

Phylogenetic analysis of tribe Neptini Newman, 1870 based on COI gene (Lepidoptera: Nymphalidae, Limenitidinae)

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Abstract

Papilionoidea Latreille, 1802 are widely recognized for their ecological significance, diverse morphology, and role as bioindicators of environmental health. Among the numerous tribes within the subfamily Limenitidinae Behr, 1864, the tribe Neptini Newman, 1870 comprises a distinct group of brush-footed species predominantly distributed across the Oriental and Palearctic regions. The tribe includes several genera, with *Neptis* Fabricius, 1807 being the most speciose and widespread. The current research aims to reconstruct the phylogenetic trees by amplifying the partial sequences of the mitochondrial gene COI to produce a DNA reference library and determine the evolutionary relationships among the species of tribe Neptini. Sequences of COI gene of eight species have been submitted to NCBI. The examination of nucleotide composition at the first, second, and third codon locations revealed a considerable A + T bias, particularly at the third codon position. Genetic divergence based on COI ranges from 5.0% to 11.4% of presently studies species. 1000 bootstrapped Neighbor-joining with Kimura-2 parameter and Maximum likelihood phylogenetic tree constructed based on COI gene sequences.

Keywords: Lepidoptera, Nymphalidae, Limenitidinae, Neptini, Phylogeny, COI gene, DNA Barcoding, India.

Análisis filogenético de la tribu Neptini Newman, 1870, basado en el gen COI (Lepidoptera: Nymphalidae, Limenitidinae)

Resumen

Papilionoidea Latreille, 1802 son ampliamente reconocidas por su importancia ecológica, su morfología diversa y su papel como bioindicadores de la salud ambiental. Entre las numerosas tribus de la subfamilia Limenitidinae Behr, 1864, la tribu Neptini Newman, 1870 comprende un grupo distinto de especies de patas peludas distribuidas predominantemente en las regiones Oriental y Paleártica. La tribu incluye varios géneros, siendo *Neptis* Fabricius, 1807 el más diverso y extendido. La presente investigación tiene como objetivo reconstruir los árboles filogenéticos mediante la amplificación de secuencias parciales del gen mitocondrial COI para crear una biblioteca de referencia de ADN y determinar las relaciones evolutivas entre las especies de la tribu Neptini. Se han enviado al NCBI las secuencias del gen COI de ocho especies. El examen de la composición de nucleótidos en las posiciones del primer, segundo y tercer codón reveló un considerable sesgo A + T, especialmente en la posición del tercer codón. La divergencia genética basada en el COI oscila entre el 5,0 % y el 11,4 % de las especies estudiadas actualmente. Se construyó un árbol filogenético de 1000 bootstraps Neighbor-joining con el parámetro Kimura-2 y la máxima verosimilitud basado en las secuencias del gen COI.

Palabras clave: Lepidoptera, Nymphalidae, Limenitidinae, Neptini, filogenia, gen COI, código de barras de ADN, India.

Introduction

With more than 18,000 species described and extraordinary efforts made to infer phylogenetic

hypotheses based on molecular data. Papilionoidea Latreille, 1802 have become a model system for insect diversification studies (Chazot et al. 2019). Lepidoptera play an important role in ecosystem as pollinators and as food. Papilionoidea employ several survival strategies to escape predators. One is aposematism, which involves chemical defence and associated conspicuous wing colour patterns to warn predators. Another is mimicry, where multiple species share a warning signal (Powell, 2009).

Over the years, the Nymphalidae Rafinesque, 1815 family of Papilionoidea has been the focus of research in numerous biological domains. However, the definition of subfamilies has been just recently defined, and the higher categorization of the family is currently being worked on (Wahlberg et al. 2009). As subfamily Limenitidinae is currently delineated, the subfamily Limenitidinae Behr, 1864 comprises a little over 800 species placed in 46 genera and four tribes: Parthenini Reuter, 1896, Adoliadini Doubleday, 1845, Limenitidini Behr, 1864, and Neptini Newman, 1870 (Wahlberg, 2007).

The tribe Neptini, within the subfamily Limenitidinae of the family Nymphalidae, comprises a diverse group. This tribe is notable for its widespread distribution across Asia, Africa, and parts of Europe and Australia, with a significant concentration in Southeast Asia. The species of the tribe Neptini are distributed throughout the Old-World tropics (Mullen et al. 2011). Some Limenitidinae butterflies, including those in Neptini, exhibit a distinct flight pattern of alternating bright uppersides and cryptic undersides, then gliding with outstretched wings. Characterized by their strong flight, territorial behavior, and often vividly patterned wings, Neptini species occupy a variety of forested habitats. The tribe includes several genera, with *Neptis* being the most speciose and widespread.

The tribe Neptini includes following genera worldwide: *Lebadea* Felder, 1861; *Neptis* Fabricius, 1807; *Pantoporia* Hübner, [1819]; *Lasippa* Moore, 1898; *Phaedyra* Felder, 1861; *Aldania* Moore, [1896] and 200 species (<https://ftp.funet>). In India, tribe Neptini includes four genera *Lasippa*, *Neptis*, *Pantoporia*, *Phaedyra* and 35 species (<https://www.ifoundbutterflies.org>).

The mitochondrial cytochrome oxidase I (COI) gene, comprising a 658 bp fragment, is the standard molecular marker used in DNA barcoding for species identification and cryptic species delimitation because of its conserved sequence and low intraspecific variability (Hebert et al. 2003; Kumar et al. 2019). Furthermore, mitochondrial genes, characterized by maternal inheritance and rapid evolutionary rates, are pivotal in molecular systematics and species delimitation (Yang et al. 2018). In the eastern USA and northwestern Costa Rica, DNA barcodes for the COI region have been highly reliable for identification upto species level within the Lepidoptera, with a success rate of >97% for approx. 2000 morphologically defined taxa (Hajibabaei et al. 2006; Hebert et al. 2003, 2010; Janzen et al. 2005). The present paper deals with the phylogenetic analysis of tribe Neptini from India and all over the world.

Material and Methods

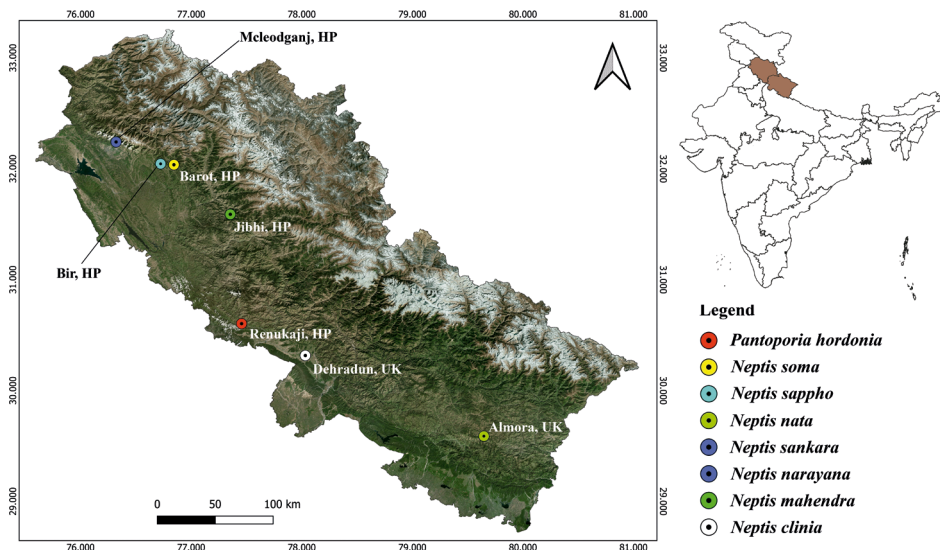
COLLECTION AND PRESERVATION

In the present study, eight samples of the genera *Neptis* and *Pantoporia* were collected from Himachal Pradesh and Uttarakhand by sweeping net and some of the sequences were downloaded from NCBI (Table 1). Specimens were photographed (Figures 1-8), wings were detached and placed in envelopes as vouchers. The bodies of the samples were placed in glass vials containing 100% ethanol. All ethanol-preserved samples were also stored at -20°C until further use. Mapping was done using QGIS software (Figure 9).

DNA Extraction: Genomic DNA was extracted from thoracic muscles and legs of Papilionoidea using Qiagen DNeasy Blood and Tissue kit. The procedure was as following: Tissue was grinded with the help of 180 µl Buffer ATL and 20 µl proteinase K, followed by vortexing and incubation at 56°C until completely lysed. Mixture was vortex occasionally during incubation. Then 200 µl Buffer AL was added. Sample was mix thoroughly by vortexing followed by incubation at 56°C for 10 minutes. Then 200 µl chilled ethanol was added followed by mixing by vortex. Mixture was then pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube, sample was centrifuged at 8000 rpm for 1 minute, flow through and collection tube was discarded. Spin column was placed in a new 2 ml collection tube, 500 µl Buffer AW1 was added, sample was centrifuged at 8000 rpm for 1 minute; flow through and collection tube were discarded. Spin column was again placed in a new 2 ml collection tube, 500 µl Buffer AW2 was added, sample was centrifuged at 14,000 rpm for 3 minutes; flow through and collection tube were discarded. Spin column was then transferred to

a new microcentrifuge tube. DNA was eluted by adding 100 µl Buffer AE to the centre of the spin column membrane. Sample was incubated for 1 minute at room temperature and sample was centrifuged for 1 minute at 8000 rpm.

Figure 9. Map showing collection localities of presently studied species.



Amplification and Sequencing of the COI gene: Extracted DNA was amplified using universal primers (LCO1490-F5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198-R5'-AAACTTCAGGGTGACCAAAAAA-TCA-3') described by Folmer et al. (1994). Amplification was performed in a total volume of 30 µl, containing 8 µl of PCR water, 15 µl of Master Mix, 1.5 µl of each primer (LCO-1490 and HCO-2198), 2 µl of BSA and 2 µl of DNA. The thermal cycling program of the PCR procedure was as follows: 1 cycle for initial denaturation at 95°C for 5 minutes; 35 cycles for denaturation at 95°C for 1 minute, annealing at 45°C for 1 minute, and extension at 72°C for 1 minute; and 1 cycle for a final extension at 72°C for 5 minutes. All the PCR products were visualized via 1% agarose gel electrophoresis with ethidium bromide (EtBr) staining under UV light via a gel documentation system to confirm successful DNA amplification. The amplification of the COI gene was performed. Amplified products were sent to the Research Laboratory Biology, New Delhi, for sequencing via the Sanger dideoxy sequencing method. The obtained sequences were submitted to the NCBI database, and accession numbers were obtained. The NCBI BLAST search engine was employed to identify conspecific sequences by analysing genetic similarities. Unique accession numbers were assigned to these sequences, making them accessible for future research and comparative analysis.

Molecular analysis: Molecular analysis was conducted in MEGA v 12. All the sequences including outgroup were aligned using Multiple Sequence Alignment. The Kimura 2-parameter (K2P) model of base substitution was used to calculate pairwise genetic distance. Nucleotide composition and patterns of nucleotide substitution were calculated in MEGA v. 12. Phylogenetic trees were constructed using Neighbour-Joining method and Maximum Likelihood method in MEGA v 12 (Figure 12). The NJ analyses were conducted using the Kimura 2-parameter distance estimate, with 1000 bootstrap replications.

Results and Discussion

Nucleotide Composition, Conserved, Variable, Parsimony Informative Sites and Singleton Sites: Across the final alignment of COI sequences, there are 343 conserved sites, 236 variable sites, 211 parsimony

informative sites and 25 singleton sites in the dataset, which confirms that the COI gene is highly conserved. In the present study, the nucleotide composition has been evaluated (Fig:10) as this is important for studying the variation among different species. The average percentage of each nucleotide for the studied fragment of COI gene was observed to be T = 39.7%, A = 29%, C = 17.3%, and G = 14.1%. This clearly shows that A + T content of the nucleotide sequences was higher than G + C. Higher A + T frequency is the distinguishing make-up of insect mitochondrial DNA (Lunt and Hyman, 1997). The nucleotide composition across codon positions in insects follows a distinct and consistent pattern, with a strong A + T bias, particularly in the first and third codon positions, whereas the second codon position has a more balanced composition.

Yang et al. (2021) reported that in the mitochondrial genomes of Tortricidae, adenine (A) is the most frequent nucleotide at the first codon position, followed by thymine (T), guanine (G), and cytosine (C). This aligns with the observed values (A-1: 31.4; T-1: 26.9; G-1: 25.8; C-1: 15.9), suggesting that the first codon position is relatively conserved, with a preference for purines (A, G) over pyrimidines (T, C). At the second codon position, thymine (T) is dominant (43.1%), followed by cytosine (C) (26.7%), adenine (A) (15.6%), and guanine (G) (14.6%), which is consistent with the findings of Kokate et al. (2021) in *Drosophila* Fallén, 1823. The second codon position plays a crucial role in determining amino acid properties, as it directly affects protein function, which may explain the greater presence of T and C, as these bases are often associated with hydrophobic or structurally stable amino acids.

The third codon position exhibited the greatest variability, with Thymine (49%) and adenine (40%) being dominant, whereas cytosine (9.2%) and guanine (1.8%) were significantly underrepresented. This finding supports the findings of Näsval et al. (2023), who highlighted a strong G/C to A/T substitution bias in butterflies. This suggests that natural selection and mutational pressures favour A + T richness at the third codon position, which is known to be the most synonymous and variable site in codon evolution. These findings indicate that the A + T content is highest in the third codon position, followed by the first, whereas the second position is relatively balanced. This pattern is consistent across different insect taxa, reflecting the influence of mutation pressure, translational efficiency, and natural selection in shaping codon usage bias.

Maximum Composite Likelihood Estimate of the Pattern of Nucleotide Substitution: The transition/transversion rate ratios were also calculated. For simplicity, the sum of r values is made equal to 100. The transition/transversion rate ratios are $k1 = 1.752$ (purines) and $k2 = 3.97$ (pyrimidines). The overall transition/transversion bias is $R = 1.398$, where $R = [A * G * k1 + T * C * k2] / [(A + G) * (T + C)]$. The pattern of nucleotide substitutions was also checked and shown in Table 2; which included codon positions 1st + 2nd + 3rd + Noncoding; all positions containing gaps and missing data were eliminated. All ambiguous positions were removed for each sequence pair (pairwise deletion option). The pairwise deletion option was applied to all ambiguous positions for each sequence pair resulting in a final data set comprising 579 positions. Evolutionary analyses were conducted in MEGA12 (Figure 12).

Table 2. Each entry shows the probability of substitution (r) from one base (row) to another base (column). Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics.

	A	T	C	G
A	-	<i>7.91</i>	<i>3.44</i>	4.92
T	<i>5.79</i>	-	13.65	<i>2.81</i>
C	<i>5.79</i>	31.39	-	<i>2.81</i>
G	10.15	<i>7.91</i>	<i>3.44</i>	-

Genetic divergence: To ensure the COI gene's identification reliability, interspecific divergences should be greater than 3% and intraspecific divergences should not exceed 3% (Wells & Sperling, 2001). Hebert et al. (2004) developed a DNA barcode analysis technique that contrasts intraspecific and interspecific genetic divergence (bar-coding gap) for species classification. To bridge the barcoding gap for species recognition in insects, a 3% interspecific genetic divergence limit was proposed (Hebert et al. 2003). Later, it was proposed that there is no universal cut off and that intraspecific and interspecific genetic divergences vary by species (Meyer & Paulay, 2005). The interspecific distance ranged from 5.0% to 11.4% (Table 3) of presently studies

species (Table 3), excluding outgroup. Minimum interspecific genetic divergence (5.0%) is observed between *Neptis soma* and *Neptis sappho*. Maximum interspecific divergence (11.4%) is observed between *Neptis nata* and *Neptis narayana*. Minimum intergeneric divergence (9.2%) is observed between *Pantoporia hordonia* and *Neptis sappho*. Maximum intergeneric (11.4%) divergence is observed between *Pantoporia hordonia* and *Neptis nata*. Species with low genetic distance indicate closely related species and have a recent common ancestor, while high genetic divergence reflects the distant relationships.

Table 3. Interspecific divergence among presently studied species.

Name of the species	1	2	3	4	5	6	7
<i>Neptis clinia</i> Moore, 1872							
<i>Neptis mahendra</i> Moore, 1872	6.5%						
<i>Neptis narayana</i> Moore, 1858	9.0%	11.0%					
<i>Neptis nata</i> Moore, [1858]	5.6%	6.3%	11.4%				
<i>Neptis sankara</i> (Kollar, [1844])	8.1%	9.7%	8.6%	7.5%			
<i>Neptis sappho</i> (Pallas, 1771)	5.4%	5.4%	9.9%	6.5%	8.8%		
<i>Neptis soma</i> Moore, 1858	5.4%	4.8%	10.1%	5.2%	8.6%	5.0%	
<i>Pantoporia hordonia</i> (Stoll, [1790])	9.9%	11.0%	10.1%	11.4%	9.9%	9.2%	10.7%

Phylogenetic analysis: A phylogenetic tree was reconstructed based on the mitochondrial gene COI gene sequences of 97 species along with the outgroup (*Aulocera swaha*). Tree was generated via the Maximum likelihood (ML) method and Neighbour- Joining (NJ) method. In the tree made by NJ method, there is the formation of 13 clades and in the tree made by ML method there is the formation of 11 clades, each representing different bootstrap value. We found genus *Lasippa* and genus *Pantoporia* to be the sisters of genus *Neptis* in NJ tree, as also reported by Dhungel & Wahlberg (2018); but these results are not congruent in ML tree. In ML tree, genus *Lasippa* and genus *Pantoporia* are nested within the species of *Neptis*. *Pantoporia venilia* is nested within genus *Neptis* making the genus *Neptis* paraphyletic as also reported by Dhungel & Wahlberg (2018).

In both the trees *Phaedyas aspasia* is placed in the genus *Neptis*, as also reported by Zhang et al. (2011). According to Zhang et al. (2011), the genera *Phaedyas* and *Neptis* are very similar in some main morphological characters as follows: 1) eyes large, prominent, and naked; 2) antenna rather short, not half the length of the fore wing; 3) fore wings elongate, triangular; 4) fore wings: inner margin three fourths of the length of the anterior, more or less emarginated towards the middle; 5) fore wings: vein R5 arises from the median cell; 6) hind wings very oval; 7) hind wings: the outer margin rounded, slightly scalloped; 8) vein Sc+R1 long in the male hind wings; 9) fore legs of the male very slender and short, more or less clothed with very delicate white hairs; 10) abdomen slender, elongated. Based on these findings, while placing *Phaedyas aspasia* in *Neptis* is not surprising, further extensive investigations with additional genes will be required to strengthen robustness further. Species *Lebadea martha* found to be sister to the genus *Neptis* in both the trees as also supported by Dhungel & Wahlberg (2018).

It is concluded that the COI gene can be used to identify Indian Papilionoidea and to determine their phylogeny. Because of its variety, the barcoding gene COI is an excellent marker for phylogenetic study and confirming geographical population distribution patterns. Molecular identification is valuable for those species that are difficult to identify using morphologic characters and help to identify the unidentified species.

Conclusion

The current study presents a detailed DNA analysis of the Neptini. The combination of DNA barcoding with phylogenetic analysis has allowed for more precise classification, uncovered new clades, and highlighted substantial genetic connections across species. The examination of nucleotide composition at the first, second, and third codon locations revealed a considerable A + T bias, particularly at the third codon position. This is consistent with reported patterns in insect mitochondrial genomes. Identifying conserved, variable, parsimony-informative, and singleton sites enhances the genetic structure of these species. As there is a complex genus in tribe Neptini, sometimes it is difficult to identify on the basis of morphology alone. So, these findings underscore the necessity of integrative taxonomic approaches that combine morphological, molecular and phylogenetic data to resolve species relationships accurately. This study not only enhances our understanding of Papilionoidea phylogenetics but also contributes to future conservation strategies by refining species delineations within the tribe Neptini.

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Conflict of Interest

The authors declare that there are no known financial interest or personal relationship that could have influenced the work presented in this article.

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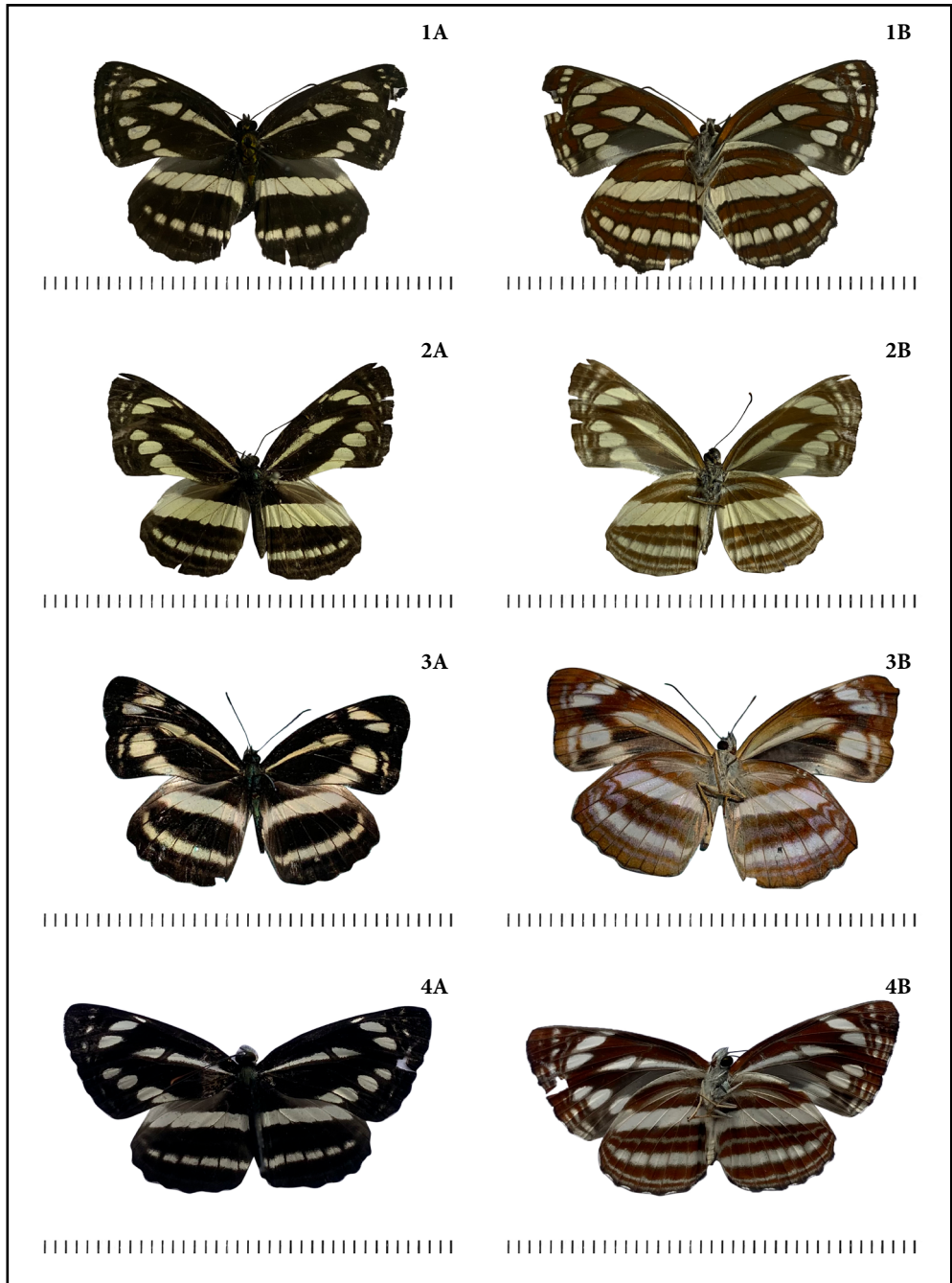
Table 1. List of presently studied species and sequences downloaded from NCBI

Reference	Species	Voucher number	Accession number	Locality
Present study	<i>Neptis clinia</i>	NA33	PV124830	Dehradun, Uttarakhand
Present study	<i>Neptis mahendra</i>	NA48	PV362688	Jibhi, Himachal Pradesh
Present study	<i>Neptis narayana</i>	NA11	PQ657142	McLeodganj, Himachal Pradesh
Present study	<i>Neptis nata</i>	NA34	PV125261	Almora, Uttarakhand
Present study	<i>Neptis sankara</i>	NA9	PQ657136	McLeodganj, Himachal Pradesh
Present study	<i>Neptis sappho</i>	NA8	PV731523	Bir, Himachal Pradesh
Present study	<i>Neptis soma</i>	NA45	PV362625	Barot, Himachal Pradesh
Present study	<i>Pantoporia hordonia</i>	NA41	PV125316	Renukaji, Himachal Pradesh
Dhungel & Wahlberg, 2018	<i>Neptis anjana</i>	PM16-13	MG741113	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis satina</i>	PM15-11	MG741106	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis agouale</i>	JM6-4	MG741057	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis nicobule</i>	JM6-3	MG741056	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis praslini</i>	JM3-2	MG741054	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis nicomedes</i>	JM2-5	MG741053	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis biafra</i>	JM2-3	MG741052	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis armandia</i>	JM2-24	MG741051	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis miah</i>	JM2-23	MG741050	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis cydippe</i>	JM2-22	MG741049	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis woodwardi</i>	JM2-21	MG741048	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis saclava</i>	JM2-17	MG741047	Sweden

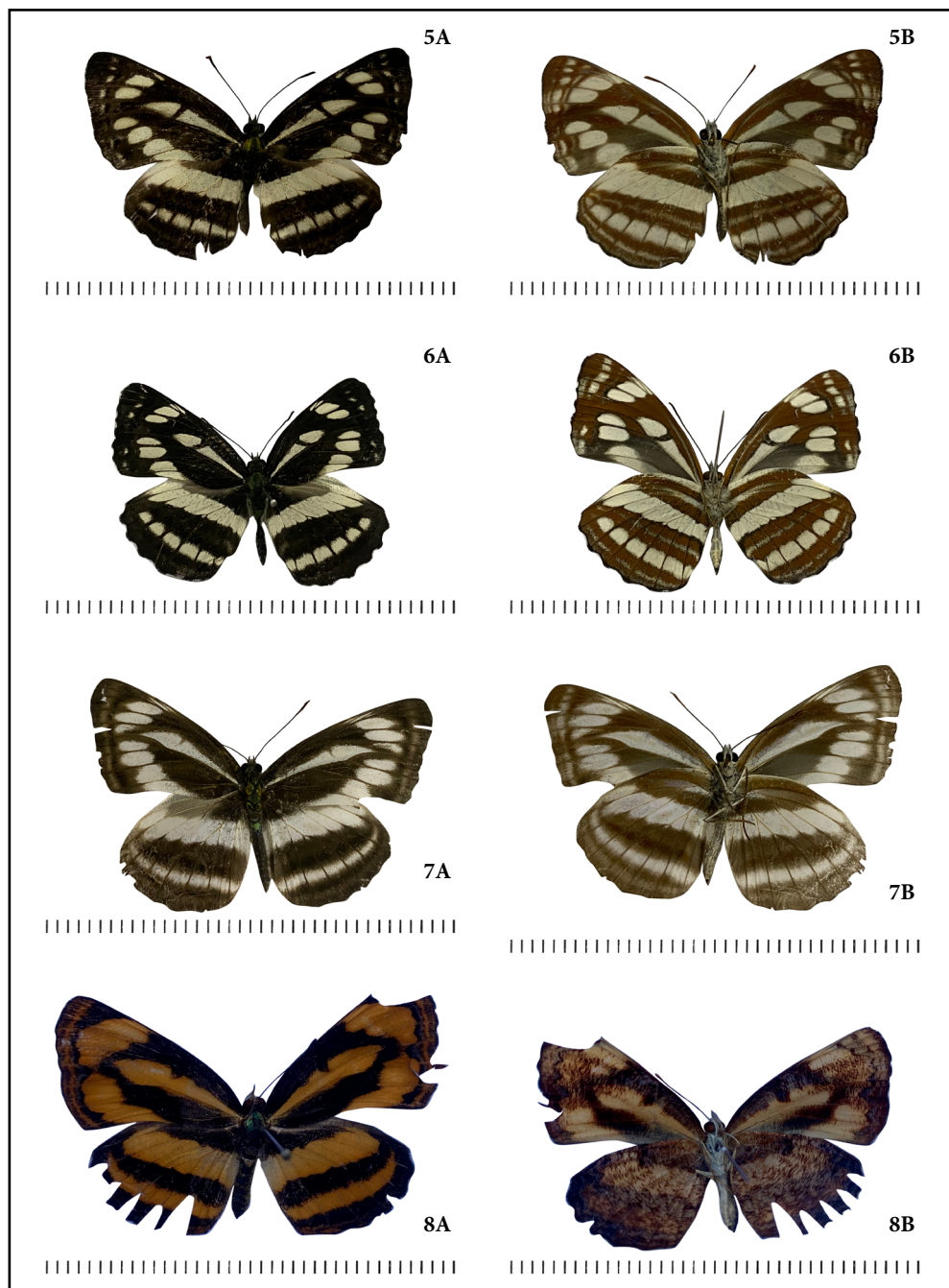
Dhungel & Wahlberg, 2018	<i>Neptis exaleuca</i>	JM2-15	MG741046	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis melicerta</i>	JM2-10	MG741045	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis nysiades</i>	JM2-1	MG741044	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis antilope</i>	JM1-7	MG741043	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis alwina</i>	JM1-5	MG741042	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis yunnana</i>	JM1-4	MG741041	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis divisa</i>	JM1-3	MG741040	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis arachne</i>	JM1-2	MG741039	Sweden
Dhungel & Wahlberg, 2018	<i>Neptis philyra</i>	JM1-11	MG741037	Sweden
Chazot et al. 2020	<i>Neptis tshetverikovi</i>	JM1_8	MW318578	Sweden
Chazot et al. 2020	<i>Neptis quintilla</i>	IS_90	MW318576	Sweden
Chazot et al. 2020	<i>Neptis nemetes</i>	JM2_4	MW318574	Sweden
Chazot et al. 2020	<i>Neptis metella</i>	JM3_4	MW318572	Sweden
Chazot et al. 2020	<i>Neptis laeta</i>	JM2_18	MW318570	Sweden
Ma et al. 2020	<i>Neptis zaida</i>	YI_18_U124	MT283669	China
Ma et al. 2020	<i>Neptis harita</i>	YI_18_U122	MT283667	Thailand
Ma et al. 2020	<i>Neptis magadha</i>	YI_17_V369	MT283665	Thailand
Ma et al. 2020	<i>Neptis namba</i>	YI_17_V164	MT283663	Thailand
Ma et al. 2020	<i>Neptis beroe</i>	MLJ06	MT283660	Thailand
Ma et al. 2020	<i>Neptis thisbe</i>	MLJ03	MT283659	China
Ma et al. 2020	<i>Neptis obscurior</i>	MA079	MT283658	China
Ma et al. 2020	<i>Neptis andetria</i>	MA065	MT283656	China
Ma et al. 2020	<i>Neptis brebissonii</i>	HD_17_Q197	MT283651	Indonesia
Ma et al. 2020	<i>Neptis clinioides</i>	HD_17_Q175	MT283649	Indonesia
Ma et al. 2020	<i>Neptis amphion</i>	HD_17_Q004	MT283648	Indonesia
Ma et al. 2020	<i>Neptis ida</i>	HD_17_F570	MT283646	Indonesia
Ma et al. 2020	<i>Neptis vikasi</i>	HD_17_F561	MT283645	Indonesia
Ma et al. 2020	<i>Neptis heliodore</i>	DL_16_N098	MT283632	Viet Nam
Ma et al. 2020	<i>Neptis pampanga</i>	DL_13_F046	MT283631	Philippines
Ma et al. 2020	<i>Neptis mindorana</i>	DL_11_E033	MT283628	Philippines
Ma et al. 2020	<i>Neptis gracilis</i>	DH_18_R344	MT283626	Indonesia
Ma et al. 2020	<i>Neptis imitans</i>	CM685	MT283624	China
Ma et al. 2020	<i>Neptis reducta</i>	CM684	MT283623	China
Ma et al. 2020	<i>Neptis choui</i>	CM682	MT283621	China
Ma et al. 2020	<i>Neptis qianweiguoi</i>	CM679	MT283618	China
Ma et al. 2020	<i>Neptis raddei</i>	CM677	MT283616	China
Ma et al. 2020	<i>Neptis radha</i>	CM676	MT283615	Viet Nam
Ma et al. 2020	<i>Neptis cymela</i>	CM661	MT283614	Philippines

Ma et al. 2020	<i>Neptis camarensis</i>	CM655	MT283610	Republic of the Congo
Ma et al. 2020	<i>Neptis aurivillii</i>	CM653	MT283609	Kenya
Ma et al. 2020	<i>Neptis strigata</i>	CM652	MT283608	Republic of the Congo
Ma et al. 2020	<i>Neptis conspicua</i>	CM521	MT283599	Republic of the Congo
Ma et al. 2020	<i>Neptis dejeani</i>	CM440	MT283597	China
Ma et al. 2020	<i>Neptis noyala</i>	CM189	MT283591	China
Ma et al. 2020	<i>Neptis hesione</i>	CM163	MT283590	China
Ma et al. 2020	<i>Neptis cartica</i>	CM159	MT283589	China
Ma et al. 2020	<i>Neptis philyroides</i>	CM145	MT283588	China
Ma et al. 2020	<i>Neptis ananta</i>	BM948	MT283587	China
Ma et al. 2020	<i>Neptis meloria</i>	BM681	MT283584	China
Ma et al. 2020	<i>Neptis kuangtungensis</i>	BM666	MT283582	China
Ma et al. 2020	<i>Neptis nemorum</i>	BM591	MT283580	China
Ma et al. 2020	<i>Neptis speyeri</i>	BM289	MT283578	China
Ma et al. 2020	<i>Neptis nemorosa</i>	BM202	MT283575	China
Ma et al. 2020	<i>Neptis thetis</i>	BM200	MT283574	China
Ma et al. 2020	<i>Neptis pryeri</i>	BM185	MT283573	China
Ma et al. 2020	<i>Neptis yerburii</i>	BM161	MT283567	China
Ma et al. 2020	<i>Neptis dumetorum</i>	BM_18_L008	MT283555	France
Zhang et al. 2011	<i>Neptis sinocartica</i>	-	HM999888	China
Wu et al. 2016	<i>Neptis sylvana</i>	N0131	KX900549	Taiwan
Dhungel & Wahlberg, 2018	<i>Lasippa monata</i>	NW17808	MG741098	Sweden
Wilson et al. 2013	<i>Lasippa tiga</i>	UMKL-JJW0262	KF226510	Malaysia
Wilson et al. 2013	<i>Lasippa heliodore</i>	UMKL-JJW0127	KF226508	Malaysia
Ma et al. 2020	<i>Lasippa ebusa</i>	DL_12_E148	MT283630	Philippines
Ma et al. 2020	<i>Lasippa illigera</i>	CM683	MT283622	Philippines
Wilson et al. 2013	<i>Lebadea martha</i>	UMKL-JJW0240	KF226512	Malaysia
Dhungel & Wahlberg, 2018	<i>Pantoporia venilia</i>	PM15-12	MG741107	Sweden
Dhungel & Wahlberg, 2018	<i>Pantoporia consimilis</i>	NW137-3	MG741085	Sweden
Wilson et al. 2013	<i>Pantoporia sandaka</i>	UMKL-JJW0296	KF226557	Malaysia
Dhungel & Wahlberg, 2018	<i>Phaedyma aspasia</i>	JM1-1	MG741036	Sweden
Bassett et al. 2011	<i>Phaedyma shepherdii</i>	USNM:ENT:00649640	HQ570615	Papua New Guinea

Figures 1-4. 1. *Neptis sappho* (Pallas, 1771), **A.** Dorsal side, **B.** Ventral side. 2. *Neptis clinia* Moore, 1872, **A.** Dorsal side, **B.** Ventral side. 3. *Neptis narayana* Moore, 1858, **A.** Dorsal side, **B.** Ventral side. 4. *Neptis nata* Moore, [1858], **A.** Dorsal side, **B.** Ventral side



Figures 5-8. 5. *Neptis soma* Moore, 1858, **A.** Dorsal side, **B.** Ventral side. 6. *Neptis mahendra* Moore, 1872, **A.** Dorsal side, **B.** Ventral side. 7. *Neptis sankara* (Kollar, [1844]), **A.** Dorsal side, **B.** Ventral side. 8. *Pantoporia hordonia* (Stoll, [1790]), **A.** Dorsal side, **B.** Ventral side.



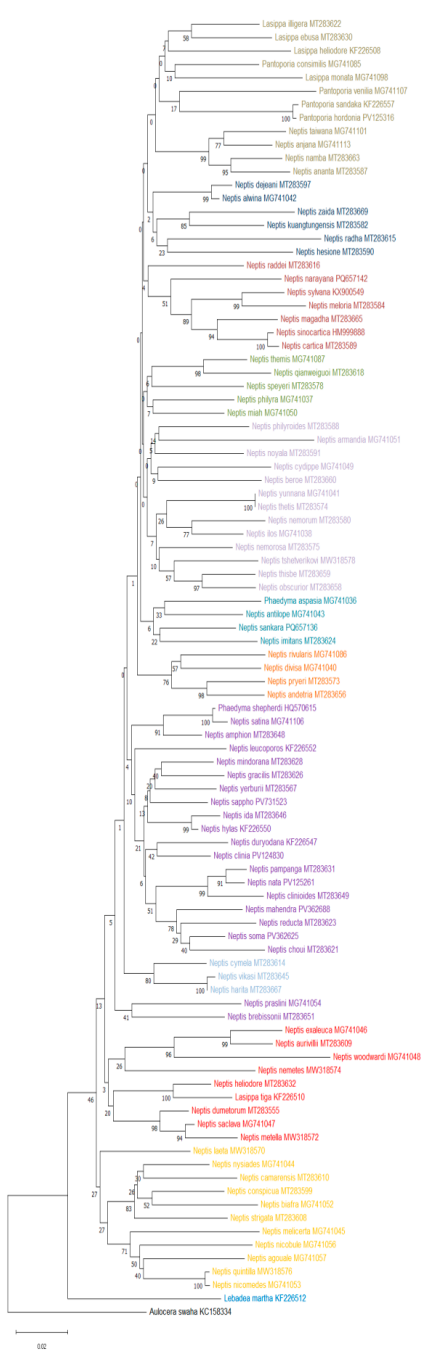


Figure 11. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree is shown. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Evolution analyses were conducted in MEGA12

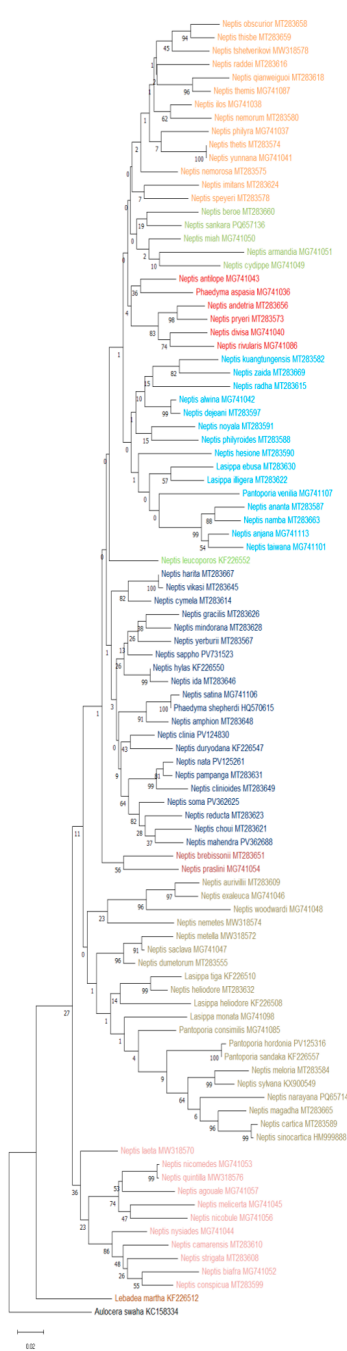


Figure 12. Evolutionary analysis by Maximum likelihood method