Otherwise, species with multiple samples were re-extracted with a different individual. Once the quantity of DNA was determined (8 ng/uL minimum and 100 ng/uL maximum), extracted samples were sent for Anchored Hybrid Enrichment (AHE) library preparation at RAPiD Genomics in Gainesville, Florida, USA. Higher concentration samples were prioritized. Out of 97 extracted samples, 95 showed promising DNA concentration of ≥ 8 ng/uL. Library preparation, hybridization enrichment, and Illumina HiSeq 2500 sequencing (PE100) was carried out at RAPiD Genomics.

DNA sequencing and assembly

We used the "BOM1" Anchored Hybrid Enrichment probe set [18], which was developed to target 921 loci across Bombycoidea. We used this probe set because it has been proven effective in capturing sequence data from both dry-pinned and ethanol-preserved specimens [18, 19, 20]

This probe kit includes 58 loci across 24 “legacy” Sanger sequencing-based genes [21], eight bombycoid vision-related loci, and 855 loci designated as the Lepidoptera Agilent Custom SureSelect Target Enrichment “LEP1” probe kit from Breinholt et al. [22]. Anchored Hybrid Enrichment is a targeted-sequencing methodology designed to capture hundreds of unique orthologous loci (i.e., single copy, phylogenetically-informative markers) from across the genome, for resolving both shallow and deep-level evolutionary relationships. Probes are designed to anchor in conserved regions that are flanked by variable regions randomly spread throughout the genome. This approach creates a diverse set of informative loci that include exons, introns, intergenic, and conserved regions of the genome [23].

Raw AHE sequences were assembled following the methods of Breinholt et al. [22], which implements an Iterative Baited Assembly (IBA) approach. This involves using the original sequencing probes to identify matching raw reads, which are then assembled into a novel probe. The newly assembled probe is subsequently used as a query to match against the remaining raw reads, and the process is repeated iteratively until confident assemblies can no longer be obtained. The pipeline also checks for quality and cross contamination due to barcode leakage and removes paralogs. Resultant assemblies extend beyond the boundaries of the initial sequencing probe, thereby leading to the production of two distinct datasets - one comprising sequences solely from the probe region of the assembly (Probe dataset), and the other comprising sequences from the complete assembly, encompassing both the probe and outer flanking regions (PF dataset).

Because the IBA approach often resulted in multiple assembled sequences for each locus per specimen, sequences were aligned using MAFFT v7.245 [24] and a 50% consensus generated using FASconCAT-G v1.02 [23] with the ‘-c -c -c’ command to result in one sequence per locus per specimen. To minimize the extent of missing data in the final concatenated dataset, loci that were only obtained from three or fewer species were excluded from the datasets. Loci were concatenated across all species into one supermatrix using FASconCAT-G with the ‘-s’ command.

In addition to specimens newly sequenced with AHE (95 taxa), we also included 18 previously sequenced hemileucine taxa in our lab that were sequenced and assembled via the same described methodologies. Four outgroup species were chosen to provide secondary calibrations for the divergence time analysis and to provide a root for the tree. All outgroups chosen were genera included in the [16] phylogeny and all had available raw transcriptomes or genomes. We chose two outgroup taxa in Saturniidae, *Attacus atlas* and *Therinia lactucina*, one from Sphingidae, *Manduca sexta*, and one from Bombycidae, *Bombyx mori* [26, 27, 28, 29]. The two transcriptomes (Saturniidae) and two genomes (*Bombyx* and *Manduca*) that we used for the outgroups were assembled to the BOM1 AHE probe regions using the methods described above. This enabled us to combine the transcriptome and genome datasets with our newly sequenced data that were also assembled to the BOM1 AHE probe regions.

Alistat v1.6 [30] was utilized to evaluate the completeness of the concatenated alignment for Probe and PF (hereon termed “PF”) datasets. We created Probe only and PF datasets because PF datasets can sometimes yield greater robustness to phylogenetic analyses of AHE data (e.g., [18,

31, 32, 33]). Completeness scores (C-scores) were computed for each taxon (Cr), and those taxa with a C-score < 0.15 were removed to avoid specimens with poor capture quality. We also removed loci that were captured across ≤ 3 taxa to improve dataset quality.

Phylogenetic analysis

Phylogenetic inference was conducted on concatenated supermatrices using a maximum likelihood analysis with IQTREE v. 1.5 [34]. We constructed two datasets, one with just the probe region, and another with both probe and PF regions. Both datasets were analyzed as nucleotides, and we determined the best substitution model and partitioning scheme using the command '-m MFP+MERGE' using ModelFinder [35] as implemented in IQTREE. The command '-B 1000 -bnni' was used to perform 1000 ultrafast bootstrap (UFBS) replicates, while optimizing each bootstrap tree using a hill-climbing nearest neighbor interchange (NNI) search to reduce the risk of overestimating branch supports. All trees were rooted to *Bombyx mori*. We refer to high node support as those with UFBS ≥ 95.

Divergence time estimation

We performed divergence time estimation in BEAST v2.6.2 [36] using a fixed ultrametric tree generated by IQTREE. We added a MultiMonophyleticConstraint prior to increase precision and accuracy. We ran the divergence time estimation analysis using the topology returned from the PF dataset. We used the Probe dataset for dating because the PF dataset returned a phylogeny with substantially higher bootstrap values when compared to the phylogeny constructed using the Probe dataset, and when running BEAST with this topology using the PF dataset for dating, the resulting 95% confidence intervals were disproportionately small, indicating the high likelihood that the extremely large dataset caused the underlying Bayesian analysis to become easily stuck at a local minima, while taking much longer to run [16, 35].

Among insects, there are disproportionately few Lepidoptera fossils [38]. Therefore, our tree was calibrated using fixed dates obtained from Kawahara et al. [16]. We used four secondary calibration points with uniform distributions to constrain the most recent common ancestors (MRCAs) of Hemileucinae and each chosen outgroup taxon. We used the Yule model of speciation [39] as the tree prior, applied a strict clock rate, and ran the analyses for 10 million generations, sampling every 1,000 generations. Runs were assessed using TRACER v1.6.0 [40] to examine convergence, and a tree with mean heights was created from 9,000 trees after discarding a burn-in of 1,000 trees. All pipeline steps and phylogenomic analyses were conducted on the University of Florida HiPerGator high-performance computing cluster (http://www.hpc.ufl.edu/).

Results and Discussion

We were able to sample 85 species and subspecies of *Automeris*, two species of *Molippa*, four species of *Leucanella*, and six species of *Pseudautomeris*, resulting in a total of 97 sampled specimens. Of the 97, 74 were dry-pinned specimens and 23 were ethanol-stored specimens. The

resulting DNA concentration range was 0.158-133 ng/uL. Samples that passed the DNA concentration cutoff (>8 ng/µL) were included in further analyses and sequencing.

In total, 95 newly-sequenced samples had sufficient data for sequence assembly. We also supplemented these sequences with 22 sequences that were sequenced and assembled prior to this project. These included 18 ingroup sequences and 4 outgroups, resulting in a total of 117 taxa that were included in the beginning data matrix. After removing low quality sequences from each dataset, the Probe dataset contained 589 loci across 150,646 nucleotide sites, covering 105 taxa and the PF dataset contained 906 loci across 106 taxa. The average length of each locus, as well as the percentage of missing data, were observed to be 256 nucleotides and 25% for the Probe dataset, and 2,236 nucleotides and 84% for the PF dataset, respectively. The high missing data percentage of the PF dataset is due to the nature of the flanking regions. These regions differ vastly among taxa, since they extend as far as possible across unconserved regions, and the terminus varies depending on the coverage and quality of raw reads.

Both probe and PF topologies support the monophyly and general placement of genera *Molippa*, *Leucanella* and *Pseudautomeris* and the paraphyly of *Automeris*. All taxa represented by multiple subspecies were recovered as monophyletic with strong support. Considering the methodology employed and the higher support values, we favor the phylogeny derived from the PF dataset (Fig. 1). However, it is important to note that both phylogenies exhibit nearly identical topologies, differing only in a few relationships at the tips while maintaining the same backbone relationships.

Recently, Rougerie et al. [37] reconstructed a phylogeny of Saturniidae, which was the only modern analysis providing a hypothesis of relationships of *Automeris*, *Leucanella, Pseudoautomeris* and *Molippa*. We find general congruence with the relationships uncovered by Rougerie et al. [37], although *Automeris* is paraphyletic our study contained more species of *Automeris* included in the analysis. There was strong support in our study for a monophyletic *Leucanella* + *Pseudautomeris*, which splits *Automeris* into two distinct groups - a larger clade (*Automeris* Clade B) branching around 21 million years ago (mya) and a smaller clade, including *Automeris io* (Io Clade), branching earlier at approximately 23 mya (see Table 1). Notably, none of the species within the smaller clade were included in the analysis by Rougerie et al. [35]. However, in both analyses, the relationships between the species included in both studies and *Leucanella*, *Molippa,* and *Pseudautomeris* were found to be consistent. We also found *Molippa* to be monophyletic, emerging as the sister group (UFBS = 100) to the remaining Hemileucines. This placement suggests that the most recent common ancestor of this clade (*Automeris* + *Leucanella* + *Molippa* + *Pseudautomeris*) dates to approximately 26 mya. This timeframe aligns closely with the findings of Rougerie et al. (2022) [37], who estimated a divergence of approximately 22 mya. It should be noted that our analyses specifically targeted four genera within a subfamily characterized by significant diversity, species complexes and apparent paraphyly. However, to gain a comprehensive understanding of relationships of these genera within and among the rest of the subfamily, broader sampling would be beneficial.

Eyespots of *Leucanella* and *Pseudautomeris* are elaborate in their shape and color, and we hypothesized that these two genera are more closely related to *Automeris* than *Molippa*, as the

latter two species have smaller, drab, potentially vestigial eyespots, or lack eyespots entirely. Our tree revealed that both *Leucanella* and *Pseudautomeris* are nested within *Automeris* with strong branch support. We postulate that predation pressure in the Neotropics drove the diversification and complexity of eyespots in species in this clade. Like many effective anti-predatory traits, eyespots in this group are likely under positive selection due to a selective benefit for survival. Previous research suggested that eyespots may be adaptive in some geographic regions, and maladaptive in others, which may explain secondary losses of eyespots in *Molippa* sp. [41,42]. There may be greater selective pressure on eyespots in the Neotropics than eyespots in North American species, driving the elaborate features and colors in these species. More complete sampling of *Leucanella* and *Pseudautomeris* would help understand the evolutionary pattern and processes that led to the diversification of *Automeris* and related species. Future studies should focus on investigating eyespot trait morphology, to further elucidate the diversification across species within the genus and sister groups.

Figure 1. Time calibrated tree showing relationships of*Automeris species and relatives*, *Leucanella*, *Pseudautomeris*, *Molippa* and outgroups. Blue circles represent nodes where UFBoot ≥ 90, though all are above 95. Red circles represent nodes where UFBoot ≤ 69. Divergence dates with 95% confidence intervals for each labeled node are in Table 1. Asterisks (*) notate pictured species.

Table 1. Minimum 95% confidence interval (CI), median, and maximum 95% CI ages of major clades and outgroup taxa in Millions Years Ago (mya). Node numbers correspond to those in Figure 1.

	Node Number	Minimum - 95%	Median	Maximum - 95%
***Automeris* Clade A**	1	0.202	0.2089	0.2163
Pseudautomeris	2	0.1627	0.1685	0.1747
Leucanella	2	0.1627	0.1685	0.1747
***io* Clade B**	3	0.2167	0.2241	0.232
Molippa	4	0.2487	0.2576	0.2665
Attacus atlas	5	04759	0.4917	0.508
Therinia lactucina	6	0.5686	0.5864	0.6035
Manduca sexta	7	0.6606	0.6822	0.7053
Bombyx mori	8	0.6607	0.6825	0.7056

Data Accessibility

All raw sequence data generated from this project are available on GenBank and will be made public upon paper acceptance. DNA alignments, phylogenies and specimen metadata will be made available via a Dryad Deposition. The final phylogeny will be uploaded to Open Tree of Life (https://tree.opentreeoflife.org/).

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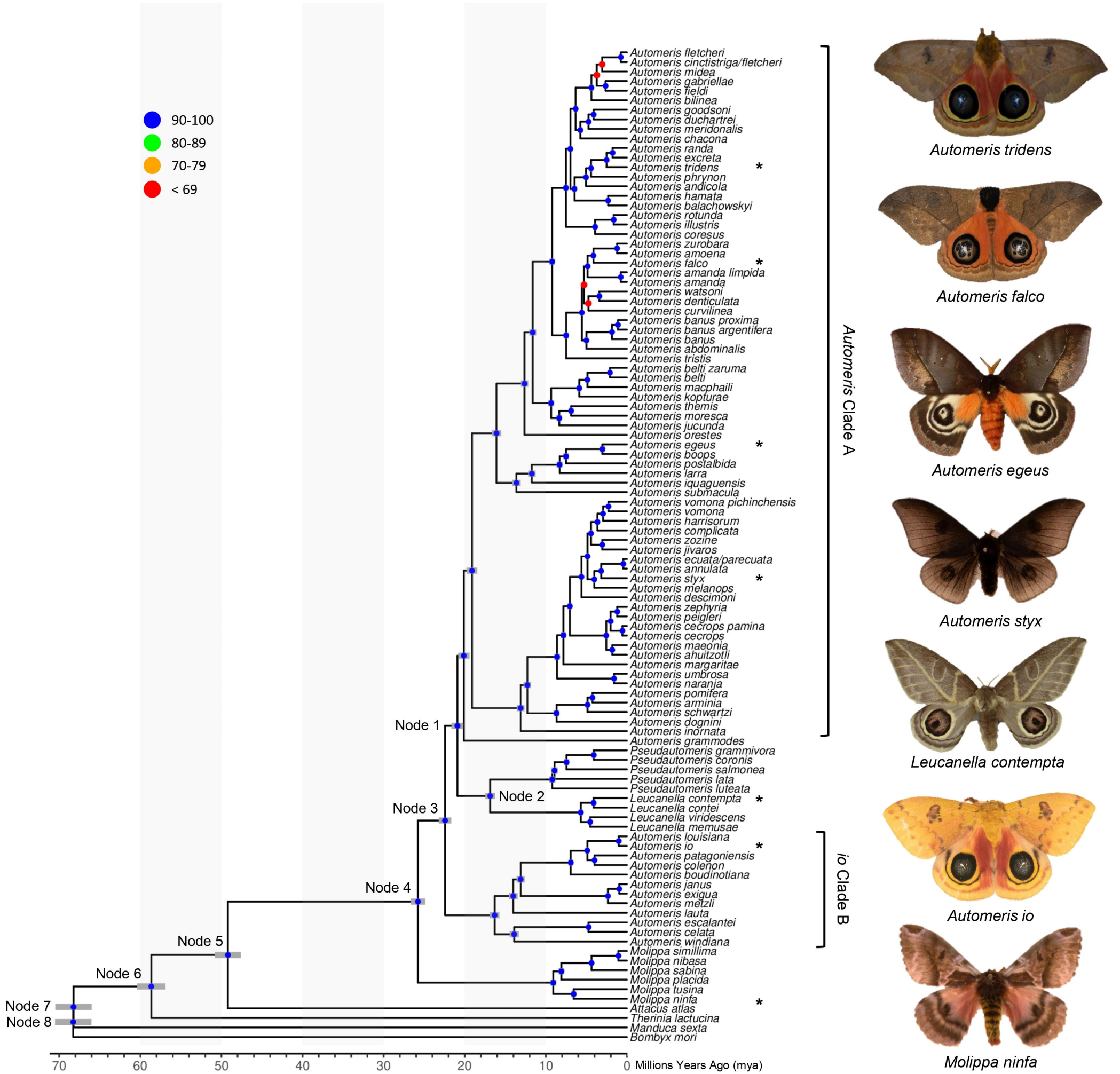
90-100
80-89
70-79
< 69
Automeris fletcheri
Automeris cinctistriga/fletcheri
Automeris midea
Automeris gabriellae
Automeris fieldi
Automeris bilinea
Automeris goodsoni
Automeris duchartrei
Automeris meridonalis
Automeris chacona
Automeris randa
Automeris excreta
Automeris tridens *
Automeris phrynon
Automeris andicola
Automeris hamata
Automeris balachowskyi
Automeris rotunda
Automeris illustris
Automeris coresus
Automeris zurobara
Automeris amoena
Automeris falco *
Automeris amanda limpida
Automeris amanda
Automeris watsoni
Automeris denticulata
Automeris curvilinea
Automeris banus proxima
Automeris banus argentifera
Automeris banus
Automeris abdominalis
Automeris tristis
Automeris belti zaruma
Automeris belti
Automeris macphaili
Automeris kopturae
Automeris themis
Automeris moresca
Automeris jucunda
Automeris orestes
Automeris egeus *
Automeris boops
Automeris postalbida
Automeris larra
Automeris iquaguensis
Automeris submacula
Automeris vomona pichinchensis
Automeris vomona
Automeris harrisorum
Automeris complicata
Automeris zozine
Automeris jivaros
Automeris ecuata/parecuata
Automeris annulata
Automeris styx *
Automeris melanops
Automeris descimoni
Automeris zephyria
Automeris peigleri
Automeris cecrops pamina
Automeris cecrops
Automeris maeonia
Automeris ahuitzotli
Automeris margaritae
Automeris umbrosa
Automeris naranja
Automeris pomifera
Automeris arminia
Automeris schwartzi
Automeris dognini
Automeris inornata
Automeris grammodes
Pseudautomeris grammivora
Pseudautomeris coronis
Pseudautomeris salmonea
Pseudautomeris lata
Pseudautomeris luteata
Leucanella contempta *
Leucanella contei
Leucanella viridescens
Leucanella memusae
Automeris louisiana
Automeris io *
Automeris patagoniensis
Automeris colenon
Automeris boudinotiana
Automeris janus
Automeris exigua
Automeris metzli
Automeris lauta
Automeris escalantei
Automeris celata
Automeris windiana
Molippa simillima
Molippa nibasa
Molippa sabina
Molippa placida
Molippa tusina
Molippa ninfa *
Attacus atlas
Therinia lactucina
Manduca sexta
Bombyx mori
Automeris Clade A
io Clade B
Node 1
Node 2
Node 3
Node 4
Node 5
Node 6
Node 7
Node 8
70
60
50
40
30
20
10
0 Millions Years Ago (mya)
Automeris tridens
Automeris falco
Automeris egeus
Automeris styx
Leucanella contempta
Automeris io
Molippa ninfa