The phylogenetic positions of *Bhagadatta* Moore, 1898, *Kumothales* Overlaet, 1940 and *Harmilla* Aurivillius, 1892 (Lepidoptera, Nymphalidae, Limenitidinae) based on molecular data

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Abstract. We sequenced multiple genes from the enigmatic genera *Bhagadatta* Moore, 1898, *Kumothales* Overlaet, 1940 and *Harmilla* Aurivillius, 1892 (Nymphalidae, Limenitidinae) and analysed them together with a large published dataset. We find that *Bhagadatta* is sister to the genera *Cymothoe* Hübner, 1819+*Harma* Doubleday, 1848, and that *Kumothales* is sister to these three. *Harmilla* is nested within the genus *Euriphene* Boisduval, 1847. We thus transfer *Kumothales* and *Bhagadatta* to the tribe *Cymothoini*, and we synonymise *Harmilla* **syn. nov.** with *Euriphene*.

Introduction

The systematics of the butterfly subfamily Limenitidinae has only recently started to unravel (Wahlberg et al. 2009; Dhungel and Wahlberg 2018; Wu et al. 2019). For a long time the subfamily was used as the "trash can" taxon for taxa that could not easily be placed in other subfamilies of the butterfly family Nymphalidae, including species now placed in the unrelated subfamilies Biblidinae, Pseudergolinae and Cyrestinae. Molecular data have clearly defined the subfamily (Wahlberg et al. 2003, 2009), and the latest comprehensive study has shown that the subfamily can be divided into seven strongly supported tribes, three of which were only recently described (Dhungel and Wahlberg 2018).

The study by Dhungel and Wahlberg (2018) attempted to sample all genera putatively belonging to Limenitidinae, but they were unable to sample the genera *Kumothales* Overlaet, 1940, *Harmilla* Aurivillius, 1892, *Euryphurana* Hecq, 1992, *Euryphaedra* Staudinger, 1891, and *Neurosigma* Butler, 1868. The phylogenetic positions, and hence the tribal affiliations, of these five genera have never been studied explicitly before, and they have all been provisionally placed in the tribe Adoliadini. The morphology of *Harmilla*, *Euryphurana* and *Euryphaedra* clearly place them close to other African Adoliadini of the genera *Euriphene* Boisduval, 1847, *Euryphura* Staudinger, 1891, and *Euphaedra* Hübner, 1819 (as suggested by the names of the latter two). The position of *Neurosigma* is likely close to the Asian members of Adoliadini, but this remains to be determined. The position of the monotypic *Kumothales* is not clear at the moment (Williams 2018).

In addition, Dhungel and Wahlberg (2018) could only include the COI sequences from a published mitogenome of *Bhagadatta* Moore, 1898 (Wu et al. 2014). The position of *Bhagadatta* as sister to *Cymothoe* Hübner, 1819+*Harma* Doubleday, 1848 was surprising, but poorly supported (Dhungel and Wahlberg 2018). *Bhagadatta* is a monotypic genus that is found from Assam to North of Indochina (Igarashi and Fukuda 2000), while *Harma* and *Cymothoe* comprise 83 species found only in sub-Saharan Africa (van Velzen et al. 2013).

Here we determine the phylogenetic positions of the genera *Bhagadatta*, *Kumothales* and *Harmilla* based on a multigene dataset. We analyse the new sequences with the dataset published by Dhungel and Wahlberg (2018) and revise the classification of Limenitidinae accordingly.

Material and methods

We sequenced specimens of *Bhagadatta austenia* (Moore, 1898), *Kumothales inexpectata* Overlaet, 1940, *Harmilla elegans* Aurivillius, 1892 and *Harmilla hawkeri* Joicey & Talbot, 1926 for one mitochondrial gene and several nuclear genes, depending on available resources. The following genes were sequenced for *Bhagadatta*: COI, EF1a, RpS5 and wingless. *Kumothales* was sequenced for ArgKin, COI, CycY, EF1a, GAPDH, MDH, PSb, RpS2 and wingless. *Harmilla elegans* was sequenced for ArgKin, COI, CycY, EF1a, GAPDH,RpS5 and wingless. *Harmilla hawkeri* for ArgKin, COI, CycY, EF1a, GAPDH, MDH, PolII, PSb, RpS2, RpS5, UDPG6DH and wingless. Molecular protocols followed published studies (Wahlberg and Wheat 2008; Wahlberg et al. 2016; Dhungel and Wahlberg 2018) and the data were combined with the dataset of Dhungel and Wahlberg (2018), which is based on 18 gene regions. All new sequences are available on NCBI GenBank with the accession numbers MT267731–MT267775. Sequences were curated and managed using VoSeq (Peña and Malm 2012).

Phylogenetic analyses were carried out using IQ-TREE 1.6.10 (Nguyen et al. 2015) in a maximum likelihood framework. The data were partitioned by gene and analysed with the partition finding (Chernomor et al. 2016) and model finding (Kalyaanamoorthy et al. 2017) algorithms of IQ-TREE (using the command MFP+MERGE). Robustness of the results were assessed using UFBoot2 (Hoang et al. 2018) and a SH-like approximate likelihood ratio test (Guindon et al. 2010), each with 1000 replicates. Analyses were run on the CIPRES server (Miller et al. 2010).

Results

The phylogenetic positions of the three focal taxa were well supported (Fig. 1). *Bhagadatta* remains sister to *Cymothoe*+*Harma* with strong support, with *Kumothales* coming out sister to these three genera. *Harmilla* is clearly nested within the genus *Euriphene*, with *H. elegans* and *H. hawkeri* coming out expectedly as sister species.

Discussion

Based on our results, the recently described Cymothoini (Dhungel and Wahlberg 2018) includes the monotypic genera *Bhagadatta* and *Kumothales*. *Kumothales* has been considered to be *incertae*

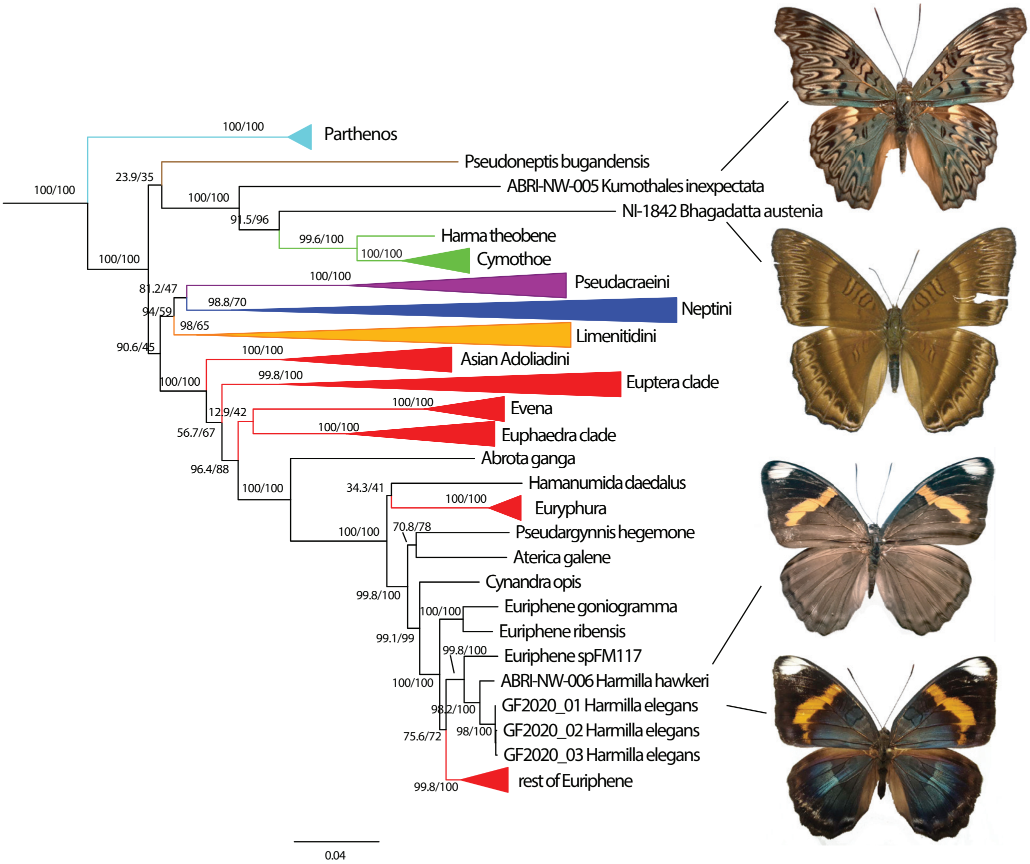

Figure 1. Phylogenetic positions of *Kumothales inexpectata*, *Bhagadatta austenia*, *Harmilla elegans* and *Harmilla hawkeri* based on a multigene analysis. All other taxa taken from the study by Dhungel and Wahlberg (2018). Numbers to the left of each node are the values for the SH-like approximate likelihood ratio test/UltraFast Bootstrap calculated in IQ-TREE.

sedis in Limenitidinae (Williams 2018). Our results place it without a doubt as sister to the rest of Cymothoini. The single species, *K. inexpectata*, is found in the rainforests of the Congo Basin. The position of the monotypic *Bhagadatta* as sister to *Harma*+*Cymothoe* continues to be surprising, but is now corroborated with more data. It appears that *Bhagadatta* has colonised Asia from Africa, as it is nested within the African clade Cymothoini.

Harmilla is nested well within *Euriphene* and thus we synonymise the former (syn. nov.) with *Euriphene*, making *Euriphene elegans* (comb. nov.) and *Euriphene hawkeri* (comb. nov.) the valid combinations for the species. *Euriphene elegans* and *E. hawkeri* are also restricted to the Congo Basin of Africa. According to Williams (2018), the taxon *hawkeri* is considered to be a subspecies of *Euriphene elegans* Aurivillius, 1892. However, our molecular results suggest that *E. hawkeri* is distinct genetically from *E. elegans*, with the DNA barcode (COI gene) showing a 4% K2P distance. This is comparable to other closely related sister species in Lepidoptera (Mutanen et al. 2016). Clearly this requires further study, but for now we suggest that the two taxa are considered as separate sister species.

Euriphene is a large genus with 75 (including *E. elegans* and *E. hawkeri*) described species (Williams 2018). Only seven species have been included in earlier molecular studies. Dhungel and Wahlberg (2018) show that several genera are closely related to *Euriphene*, with the monotypic *Cynandra* Schatz, 1887, being sister to the genus. The species poor *Pseudargynnis* Karsch, 1892, and *Aterica* Boisduval, 1833 (a total of three described species) form the sister clade to *Euriphene*+*Cynandra*. Whether *Euriphene* is monophyletic with regard to these three genera needs to be determined with greater taxon sampling of *Euriphene*. In addition, the genera *Euryphura* and *Hamanumida* Hübner, 1819 are sister to the aforementioned genera, and together all six genera form a strongly supported clade which is sister to the Asian *Abrota ganga* Moore, 1857. The so far unsampled monotypic genus *Euryphurana* is likely to belong to the *Euriphene* clade.

The pattern of a monotypic Asian genus being sister to a species rich African clade (*Abrota* – *Euriphene* clade) is mirrored in the *Bhagadatta* – *Cymothoe* case, and it would be interesting to investigate biogeographic processes behind these two cases. Did the common ancestor of both clades colonise Asia from Africa (both are nested within African taxa)? Did this happen at around the same time? Answers to these questions would enhance our understanding of the evolutionary history of Nymphalidae as a whole.

In conclusion, molecular data have allowed us to determine the phylogenetic positions of previously enigmatic taxa, and through that given us a more stable classification. Within Limenitidinae, much work remains to be done. Three described genera have yet to be sequenced (*Euryphurana*, *Euryphaedra*, and *Neurosigma*). In addition, several recent studies have shown that various genera within Limenitidinae are not monophyletic (Ebel et al. 2015; Dhungel and Wahlberg 2018; Wu et al. 2019; Toussaint et al. 2020), suggesting that we have some way to go before we reach a stable classification for the second most species rich subfamily of Nymphalidae.

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