Systematic Entomology

Systematic Entomology (2020), DOI: 10.1111/syen.12435

A phylogenomic tree inferred with an inexpensive PCR-generated probe kit resolves higher-level relationships among *Neptis* butterflies (Nymphalidae: Limenitidinae)

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Abstract. Recent advances in obtaining reduced representation libraries for next-generation sequencing permit phylogenomic analysis of species-rich, recently diverged taxa. In this study, we performed sequence capture with homemade PCR-generated probes to study diversification among closely related species in a large insect genus to examine the utility of this method. We reconstructed the phylogeny of *Neptis* Fabricius, a large and poorly studied nymphalid butterfly genus distributed throughout the Old World. We inferred relationships among 108 *Neptis* samples using 89 loci totaling up to 84 519 bp per specimen. Our taxon sample focused on Palearctic, Oriental and Australasian species, but included 8 African species and outgroups from 5 related genera. Maximum likelihood and Bayesian analyses yielded identical trees with full support for almost all nodes. We confirmed that *Neptis* is not monophyletic because *Lasippa heliodore* (Fabricius) and *Phaedyma amphion* (Linnaeus) are nested within the genus, and we redefine species groups for *Neptis* found outside of Africa. The statistical support of our results demonstrates that the probe set we employed is useful for inferring phylogenetic relationships among *Neptis* species and likely has great value for intrageneric phylogenetic reconstruction of Lepidoptera. Based on our results, we revise the following two taxa: *Neptis heliodore* **comb. rev.** and *Neptis amphion* **comb. rev.**

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Introduction

Recent advancements in sample preparation for next-generation sequencing permit the isolation and sequencing of multiple homologous loci from many individuals in a fast and cost-effective manner (McCormack *et al.*, 2013). Molecular data have helped resolve relationships among many taxa for which morphological information is limited or misleading (San Jose *et al.*, 2018; Żyła & Solodovnikov, 2019), and increased numbers of markers generally result in more strongly supported phylogenies (Rokas *et al.*, 2003; Zwick *et al.*, 2011). Several phylogenomic laboratory methods have been used to infer phylogenetic relationships in arthropods (Blaimer *et al.*, 2015; Che *et al.*, 2017; Breinholt *et al.*, 2018; Espeland *et al.*, 2018; Klopfstein *et al.*, 2019; Machado *et al.*, 2019). One of these reduced representation methods for isolating homologous loci is sequence capture, also known as targeted capture or hybrid enrichment. This method can isolate many orthologous loci, even from low quality or degraded DNA samples (Hawkins *et al.*, 2016; Knyshov *et al.*, 2019), and has been increasingly used in phylogenetic research of many different groups (Haddad *et al.*, 2018; Courcelle *et al.*, 2019; Liu *et al.*, 2019; Van Dam *et al.*, 2019; Zhang *et al.*, 2019). This technique is particularly useful when working with rare or geographically isolated species represented only by dried specimens in museum collections that would be difficult to collect as fresh specimens. Since DNA in museum specimens is already fragmented, approaches that use a restriction enzyme or other technique to further fragment the genome, such as genotyping-by-sequencing or RADseq (de la Harpe *et al.*, 2017), cannot be employed.

Many approaches for sequence capture have been developed, including *de novo* transcriptome-based exon capture (Bi *et al.*, 2012; Portik *et al.*, 2016), anchored enrichment (Lemmon *et al.*, 2012) and sequencing of ultraconserved elements (Faircloth *et al.*, 2012; McCormack *et al.*, 2013). Using PCR-generated probes to capture target loci is cost effective because it only requires standard lab equipment and reagents and does not rely on a commercially produced probe kit. Further, it allows targeting of specific, known loci that can be combined with data obtained through Sanger sequencing (Maricic *et al.*, 2010; Peñalba *et al.*, 2014). Zhang *et al.* (2019) employed a novel sequence capture method to develop PCR-generated probes to capture 94 single-copy, protein coding nuclear markers for Lepidoptera (butterflies and moths) – the second largest order of insects. While Zhang *et al.* (2019) demonstrated the usefulness of these markers for inferring deep relationships among families; it is not yet known whether they are suitable for inferring lower-level phylogenetic relationships among species within lepidopteran genera. To test the utility of these markers at lower taxonomic levels, we used a subset of 89 loci obtained by Zhang *et al.'s* (2019) sequence capture technique to unravel the phylogenetic relationships of the butterfly genus *Neptis* Fabricius.

With over 160 species, *Neptis* butterflies (Nymphalidae: Limenitidinae: Neptini) are a species-rich and abundant component of many butterfly faunas, with around 83 species in Africa (including islands of the Indian Ocean), a few (ca 3) in Australasia, ∼44 species from the Oriental region, and ∼36 species found in the Palearctic region (Eltringham, 1921; Eliot, 1969; D'Abrera, 1985, 1990, 1993, 2004; Bozano, 2008; Brower, 2008; Lang, 2012; Wu & Xu, 2017; Savela, 2018; Wahlberg, 2018; Williams, 2019). Some species such as *N. sappho* (Pallas) range across millions of square kilometres in the Palearctic, while others, including *N. gracilis* (Kirsch) from Tanimbar, are restricted to single islands.

Adults of this genus typically fly with their wings held open and parallel to the ground with few wing beats, and are commonly called 'sailors' or 'gliders'. Most species are characterized by black wings with white or orange markings on the upper side (Eltringham, 1921). Larvae generally bear two small conical processes on the vertex of the head, and a pair of elongated, tubercular processes on the second and third segments (Roberts, 2001). The genus has a wide range of host plants, but most *Neptis* feed on species in the families Fabaceae, Rosaceae, Ulmaceae or Betulaceae.

Much effort has been made to classify *Neptis* using morphology, but most existing studies focus on regional taxonomic revisions (Fruhstorfer, 1908a, 1908b; Eltringham, 1921; Eliot, 1969; D'Abrera, 1985, 1990, 1993, 2004; Bozano, 2008; Lang, 2012; Wu & Xu, 2017). Eliot (1969) studied the tribe Neptini outside of Africa and divided these non-African *Neptis* into 23 species groups based on external characters and adult genitalia. This species-group classification was adopted by Bozano (2008) and Lang (2012), with some minor modifications. While Eliot (1969) described the distributions of these *Neptis* as 'Eurasian' or 'Australian', we adopt the more commonly used biogeographic terms Palearctic (referring to temperate Asia and Europe), Oriental (referring to tropical and subtropical Asia) and Australasian (referring to Australia, New Guinea and other islands of Melanesia).

Many species within *Neptis* are morphologically similar to each other and have wing patterns similar to species in related genera within Limenitidinae, including *Phaedyma* Felder, *Lasippa* Moore and *Pantoporia* Hübner. Recent molecular phylogenetic studies demonstrated that the genus *Neptis* is not monophyletic (Zhang *et al.*, 2011; Chen *et al.*, 2017; Dhungel & Wahlberg, 2018), nor are the traditional species groups within it as originally described by Eliot (1969). Phylogenetic studies including *Neptis* have failed to arrive at a convincing evolutionary picture of the genus because they sampled too few *Neptis* species and/or used a small number of loci, resulting in weakly supported relationships (Chen *et al.*, 2007; Wu *et al.*, 2007a, 2007b; Zhang *et al.*, 2008a, 2008b; Wahlberg *et al.*, 2009; Mullen *et al.*, 2010; Zhang *et al.*, 2011; Wu *et al.*, 2016; Dhungel & Wahlberg, 2018). In the present study, we used 89 loci to reconstruct phylogenetic relationships among *Neptis* butterflies and to test the usefulness of Zhang *et al.*'*s* (2019) PCR-generated probes for inferring relationships among species.

Materials and methods

Taxon sampling

We sampled 100 individuals from 65 species of *Neptis* from the Palearctic, Oriental and Australasian regions representing 22 of the 23 major species groups and including *N. sappho*, the type species of the genus (Table S1). It should be noted that *Papilio aceris* Esper was originally designated as the type species of *Neptis*, but was later confirmed to be a junior synonym of *N. sappho* (Eliot, 1969). We additionally sampled: (i) eight African *Neptis* species to verify their monophyly and phylogenetic position; (ii) type species from three other genera in the related tribe Neptini, *Pantoporia hordonia* (Stoll), *Lasippa heliodore* (Fabricius), *Phaedyma amphion* (Linnaeus) and (iii) outgroup species from two genera in the related tribe Limenitidini, *Limenitis* Fabricius and *Chalinga* Moore (Table S1). Most of the samples used in this study were from dry, pinned specimens collected fewer than 10 years ago, and the oldest was collected from 17 years ago. In addition, we added Sanger sequence data from 32 *Neptis* and 6 outgroup samples included in the study by Dhungel & Wahlberg (2018) to compare phylogenetic results based on the three commonly used loci employed by this recent Sanger study (cytochrome c oxidase subunit I, *COI*; elongation factor-1a, *EF-1a*; glycer-aldehyde-3-phosphate dehydrogenase, *GAPDH*) with the results were obtained using nearly 30 times more loci (Zhang *et al.*, 2019). Our sample represents >80% of recognized Palearctic *Neptis* species, two-thirds of recognized Oriental and Australasian *Neptis* species, and ~20% of recognized African *Neptis* species (Lamas, 2015).

DNA extraction, library preparation, probe preparation, hybridization and sequencing

Genomic DNA for each sample was extracted from leg, thoracic or abdominal tissues using a Hipure Insect DNA Kit (Magen, Guangzhou, China) following the manufacturer's protocol. Libraries and probes were prepared following the methods of Zhang *et al.* (2019). We prepared PCR-generated probes of 88 protein-coding nuclear loci for hybridization (Table S1). Each hybridization reaction was carried out with 500 ng of pooled libraries and 2.5 μL of bait-coated beads using a touch-down hybridization program. The reaction, with a total duration of 30 h, was started from 65°C after denaturation, decreased by 5°C every 6 h and ended at 45°C (Zhang *et al.*, 2019). The captured libraries were amplified and pooled in equal concentrations before sequencing on an Illumina HiSeqX10 sequencer using the paired-end 150-bp mode.

Data processing and datasets assembly

Illumina paired-end reads were quality controlled using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), Trimmomatic (Bolger *et al.*, 2014) and FastUniq (Xu *et al.*, 2012). For each sample, clean reads were assembled into contigs using the SPAdes 3.8.1 genome assembler (Bankevich *et al.*, 2012). The sequencing depths of the filtered contigs were calculated by SAMtools 1.4.1 (Li *et al.*, 2009). Only contigs with an average sequencing depth ≥5× were retained for further analyses. The mitochondrial locus cytochrome c oxidase subunit I (*COI*) was obtained from the captured libraries after sequencing. Orthologous sequences were called from the contig set of each sample using tBLASTN, with the 88 locus sequences retrieved from the *Danaus plexippus* (Linnaeus) genome as references. A reversed BLASTN was performed with the orthologous contigs to identify potential chimeric sequences. After cleaning, a single sequence from each locus was retained to create a contig set from each sample. Sequenced contigs of the 88 nuclear loci were converted to amino acid sequences with MAFFT version 7.0.1 (Katoh & Standley, 2013) and aligned to reference protein sequences from *D. plexippus*. The aligned sequences were trimmed at both ends to keep only the probe regions of each locus, and the trimmed alignment of each locus was refined using the 'codon' mode of Gblocks 0.91b (Castresana, 2000). Two datasets were concatenated from aligned nuclear and mitochondrial loci using SequenceMatrix 1.8 (Vaidya *et al.*, 2011): (i) a dataset with *COI* and the 88 successfully captured nuclear loci; (ii) a subset of the sequence capture data comprising the three loci *COI*, *EF-1a* and *GAPDH*; and Sanger sequence data from Dhungel & Wahlberg (2018) combined and aligned with the three-locus, sequence capture data subset. The 89-locus and 3-locus matrices are provided in Files S1 and S2, respectively. All sequences and their accession numbers used in this study are listed in Table S1.

Phylogenetic analyses

Phylogenetic analyses were conducted using Maximum Likelihood (ML) and Bayesian Inference (BI). For both datasets, a three-partition scheme with one partition for each codon position was selected using PartitionFinder2 (Lanfear *et al.*, 2016). Maximum likelihood analyses were run under the GTR + I + G model and 5000 ultrafast bootstrap replicates in IQ-TREE 1.6 (Nguyen *et al.*, 2014) and under the GTRGAMMA model with 1000 bootstrap replicates in RAxML-HPC2 8.2.12 (Stamatakis, 2014) implemented on XSEDE in the CIPRES Science Gateway 3.3 (Miller *et al.*, 2010). Bayesian analyses were performed in MrBayes 3.2.6 (Ronquist *et al.*, 2012) on the CIPRES Science Gateway, with two independent MCMC (Markov Chain Monte Carlo) runs with four chains (one cold and three heated) for 5 million generations sampled by every 1000 generations, and the first 25% of trees were discarded as burn in. Tracer 1.7 (Rambaut *et al.*, 2018) was used to examine the rate multiplier of each locus. The final trees were visualized using FigTree 1.4.3 (Rambaut, 2017) and edited in Adobe Photoshop CC and Adobe Illustrator CS6 (adobe.com).

Results

Sequence capture and data matrix statistics

A total of 264 108 999 paired-end reads were generated after hybrid capture and Illumina sequencing. For each sample,

we recovered an average of 1 792 566 paired-end reads and 6161 contigs, with a mean read-to-target mapping percentage of 3.68% (Table S2). The average sequence coverage of the orthologous contigs was 137×, with a wide range from 5 to 793× (Table S3). We successfully captured 89 loci from 117 samples, totalling up to 84 519 bp per sample. The sequence length of each locus varied from 264 bp to 2058 bp, with an average length of 950 bp. Most loci were captured from over 97% (114/117) of samples except hyp20 and NEDD1, which were captured from 94% and 26% of samples, respectively. Each locus was nearly complete, with an average of 3.27% missing data ranged from 0.3 to 81.07%. Our super matrix includes 30 019 (35.5%) parsimony informative characters and a GC content of 41%. The general features of the 89-locus dataset, including identity and conservativeness of each locus, are summarized in Table S4.

Phylogenetic analyses and taxonomic changes

Topologies of the 89-locus trees derived from ML and BI analyses are highly congruent and strongly supported (BS/UFBoot = 100/100, PP = 1) at nearly all nodes (Fig. 1); these topology of these trees is similar to those inferred with the three ‘traditional’ loci (Figs. S1, S2), but provide markedly higher support and resolution than the three-locus trees. Our phylogenetic analyses based on 89 loci recover *Neptis* as not monophyletic with strong support (BS/UFBoot = 100/100, PP = 1) because *Phaedyma amphion* (Linnaeus) and *Lasippa heliodore* (Fabricius) are nested within the genus. Of the 22 species groups we sampled, eight are represented by a single species and six of the remaining 14 species groups are strongly supported as being nonmonophyletic: *N. hylas* group, *N. nata* group, *N. vikasi* group, *N. sankara* group, *N. antilope* group, and *N. armandia* group.

We designate 13 strongly supported (BS/UFBoot = 100/100, PP = 1), revised species groups which each include at least two Palearctic, Oriental or Australasian *Neptis* species. Detailed information on these species groups is provided in Table S5. To discuss our phylogenetic results, we divided the topology into five clades labelled A to E (Fig. 1). Clade A comprises two sister subclades: *N. narayana* Moore + (*N. meloria* group + *N. cartica* group) and *N. radha* Moore + *N. ananta* group. This clade is strongly supported with high support values (BS/UFBoot = 100/100, PP = 1), and forms the sister group to the remaining *Neptis* species (BS/UFBoot = 97/98, PP = 1): Clade B, which only contains one species, *Neptis* (=*Lasippa*) *heliodore*, and the remaining three clades, Clades C–E. Clade C is the most diverse lineage and includes 7 species groups: *N. alwina* group, *N. sankara* group, *N. pryeri* group, *N. zaida* group, *N. antilope* group, *N. arachne* group and *N. thisbe* group. Clade D with all African *Neptis* samples is monophyletic and sister to Clade E, which makes *Neptis* outside of Africa paraphyletic. Within Clade E, *N. venilia* (Linnaeus) occupies the basal position, followed by *N. leucoporos* Fruhstorfer and the subclade of *P. amphion* + *N. vikasi* group which is sister to the subclade of *N. praslini* group + *N. hylas* group.

Discussion

Performance of the sequence capture approach

The sequence capture probe set, which was initially developed to study interfamilial lepidopteran relationships (Zhang *et al.*, 2019), is highly efficacious in recovering relationships among closely related species of *Neptis*. Relationships among *Neptis* taxa were successfully recovered with strong support for all clades using the 89-locus dataset. This tree had far better resolution and stronger support than the tree based on the three-locus dataset, which failed to yield strong support for basal splits within *Neptis* and had many poorly supported branches. Increased numbers of loci and a broader taxonomic sample resulted in a more strongly supported phylogenetic framework than the phylogeny based on limited loci in previous studies (Wu *et al.*, 2007a, 2007b; Zhang *et al.*, 2011; Wan *et al.*, 2013; Wu *et al.*, 2016; Dhungel & Wahlberg, 2018). Sequence capture is also cost-effective and furthermore allows generating sequences from degraded samples such as pinned, dry specimens (St. Laurent *et al.*, 2018). Most (86%) of the samples in this study are dry specimens, and 99% of these captured successfully. In contrast, the efficiency of sequence acquisition is often low for dry, degraded samples using Sanger sequencing, largely due to the difficulties of PCR amplification of target DNA regions.

The monophyly of Neptis and position of African Neptis

Results from previous studies have shown that *Neptis* is not a monophyletic group, with *Phaedyma columella* (Cramer), *P. aspasia* (Leech) and *Pantoporia venilia* (Linnaeus) clustered within the genus (Wu *et al.*, 2007b; Zhang *et al.*, 2011; Chen *et al.*, 2017; Dhungel & Wahlberg, 2018). Of these three species, *P. aspasia* and *P. venilia* have been formally moved to *Neptis* (Dhungel & Wahlberg, 2018). Our molecular analyses indicate for the first time that *Lasippa heliodore* and *Phaedyma amphion* are nested within *Neptis* with strong support, which demonstrates that the genus is still not monophyletic. Linnaeus (1758) first described the taxon *amphion* as a species of *Papilio* Linnaeus. Subsequent authors placed it in different genera: *Nymphalis* Kluk (Godart, 1824), *Limenitis* (Boisduval, 1832), *Athyma* Westwood (Butler, 1866), *Neptis* (de Nicéville, 1900; Grose Smith, 1900; Klunder van Gijen, 1912; Fruhstorfer, 1913) and *Phaedyma* (Felder, 1861; Eliot, 1969). Such unstable placement is likely due the high degree of morphological similarity among these genera. Many contemporary authors treat taxon *amphion* as a *Phaedyma* species, as it has well-developed specula on the upper surface of male hindwing and has enormous sickle-shaped terminal processes on the clasps. Fabricius (1787) originally described taxon *heliodore* as a species of *Papilio*, then Moore (1898) placed it in the genus *Lasippa*, which was adopted by Eliot (1969) and many subsequent authors. However, various other authors (Butler, 1879; Fruhstorfer, 1908a; Roepke, 1938; Eliot, 1959) have included it in *Neptis* as a consequence of its morphological similarity. A phylogenetic study based on barcode sequencing revealed taxon

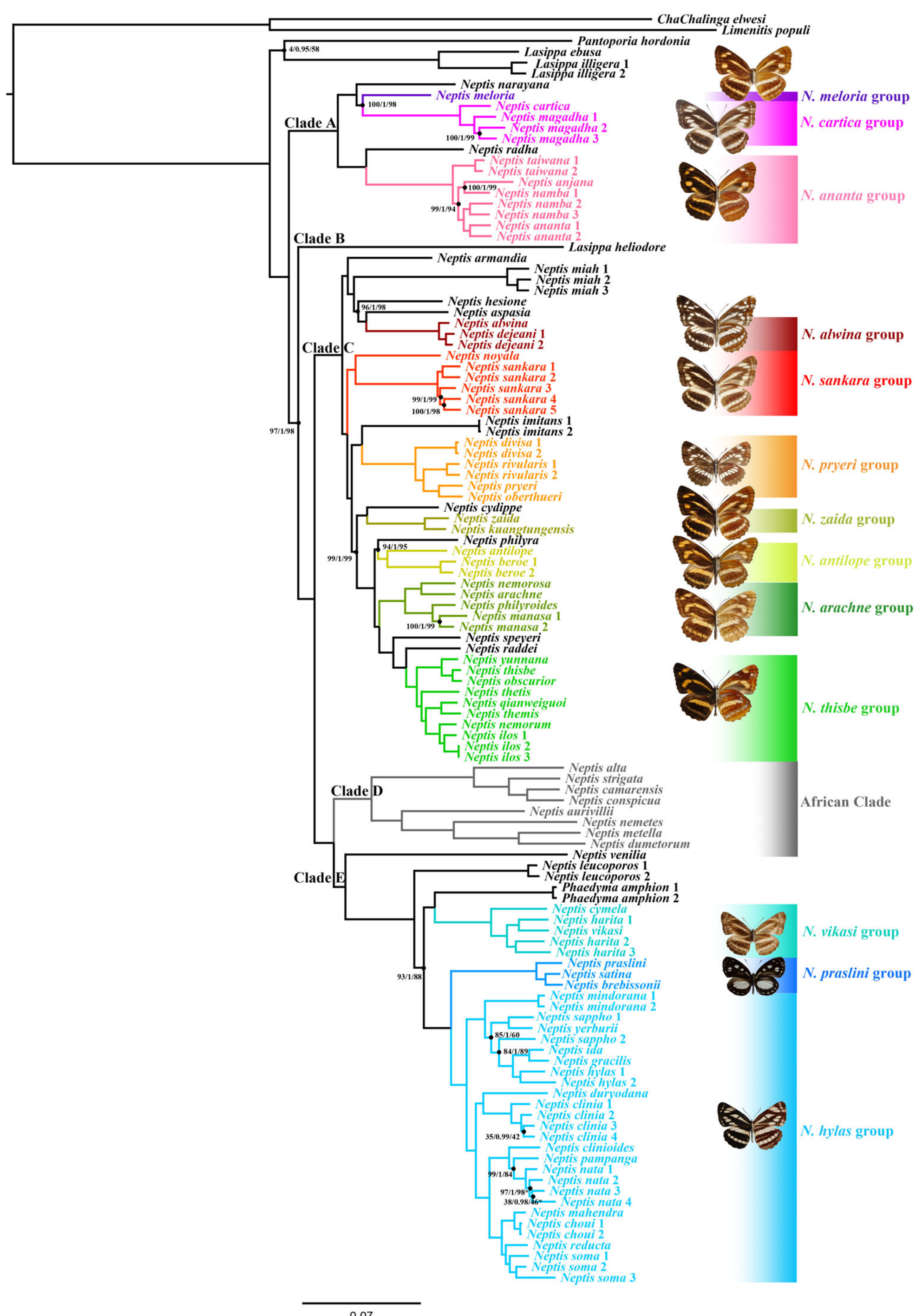

Fig. 1. Maximum likelihood phylogram showing relationships within *Neptis* sampled for this study. The phylogeny is inferred by RAxML, MrBayes and IQTREE based on 89 loci from 117 samples partitioned by codon. Numbers beside nodes from left to right are RAxML bootstrap values (BS), Bayesian posterior probabilities (PP) and IQTREE ultrafast bootstrap values (UFBoot), respectively. Nodes without numbers had the highest possible support by every method (100/1/100); these values are omitted for clarity. Highlighted clades show current recognized species groups and the African clade. [Colour figure can be viewed at wileyonlinelibrary.com].

heliodore to be clustered within a clade formed by species of *Neptis* (Wu *et al.*, 2016). Although *heliodore* is the type species of genus *Lasippa*, it does not form a clade with the two other *Lasippa* species sampled in this study: *L. ebusa* (C. & R. Felder) and *L. illigera* (Eschscholtz). We therefore revive the combinations *Neptis amphion* comb. rev. and *Neptis heliodore* comb. rev.

Eliot (1969) regarded African *Neptis* species as distantly related to species found outside of Africa, but our results and those of Dhungel & Wahlberg (2018) demonstrate that African *Neptis* form a monophyletic clade nested within *Neptis* from the Palearctic, Oriental, and Australasian regions.

Eliot (1969) placed *N. raddei* and *N. imitans* (Oberthür) in the genus *Aldania*; Kurosawa (1976) and Tuzov *et al.* (2000) added *N. thisbe* Ménétriès, *N. thetis* Leech, *N. yunnana* Oberthür, *N. themis* Leech, and *N. ilos* Fruhstorfer. Our results demonstrate that these species do not form a monophyletic group – even *N. raddei* and *N. imitans* are distantly related. *Aldania* is thus retained as a junior synonym of *Neptis*. *Neptis imitans* seems to mimic *Parantica sita* (Kollar), and *N. raddei* vaguely resembles several species in Danaini Boisduval (Eliot, 1969) or possibly *Calinaga* spp.

Neptis species groups in the Palearctic, Oriental and Australasian regions

With strong support, our phylogenetic hypothesis shows that *Neptis* outside of Africa are in need of revision, including the species groups. Here, we propose a total of 13 species groups based primarily on our phylogenetic results and the comprehensive morphological classification of Eliot (1969) in combination with regional taxonomic studies (Bozano, 2008; Lang, 2012) and larval morphology (Igarashi & Fukuda, 1997; Igarashi & Harada, 2015). Two of our species groups are identical to Eliot's (1969) arrangements, which are confirmed by our molecular results: *N. cartica* group and *N. alwina* group. The establishment of group names follows those in previous morphological studies, except the newly named *N. meloria* group. We discuss the arrangements of the 13 species groups below.

Neptis meloria *group.* Eliot (1969) grouped *N. antilope* Leech, *N. meloria* Oberthür and *N. sylvana* Oberthür based on the unmarked basal area of their hindwings. This taxonomic treatment was later adopted by Lang (2012). We sampled the former two species in our study and found that *N. antilope* was not closely related to *N. meloria*. We redefine this species group to include only *N. meloria* and *N. sylvana*, as both species share a distinct morphological trait on their forewings (Table S5), though the latter species was not sampled in our molecular analyses.

Neptis cartica *group.* Eliot (1969) included *N. cartica* Moore and *N. magadha* C. & R. Felder in this group. Our results indicate that the two species form a strongly supported clade, which is consistent with morphology. We also recover a sister relationship between the *N. cartica* and *N. meloria* groups, and these two groups are sister to *N. narayana*. This study is the first to include *N. magadha* from China; the Chinese sample is more closely related to the Thai specimen than the sample from Belitung, Indonesia.

Neptis ananta *group.* This group contains six closely related and morphologically similar species, four of which were sequenced in this study (Table S5). The taxon *N. taiwana* Fruhstorfer, which is endemic to Taiwan, China, was previously regarded as a subspecies of *N. ananta* Moore (Fruhstorfer, 1908c). Eliot (1969) treated the taxon *taiwana* as a distinct species, which is confirmed by our analysis in which *N. taiwana* is not sister to *N. ananta*. In our phylogeny, three *N. namba* Tytler samples are not monophyletic. The *namba* specimen from Thailand and *N. anjana* Moore form a clade, indicating the taxonomy of *N. namba* is problematic. Based on analyses of one mitochondrial gene, Zhang *et al.* (2011) recovered *N. radha* as the sister of *N. namba* with weak support. Our analysis shows that the two taxa share a close relationship, and strongly supports that *N. radha* is the sister taxon of *N. ananta* group.

Neptis alwina *group.* This group includes *N. alwina* (Bremer & Grey) and *N. dejeani* Oberthür, which is consistent with the arrangement of Eliot (1969). Previous molecular studies placed *N. alwina* in a variety of positions. Based on sequence data from three loci, Wan *et al.* (2013) placed it as sister to the species of *N. pryeri* group. In contrast, the results of Wu *et al.* (2016) and Dhungel & Wahlberg (2018) suggested that the latter species belong in a distant, dichotomous clade. However, they were unable to infer a well-resolved sister relationship between *N. aspasia* and the *N. alwina* group, but our research strongly supports their sister relationship. The short branches in our analysis suggest that *N. alwina* and *N. dejeani* might not be separate species, but more data are required to verify this hypothesis.

Neptis sankara *group.* Eliot (1969) and Bozano (2008) included *N. sankara* (Kollar), *N. philyra* Ménétriès and *N. speyeri* Staudinger in this group. Lang (2012) later added *N. kuangtungensis* Mell based on adult morphological characters. However, the first three species have distinctly different larval morphology (Igarashi & Harada, 2015) and a DNA barcoding study demonstrated that the species do not form a clade (Wu *et al.*, 2016). We sampled all four species and found that none of them are sister taxa. Thus, as previously designated, the *N. sankara* group is wholly polyphyletic. We thus revise this group based on our phylogenetic results to include *N. sankara* and *N. noyala* Oberthür, as they form a well-supported clade.

Neptis pryeri *group.* Eliot (1969) grouped *N. divisa* Oberthür, *N. rivularis* (Scopoli) and *N. pryeri* Butler together, Bozano (2008) subsequently added *N. andetria* Fruhstorfer and named it the *rivularis* group to name the group. Recently, Fukúda & Minotani (2017) did an extensive morphological study on the *N. pryeri* group, and concluded that the group consisted of seven recognized species and an undescribed

one, but *N. divisa* and *N. rivularis* were not included in their study. Our results support the morphological treatments of four species from Eliot (1969), Bozano (2008) and Fukúda & Minotani (2017). Combining the previous morphological studies with our present phylogenetic results, we propose that the *N. pryeri* group comprises nine species (Table S5), with *N. imitans* as its sister taxon.

Neptis zaida *group.* This group includes *N. thestias* Leech, *N. zaida* Westwood and *N. kuangtungensis*, which was classified by Eliot (1969), although he tentatively treated *N. kuangtungensis* as a subspecies of *N. zaida*. In subsequent morphological studies, *N. zaida* and *N. kuangtungensis* were separately placed in the *N. noyala* group (Bozano, 2008) and *N. sankara* group (Lang, 2012), respectively. However, a sister relationship between them is strongly supported in our analyses. We follow Eliot's (1969) classification and retain *N. thestias* (unsampled) in this group based on morphological characters (Table S5).

Neptis antilope *group.* We revise this group to contain only two species: *N. antilope* and *N. beroe* Leech. The former species was previously placed in Eliot's (1969) *N. antilope* group together with *N. meloria* and *N. sylvana*. This taxonomic treatment was later adopted by Bozano (2008). Our analyses recover *N. antilope* as sister to *N. beroe*; Eliot (1969) did not recognize affinities between this latter species and any other *Neptis*. The two species' close relationship is underscored by their similar life histories (Igarashi & Harada, 2015). Without sampling *N. beroe*, Dhungel & Wahlberg (2018) indicated that *N. philyra* and *N. antilope* were closely related, albeit with poor support. We treat *N. philyra* as a sister taxon of *N. antilope* group based on our molecular results and the species' distinctive morphological traits.

Neptis arachne *group.* This group consists of *N. arachne* Leech, *N. nemorosa* Oberthür, *N. philyroides* Staudinger and *N. manasa* Moore, supported strongly by the molecular results of this study. Eliot (1969) included only the former two species in this group and treated the other two as separate monotypic groups. Igarashi & Fukuda (1997) suggested a close relationship between *N. nemorosa* and *N. philyroides* based on larval and pupal characters, shared also by *N. manasa* in a subsequent study of life histories (Igarashi & Harada, 2015).

Neptis thisbe *group.* Eliot (1969) identified two divisions within this group: (i) *N. thisbe*, *N. yunnana*, and *N. nycteus* de Nicéville; and (ii) *N. themis*, *N. thetis* and *N. nemorum* Oberthür. His classification is broadly supported by our molecular study. This group comprises a total of nine species, and we sampled all but *N. nycteus*. Two taxa (*N. obscurior* and *N. ilos*) were treated as subspecies within this group by Eliot (1969), and five species (*N. yunnana*, *N. thisbe*, *N. thetis*, *N. themis* and *N. ilos*) were regarded as members of *Aldania*, which we demonstrate to be a synonym of *Neptis* (Kurosawa, 1976; Tuzov *et al.*, 2000). The sister relationship between the *N. thisbe* group and *N. raddei* is firmly supported by our results. This group is split into two lineages according to our phylogeny, the first contains *N. yunnana*, *N. thisbe* and *N. obscurior* Oberthür, and the second includes the other five species. The taxon *ilos* was treated as a subspecies of *N. themis* in Eliot (1969), but is regarded as a species with a close relationship to *N. nemorum* in our study.

Neptis vikasi *group.* We mainly follow Eliot's (1969) arrangement to assign nine species to the present group (Table S5) except for taxon *N. cyra* (synonym of *N. clinia* Moore) and *N. miah* Moore, which our analyses demonstrate to be distantly related to other members of Eliot's *N. vikasi* group. The phylogenetic results in this study indicate that *P. amphion* shares a sister affinity with the *N. vikasi* group, and three *N. harita* Moore samples are paraphyletic with a single sample of *N. vikasi* Horsfield nested within them. We provisionally treat *N. vikasi* as a distinct species, but further investigations are needed to clarify the status of the two species.

Neptis praslini *group.* This group is endemic to the Australasian region. Eliot (1969) divided it into two minor groups: (i) *N. praslini* (Boisduval) and *N. nausicaa* de Nicéville; (ii) *N. brebissonii* (Boisduval) and *N. satina* Grose-Smith. However, Dhungel & Wahlberg (2018) found that *N. praslini*, *N. sappho*, *N. ida* Moore, *N. nata* Moore, and *N. clinia* (the latter two were both called *Neptis* sp. in the paper, but subsequently identified) form a clade that is sister to *N. satina* with strong support. We recovered *N. praslini*, *N. brebissonii* and *N. satina* in a strongly supported clade, in agreement with Eliot (1969), and found that *N. sappho*, *N. ida*, *N. nata* and *N. clinia* are not sister to members of the *N. praslini* group or to each other. We were unable to sample *N. nausicaa*. Members of this group mimic *Tellervo* spp. (Eliot, 1969).

Neptis hylas *group.* This group comprises 18 species (Table S5) in two major groups (*N. hylas* group and *N. nata* group) designated by Eliot (1969) and two other species recognized by subsequent authors (Sasaki, 1982; Yuan & Wang, 1994). We demonstrate that the *N. hylas* (Linnaeus) and *N. nata* groups of Eliot (1969) are not monophyletic. Our results strongly support the *N. praslini* group as sister to this group, similar to Dhungel & Wahlberg (2018), although they did not find a monophyletic *N. praslini* group. The species *N. clinioides* de Nicéville and *N. clinia* were placed together (Eliot, 1969). However, Wu *et al.* (2007b) did not regard them as sister taxa, which is corroborated by our study. Yuan & Wang (1994) described *N. choui* Yuan & Wang, which was included in the *N. nata* group by Lang (2012). This species is nested within the *N. hylas* group and is close to *N. mahendra* Moore according to our results. The Taiwanese population of *N. sappho* has long been recognized as the distinct subspecies *N. sappho formosana* Fruhstorfer. In our study, it is sister to *N. yerburii* Butler and does not group with a sample of *N. sappho* from Jiangxi in China. Further sampling is required to determine potential cryptic species within this widespread taxon. The species *N. yerburii* has been regarded as a subspecies of *N. nata* (Lamas, 2015), but we find that they are

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not closely related, and thus confirm that *N. yerburii* is a valid species.

Species unassigned to species groups

We sequenced 15 species that are not included in our revised species group scheme described above (Fig. 1, black branch labels); these fall into three categories. The first category includes the four species *N. narayana*, *N. radha*, *N. cydippe* Leech and *N. leucoporos*, each of which was treated by Eliot (1969) as a monotypic group. Since these species are each morphologically distinctive and because more than one species is needed to constitute a group, these are left as 'orphaned' species without any species group. The second category includes the five species *N. armandia* (Oberthür), *N. miah*, *N. hesione* Leech, *N. philyra* and *N. speyeri*, each of which is morphologically similar to one or more unrelated congeners. This prompted Eliot (1969) to include them in species groups that we found to be para- or polyphyletic, such as Eliot's inclusion of the non-sister species *N. armandia* and *N. hesione* into the now defunct *N. armandia* group. The last category comprises six species that were placed in other Neptini genera at the time of Eliot's (1969) revision. The species *N. aspasia*, *N. imitans*, *N. raddei* and *N. venilia* were moved to *Neptis* by previous authors (Bozano, 2008; Lang, 2012; Dhungel & Wahlberg, 2018), and *N. heliodore* and *N. amphion* are transferred to the genus as revived combinations in this study. Since the members of species groups are generally morphologically similar, it does not make sense to include these distinctive taxa in our species groups, even if they are closely related. We note that the Australasian species *N. venilia*, with unique larval and adult characters (Igarashi & Fukuda, 1997), is recovered as the earliest diverging branch of Clade E in our phylogenies, which disagrees with the placement in the study of Dhungel & Wahlberg (2018), where it was recovered as sister to the African lineage.

Conclusion

Our analyses, based on an 89-locus dataset generated through sequence capture via a PCR-generated probe set, provides a comprehensive, robust phylogeny for *Neptis*, particularly the non-African species. We confirm that the genus is not a monophyletic group because *Lasippa heliodore* and *Phaedyma amphion* are nested within the group. As a result, we propose 13 species-groups of non-African *Neptis*. The PCR-generated probe set that we employed was developed to study deep, higher-level relationships between families, but we demonstrate its utility for resolving lower level interspecific phylogenetic relationships in the genus *Neptis*, and presumably other Lepidoptera.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

File S1. Aligned 89-locus matrix used in the phylogenetic analyses.

File S2. Aligned 3-locus matrix used in the phylogenetic analyses.

Fig. S1. Maximum-likelihood phylogeny of *Neptis* based on sequences of 3 loci.

Fig. S2. Bayesian phylogeny of *Neptis* based on sequences of 3 loci.

Table S1. List of taxa used in this study, with respective GenBank accession numbers.

Table S2. Summary of the sequencing results from 108 individuals of *Neptis* and nine outgroups.

Table S3. Summary of the sequencing coverage per locus per sample.

Table S4. Summary information for the 89 loci used in this study.

Table S5. Redefined *Neptis* species groups and a list of species not associated with any group.

Acknowledgements

We are grateful to Yu-Feng Hsu, Yutaka Inayoshi, Petr Vlasanek and Hideyuki Chiba for providing identified specimens for sequencing. We thank Xiaoling Fan and Zhenfu Huang for kindly help in data processing. This work was supported by grants from: (i) the National Natural Science Foundation of China (No. 31572303 and No. 31711530024 to M.W., No. 31872205 to P.Z.); (ii) the Swedish Foundation for International Cooperation in Research and Higher Education (STINT) and the Swedish Research Council (2019-03441) to S.N.; (iii) the US National Science Foundation (DEB-1120380, DEB-1541557) and National Geographic Society Committee for Exploration and Research (CRE 9285-13, WW-227R-17) to D.J.L.; and (iv) the biodiversity investigation, observation and assessment program of the Ministry of Ecology and Environment of China. Specimens were collected in compliance with relevant laws. Research in Indonesia was conducted under permits from RISTEK and other pertinent authorities. Specimen collection in the Philippines was done in collaboration with the National Museum of the Philippines as authorized by the National Cultural Heritage Act of 2009. Research in Thailand was authorized by permits from the National Research Council of Thailand and the Department of National Parks, Wildlife and Plant Conservation. Specimen collection in Vietnam was conducted in collaboration with the Vietnam Forest Museum under an MoU with Cat Tien National Park, and sampling in Nepal was undertaken by Tenzing Doleck with permits from the Department of National Parks and Wildlife Conservation. The remaining specimens used in this study were from the Insect Collection, Department of Entomology, South China

Agricultural University, Guangzhou, China. The authors declare no conflicts of interest.

Data availability statement

All sequences obtained in this study are available in GenBank, their accession numbers are listed in Table S1. Tree files are available in TreeBASE with a study accession URL: http://purl.org/phylo/treebase/phylows/study/TB2:S26101.

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Accepted 28 April 2020