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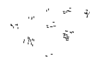
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Phylogenetic relationship and morphological evolution in the subfamily Limenitidinae (Lepidoptera: Nymphalidae)

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Abstract

The mitochondrial cytochrome oxidase subunit I (*COI*) gene and the nuclear elongation factor 1α (*EF-1α*) gene were sequenced from 29 species of Nymphalidae (Nymphalidae, Lepidoptera). Phylogenetic trees were constructed based on the sequences determined from the 29 species and sequences of other 36 species deposited in GenBank using the neighbor-joining (NJ), maximum likelihood (ML) and Bayesian methods with *Libythea celtis* (Libytheinae) as the outgroup. Our phylogenetic trees indicated four major clades. Clade A includes three subfamilies: Apaturinae, Nymphalinae, and Limenitidinae, excluding the tribe Limenitidini; Clade B includes the subfamilies Heliconiinae and the tribe Limenitidini; Clade C includes Satyrinae, Calinaginae, Charaxinae and Morphinae; and Clade D includes subfamily Danainae. Our study suggested that the tribes Pseudergolini, Biblidini, Limenitidini and Cyrestidini should be considered as subfamilies and confirmed the interspecific relationships within the subfamily Pseudergolinae, namely *Amnosia* + (*Pseudergolis* + (*Stibochiona* + *Dichorragia*)). We then mapped three morphological characters (spot of anal angle, eyespots, and process from outer margin of hind wing) onto the phylogenetic tree constructed by ML analysis using the combined sequence data. Based on this the evolutionary patterns of these morphological characters were identified, they indicated that the three characters evolved repeatedly in the family Nymphalidae.
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Keywords: Nymphalidae; Limenitidinae; Mitochondrial DNA cytochrome oxidase subunit I gene (*COI*); Nuclear elongation factor 1α gene (*EF-1α*); Molecular phylogeny; Character evolution

1. Introduction

The family Nymphalidae (Lepidoptera), comprising approximately 7200 species of butterflies, are distributed in all continents of the world except Antarctica [1–3]. Although a large number of the nymphalid species have been taxonomically described, their phylogenetic relationships remain controversial [4,5]. Specifically, there have been great uncertainties regarding the monophyletic nature of the subfamily Limenitidinae and its closest relatives. Harvey [6] reported that Limenitidinae should include the four tribes, Coloburini, Biblidini, Limenitidini and Cyrestidini, as well as the two genera *Pseudergolis* and *Stibochiona*. He also suggested that the tribe Limenitidini contained Limenitiditi, Neptiti, Partheniti and Euthaliiti. In contrast, Paul [7] placed the species of Limenitidinae into the subfamily Nymphalinae, whereas Ackery [8] included the tribe Coloburini in his Nymphalinae. As a result, Limenitidinae has long been abused and has acted as a "trash can" subfamily for many

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species with unclear affinities. In 1999, Ackery et al. [9] suggested that Biblidini should be considered as a subfamily (Biblidinae) rather than a tribe of Limenitidinae, which was supported by Wahlberg et al. and Victor et al. [10,11] Later on, it was found that the Limenitidinae (*sensu* Harvey, 1991) is polyphyletic, and does not include the tribes Cyrestini, Pseudergolini and Biblidini [10]. The finding that Limenitidini is a sister to Cyrestidini ended the debate about the unity of the Limenitidiane *sensu latu*, partitioning this paraphyletic group into at least two (or three if Coeini is considered) monopyletic clades that are not obligatory sister groups [11].

Despite the extensive use of Limenitidinae species in basic research [12,13], the phylogenetic structure of Limenitidinae remains unclear, especially at the levels of tribes and genera, which make it difficult to interpret the evolutionary significance of these results. Phylogenetic studies of taxa that exhibit adaptive phenotypic variation provide valuable insights into the evolutionary mechanisms driving the origins of biodiversity. For example, mapping morphological characters onto phylogenetic trees can be an effective tool for recreating individual character states and investigating the evolution of the morphological characters. Historically, the wing pattern diversity in butterflies has been an important characteristic for studies of evolution. Brakefield et al. [14] investigated development, plasticity and evolution of butterfly eyespot patterns and suggested that the evolution of eyespot patterns can occur rapidly through modulation of different stages of this pathway, and requires only single or very few changes in regulatory genes. Fric et al. [15] reconstructed the phylogeny of the genera *Araschnia, Mynes, Symbrenthia* and *Brensymthia* and investigated evolution of seasonal polyphenism by analyzing the link between the wing colour-pattern characters and the seasonal polyphenism in the *Araschnia* species. In this study, three morphological characters (i.e., spot of anal angle, eyespots of hind wings, and process from outer margin of hind wing) that deal with predator avoidance were selected. Of the three characters, the spot of anal angle and the process from outer margin of hind wings have similar functions, i.e., inducing the predators to attack unimportant or abdicable part of the butterflies; while eyespots of hind wings frighten the predator away as they will be suddenly exposed when the butterflies are attacked. To infer whether the three characters have evolved multi-times in Limenitidinae and other subfamilies, a robust phylogenetic framework of Nymphlidae, especially the monophyly of subfamily Limenitidinae, is needed.

In this study, we sequenced the mitochondrial DNA (mtDNA) cytochrome oxidase subunit I (*COI*) gene and nuclear elongation factor 1α (*EF-1α*) gene from 29 species of Nymphalidae to reconstruct the phylogenetic relationships of the subfamily Limenitidinae by the neighbor-joining (NJ), maximum likelihood (ML), and Bayesian methods. We also investigated the evolutionary histories of the morphological characters by mapping them onto the phylogenetic trees.

2. Materials and methods

2.1. Materials

Sixty-five samples representing five subfamily of the family Nymphalidae were used to resolve the relationships of the major lineages in Nymphalidae and the subfamily Limenitidinae. All specimens were preserved by dehydration in small envelopes in the Insect Collection Center of Shanxi University. The method of preservation has been proven to be effective for DNA isolation and PCR amplification. In addition, 74 referential DNA sequences were acquired from GenBank (Table 1).

2.2. DNA sequencing

The genomic DNA was extracted from the legs of adult butterflies by routine phenol/chloroform extraction method.

Two pairs of primers, HCO and LCO [16], and C1-J-2183 and TL2-N-3014 [17], were used to amplify the cytochrome oxidase I (*COI*) gene of mtDNA. The other two pairs of primers, Starsky and Luke, Cho and Verdi, were used to amplify the elongation factor 1α (*EF-1α*) gene sequence [18]. The primer sequences are shown in Table 2. PCR amplification was carried out in 50 μl of solution containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.1% glutin, 0.25 mM dNTP, 0.6 μM of each primer, 1.5U *Taq* DNA polymerase (SABC Inc.), and 3 μl template DNA (containing 20–50 ng DNA). Thermal cycle parameters for *COI* were as follows: 5 min at 95 °C (1 cycle); 30 s at 94 °C, 30 s at 47 °C, 1 min 30 s at 72 °C (35 cycles); 10 min at 72 °C (1 cycle). The cycling profile for *EF-1α* was 95 °C for 7 min, 35 cycles of 95 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. Amplified products were separated on a 1.2% agarose gel, and the target DNA fragment was purified by Gel Extraction Mini Kit (Watson Biotechnologies, Inc.) and sequenced using the primers mentioned above on an ABI 377 DNA sequencer at Takara Inc. in Dalian, China.

2.3. Morphological observation

The wing color and patterns were observed. The wing pattern data set was established from photographs of 65 species of the Nymphalidae butterflies. The spot of anal angle and process from outer margin of hind wings of butterflies were identified as either absence or presence. Eyespot of hind wings was graded as absent, present or degraded (the case where hind wings have a few unconspicuous eyespots defined as degraded).

2.4. Data analyses

2.4.1. Molecular data analyses

All the sequences obtained have been deposited to the GenBank, which are shown in Table 1. The assembled

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Table 1
List of species and GenBank accessions used in this study

Subfamily	Tribe	Species	COI	*EF-1α*
Apaturinae		*Apatura iris**	EF534090	EF683655
		*Mimathyma schrenckii**	EF534088	EF683659
		*Chitoria ulupi**	EF534093	EF683660
		*Timelaea maculate**	EF534099	EF683661
		*Sephisa chandra**	EF534084	EF683656
		*Euripus nyctelius**	EF534096	EF683658
		*Hestina assimilis**	EF534097	EF683641
		*Sasakia charonda**	EF534092	EF683657
Nymphalinae	Nymphalini	*Polygonia canace**	EF683672	EF683649
		*Polygonia c-aureum**	EF683673	EF683650
		*Nymphalis vau-album**	EF683675	EF683651
		*Aglais io**	EF683674	EF683652
		*Aglais urticae**	EF683676	EF683666
		*Araschnia doris**	EF683671	EF683654
		*Vanessa cardui**	EF683677	EF683653
	Kallimini	*Junonia orithya**	EF683678	EF683642
		*Doleschallia bisaltide**	EF683667	EF683662
		*Hypolimnas bolina**	EF683668	AY090190
	Melitaeini	*Melitaea scotosia**	EF683669	EF683663
		*Melitaea jezabel**	EF683670	EF683664
	Coeini	*Baeotus beotus*	AY788615	AY788720
Calinaginae		*Calinaga buddha**	EF683684	EF683647
Limenitidinae	Coloburini-Biblidini	*Colobura dirce*	AY090228	AY090196
		Byblia anvatara	AY788595	AY788697
		Eurytela dryope	AY218242	AY218262
		Mesoxantha esothea	AY788598	AY788700
		Ariadne enotrea	AY218237	AY218256
		Hamadryas februa	AY090216	AY090182
		Sevenia boisduvali	AY218247	AY218267
		Callicore pacifica	AY788596	AY788698
		Nica flavilla	AY218245	AY218265
		Catonephele numilia	AY090215	AY090181
		Myscelia capensis	AY788599	AY788701
	Limenitidini	*Parthenos sylvia*	AY090218	AY090184
		Euphaedra herberti	AY218241	AY218261
		Limenitis reducta	AY090217	AY090183
		Adelpha bredowi	AY788591	AY788693
		*Litinga mimica**	EF683679	EF683643
		*Athyma jina**	EF534100	EF683644
		*Auzakia danava**	EF683683	EF683646
		*Neptis sappho**	EF683682	EF683648
		*Euthalia monina**	EF683680	EF683645
	Cyrestidini	*Cyrestis thyodamas*	AY218240	AY218260
		Chersonesia rahria	AY788601	AY788703
		Marpesia orsilochus	AY788604	AY788706
		Marpesia chiron	AY788603	AY788705
	Pseudergolini	*Amnosia decora*	AY218235	AY218254
		Stibochiona nicea	AY218249	AY218269
		Pseudergolis wedah	AY788605	AY788707
		Dichorragia nesimachus	AY788602	AY788704
Heliconiinae	Heliconiini	*Argynnis paphia*	AY090200	AY090166
		*Argyreus hyperbius**	EF683681	EF683665
		Clossiana selene	AY090201	AY090167
		Heliconius hecale	AY090202	AY090168
		Vagrans egista	AY090203	AY090169
	Acraeini	*Actinote stratonice*	AY218233	AY218252
Charaxinae	Charaxini	*Charaxes castor*	AY090219	AY090185
		*Charaxes bernardus**	EF534101	EF683640
Satyrinae	Melanitini	*Melanitis leda*	AY090207	AY090173
	Tribe unknown	*Manataria maculata*	AY218244	AY218264

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Table 1 (*continued*)

Subfamily	Tribe	Species	COI	*EF-1α*
Morphinae	Morphini	*Morpho peleides*	AY090210	AY090176
	Amathusiini	*Stichophthalma howqua*	AY218250	AY218270
Danainae	Ithomiini	*Greta oto*	AY090206	AY090172
	Danaini	*Euploea camaralzeman*	AY090205	AY090171
Libytheinae		*Libythea celtis*	AY090198	AY090164

Note: The species sequenced in this study are indicated by an asterisk.

Table 2
Sequences of the primers used in this study

Primer	Sequence
COI	
HCO	5′-TAA ACT TCA GGG TGA CCA AAA AAT CA-3′
LCO	5′-GGT CAA CAA ATC ATA AAG ATA TTG G -3′
C1-J-2183	5′-CAA CAT TTA TTT TGA TTT TTT GG -3′
TL2-N-3014	5′-TCC AAT GCA CTA ATC TGC CAT ATT A -3′
EF-1α	
Starsky	5′-CAC ATY AAC ATT GTC GTS ATY GG-3′
Luke	5′-CAT RTT GTC KCC GTG CCA KCC-3′
Cho	5′-GTC ACC ATC ATY GAC GC-3′
Verdi	5′-GAC ACC AGT TTC IAC TCT GCC-3′

sequences were aligned using Clustal X version 1.83 [19] and checked manually. The presence of substitution saturation was determined with DAMBE version 4.5.18 [20]. The genetic distance versus the number of transitions and transversions at the first two codon positions and at the third codon position in all taxa was plotted to examine the saturation at three codon sequences.

We have analyzed three data sets, which are the mtDNA *COI* gene, the nuclear *EF-1α* gene and the combined data set of mitochondrial and nuclear sequences. To test the robustness of phylogenetic relationships among the members in subfamily Limenitidinae, phylogenetic analyses were performed using neighbor-joining (NJ; PHYLIP v3.66) [21], maximum likelihood (ML; RAxML 7.0.0) [22] and Markov Chain Monte Carlo (MCMC; MrBayes v3.1.2) [23] methods. For NJ analysis, a distance matrix was constructed from the aligned sequences using Kimura two-parameter formula. Support values of the distance tree were obtained by bootstrapping [24] 1000 replicates using SEQBOOT in the PHYLIP package. The phylogenetic trees were constructed by maximum likelihood method using the program RAxML 7.0.0 [22]. The optimum substitution models were first determined by the programs Modeltest, version 3.7 [25], and PAUP*, version 4.0b4a [26]. The AIC results from Modeltest provided the GTR + I + G model as the best fit for substitution model. The ML tree was then constructed in RAxML using the GTR + I + G model. Bayesian phylogenetic analyses were performed with MrBayes version 3.1.2 [27]. The optimum substitution models were determined by using the programs MrModeltest (version 2.2 [28]) and PAUP* (version 4.0b4a [26]). The MrBayes analysis was run with four MCMC chains and a burn-in of 20,000 generations followed by a search of 10,000,000 generations for the best tree. These sensitivity analyses are helpful for identifying potential instances of long-branch attraction [29] and can provide a valuable heuristic tool for guiding subsequent sampling strategies to refinement of the current hypothesis.

2.4.2. Morphological data analyses

Using MacClade 3.0, we mapped the three morphological characters (i.e., spot of anal angle, eyespots and process from outer margin of hind wing) on the internal nodes of the combined phylogenetic tree that was constructed based on a maximum likelihood of divergent DNA sequences of the *COI* and *EF-1α* genes.

3. Results

3.1. General properties of the sequences

The complete alignment of the partial mtDNA *COI* gene sequence from the 65 nymphalid species used in this study resulted in an alignment containing 1487 sites, among which 675 sites were variable and 555 sites were parsimony-informative. The mean frequency of nucleotides in the compared *COI* sequences showed a bias of A + T (T 40.5%, C 14.7%, A 31.3% and G 13.5%), which is commonly found in insect mitochondrial genomes [18,30]. The *COI* region showed no indels. The A + T content at the third, second and first codon positions from the *COI* fragment was 92.3%, 61.1% and 62.2%, respectively. The distribution of variable sites showed that the majority of substitutions were at synonymous sites. For the nuclear *EF-1α* region, the total data set consisted of 1240 nucleotides, among which 443 sites were found with variations and 351 sites were parsimony-informative characters. The base composition of the *EF-1α* fragment varied among the individuals and the averages were T 22.7%, C 26.8%, A 26.5% and G 24.0%.

All three codon positions in the mtDNA *COI* and nuclear *EF-1α* genes were tested independently for saturation, achieved by plotting the number of observed substitutions against the genetic distance estimates. The scattergrams (Fig. 1a–c) showed that transitions and transversions for the first, second and third codons of the nuclear *EF-1α* gene as well as the first and second codons

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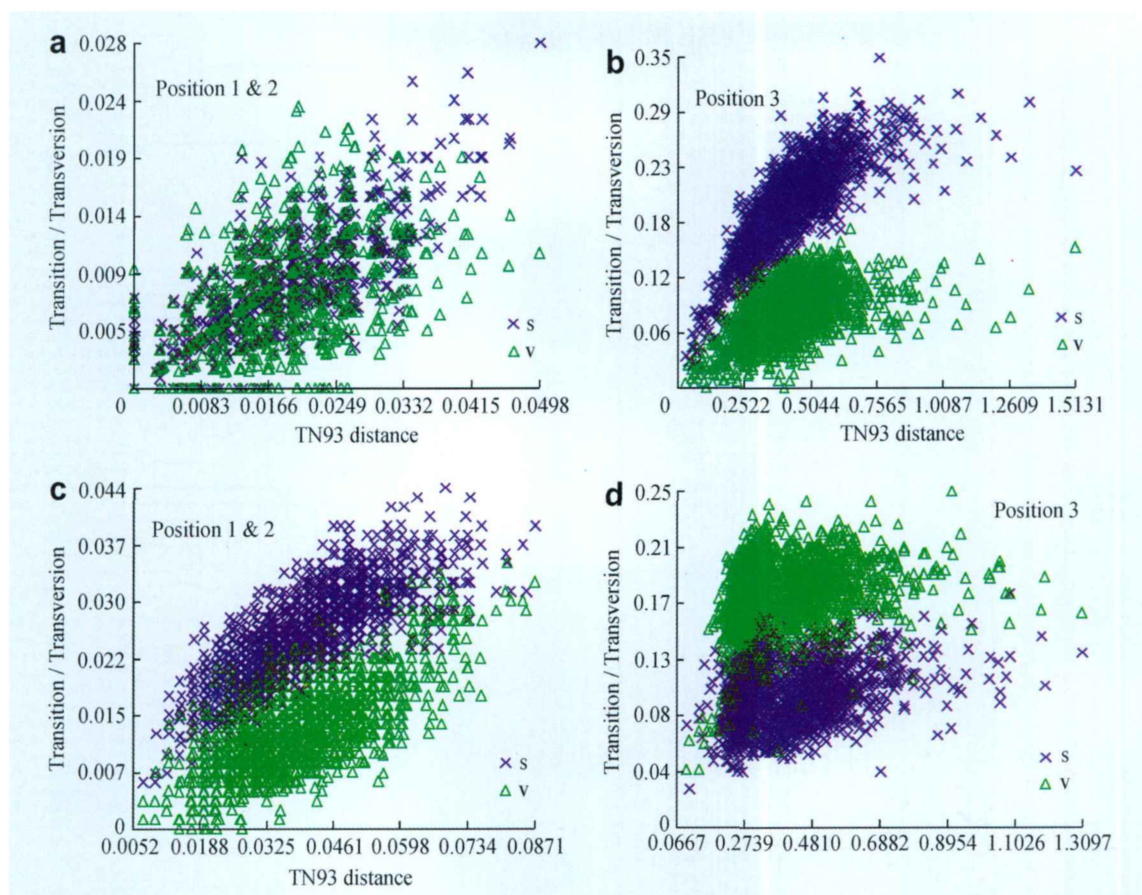

Fig. 1. Scatterplots showing the number of substitutions (Transition, TS; Transversion, TV; *y* axes) vs genetic distance (TN93; *x* axes) at the 1st and 2nd codon positions, and the 3rd codon positon. (a) The 1st and 2nd position TS and TV of *EF-1α*; (b) the 3rd position TS and TV of *EF-1α*; (c) the 1st and 2nd position TS and TV of *COI*; (d) the 3rd position TS and TV of *COI*.

of the mtDNA *COI* gene increased with the genetic distance, but considerable scattering was also observed. In addition, a similar plot of the third codon transition of the *COI* gene (Fig. 1d) suggested that saturation of transition occurred between certain pairs of the taxa, which may lead to higher levels of homoplasy [30]. Note that multiple substitutions at a site may potentially obscure true phylogenetic signals.

3.2. Phylogenetic analyses

In order to investigate the phylogenetic relationship of the subfamily Limenitidinae and obtain a phylogenetic framework of the family Nymphalidae, we constructed the phylogenetic trees for the species in the subfamilies Apaturinae, Nymphalinae, Satyrinae, Danainae, Calinaginae, Heliconiinae, Charaxinae, Morphinae and Libytheinae. Using different methods, phylogenetic reconstructions based on the three data sets (*i.e.*, the mtDNA *COI* gene, the nuclear *EF-1α* region and the combined data) revealed similar topologies. Because of the relatively low resolution of the trees constructed from the separated data, phylogenetic trees based on the combined data sets were further analyzed. Using NJ, ML and Bayesian methods, the analyses revealed similar topologies, especially for the topologies reconstructed by ML and Bayesian methods (Fig. 2).

Using Libytheinae as an outgroup, the phylogeneitc tree (Fig. 2) revealed four main clades. Clade A contains species of the subfamilies Apaturinae, Nymphalinae and Limenitidinae, excluding Limenitidini; Clade B contains species of Heliconiinae and the tribe Limenitidini of the subfamily Limenitidinae (*sensu* Harvey, 1991); Clade C contains species of Charaxinae, Calinaginae, Satyrinae and Morphinae; and Clade D contains species of Danainae. The positions of Heliconiinae and Limenitidini in Limenitidinae (*sensu* Harvey, 1991) and the closer relationships of Charaxinae and Calinaginae with Satyrinae and Morphinae, were generally consistent with the previously reported results of Wahlberg [10] who used MP method, and the analysis was based on the mtDNA *COI*, nuclear *EF-1α* and *Wingless* genes. In the NJ tree, Clade A is subdivided into five subclades and two of which were strongly supported (bootstrap values > 89%, Fig. 2). The five distinct subclades are the species *Colobura dirce* and the species of Nymphalinae (A1), *Baeotus beotus*, the species of Apaturinae (A2), the tribe Biblidini of Limenitidinae (A3), the tribe Cyrestidini of Limenitidinae (A4), and the species *Amnosia decora, Stibochiona nicea, Pseudergolis wedah* and *Dichorragia nesimachus* (A5). In Clade B, two subclades, belonging to the species of the tribe Limenitidini in Limenitidinae, are clustered with a strong support (B1) and the species of the subfamily Heliconiinae form a cluster (B2). The species of the

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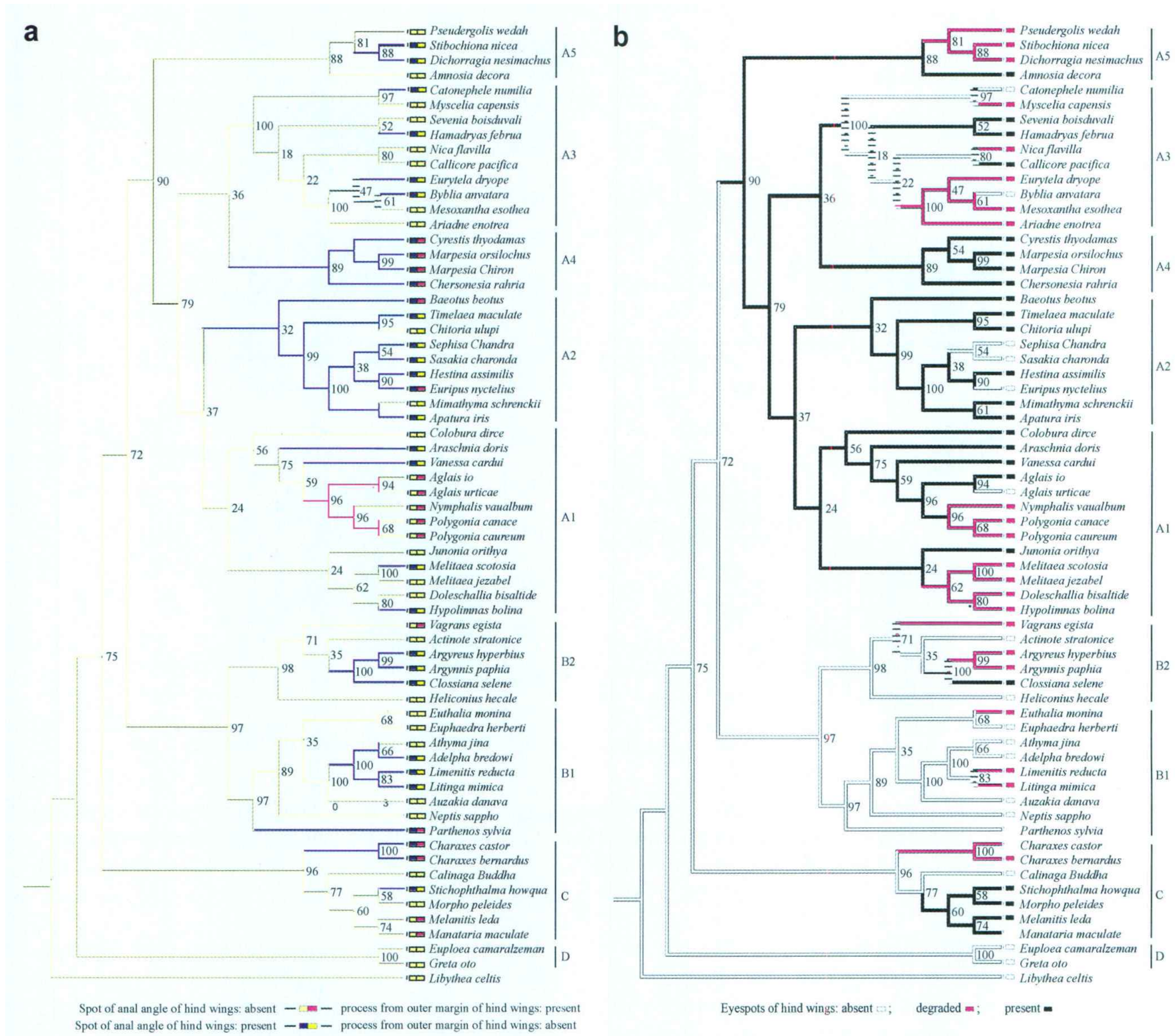

Fig. 2. Evolution of three wing morphological characters in Nymphalidae. The phylogenetic tree was reconstructed using ML method based on 65 species of Nymphalidae, with *Libythea celtis* as an outgroup. (a) The two morphological characters, namely pot of anal angle and process from outer margin of hind wings, were mapped on the phylogenetic tree by ML method using the MacClade 3.0. The bootstrap values are shown at branching point. (b) The character of eyespots of hind wings was mapped on the phylogenetic tree by ML method using the MacClade 3.0.

subfamily Morphinae, Satyrinae, Charaxinae and Calinaginae clustered together with the strong support (C), but the species of Danainae form a separate cluster (D). The same clades were recovered on the Bayesian trees, except for a few positions of several species on these trees (data not shown). Furthermore, the topologies are also very similar to those of the NJ tree except for the positions of Clades C and D.

3.3. Mapping of morphological characters

The three morphological characters were mapped onto the phylogenetic tree that was constructed using the combined dataset (Fig. 2). The mapping result suggested possible repeated origins of these characters in the family Nymphalidae. We also mapped the three characters onto each well-supported subclade, including A1 (except *Colobura*) and A2 (except *Baeotus*). In Subclade A1, the spot of anal angle of hind wings had evolved four times, the process from outer margin of hind wings had evolved one time, and eyespots of hind wings evolved one time and had been degraded in some species. In Subclade A2 excluding *Baeotus*, A3, B1 and Clade C, the spot of anal angle of hind wings had evolved several times. However, it evolved only once in Subclade A4, A5 and B2. Process from outer margin of hind wings evolved once in Subclade A2 except *Baeotus*, A3, B1 and B2, but twice in Clade C. Within A4 and C, the eyespots of hind wings evolved only once and were a basal character for the two subclades. In contrast, the eyespots of hind wings were absent or degraded in most species of Subclade B1. The eyespots of hind wings were present or degraded in most species of Clade A, but absent in most species of Clades B, C and D.

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4. Discussion

4.1. Phylogenetic relationships

Phylogenetic analyses based on the combined sequence data set using NJ, ML and Bayesian methods produced trees with four main clades (A, B, C and D). Clades A and B were further separated into several subclades.

Our data suggested that Subclades A1 and A2 are sister groups, Subclade A3 was a sister to A4 and Subclade A5 was a sister to the group ((A1 + A2) + (A3 + A4)). This pattern was inconsistent with the results of Wahlberg [10], who reported that the tribes Cyrestini and Pseudergolini were sister groups. However, the tribe Cyrestini was the sister to Biblidini and the tribe Pseudergolini had a further relationship with Cyrestini in our results, suggesting that the tribes Cyrestini and Pseudergolini cannot be placed into the same subfamily Cyrestinae. Instead, they should be classified into two subfamilies (i.e. Cyrestinae and Pseudergolinae). In Subclade A5, *Stibochiona nicea* and *Dichorragia nesmachus* clustered together with a high bootstrap value (88%). *Pseudergolis wedah* was a sister to the genus *Stibochiona* + *Dichorragia* and *Amnosia decora* clustered with them. The species *Colobura dirce* of the tribe Coloburini was closer with Nymphlinae.

The phylogenetic positions of the species *Aglais io* and *Polygonia canace* have long been controversial. Paul [7] placed the species *Aglais io* and *Polygonia canace* in the genus *Vanessa*. However, Chou [31,32], Li and Zhu [33] and Bai et al. [34] suggested that the species *Aglais io* and *Polygonia canace* should be included in the genus *Inachis* and *Kaniska*, respectively. In this study, the species *Aglais io* was found to be a sister to *Aglais urticae* with a strong support (bootstrap value 94%), which agreed with Harvey's suggestion [6] that moving the species *Aglais io* into the genus *Aglais*. Nevertheless, the species *Polygonia canace* and *Polygonia caureum* clustered together with a low bootstrap value (68%), showing that the two species should be placed into different genera. This confirmed the concept of Chou [31,32]. In Subclade A2, *Chitoria ulupi* and *Timelaea maculate* were sister species and the other species formed a cluster with very strong support (bootstrap value 100%). Accordingly, it seems reasonable to divide the subfamily into two tribes Apaturini and Chitorini.

According to Harvey's classification, the tribe Limenitidini included four subtribes, which are Limenitiditi, Neptiti, Partheniti and Euthaliiti. However, Chou [31,32] suggested that Limenitidinae included the tribes Euthaliini, Limenitini, Chalingini, Neptini and Parthenini. However, it follows from our study that *Auzakia danava* was a sister to *Adelpha* + (*Limenitis* + (*Litinga* + *Athyma*)) with a strong supported bootstrap value (100%), thus confirming Harvey's opinion. If it is the case, the subfamily Limenitidinae should consist of Limenitiditi, Neptiti, Partheniti and Euthaliiti. In Clade C, Calinaginae was the sister group to Satyrinae + Morphinae with a good supported bootstrap value (77%) and clustered with Charaxinae as well (bootstrap value 96%). Obviously, this result was different from the findings of recent studies [10,11], which found that Calinaginae and Charaxinae were sister groups and Calinaginae was basal to Charaxinae.

Based on the above results, one can draw the following conclusions.

1) The tribes Pseudergolini, Biblidini, Limenitidini and Cyrestidini formed monophyletic groups. Therefore, they should be considered as different subfamilies, namely Pseudergolinae, Biblidinae, Limenitidinae and Cyrestidinae.
2) The subfamily Apaturinae should be divided into two tribes, Apaturini and Chitorini.
3) If Limenitidini is identified as a subfamily, Limenitidinae could be divided into four rather than five tribes.
4) In Pseudergolinae, the relationships of the genera should be considered as *Amnosia* + (*Pseudergolis* + (*Stibochiona* + *Dichorragia*)).

4.2. Evolution of morphological characters

The evolution of morphological characters is a difficult phenomenon to study because it is rarely fast enough to be observed directly. However, the evolution often leaves its footprint in the distribution of the characters among living organisms. Therefore, for a given phylogenetic tree, one may reconstruct the evolutionary history of individual characters of interest. Currently, many studies in this field still rely on explicit or implicit parsimony mapping of characters onto a single phylogenetic tree [35].

All existing different morphological characters of various butterflies have certain connections with the survival environment and the natural selection. In the long-term struggle between butterflies and predators, the morphological characters that benefit to survival are usually preserved, and thus have important evolutionary significance. Our results of mapping characters on the phylogenetic tree suggested that the three characters might have repeatedly evolved in the family Nymphalidae. In particular, the spots on anal angle of hind wings might also have originated more than once in Subclades A1 excluding *Colobura*, A2 excluding *Baeotus*, A3, B1 and Clade C. The spot on anal angle of hind wings had originated many times in Clade C, Subclades A1, A3 and B1. In contrast, it may have originated only once in Subclades A2, A4, A5 and B2, and the absence of the spot on anal angle of hind wings was a basal character in each Subclade. The eyespots of hind wings were identified as presence, degraded or absence. The diversity of the eyespots of hind wings and anal angle of hind wings generated by butterflies may be attributed to the avoidance of predation and adaptation to different environments. For example, some species in the subfamily Satyrinae escape from their predators by abruptly exposing their eyespots threateningly, which have led to mim-

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icry by other species within the subfamily. This character is present or degraded in most species of Clade A, but absent in most species of Clades B, C and D, suggesting that absence of eyespots of hind wings is a basal character. To adapt the new or changed environments and avoid from predators, some hind wings of butterfly species presented eyespots and entail on their offspring, though this character of some species degraded or even dispeared again. Each species in Subclade A4 has all of the three characters, but their distribution, host plants and habitats are different, it implies that the three characters might be generated by the mode of convergent evolution.

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