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A phylogenomic analysis of lichen-feeding tiger moths uncovers evolutionary origins of host chemical sequestration

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ABSTRACT

Host species utilize a variety of defenses to deter feeding, including secondary chemicals. Some phytophagous insects have evolved tolerance to these chemical defenses, and can sequester secondary defense compounds for use against their own predators and parasitoids. While numerous studies have examined plant-insect interactions, little is known about lichen-insect interactions. Our study focused on reconstructing the evolution of lichen phenolic sequestration in the tiger moth tribe Lithosiini (Lepidoptera: Erebidae: Arctiinae), the most diverse lineage of lichen-feeding moths, with 3000 described species. We built an RNA-Seq dataset and examined the adult metabolome for the presence of lichen-derived phenolics. Using the transcriptomic dataset, we recover a well-resolved phylogeny of the Lithosiini, and determine that the metabolomes within species are more similar than those among species. Results from an initial ancestral state reconstruction suggest that the ability to sequester phenolics produced by a single chemical pathway preceded generalist sequestration of phenolics produced by multiple chemical pathways. We conclude that phenolics are consistently and selectively sequestered within Lithosiini. Furthermore, sequestration of compounds from a single chemical pathway may represent a synapomorphy of the tribe, and the ability to sequester phenolics produced by multiple pathways arose later. These findings expand on our understanding of the interactions between Lepidoptera and their lichen hosts.

1. Introduction

To defend against enemies, such as phytophagous insects and pathogens, plants and fungi have evolved a wide variety of defenses including secondary metabolites (allelochemicals (Fraenkel, 1959)). Secondary metabolites act by making the host distasteful or toxic to herbivores. However, the impact of these chemical defenses can vary depending on the feeding strategy of an organism (*e.g.*, specialist or generalist (Reudler et al., 2011)). Unique and highly toxic allelochemicals (*e.g.*, cardiac glycosides) typically act to exclude non-specialized feeders that are unable to detoxify or sequester the compound (Becerra, 1997; Krieger et al., 1971; Whittaker and Feeny, 1971). Theoretically, generalist feeders evolve broad-spectrum mechanisms of detoxification that can be applied to an array of compounds traditionally thought of as less acutely toxic or "digestibility-reducing" (Ali and Agrawal, 2012), while organisms that specialize on hosts containing highly toxic compounds can lose the ability to feed on many host plants (Whittaker and Feeny, 1971). In addition to evolving tolerance to their host(s) allelochemicals, some phytophagous insects sequester the secondary metabolites for use as a chemical defense against their own predators and parasitoids (Opitz and Müller, 2009).

With more than 157,000 described species (van Nieukerken et al., 2011), butterflies and moths (Lepidoptera) are the largest radiation of phytophagous animals on the planet (Scoble, 1992), specializing on photosynthetic (vascular, non-vascular plants) and saprophytic (macrofungi, lichens) food sources alike. Within the order, the tiger moths

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(Arctiinae), particularly the tiger moth tribe (Arctiini), are emerging as an evolutionary model for understanding the interactions of larval host chemistry, recruitment of detoxification enzymes (Hartmann, 2004; Langel and Ober, 2011), and the effect of stored host-derived compounds on predators (*e.g.*, bats (Barber and Conner, 2007; Corcoran et al., 2009; Hristov and Conner, 2005)) and parasitoids (Bernays, 2001; Singer et al., 2009; Singer et al., 2004). Larvae of some Arctiini sequester an array of secondary metabolites from their host plants including pyrrolizidine alkaloids, cardiac glycosides, and iridoid glycosides (Bowers and Stamp, 1997; Hartmann et al., 2005; Rothschild et al., 1977; Rothschild et al., 1973). These compounds may be used for self-medication, as a chemical defense, or in chemical courtship (Conner, 2009; Weller et al., 1999). Chemically defended Arctiini species advertise their chemical defenses and distastefulness using warning signals (aposematism) that are visual, acoustic, or a combination of both (Conner, 2009; Corcoran et al., 2009). Furthermore, the ability to retain sequestered pyrrolizidine alkaloids from the larva to adult is a synapomorphy of Arctiini (Weller et al., 1999; Zaspel et al., 2014). Although numerous studies have examined plant-insect interactions (*e.g.,* chemical defense and larval feeding behavior) in the subfamily Arctiinae (Bowers and Stamp, 1997; Conner, 2009; Hartmann et al., 2005; Rothschild et al., 1977; Rothschild et al., 1973; Weller et al., 1999), very little is known about the lichen-insect interactions in the lichen moths, Lithosiini.

Lithosiini are a cosmopolitan group of approximately 3000 described species best known for their larval feeding behavior, lichenivory (Rawlins, 1984), which is thought to be ubiquitous among the described species. While the tribe is considered to be the most biodiverse lineage of lichenivores, some species primarily graze on algae (McCabe, 1981). Like the more derived Arctiini, Lithosiini (Fig. 1) utilize chemical defenses against predators. Palatability studies that included lichen moth species found that the adults were distasteful to both bats and birds (Acharya and Fenton, 1992; Collins and Watson, 1983; Dowdy and Conner, 2016; Hristov and Conner, 2005), but did not include analyses to identify the source of their chemical defenses. Furthermore, unpalatable adult lichen moths appear to exhibit visual and acoustic aposematism, homologous to strategies in Arctiini (Dowdy and Conner, 2016; Simmons, 2008). For example, the lichen moth *Hypoprepia fucosa* is a visual mimic of cyanogen-sequestering fireflies (Coleoptera: Lampyridae) (*e.g.*, the adult color patterns of the moth resemble those of the fireflies; Forbes, 1960), and *H. fucosa* is chemically defended with compounds that are as unpalatable to bats as non-lichen feeding tiger moths defended with cardiac glycosides, the most potent secondary metabolite sequestered by Arctiini (Hristov and Conner, 2005). In addition, species in the lithosiine genus *Cisthene* engage in acoustic aposematism and are as unpalatable as *Pygarctia roseicapitis* (Dowdy and Conner, 2016), an unrelated, non-licheniverous arctiine that sequesters cardiac glycosides. While the chemical basis of unpalatability in adult Lithosiini has not been identified, it is hypothesized that the source of defense is phenolic compounds sequestered from the lichen host by lichen moth larvae (Hesbacher et al., 1995; Wagner et al., 2008). Thus, examining the adult metabolome not only provides a mechanism for identifying lichen derived phenolics that potentially contribute to unpalatability, it also serves as a means of assessing larval feeding behavior in the adult stage.

In two previous metabolomic studies (Hesbacher et al., 1995; Scott et al., 2014) that collectively examined 35 species of adult Lithosiini representing four of the seven lichen moth subtribes, lichen phenolics were identified in the metabolite profiles of 32 species. Both studies identified multiple phenolics in adults and found intraspecific variation in the compounds present. These phenolics are products of lichens, symbiotic organisms comprised of photosynthetic and fungal components. The fungal component of lichens produces numerous unique secondary metabolites (> 1000 metabolites identified; Stocker-Wörgötter (2008)) through three, primary chemical pathways (Ranković and Kosanić (2015); Fig. 2): acetate–polymalonate, shikimic

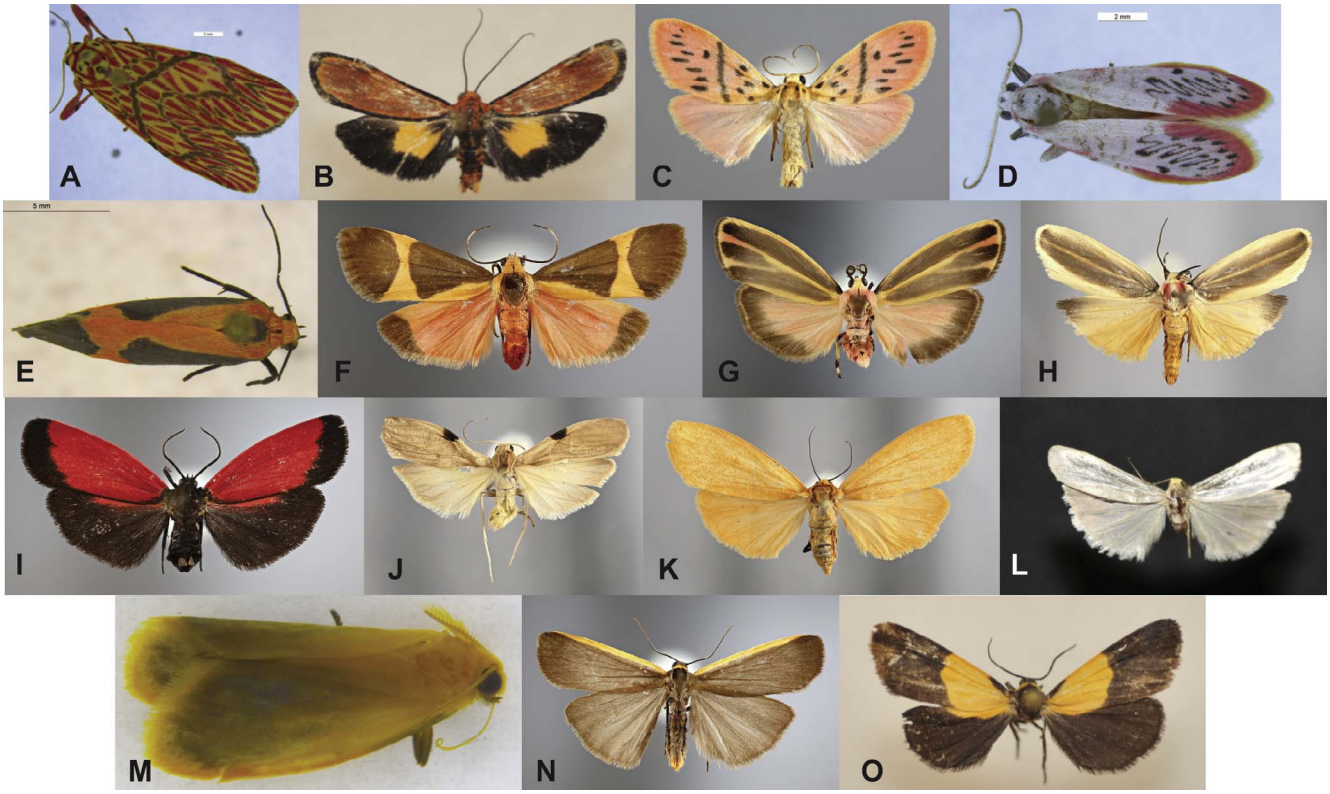

Fig. 1. Species of Lithosiini examined for phenolic compounds. (A) *Miltochrista* sp.; (B) *Cyana meyricki*; (C) *Lyclene acetola*; (D) *Miltochrista ziczac*; (E) *Cisthene martini*; (F) *Cisthene tenuifascia*; (G) *Hypoprepia fucosa*; (H) *Rhabdatomis laudamia*; (I) *Ptychoglene phrada*; (J) *Macotasa nubeculoides*; (K) *Nishada sambara*; (L) *Crambidia cephalica*; (M) *Eilema plana*; (N) *Manulea bicolor*; (O) *Euryptidia ira*.

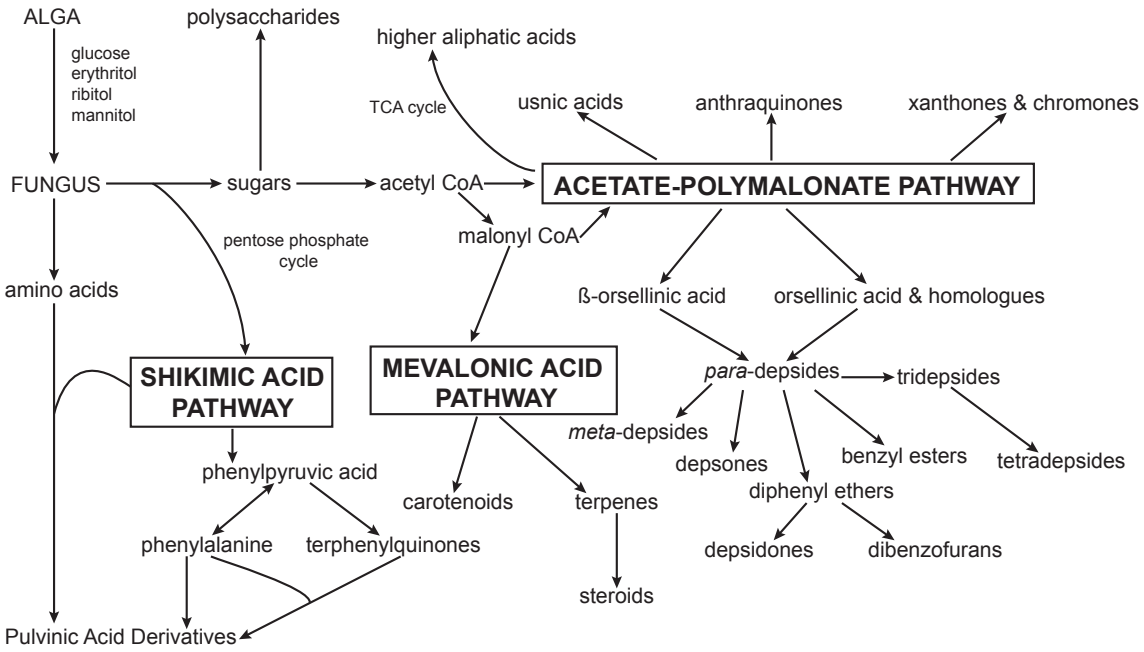

Fig. 2. Three metabolic pathways that produce the majority of lichen phenolics. Modified from Ranković and Kosanić (2015).

acid, and mevalonic acid pathways. The most common phenolics (*e.g.*, depsides, depsidones, and dibenzofurans) are products of the acetate–polymalonate pathway (Ranković and Kosanić, 2015), within which the majority of phenolics arise from the orcinol series through the oxidative coupling of small phenolic units related to orcinol and β-orcinol. The products of the orcinol series can be further modified to form other classes of phenolics including diphenyl ethers (Fig. 2). The phenolics produced by the three pathways are distributed throughout the layers of the lichen. They act to defend the photosynthetic component from the impacts of herbivores and solar radiation (Solhaug and Gauslaa, 2012) and possess antimicrobial, anticancer, antiviral, and antioxidant properties (Ranković and Kosanić, 2015). Lichen metabolites have been found to deter herbivory in gastropods and other invertebrates (Asplund, 2011; Asplund et al., 2009), yet the precise mechanisms and ecological functions of lichen-invertebrate defenses are virtually unknown to science.

While metabolomic studies (Hesbacher et al., 1995; Scott et al., 2014) and the behavior of lichen moth adults (Dowdy and Conner, 2016) suggest that lichenivory and subsequent phenolic sequestration is widespread within the tribe, the origins of this association and precise mechanisms of sequestration are currently unknown. In order to recover broad patterns of this historical relationship, a more comprehensive investigation of the compounds sequestered and the reliability with which they can be recovered both within biological replicates and across major clades is needed. For example, can the sequestered lichen phenolics be recovered reliably using metabolomic analyses of adult specimens stored under a range of conditions? Are chemical profiles consistent within and among closely related species, or do the metabolomes exhibit significant intraspecific variation? Given that lichens can contain a variety of phenolics derived from different chemical pathways, is lichen metabolite sequestration selective or more generalized (*i.e.*, do tiger moth larvae sequester lichen compounds produced by one or more chemical pathways)?

Our ability to examine the evolution of the relationship between these moths and their lichen hosts is hampered by the lack of a robust phylogenetic framework. While six separate phylogenetic studies (Jacobson and Weller, 2002; Scott and Branham, 2012; Scott et al., 2014; Wink and von Nikisch-Rosenegk, 1997; Zahiri et al., 2012; Zaspel et al., 2014) recovered the monophyly of the lichen moth tribe using molecular or morphological data, they did not resolve relationships among tribes and genera. In the most recent study of Lithosiini, Scott et al. (2014) assessed the evolutionary relationships among 65 lichen moth species representing four of the subtribes using four gene fragments (*cytochrome oxidase C subunit I, cytochrome oxidase B*, *ribosomal protein S5*, and *nuclear large subunit rRNA 28S D2 loop*). Although these gene fragments had been shown to be phylogenetically informative at the higher level in Lepidoptera and Noctuoidea (Mutanen et al., 2010; Wahlberg and Wheat, 2008; Zahiri et al., 2012), they did not recover strong branch support for the deeper relationships within Lithosiini. Furthermore, the monophyly of each of the three subtribes represented by multiple taxa was not supported. Thus, there is need to develop a novel dataset that can provide a reliable evolutionary framework for further testing of the phylogenetic relationships within Lithosiini.

One of the main goals of the present study is to utilize a massively large transcriptomic dataset for lichen moths in order to establish the phylogenetic architecture of the group and combine it with data from a metabolomic analysis of lichen host compounds. With these data, we inferred a phylogeny of the tribe. We used hierarchical clustering and a permutational multivariate analysis of variance to assess the similarity of the overall phenolic profiles among and within species included in our metabolomic dataset. To test whether lichen moth clades exhibit a pattern of specialized versus generalized phenolic sequestration, we conducted an initial ancestral reconstruction of lichen chemical pathways known to produce compounds sequestered in the moth species we sampled. Our results provide strong support for the monophyly of two subtribes and demonstrate that closely related species in our sample consistently share chemical profiles regardless of specimen age or origin. Our analysis of chemical pathways provides initial evidence of chemical specialization vs generalization in this hyperdiverse moth lineage.

2. Materials and methods

2.1. Phylogenomic taxon sampling

Thirty-seven species, 27 from Arctiinae (22 Lithosiini, one Syntomiini, and four Arctiini), nine from the erebid subfamilies Aganainae, Calpinae, and Lymantriinae were sampled for the phylogenetic analysis. The 1 K Insect Transcriptome Evolution Bioproject (1KITE; http://www.1kite.org/1kite_species.php; https://www.ncbi.

nlm.nih.gov/bioproject/183205) provided the transcriptomes of three of the species sampled (*e.g. Phragmatobia fuliginosa, Amata phegea,* and *Phyllodes eyndhovii*). We selected these 37 species to test the monophyly of the tribe Lithosiini and its subtribes, as proposed by Bendib and Minet (1999), and the *Eugoa* group of Holloway (2001). The *Eugoa* group represents a generic grouping that was established to place some genera that were not treated by the work of Bendib and Minet (1999) and includes genera found in the Old World Tropics, which is an area of high diversity. The 22 Lithosiini represented 19 genera, four of the seven subtribes (Cisthenina, Endrosina, Lithosiina, and Nudariina), and the *Eugoa* group (Table S1, supporting information). The sampling included 15 Old World (OW) and seven New World (NW) species (Table S1, supporting information). Diverse genera whose wing color patterns have caused taxonomic confusion (*e.g.*, *Miltochrista*) or have been found to be polyphyletic in previous studies (*e.g.*, *Lyclene* (Scott and Branham, 2012; Scott et al., 2014)) were represented by multiple species. Phylogenetic analyses were rooted using *Bombyx mori*, whose position well outside the Arctiinae is supported by many studies (Kawahara and Breinholt, 2014; Mutanen et al., 2010; Regier et al., 2013; Regier et al., 2009).

2.1.1. RNA-Seq

We generated 33 novel transcriptomes for this study. These transcriptomes were produced from total RNA extracted from whole body flash-frozen or RNAlater-preserved adult specimens using either an RNeasy MiniKit (Qiagen) or a Trizol-Chloroform extraction. For *Haematomis uniformis*, total RNA was extracted from a larval specimen. Wing vouchers and collection location data for each specimen were deposited into the Purdue Entomological Research Collection. Extractions obtained using the Trizol – Chloroform protocol were purified with a DNA-Free RNA Kit (Zymo Research). Transcriptome data (ca. 2.5 GB raw data per sample) available from the 1KITE initiative was used for species representing Arctiina (*Phragmatobia fuliginosa*: BioProjectID: 267893, TSA: GCJS01000000), Syntomiini (*Amata phegea*: BioProjectID: 267859, TSA: GCNU01000000), and Phyllodini (*Phyllodes eyndhovii*: BioProjectID: 267894, TSA: GCOO01000000). Sequence data was obtained from the NCBI Short Read Archive for *Lymantria dispar* (Bazinet et al., 2013). The most recent proteome of *Bombyx mori* was obtained from Ensembl Genomes (Kersey et al., 2014).

For the 33 novel transcriptomes generated in this study, RNA was quantified using a Bioanalyzer 2100 (Agilent Technologies) at the Purdue University (PU) Genomics Core. RNA-Seq library construction and paired end sequencing on an Illumina HiSeq 2000 was also completed at the PU Genomics Core. Library construction was performed using the Illumina TruSeq Stranded Total RNA with the Ribo-Zero™ Gold Kit (epicentre). The libraries were barcoded and sequenced on five lanes (six or eight pooled samples in a lane). Adapters were removed and poor quality bases (Phred $<$ 20) trimmed from the 5′ and 3′ ends of reads using Trimmomatic (Bolger et al., 2014). Following this processing, any reads of less than 30 bases were discarded. *De novo* assembly of the transcriptomes from these quality and adapter clipped reads was completed using Trinity (Grabherr et al., 2011). Trans-chimeras were removed from each of the novel transcriptomes using the pickH-capblast method that produces a dataset with fewer redundancies (Yang and Smith, 2013). Coding regions of 100 amino acids or longer in the sequences were then determined using the TransDecoder plugin within Trinity. The new sequence data is available from GenBank (NCBI BioProject PRJNA312728; BioSamples SAM04569175-04569206). Data provided by 1KITE is available under the Umbrella BioProject PRJNA183205 (for full description of protocol used to sequence, assemble, and subsequently check for cross contamination in 1KITE transcriptomes see Appendix S1, supporting information based on Peters et al., 2017).

2.1.2. Phylogenetic methods

Single copy ortholog groups were identified in OrthoMCL (Fischer et al., 2011) using the Basic Protocol 2 outlined in Fischer et al. (2011). In total, 1328 single copy orthologous loci groups were identified that were present in more than 75% (28) of the taxa in the study. The sequences within these groups were aligned using the L-INS-i algorithm in MAFFT v.7 (Katoh and Standley, 2013). After trimming the alignments using the 'automated1' setting in trimAl v1.4 (Capella-Gutiérrez et al., 2009), a consensus sequence for representative sequences of each ortholog group was extracted with EMBOSS v6.5.7 (Rice et al., 2000). To identify any contamination in the ortholog groups, the consensus sequence of each group was blasted against the NCBI non-redundant protein database using BLASTp, and the five most likely hits were retained along with the 'qseqid, qlen, sallseqid, sscinames, salltitles, pident, length, mismatch, gapopen, evalue, and bitscore' for each hit. Groups with consensus sequences that did not align with any lepidopteran sequences or best aligned with non-invertebrate sequences were examined further. Individual sequences from these groups identified in the previous step were BLASTp searched against the non-redundant protein database to assess the identity using the above settings. All sequences from each of seven ortholog groups identified as prokaryotic or mammalian sequences were excluded using this method. We concatenated the remaining 1321 orthologs into a supermatrix using FASconCAT (330542 amino acids; Appendix S2, supporting information; Kuck and Meusemann, 2010). See Appendix S3 (supporting information) for a complete list of ortholog groups and their putative annotation. Table S2 (supporting information) provides a list of the coverage of each ortholog group in the species sampled. Substitution models and data partitions were determined using the r-cluster search strategy (rcluster-percent = 10; rclustermax = 1000) implemented in PartitionFinder v2 with RAxML (Lanfear et al., 2017; Lanfear et al., 2014; Stamatakis, 2014). All 128 models of amino acid evolution were considered using the '–raxml' option. Following the program recommendation for analyses that will use RAxML, two additional searches were completed in PartitionFinder to identify the optimal model of rate heterogeneity (+I+G or +G) for all partitions (*i.e.*, the model of rate heterogeneity that produces the lowest BIC score when applied to all partitions). These two runs were completed using the best partitioning scheme identified in the first PartitionFinder analysis. See Appendix S4 (supporting information) for the partitioning scheme used and the model of evolution applied to each partition. An initial ML analysis was conducted in RAxML – HPC2 (Stamatakis, 2006) on the CIPRES Science Gateway (Miller et al., 2010) using the '-f a' option with 1000 bootstrap replicates. We ran an additional 100 searches for the best likelihood tree in RAxML using the '-f d' option and mapped bootstrap support values onto the best tree using the '-f b' option and the previously obtained bootstrap replicates. The tree was rooted using *Bombyx mori*.

2.2. Metabolomic taxon sampling

Fifteen species were sampled from OW and NW taxa to represent the four subtribes of Bendib and Minet (1999) (Cisthenina, Eudesmiina, Lithosiina, and Nudariina; Table S1, supporting information; Fig. 1). When possible, taxa that were sampled for the phylogenomic analyses were included. Eleven of the 22 Lithosiini included in the phylogenomic study were sampled. These species were also chosen to exemplify the variation of wing color patterns found in the tribe. Both brightly colored species and drably colored species were assessed. When material was available, three biological replicates were sampled for each species.

2.2.1. Metabolomic analysis

Fifteen species of adult Lithosiini were assessed for the presence of 129 lichen phenolics (Appendix S5, supporting information). Metabolites were obtained using the entire body of each specimen. Dorsal and ventral photographs of these specimens and their labels

were taken as vouchers and deposited within the Purdue Entomological Research Collection. The specimens were homogenized using a Precellys® 24 tissue homogenizer. Metabolites were obtained from the homogenate using a methanol-water: chloroform extraction (1.5 ml methanol; 0.5 ml water; 1 ml chloroform). The targeted phenolics are polar compounds; therefore, only the methanol-water phase of the extraction, which would contain polar compounds, was used in the final analysis. After the phases separated, the methanol-water phase was removed with a pipette and dried by spinning at room temperature for 14–16 h in a Savant SpeedVac Concentrator (Thermoscientific). The concentrated pellets of metabolites were resuspended in 50 µl of a 95% water: 5% acetonitrile solution.

The LC-MS analysis of these metabolites was performed with an Agilent 1100 system (Palo Alto, CA) using a Waters T3 column (3 µm, 150 × 2.1 mm i.d) and an Agilent MSD-TOF spectrometer following the methodology of Scott et al. (2014). These analyses were conducted in the Metabolite Profiling Facility within the Bindley Bioscience Center at Purdue University. Reads were analyzed using Agilent MassHunter Qualitative Analysis software. For each sampled species, this software was used to compare the peaks that were identified to determine differences in quantity and types of compound present (Appendix S6, supporting information). All replicates from each species were then assessed for 129 lichen phenolics, which have previously been reported to exhibit pharmaceutical activity and have a known chemical formula. Any peak with a mass that differed by less than 10 ppm from that of a given phenolic was considered a presumptive positive. Commercially available, chemical standards (*e.g.*, pure compounds) for five of the candidate phenolics (resorcinol, ß-thujone, divarin, didymic acid, and methyl ß-orcinolcarboxylate) were also analyzed at the Metabolite Profiling Facility using the same conditions as the biological samples.

2.2.2. Clustering analysis of phenolic compounds and ancestral state reconstruction

Among tiger moths, lichen phenolic sequestration is only known from species in Lithosiini. To date, sequestered lichen-derived metabolites have been documented in five of the seven subtribes (Hesbacher et al., 1995; Scott et al., 2014). Our metabolomic examination of 15 of 22 Lithosiini species (68% of ingroup sampling) included in the phylogenetic dataset identified presumptive positives for 33 unique lichen phenolics. To further examine this dataset, and test whether replicates within species shared similar patterns of chemical sequestration, we completed a hierarchical clustering analysis that grouped individuals based only on their phenolic profiles. The presence/absence of the compounds was used to calculate the Jaccard's dissimilarity (Legendre and Legendre, 1998) between chemical profiles of individuals. This asymmetric association measure works well with presence/absence data when double negatives are not considered to represent similarity. We clustered the profiles using Ward's clustering algorithm (Murtah and Legendre, 2014). To determine the pruning height, and thus cluster delineation, we subjected the same data to k-means clustering with k varying 1–20. The resulting curve of within-group error sum of squares versus k showed a weak elbow at k = 8. We used this pruning height to assign individuals to eight chemical profile groups. We used a permutational multivariate analysis of variance (adonis) to test for a significant different among the chemical profiles. Cluster and adonis analyses were carried out with adonis using the 'vegan' package (Oksanen et al., 2015) in R (R Core Team, 2014).

Due to the high diversity of compounds recovered in a relatively small number of taxa sampled for our phylogeny, coupled with the unavailability of chemical standards for a large majority of phenolic compounds (Betz et al., 2011), we took a more conservative and biologically meaningful approach, and coded terminal taxa according chemical pathways instead of individual compound. The chemical pathways are based on the groupings identified and described by lichen biologists (Culberson, 1969; Ranković and Kosanić, 2015). A denser taxon sampling and comprehensive testing against chemical standards will be important in future comparative work, but beyond the scope of this initial study. The chemical pathways are A–F: (A) "orcinol" (phenolics that are members of the orcinol pathway including precursors, monocyclic derivatives, *para*-depsides, depsidones, dibenzofurans, depsones, and *meta*-depsides); (B) "aliphatic acids"; (C) "anthraquinones"; (D) "chromones"; (E) "diphenyl ethers"; (F) "mono-terpenes" (Appendix S7, supporting information).

Published feeding records for tiger moths suggest patterns of chemical acquisition and sequestration are generally conserved at the genus level (Conner and Jordon, 2009); our clustering analysis of individual phenolic compounds by taxon provides additional support for this claim. Therefore, in cases where a chemical profile for a given species were absent, available profiles for congeners were used (approach as in Hwang and Weirauch, 2012; Zaspel et al., 2014). Chemical pathway profiles of ancestral nodes were reconstructed using a Bayesian approach. We reconstructed ancestral areas using the Bayesian Binary MCMC ancestral state reconstruction (BBM) method implemented in RASP (Yu et al., 2015). The analysis was run for 5,000,000 cycles using 4 chains, which were sampled every 100 generations; the first 1000 iterations were discarded as burn-in, and the Fixed Jukes-Cantor model was used with a null root distribution.

3. Results

3.1. Phylogenetic analysis

To examine the evolutionary relationships among lithosiine species, we constructed a dataset of single copy orthologs identified from the transcriptomic data of 22 lithosiine species, 14 non-lithosiine erebid outgroups, and rooted the tree with *Bombyx mori* (Bombycidae). The final dataset contained 1321 single copy orthologs (330,542 amino acids). A maximum likelihood (ML) analysis of the partitioned, dataset recovered a well-supported tree with all but one node receiving non-parametric bootstrap (BS) support score of greater than 80% (Fig. 3; See Appendix S4 (supporting information) for partitioning scheme used and substitution model applied to each partition).

The outgroup taxon sampling included four erebid subfamilies (Arctiinae, Aganainae, Calpinae and Lymantriinae). The analysis recovered Lymantriinae as the most basal subfamily. Calpinae was reciprocally monophyletic with BS = 100 to Aganainae + Arctiinae (BS = 100). Within Arctiinae, the ML analysis recovered the monophyletic Lithosiini (BS = 100) sister to Arctiini + Syntomiini (BS = 100).

Within the Lithosiini, we included species representing four of the seven subtribes and the *Eugoa* species group. The ML analysis recovered the subtribes Nudariina and Lithosiina as well-supported, monophyletic clades (both BS = 100; Fig. 3). Cisthenina was polyphyletic: the OW Cisthenina species are recovered as a monophyletic clade (BS = 100) at the base of Lithosiini (BS = 100). The NW Cisthenina species were recovered in a more derived position (BS = 100) as the sister group to the OW Nudariina clade (BS > 87). The ML analysis found the single representative of the subtribe Endrosina (*Stigmatophora roseivena*) as the sister group to *Trischalis subaurana* (BS = 100) a representative of the *Eugoa* group (BS = 100), which was represented by two species. The Lithosiina are sister to the *Eugoa* group/Endrosina clade (BS > 81).

We also included multiple representatives of two diverse Lithosiini genera associated with the subtribe Nudariina, which previous studies (Scott and Branham, 2012; Scott et al., 2014; Zaspel et al., 2014) found not to be monophyletic: *Lyclene* and *Miltochrista*. Although representatives of these two genera were both confirmed as Nudariina in our ML analysis, only *Lyclene* was recovered as monophyletic (BS = 100). The ML analysis recovered a polyphyletic *Miltochrista* within Nudariina.

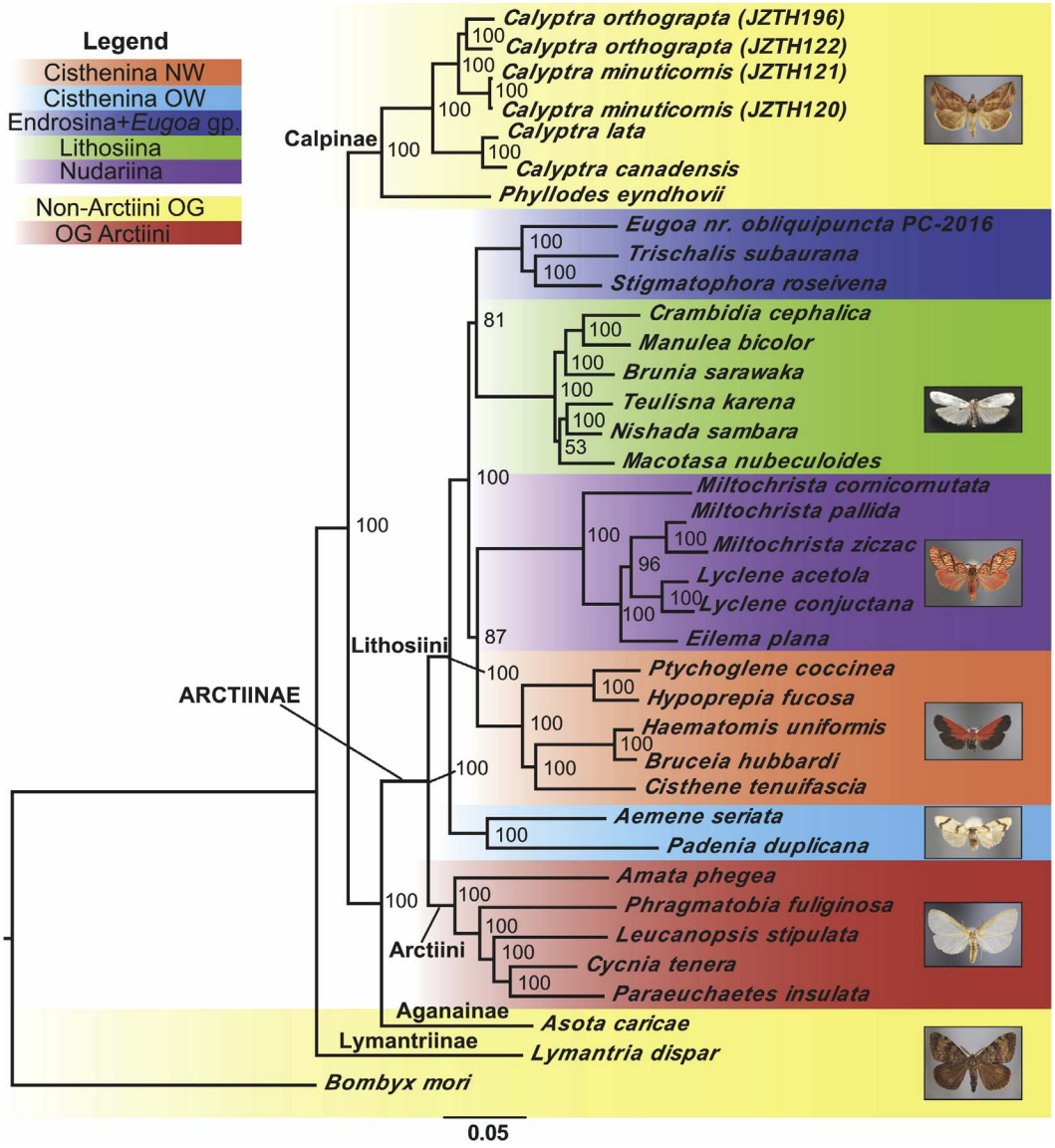

Fig. 3. Phylogeny of the lichen moth tribe. The maximum likelihood tree recovered from the RAxML analysis, rooted with *Bombyx mori* (lnL = −3478648.753226). Bootstrap support values ≥ 50 shown beside each node.

3.2. Metabolomic analysis

The metabolomic analysis of 15 lichen moth species (Fig. 1) revealed presumptive positives for 33 compounds representing 10 classes of lichen phenolics derived from two of the major metabolic pathways that produce phenolics (Fig. 4; Table S3, supporting information). When possible, three biological replicates were included for each species to assess for intraspecific variation due to preservation techniques and possible host switching by larvae. We also analyzed the spectra of commercially available standards of five of the compounds: resorcinol, ß-thujone, divarin, didymic acid, and methyl ß-orcinolcarboxylate. The retention times of these standards were compared to those obtained for the presumptive positives; however, the retention times of the standards did not match those found in the moths. Thus, we limit the rest of our results from the metabolomic analysis to the remaining 28 phenolics.

Of the remaining 28 compounds identified in this study, 17 are precursors, products, or monocyclic derivatives (*i.e.,* breakdown products) of the orcinol series. In addition to being the most common type of phenolic class, the orcinol series is also the only class of phenolics present in all of the sampled species. With the exception of the aliphatic acids and anthraquinones, we did not identify multiple presumptive positives for any other phenolic compound class. The most commonly identified phenolics were anziac acid (orcinol series: *para*-depside) and micareic acid (diphenyl ether). We identified presumptive positives for each of these phenolics in the metabolomic profiles of six species. Eleven of the phenolic compounds identified were unique to a single species.

In addition to identifying a wide range of phenolic compounds, our metabolic analysis identified interspecific variation in the phenolics present in the sampled species (Table S3, supporting information). The species with the highest number of phenolics identified, *Cyana meyricki* (13; Fig. 1B) represents the subtribe Nudariina, which has the highest, per-species average number of phenolics (M = 8.23 ± 3.77). The species with the lowest number of phenolics is *Manulea bicolor* (2; Fig. 1 N), a member of Lithosiina. We identified six or more phenolics in three Cisthenina species known to be unpalatable to bats and birds (*Cisthene martini*, *C. tenuifascia*, and *Hypoprepia fucosa*; Fig. 1E–G; (Dowdy and Conner, 2016; Hristov and Conner, 2005)). The metabolomic profile of the firefly mimic, *Rhabdatomis laudamia* contained presumptive positives of seven phenolics.

Based on prior studies (Hesbacher et al., 1995; Scott et al., 2014),

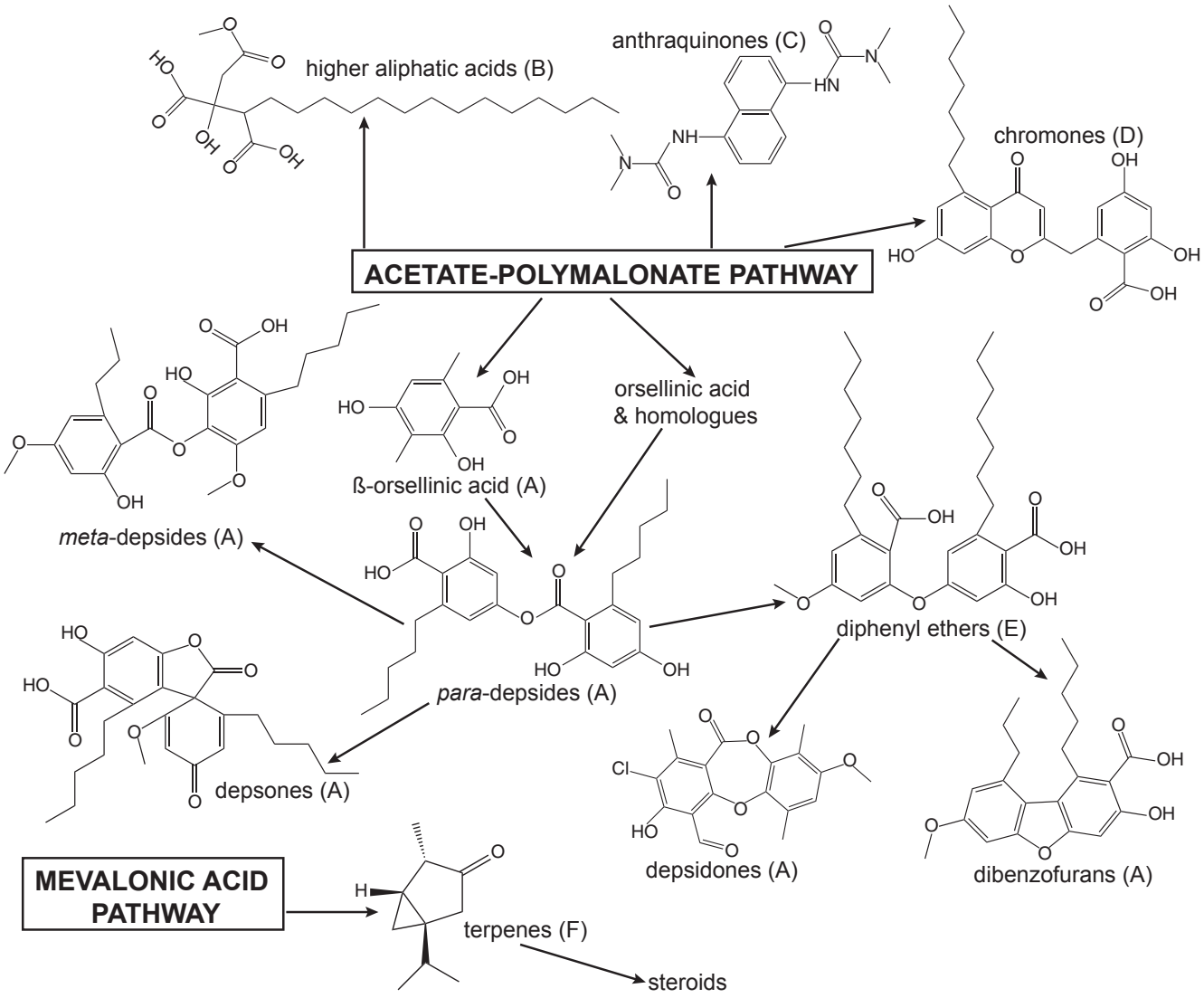

Fig. 4. Phenolic classes identified in the metabolomic analysis. Examples of the chemical classes of phenolics identified in our analysis, the metabolic path they arise from, and the coding used for each in the RASP analysis. Acetate-Polymalonate Pathway: ß-Orsellinic Acid (A), *para*-depside (A)-anziac acid, *meta*-depside (A)-homosekikaic acid, depsone (A)-picrolichenic acid, depsidone (A)-pannarin, dibenzofuran (A)-didymic acid, aliphatic acid (B)-caperatic acid, anthraquinone (C)-solorinic acid, chromone (D)-siphulin, diphenyl ether (E)-micareic acid. Mevalonic Acid Pathway: mono-terpene (F)-ß-thujone.

we hypothesized that lichen moths selectively sequester phenolics from specific chemical classes, but the phenolics sequestered can vary intraspecifically. To examine the pattern of overall phenolic sequestration among and within our species, and to test our hypothesis of specialized chemical sequestration, we used a hierarchical clustering analysis to group individuals based solely on their phenolic profiles. We found that the chemical profile clustering grouped individuals within ten species with conspecifics, while individuals of *Cisthene martini*, *Eilema plana*, *Euryptidia ira*, *Hypoprepia fucosa*, and *Nishada sambara* were spread among more than one group (Fig. 5A). In all clusters except Cluster 3, species from the same geographic region were grouped together. Furthermore, the phenolic profiles of the different clusters were significantly different (adonis: df = 833, F = 7.32, p-value = 0.0001) as shown in the NMDS plot (Fig. 5B).

3.2.1. Evolution of phenolic sequestration

A representation of the most likely states from the BBM of chemical profiles for Lithosiini is presented in Fig. 6. Outgroup taxa included in our study are not lichenivores, and are not known to sequester lichen-derived compounds. Therefore, we only report ancestral reconstructions for clades within Lithosiini. The ancestral state for the Lithosiini node could not be reconstructed. This ambiguity is most likely due to a lack of metabolic profile data (*e.g.*, phenolics sequestered) in the earlier lithosiine lineages. However, while the most probable ancestral state at the node uniting all Lithosiini except the OW Cisthenina could not be reconstructed (60.87%), sequestration of orcinol series-associated compounds was the next most probable state (25.28%). We also reconstructed a single origin of orcinol series-associated lichen compounds (40.74%) at the node uniting Nudariina and NW Cisthenina (P = 0.20). Although the ancestral state reconstructed at this position represents an origin of phenolic sequestration for compounds in the orcinol series, the chemical profiles obtained for the subtribes (and species) therein contain a diverse array of phenolics. For example, 20 of the 33 lichen-derived compounds reported were recovered in the Nudariina, and 18 were detected in the NW Cisthenina. Within the NW Cisthenina, support at the node uniting *Hypoprepia* and *Ptychoglene* in this reconstruction was relatively strong (P = 0.79). A stark transition from primarily orcinol associated compound sequestration (*i.e.*, specialized) to a more "generalized" pattern of lichen derivative sequestration (*i.e.*, sequestration of lichen products arising from more than one chemical pathway) was observed in the common ancestor of Nudariina. Although not strongly supported (P = 0.35), the putative

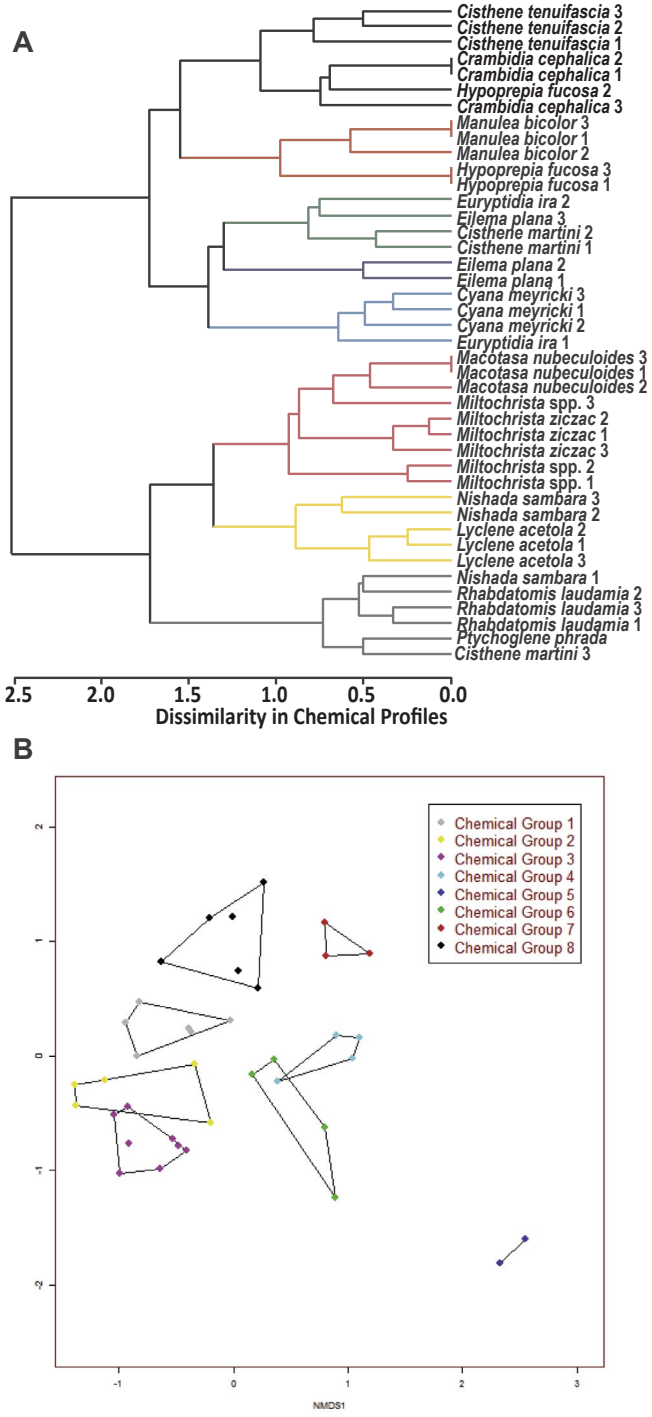

Fig. 5. Statistical analyses of the lichen phenolic profiles of all analyzed moths. (A) Cluster analysis of individual chemical profiles of individual moths. Ward's clustering algorithm (Murtah and Legendre, 2014) was used on the Jaccard's dissimilarity (Legendre and Legendre, 1998), which measured the overall dissimilarity in the occurrence of the individual chemicals. To delineate groups of similar metabolic profiles, the pruning height was determined with a scree plot of the within-groups error sums of squares from k-means clustering. (B) Nonmetric multidimensional scaling of individual moths colored according to chemical group. Individuals with identical metabolic profiles treated as a single point. Dissimilarity ranks were ordered according to Jaccard's dissimilarity of the chemical profiles. The chemical groups correspond to the species groups found in Fig. 5A.

ancestor of Nudariina is inferred to have sequestered a broad range of lichen compounds. The novel character state reconstruction for the putative ancestor of Nudariina included orcinol compounds, diphenyl ethers, and mono-terpenes (69.68%); these lichen phenolics were particularly prevalent in the OW genera *Lyclene* (P = 0.69) and *Miltochrista* (P = 0.98). There was no definitive ancestral reconstruction for the *Eugoa* group or subtribe Lithosiina, again, perhaps due to missing chemical profile data in those terminal taxa. However, among the Lithosiina, *Crambidia* and *Manulea* species both tested positive for compounds associated with the orcinol series resulting in strong support (P = 0.86) for reconstruction of this state at the node uniting the two genera. Our results yielded weak support (P = 0.05) for the reconstruction of orcinol-associated compounds at the node joining genera *Macotasa, Nishada*, and *Teulisna*. This state was recovered in 21.61% of the topologies, and the next most probable state was sequestration of both orcinol- associated compounds and diphenyl ethers (18.44%). Sequestration of diphenyl ether compounds (21.11%) was reconstructed with weak to moderate support (P = 0.21) for the common ancestor of *Nishada* and *Teulisna*.

4. Discussion

Elucidating the deeper evolutionary relationships within Lithosiini has proven to be a difficult problem. This inhibits our ability to use this lineage as a model system for understanding the relationships between Lepidoptera and lichens. By analyzing a phylotranscriptomic dataset, we are able to recover strong support for the early relationships within the lichen moths. These findings provide the first evidence that the chemical profiles within species and among closely related species are more similar to each other than expected by chance, and species sequester specific classes of phenolics. Furthermore, our initial metabolomic examination and reconstruction of chemical pathways support the idea that lichen phenolics are consistently, and perhaps even selectively, sequestered in Lithosiini.

4.1. Evolution of lichen moths

Previous phylogenetic studies of the evolutionary relationships of Lithosiini used either adult morphological characters (Scott and Branham, 2012) or molecular datasets (Scott et al., 2014) that could not resolve the deeper relationships of the lithosiine genera or subtribes. However, transcriptome sequencing provides a means to obtain large quantities of data even for non-model organisms (Metzker, 2010). Transcriptome-based datasets have been found to be informative in resolving the deeper relationships at a range of taxonomic levels and a variety of organisms (Hittinger et al., 2010; Misof et al., 2014; Wen et al., 2013). Our study is the first to use a phylogenomic (*e.g.*, transcriptome-based) dataset to reconstruct the evolutionary history of lichenivory and sequestration of lichen based secondary metabolites. The ML analysis of Lithosiini transcriptomes resulted in 20 of the 21 internal relationships that are well supported, adding to a growing body of evidence that transcriptome based datasets are a powerful approach to resolve previously intractable phylogenetic questions, and confirming other studies that have used transcriptomes for lepidopteran phylogenetics (*e.g.*, Kawahara and Breinholt, 2014, Breinholt and Kawahara, 2013, and Bazinet et al., 2013).

Among the four subfamilies represented in our taxon sampling for the phylogenomic analysis, the most likely topology supported the monophyly of all subfamilies. Relationships recovered among these subfamilies are congruent with those recovered in recent phylogenetic analyses of Erebidae (Zahiri et al., 2012) and Arctiinae (Zaspel et al., 2014). Within Arctiinae, the topology we recovered provides further support for the hypothesis that Lithosiini is basal to the Arctiini + Syntomiini clade (Zahiri et al., 2012; Zaspel et al., 2014).

Our study provides a framework phylogeny that supports some of the previously proposed subtribal classifications of Lithosiini (Bendib and Minet, 1999; Holloway, 2001) and also highlights groups that will need further investigation. Our results support the subtribal hypotheses of Bendib and Minet (1999) for two lineages (Lithosiina and Nudariina). However, we found the subtribe Cisthenina to be polyphyletic. As

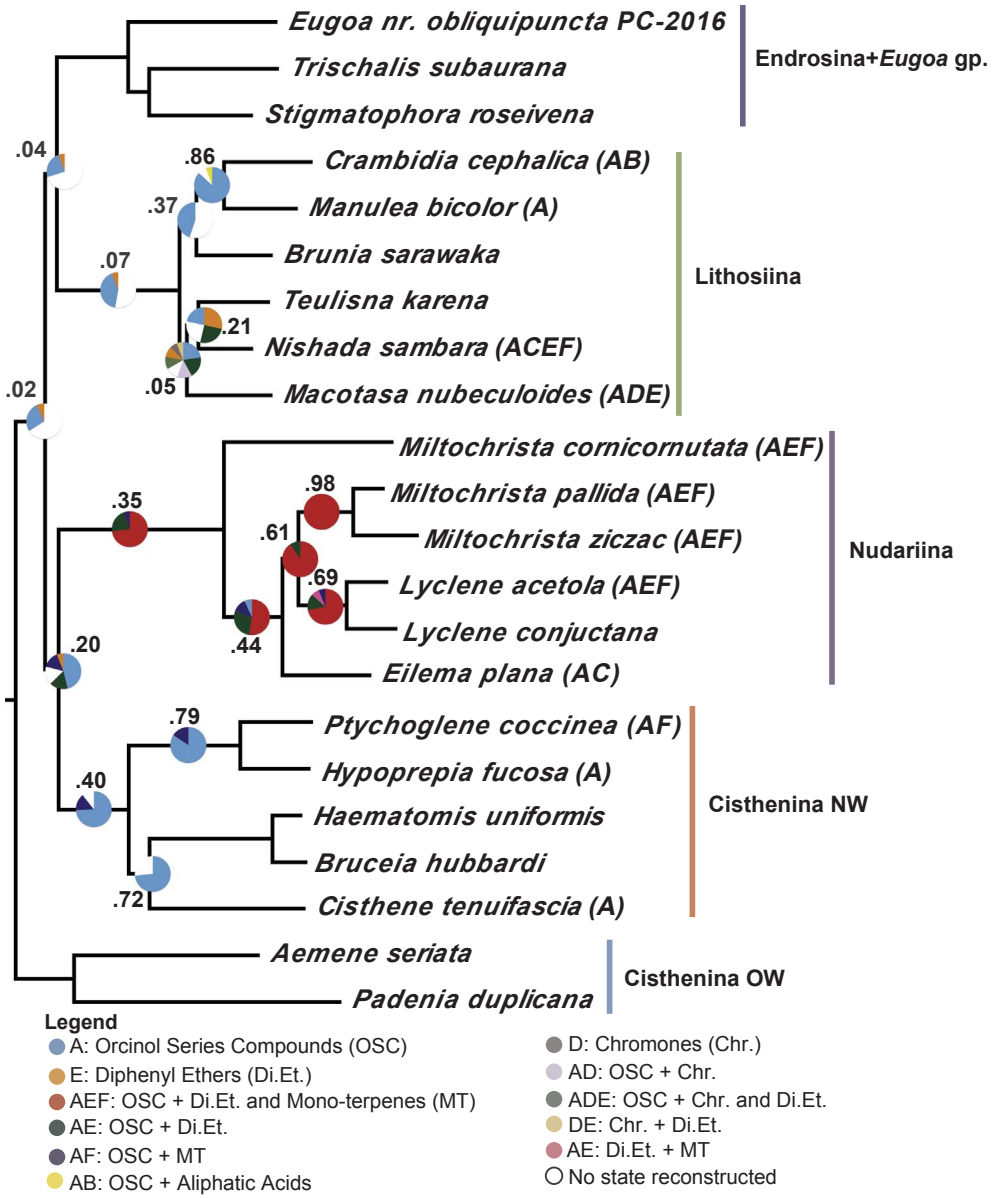

Fig. 6. Evolutionary hypothesis of lichen phenolic sequestration in the lichen moth tribe. The most likely ancestral lichen phenolic sequestration strategy reconstructions and alternative reconstructions with a likelihood > 5% are indicated on each branch of the Lithosiini clade along with the probability of the most likely ancestral state. The probability (P) of the most likely ancestral state is provided at each node. The topology shows only the relationships among the lichen moths and is reformatted from the phylogeny in Fig. 3. The coding used for each species in the analysis is provided next to the species name. A = orcinol series compounds, B = aliphatic acids, C = anthraquinone, D = chromone, E = diphenyl ether, F = mono-terpene.

currently defined, Cisthenina contains OW and NW species (Bendib and Minet, 1999; Holloway, 2001), and our analysis included representatives of both geographic regions. In our result, the two, non-adjacent clades representing Cisthenina are composed exclusively of only OW or NW taxa. Scott et al. (2014) also recovered a polyphyletic Cisthenina represented by two clades that contained only NW taxa. These findings suggest that as currently defined the taxa placed within Cisthenina could represent three independent lineages. Thus, an intensive study is needed to determine the composition of Cisthenina and what morphological characters may reliably diagnose taxa to this subtribe. The fourth subtribe, Endrosina, is found in the clade containing the two representatives of the *Eugoa* group of Holloway (2001).

We included multiple representatives of two diverse genera (*e.g.*, *Lyclene* and *Miltochrista*). Each of these genera is composed of a large number of species with similar wing color patterns (within each genus) that have caused confusion and led to previous studies identifying them as either paraphyletic (*e.g.*, *Miltochrista* (Scott and Branham, 2012)) or polyphyletic (*e.g.*, *Lyclene* (Scott et al., 2014; Zaspel et al., 2014), *Miltochrista* (Zaspel et al., 2014)). Our analyses recovered only *Lyclene* as monophyletic. We found all representatives of *Miltochrista* placed in the Nudariina. Our findings suggest that monophyletic groups might be recovered from these large genera; however, an in-depth analysis of these lineages will be needed to determine their exact composition.

4.2. Phenolic sequestration

Metabolomic analysis demonstrated that sequestration of lichen phenolics in the larval stage and successful retention of these compounds through diapause to the adult stage occurs in four of the lithosiine subtribes: Cisthenina, Eudesmiina, Lithosiina, and Nudariina. Hesbacher et al. (1995) identified lichen phenolics in the metabolome of species representing Endrosina. However, to our knowledge, no metabolomic studies examined the chemical profiles of the remaining two subtribes (Acsalina and Phryganopterygina). We identified presumptive positives for 33 unique phenolics in the Lithosiini sampled; pyrrolizidine alkaloids, cardiac glycosides, iridoid glycosides, or tetrahydrocannabinol were not found in the metabolome of any species included in our analysis. While Scott et al. (2014) identified physodic acid in the metabolic profile of all sampled species, we did not find any peaks whose mass was within 10 ppm to this phenolic. Thus, the more stringent protocol for identifying presumptive positives utilized in this study decreases the probability of false positive. Genera within Arctiini

sequester these chemicals for defense against predators and parasitoids (Bowers and Stamp, 1997; Hartmann et al., 2005; Rothschild et al., 1977; Rothschild et al., 1973). Untargeted metabolic analyses such as the ones completed for this study generate large numbers of unknown metabolites (Goodacre et al., 2004). Preliminary identifications of unknown peaks can be made based on the mass of compounds of interest, and then these identifications should be confirmed using chemical standards for each unknown compound. Our spectral analysis of chemical standards representing five of the compounds identified with presumptive positives did not confirm their identities with matching retention times. However, due to a limited number of chemical standards for botanical products (Bowen and Northern, 2010) and a lack of available databases, the best option for establishing a preliminary identification of phenolics is to use their mass. Furthermore, retention times can vary between analyses due to experimental drift (Patti et al., 2012). The number of samples run in an analysis or running samples at different times on the same machinery can cause this variation. Thus, the difference in retention time between the peaks identified as presumptive positives and the chemical standards could be due in part to this phenomenon. In addition, the matrices in which botanical compounds, such as lichen phenolics, occur are by nature highly variable (Betz et al., 2011), which can also contribute to differences in retention times and make chemical confirmation of them using standards difficult. To confirm the presence of the remaining phenolics identified, analyses of additional standards will be needed. For many of these compounds, chemical standards are not commercially available. Thus, absolute certainty of the compound identities is limited and will require more studies and the use of additional techniques, such as H^+ NMR. Studies such as this add to a growing body of literature regarding the association between lithosiines and lichens and can be used to guide future studies examining questions related to phenolic sequestration.

Our examination of the metabolomes combined with those of previous studies (Hesbacher et al., 1995; Scott et al., 2014) found intraspecific variation in the phenolic profiles of adult lichen moths. However, our clustering analysis demonstrated that within the majority of species, profiles are more similar within than among species. Thus, our findings demonstrate proof of concept that the use of adult specimens preserved in a variety of conditions and LC-MS provides a potentially reliable means of confirming lichen compound sequestration in the absence of known or observed lichenivory. Given the consistency of the chemical adult profiles and the number of presumptive positives identified, we reconstructed the evolution of sequestration by pathway, not compound. This protocol allowed us to look for broader patterns of evolution within the context of chemical specialization vs generalization. Since lichen host chemistry can vary considerably within and among species depending on environmental conditions (Betz et al., 2011) but the chemical pathways do not, a reconstruction of chemical pathways is potentially more biologically meaningful than by individual compound.

Our initial reconstruction of the origins of lichen phenolic sequestration demonstrated that the ability to selectively sequester phenolics from the orcinol series is the ancestral state for the NW Cisthenina + Nudariina clade and some members of Lithosiina as currently defined (Fig. 6). While this analysis could not resolve whether sequestration of orcinol series phenolics occurs in the more basal OW Cisthenina clade or in the *Eugoa* group/Endrosina, these taxa have not been sampled in any metabolomic studies. Thus, the ability to selectively sequester phenolics from the orcinol series is a strong candidate for a metabolic synapomorphy of Lithosiini.

Regardless of whether this is the case, results from our analyses indicated generalized sequestration of phenolics produced by more than one chemical pathway represents the derived condition. Species of Nudariina sequester compounds from two of the three main metabolic pathways (Figs. 2, 4 and 6) that produce phenolics: acetate-palmolate and mevalonic acid pathway. It is possible that generalist sequestration of phenolics would allow the species in Nudariina to utilize a broader range of host lichens. A similar pattern of evolution of "specialist to generalist" feeding behaviors was also supported in the derived tiger moth clades (Zaspel et al., 2014), yet whether the detoxification and sequestration systems of herbivorous vs. lichenivorous tiger moths are similar, remains unknown.

In all species included in our metabolomic analysis, we found presumptive positives for lichen phenolics derived from the orcinol series. This result holds both for brightly colored, presumptively aposematic lineages (*Cisthene* spp. and *Ptychoglene phrada*) and for firefly mimics (*e.g.*, *Rhabdatomis laudamia*). The orcinol series refers to lichen phenolics that are produced by the acetate-polymalonate pathway and occur as two types: orcinol and ß-orcinol (Ranković and Kosanić, 2015). Phenolics from the orcinol series have antibacterial properties (Cheng et al., 2013) and cause contact dermatitis in humans. Hesbacher et al. (1995) and Wagner et al. (2008) hypothesized that sequestered lichen phenolics may be used by Lithosiini as a defense against predators and/or pathogens. As such, phenolics from the orcinol series could provide the chemical basis for the observed unpalatability in adult lithosiines.

These results suggest that lichen feeding is common and lithosiine larvae may selectively forage on a broad range of lichens, potentially making use of a variety of chemical defenses. We recognize the relatively small sample size used in our reconstruction analysis, but we have selected exemplar taxa that represent the major lithosiine lineages across their geographic ranges. Our approach to reconstructing the states is relatively basic, yet it provides a way to interpret the data in a phylogenetic context. Additionally, the results of our clustering analyses (Fig. 5) provide strong evidence that our chemical data is consistent and reliable both within and among the related taxa, thus even with an increased sampling, we predict close relatives will share similar chemical profiles. While further studies will be needed to determine what compounds are sequestered among early lithosiines, and to confirm whether the phenolics identified effectively deter predators and parasitoids, these findings help to broadly expand upon the poorly understood interactions between Lepidoptera and lichens.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ympev.2017.12.015.

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