The Evolution of Androconia in Mimetic Tiger Moths (Noctuoidea: Erebidae: Arctiinae: Ctenuchina and Euchromiina)

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ABSTRACT Tiger moth courtship involves an intricate interplay of female calling and male responses, involving pheromones, ultrasound, or both. A comparative phylogenetic approach is needed to separate proximal (ecological) from ultimate (evolutionary) explanations for observed behaviors. This study focused on mimetic tiger moths (Ctenuchina and Euchromiina) to provide a phylogeny to understand the evolution of male courtship structures (androconia). Genetic data from one mitochondrial gene (1,173 basepairs [bp] of COI) and two nuclear genes (238 bp of 28S rRNA D1 loop; 650 bp of EF1-α) were sampled for 29 species and analyzed using maximum parsimony, maximum likelihood, and Bayesian methods to estimate phylogenetic relationships. The ancestral reconstruction of androconia was optimized using parsimony and Bayesian approaches. Excluding three species, *Euchromiina* and *Ctenuchina* were recovered as reciprocally monophyletic, contradicting earlier molecular phylogenies. The genus *Cosmosoma* was found to be polyphyletic, as was *Eucereon*. Reconstruction of androconial structures revealed that these structures were acquired once, with subsequent losses in particular species.

KEY WORDS androconia, sexual selection, comparative biology, phylogenetic constraint, life history

Sexual selection is one of the most important evolutionary forces driving diversification and speciation. Because of female selectivity during courtship, male behaviors often convey important information about species identity and mate quality (Eberhard 1985, 1996, 1997). Tiger moth courtship is particularly complex and may involve multiple sensory systems: chemoreception, auditory, and tactile mechanoreception (Conner 1987, Sanderford and Conner 1990, Simmons and Conner 1996). Moths in the *Ctenuchina* and *Euchromiina* (Erebidae: Arctiinae) are no exception; these species possess diverse male behaviors and structures (androconia). Approximately 3,000 species currently are placed in these groups, but their phylogenetic relationships are not well understood (Fig. 1; Kitching and Rawlins 1998; Jacobson and Weller 2002; Lafontaine and Schmidt 2010; Zahiri et al. 2011, 2012). Elucidating the evolution of androconia provides an important step to understand the proximate causes of chemoecology and behavior in these lineages.

Phylogenetic studies slowly have been accumulating for the ctenuchines and euchromiines. Results based on morphology alone can be subject to convergence because of adult mimicry; these moths typically have reduced hind wings (and venation), and tympanal and abdominal modifications to produce narrow-looking waists (Jacobson and Weller 2002, Weller et al. 2000, Simmons and Weller 2006). These modifications allow for precise mimicry of wasps, beetles, and other noxious models. The lack of fresh or ethanol-preserved material hampers molecular phylogenetic studies, because museum material necessarily results in small DNA fragments, limiting the universe of gene markers that can be used. With these limitations, Simmons and Weller (2001) found that Ctenuchini and Euchromiini were not reciprocally monophyletic. The evolution of communication systems could not be addressed because the study did not include species with documented behaviors.

We undertook an enlarged molecular study to address the evolution of courtship communication. Here, we present the first reconstruction of the Euchromiina and Ctenuchina that observes evolution of highly complex androconia. We provide a review of the relevant structures and behaviors, as well as an updated review of their complicated taxonomic history. Although most genera in Ctenuchina and Euchromiina are small (32 of 141 genera contain >10 species), this study includes 21 species representing not only the large genera within these tribes, but also the three major lineages posited by Hampson (1898). The monophyly of each subtribe and their relationship to each other and the putative sister lineage, Phaegopterina, is examined through expanded outgroup sam-

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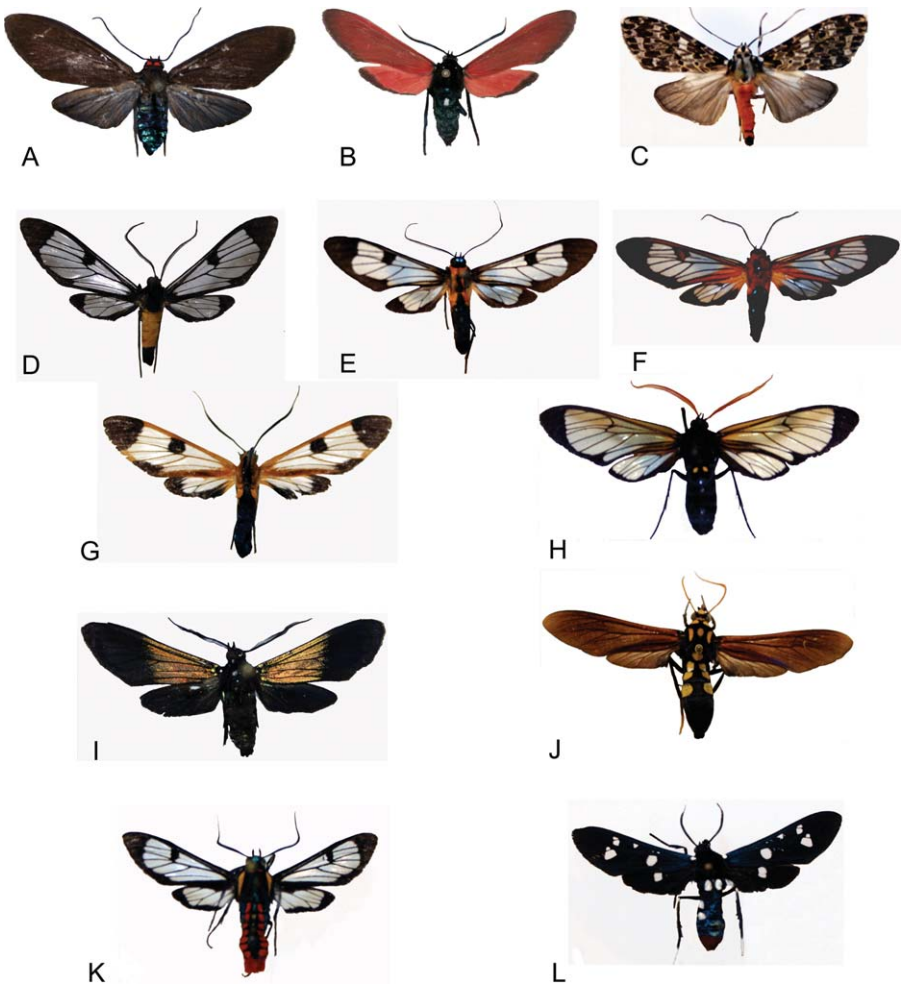

Fig. 1. Representatives of Ctenuchina and Euchromiina (all specimens courtesy of FLMNH). A. *E. lamia*; B. *E. heros*, C. *E. dentata*; D. *Cosmosoma braconoides* (Walker); E. *Cosmosoma subflamma* (Walker); F. *Cosmosoma teuthras* (Walker); G. *Dycladia correbioides* Felder; H. *Isantherene perbosci* (Guerin); I. *M. vittata*; J. *O. calcarata*; K. *Phoenicoprocta lydia* (Druce); L. *S. epilais.* (Online figure in color.)

pling. Parsimony and likelihood analyses (maximum likelihood, Bayesian inference) were preformed on a concatenated molecular data set of three gene regions, Elongation Factor 1-α (EF1-α), D1 loop of the 28S ribosomal RNA (D1), and mitochondrial gene cytochrome oxidase I (COI). To understand the evolution of androconia, observed structures were optimized on the resulting phylogeny by using parsimony and likelihood models available in Mesquite (Maddison and Maddison 2009).

Review of Systematic Issues. Hampson (1898) described four lineages within Ctenuchinae (his Syntomidae) based on hindwing venation. Forbes (1939, 1960) maintained this classification and formally designated three tribes: Syntomini (restricted to the Old World), Ctenuchini (restricted to the New World), and Euchromiini (primarily New world with the exception of *Euchromia*). Based on morphological characters, Jacobson and Weller (2002) found a polyphyletic Ctenuchinae (sensu Holloway 1988); the syntomiines were found to be more closely related to the lichen moths (Lithosiinae), whereas the ctenuchines and euchromiines were hypothesized to be reciprocally monophyletic sister groups. By using the recommendations of Kitching and Rawlins (1998), Jacobson and Weller retained Syntomiinae, and placed Ctenuchini and Euchromiini as sister tribes within their revised concept of Arctiinae.

The past two decades have produced several molecular studies of Noctuidae and a reappraisal of family limits (Weller et al. 2009; Lafontaine and Schmidt 2010; Zahiri et al. 2011, 2012). These studies affect the familial status of Lymantriidae and Arctiidae. Lafontaine and Fibiger (2006) proposed a new taxonomy for the Noctuoidea that placed most members of the LAQ clade (Lymantriidae, Arctiidae, and quadrifine noctuids) as subfamilies of Noctuidae. Lafontaine and Schmidt (2010) later recommended that the Erebidae

should include members of the LAQ clade. The monophyly and composition of the Erebeidae then was confirmed with subsequent molecular results (Zahiri et al. 2011, 2012). Thus, the ranks of all tiger moth taxa are as follows: Arctiidae become Arctiinae; subfamilies Lithosiinae, Syntominae, and Arctiinae become tribes (Lithosiini, Syntomini, Arctiini); and current tribes become subtribes (e.g., Ctenuchina, Euchromiina). For the remainder of this paper, we will use the recommended, revised taxonomic categories for Arctiinae (van Nieukerken et al. 2011).

Review of Arctiine Mating Behaviors and Structures. Through behavioral studies (Conner et al. 1981, Coro et al. 1983, Cardé and Baker 1984, Conner 1987, Portilla et al. 1987, Sanderford and Conner 1995, Simmons and Conner 1996, Davidson et al. 1997, Conner et al. 2000), several elements have been found to be common to most arctiine courtship scenarios. Females initiate courtship by releasing a pheromone blend, usually comprised of long alkanes with a variety of functional groups (Roelofs and Cardé 1971, Hill et al. 1982, Simmons et al. 1998). Upon sensing the pheromone, males then fly upwind, in a zigzag path in and out of the pheromone plume (Cardé and Millar 2009). Once the male reaches the female, he produces a counter-signal of some kind. If the signal is appropriate, the female allows the male to mate; if not, the female will take flight (Simmons and Conner 1996). Successful copulation often is prolonged, ensuring the transfer of both the sperm and the associated spermatophore (Dussourd et al. 1991, LaMunyon and Eisner 1994).

Variations on this basic courtship sequence are numerous, but mostly involve the male signal. In many cases, males release a short-range pheromone, usually hydroxydanaidal (Conner et al. 1981, Boppré and Schneider 1985, Eisner and Meinwald 1987, Boppré 1995, Jordan e al. 2005, Jordan and Conner 2007). Hydroxydanaidal is derived from pyrrolizidine alkaloids (PAs), either sequestered by the male during larval feeding (Conner et al. 1990, Elmke et al. 1990) or adult visits to a PA plant where they engage in pharmocophagy—actively dissolving PA crystals on withered plant tissues with their saliva and imbibing them (Pliske 1975; Boppré 1981, 1984, 1986; Boppré and Schneider 1985).

Although the male pheromone is chemically the same in many species, its means of dissemination varies (Fig. 2). Coremata, which are paired, inflatable tubes located between the seventh and eighth sterna, are most common in tiger moth males (Fig. 2A). The coremata, which are everted upon reaching the female, are covered in scent scales that are modified to disseminate male pheromone (Birch et al. 1990, Weller et al. 2000). Coremata can be very simple or highly complex. In two unrelated species, *Creatonotos gangis* L. and *Estigmene acrea* (Drury), the size and complexity of the organ are determined by the PA diet of the male larvae (Boppré and Schneider 1985, Jordan and Conner 2007). However, not all coremata produce PA-based pheromones; *Cycnia tenera* Hübner produces a non-PA pheromone (Conner 1987). Found throughout Arctiinae, coremata are hypothesized to

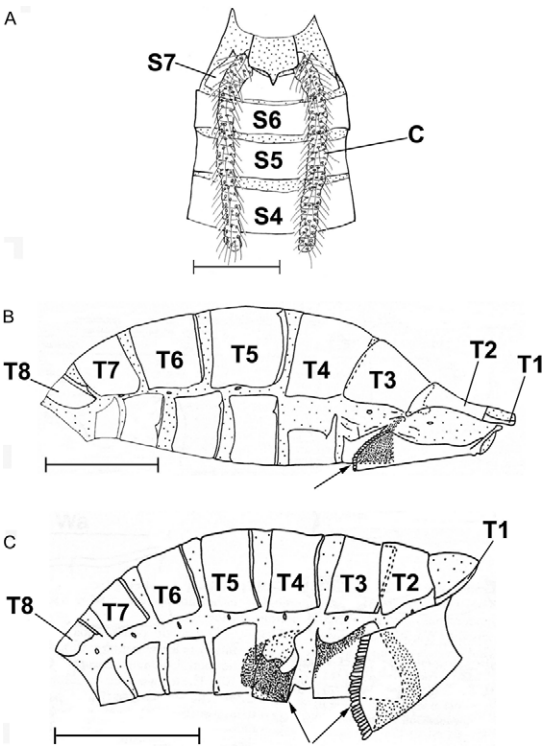

Fig. 2. Androconia found in Ctenuchina and Euchromiina. A. Ventral view of segments 4–7 (S4–S7) of male *Pleurosoma angustata* (Möschler), showing coremata (C) (scale bar = 10 mm) B. Abdominal scent pouch of male *M. novercida*, involving sternites 2–4 (T 1–8 = abdominal tergites, arrow = location of pouch, scale bar = 20 mm) C. Abdominal scent pouch of male *Myrmecopsis ruatana* (Druce), involving sternites 2–4 (scale bar = 20 mm).

be evolutionarily labile, because their presence and functionality can vary within and among genera (Boppré and Schneider 1985, Jordan and Conner 2007).

Although coremata are common throughout Ctenuchina (Weller et al. 2000), members of Euchromiina possess an alternative structure, the subabdominal scent pouch (also referred to as a "ventral valve") (Barth 1953, 1956; Blest 1964; Weller et al. 2000; Figs. 2B and C). Males explosively release deciduous scales (flocculent) impregnated with hydroxydanaidal-based pheromone from this pouch upon reaching the female, covering her in a mass of scent scales (Conner et al. 2000). Experiments reveal that the female is instantly protected from predators by her chemically impregnated nuptial veil (Conner et al. 2000). Two types of pouches exist and both can be found within a single genus. In the first, a shallow single pouch covered by an elongated abdominal sternite (S2), such as those displayed in *Myrmecopsis novercida* (Kaye) (Fig. 2B) or *Syntomeida ipomoeae* Harris (not shown) can be found. The double-pouch morphology is comprised of a duplicated antecosta at the third sternite (S3) that results in two larger pouches, such as those found in *Myrmecopsis ruantana* Druce (Fig. 2C; Weller et al. 2000).

A third type of abdominal androconia is found in members of *Antichloris*, *Ceramidia*, and *Ceramidiodes*. Some of these species possess bilobed pockets containing nondeciduous hair pencils between the seventh and eighth sternites that presumably are everted upon extrusion of the genitalia (Field 1975). Male *Macrocneme* possess a ventral pouch found at the base of the genitalia that also contains scent scales (Dietz 1994). These structures differ slightly from coremata as they are not tubular and are greatly reduced, but may be homologous to coremata in other species of Ctenuchina. Although the diversity of these structures continues to be documented, much remains to be discovered about their use.

Finally, nonabdominal androconia can occur on legs and wings. Males of *Orcynia calcarata* (Walker), for example, have internal pouches with scent scales on the metathoracic tibia (Simmons and Weller 2006). *Melese* males typically have a folded forewing tip that conceals a wing hair pencil. Although nonhomologous structurally, all androconia share the function of disseminating male courtship pheromones.

Pheromone-based communication in males appears to be predicated on the ability of species (or their most recent common ancestor) to sequester PAs (Simmons and Conner 1996, Weller et al. 1999, Conner and Weller 2004). Larval PA feeding often is replaced with cardenolide (CG) feeding across Arctiinae (Weller et al. 1999, Conner and Weller 2004). In CG-larval feeding species, male pheromone structures usually are reduced or lost (Conner 1987, Portilla et al. 1987, Sanderford and Conner 1990, Simmons and Conner 1996). Males may then use another sensory modality, ultrasound, during courtship (Coro et al. 1983, Sanderford and Conner 1990, Simmons and Conner 1996, Rodríguez-Loeches et al. 2009). Unfortunately, lack of behavioral studies in key lineages limits study of when and how ultrasound use evolved across the tiger moths. Certainly, further examples of ultrasonic courtship await discovery within Arctiinae.

Materials and Methods

Sampling and Sequencing. Specimens used in this study either were dried, pinned specimens, or collected directly into absolute ethanol and stored at −70°C. Voucher specimens were deposited at the McGuire Center for Lepidoptera and Biodiversity, FL Museum of Natural History, Gainesville, FL (FMNH); and University of Minnesota Insect Collection, Department of Entomology, University of Minnesota, St. Paul, MN (UMN; Table 1).

Twenty-nine species representing 24 genera distributed among four tribes were included in our study (Table 1). Of the 21 ingroup species, seven of these traditionally are placed within Ctenuchina and 14 within Euchromiina (Table 1). Eleven of 32 large genera (>10 species; 34%) and all major lineages posited by Hampson (1898) are represented except Syntomini (Table 1). Nine outgroup species from Callimorphina, Pericopina, and Phaegopterina also were sampled.

DNA extractions were performed using the DNeasy Tissue Kit (Qiagen, Germantown, MD) and the Insect Extraction Protocol B (DNeasy Tissue protocol 2007) with a 10-min incubation at 70°C and final elution into 70 μl of 70°C EB buffer from the Qiagen polymerase chain reaction (PCR) purification kit (part number 28106). This protocol was followed with frozen material stored dry or in absolute ethanol, as well as pinned specimens. Extraction control blanks were performed along with each extraction set. Verification of these blanks and quantification of DNA concentration in the extractions were performed via a NanoDrop (Thermo Scientific, Wilmington, DE). Extraction blanks, when used as template for PCR, were also negative, indicating that no foreign DNA was present in the extractions.

Gene fragments from one mitochondrial (cytochrome oxidase I [COI]) and two nuclear genes (D1 region 28S ribosomal RNA [D1], Elongation Factor 1-α [EF1-α]) were amplified and sequenced for all 29 species, as published previously (Simmons and Weller 2001). Combinations of five primers, four published and one novel (Table 2), were used to amplify and sequence a 1,175-bp portion that spanned the COI, leaving a 200-bp gap in the middle (position on the *Drosophila yakuba* Fallén genome given before sequence; Clary and Wolstenholme 1985). Primers and annealing temperatures for each gene region are given in Table 2.

PCR products were cleaned for automated sequencing with the Qiaquick PCR purification kit (Qiagen) according to the protocol, except that the product was eluted with 40-μl ddH_2O. Sequencing reactions were performed using the BigDye Terminator version 3.1 Cycle Sequencing Ready Reaction Sequencing Kit (Applied Biosystems, Foster City, CA) by using the recommended protocol. Sequencing reactions were purified with the ABI BigDye Xterminator kit (Foster City, CA). An ABI 3100 system was used to visualize and record the sequence.

Data were imported into Sequencher 4.5 (Gene Codes, Ann Arbor, MI). Sequences for each individual then were assembled to produce a consensus sequence for each individual. Protein coding sequences (COI, EF1-$\propto$) were translated and checked for stop codons. Consensus sequences for individuals then were aligned by conserved motifs for each gene region and adjusted by eye when necessary. The three gene regions then were concatenated into a total evidence matrix for phylogenetic analyses.

Phylogenetic Analyses. Phylogenetic trees were generated using three optimality criteria (parsimony, maximum likelihood, and Bayesian) to measure whether tree topology was sensitive to tree reconstruction methods. Parsimony trees were constructed via PAUP* (Swofford 2002) by using heuristic searches with 1,000 random addition replicates. Sequence data were not weighted by gene or by codon position for protein-coding genes. Robustness of resulting nodes was assessed via 1,000 bootstrap replicates that produced a majority rule consensus tree.

Table 1. Species sampled for the three gene regions (2,098 bp total), with their label data and information about male androconia or courtship behavior

Subtribe	Genus species author Androconia Voucher location	Collection data	COI 1,174 bp	D1 283 bp	EF1-a 641 bp
Outgroup species					
Callimorphina	*Utetheisa ornatrix* (L.)	MEXICO: Puebla: near Las	GU258440	GU258469	GU258498
	Genital coremata	Vegas; June 2007; R. B.			
	UMN	Simmons			
Pericopina	*C. fidelissima* H-S	BAHAMAS: Cat Island;	GU258414	GU258443	GU258472
	Unknown	Fernandez Bay Village;			
	UMN	June 2005; R. B. Simmons			
Phaegopterina	*Bertholdia detracta* Seitz	ECUADOR: Napo:	GU258413	GU258442	GU25847
	Ventral coremata	Mishualli; May 1997;			
	FMNH	A. D. Warren			
	E. bolteri	MEXICO: Puebla: near Las	GU258425	GU258454	GU258483
	None	Vegas; June 2007; R. B.			
	UMN	Simmons			
	Halysidota sp.	BAHAMAS: Cat Island;	GU258428	GU258457	GU258486
	Ventral coremata	Fernandez Bay Village;			
	UMN	June 2005; R. B. Simmons			
	Melese drucei Rothschild	ECUADOR: Napo:	GU258431	GU258460	GU258489
	Ventral coremata	Mishualli; May 1997;			
	FMNH	A. D. Warren			
	Melese flavimaculata Dognin	ECUADOR: Napo:	GU258432	GU258461	GU258490
	Ventral coremata	Mishualli; May 1997;			
	FMNH	A. D. Warren			
	Munona iridescens Schaus	ECUADOR: Pichincha:	GU258433	GU258462	GU258491
	Ventral coremata	Tinalandia; May 1997;			
	FMNH	A. D. Warren			
	O. cingulata	ECUADOR: Napo:	GU258435	GU258464	GU258493
	Ventral coremata	Mishualli; May 1997;			
	FMNH	A. D. Warren			
Ingroup species					
Ctenuchina	*Aclytia heber* (Walker)	ECUADOR: Napo:	GU258412	GU258441	GU258470
	Ventral coremata	Mishualli; May 1997;			
	FMNH	A. D. Warren			
	Correbia lycoides Walker	COSTA RICA: San	GU258415	GU258448	GU258473
	Ventral coremata	Vito: Las Alturas Field			
	UMN	Station; June 1997; R. B. Simmons			
	Ctenucha virginica Esper	USA: Minnesota:	GU258420	GU258449	GU258478
	Ventral coremata	Polk County: East			
	UMN	Grand Forks; June 2006; K. R. Simmons			
	E. heros	BAHAMAS: North Andros	GU258422	GU258451	GU258480
	None	Island; Love at First Sight;			
	FMNH	June 2006; M. Simon, L. D. and J. Y. Miller			
	E. lamia	ECUADOR: Pichincha:	GU258423	GU258452	GU258481
	Ventral coremata	Tinalandia; May 1997; A. D.			
	FMNH	Warren			
	E. dentata	COSTA RICA: San Vito;	GU258424	GU258453	GU258482
	Ventral coremata	Las Alturas Field Station;			
	UMN	June 1997; R. B. Simmons			
	G. consorta	COSTA RICA: San Vito;	GU258427	GU258456	GU258485
	Ventral coremata	Las Alturas Field Station;			
	UMN	June 1997; R. B. Simmons			
Euchromina	*C. braconoides*	NICARAGUA: Jinotega;	GU258416	GU258444	GU258474
	Abdominal scent pouch	Aréa Protegida Bayanilí-			
	UMN	El Diablo; La Quabradona; 1 KM NE of Santa Maura; 13° 10.389′ N, 85° 51.404′ W; el. 1,050 m; July 2000; Chamorro; Lacayo, Christensen			
	Cosmosoma stibostictum (Butler)	ECUADOR: Napo:	GU258417	GU258445	GU258475
	Abdominal scent pouch	Mishualli; May 1997;			
	FMNH	A. D. Warren			
	C. subflamma	ECUADOR: Napo:	GU258418	GU258446	GU258476
	Abdominal scent pouch	Mishualli; May 1997;			
	FMNH	A. D. Warren			
	C. teuthras	MEXICO: Tamaulipes;	GU258419	GU258447	GU258477
	None	June 2004; J. K. Adams			
	UMN				
	D. correbioides	MEXICO: Tamaulipes;	GU258421	GU258450	GU258479
	None	June 2004; J. K. Adams			

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Table 1. Continued

Subtribe	Genus species author Androconia Voucher location	Collection data	COI 1,174 bp	D1 283 bp	EF1-a 641 bp
	UMN				
	Eunomia latenigra (Butler)	BAHAMAS: Cat Island;	GU258426	GU258455	GU258484
	Abdominal scent pouch	Fernandez Bay Village;			
	UMN	June 2005; R. B. Simmons			
	I. perbosci	MEXICO: Tamaulipes;	GU258429	GU258458	GU258487
	Abdominal scent pouch	June 2004; J. K. Adams			
	UMN				
	M. vittata	ECUADOR: Pichincha:	GU258430	GU258459	GU258488
	Genital scent pockets	Tinalandia; May 1997;			
	FMNH	A. D. Warren			
	O. calcarata	BRAZIL: Rhondonia:	GU258434	GU258463	GU258492
	Tibial scent pouches	Fazenda Rancho Grande;			
	FMNH	Aug. 1993; A. D. Warren			
	P. lydia	MEXICO: Tamaulipas;	GU258436	GU258465	GU258494
	Abdominal scent pouch	June 2004; J. K. Adams			
	UMN				
	Poliopastea clavipes (Boisduval)	MEXICO: Tamaulipas;	GU258437	GU258466	GU258495
	Abdominal scent pouch	June 2004; J. K. Adams			
	UMN				
	Sphecosoma felderi (Druce)	MEXICO: Tamaulipas:	GU258438	GU258467	GU285496
	Abdominal scent pouch	El Lobo; June 2004; J. K.			
	UMN	Adams			
	S. epilais	USA: Florida: Weslev	GU258439	GU258468	GU285497
	None	Chapel June 2006; L. D.			
	FMNH	and J. Y. Miller			

Classification follows Hampson (1898), Zerny (1912), Forbes (1939), Jacobson and Weller (2001), and Simmons and Weller (2006). GenBank numbers are given for each gene fragment, and voucher location is designated.

To select a model of sequence evolution for the maximum likelihood analyses, the model test block was executed in PAUP* (Posada and Crandall 1998). The model that best fit the concatenated matrix was GTR + I + G. Based on this result, we implemented ML using the default settings in GARLI to construct the ML trees (Zwickl 2006) (number of substitution types, 6; gamma distribution of variable sites with an estimated alpha parameter; number of rate categories, 4). Once a stable topology was constructed, one million generations of trees were constructed to ensure the reliability of the resulting tree. One thousand replications of parametric bootstrapping were performed to assess the robustness of resulting nodes in the best tree produced by GARLI.

For the Bayesian analysis, nucleotide substitution models for the combined dataset were obtained using AIC as implemented in Modeltest 3.7 (Posada and Crandall 1998; Posada and Buckley 2004). Five million generations of random trees were used to initiate all searches. These trees were the starting point for two simultaneous analyses of four Markov Chain Monte Carlo chains (one heated, three cold) in MRBAYES (Huelsenbeck and Ronquist 2001, Ronquist and Huelsenbeck 2003). Cold chains were sampled every 100 runs, and the analysis was stopped when runs converged on results that had <0.01 split frequencies. Posterior probabilities were used to assess the strength of relationships found via these methods.

Comparative Methods. Androconial type was treated as an attribute based on function (Mickevich and Weller 1990) and scored as a standard character in a matrix created in Mesquite 2.5 (Maddison and Maddison 2009). Coding does not reflect homology of morphological structures, but rather their function. Further, we did not distinguish between degree of

Table 2. Primers used for amplification and sequencing of each gene region, listed from 5′ to 3′

Gene region annealing temperature	Direction: primer name: position	Primer sequence	Reference
mtDNA: COI 45°	Sense: K698: 1435	TACAATTTATCGCCTAAACTTCAGCC	Simon et al. (1994)
	Sense: Mace: 1612	GGAGATGATCAAATTTATAATACTATTG	This paper
	Sense: revNancy: 2191	GAAGTTTATATTTTAATTTTACCGGG)	Simmons and Weller (2001)
	Antisense: Nancy: 2191	CCCGGTAAAATTAAAATATAAACTTC	Simon et al. (1994)
	Antisense: Pat: 3015	TCCATTACATATAATCTGCCATATTAG	Simon et al. (1994)
28S rRNA 47°	Sense: D1UP	GGGGAGGAAAAGAACTAAC	Larsen (1992), Kjer (1995)
	Antisense: D1DN	CAACTTTCCCTTACGGTACT	Larsen (1992), Kjer (1995)
nuDNA EF1-α	Sense: 94F	GGTCACAGACATTTCATCAAGAACATGAT	Cho et al. (1995)
53°	Sense: Jack	GGGTGGGTTGTTCTTGGAG	This paper
	Antisense: 53R	GCGAACTTGCAAGCAATGTGAGC	Cho et al. (1995)
	Antisense: Riddick	CCACTGAGCCCCCATAC	This paper

development or types of abdominal coremata. We were interested in the large scale pattern of chemical communication as opposed to tracing unique occurrences of homologous structures.

Androconial type was determined by direct observation of specimens and four classes were scored: 0: coremata located on sternites 7 and 8; 1: abdominal scent pouch, either single or double; 2: androconial structure found at various locations on males, including legs and the bilobed pouch condition on sternites 7 and 8; and 3: no observable androconial structure. Observational data were largely incomplete for species included in our study. We used morphology as a proxy for male courtship behavior. In this manner, androconial structures were mapped on the strict consensus of the Bayesian topologies by using parsimony models and likelihood models in Mesquite to reconstruct ancestral character states.

Results

Phylogenetic Results. The three gene regions were amplified successfully and sequenced for all 29 species, including museum specimens. For the D1 loop (28S rRNA), we obtained 238 basepairs (bp) that averaged 2.8% divergence (uncorrected pair-wise) across all species. We obtained 650 bp of EF1-α that was 6.8% divergent. The COI region was successfully sequenced for two sequential regions for 1,173 bp in total that averaged 14% sequence divergence. The COI exhibited typical AT compositional bias for insect mitochondrial DNA (T: 40%, A: 30%, C: 16%, G: 14%, average).

The complete matrix for all three genes yielded 554 parsimony-informative characters of the 2,098 total base pairs (26% of the characters). A partitioned homogeneity test indicated that each gene region had characters that were in agreement with those of other regions ($P = 1.00$). We show the Bayesian topology with the MP and ML statistics added because all analyses were largely congruent except in some placements of taxa as noted (Fig. 3). Maximum parsimony analysis resulted in one fully resolved, most parsimonious tree (L: 3,041 steps, CI: 0.34, RI: 0.28, not shown). Maximum likelihood results found well-supported relationships, mostly between species pairs (-ln likelihood: 18,920.34). The two simultaneous Bayesian analyses converged ($P < 0.01$) after 20,000,000 generations (=burnin of 50,000 trees; -ln likelihood: 18,954.18). The analyses under the different optimality criteria resulted in topologies that support four general conclusions, but only the Bayesian analysis had several internal nodes with strong support (above 90%).

First, none of the results support a monophyletic Phaegopterina, confirming earlier phylogenetic studies (Jacobson and Weller 2002). Only the association of phaegopterine genera *Melese* and *Bertholdia* was strongly supported. The relationship of *Melese* and *Bertholida* with *Composia fidelissima* Herrich-Schäffer (Pericopina) and *Ordishia cingulata* Rothschild (Phaegopterina) also had strong support (98% posterior probability) in the Bayesian topology; these relationships did not receive bootstrap support in the ML and MP analyses (Fig. 3). Another general result was that three species, two ctenuchine (*Galathea consorta* Walker and *Eucereon dentata* Dognin) and one euchromiine (*Orcynia calcarata*), are imbedded within the phaegopterine grade (Fig. 3).

All analyses recovered a core clade of 11 euchromiine genera (with reassignment of *Empyreuma* to Euchromiina) and a core clade of four ctenuchine genera with moderate support in the Bayesian analysis (92 and 83% posterior probability, respectively; Fig. 3). These are supported as sister groups (96% posterior probability). Within the core Euchromiina, the large genus *Cosmosoma* is polyphyletic (Fig. 3), with species found in three well-supported subclades. In addition, the Bayesian topology reconstructs four ctenuchine genera (*Ctenucha*, *Episcepsis*, *Correbia*, and *Aclytia*) as a clade with moderate support (83% posterior probability; Fig. 3). Further, support is obtained for *C. virginica* and *Episcepsis lamia* (Butler), reflected in moderate bootstrap support and a high posterior probability (Fig. 3).

Relationships of these remaining taxa vary depending on the type of analytical approach. Other putative sister taxa are only found in a subset of analyses (i.e., *Syntomeida epilais* (Walker), *Macrocneme vittata* (Walker), and *Empyreuma heros* Bates. The placement of these species as a clade in the Bayesian analysis is only weakly supported (53% posterior probability). Inspection of the ML phylogram does not support the hypothesis of long-branch attraction for these species. The mean branch length of these three species to node C + E is 7.6 [± 0.04]%; these branch lengths are similar to other species in the analysis. The mean branch length for the remainder of ingroup taxa to node C + E (excluding *G. consorta*, *E. dentata*, and *O. calcarata*) was 7.1 [± 0.01]%; the branch lengths of all ingroup taxa were found to be similar based on a Student *t*-test ($\propto > 0.05$).

Comparative Results. Under both MP and ML, presence of abdominal coremata was reconstructed as the ancestral condition of the ingroup with a 99% probability (Fig. 4). Coremata were lost or modified three times. Origin of tibial androconia was reconstructed at the node connecting *Orcynia calcarata* and *Galathea consorta* to the remaining species, but this occurrence has a low probability ($P = 0.06$; Fig. 4).

Within a redefined Euchromiina (inclusive of *Empyreuma*, Fig. 3), chemical communication was lost completely in the *Empyreuma* clade (p for loss of androconia: 0.90, p for acquisition of genital coremata: 0.06; Fig. 4); if chemical communication is still present, abdominal (P) or genital (V) androconia replaced coremata. The MP reconstruction placed the loss of coremata or gain of the abdominal scent pouch at the putative euchromiines [*Phoenicoprocta lydia* (Druce) and remainder of tree; Fig. 4]. The ML results supported this reconstruction ($P = 0.95$). Abdominal scent pouches were lost twice, according to both reconstruction methods (Fig. 4; *D. correbioides* and *C. teuthras*), although ML results indicated that these

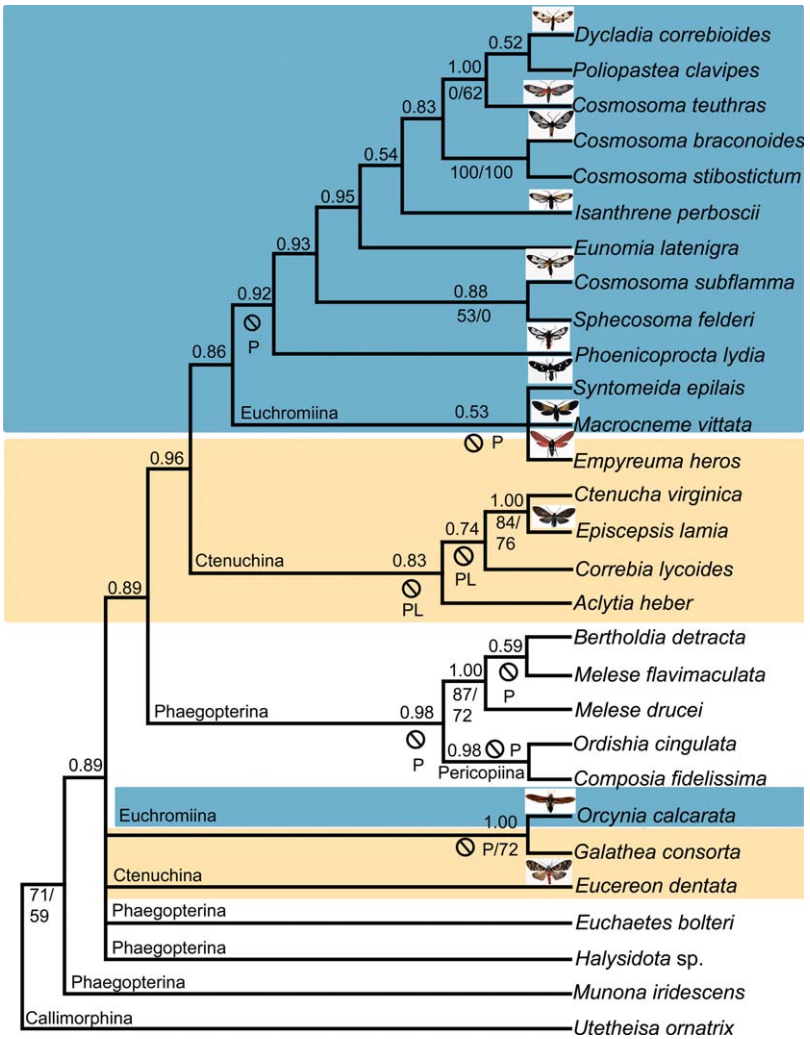

Fig. 3. Strict consensus of Bayesian topologies, with current taxonomy and distribution (-ln likelihood = 18954.18). Shading indicates distribution of ingroup species. Posterior probabilities >50% given above appropriate nodes; MP and ML bootstrap support >50% given below appropriate nodes (MP/ML); ⃠ = node not found in MP (=P) or ML (=L) topologies. (Online figure in color.)

losses may not be independent with loss reconstruction occurring before the node connecting *Dycladia*, *C. teuthras*, and *Poliopastea* (p of androconial loss: 0.51; Fig. 4).

Discussion

Challenges of Phylogeny Reconstruction. Estimating the phylogeny of mimetic arctiines is complicated by their relatively recent origin (<20 million years ago, Grimaldi and Engel 2008); availability of quality material for genetic study; and mimetic adult habit that affects key character systems used by early taxonomists (e.g., wing venation, wing pattern). Recent studies of the phylogeny of Lepidoptera (review Roe et al. 2009) suggest that Lepidopteran phylogeny is particularly recalcitrant to estimation with molecular data because of the rapid speciation of this hyperdiverse order. Arctiinae represents another burst of lepidopteran speciation (11,000 species or more) within a relatively short time frame. The oldest putative fossil for Arctiinae dates to the mid-Miocene (Grimaldi and Engel 2008). The phylogenetic pattern is inherently difficult to estimate, consequently, and will require multiple genes and taxa to elucidate.

Another challenge is the availability of quality material for molecular study. In general, collection of fresh or frozen material for DNA studies of these species is difficult, because many species, when collected, are found in low abundance. Over 50% of our species was amplified from traditional museum prepared specimens. Our recovery of a reciprocally monophyletic Euchromiina and Ctenuchina (with exclusion of three species and reassignment of *Empy-*

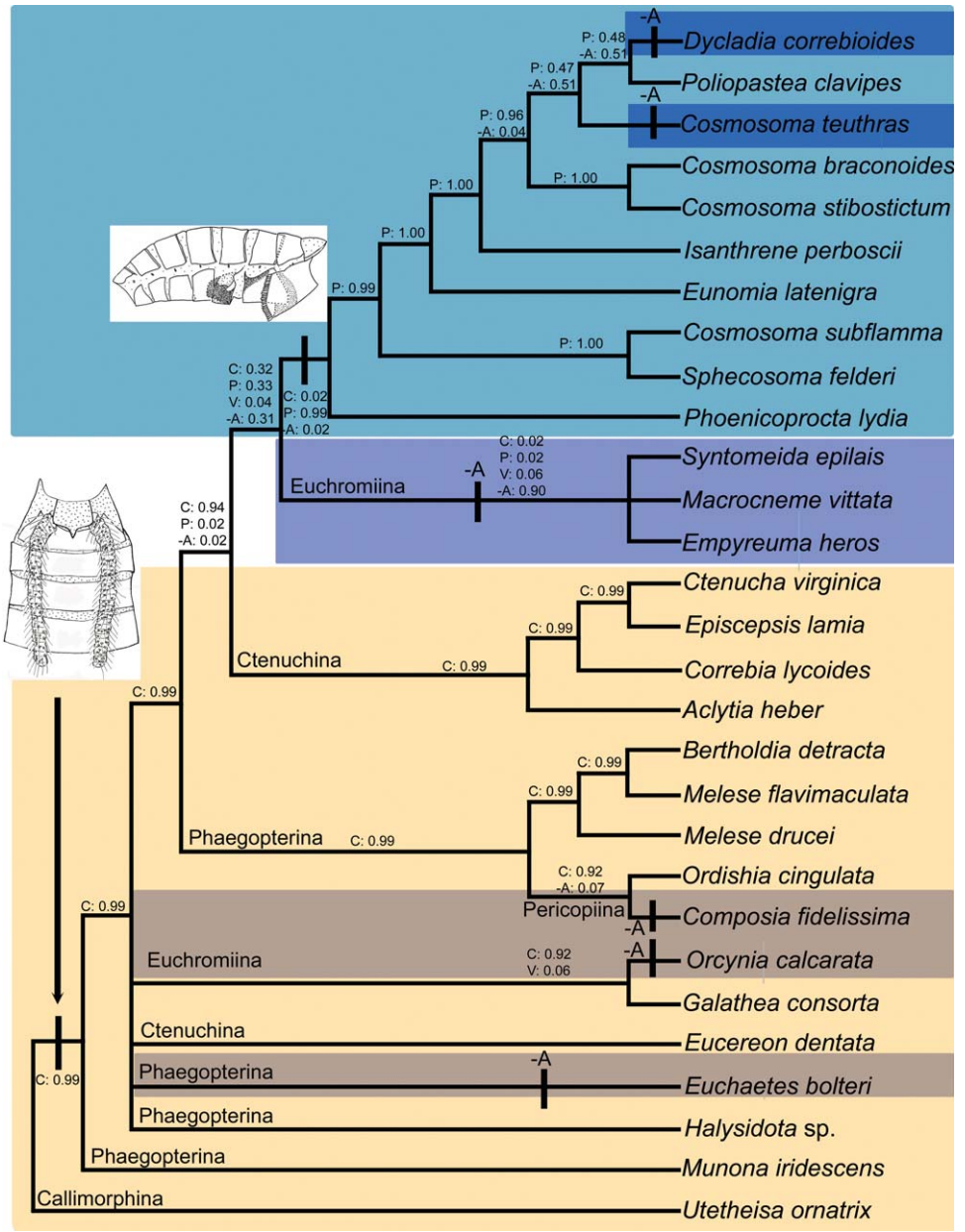

Fig. 4. Reconstruction of androconia evolution. Dark bars indicate MP reconstruction of character evolution. Probability of ancestral character condition given at node for ML reconstruction. P = abdominal scent pouch, C = coremata, -A = no observable androconia, V = androconia found in various, nonabdominal location. (Online figure in color.)

reuma) demonstrates the importance of gene and species sampling. These results support an earlier study based on adult and immature morphology (Jacobson and Weller 2002) but contradict the first molecular phylogeny published for the Euchromiina based on the 28S D1 loop and mitochondrial genes COI and cytochrome *b* (Simmons and Weller 2001). The addition of the EF1-α fragment and sampling of taxa is most likely the cause of the contradicting results. In the 2001 study, an internal root ctenuchine *Hyalaceria* was used; here the root was comprised of 9 outgroup species including 6 selected from Phaegopterina, 2 Pericopina, and 1 callimorphine. Of the 20 ingroup genera included here, only six were included in the original study and of those, only three species of the original study are represented here. Further, only one node (supporting monophyly of a genus) was supported by a significant bootstrap (Simmons and Weller 2001) and Bayesian analyses were not generally available. We conclude that inclusion of an addi-

tional genetic marker EF1-α, balanced outgroup sampling and additional analytical approaches have improved the phylogenetic estimation.

The resulting phylogeny challenges the classifications proposed by early lepidopteran taxonomists (Hampson 1898, Forbes 1960). These arrangements relied heavily on easily viewed characters such as wing venation, coloration, antennal modifications, and leg spurs. Wing venation was the primary character system; however, venation is often reduced (veins lost or fused) in mimics of aculeate hymenopterans (Simmons and Weller 2006). Thus, we were not surprised that we did not recover groups based upon wing venation. For example, in the Bayesian analysis, *Ctenucha*, *Episcepsis*, *Correbia*, and *Aclytia* are recovered as a clade with moderate support (83%; Fig. 3). This clade includes representatives of two of three Hampson wing venation lineages (Hampson 1898). Placement of *Empyreuma* and two other ctenuchine species contradicts the classification of Hampson (1898). *Empyreuma* is associated with *Syntomeida* and other Euchromiina (not Ctenuchina of Hampson) confirming earlier studies (Simmons and Weller 2001). The species *Galathea consorta* and *Eucereon dentata* arise within Phaegopterina in all analyses. *Eucereon* and *Galathea* were treated as "transitional" ctenuchine genera to the very accurate wasp mimics by Hampson (1898). More extensive sampling of Hampson's lineages I and II, especially of "transitional" ctenuchine genera and additional sampling of phaegopterines is warranted given these results.

Results for Hampson's Euchromiina also showcase problems of past reliance on wing venation and coloration patterns for classifications from delimiting genera to the definition of the subtribe. *Cosmosoma* is comprised of approximately 155 species and defined solely by wing venation. The genus was polyphyletic in all analyses (Fig. 3). Placement of putative euchromiines *Syntomeida*, *Empyreuma*, and *Macrocneme* varies depending on the reconstruction method; none of these relationships have strong bootstrap support (Fig. 4). Species of these genera are of interest because males have highly modified genital valves (Dietz 1994; Weller et al. 2004), several use CG-based larval host plants (Conner 1987), and some use ultrasound in male courtship (Portilla et al. 1987; Sanderford and Conner 1990, 1995; Sanderford et al. 1998). Only if *Syntomeida epilais* and *O. calcarata* are excluded, can a monophyletic Euchromiina be defined that is recovered in both ML and Bayesian topologies (Fig. 4), with strong support in the latter (posterior probability: 0.92). These species represent the diversity of group 3 (Hampson 1898), indicating that further sampling in this group likely will strengthen support for its monophyly.

Despite the challenges of obtaining material for molecular studies, these data are critical to address arctiine relationships, particularly for mimetic species. Although more recent study of characters associated with mimicry at the species and generic level successfully have established key homologies for phylogeny reconstruction (Simmons and Weller 2006), at the infratribe level, adult morphology alone cannot provide sufficient informative variation (e.g., Callimorphina, DaCosta and Weller 2005). Immature stages often are lacking, especially for Euchromiina and Ctenuchina (Jacobson and Weller 2002). Molecular studies are a desired complement to morphological work.

Evolution of Androconia. Clear patterns emerge in the evolution of androconial structures with some appearing phylogenetically constrained. As studies of behaviors are undertaken, the search for proximal explanations will need to acknowledge the evolutionary "antiquity" of the underlying structures. Our results are consistent with earlier studies (Jacobson and Weller 2002, DaCosta and Weller 2005) that ventral abdominal coremata are the ancestral state to this clade comprised of Phaegopterina, Pericopina, Ctenuchina, and Euchromiina (PPCE clade of Jacobson and Weller 2002). Four losses of ventral coremata are estimated, with three being unique to particular species (Fig. 4; *C. fidelissima*, *Euchaetes bolteri* Stretch, *O. calcarata*); the fourth is a potential synapomorphy of Euchromiina.

Abdominal scent pouches originate early with our redefined Euchromiina but are subsequently lost in *D. correbioides* and *C. teuthras* (Fig. 4). The morphology varies across the group, from a single shallow pouch to large two chambered pouches (Figs. 2B and C; Weller et al. 2000). *Macrocneme* males bear androconial scales at the base of their genital capsule; however, courtship behavior is undocumented. The addition of exemplar species and more behavioral observations within *Syntomeida–Empyreuma–Macrocneme* clade will be needed to clarify the pattern of courtship evolution.

On the surface, the conserved nature of these androconia appears to contradict the general characterization of sexual selection: males display signal variation that allows females to assess and choose their mates, and therefore these signals are not phylogenetically useful. In Lepidoptera, however, selection initially occurs when the female releases diffuse amounts of pheromone, which only allows males that are "tuned" to that signal to respond (Millar 2000, Cardé and Haynes 2004, Cardé and Millar 2009). Further, the structure itself (i.e., coremata, abdominal scent pouch, genital capsule scales) is not the basis of female choice, but rather the amount and chemical structure of the pheromone emitted from that structure (review Cardé and Millar 2009). Hypotheses concerning proximal cues (quantity of pheromone, chemistry) and their rapid evolution can be reconciled with ultimate explanations of a dispersing structure's conserved evolutionary history. Thus, it may be easier, in an evolutionary sense, to lose or change the use of these structures than to produce androconia de novo. The species' diversification of arctiines since the Miocene may be driven as much or more by microchanges in pheromone chemistry rather than structural diversification of its delivery systems.

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