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

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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 species 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
whether the blood-feeding habit in nine molecular markers. The resulting phylogeny was used to test the monophyly of Calpinae (Zaspel and Branham, 2008), generic checklists (Nye, 2004), and we rooted the topologies with four outgroup species representing two related subfamilies, Eupleuropteryginae and Hypocalinae (Fibiger and Lafontaine, 2005; Zahiri et al., 2011). This program is an updated version of Statistical Dispersal-Vicariance Analysis (S-DIVA v.1.5; Yan et al., 2010). RASP determines the probabilities of each feeding behavior categories 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

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

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 (Kitting 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 Calpini 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 (Fibiger and Lafontaine, 2005; 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 for 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 Ill 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 pseudo-replicates (Felsenstein, 1985), parsimony bootstrap with 1000 pseudo-replicates 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-1a, 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 represented by the large tropical genus Eudocima clade consisting of genera assigned to Ophiderini and Phyllodini and Oraesia. 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-1a, 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).

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, Onesia, 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 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 Phylldodini + 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 Phylldodini (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 sitiaca 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 Minioidea, is placed with strong support as sister to tribe Ophiderini, consisting of the pan-tropical genus Eudocima (of which Ophideres Boisdval, 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 sitiaca placed as a member of the Phylldodini–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
<table>
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<th>Family</th>
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<th>Tribe</th>
<th>Species</th>
<th>GenBank accession numbers</th>
<th>Locality</th>
<th>Type status</th>
<th>Type Locality</th>
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<td>TG Costa Rica</td>
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**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.
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.

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

<table>
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<th>Clade</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
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<tbody>
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<td>Feeding behavior (s)</td>
<td>Non-piercing fruit-sucking</td>
<td>Primary piercing of soft-skinned fruit</td>
<td>Primary piercing of thick-skinned fruit</td>
<td>Primary piercing of soft-skinned fruit</td>
<td>Primary piercing of soft-skinned fruit</td>
<td>Primary piercing of hard-skinned fruit</td>
<td>Primary piercing of thick-skinned fruit</td>
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<tr>
<td>Marginal probabilities (P)</td>
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<td>44%</td>
<td>98%</td>
<td>72%</td>
<td>66%</td>
<td>62%</td>
<td>85%</td>
<td>93%</td>
</tr>
</tbody>
</table>

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: *Hysorophora hormos* Hübner, *Miniodes phaeosoma* Hampson (Phyllodini), *Phyllodes imperialis* Drude (Phyllodini), *Eudocima salaminia* (Cramer) (Ophiussini), *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.
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Zaspel, M.J., Branham, M.A., 2008. World checklist of tribe Calpini (Lepidoptera: Erebidae) and the evolution of blood feeding in the vampire moths based on morphological data. 
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