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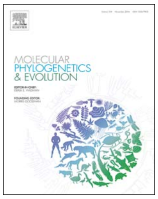

Evolution of *Hypolimnas* butterflies (Nymphalidae): Out-of-Africa origin and *Wolbachia*-mediated introgression

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ABSTRACT

Hypolimnas butterflies (Nymphalidae), commonly known as eggflies, are a popular model system for studying a wide range of ecological questions including mimicry, polymorphism, wing pattern evolution, and *Wolbachia*-host interactions. The lack of a time-calibrated phylogeny for this group has precluded understanding its evolutionary history. We reconstruct a species-level phylogeny using a nine gene dataset and estimate species divergence times. Based on the resulting tree, we investigate the taxon's historical biogeography, examine the evolution of host plant preferences, and test the hypothesis that the endosymbiotic bacterium *Wolbachia* mediates gene transfer between species. Our analyses indicate that the species are grouped within three strongly supported, deeply divergent clades. However, relationships among these three clades are uncertain. In addition, many *Hypolimnas* species are not monophyletic or monophyletic with weak support, suggesting widespread incomplete lineage sorting and/or introgression. Biogeographic analysis strongly indicates that the genus diverged from its ancestor in Africa and subsequently dispersed to Asia; the strength of this result is not affected by topological uncertainties. While the larvae of African species feed almost exclusively on Urticaceae, larvae of species found further east often feed on several additional families. Interestingly, we found an identical mitochondrial haplotype in two *Hypolimnas* species, *H. bolina* and *H. alimena*, and a strong association between this mitotype and the *Wolbachia* strain *w*Bol1a. Future investigations should explore the plausibility of *Wolbachia*-mediated introgression between species.

1. Introduction

The Old World butterfly genus *Hypolimnas* is remarkable for the highly varied wing patterns of the taxon's 29 described species (Lamas, 2015; Table A1), which are commonly known as eggflies. Interspecific variability is so marked because different *Hypolimnas* species are Batesian and/or Müllerian mimics of different, unpalatable model species (Marsh et al., 1977; Vane-Wright et al., 1977). For this reason, the genus has been a model for ecological and evolutionary studies, beginning with Poulton's (1897) writings on mimicry in *H. bolina* and *H. misippus*, and Marshall's (1902) observations on mimicry and polymorphism of *H. dubius* (now known as *H. anthedon*). Since then, field and laboratory studies on members of this genus have provided insights into the selective forces that change frequencies of polymorphic forms in populations (Edmunds, 1966, 1969; Smith, 1973, 1976; Turner, 1978; Gordon, 1987; Gordon and Smith, 1998; but see Clarke et al., 1989). Classical genetics research (Clarke and Sheppard, 1975; Smith and Gordon, 1987) and pharmacological investigations (Marsh et al.,

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1977) on *Hypolimnas* species have furthered understanding of wing pattern formation in butterflies. Although investigations on the relevance of wing patterns are mostly limited to two species, *H. bolina* and *H. misippus*, field observations suggest geographic patterns for model-mimic relationships among different lineages. While African species usually mimic *Amauris* and/or *Acraea*, Asian species generally mimic *Danaus* and/or *Euploea* (Table A1 and references therein). Females of the exceptionally widespread, pantropical species *H. misippus* mimic *Danaus chrysippus* through out the Old World - a species that is itself unusually widespread (Vane-Wright et al., 1977; Braby et al., 2015).

Hypolimnas butterflies have been the subject of research on reproductive diapause (Kemp, 2001a; Pieloor and Seymour, 2001), seasonal polyphenism (Kemp, 2000; Kemp and Jones, 2001), territorial behaviour (Stride, 1956; Kemp, 2001b; Kemp and Rutowski, 2001), and parental care (Nafus and Schreiner, 1988; Schreiner and Nafus, 1991). Recent studies on *Hypolimnas* have focused on the microscopic ornamentation of wing scale arrangements (Saito, 2002; Kemp and Macedonia, 2006; Siddique et al., 2016). Moreover, decade-long investigations on *Hypolimnas* populations (Charlat et al., 2009; Duplouy et al., 2010) and breeding experiments (Dyson et al., 2002) have made *Hypolimnas* a model for studying population-level and evolutionary interactions between insects and alpha-proteobacterial lineage *Wolbachia*. This intracellular, bacterial parasite is found in more than half of all insect species. It is generally inherited maternally through its presence in the cytoplasm of the ovum, and the bacterium can manipulate reproduction of its host to favor production of females (Werren et al., 2008). Interestingly, studies on *Hypolimnas-Wolbachia* interactions have provided clear evidence for interference among selfish genetic elements (Charlat et al., 2006), and W.D. Hamilton's theory on rapid sex-ratio flux due to an arms race between sex-ratio distorters and counteracting suppressor genes (Hornett et al., 2006; Charlat et al., 2007).

However, the absence of information on phylogenetic relationships among *Hypolimnas* species precludes broader understanding of evolutionary patterns in this group (Vane-Wright et al., 1977; Kemp et al., 2014). For instance, studies on butterfly-hostplant associations, historical biogeography, and wing-pattern evolution within the genus are hindered by the lack of a robust phylogeny.

Hypolimnas species are found in all tropical regions. Only one species, *H. misippus*, is found in Central and South America (where it is quite likely introduced) and it is distributed on every continent but Europe and Antarctica. *Hypolimnas bolina* is also unusually widespread from Oceania west to tropical Asia and Madagascar (Vane-Wright et al., 1977; Tsukada 1985; Parsons, 1998; Larsen, 2005). Although members of the genus are morphologically similar to each other, there is no known synapomorphic morphological character that validates the monophyly of the genus (Vane-Wright et al., 1977). Previous phylogenies of Nymphalinae (Wahlberg et al., 2005, 2009; Su et al., 2017) included few representative species of *Hypolimnas*, and recovered the genus as monophyletic and sister to the African genus *Precis*. The most comprehensive phylogeny of the group (Kodandaramaiah, 2009) included data from a maximum of three genes from 13 species, and was unable to resolve intrageneric relationships with strong support. Furthermore, the taxonomy of many species (*e.g., H. bolina*) has been challenging owing to their extensive wing pattern polymorphism (Vane-Wright et al., 1977).

In this study, we reconstruct phylogenetic relationships among 113 *Hypolimnas* specimens collected throughout the genus' entire range using up to 7013 base pairs (bp) comprising sequences from one mitochondrial and eight nuclear loci. We also estimate divergence times of the species. Based on our results, we investigate the historical biogeographic patterns, evolution of larval host plant preferences, and examine the veracity of current species-level taxonomy. We also investigate patterns of *Wolbachia* infection in the group and evaluate the plausibility of *Wolbachia*-mediated gene transfer between species.

2. Methods

2.1. Taxon and marker sampling

We sampled 26 out of 29 recognized *Hypolimnas* species (Table A1). We were unable to sample *H. aubergeri, H. chapmani,* and H. *euploeoides*. The latter species is found on the Admiralty Islands north of New Guinea, but the others are African. Note that Lamas (2015) regards *H. sumbawana* as a subspecies of *H. anomala*, but our results demonstrate that *sumbawana* is clearly distinct from *anomala* (they are not sister taxa). Wherever possible, we included multiple specimens of each species from different geographic areas, for a total of 113 specimens. Preliminary species-level identifications were based on traditional morphological characters. We attempted to amplify eight nuclear loci (ArgKin, CAD, EF1a, GAPDH, MDH, RpS2 and RpS5, Wgl) totaling 5538 bp and one 1475 bp mitochondrial locus (COI) from each specimen using the primers and protocols described in Wahlberg and Wheat (2008). A three loci dataset (COI, EF1a, and Wgl) from 13 specimens (a subset of the species used here) that was previously used for phylogenetic analysis (Kodandaramaiah, 2009) was compiled from GenBank. Sequences of the sister genus *Precis* (Wahlberg et al., 2005, 2009) were also obtained from GenBank and served as the outgroup.

2.2. Phylogenetic analyses

We used PartitionFinder v1.1.1 (Lanfear et al., 2012) to determine optimal data partitioning schemes and evolutionary models before performing ML analyses using RAxML v8 (Stamatakis, 2014) on the CIPRES web portal (Miller et al., 2010). Nodal support values were calculated from 1000 bootstrap trees. We performed separate ML analyses for (a) the mitochondrial dataset, (b) the nuclear dataset, and (c) the combined nuclear and mitochondrial dataset of all sequenced loci.

We used MrBayes v3.2 (Ronquist et al., 2012) on the CIPRES web portal (Miller et al., 2010) for Bayesian Inference (BI) of phylogeny of the combined nuclear and mitochondrial dataset and evolutionary models from PartitionFinder v1.1.1 (Lanfear et al., 2012). The program MrBayes was set to estimate the base frequencies and shape parameters from the data. We performed two independent runs with two chains per run for 10 million generations, sampling trees every 1000 generations. The convergence of independent runs was analyzed from the values of Potential Scale Reduction Factors (value close to one indicates convergence) (Gelman and Rubin, 1992). The consensus tree was reconstructed after discarding the first 25% of trees as burn-in. Posterior probability (PP) values provided a measure of nodal support.

2.3. Molecular dating and diversification analyses

For the molecular dating analysis, we included sequences of all other genera in Junoniini (the tribe to which *Hypolimnas* belongs) (Wahlberg et al., 2005; Kodandaramaiah and Wahlberg, 2007) and used four members of the sister tribe Victorinini (Wahlberg et al., 2005; Su et al., 2017) as outgroup. We estimated divergence times using BEAST v2.4 (Bouckaert et al., 2014) taking into account the secondary calibrations from Wahlberg et al. (2009) which is a family-level study of Nymphalidae that used seven fossils and host plant ages for node calibrations, and is based on a dataset of 10 gene regions. A recent paper by Su et al. (2017) on the subfamily Nymphalinae suggests that all clades are older than in Wahlberg et al. (2009), using a dataset of 3 gene regions calibrated with only two of the above mentioned seven fossils (*Vanessa amerindica* and *Prodryas persephone*: late Eocene) and host plant ages. The placement of these two fossils in the butterfly tree has recently been questioned in a morphological revision of all butterfly fossils (de Jong, 2017), with a recommendation to place the former fossil at the base of the subfamily Nymphalinae (rather than within the tribe Nymphalini) and not to use the later fossil at all, due to ambiguous characters. In a very recent study taking these recommendations into