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PHYCITINAE PHYLOGENY BASED ON TWO GENES, WITH IMPLICATIONS FOR MORPHOLOGICAL TRAIT EVOLUTION AND HEINRICH'S TRIBAL CLASSIFICATION (LEPIDOPTERA: PYRALIDAE)

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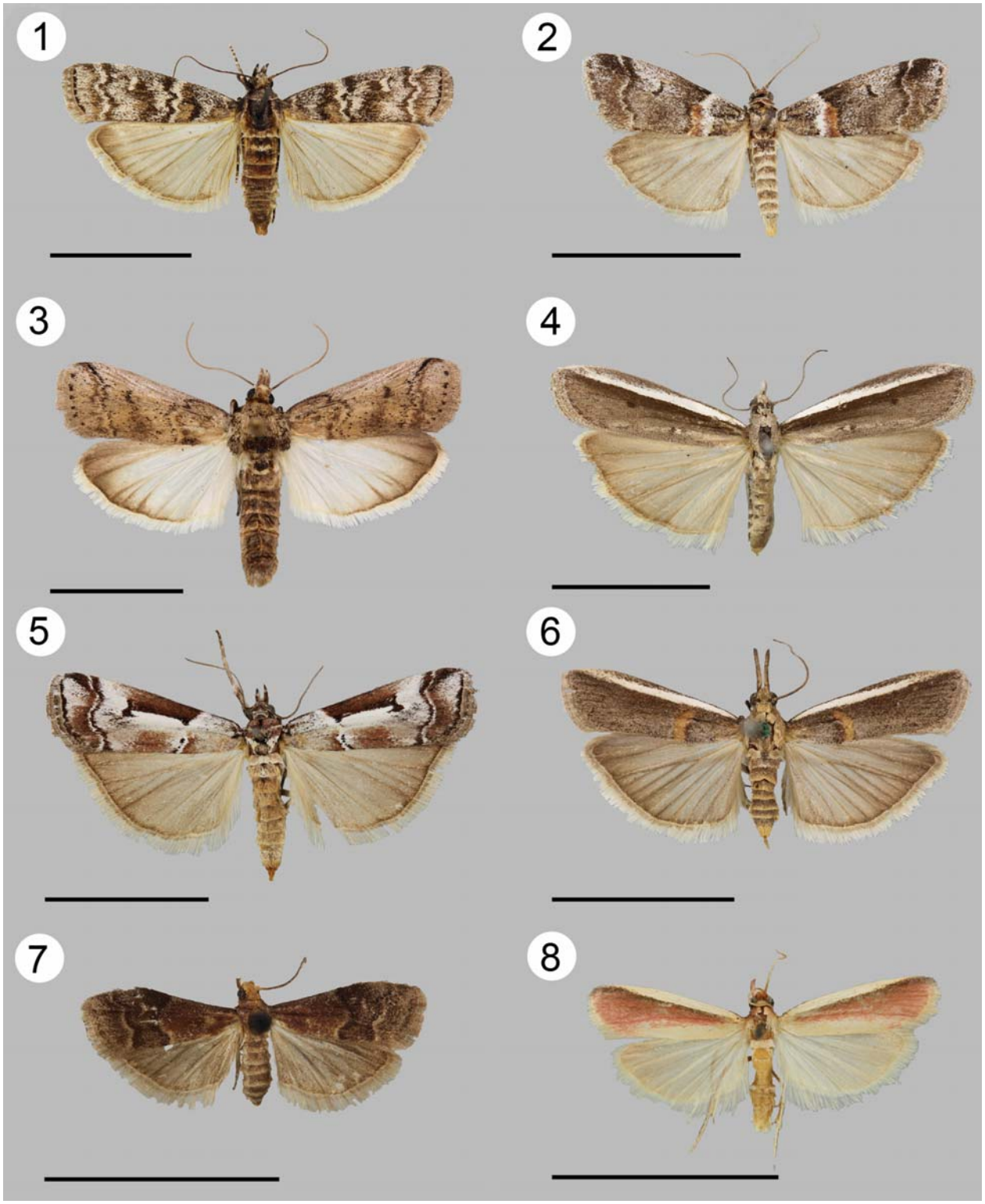
ABSTRACT. Phycitinae are a morphologically and ecologically diverse group of Lepidoptera with numerous pest species. Establishment of a stable classification system for the subfamily has been challenging due to complex evolutionary patterns in adult morphological structures and difficult species identifications. Currently, Carl Heinrich's dual system, published in 1956, serves as the main reference point for tribal classification, but its inherent ambiguity and geographic constraints have meant that no system is widely accepted for the subfamily. Here we present the first molecular phylogeny of the Phycitinae, based on two independent gene regions (cytochrome oxidase I and elongation factor 1 alpha). We use this molecular phylogeny to examine evolutionary trends in four key morphological structures (hind wing venation, male antennae, male maxillary palpi and male abdomen 8 modifications for pheromone dispersion) and determine their phylogenetic utility. Our results indicate two major groups of genera in the Phycitinae and that morphological traits appear to correspond to these relationships, although some homoplasy exists.

Additional key words: Lepidoptera, androconia, wing venation, mitochondrial DNA, phylogeny

Phycitine moths are notable for their diverse ecological and economic impacts on a global scale. Phyctinae comprise the most species-rich subfamily of the Pyralidae, with over 3400 species and about 600 genera (Figs 1–8). They occur in habitats such as temperate forests (e.g., *Dioryctria* Zeller) (Du et al. 2005, Roe et al. 2006), lowland tropical forests (e.g., *Hypsipyla grandella* (Zeller)) (Heinrich 1956), deserts (e.g., *Cactoblastis* Ragonot, cactus-feeders) (Heinrich 1939, Mann 1969), and grasslands (e.g., *Pima* Hulst) (Neunzig 2003), and are an important component of most terrestrial ecosystems (e.g. Common 1990). The cactus moth, *Cactoblastis cactorum* (Berg) may be the best known phycitine (e.g. Common 1990). Introduced to Australia, South Africa and other regions to control introduced *Opuntia* cacti, this species has been hailed as one of the great examples of successful biological control (Dodd 1940, Moran & Zimmermann 1984, Zimmermann et al. 2000, Walton 2005). However, subsequent accidental introduction of the species into southern USA has demonstrated how easily a biological control agent can become a serious pest (Zimmermann et al. 2000, Mahr 2001, Hight et al. 2002, Solis et al. 2004, Pemberton and Liu 2007, Simonsen et al. 2008). A few other important phycitine pests include: *Dioryctria* on conifers, *Hypsipyla robusta* (Moore) on Red Cedar (Common 1990); *Etiella* Zeller species on legumes including soybeans (Segarra-Carmona & Barbosa 1990, Common 1990); *Acrobasis tricolorella* Grote in prune and cherry orchards (Neunzig 1986); *Zophodia*



FIGS. 1–8. Exemplars of Phycitinae genera included in this study. **1.** *Dioryctria abietella*; **2.** *Acrobasis tricolorella*; **3.** *Cactoblastis cactorum;* **4.** *Pima albocostalialis;* **5.** *Ambesa laetella;* **6.** *Etiella zinckenella;* **7.** *Eulogia ochrifrontella;* **8.** *Peoria approximella*. Scale bars = 10mm

grosssulariella (Hübner) on *Ribes* sp. (Neunzig 1997); and *Homoeosoma electellum* (Hulst) on sunflowers (Neunzig 1997). Their economic importance has led to deeper study of some taxa as model species. For example, Indian meal moths (*Plodia interpunctella* (Hübner)) and Mediterranean Flour Moth (*Ephestia kuehniella* Zeller) are cosmopolitan stored product pests as well as useful lepidopteran models for gamma radiation effects, gut physiology, and wing pattern development (Robinson 1971; Leibenguth 1989; Srinivasan et al. 2006; Shim et al. 2009; Mansour 2010).

While the phylogenetic relationships among families and subfamilies of Pyraloidea have been convincingly addressed recently (Regier et al. 2012), there has been little consensus about relationships within Phycitinae (Minet 1985, 1982; Neunzig 1986, 1990, 1997, 2003; Solis & Mitter 1992; Horak 1997, 2003; Simonsen 2008). Most taxonomic treatments rely on the foundational work of Heinrich (1956) who revised all known New World species and provided informal groupings of genera based on wing venation and male genitalia morphology. This work remains the primary systematic treatment of the subfamily.

Despite Heinrich's extensive 25-year study of New World phycitine moths, he was unable to establish a tribal classification system based on 'natural' (i.e. monophyletic) groups. To satisfy the expectation that his proposed classification should both serve a taxonomic purpose (accurately define, delineate and name categories that "represent objective realities in nature") and a practical purpose ("to arrange these categories in an order that permits their ready identification"), he adopted a dual classification (Heinrich 1956, p. vi). The first classification was based on genitalia characters and considered more natural; the second was based on wing venation, considered wholly artificial, and "proposed merely for key purposes".

The resultant classification at the genus level was presented as a complex 2-dimensional diagram, where genera were arranged on the basis of wing venation into vertical columns representing three main groups, with Groups 1 and 2 each containing several subdivisions (Heinrich 1956, p vii; redrawn here as Fig. 9—note that we do not attempt to test all the groups in the table, or explore most of the characters they are based on. The table is reproduced here to provide readability and access to Heinrich's revision). Horizontal lines joined genera or groups of genera where genitalia characters were thought to indicate natural relationships. The generic groupings presented in the diagram were, in his view, "divisions of convenience" (pp. vi, Heinrich 1956) rather than definitions of taxonomic groups. However, evolutionary relationships, and thus natural classifications, should be reflected in all character systems (Schuh and Brower 2009). Therefore, rather than setting up two different classifications based on supposedly independent character systems and subsequently amalgamating them, most systematists now try to combine all character sets into one unified classification. Furthermore, the characters Heinrich applied in his wing venation system were themselves arbitrary (and thus liable to produce an artificial system): the veins were simply numbered sequentially without any consequent nomenclature. Therefore, no homology of veins can confidently be proposed. That is, FW vein 7 in one group of genera may or may not be homologous with FW vein 7 in another group—this is not helped by the fact that subdivisions of venation groups 1 and 2 are highly complex and likely comprise several characters each (characters that could have phylogenetically contradicting signals). Finally, while some characters may be more prone to homoplasy (and thus of less value to higher-level taxonomy), it cannot be decided a priori which system is more artificial. The taxonomic value of characters can only be determined based on a well-supported phylogenetic hypothesis. Nevertheless, Heinrich's work has proved to be extremely useful, because it provided the first (and still most useful) overview of phycitine classification and morphology in any major geographic region.

More recent attempts at generic classification have resulted in as many as 3 tribes and 14 subtribes (Agenjo 1958) and as few as one tribe with two subtribes (Roesler 1973) within the Phycitinae. Attempts to reconstruct phylogenetic relationships within the Phycitinae are hampered by the sheer number of taxa (both genera and species), and the diversity and rampant homoplasy of adult morphological structures (Heinrich 1956; Roesler 1986; Horak 1997; Simonsen 2008).

Here, we provide the first molecular phylogeny of phycitine genera, using two markers, mitochondrial cytochrome oxidase I (~1500 bp) and nuclear elongation factor 1 alpha (~500 bp). Like Heinrich's work, taxon sampling was biased toward the Americas, and we focused on comparing our results to the groupings proposed by Heinrich (1956). We also test two other hypotheses: 1), the monophyly of true cactus-feeding genera (Neunzig 1997; Simonsen 2008), and 2) the inclusion of "Anerastiini" within Phycitinae (Horak 1997, 2003). Finally, we examine the correspondence of our molecular phylogeny to the distributions of four well-studied morphological traits: hind wing venation, male antennae, male maxillary palpi and male abdomen 8 modifications for pheromone dispersion.

MATERIALS AND METHODS

Collection of Specimens. Adult specimens were collected from sites across North America by collectors who used a variety of sampling methods, including trapping at lights, pheromone lures and rearing (Supplementary Material Table 1). Pheromone trapping was conducted in the southeastern USA as described by Miller et al. (2010). Ingroup Phycitinae were represented by 32 genera and 45 species. Tribe Phycitini was represented by 24 species, Anerastiini by one species, and 20 species were unassigned. Two species from China (*Ceroprepes ophthalmicella* (Christoph) and *Oncocera faecella* Stephens) were provided by collaborators (Du et al. 2005). Nine outgroup species represent other subfamilies of Pyralidae and Crambidae. Identifications were performed by Roe, Scholtens, or Simonsen and voucher material was deposited at University of Alberta E. H. Strickland Museum (images at http://www.biology.ualberta.ca/facilities/strickland/Vouchers/index.html).

Molecular Methods. Total genomic DNA was extracted using a Qiagen DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) using manufacturer's instructions. The mitochondrial cytochrome c oxidase I gene (COI; ~1500 bp) was sequenced for all species (Table 1) using primers described in Roe et al. (2006). For a subset of specimens (29 ingroup, 9 outgroup; Table 1), a 534 bp fragment of elongation factor 1 alpha (EF1a, ~500 bp) was amplified and sequenced using two overlapping sets of primers: E15f (5' CGGACACGTCGACTCCGG 3') to rcM44.9 (5' CTTCATCAAATCYCTGTGTCC 3') and M44-1 (5' GCTGAGCGYGARCGTATCAC 3') to E600rc (5' TCCTTACGCTCAACATTCC 3') (Cho et al. 1995; Reed & Sperling, 1999). Protocols are described by Roe et al. (2011) and Simonsen et al. (2011). Sequences were analyzed with Sequencher v. 4.8 (Gene Codes Corp., Ann Arbor, MI) and submitted to GenBank under accession numbers KP693908-KP693998.

Sequence alignment and phylogenetic analysis. All sequences were initially aligned in Sequencher v. 4.8, followed by manual adjustments. Sequence fragment lengths were not equal and gaps were treated as missing data. Alignments of mtDNA and EF1a data sets were deposited in TreeBase (http://www.treebase.org; accession number S17014).

Maximum likelihood analyses were performed on the concatenated COI +EF1a data matrix using RaxML accessed via the CIPRES Science Gateway (Miller et al. 2009) (www.phylo.org/portal2/) using default settings. ML analysis was partitioned into first, second, and third codon positions (nt1, 2, 3) for both gene fragments, resulting in six data partitions. Clade support was assessed with 1000 bootstrap inferences and all free model parameters were assessed by RaxML (Supplementary Material Table 2). RaxML simultaneously searches ML tree-space and uses a rapid bootstrapping algorithm to complete a full ML analysis in a single run (Stamatakis et al. 2008). Partitioned Bayesian likelihood analyses were also performed with Mr Bayes via the CIPRES Science Gateway with default settings. ML analysis used six data partitions and a GTR+I+Γ model, with model parameters estimated by Mr Bayes and allowed to vary between partitions. In RaxML the model was determined within the likelihood framework of the program, and the Bayesian model chosen based on the ModelTest results from RaxML. Four MCMC chains were run for 10 million generations with the chains sampled every 1000 generations. The lnL probability plot was checked for stationarity, with the first 25% of trees discarded as burnin.

Morphological trait MP reconstruction. Four characters were selected for study based on prior morphological studies and previous classifications (Heinrich 1956; Roesler 1973; Horak1997, 2003; and Neunzig1986, 1990, 1997, 2003). Characters were defined as follows: **Character 1. Hind wing veins M_2 and M_3** : 0. separate, 1. Fused; **Character 2. Base of male antennal flagellum**: 0. Unmodified, 1. Flat sinus with sensory scales, 2. Short sinus surrounded by raised scales; **Character 3. Male maxillary palpus**: 0. Unmodified, 1. Terminal segment with a conspicuous tuft of elongate scales; **Character 4. Male abdominal segment 8.** 0. Without modified scale tufts, 1. Paired dorsal scale tufts present, 2. Paired latero-ventral scale tufts present, 3. Paired ventral scale tufts or ventral composite brushes present, 4. Unpaired ventral scale tufts. Here 'dorsal' refers to a position confined to the region of the tergite; 'latero-ventral' refers to a position in the pleural region below the dorso-ventral midline; and ventral refers to a position confined to the region of the sternite. Character 1 was chosen to test Heinrich's two main wing venation groups as mentioned above. We do not explore any of Heinrich's other wing venation characters as homology of the veins used in these characters is highly uncertain (as outlined above). Characters 2–4 were chosen based on preliminary results from a study of secondary sexual characters across phycitine genera (Simonsen unpublished). The term 'composite scale brushes' refers to the fact that these structures are made up by a number of components (see Heinrich 1956 and Simonsen & Roe 2008 for details). We do not attempt to evaluate the phylogenetic utility of

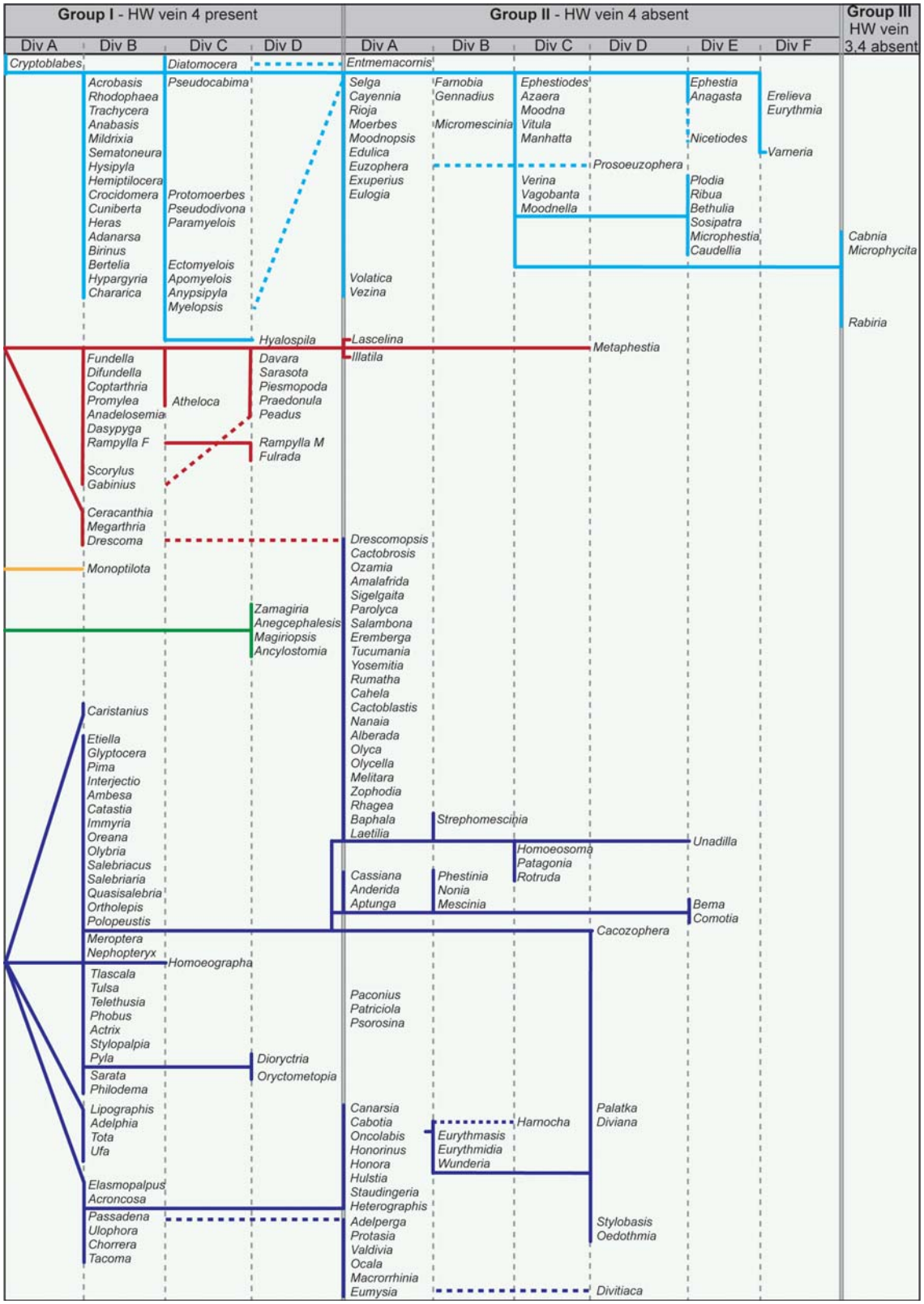


FIG. 9. A redrawn version of Heinrich's dual classification (1956, p. vii) showing the relationships of genera of American Phycitinae grouped according to genitalia and wing venation. Relationships based on genitalic characters are shown in horizontal arrangement and joined with colored lines. Wing venation groupings are presented in vertical columns.

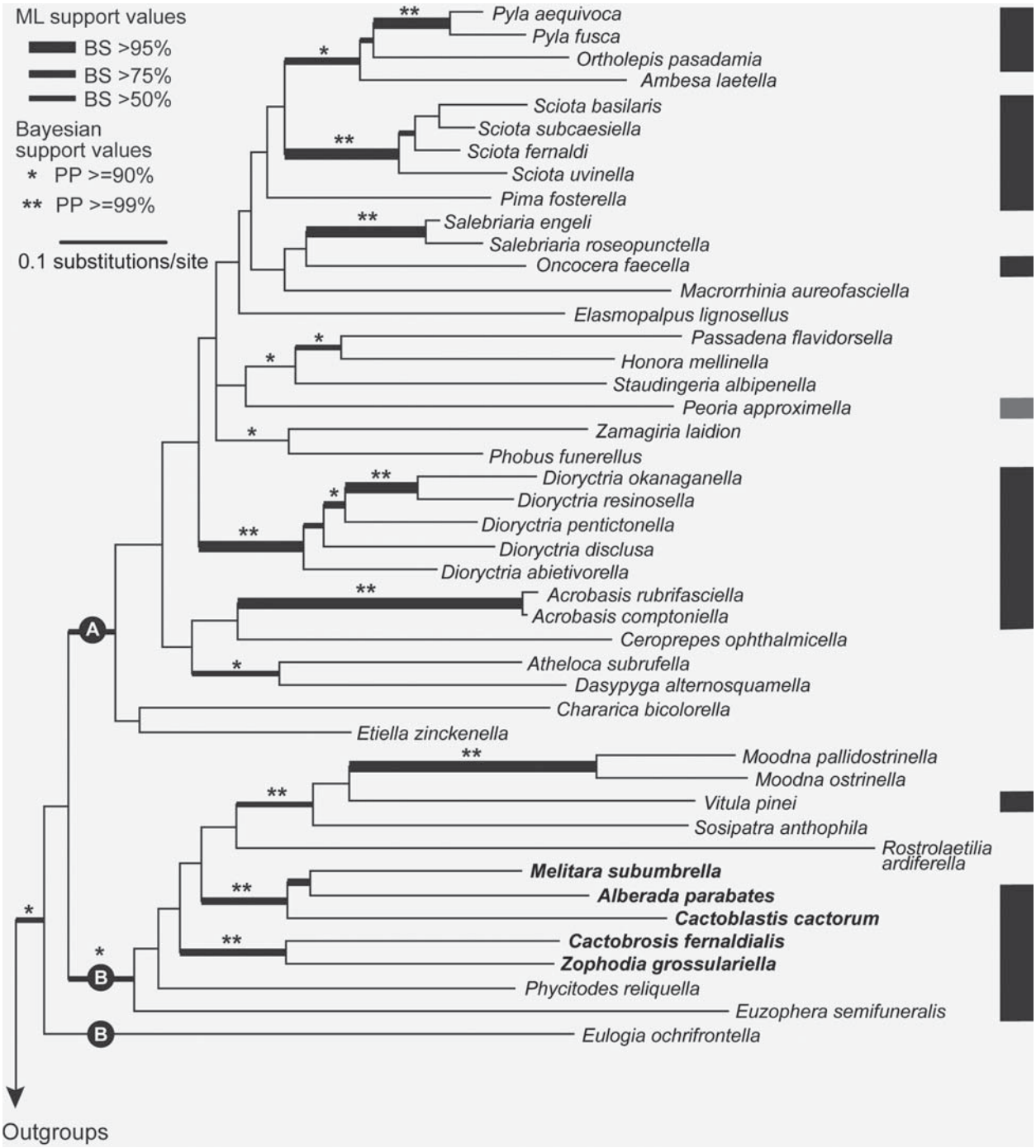


FIG. 10. Maximum likelihood (ML) tree for the concatenated COI + EF1a matrix of 34 genera in the subfamily Phycitinae. ML clade support (line thickness) is based on 1000 bootstrap runs. Bayesian support values are indicated on branches. Tribal affiliation (Zeller 1839) is shown to the right of the tree: Phycitini (black), Anerastiini (grey), and unassigned genera (unlabeled). Clade A and Grade B are discussed in the text. Taxa in bold represent cactus-feeding genera based on Simonsen (2008).

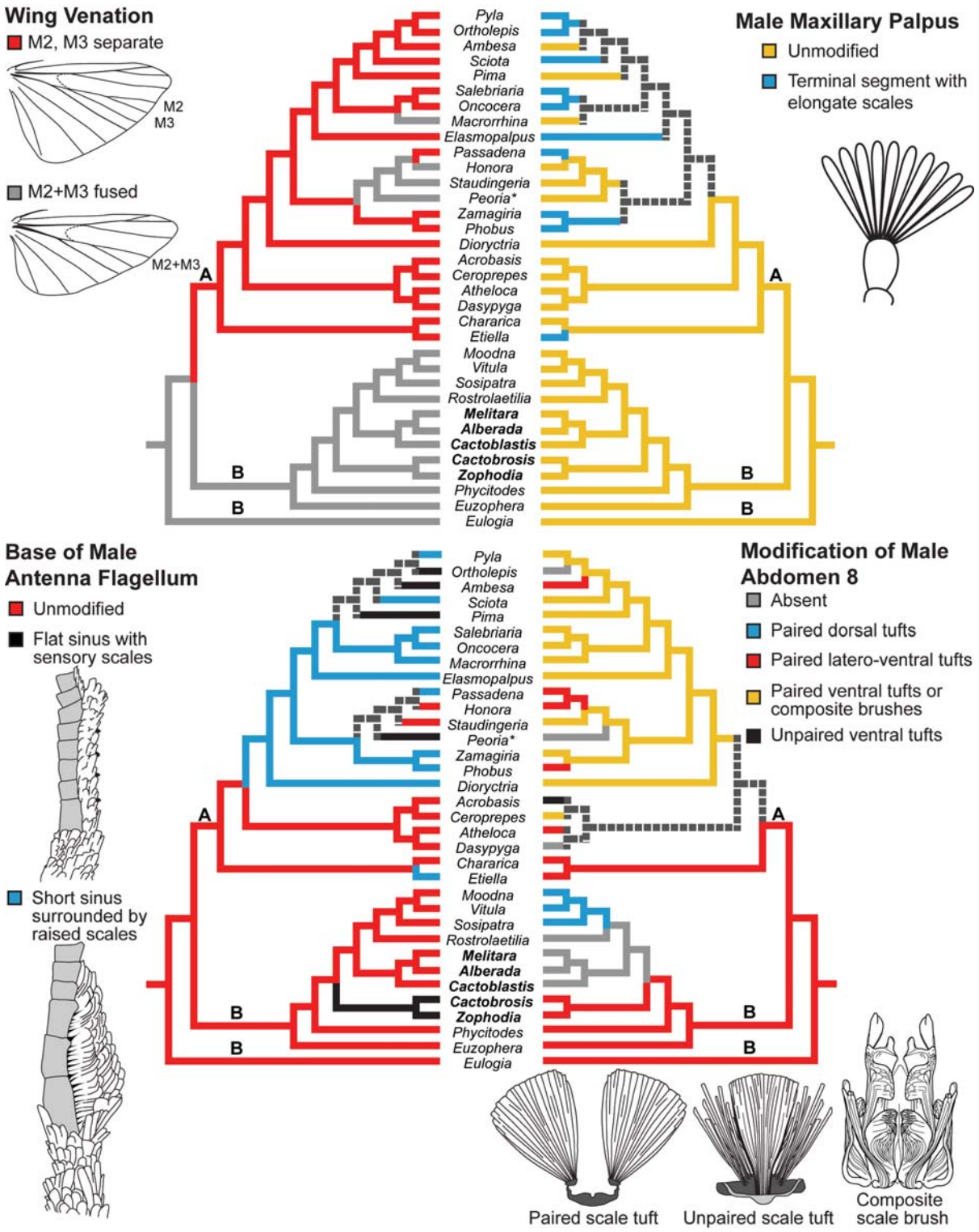


FIG. 11: Morphological trait reconstruction on the ML tree (Fig 10) for Phycitinae genera. Taxa in bold represent cactus-feeding genera. Groupings of genera are labeled for discussion in the text. Peoria is the single representative of Anerastiini.

TABLE 1. Specimen collection localities, voucher numbers, and GenBank accession numbers.

Species	Authority	Locality
Pyralidae, Phycitinae		
Acrobasis comptoniella	Hulst	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Acrobasis rubrifasciella	Packard	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Alberada parabates	(Dyar)	USA: AZ: Santa Cruz Co., Sycamore Canyon
Ambesa laetella	Grote	USA: UT: Cache Co., Cache National Forest Logan Canyon
Atheloca subrufella	(Hulst)	USA: FL: Highlands Co. Archbold Biological Station
Cactoblastis cactorum	(Berg)	USA: FL: Tallahassee
Cactobrosis fernaldialis	(Hulst)	USA: AZ. Pima Co., Box Canyon Rd.
Ceroprepes ophthalmicella	(Christoph)	CHINA: Henan Prov. Mt. Baiyun
Chararica bicolorella	(Barnes & McDunnough)	USA: AZ: Maricopa Co. Sycamore Creek nr. Phoenix
Dasypyga alternosquamella	Ragonot	USA: CA: Toulumne Co. Upper Chiquito Campground
Dioryctria abietivorella	(Grote)	USA: CA: Butte Co. Chico
Dioryctria disclusa	Heinrich	USA: MI: Cheboygan Co. Carp Creek
Dioryctria okanaganella	Mutuura, Munroe & Ross	USA: CA: Eldorado Co. Placerville
Dioryctria pentictonella	Mutuura, Munroe & Ross	USA: CA: Eldorado Co. Placerville
Dioryctria resinosella	Mutuura	USA: MI: Cheboygan Co. Carp Creek
Elasmopalpus lignosella	Zeller	USA: FL: Monroe Co. W Summerland Key, cellphone Tower
Etiella zinckenella	(Treitschke)	CAN: BC: Tranquille Ecological Reserve
Eulogia ochrifrontella	Zeller	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Euzophera semifuneralis	(Walker)	USA: MI: Cheboygan Co. Carp Creek
Honora mellinella	Grote	USA: OR: Jefferson Co. Deschutes National Forest Jack Creek
Macrorrhinia aureofasciella	Ragonot	USA: AZ: Santa Cruz Co. Madera Canyon
Melitara subumbrella	(Dyar)	CAN: Sask: Grasslands National Park
Moodna ostrinella	(Clemens)	USA: MI: Cheboygan Co. Carp Creek
Moodna pallidostrinella	Neunzig	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Oncocera faecella	Zeller	CHINA: Inner Mongolia, Mt. Manhan
Ortholepis pasadamia	(Dyar)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Passadena flavidorsella	(Ragonot)	USA: AZ: Maricopa Co. Sycamore Creek nr. Phoenix
Peoria approximella	(Walker)	USA: MI: Cheboygan Co. Wildwood Rd.

Lat.	Long.	Date	Collector	Voucher#	COI	EF1a
45.563	-84.673	13-VII-2006	B Scholtens	AR415	KP693945	KP693908
45.562	-84.679	26-VI-2006	B Scholtens	AR416	KP693946	
		20-VIII-05	TJ Simonsen	FS-b-2443	KP693947	
		5-6-VII-2007	TJ Simonsen	TJS-08-003	KP693948	KP693909
		9-10-VI-2006	TJ Simonsen	TJS-06-312	KP693949	
		Reared	SD Hight	CC-006	KP693950	KP693910
		10-VIII-2005	TJ Simonsen	TJS-05-367	KP693951	KP693911
		24-VII-2002	X Wang	Du79	KP693952	KP693912
		8-V-2007	TJ Simonsen	TJS-08-008	KP693953	KP693913
		10-VII-2007	TJ Simonsen	TJS-08-001	KP693954	
39.728	-121.837	25-VII-2000	C Rudolf	AR22	KP693955	KP693914
45.551	-84.685	31-VII-2006	B Scholtens	AR414	KP693956	KP693915
38.73	-120.799	16-VI-2001	AD Roe	AR150	KP693957	KP693916
38.73	-120.799	15-VI-2001	AD Roe	AR149	KP693958	KP693917
45.551	-84.685	31-VII-2006	B Scholtens	AR413	KP693959	KP693918
		7-VI-2006	TJ Simonsen	TJS-06-255	KP693960	KP693919
		31-V-2008	JJ Dombroskie	TJS-08-025	KP693961	
45.563	-84.673	26-VI-2006	B Scholtens	AR412	KP693962	KP693920
45.551	-84.685	31-VII-2006	B Scholtens	AR411	KP693963	KP693921
		26-VII-2007	JJ Dombroskie	TJS-08-31	KP693964	
		6-V-2007	TJ Simonsen	TJS-08-006	KP693965	
		2-VI-2006	GR Pohl	FS-b--2427	KP693966	KP693922
45.551	-84.685	31-VII-2006	B Scholtens	AR409	KP693967	
45.563	-84.673	26-VI-2006	B Scholtens	AR408	KP693968	KP693923
		8-10-VIII-2002	D Zang	Du33	KP693969	
45.563	-84.673	13-VII-2006	B Scholtens	AR407	KP693970	KP693924
		8-V-2007	TJ Simonsen	TJS-08-039	KP693971	KP693925
45.365	-84.652	2-VII-2006	B Scholtens	AR406	KP693972	KP693926

TABLE CONTINUED ON NEXT PAGE

TABLE 1. Specimen collection localities, voucher numbers, and GenBank accession numbers.(Continued from previous page)

Species	Authority	Locality
Phobus funerellus	(Dyar)	USA: CA: Toulumne Co. Upper Chiquito Campground
Phycitodes reliquella	(Dyar)	USA: MI: Cheboygan Co. Carp Creek
Pima forsterella	Hulst	CAN: AB: Jasper National Park, Maligne Canyon Hostel
Pyla fusca	(Haworth)	CAN: AB: Kootenay Plains Ecol. Res., Siffleur Falls St. Area
Pyla aequivoca	Heinrich	CAN: AB: Brown Creek Camp, 30 km NW Nordegg
Rostrolaetilia ardiferella	(Hulst)	USA: TX: El Paso Co. Franklin Mountain State Park
Salebriaria engeli	(Dyar)	USA: MI: Cheboygan Co. Wildwood Rd.
Salebriaria roseopunctella	Neunzig	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Sciota basilaris	(Zeller)	USA: MI: Cheboygan Co. Carp Creek
Sciota fernaldi	(Ragonot)	USA: ID: Teton Co. Caribou National Forest Falls Camground
Sciota subcaesiella	(Clemens)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Sciota uvinella	(Ragonot)	USA: FL: Baker Co. Osceola N.F. Fire Lookout FR202 @ Hwy 202
Sosipatra anthophila	(Dyar)	USA: TX: Brewster Co. Big Bend National Park
Staudingeria albipenella	(Hulst)	CAN: AB: Kootenay Plains Ecol. Res., Siffleur Falls St. Area
Vitula pinei	Heinrich	USA: NV: Lander Co. Toiyabe National Forest Victorine Canyon
Zamagiria laidion	(Zeller)	USA: FL: Monroe Co. No Name Key, No Name Blvd.
Zophodia grossulariella	(Hübner)	CAN: AB: Wagner Natural Area, ~30km W Edmonton
Outgroups		
Pyralidae, Pyralinae		
Aglossa costiferalis	(Walker)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Dolichomia olinalis	(Guenée)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Pyralis farinalis	(Linnaeus)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Pyralidae, Chrysauginae		
Condylolomia participialis	Grote	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Galasa nigrinodis	(Zeller)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Pyralidae, Epipaschiinae		
Pococera expandens	(Walker)	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Crambidae, Crambinae		
Crambus albellus	Clemens	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Crambidae, Scopariinae		
Scoparia biplagialis	Walker	USA: MI: Cheboygan Co. Univ. Michigan Biological Station
Crambidae, Glaphyriinae		
Dicymolomia julianalis	(Walker)	USA: MI: Cheboygan Co. Carp Creek @ Hogback Rd.

Lat.	Long.	Date	Collector	Voucher#	COI	EF1a
		10-VII-2007	TJ Simonsen	FS-b-4248	KP693973	
45.551	-84.685	15-VII-2006	B Scholtens	AR405	KP693974	KP693927
		12-VII-2007	JJ Dombroskie	TJS-08-019	KP693975	
		7-VIII-2007	JJ Dombroskie	TJS-08-017	KP693976	
52.717	-116.267	19-VII-2002	G. Anweiler	AR235	KP693977	
		17-VIII-2005	TJ Simonsen	TJS-05-236	KP693978	KP693928
45.365	-84.652	15-VII-2006	B Scholtens	AR404	KP693979	KP693929
45.562	-84.679	26-VI-2006	B Scholtens	AR403	KP693980	KP693930
45.551	-84.685	15-VII-2006	B Scholtens	AR402	KP693981	KP693931
		3-VII-2007	TJ Simonsen	TJS-08-033	KP693982	
45.562	-84.679	26-VI-2006	B Scholtens	AR401	KP693983	KP693932
		19-IX-2006	TJ Simonsen	TJS-06-227	KP693984	
		20-VIII-2005	TJ Simonsen	TJS-05-258	KP693985	
		31-V-2007	JJ Dombroskie	TJS-08-099	KP693986	
		8-VII-2008	TJ Simonsen	TJS-08-007	KP693987	KP693933
		5-VI-2006	TJ Simonsen	TJS-06-277	KP693988	KP693934
		7-V-2006	TJ Simonsen	TJS-06-52	KP693989	KP693935
45.563	-84.673	11-VII-2006	B Scholtens	AR417	KP693990	KP693936
45.562	-84.679	26-VI-2006	B Scholtens	AR418	KP693991	KP693937
45.563	-84.673	29-VI-2006	B Scholtens	AR420	KP693992	KP693938
45.563	-84.673	13-VII-2006	B Scholtens	AR423	KP693993	KP693939
45.563	-84.673	8-VII-2006	B Scholtens	AR422	KP693994	KP693940
45.564	-84.681	10-VII-2006	B Scholtens	AR421	KP693995	KP693941
45.562	-84.679	26-VI-2006	B Scholtens	AR424	KP693996	KP693942
45.562	-84.679	26-VI-2006	B Scholtens	AR432	KP693997	KP693943
45.551	-84.685	2-VI-2006	B Scholtens	AR428	KP693998	KP693944

TABLE 2. Parameters for each of six partitions in the concatenated cytochrome c oxidase 1 (COI) and elongation factor 1 alpha (EF1a) matrix. A partitioned ML analysis was conducted with RaxML on the CIPRES web portal, using GTR substitution matrix and CAT approximation for estimating rate heterogeneity. This analysis yielded a final optimized ML tree (Fig. 10) with –ln=-20476.3195.

	COI			EF1a		
	codon 1	codon 2	codon 3	codon 1	codon 2	codon 3
Base freq.						
A	0.2987	0.1801	0.4319	0.3342	0.3410	0.1398
C	0.1396	0.2309	0.05784	0.1364	0.2319	0.4170
G	0.2481	0.1603	0.01376	0.3506	0.1650	0.2489
T	0.3136	0.4288	0.4965	0.1788	0.2625	0.1943
Rate Matrix						
A-C	9.3400	0.9177	33.4836	0.00001700	0.00001700	3.7741
A-G	9.3668	1.6709	1850.3611	1.0690	0.4621	33.0491
A-T	7.5589	0.5324	34.1690	0.00001700	0.00001700	21.9160
C-G	0.7817	1.8712	644.1804	1.9336	2.8750	0.9424
C-T	304.1283	1.1718	1867.7692	28.3778	0.3805	54.5889
G-T	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
alpha	0.1803	0.02001	0.4234	0.08886	0.02001	0.7219

the individual components, but following Horak (2003) we attempt to explore whether the overall position of the structures are of phylogenetic importance. Species were coded from the literature (Heinrich 1956; Roesler 1973; Neunzig 1986, 1990, 1997, 2003; Simonsen 2008) or directly by TJS for this study based on material in the collections of NHM, London, either as observations on pinned specimens (under a stereo microscope) or from abdomen dissections macerated in 10% aqueous KOH solution (Supplementary Material Table 3). Each multistate character was coded as unordered (i.e. characters 2 and 4), and ancestral states were reconstructed by maximum-parsimony optimization onto the ML tree using MacClade v. 4.08.

RESULTS

Molecular results. The tree resulting from ML analysis (–ln=20476.3195, Fig. 10) had long terminal branches and short internodes. The topology from the Bayesian analysis was similar (tree not shown), and Bayes support values were shown on the ML tree. The subfamily Phycitinae was recovered as monophyletic (>75%, BPP>=90%) within which we designate clade A (32 species) and grade B (13 species). Tribe Anerastiini (represented by *Peoria approximella*) is nested within Clade A, rendering the Phycitini non-monophyletic. All genera with multiple representatives were recovered as monophyletic with high support (ML >95%, BPP>=99%). Relationships among genera were not highly supported, with one notable exception. All species from the true cactus-feeding group were placed in grade B, forming two well-supported clades (*Alberada* + *Cactoblastis* + *Melitara* and *Cactobrosis* + *Zophodia*) (Fig. 10).

The six data partitions based on gene and codon position (nt1,2,3) showed expected patterns of change, with most substitutions concentrated in third codon C-T transitions followed by third codon A-G transitions (Table 2) (Reed & Sperling 1999).

Morphological trait reconstruction. When the four morphological characters were mapped onto the tree, several patterns emerged, although none were without homoplasy (Fig. 11). The hind wing condition of a "fused M_2+M_3" characterized grade B, with the majority of members of clade A having M_2 and M_3 separate (18 out of 22 genera). Within clade A, fusion of M_2+M_3 occurs twice with one reversal. The modification of the male maxillary palpus is restricted to clade A, with either six independent acquisitions or a set of gain-loss-gain sequences. Of the three states observed for the base of the male antenna flagellum, only the "short sinus" (blue; Fig. 11) is restricted to clade A. The most complicated character, male abdomen 8 modifications, shows moderate correspondence with tree structure. Paired latero-ventral tufts were reconstructed as the ancestral condition for grade B. Paired ventral tufts or composite brushes are restricted to clade A, although additional states also occur within the clade (yellow, Fig. 11). Paired dorsal tufts are restricted to grade B and represent a monophyletic grouping (blue, Fig. 11). Lack of male ornamentation occurs as four independent losses.

TABLE 3: Species re-examined and morphological character matrix. The matrix was used for tracing the characters illustrated in Fig. 11, as explained in the text. **Re-examined species** indicate which species were used for scoring characters; Lit. = all characters scored from literature; **BM(NH) slide** numbers refer to slides in the Natural History Museum, London's (NMH) slide collection; **external only** = characters 1–3 scored directly, character 4 scored from literature (see: Heinrich (1956), Neunzig (1986, 1990, 1997, 2003), Roesler (1973), Simonsen (2008)); all reexamined species were obtained from the collections of NHM.

Genus	**Re-examined species**	**BM(NH)slide**	**1**	**2**	**3**	**4**
Pyla	*P. fusca* (Haworth)	Pyr22470	0	2	1	3
	P. araeneola Balogh & Wilterding	Pyr21264				
Ortholepis	*O. pasadamia* (Dyar)	external only	0	1	1	0
Ambesa	*A. laetella* Grote	external only	0	1	0	2
Sciota	*S. basilaris*,(Zeller)	external only	0	2	1	3
	S. subcaesiella (Clemens)					
Pima	*P. boisduvaliella* Guenée	external only	0	1	0	3
Salebriaria	*S. fasciata* (Dyar)	external only	0	2	1	3
Oncocera	*O. faecella* (Zeller)	Pyr22492	0	2	1	3
Macrorrhina	*M. aureofasciella* Ragonot	external only	1	2	0	3
Elasmopalpus	*E. lignosella* Zeller	external only	0	2	1	3
Passadena	Literature		0	2	1	2
Honora	*H. mellinella* Grote	external only	1	0	0	2
Staudingeria	*S. holophaceella* Rebel	external only	1	0	0	3
Peoria	*P. punctilinaella* (Hampson)	Pyr17672	1	1	0	0
Zamagiria	*Z. laidion* (Zeller)	external only	0	2	1	3
Phobus	*P. incertus* Heinrich	external only	0	2	1	2
Dioryctria	*D. abietella* (Denis & Schiff.)	Pyr22467	0	2	0	3
Acrobasis	*A. comptoniella*,Hulst	external only	0	0	0	4
	A. rubrifasciella Packard					
Ceroprepes	*C. naga* Roesler & Küppers	Pyr22491	0	0	0	3
Atheloca	*A. subrufella* (Hulst)	external only	0	0	0	2
Dasypyga	*D. alternosquamella* Ragonot	Pyr19297	0	0	0	0
Chararica	*C. hystriculella* (Hulst)	external only	0	0	0	2
Etiella	*E. zinckenella* (Treitschke)	external only	0	2	1	2
Moodna	*M. ostrinella* (Clemens)	external only	1	0	0	1
Vitula	*V. edmandsii** (Packard)	external only	1	0	0	1
Sosipatra	Literature		1	0	0	1
Rostrolaetilia	Literature		1	0	0	0
Melitara	Literature		1	0	0	0
Alberada	Literature		1	0	0	0
Cactoblastis	*C. cactorum* (Berg)	external only	1	0	0	0
Cactobrosis	*C. fernaldialis* (Hulst)	external only	1	1	0	2
Zophodia	*Z. grossulariella* (Hübner)	Pyr22489	1	1	0	2
Phycitodes	*P. mucidellum* (Ragonot)	external only	1	0	0	2
Euzophera	*E. semifuneralis* (Walker)	external only	1	0	0	2
Eulogia	*E. ochrifrontella* (Zeller)	external only	1	0	0	2

DISCUSSION

Phylogeny, comparisons to Heinrich, and classification implications. Apart from confirming the monophyly of the subfamily (which has never been seriously challenged) three interesting phylogenetic results were obtained: **1.** groupings of taxa show correspondence with the hind wing venation groups suggested by Heinrich (1956); **2.** *Peoria* (tribe Anerastiini) was recovered within clade A; and **3.** reconstruction of the larval cactus-feeding habit shows a complex pattern of either one (with several subsequent losses) or several origins of cactus-feeding.

Although Heinrich (1956) used wing venation to divide the subfamily into "practical" groups for identification purposes alone, our results indicate that this character system is phylogenetically informative. His division based on hind wing venation (M_2 and M_3 fused or separate) is supported by our molecular phylogeny and represents evolutionary groupings within Phycitinae.

Based upon adult morphology, Horak (2003) concluded that Anerastiinae was not a valid subfamily and might not even retain tribal status within Phycitinae. Our molecular results support this morphological assessment of the taxonomic validity of Anerastiini. Although its current placement was characterized by low support values, *Peoria* was deeply embedded in clade A. Our results suggest that separate subfamily status for Anerastiinae may be unwarranted, however, only a single representative was available so we were unable to fully assess its validity as a separate tribe. Additional representatives will need to be sampled.

Simonsen (2008) used adult morphology to examine the evolution of cactus-feeding among phycitine genera. The study suggested that cactus feeding arose once among phycitine genera, with one subsequent shift to a different host. Our molecular results support cactus feeding as evolving once (Grade B). The cactus feeding genera (*Melitara, Alberada, Cactoblastis, and Cactobrosis*) are paraphyletic with respect to other members of grade B. While *Zophodia* feeds on Grossulariaceae (e.g. Neunzig 1997) and is not a cactus feeder, it has been historically associated with cactus feeding genera, and in particular with *Cactobrosis* (Heinrich 1956; Roesler 1973; Neunzig 1997). This close relationship is confirmed here.

Our molecular study supports some aspects of previous studies (Heinrich 1956; Simonsen 2008), while some results are at odds with previous morphological hypotheses. For example, the enigmatic genus *Rostrolaetilia* has been associated with cactus-feeding genera by some authors based upon male genitalia (Blanchard & Knudsen 1979; Neunzig 1997), but not others (Simonsen 2008). Placement of *Rostrolaetilia* within grade B was only moderately supported in both analyses (>75% ML, >=90% Bayesian), and the genus is placed on a very large branch. This may indeed reflect the enigmatic nature of the genus, and indicates that it has an isolated position within Phycitinae. More comprehensive gene and taxon sampling are needed before the phylogenetic position of *Rostrolaetilia* should be seen as conclusive. The inclusion of *Euzophera* and close association of *Sosipatra, Vitula*, and *Moodna* was also novel. A close relationship between *Moodna, Vitula*, and *Sosipatra* was suggested by Heinrich (1956) based on male genitalia, lending credence to the phylogenetic utility of Heinrich's generic groupings. Although *Sosipatra anthophila* was not formally included in the cactus-feeding group by Heinrich (1939), this species was reported to feed on *Opuntia* cactus (Heinrich 1956), a fact overlooked by Simonsen (2008). The close relationship between *Staudingeria, Honora*, and *Passadena* (Clade A) was another example of a generic grouping predicted by Heinrich's (1956) table of relationships based upon wing venation and genitalia, lending further support to the phylogenetic utility of his "divisions of convenience". Finally, *Dioryctria*'s relatively isolated position within Clade A was in agreement with Heinrich's arrangements where the genus was isolated together with *Oryctometopia*, a genus not sampled here.

But several other relationships recovered here—especially the isolated position of *Eulogia* as sister to the remaining Phycitinae—were at odds with Heinrich's scheme in which *Eulogia* was placed in a subordinate group also comprising *Euzophera* based on both wing venation and genitalia morphology. We consider it likely that *Eulogia*'s position will change with increased taxon sampling. Another unexpected result was the close relationship between *Etiella* and *Chararica*, as well as the pair's isolated position as the sister group of the remainder of Clade A. In Heinrich's diagram, both were related to other taxa, each of which were subordinate within Clade A. Although the sister group relationship between *Dasypyga* and *Atheloca* found here was not contradicted by Heinrich's arrangements, the pair's close relationship with *Acrobasis* and *Ceroprepes* cannot be reconciled with Heinrich's results. Finally, we note that the close relationships between *Pyla, Ortholepis, Ambesa, Sciota*, and *Pima* were not in agreement with Heinrich's arrangement where all these genera except *Ambesa* and *Pima* were found in distant genitalia and/or wing venation groups. In conclusion, the disagreements between our results and Heinrich's arrangements were equally as pronounced as the similarities. We note,

however, that some of the more pronounced differences, such as the sister group relationship between *Etiella* and *Chararica* (as well as the pair's isolated position), are based in our analyses on very long branches.

Morphological Trait Reconstruction. Phycitinae morphology has generally been considered highly homoplastic and of little value in phylogenetic studies of the subfamily (e.g. Heinrich 1956; Roesler 1986). To test this widespread assumption, we mapped four structures on our phylogeny that have been central to morphological studies. Three of these character systems were easily observed without dissection (wing, antennae, palps) and are typically used for identification. The fourth, and most complex character, requires dissection of a male specimen and has been under used in the past (Horak 1997; Simonsen & Roe 2009). All character systems were consistent with the phylogeny, which contradicts assessments by earlier authors (reviewed by Simonsen 2008). In particular, wing venation and the highly complex modifications found in the 8th male abdominal segment appear to be informative (see also Horak 1997, 2003; Simonsen 2008; Simonsen & Roe 2009). The observed homoplasy does not impede the use of these traits for establishing a classification system, in spite of Heinrich's (1956) contention that his morphological groupings (particularly hind wing venation) contained no phylogenetic information. This study and others support the view that, when used carefully, morphological character systems can contribute to our understanding of phylogenetic relationships within Phycitinae (Horak 1997, 2003; Simonsen 2008, Simonsen & Roe 2009). Overall, we conclude that the major impediment to using morphology to estimate phycitine phylogeny has been the lack of rigorous analysis in the pre-cladistic era.

Our results have some implications for the higher classification of Phycitinae. Apart from the apparently isolated position of *Eulogia*, the subfamily seems to be divided into clade A and grade B. However, formally naming these is premature since some relationships are likely to change with increased gene and taxon sampling. Unfortunately, the quality of the available DNA did not allow more comprehensive gene sampling. In addition, the present study contained fewer than 10% of the named genera and was biased towards North American taxa. The use of single species to represent genera, as opposed to multiple species, also presents a challenge to our character reconstructions. Modifications of male pheromone-dispersing structures found on abdomen 8 can differ among congeners (as genera are currently defined). Loss of abdominal structures within a genus may occur (Horak 1997; TJS unpublished survey) and our study does not capture occasional polymorphic conditions within genera. Nonetheless, our study represents the first attempt to examine generic relationships of Phycitinae using DNA data and provides interesting hypotheses for future studies. We hope that it will inspire a renewed interest in these ecologically, evolutionarily, and economically important Lepidoptera.

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