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Short Communication

A molecular phylogenetic analysis of the vampire moths and their fruit-piercing relatives (Lepidoptera: Erebidae: Calpinae)

J.M. Zaspel [a,b,*], R. Zahiri [c], M.A. Hoy [d], D. Janzen [e], S.J. Weller [b], N. Wahlberg [c]

[a] Department of Biology and Microbiology, University of Wisconsin Oshkosh, 800 Algoma Blvd., Oshkosh, WI 54901, USA
[b] University of Minnesota Entomology Department, Bell Museum of Natural History, 1980 Folwell Ave., St. Paul, MN 55108, USA
[c] Laboratory of Genetics, Department of Biology, University of Turku, Turku 20014, Finland
[d] Department of Entomology and Nematology, University of Florida, Natural Area Drive Gainesville, FL 32611, USA
[e] Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA

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ABSTRACT

Within butterflies and moths, adult hematophagy is limited to species within the vampire moth genus *Calyptra*. These moths are placed within the subfamily Calpinae, whose other members are known to exhibit a broad range of feeding behaviors including those that can be considered 'piercers' of fruits or other hosts and 'tear feeders'. Here, we reconstruct a phylogenetic hypothesis of Calpinae using molecular data to test whether hematophagy in *Calyptra* arose from plant or animal-related behaviors. We use a Bayesian method of ancestral state reconstruction to determine the most likely feeding behaviors for the subtribes and genera within this lineage.

1. Introduction

Within the order Lepidoptera (butterflies and moths), the ability to pierce mammalian tissue and take a blood meal, hematophagy, is restricted to the moth genus *Calyptra* Ochsenheimer (Noctuoidea, Erebidae, Calpinae). These moths have adapted mouthparts that allow them to pierce through the skin of animals such as elephants, rhinoceros, and occasionally humans (see Bänziger, 1971, 1982, 1983, 1989, 2007). Of the seventeen species described (Bänziger, 1983), males of ten *Calyptra* species have been observed piercing mammalian skin and feeding on blood under natural or experimental conditions (Fig. 1; Bänziger, 1989; Zaspel et al., 2007). Males are facultatively hematophagous; females have not been documented feeding on blood. *Calyptra* are considered crude subcutaneous pool feeders and obtain blood through repeated piercing of blood vessels in the host (Bänziger, 1989). These species do not appear to be attracted to carbon dioxide like many hematophagous insects, nor are they biochemically adapted (e.g., anticoagulants in saliva) to overcome hemostasis (Zaspel pers. obs.). Feeding is painful for a human host (Bänziger, 1989; Zaspel et al., 2007). As far as known, they do not vector any zoonotic diseases.

Several hypotheses exist to explain the possible benefits of this facultative behavior. Hematophagous males may seek out mammalian hosts to obtain additional nutrients such as amino acids, salts, or sugars thereby increasing their longevity or fitness. Many Lepidoptera with functional mouthparts will visit feces or urine presumably to obtain amino acids or salts because salt (NaCl) and protein are limited in the herbivorous larval diet (Scoble, 1992). Hematophagous *Calyptra* species are likely not benefitting from amino acids, however. A prior study found that the blood meal itself does not increase longevity (Bänziger, 2007) nor have males tested positive for proteases; indicating proteins are not digested. Salts found in mammalian blood may be the important nutrient. Bänziger (2007) documented that males sequester up to 95% of the NaCl from their blood meals. Typically, male Lepidoptera 'puddle' or visit feces more frequently than females and some evidence exists that males transfer salts to the females during mating. These salts are used for egg production (Smedley and Eisner, 1995) or to replenish salt supplies depleted during oviposition (Adler and Pearson, 1982). Thus, a possible use of sodium is as a nuptial gift.

Adult hematophagy is limited to species of *Calyptra*, but these species belong to a larger subfamily Calpinae. As adults, members of the subfamily exhibit a broad range of feeding behaviors including those that can be considered 'piercers' of fruits or other hosts and 'tear feeders' (lachryphagy). The 'piercers' are capable of damaging fruit crops by piercing the skins to suck juices (Bänziger, 1982). For example, some species of *Eudocima* Billberg (e.g., *E. phalonia*), can occur in large numbers and cause extensive crop losses, much of which is attributable to fungi and bacteria that enter through the hole made by the moth or are introduced on the

* Corresponding author at: Department of Biology and Microbiology, University of Wisconsin Oshkosh, 800 Algoma Blvd., Oshkosh, WI 54901, USA.
E-mail address: zaspelj@uwosh.edu (J.M. Zaspel).

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Fig. 1. *Calyptra thalictri* feeding on human blood (photograph taken in Russian Federation 2008).

proboscis (Holloway, 2005). Bänziger (1971) hypothesized that the ability to pierce mammalian skin and suck blood in *Calyptra* spp. is directional and has evolved from the fruit-piercing habit.

Alternatively, some authors (Downes, 1973; Hilgartner et al., 2007) suggest that the skin-piercing, hematophagous behavior is derived from animal-associated feeding behaviors such as dung, urine, or tear feeding. Unlike generalist puddling behavior, lachryphagy tends to be a facultative behavior that has been documented in several lepidopteran families (Bänziger and Büttiker, 1969). Within Calpinae, one genus *Hemiceratoides*, has a known lachryphagous species; *Hemiceratoides hieroglyphica* was observed feeding on the tears of sleeping birds in Madagascar (Hilgartner et al., 2007).

To test whether hematophagy arose from plant (e.g., fruit piercing) or animal-related (lachryphagy) behaviors, a robust phylogeny is needed. A recent morphological study supports a monophyletic Calpini based on shared derived features of the proboscis and male–female genitalia (Zaspel and Branham, submitted for publication), another study based upon molecular markers supports a monophyletic subfamily Calpinae comprised of the tribes Calpini, Ophiderini and Phyllodini (Zahiri et al., 2012) but not the inclusion of Anomini and Scoliopterygini (Fibiger and Lafontaine, 2005; Lafontaine and Fibiger, 2006). The taxonomic sampling was insufficient to address the evolution of hematophagy in the subfamily. Here, we reconstruct the phylogeny of the Calpinae as recently redefined (Zahiri et al., 2012) with expanded taxon sampling and nine molecular markers. The resulting phylogeny was used to test whether the blood-feeding habit in *Calyptra* species evolved from plant (e.g., fruit piercing) or animal-related (lachryphagy) behaviors through reconstruction of ancestral states.

2. Materials and methods

2.1. Taxon sampling

Ingroup taxa were selected based on several sources: a checklist of Calpini (Zaspel and Branham, 2008), generic checklists (Nye, 1975; Poole, 1989), and previous species and generic associations published by other authors (Kitching and Rawlins, 1998; Fibiger and Lafontaine, 2005; Lafontaine and Fibiger, 2006). Taxa were also selected based on the results of Zahiri et al. (2012). Eight putative Calpinae genera of 20 possible (Fibiger and Lafontaine, 2005; Zaspel and Branham, 2008) were represented in the study. To test the monophyly of Calpinae, we included six outgroup species representing two related subfamilies, Eulepidoptinae and Hypocalinae and we rooted the topologies with *Panopoda rufimargo* (Eulepidotinae) based on prior results (Zahiri et al., 2011).

2.2. Molecular data

We extracted DNA from one or two legs, dried or freshly preserved in 96% ethanol, using the DNeasy tissue extraction kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. For each specimen, we sequenced portions of one mitochondrial marker (cytochrome c oxidase subunit I; COI), one ribosomal RNA gene region (28S rRNA D2 region), and seven nuclear markers: elongation factor-1α (EF-1α), ribosomal protein S5 (RpS5), carbamoylphosphate synthase domain protein (CAD), cytosolic malate dehydrogenase (MDH), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), isocitrate dehydrogenase (IDH) and wingless (Wahlberg and Wheat, 2008). PCR and sequencing protocols follow Wahlberg and Wheat (2008). Resulting chromatograms were checked and DNA sequences aligned by eye using the program BioEdit (Hall, 1999).

2.3. Phylogenetic analyses

Gene regions were combined and analyzed using various phylogenetic approaches. Parsimony analyses were undertaken using New Technology heuristic searches implemented in the program, TNT v 1.1 (Goloboff et al., 2003). New Technology searches (Goloboff, 1999) of tree space included the options Tree Fusion, Ratchet, Tree Drifting and Sectorial search (default parameters applied) until one minimal tree was found 1000 times. All characters were treated as unordered and equally weighted.

Model-based phylogenetic analyses were implemented using Maximum Likelihood (ML) and Bayesian Inference (BI). For ML analyses, a GTR + G model was selected as the most appropriate model of sequence evolution for each gene partition based on the Akaike Information Criterion using FindModel (http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html). ML analyses were conducted using the default settings on the web–server RAxML III BlackBox (Stamatakis et al., 2008). BI analyses were carried out by using the software MrBayes v3.1 (Ronquist et al., 2005). Bayesian analyses were run twice using the algorithm Markov Chain Monte Carlo (MCMC) for five million generations. Clade robustness was estimated by ML bootstrap with 1000 pseudoreplicates (Felsenstein, 1985), parsimony bootstrap with 1000 pseudoreplicates and posterior probabilities, in RAxML, TNT and MrBayes, respectively.

2.4. Reconstruction of ancestral feeding behaviors

We used a Bayesian method of ancestral state reconstruction (F81 + G model) as implemented in the newly developed program *Reconstruction of Ancestral States in Phylogenies* (RASP v.2.0) (Yan et al., 2011). This program is an updated version of *Statistical Dispersal-Vicariance Analysis* program (S-DIVA v.1.5; Yan et al., 2010). RASP determines the probabilities of each feeding behavior category for each node averaged over all sampled trees resulting from the BI phylogenetic analysis. For comparison, ancestral feeding behaviors were also reconstructed using Parsimony Ancestral

States as implemented in Mesquite v.2.75 (Maddison and Maddison, 2011). Recorded observations of feeding behaviors for the species included in the phylogenetic analysis were extracted from published reports and personal observations. Feeding behaviors were divided into the following functional feeding categories:

(A) Non-piercing fruit sucking, (B) Primary piercing of soft-skinned fruit; secondary piercing of thick- or hard-skinned fruit, (C) Primary piercing of thick-skinned fruit; secondary piercing of hard-skinned fruit, (D) Primary piercing of hard-skinned fruit, (E) Mammalian skin piercing and blood feeding, and (F) Lachryphagous. These categories have been fully described by Zaspel et al. (2011). Putative feeding behaviors were coded as binary-state characters for all terminal taxa (see Zaspel et al., 2011 for Refs.). Frequencies of feeding behaviors for clades were plotted as marginal distributions on a majority-rule consensus tree. Feeding behaviors with the highest RASP value (RV) for a given node are indicated by color and probabilities are given in Table 2.

3. Results

3.1. Phylogenetic results

Our phylogenetic analyses were based on sequence data from one ribosomal RNA gene region (662 bp of 28SD2), one mitochondrial gene region (1477 bp of COI) and seven nuclear gene regions (1240 bp of EF-1α, 400 bp of wingless, 617 bp of RpS5, 850 bp of CAD, 410 bp of MDH, 691 bp of GAPDH and 710 bp of IDH) for a total of 7069 aligned nucleotide sites. We were unable to amplify some genes for some taxa (Table 1).

Optimal topologies found by the three methods (parsimony, ML and BI) for the combined, complete datasets were identical and strongly supported monophyly of the subfamily Calpinae (BP $\geqslant$ 99; PP = 1) exclusive of the six outgroup species. The two representatives of Hypocalinae (*Hypsoropha*, *Hypocala*) formed a well-supported clade (BP $\geqslant$ 100; PP = 1). Within Calpinae, our analysis strongly supported monophyly of Calpini (BP $\geqslant$ 98; PP = 1), contained the type genus *Calyptra*, the New World *Gonodonta* Hübner, and Cosmopolitan genera *Plusiodonta* Guenée, and *Oraesia* Guenée. This clade placed sister to a well supported clade consisting of genera assigned to Ophiderini and Phyllodini (BPP71; PP = 0.98, Fig. 2A). There was strong support (BP $\geqslant$ 94; PP = 1) for the clade comprised of *Phyllodes* Boisduval and the African genus *Miniodes* Guenée. Ophiderini (BP $\geqslant$ 70; PP = 1) was represented by the large tropical genus *Eudocima* and the African genus *Hemiceratoides*.

3.2. Reconstruction of ancestral feeding behaviors

The results from the RASP analysis of adult feeding behaviors suggested a non-piercing ancestor for Calpinae + outgroups (Fig. 2B, Node I; Table 2, P = 100%). The best-supported ancestral feeding behavior for subfamily Calpinae is primary piercing of soft-skinned fruits (Fig. 2B, Node II; Table 2, P = 44%) with other feeding behaviors such as non-piercing and fruit sucking showing lower probabilities (P = 25%). The ancestral reconstruction analysis supported primary piercing of thick-skinned fruits and secondary fruit piercing of fruits for Calpini (*Plusiodonta, Oraesia, Calyptra, and Gonodonta*) (Fig. 2B, Node III; Table 2, P = 98%). The ancestral feeding behavior with the highest probability for the vampire moth genus *Calyptra* was primary piercing of thick-skinned fruits (Fig. 2B, Node VIII; Table 2, P = 93%). *Calyptra thalictri* and *C. minuticornis* have been reported feeding on blood under experimental and natural conditions, respectively (Fig. 2B, red[1] branches). However, there is only one known blood feeding incident for *C. lata* and none for *C. hokkaida*; adult feeding behaviors for *C. canadensis* are unknown. The ancestral feeding behavior for clade Phyllodini + Ophiderini is also primary piercing of soft-skinned fruit and secondary piercing of other fruit hosts (Fig. 2B, Node IV; Table 2, P = 72%). Primary piercing of soft-skinned fruits and secondary piercing of all fruits is the feeding behavior with the highest support for Phyllodini (Fig. 2B, Node V; Table 2, P = 66%). The ancestral feeding behavior with the highest probability for Ophiderini is also primary piercing of soft-skinned fruits and secondary fruit piercing (Fig. 2B, Node VI; Table 2, P = 62%), with primary piercing of hard-skinned fruits being the derived condition for species in the genus *Eudocima* (Fig. 2B, Node VII; Table 2, P = 85%). *Hemiceratoides sittaca* represents an independent origin of tear feeding in the subfamily (Fig. 2B, green branch).

The Parsimony Ancestral States analysis implemented in Mesquite v.2.75 (Maddison and Maddison, 2011) resulted in similar feeding behavior reconstructions for Calpinae. A non-piercing ancestor for Calpinae + outgroups was the reconstruction for Node I, with a primary piercing of soft-skinned fruit reconstruction for Calpinae (Fig. 2B, Node II). There were two independent origins for primary piercing of thick-skinned fruits: one for tribe Calpini (Fig. 2B, Node III) and another for Ophiderini (minus genus *Hemiceratoides*); There were two separate origins of skin piercing and blood feeding within the genus *Calyptra* (Fig. 2B, Node VIII, red branches).

4. Discussion

4.1. Phylogeny and evolution of adult feeding behaviors in Calpinae

As in Zahiri et al. (2011, 2012), we find strong support for the subfamily Calpinae that comprises three monophyletic tribes: Phyllodini, Ophiderini and Calpini (Fig. 2A). The tribe Phyllodini, consisting here of the type genus and the African genus *Miniodes*, is placed with strong support as sister to tribe Ophiderini, consisting of the pan-tropical genus *Eudocima* (of which *Ophideres* Boisduval, the type genus, is a synonym) and the African genus *Hemiceratoides*. The tribe Phyllodini share several features with Ophiderini. The adults of both tribes share the flash coloration of the hindwings coupled with cryptic, leaf-mimicking forewing facies. These two tribes together placed as sister to tribe Calpini (Fig. 2A). The proboscis of Calpini is distinctly modified, being robust, sharp, and with socketted hooks to facilitate the piercing of the tough skins of fruit and, in the case of *Calyptra*, mammals (Zaspel et al., 2011).

The results from the ancestral reconstruction of feeding behaviors analysis support the hypothesis of Bänziger (1971) that hematophagy in *Calyptra* evolved from the fruit-piercing habit as opposed to lachryphagy (Hilgartner et al., 2007) or other animal-associated feeding behaviors (Downes, 1973). *Hemiceratoides sittica* placed as a member of the Phyllodini–Ophiderini clade rather than sister to Calpini (Fig. 2A). Our results support the interpretation that lachryphagy in the genus *Hemiceratoides* represents a unique origin of this behavior within the subfamily.

While about half of the species in the genus *Calyptra* are considered to be facultative blood feeders, two were available for our molecular analysis (*C. thalictri* and *C. minuticornis*). Hematophagous *Calyptra* spp. are also obligatory fruit piercers in South and Southeast Asia (Bänziger, 2007) and thus are considered both fruit and blood feeders. The remaining *Calyptra* species included in the

[1] For interpretation of color in Figs. 1 and 2, the reader is referred to the web version of this article.

Table 1
List of taxa with voucher codes (specimen ID = specimen identity) and GenBank accession numbers. – = Gene region was not amplified for specimen; TG = type genus and TS = type species.

Family	Subfamily	Tribe	Species	Specimen ID	*28SD2*	*COI-LCO*	*COI-Jerry*	*EF1−α-begin*	*EF1-α-end*	*Wingless*	*GAPDH*	*RpS5*	*MDH*	*CAD*	*IDH*	Type status	Locality
Outgroup																	
Erebidae	Eulepidotinae	Eulepidotini	*Panopoda rufimargo*	RZ59-CWM-94-0304	–	HQ006231	HQ006934	HQ006326	HQ006418	HQ006839	HQ006488	HQ006743	HQ006651	HQ007018	HQ006564	TG	USA
Erebidae	Eulepidotinae	Eulepidotini	*Antiblemma fusciretículata*	RZ334-05-srnp-42594	JN674829	JN401297	JN401179	JN401411	–	JN400975	JN401620	JN401918	JN401828	–	–		Costa Rica
Erebidae	Eulepidotinae	Eulepidotini	*Eulepidotis rectimargo*	RZ12-05-srnp-16742	JN674830	HQ006162	HQ006960	HQ006259	HQ006354	HQ006771	–	HQ006678	HQ006588	HQ006960	HQ006511	TG	Costa Rica
Erebidae	Eulepidotinae	Unassigned	*Azeta ceramina*	RZ22-05-srnp-59274	–	HQ006182	HQ006886	HQ006278	HQ006373	HQ006790	–	HQ006697	HQ006605	HQ006978	HQ006527		Costa Rica
Erebidae	Hypocalinae		*Hypsoropha hormos*	RZ17-CWM-94-0228	–	HQ006176	HQ006880	HQ006273	HQ006367	HQ006784	HQ006449	HQ006692	HQ006600	HQ006972	HQ006521		USA
Erebidae	Hypocalinae		*Hypocala andremona*	RZ340-07-SRNP-56817	–	JN401295	JN401177	JN401410	JN401521	JN400980	JN401619	JN401917	JN401826	JN401078	JN401721	TG	Costa Rica
Ingroup																	
Erebidae	Calpinae	Phyllodini	*Miniodes phaeosoma*	RZ153-RMCA-UD-258	JN674831	HQ006173	HQ006877	HQ006270	HQ006364	HQ006782	HQ006446	HQ006689	HQ006597	HQ006969	HQ006518		Ghana
Erebidae	Calpinae	Phyllodini	*Phyllodes eyndhovii*	RZ56-AYK-04-0856	JN674832	HQ006228	HQ006931	HQ006323	HQ006415	HQ006836	–	HQ006740	HQ006648	–	HQ006561	TG	Taiwan
Erebidae	Calpinae	Phyllodini	*Phyllodes imperialis*	RZ546	–	JN674869	JN674851	JN674886	JN674902	JN674968	JN674919	JN674950	JN674934	–	JN674991	TG	Australia
Erebidae	Calpinae	Ophiderini	*Hemiceratoides sittaca*	RZ155-RMCA-UD-260	JN674833	JN401290	JN401172	JN401405	JN401516	JN400971	JN401614	JN401912	JN401820	–	JN401717		Ghana
Erebidae	Calpinae	Ophiderini	*Eudocima salaminia*	RZ338	–	JN401291	JN401173	JN401406	JN401517	JN400990	JN401615	JN401913	JN401821		JN401718	TG/TS	Hong Kong
Erebidae	Calpinae	Ophiderini	*Eudocima fullonia*	RZ16-A-0901	JN674834	HQ006174	HQ006878	HQ006271	HQ006365	HQ006783	HQ006447	HQ006690	HQ006598	HQ006970	HQ006519	TG	Malaysia
Erebidae	Calpinae	Ophiderini	*Eudocima divitiosa*	RZ210-RMCA-UD-411	–	JN674870	JN674852	JN674887	JN674903	JN674969	JN674920	JN674951	–	–	JN674992	TG	Ghana
Erebidae	Calpinae	Ophiderini	*Eudocima tyrannus*	RZ430	JN674835	JN674871	JN674853	JN674888	JN674904	JN674970	JN674921	JN674952	JN674935	–	JN674993	TG	Russian
Erebidae	Calpinae	Calpini	*Gonodonta uxor*	RZ335-05-srnp-32875	–	HQ006208	HQ006912	HQ006304	–	HQ006816	HQ006471	HQ006720	HQ006629	–	HQ006545		Costa Rica
Erebidae	Calpinae	Calpini	*Gonodonta lincus*	RZ417	JN674836	JN674872	JN674854	JN674889	JN674905	JN674971	JN674922	JN674953	JN674936	–	JN674994		Brazil
Erebidae	Calpinae	Calpini	*Gonodonta milla*	RZ421	JN674837	JN674873	JN674855	JN674890	JN674906	JN674972	JN674923	JN674954	JN674937	–	JN674995		Brazil
Erebidae	Calpinae	Calpini	*Gonodonta syrna*	RZ420	JN674838	JN674874	JN674856	JN674891	JN674907	JN674973	JN674924	JN674955	JN674938	–	JN674996		Brazil
Erebidae	Calpinae	Calpini	*Gonodonta fulvangula*	RZ423	–	JN674875	JN674857	JN674892	JN674908	JN674974	–	JN674957	JN674940	–	JN674997		Brazil
Erebidae	Calpinae	Calpini	*Gonodonta nutrix*	RZ432	JN674839	JN674876	JN674858	JN674893	JN674909	JN674975	–	JN674956	JN674939	JN674985	JN674998		USA
Erebidae	Calpinae	Calpini	*Gonodonta sicheas*	RZ419	–	JN674877	JN674859	JN674894	JN674910	JN674976	JN674925	JN674959	JN674941	–	JN674999		Ecuador
Erebidae	Calpinae	Calpini	*Plusiodonta nitissima*	RZ333-05-srnp-3890	JN674840	HQ006207	HQ006911	HQ006303	–	HQ006815	HQ006470	HQ006719	HQ006628	–	–		Costa Rica
Erebidae	Calpinae	Calpini	*Plusiodonta coelonota*	RZ106	JN674841	JN674878	JN674860	JN674895	JN674911	JN674977	JN674926	JN674960	JN674942	–	JN675000		Hong Kong
Erebidae	Calpinae	Calpini	*Plusiodonta casta*	RZ429	JN674842	JN674879	JN674861	JN674896	JN674912	JN674978	JN674927	JN674961	JN674943	–	JN675001		Russian
Erebidae	Calpinae	Calpini	*Oraesia emarginata*	RZ102	JN674843	HQ006159	HQ006864	HQ006256	HQ006351	HQ006768	HQ006439	HQ006675	HQ006586	HQ006958	HQ006508	TS	Hong Kong
Erebidae	Calpinae	Calpini	*Oraesia excavata*	RZ337	–	JN401293	JN401175	JN401408	JN401519	JN400987	JN401617	JN401915	JN401824	JN401076	JN401719		Hong Kong
Erebidae	Calpinae	Calpini	*Oraesia excavata*	RZ434	JN674844	JN674880	JN674862	–	JN674913	–	–	JN674958	–	JN674986	–		USA
Erebidae	Calpinae	Calpini	*Oraesia nobilis*	RZ422	JN674845	JN674881	JN674863	JN674897	JN674914	JN674979	JN674928	JN674962	JN674944	–	JN675002		Brazil
Erebidae	Calpinae	Calpini	*Oraesia glaucocheila*	RZ418	JN674846	JN674882	JN674864	JN674898	JN674915	JN674980	JN674929	JN674963	JN674945	JN674987	JN675003		Brazil
Erebidae	Calpinae	Calpini	*Oraesia rectistria*	RZ433	JN674847	JN674883	JN674865	JN674899	JN674916	JN674981	JN674930	JN674964	JN674946	–	JN675004		Nepal
Erebidae	Calpinae	Calpini	*Calyptra thalictri*	MM00963	JN674848	HQ006156	HQ006861	HQ006252	HQ006348	HQ006763	HQ006435	HQ006671	HQ006582	HQ006955	HQ006504	TG/TS	Finland
Erebidae	Calpinae	Calpini	*Calyptra hokkaida*	RZ336-AYK-04-0999-15	–	JN401292	JN401174	JN401407	JN401518	JN400972	JN401616	JN401914	JN401823	JN401075	JN401718	TG	Japan
Erebidae	Calpinae	Calpini	*Calyptra lata*	RZ431	JN674849	JN674884	JN674866	JN674900	JN674917	JN674982	JN674931	JN674965	JN674947	JN674988	–	TG	Russian
Erebidae	Calpinae	Calpini	*Calyptra canadensis*	CTW2	JN674850	–	JN674867	–	–	JN674983	JN674933	JN674967	JN674949	JN674989	JN675005	TG	USA
Erebidae	Calpinae	Calpini	*Calyptra minuticornis*	RZ514-N46	–	JN674885	JN674868	JN674901	JN674918	JN674984	JN674932	JN674966	JN674948	JN674990	JN675006	TG	Malaysia

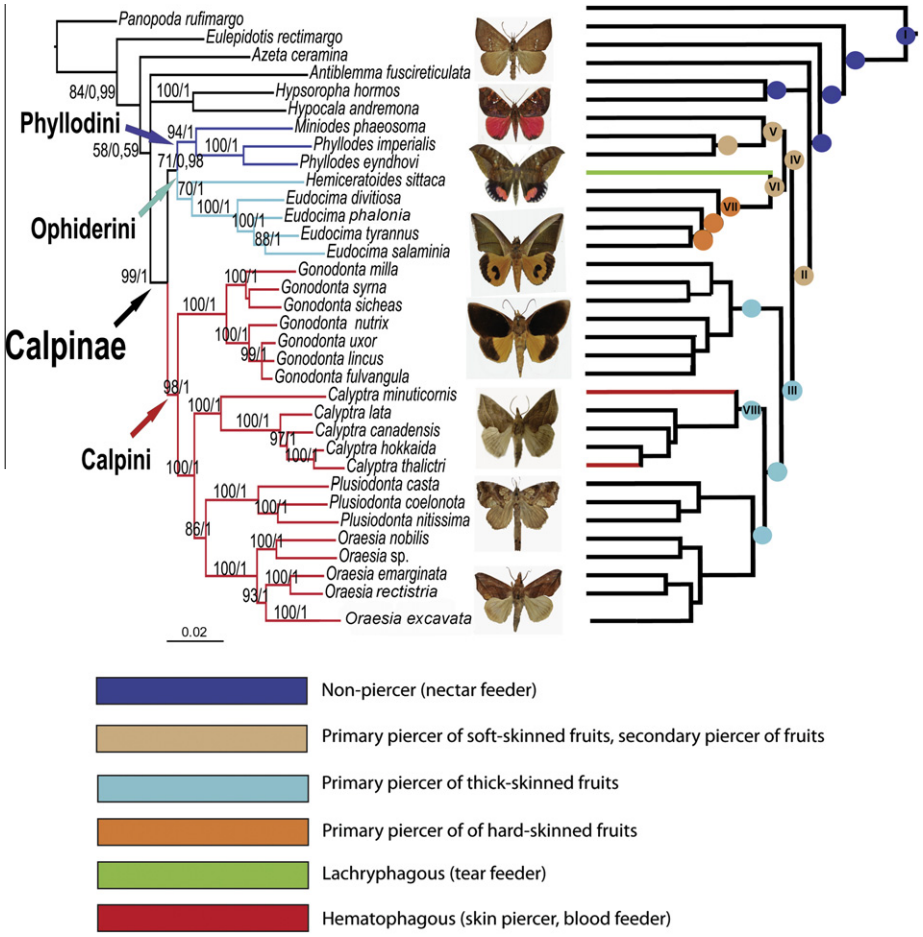

Fig. 2. (A) Phylogenetic hypothesis of the subfamily Calpinae (Noctuoidea, Erebidae) based on a Bayesian Inference analysis, along with outgroups. Clades representing tribes are coloured. Support values under the two support measures (ML Bootstrap/posterior probabilities) shown next to the branches. Names of moths shown in figure from top to bottom are: *Hypsoropha hormos* Hübner, *Miniodes phaeosoma* Hampson (Phyllodini), *Phyllodes imperialis* Druce (Phyllodini), *Eudocima salaminia* (Cramer) (Ophiusini), *Gonodonta sicheas* (Cramer) (Calpini), *Calyptra thalictri* (Borkhausen) (Calpini), *Plusiodonta casta* (Butler) (Calpini) and *Oraesia excavata* (Butler) (Calpini). (B) Summary of Bayesian ancestral state reconstruction analysis for major Calpinae lineages, optimized in the program RASP (Yan et al., 2011). Ancestral feeding reconstructions with highest marginal probabilities are indicated at each node. Red branches indicate hematophagous species; green branch indicates lachryphagous species.

Table 2
Marginal probabilities of feeding behaviors for major clades based on reconstruction of ancestral states analysis (RASP v.2.0).

Clade	I	II	III	IV	V	VI	VII	VIII
Feeding behavior (s)	Non-piercing fruit-sucking	Primary piercing of soft-skinned fruit	Primary piercing of thick-skinned fruit	Primary piercing of soft-skinned fruit	Primary piercing of soft-skinned fruit	Primary piercing of soft-skinned fruit	Primary piercing of hard-skinned fruit	Primary piercing of thick-skinned fruit
Marginal probabilities (*P*)	100%	44%	98%	72%	66%	62%	85%	93%

study have not been reported feeding on blood, however, a lack of recorded observations of feeding behavior in some species does not necessarily mean that the species in question do not pierce mammalian skin. In such situations, there is simply no evidence that suggests they do. Presently, we consider these remaining species to be exclusive piercers of thick-skinned fruits (Bänziger, 1971, 1982, 1989; Zaspel et al., 2007; Zaspel and Branham, submitted for publication). Thus, fruit-piercing and hematophagous moths in Calpinae exhibit a grade of feeding behaviors and types of piercing (e.g., primary vs. secondary and nectar feeding) some of which are not mutually exclusive. For this reason, more than one ancestral feeding behavior may be highly supported at any given node (e.g., Primary piercing of soft-skinned fruits and non-piercing/fruit sucking; Node II). Our results support a directional *addition* of feeding types from nectar feeding to fruit piercing, to skin piercing and blood feeding rather than a directional *progression* as hypothesized by Bänziger (1971). This work also suggests blood feeding is restricted to one genus within Calpinae, *Calyptra* (Fig. 2B). Blood feeding records continue to be documented in recent primary literature (*C. thalictri*, Zaspel et al., 2007) and recorded on recent collecting expeditions (*C. lata*, Zaspel unpublished field observations 2008). Thus, blood feeding may occur in other *Calyptra* species but remains to be demonstrated.

In conclusion, the origin of male adult hematophagy in Calpini is reconstructed as arising from a fruit-piercing ancestor. Selection for salt collection and transfer to females is the most likely explanation for this facultative behavior. Additional physiological and behavioral work is needed to confirm this explanation.

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