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Short Communication

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* 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

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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