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When caterpillars attack: Biogeography and life history evolution of the Miletinae (Lepidoptera: Lycaenidae)

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Of the four most diverse insect orders, Lepidoptera contains remarkably few predatory and parasitic species. Although species with these habits have evolved multiple times in moths and butterflies, they have rarely been associated with diversification. The wholly aphytophagous subfamily Miletinae (Lycaenidae) is an exception, consisting of nearly 190 species distributed primarily throughout the Old World tropics and subtropics. Most miletines eat Hemiptera, although some consume ant brood or are fed by ant trophallaxis. A well-resolved phylogeny inferred using 4915 bp from seven markers sampled from representatives of all genera and nearly one-third the described species was used to examine the biogeography and evolution of biotic associations in this group. Biogeographic analyses indicate that Miletinae likely diverged from an African ancestor near the start of the Eocene, and four lineages dispersed between Africa and Asia. Phylogenetic constraint in prey selection is apparent at two levels: related miletine species are more likely to feed on related Hemiptera, and related miletines are more likely to associate with related ants, either directly by eating the ants, or indirectly by eating hemipteran prey that are attended by those ants. These results suggest that adaptations for host ant location by ovipositing female miletines may have been retained from phytophagous ancestors that associated with ants mutualistically.

KEY WORDS: **Ant association, aphytophagy, coevolution, myrmecophagy, myrmecophily, social parasitism.**

Evolutionary shifts to herbivory are associated with increased diversification in insects (Farrell et al. 1992). More than one-quarter of the earth's described species are phytophagous insects that feed obligately on living plant tissue during at least part of their life cycle (Strong et al. 1984; Grimaldi and Engel 2005). Although less than one-third of insect orders include

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herbivores (Orthoptera, Phasmatodea, Blattodea (including termites), Hemiptera, Thysanoptera, and the four "mega diverse" orders: Hymenoptera, Diptera, Coleoptera, and Lepidoptera), these orders are disproportionately species rich (Strong et al. 1984; Mitter et al. 1988; Winkler and Mitter 2008; Futuyma and Agrawal 2009). Conversely, insect lineages that have shifted from herbivory to parasitism tend to be less diverse than their plant feeding relatives, perhaps because ascending the trophic pyramid restricts population sizes and densities, thereby decreasing opportunities for speciation while increasing extinction likelihood (Wiegmann et al. 1993).

Of the four largest holometabolous orders, only Lepidoptera are almost exclusively phytophagous. Although a significant proportion of Hymenoptera, Diptera, and Coleoptera derive their nutrition from animal sources at some point during their life cycle, less than 2% of Lepidoptera, or as few as 200 to 300 species, have been recorded as "aphytophagous" and feed obligately on something other than living plants for at least some portion of their life cycle (Pierce 1995). Moreover, these few hundred species are taxonomically widespread throughout Lepidoptera, compared with the other megadiverse orders in which parasitic or carnivorous behavior is restricted to a few lineages. The taxonomic distribution of aphytophagy suggests that the habit has evolved multiple times independently in the Lepidoptera, particularly in the butterfly family Lycaenidae (Cottrell 1984; Pierce 1995). Some aphytophagous Lepidoptera are predators that eat other animals, primarily insects, and others are parasites, that potentially lower their host's fitness without killing them (e.g., via trophallaxis with ants). Other aphytophagous taxa feed on detritus, lichen, and keratin. Despite multiple shifts away from herbivory within the Lepidoptera, these shifts appear to be evolutionarily transient, or "tippy" in their distribution—aphytophagous lepidopteran lineages rarely persist and radiate (Pierce 1995). Most shifts to predation/parasitism in the family Lycaenidae have occurred within otherwise phytophagous clades, whose species associate mutualistically with ants, and have given rise to only one or two parasitic species.

The caterpillars of approximately three-quarters of the species in the family Lycaenidae associate with ants (Pierce et al. 2002). A significant number of species in the sister family of the Lycaenidae, the Riodinidae, are also known to associate with ants (e.g., DeVries 1991b; DeVries and Penz 2000; Kaminski et al. 2013), whereas species in other lepidopteran families, with rare exceptions, do not. These associations can range from facultative to obligate interactions, and from mutualism to parasitism. Ant-associated Hemiptera are often involved. The first innovation in the evolution of myrmecophily in the Lycaenidae is likely to have involved tolerance of the caterpillar by ants (termed "myrmecoxeny"). In most circumstances, foraging ants encountering a caterpillar will regard it as potential prey. However, once lycaenid caterpillars evolved a means to appease aggressive ants, myrmecophilous lycaenids would have had the great advantage of occupying "enemy free space" (Atsatt 1981), and more complicated interactions would have been possible, both with ant-associated Hemiptera and with the ants themselves. Presumably at the same time, or shortly thereafter, lycaenid caterpillars that could appease ants also began to reward them with nutritious secretions in exchange for defense against parasites and predators. These interactions were facilitated by a number of adaptations, including specialized exocrine glands, an unusually thick cuticle, a retractable head, and various stridulatory organs used to communicate with ants and conspecifics (Hinton 1951; Malicky 1970; DeVries 1991a; Travassos and Pierce 2000). The great majority of lycaenid–ant interactions involve ants associating apparently mutualistically with caterpillars feeding on plants, but a smaller proportion—less than 5% of the species with described life histories—associate parasitically with ants and are aphytophagous, feeding either on Hemiptera attended by ants or on the ants themselves (Pierce et al. 2002).

In general, predatory and parasitic Lepidoptera consume organisms that cohabit the plants on which they live: ants, Hemiptera, and insect eggs. It is perhaps because of their proximity to ants and ant-attended Hemiptera that shifts to parasitism and predation have occurred so frequently in the Lycaenidae relative to other lepidopteran taxa (Pierce 1995; Pierce et al. 2002). The lycaenid subfamily Miletinae, which comprises approximately 190 species in 13 currently recognized genera, is the largest radiation of aphytophagous butterflies. The larvae of all Miletinae whose life histories have been described are predatory, parasitic, or otherwise aphytophagous, and it is expected that all species in this subfamily share this trait (Cottrell 1984; Savela 2014).

Several hypotheses have been proposed to explain the evolutionary steps leading to this unusually successful radiation and to the diversity of diets within it (Table 1). Balduf (1938) speculated that the Miletinae arose from the lichen-feeding subfamily Liptenainae. Cottrell (1984) argued that lycaenid larvae and Hemiptera both prefer to eat and occasionally live on nitrogen-rich plant parts, and that a shift to carnivory may have followed. Maschwitz et al. (1988) hypothesized that feeding on ant-attended aphids was the ancestral pattern in the subfamily that gave rise to other derived strategies. A phylogenetic estimate of the Miletinae and determination of their sister taxon facilitates an evaluation of these hypotheses.

The biogeographic history of the Miletinae appears to be complex. Of the 13 genera, four genera are wholly Asian (*Allotinus*, *Lontalius*, *Miletus*, and *Taraka*), two are primarily distributed in Southeast Asia, but have species that inhabit the Australian region including New Guinea (*Liphyra* and *Logania*), five are entirely Afrotropical (*Aslauga*, *Euliphyra*, *Lachnocnema*, *Megalopalpus*, and *Thestor*), *Spalgis* species are found in all three of these regions (Oriental, Australian, and Afrotropical),

Table 1. **Distribution, dietary, and ant associate information for all miletine species with known life histories, including distribution information for species that are included in this study but have no life-history information.**

			Associated ants				Geographic distribution									Larval food											
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphidae	Pemphigidae	Hormaphididae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
Miletinae: Lachnocnemini																											
Lachnocnema bibulus	X			2	2	*Camponotus acvapimensis, C. maculatus, Crematogaster* sp., *Pheidole* sp.		X													X	X	X	X		X		1–6
L. brimo	X			1		*Camponotus* sp.		X													X		X					6, 7
L. divergens	X	X						X																				8
L. durbani	X							X												X	X							5, 7
L. magna								X													X			X				7
Thestor basutus				1		*Anoplolepis custodiens*		X												X			X	X				5, 9
T. brachycerus				1		*A. custodiens*		X																				5
T. braunsi	X			1		*A. custodiens*		X																				9
T. dicksoni				1		*A. custodiens*		X																X				5, 7
T. holmesi				1		*A. custodiens*		X																X				7, 9
T. kaplani	X			1		*A. custodiens*		X												[X]			[X]	[X]				9
T. montanus	X			1		*A. custodiens*		X																				9
T. overbergensis	X			1		*A. custodiens*		X																				9
T. penningtoni	X			1		*A. custodiens*		X																[X]				9
T. protumnus				1		*A. custodiens*		X												X				X				5, 10
T. rileyi				1		*A. custodiens*		X																X				7
T. rooibergensis	X			1		*A. custodiens*		X																				9
T. stepheni	X			1		*A. custodiens*		X																				9
T. swanepoeli	X			1		*A. custodiens*		X																				9
T. yildizae	X			1		*A. custodiens*		X																X				11
Miletinae: Liphyrini																												
Aslauga aura	X	X		[1]	[2]			X												[X]	[X]							
A. lamborni				1	2	*Oecophylla longinoda, Crematogaster buchneri, Pheidole rotundata*		X												X	X								1, 4

(*Continued*)

Table 1. Continued.

			Associated ants				Geographic distribution									Larval food												
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphididae	Pemphigidae	Hormaphidiidae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
A. latifurca								X												X	X							7, 12, 13
A. orientalis	X				[1]			X												X								12
A. purpurescens								X												X	X							4, 12, 13
A. vininga					1	*C. buchneri*		X												X								1, 7
Euliphyra hewitsoni	X			1		*O. longinoda*		X																X				8
E. leucyania				1		*O. longinoda*		X																X	X			8, 14
E. mirifica				1		*O. longinoda*		X																X	X			14, 15
Liphyra brassolis	X			1		*Oecophylla smaragdina*			X	X	X	X			X									X				16–18
L. grandis				1		*O. smaragdina*								X										X				19
Miletinae: Miletini																												
Allotinus apries	X				1	*Myrmicaria arachnoides*					X	X								X				X				20
A. borneensis	X	X									X																	21
A. corbeti	X	X									X	X																21
A. davidis	X			1	1	*Anoplolepis gracilipes*, *Crematogaster difformis*					X					X													21, 22
A. drumila	X	X							X	X																		21
A. fallax	X			1		*A. gracilipes*					X	X										X						21, 23
A. horsfieldi	X										X					X												7, 21, 24
A. leogoron	X	X									X																	21
A. major	X			1		*A. gracilipes*							X								X							21, 25
A. nicholsi	X	X									X																	21
A. nivalis	X	X									X	X																21
A. portunus	X	X									X																	21
A. punctatus	X	X										X																21
A. samarensis	X	X										X	X															21

(*Continued*)

Table 1. Continued.

			Associated ants				Geographic distribution									Larval food													
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphididae	Pemphigidae	Hormaphididae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
A. sarrastes	X	X									X	X																21
A. strigatus	X	X									X																	21
A. substrigosus	X		1		1	*Technomyrmex* sp., *Crematogaster* sp.				X	X	X						X		X	X							20–22
A. subviolaceus	X			1		*A. gracilipes*				X	X	X									X							20, 21
A. unicolor	X			1		*A. gracilipes*				X	X	X	X			X		X		X	X		X			X		7, 21–23, 26
Logania distanti	X	X							X	X	X	X												[X]				21
L. hampsoni			2			*Anonychomyrma nitidiceps*, *Technomyrmex albipes*								X											X				21, 27
L. malayica	X				2	*Rhoptromyrmex wroughtonii*					X	X				X		X		X	X		X	X		X	X	20, 21, 28
						Leptothorax sp.																						
L. marmorata	X		1			*Dolichoderus* sp.					X	X									X					X	X	21, 28
L. regina	X	X	[2]								X																	21
Lontalius eltus	X	X									X																	21
Megalopalpus zymna	X			1	1	*Pheidole aurivillii, C. acvapimensis*		X													X	X						1, 7, 29, 30
Miletus ancon	X									X	X									X								7, 21
M. biggsii	X		1			*Dolichoderus* sp.				X	X					X		X		X								20, 22, 23, 31
M. boisduvali			1			*Dolichoderus bituberculatus*					X		X	X				X		X								31, 32
M. cellarius	X	X									X																	31
M. chinensis	X		2	1		*Dolichoderus* sp., *D. bituberculatus*, *Polyrhachis dives*				X	X					X													31, 33, 34
M. drucei	X	X									X	X																31
M. gaesa	X	X									X																	31
M. gallus	X	X									X																	31
M. gopara	X	X									X																	31
M. heracleion	X	X									X																	31
M. leos	X	X											X	X														31

(*Continued*)

Table 1. Continued.

			Associated ants				Geographic distribution									Larval food													
Taxon	Sampled	Immatures unknown	Dolichoderinae	Formicinae	Myrmicinae	Species	Eastern North America	Africa	India/the Himalayas	China/Indo-China	Sundaland	Philippines	Sulawesi/lesser Sundas	Maluku and New Guinea	Australia	Aphidae	Pemphigidae	Hormaphidae	Greenideidae	Coccidae/Pseudococcidae	Membracidae	Cicadellidae	Psyllidae	Ant brood/trophallaxis	Insect prey in ant nests	Hemipteran honeydew	Extrafloral nectar	References
M. mallus	X		1			*Dolichoderus* sp.				X						X			X									23, 31
M. nymphis	X									X	X									X								6, 31
M. symethus	X		1			*Dolichoderus* sp.					X	X								X								31, 35
Miletinae: Spalgini																												
Feniseca tarquinius	X			3	1	*Camponotus* spp., *Formica* spp., *Lasius pallitarsus, Myrmica* spp.	X										X											36, 37
Spalgis epius	X			2	1	*A. gracilipes, O. smaragdina, Crematogaster* sp.			X	X	X	X	X	X						X									29, 38, 39
Spalgis lemolea	X			2	1	*Anoplolepis* sp., *O. longinoda, Crematogaster* sp.		X												X									1, 29
Spalgis substrigata																				X								40
Miletinae: Tarakini																												
Taraka hamada	X			1		*Formica japonica*			X	X	X						X	X								X		29, 34, 39, 41
Taraka mahanetra	X	X								X	X																	42, 43

Sampled = species that were included in the present phylogenetic study; Dolichoderine, Formicinae, Myrmicinae = number of species from each of these subfamilies that have been recorded in association with a particular miletine species. Occasionally, life-history traits were inferred from closely related species because information about a sampled species was unavailable. Symbols indicating these inferred life-history traits are surrounded by square brackets []. Where necessary, ant names have been updated to reflect current taxonomy.

References: 1 = Lamborn et al. (1914); 2 = Cripps and Jackson (1940); 3 = Farquharson (1922); 4 = van Someren (1974); 5 = Clark and Dickson (1971); 6 = Fiedler (1991); 7 = Robinson et al. (2009); 8 = Larsen (200)5; 9 = Heath and Claassens (2003); 10 = Migdoll (1988); 11 = Heath and Claassens (2000); 12 = Cottrell (1981); 13 = Jackson (1937); 14 = Dejean and Beugnon (1996); 15 = Hinton (1951); 16 = Cottrell (1987); 17 = Johnson and Valentine (1986); 18 = Dodd (1902); 19 = Parsons (1991); 20 = Maschwitz et al. (1988); 21 = Eliot (1986); 22 = Maschwitz et al. (1985); 23 = Lohman and Samarita (2009); 24 = Bingham (1907); 25 = Kitching (1987); 26 = Fiedler and Maschwitz (1989); 27 = Parsons (2000); 28 = Fiedler (1993); 29 = Cottrell (1984); 30 = Ackery (1990); 31 = Eliot (1961); 32 = Roepke (1919); 33 = Kershaw (1905); 34 = Bascombe et al. (1999); 35 = Eliot (1980); 36 = Mathew et al. (2008); 37 = Lohman et al. (2006); 38 = Venkatesha et al. (2004); 39 = Hsu (2006); 40 = Smith (1914); 41 = Banno (1990); 42 = Corbet et al. (1994); 43 = Ek-Amnuay (2006).

and the monotypic genus *Feniseca* is strictly Nearctic (Eliot 1973, 1986). The distribution of various genera and higher taxa within Miletinae implies that lineages have dispersed between Africa and Asia repeatedly; however, the number and directionality of dispersal events are unclear.

Phylogenetic patterns of association between the three interacting taxa—butterflies, ants and Hemiptera—may likewise be complex. Related phytophagous butterfly caterpillars tend to feed on related plants (Ehrlich and Raven 1964; Janz et al. 2006) in part because adaptations to the chemical defenses of particular plant lineages restrict the dietary choices of herbivorous insects (e.g., Berenbaum 1995; Futuyma and Agrawal 2009). Predacious insects (not including more specialized parasites and parasitoids) tend not to be dietary specialists, and individual miletine species have been recorded feeding on a variety of different hemipteran taxa. For example, three miletine species in three different genera have been reported eating members of all four superfamilies of Hemiptera (Table 1). However, some species are notably selective in their prey choice, including *Feniseca tarquinius*, which specializes on Woolly Alder Aphids, *Paraprociphilus tessellatus* (Mathew et al. 2008).

The species of Hemiptera eaten by miletine caterpillars have several important similarities. They are usually soft bodied, restricted to their host plants by limited mobility, and are typically attended by ants. Thus, once miletine caterpillars began to consume Hemiptera, they presumably could easily switch to eating any kind of Hemiptera. It is also possible that because attendant ants defend Hemiptera against predators, selection has favored caterpillars that are able to fool ants semiochemically to elude detection by their prey, as has been demonstrated in the species *F. tarquinius* (Youngsteadt and DeVries 2005; Lohman et al. 2006).

Some taxonomic associations between miletine butterfly larvae and ants are apparent. *Miletus* caterpillars, for example, have always been found in association Hemiptera attended by *Dolichoderus* ants, and *Liphyra* and *Euliphyra* feed exclusively on the immatures of *Oecophylla* ants. However, it is unclear whether these taxonomic patterns translate into a relatively small number of transitions to novel ant associations with the family, or whether ant associations are more evolutionarily labile. Unlike mutualistic interactions between lycaenid larvae and ants, miletine larvae do not interact directly with the ants attending their hemipteran prey. Although mutualistically myrmecophilous lycaenids entrain the defensive assistance of ants with nutritious rewards offered from specialized glands, the caterpillars of miletine species universally lack a dorsal nectary organ for provisioning nutritious secretions, and only a few retain tentacle organs (Cottrell 1984). Nevertheless, they all retain the single celled "pore cupola organs" thought to be critical for ant appeasement (Cottrell 1984), suggesting that adaptation for some kind of association with ants may still be present in this group.

The species of ants associated with miletine caterpillars are in the largest and most common subfamilies. They share characteristics common to many "agricultural" ants that associate closely with other Lycaenidae (Pierce and Elgar 1985; Eastwood and Fraser 1999; Fiedler 2001). They tend to be dietary generalists; spend much of their time above ground, frequently in tree canopies and sometimes nesting in trees; and possess large, polydomous colonies with impressive mass recruitment systems of defense (Hölldobler and Wilson 1990). The workers are typically opportunistic foragers, and representatives of each subfamily have been recorded attending many different species of Hemiptera. Because aphytophagous lycaenids rely predominantly on Hemiptera or on Hemiptera-associated ants for their sustenance, it seems likely that associating with dominant, ecologically "apparent" ants with large colonies may be important for maintaining parasitic relationships over long periods of time.

We therefore hypothesize that there will be strong phylogenetic associations between Miletinae and their hemipteran hosts. Despite the fact that the larvae of Miletinae do not possess a dorsal nectary organ to reward attendant ants, we also speculate that a relationship with ants may nevertheless persist as the "ghost of ant association past." Miletinae are likely to have evolved from a lycaenid lineage that associated with ants mutualistically, and behavioral or other adaptations for maintaining these interspecific interactions may have been retained because of at least two main selective advantages that they conferred: to enable miletines to avoid detection and attack by ants, which normally defend their hemipteran mutualists, and to facilitate ovipositing females in the location of suitable host prey, because ant attendance typically makes associated Hemiptera easier to find.

In this study, we reconstruct the phylogeny of the Miletinae (Lepidoptera: Lycaenidae) using 90 specimens comprising 68 exemplar ingroup taxa and 22 taxa representing a taxonomically broad sample of all possible outgroup lineages. We use this phylogeny to examine the evolution of aphytophagy, shifts in diet breadth and preferences, and ant associations with hemipteran prey. In addition, we examine the biogeographic history of the group and discuss the causes and effects of dramatic dietary shifts between different trophic levels.

Methods

SPECIMEN COLLECTION AND TAXON SAMPLING

Wings were removed from wild-caught specimens and stored in paper envelopes as vouchers; bodies were immediately transferred into 100% ethanol and ultimately stored at −80°C. All specimens and their genomic DNA are deposited in the DNA and Tissues Collection of the Museum of Comparative Zoology at

Harvard University in Cambridge, Massachusetts. The specimens sequenced for this study include 63 species from all 13 currently recognized genera (Table S1). The two large genera, *Allotinus* and *Miletus*, were sampled most extensively and enabled us to evaluate the monophyly of Eliot's subgeneric designations (1986; Corbet et al. 1994). Our ingroup sample includes representatives of approximately two-thirds of the species for which life histories have been documented (Table 1), and one-third of all valid Miletinae species that have been described (Bridges 1988). Representatives of all putative miletine sister groups were included as outgroups (Lipteninae, Poritiinae, Aphnaeinae) and the tree was rooted with two specimens from the subfamily Curetinae.

DNA EXTRACTION, SEQUENCING, AND ALIGNMENT

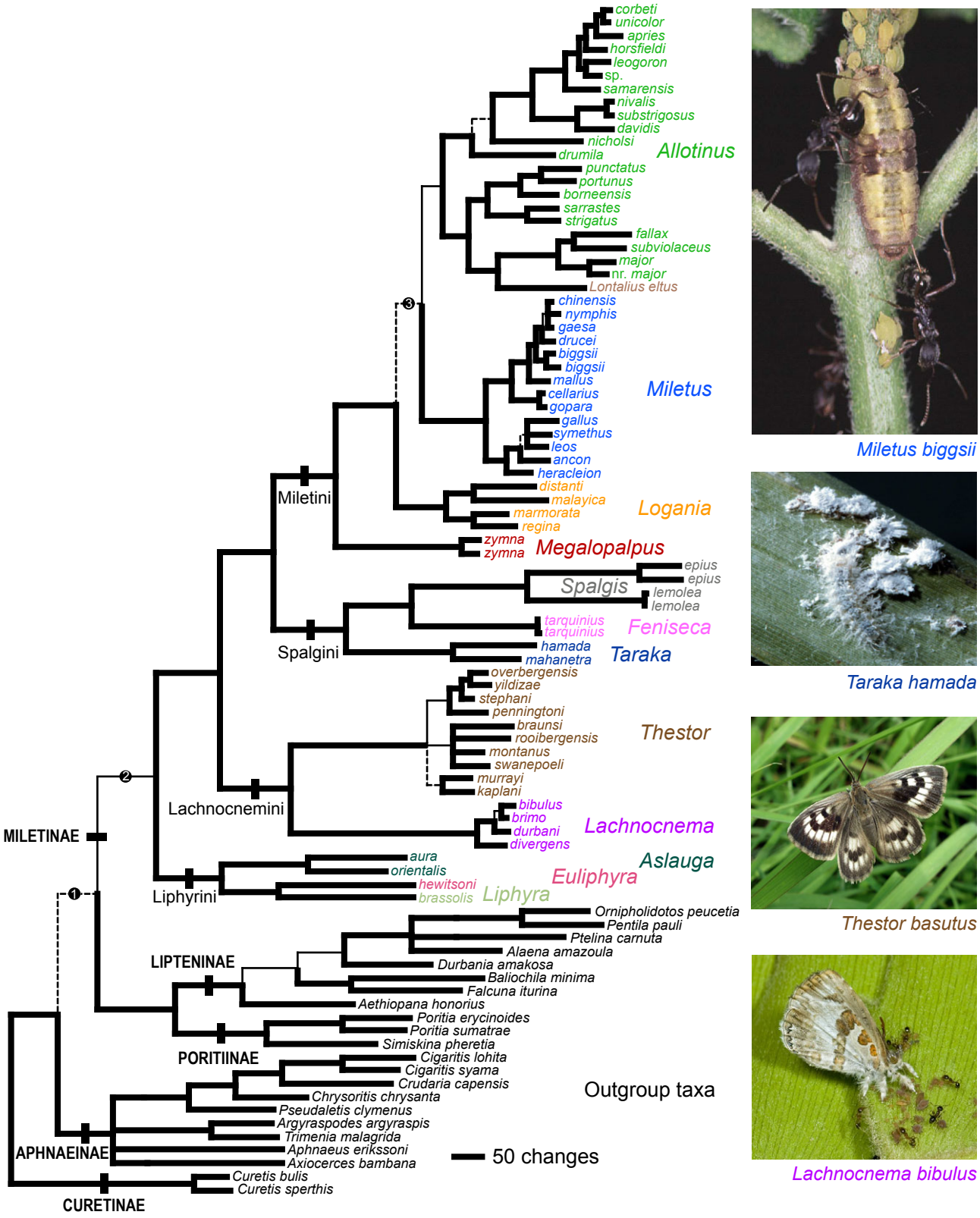
Genomic DNA was extracted from three legs or a small piece of abdominal tissue using a DNeasy Tissue Kit (Qiagen, Inc., qiagen.com). Seven markers comprising 4915 bp were amplified using complementary primer pairs (Table S2): mitochondrial *cytochrome c oxidase* I (1197 bp, COI); nuclear rDNA 28S (580 unambiguously aligned bp out of about 820 bp sequenced); and the five nuclear, protein-coding markers: *elongation factor* 1α (1065 bp, EF1α), *wingless* (402 bp, wg), *histone 3* (327 bp, H3), *carbamoylphosphate synthase* (747 bp, CAD), and *glyceraldehyde-3-phosphate dehydrogenase* (597 bp, G3PD). All PCRs comprised 16.65 μl ultra pure water, 1 μl 25 mM $MgCl_2$, 2.5 μl 10X PCR buffer, 1 μl 10 mg/mL bovine serum albumin, 0.25 μl 100 mM dNTPs, 0. 2μl 5 U/μl Taq polymerase (Qiagen, Inc., qiagen.com), and 1.2 μl of each primer (10 mM) for a total volume of 25 μl. The reactions were run with a touchdown cycling profile. Typical reaction conditions were: 2 min at 94°C followed by 20 cycles of 50 sec at 94°C, 40 sec at 48°C (decreasing by 0.5°C per cycle), and 80 sec at 70°C followed by 20 similar cycles with the annealing temperature constant at 50°C and ending with a final annealing step of 73°C for 5 min. The only exception was histone 3, in which the third phase of each cycle (the extension phase) was decreased to 60 sec. PCR products were purified by incubating samples at 37°C for 35 min with *Escherichia coli* enzyme exonuclease I and Antarctic phosphatase (EXO-AP), and subsequently raising the temperature to 80°C for 20 min to deactivate the enzymes. Cycle sequencing was done using BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kits, and sequencing was performed on Applied Biosystems 3100 or 3470 automated sequencers. The resulting electropherograms were assembled and edited in Sequencher 4.2 (Gene Codes Corp., genecodes.com). All markers were aligned using MAFFT 5 (Katoh et al. 2005) and concatenated with MacClade 4.06 (Maddison and Maddison 2003). Several portions of 28S could not be aligned unambiguously, and about 240 bp were excised from the alignment in MacClade and not used in the analyses, resulting in a total of about 580 bp of 28S sequence. Although 28S rDNA is present in multiple copies in most genomes, these copies generally evolve synchronously via concerted evolution (Hillis and Dixon 1991). This was not the case in the genus *Thestor*; different copies of 28S were amplified when using different primer sets for several individuals. The marker 28S could only be amplified in four of ten *Thestor* species using the S3660-A335 primer pair, and only these sequences are included in our dataset. GenBank numbers for all sequences are provided in Table S1 and the DNA sequence alignment is provided as online Supporting Information.

PHYLOGENETIC ANALYSES

Maximum likelihood, Bayesian, and maximum parsimony methods were used to infer the phylogeny of Miletinae. Maximum likelihood (ML) trees were inferred for individual genes and the full dataset using GARLI 0.951 (Zwickl 2006). The GTR+I+G model of sequence evolution was selected by Modeltest 3.7 (Posada and Crandall 1998) for each gene and the concatenated dataset using the Akaike information criterion (AIC). All model parameters were estimated from the data. Confidence in the most likely tree based on all genes was assessed with 1000 bootstrap replicates performed in GARLI. Each replicate automatically terminated after the search algorithm progressed 10,000 generations without improving the tree topology by a log likelihood of 0.01 or better. A majority-rule consensus tree was calculated with PAUP* 4.0b10 (Swofford 2002).

Bayesian phylogenetic analyses were performed with MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003). The data were partitioned by gene, using the GTR+I+G model for each gene. The substitution rates, character state frequencies, gamma shape parameters, and proportions of invariant sites were unlinked among each of the seven partitions. An analysis of 10 million generations consisted of two independent runs of four chains each with the heating temperature (temp) constrained to 0.2. Trees were sampled every 100 generations, resulting in 100,001 trees. The first 500 trees (0.5%) were discarded before a majority rule consensus tree and posterior probability branch support values were calculated from the remaining trees. Changes in the posterior probabilities of up to 20 splits were plotted over the generations of the analysis with the computer program "Are We There Yet?" (Nylander et al. 2008) to assess whether the chains had converged by the end of the analysis. The phylogenetic tree presented in Figure 1 is archived on treebase.org (submission 17026).

PAUP* was used to find the most parsimonious tree using the concatenated dataset of all seven markers. One hundred random addition searches were conducted using heuristic search methods with the TBR branch swapping, collapsing zero-length branches, and weighting all characters equally. Branch support was assessed using 1000 bootstrap replicates. To visualize genetic distances among and within genera, uncorrected pairwise (p-) distances were calculated between all ingroup samples using PAUP*,



Miletus biggsii

Taraka hamada

Thestor basutus

Lachnocnema bibulus

Figure 1. Maximum likelihood phylogenetic estimate of 63 Miletinae and 22 outgroup species based on seven markers totaling 4915 bp. Thick branches indicate maximum likelihood bootstrap support (ML) ≥ 90, Bayesian posterior probability (B) ≥ 0.99, and parsimony bootstrap (MP) ≥ 80. Thin branches indicate ML ≥ 70, B ≥ 0.90, and MP ≥ 55; dashed branches denote ML ≥ 50, B < 0.50, and MP < 50. Species names at each terminal node are color-coded by genus. Support for numbered nodes: ❶ ML = 62, B = 0.98, and MP < 50; ❷ ML = 78, B = 1, and MP = 64; ❸ ML = 52, B < 50, and MP < 50. Taxonomy follows Eliot (1973).

and frequency distributions of p-distances between congeneric species were plotted along with the distribution of p-distances between species from different genera. These histograms were then redrawn after all *Allotinus* intrageneric distances were removed.

BIOGEOGRAPHIC INFERENCE AND DIVERGENCE TIME ESTIMATION

Ancestral areas and ingroup dispersal events were inferred using the programs DIVA version 1.2 (Ronquist 1997) and LAGRANGE (Ree and Smith 2008) in conjunction with a time-calibrated version of the most likely tree inferred with the software BEAST 1.7.5 (Sanderson 2003). All possible area combinations were permitted, and the biogeographic model used to infer historical patterns was constant through time. Genera were coded as belonging to one of three biogeographic regions: Afrotropical, Oriental, or Nearctic. The only exception was *Spalgis*, which is the only genus found in more than one of these regions: *Spalgis epius* was coded as Oriental and *S. lemolea* as African. Note that *Liphyra*, *Logania*, and *S. epius* extend from the Oriental into the Australian region (Parsons 2000), but, for simplicity, we classified these as Oriental, as their distributions are centered there.

Unfortunately, there are no fossilized Miletinae to aid in tree calibration. Thus, we used normally distributed tmrca (time to the most recent common ancestor) priors including maximum and minimum ages within the 95% HPD (highest posterior density) distribution on five nodes as calibrated by Heikkilä et al. (2012; Table S3), and a Bayesian phylogeny was inferred with BEAST. Heikkilä et al. (2012) used BEAST to calibrate divergence times using four fossils within the Nymphalidae and Pieridae to calibrate their Bayesian tree to estimate dates for the origin and diversification of the seven butterfly families. The uncorrelated relaxed clock (Drummond et al. 2006) and a constant population size under a coalescent model were set as priors. Two independent chains were run for 50 million generations each, sampling values every 5000 steps. A conservative burn-in of 500,000 generations was applied after checking Markov chain Monte Carlo (MCMC) convergence in Tracer version 1.5 (Rambaut and Drummond 2007).

CHARACTER MAPPING AND ANCESTRAL CHARACTER STATE RECONSTRUCTION

Larval food source(s), taxa of associated Hemiptera, and taxa of associated ants (where known; Table 1) were mapped onto the best ML tree. Ant associations were coded by ant subfamily. If a given lycaenid species was known to associate with ant species from two or more subfamilies, then it was scored as being associated with multiple subfamilies. If a particular lycaenid species was known to associate with ants from multiple genera within a single ant subfamily, then it was scored in the same way as a lycaenid that associated with only one species of ant within that subfamily. Field observations of miletine caterpillars are few, and we were not always able to sample species with available life-history information. In a few cases, we inferred life-history information from close relatives; these life-history inferences are marked in Table 1. Hemiptera associations were coded by both hemipteran family and superfamily.

Phylogenetic distributions of life-history characters (feeding habit, ant association, and taxon of hemipteran associate) were examined using MacClade 4.06, and ancestral states were reconstructed using the ACCTRAN algorithm. The directionality of character shifts between different feeding habits and between associating with ants from different subfamilies was confirmed with a reversible jump MCMC analysis implemented in the BayesMultiState module of BayesTraits 1.1 (Pagel et al. 2004). A regular MCMC analysis was done first, and the ratedev parameter was varied until the acceptance rate was around 30% to estimate priors to be used in the reverse jump analysis. The ratedev parameter used in the final reverse jump analysis was 55 with the prior set to exp(0, 50). The number of rates allowed was 6. The analysis was run for 5,050,000 generations, the first 50,000 of which were discarded as burnin. The BayesTraits results were then compiled in Microsoft Excel and graphed in JMP 7.0 (SAS 2007).

The permutation tail probability test (PTP) implemented in PAUP* was used to determine whether characters had a random distribution on the phylogeny or whether they tended to cluster. More specifically, this method was used to determine whether the diets of Miletinae are phylogenetically conserved by addressing the question: Do related miletine species feed on prey from the same hemipteran superfamily? A clustered character distribution would suggest that transitions between character states (e.g., feeding on Coccidoidea vs. Aphioidea) requires some degree of evolutionary adaptation and is not labile. Each analysis was replicated 1000 times using all ingroup taxa. For easier interpretation, the inferred character states were then mapped onto a penalized likelihood rate-smoothed version of the most likely tree.

Results

PHYLOGENETIC RELATIONSHIPS OF THE MILETINAE

The best ML tree ($-\ln = 71009.4$), the Bayesian consensus tree, and the most parsimonious tree were all highly congruent. Low branch support and slight differences in topology are indicated with thin or dashed lines in Figure 1. None of the gene trees recovered the topology of the full dataset or had strong support at deep nodes (Fig. S2), underscoring the importance of our

multigene dataset. Most nodes were strongly supported; more than half of all nodes had Bayesian posterior probabilities of 1, and all but three nodes had posterior probabilities >0.90. Notably, however, the sister-group relationship between the Miletinae and Lipteninae + Poritiinae was poorly supported, as was the sister-group relationship of *Allotinus* and *Miletus*. The inclusion of monotypic *Lontalius eltus* within the genus *Allotinus* is strongly supported, indicating that this species should hereafter be known as *Allotinus eltus* (Eliot). All other genera are monophyletic. *Allotinus* comprises two strongly supported clades that are united by weak parsimony bootstrap (62) and ML (78) support. The Miletinae as a whole are monophyletic with strong Bayesian posterior probability (1) and weaker ML (78) and parsimony (64) support. Taxonomic and systematic implications of this work are discussed in Appendix S1.

Comparison of inter- and intrageneric pairwise distances revealed that genetic distances between species are similar in magnitude, and overlap with distances between other species in different genera within the Miletinae. For example, there was, in some cases, a greater genetic distance between two species of *Allotinus* than between species in two different miletine genera. This was also true of species of *Spalgis*, which is the only genus with species in both Afrotropical and Oriental regions.

BIOGEOGRAPHIC INFERENCE AND DIVERGENCE TIME ESTIMATION

Ancestral area reconstruction analyses were performed with LAGRANGE and DIVA. DIVA analyses frequently suggested several possible biogeographic scenarios. However, in all instances, at least one of the optimal solutions from the DIVA analysis was consistent with the most optimal solution in LAGRANGE. Both methods agreed that the extant distribution of Miletinae taxa required five dispersal events (four between the Afrotropical and Oriental region and one from the Oriental into the Nearctic). According to LAGRANGE, the Miletinae originated in Africa with a relative probability greater than 0.98 and then several lineages dispersed to the Orient, where they radiated (Fig. 2). DIVA analysis suggested that an Afrotropical or an Afrotropical + Oriental origin were equally likely.

Our analyses place the origin of the Miletinae near the start of the Eocene, 57 (95% CI, 49–64) million years ago. The *Liphyra* lineage dispersed out of Africa 32 (23–41) million years ago, *Taraka* and its relatives dispersed out of Africa about 38 (32–45) million years ago, and the monophyletic Oriental Miletini (*Allotinus*, *Miletus*, and *Logania*) clade migrated out of Africa 30 (24–36) million years ago. The *Spalgis lemolea* lineage dispersed back into Africa from Asia approximately 18 (13–23) million years ago, and the *Feniseca* lineage dispersed into North America from Asia 32 (26–39) million years ago (Fig. 2).

CHARACTER MAPPING AND ANCESTRAL CHARACTER STATE RECONSTRUCTION

The immature stages of many miletine species are unknown, and ancestral state reconstruction was therefore used to infer probable life-history characteristics (food type and taxon of ant associate) of species for which information is lacking (Fig. 3). Miletine larvae have been recorded feeding on at least seven different types of food: Hemiptera, ant brood, ant trophallaxis, detritus, insect prey in ant nests, hemipteran honeydew, and extrafloral nectar (Table 1). Most feed on Hemiptera, although many supplement this with additional food types. When we mapped all food types onto the best ML tree, the distribution of these seven feeding habits was not significantly clustered ($P = 1.0$); grouping detritus with insect prey and ant trophallaxis with ant brood resulted in five feeding categories that were significant ($P = 0.017$). However, when we grouped feeding behaviors into three categories: "Hemiptera only," "Hemiptera + Other," and "Other only" (where "Other" refers to ant brood, ant trophallaxis, hemipteran honeydew, extrafloral nectar and/or detritus), then the phylogenetic association was highly significant ($P = 0.005$) and several trends in feeding behavior became evident.

The prey taxon on which miletine larvae feed is phylogenetically conserved: the larvae of closely related butterfly species tend to feed on related prey taxa. A significant correlation exists between Miletinae phylogeny and the families of Hemiptera consumed ($P = 0.046$), as well as between Miletinae and hemipteran superfamily ($P = 0.018$; Fig. 3). Moreover, a strong association was recovered between Miletinae phylogeny and the subfamily of ants with which they associate, either directly because the miletines consume the ants, or indirectly because the ants attend their hemipteran prey (PTP test, $P = 0.008$; Fig. 3).

Discussion

BIOGEOGRAPHY

Dispersals of miletines between geographic regions may have been driven by climatic changes. The Miletinae originated in Africa about 57 (95% CI, 50–64) million years ago near the beginning of the Eocene when global temperatures were higher and the Earth was covered by forests (Zachos et al. 2001). Even sections of Northern Africa that are currently desert were then covered by rainforest (Jacobs 2004). In the mid-Eocene, global climates and ecosystems began undergoing drastic transformations: there was significant cooling and a reduction in the prevalence of global tropical forests (Zachos et al. 2001). This led to mass global extinctions from around 40 to 33 million years ago (Jacobs 2004). It was during this period, specifically between 30 and 38 (23–45) million years ago, that three clades of the Miletinae dispersed out of Africa. It is possible that they shifted their ranges to cope with the transformation of their previous ranges from warm and humid

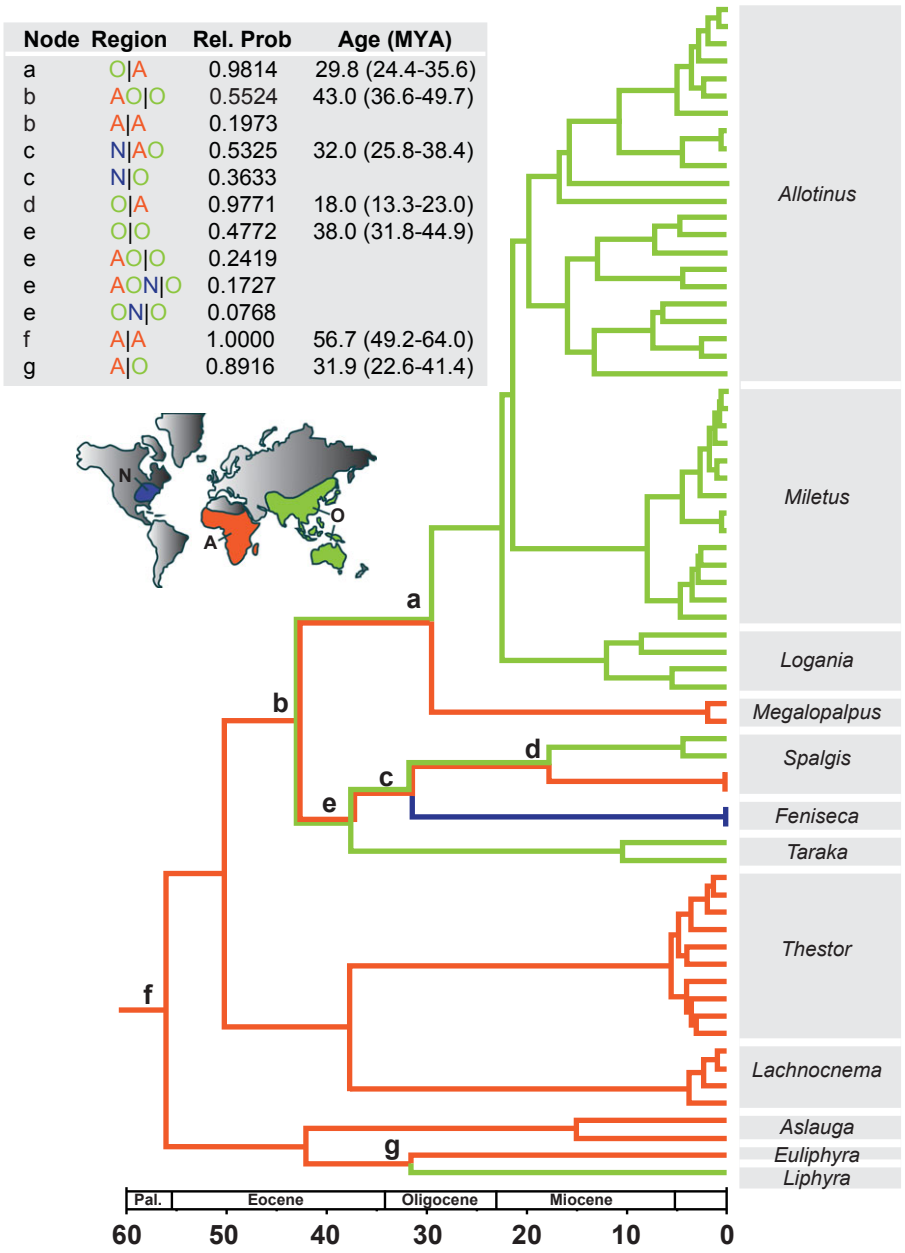


Figure 2. **Geographic distribution of the Miletinae inferred with LAGRANGE (Ree et al. 2005; Ree and Smith 2008). Nodes labeled a–g refer to probabilities of daughter lineages inheriting particular ranges as described in the inset table. These are presented as probabilities that [upper branch inherited given range] | [lower branch inherited given range]. At node d, for example, there is a 98% probability that the ancestor of *Spalgis epius* (upper branch) inherited an Oriental distribution and the ancestor of *S. lemolea* (lower branch) inherited an African distribution from their common ancestor. Colored branches indicate the inferred ancestral areas inherited by each lineage.**

climates to the relatively harsh and dry ones of the late Eocene and early Oligocene.

Following this initial dispersal of Miletinae out of Africa, ancestors of *Spalgis lemolea* appear to have dispersed back into Africa approximately 18 (13–23) million years ago. This corresponds closely to the time when the Tethys Sea closed and the Gomphotherium land bridge formed (Harzhauser et al. 2007). The closure of the Tethys Sea was associated with another global cooling event. The cooler temperature reduced the atmosphere's ability to absorb moisture and as a consequence most of Africa's forests became grasslands (Zachos et al. 2001). After the collision of the Afro-Arabian plates with Eurasia, there was a significant faunal exchange between Africa and Eurasia. The best-known example of this is the dispersal of proboscideans that migrated from Africa to Eurasia around 19–18.5 million years ago (Harzhauser et al. 2007). The land bridge became a

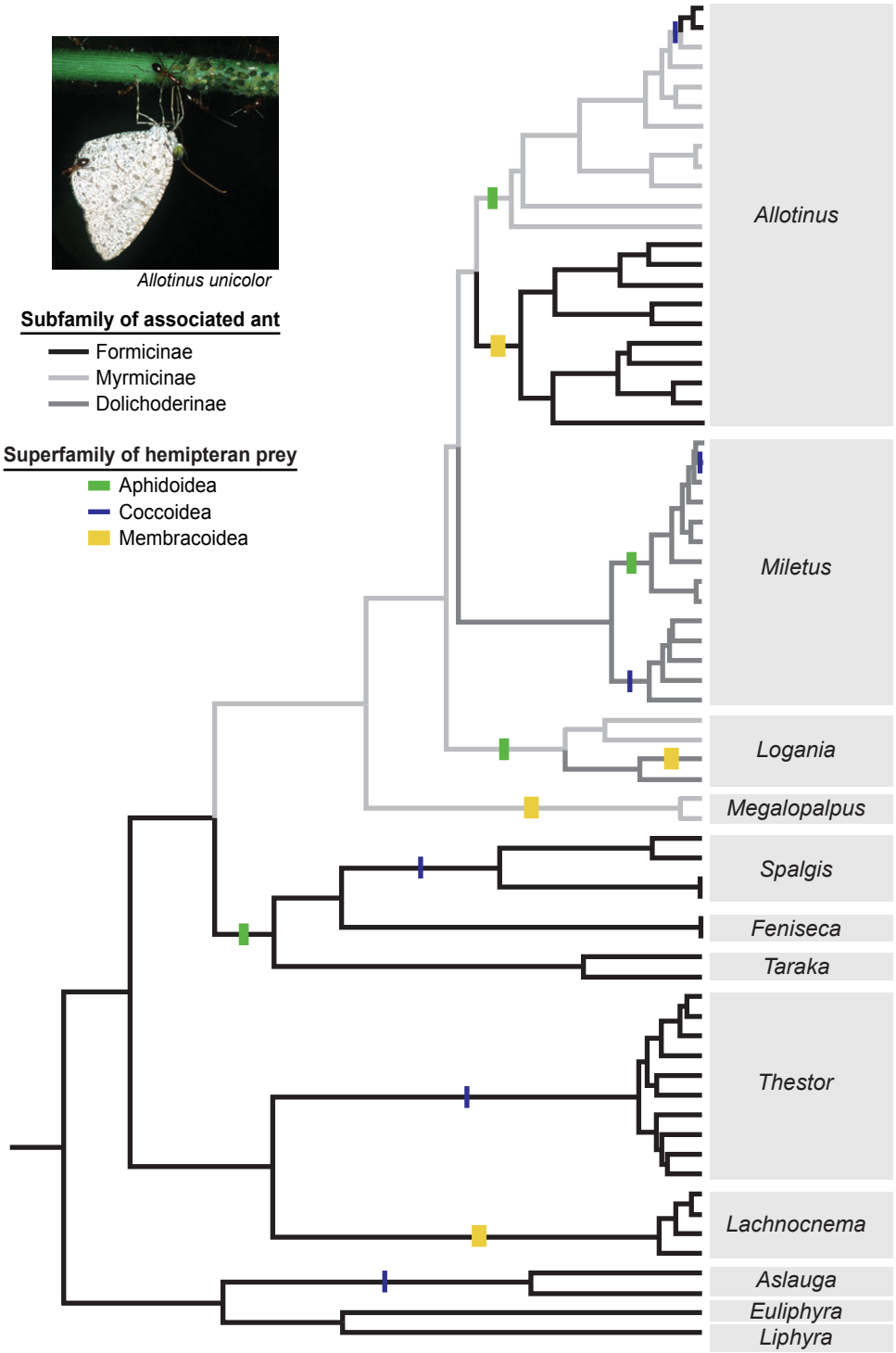


Figure 3. Mapping of ancestral character state reconstructions to estimate the most likely ant subfamily and Hemiptera superfamily associated with the larvae of miletine ancestors. Ant-associated subfamilies were treated as a multistate unordered character; state transitions were equally weighted. Inferred character states of the ant-associated subfamilies are indicated by branch colors and the colored bar to the right of the phylogram indicates the reconstructed Hemiptera superfamily association.

corridor not only for land mammal movement, but also for insect dispersal, including *Junonia* butterflies 19 million years ago (Kodandaramaiah and Wahlberg 2007).

EVOLUTION OF APHYTOPHAGY

Although the Miletinae are monophyletic (Fig. 1), we cannot determine from the phylogeny whether lichen-feeding evolved before the evolution of aphytophagy, as hypothesized by Balduf (1938). The lichen-feeding Lipteninae are sister to the Poritiinae, and this clade is sister to the Miletinae. All liptenines with known life histories feed on lichens, but the few poritiine life histories that have been described suggest that they consume the leaves of vascular plants. Maschwitz et al. (1988) hypothesized that aphid-feeding (superfamily Aphidoidea) was the ancestral condition in the Miletinae, and that other feeding strategies evolved subsequently. The two earliest diverging lineages, Liphyrini and Lachnocnemini, are predominantly coccid feeders (superfamily Coccidoidea; Fig. 3), and it thus seems likely that this was the ancestral condition. The exact role of ant association in the route to Hemiptera feeding is difficult to determine because either simple tolerance (myrmecoxeny) or mutualism (myrmeocophily, involving the production of nutritious food rewards) may have preceded or evolved concurrently with the entomophagous feeding strategy observed today. Perhaps significantly, reversal from a predatory or parasitic lifestyle to feeding once again on plants is not observed in the Miletinae. It is unclear why this transition has been unidirectional, but physiological changes associated with a shift from consuming nitrogen-poor tissue in plants to nitrogen-rich tissue in animals may be difficult to reverse.

A simplified categorization of feeding habits mapped onto a rate-smoothed version of the most likely tree (Fig. 3) reveals that most Miletinae feed on Hemiptera, including the earliest diverging ("basal") lineages. Species that include ant brood, ant trophallaxis, insect prey within ants nests, extrafloral nectar, or hemipteran honeydew in their diets seem to be randomly distributed on the phylogeny, suggesting that these habits are evolutionarily labile and/or facultative.

PHYLOGENETIC CONSERVATISM OF SPECIES INTERACTIONS

Interactions between Miletinae and ants were strongly evolutionarily conserved with few transitions among ant subfamilies. Relationships with Hemiptera were also conserved, but this pattern was less distinct. Character reconstruction suggests that feeding on Hemiptera was the ancestral state for the Miletinae. The collective prey species eaten by miletines include species from 10 families in four superfamiles that do not form a monophyletic group (von Dohlen and Moran 1995; Lee et al. 2009). When these hemipteran taxa are mapped onto the miletine tree, a relationship between Miletinae phylogeny and prey taxon is recovered (PTP test, $P = 0.018$; Fig. 3): the prey eaten by the larvae of a miletine species are usually related to the prey eaten by a related miletine species.

Although closely related miletines may associate with a variety of ant species and genera, the subfamilies to which those ants belong are highly constrained across the phylogeny: sister miletines are more likely to associate with ants from the same subfamily (PTP test, $P = 0.008$; Fig. 3). This is true both for ants that are consumed directly by caterpillars (such as *Liphyra* and *Thestor* species), or those that attend a caterpillar's prey species. The reason for this is not immediately clear, but could result from historical contingency, or possibly because adaptation to associating with a novel ant subfamily requires adaptation to a new suite of pheromones or recognition chemicals characteristic of that subfamily (Morgan 2008). Nevertheless, transitions between ant subfamilies have occurred, and the frequency of these shifts between ant subfamilies appears to be constant (BayesTraits reversible jump analysis; number of parameters $= 1.2 \pm 0.4$). To date, Miletinae caterpillars have only been found associating with ants in the subfamilies Formicinae, Myrmicinae, and Dolichoderinae. Switching between certain ant subfamilies are equally probable: Myrmicinae to Dolichoderinae; Myrmicinae to Formicinae; Dolichoderinae to Myrmicinae; Dolichoderinae to Formicinae; and Formicinae to Myrmicinae. However, switching from Formicinae to Dolichoderinae is not likely to have occurred.

The phylogenetic conservatism in ant association appears counterintuitive because most miletine caterpillars have a direct interaction with Hemiptera (most species eat them), but only an indirect association with ants that attend the Hemiptera. However, the significant conservatism of ant subfamily in associations recorded across the miletine family, in concert with a number of other behavioral observations, suggests that ants are a more important participant in these interactions than previously appreciated. Maschwitz et al. (1988) observed that fluttering female butterflies seem to be able to detect ants, even when they are not readily visible (e.g., behind a leaf). Moreover, miletine adults may be able to detect aggregations of the appropriate attending ant species even when there are no Hemiptera present (e.g., at sap flows; Fiedler and Maschwitz 1989; Lohman and Samarita 2009). Aggregations of Hemiptera are liable to be ephemeral in space and time, and an ovipositing female butterfly is challenged with locating sites with adequate numbers of hemipteran prey where she can deposit her eggs. Many aphids are known to produce alarm pheromones under duress (Nault and Montgomery 1979), but these chemical signals are not produced without provocation, and would therefore be an unreliable cue for ovipositing females to use in locating hemipteran colonies. Ants, however, produce a wide variety of different semiochemicals in different contexts (Vander Meer et al. 1998), and some compounds, such as trail pheromones, are

released with sufficient frequency to be a reliable cue indicating the presence of ants. Because of their semiochemical and visual apparency, ants might thus act as homing beacons for ovipositing female miletine butterflies that can smell and see the ants and use them to find Hemiptera that are frequently in the company of ants. Because different ant taxa communicate with different suites of chemical compounds, particular miletine species or genera may be adapted to detect some but not all ant taxa. These specialized ant associations suggest the possibility of a "ghost of ant association past" through which associations with specific ants may have facilitated the evolution and/or maintenance of a parasitic or predatory lifestyle.

Ants normally protect the Hemiptera that they attend, and fend off predatory insects, but miletine caterpillars can employ chemical camouflage to avoid detection by semiochemically resembling their surroundings. Ants use a mixture of cuticular hydrocarbons (CHCs) in their epicuticular wax as recognition cues (Van Zweden and d'Ettorre 2010). These identifying labels can be species-, caste-, or colony-specific, and ants appear to use CHCs to identify other insects as well. Lohman et al. (2006) showed that larvae of the North American miletine, *Feniseca tarquinius*, resemble the CHC profile of their woolly aphid prey rather than that of the attendant ants, thereby avoiding attack by the ants and detection by their prey. These and additional studies suggest that CHCs are used as recognition cues by ants to discriminate trophobionts from invaders and that predacious, hemipteran-feeding miletine larvae are able to produce or acquire a sufficient subset of semiochemicals to dupe ants (and possibly also aphids) to avoid detection (Lohman 2004; Youngsteadt and DeVries 2005; Lohman et al. 2006).

A large proportion of lycaenids that are recognized as endangered species have predatory or parasitic lifestyles. This demographic and phylogenetic pattern is similar in other insects. Weigmann et al. (1993) observed that insect lineages with highly specialized carnivorous and parasitic lifestyles tend to be less diverse than their relatives with more general feeding behaviors, and suggested that one explanation for the evolutionary success of phytophagous compared to aphytophagous insects is simply the trophic pyramid, with its differences in the quantity and availability of resources at each level. Aphytophagy has arisen multiple times within the Lepidoptera, but has rarely resulted in radiation (Pierce 1995). Miletinae are a conspicuous exception to this general pattern, and it seems that their limited success as aphytophagous Lepidoptera is likely to be due to their adaptations for finding prey. The ability to use ants as cues in locating ephemeral hemipteran prey may have been especially important. Both ant and hemipteran resources must have been sufficiently abundant, predictable, and ecologically apparent to have enabled the persistence and diversification of this unusual group.

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DATA ARCHIVING

Data Archival Location: Specimens and extracted genomic DNA are vouchered in the Museum of Comparative Zoology DNA and Tissues Collection. DNA sequences are archived in GenBank (accession numbers AF279218, AF279223, DQ018905-DQ018909, DQ018938-DQ018942, KF787151, KF787166, KF787171, KF787173, KF787184, KF787191, KF787203, KF787206, KF787209, KF787214, KF787220-KF787222, KF787237, KF787245, KF787273, KF787285, KF787288, KF787291, KF787296, KF787409-KF787411, KF787426, KF787432, KF787434, KF787449, KF787462, KF787473, KF787476, KF787479, KF787484, KF787490-KF787492, KF787507, KF787513, KF787515, KF787530, KF787543, KF787555, KF787558, KF787561, KF787566, KP215665-KP215816, KP215818-KP215828, and KP215906-KP216198), the DNA sequence alignment is provided as online Supporting Information, and phylogenetic trees are archived on treebase.org (submission 17026).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix S1. Taxonomy and Systematics of the Miletinae (Lepidoptera: Lycaenidae).
Figure S1. Maximum likelihood phylogenies based on single genes do not recover the topology of the tree based on the full data set.
Table S1. Specimen information and GenBank accession numbers for species included in this study.
Table S2. Primers used in this study.
Table S3. Calibration points.