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Phylogeny, biogeography, and host–plant association in the subfamily Apaturinae (Insecta: Lepidoptera: Nymphalidae) inferred from eight nuclear and seven mitochondrial genes

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ABSTRACT

The subfamily Apaturinae consists of 20 genera and shows disjunct distributions and unique host–plant associations. Most genera of this subfamily are distributed in Eurasia South-East Asia and Africa, whereas the genera *Doxocopa* and *Asterocampa* are distributed mainly in South America and North America, respectively. Although the Apaturinae larvae mainly feed on the Cannabaceae, those of the genus *Apatura* are associated with *Salix* and *Populus* (Salicaceae), which are distantly related to the Cannabaceae. Here, we infer the phylogeny of Apaturinae and reconstruct the history of host shifting and of colonization in the New World. We analyzed 9761 bp of nuclear and mitochondrial DNA sequence data, including the genes encoding EF1a, Wg, ArgK, CAD, GAPDH, IDH, MDH, RpS5, COI, COII, ATPase8, ATPase6, COIII, ND3, and ND5 for 12 apaturine genera. We also inferred the phylogeny with six additional genera using mitochondrial sequence data alone. Within the Apaturinae, two major clades are recovered in all the datasets. These clades separate the New World genera, *Doxocopa* and *Asterocampa*, indicating that dispersal to the New World occurred at least twice. According to our divergence time estimates, these genera originated during the Early Oligocene to the Early Miocene, implying that they migrated across the Bering Land Bridge rather than the Atlantic Land Bridge. The temporal estimates also show that host shifting to *Salix* or *Populus* in *Apatura* occurred more than 15 million years after the divergence of their host plants. Our phylogenetic results are inconsistent with the previously accepted apaturine genus groups and indicate that their higher classification should be reconsidered.

1. Introduction

The butterfly family Nymphalidae contains about 6000 described species, which have been used in diverse evolutionary studies, such as metapopulation dynamics (Thomas and Hanski, 2004), hybridization (Mallet et al., 2007; Blum, 2008), chemical interactions (Dapporto, 2007), chromosomal rearrangements (Brown et al., 2007), and insect–plant interactions (Janz et al., 2001; Wahlberg, 2001; Nygren et al., 2006; Nylin and Wahlberg, 2008). Thus, the systematics of Nymphalidae has been under intense scrutiny, and recent studies using molecular markers have inferred robust and comprehensive phylogenies (Wahlberg et al., 2003, 2005, 2009a; Wahlberg and Wheat, 2008). These studies divide the Nymphalidae into five major lineages, comprising 12 subfamilies, with fairly strong statistical supports. However, the systematic relationships within each subfamily are less well known, except for Nymphalinae (Wahlberg et al., 2005; Wahlberg, 2006), Satyrinae (Peña et al., 2006), and Danainae (Willmott and Freitas, 2006; Brower et al., 2010).

The subfamily Apaturinae is one of the unresolved nymphalid groups. This subfamily comprises 430 described species belonging to 20 genera (Le Moult, 1950; Ackery et al., 1999) and is defined by unique morphological character states. Apaturine species have male genitalia with an extremely elongated phallus and saccus, character states that are absent from other butterfly species (Le Moult, 1950; Shirôzu, 1960; Shirôzu and Saigusa, 1971; Ackery et al., 1999). These unique character states support monophyly of the Apaturinae (Shirôzu and Saigusa, 1971; Ackery et al., 1999) which has also been supported by molecular data

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(Wahlberg et al., 2003, 2005, 2009a). Although monophyly is well established, relationships among the genera within Apaturinae have been little studied. Le Moult (1950) divided the subfamily into three groups, on the basis of wing venation and male genital characters. The first group comprises the genera, *Apatura*, *Sephisa*, *Chitoria*, *Bremeria*, *Hestinalis*, *Hestina*, *Euripus*, *Sasakia*, *Rohana*, and *Narsenga*, and is characterized by an opened discoidal cell and a straight phallus. The second group comprises *Apaturina*, *Dilipa*, and *Thaleropis*, and is characterized by a closed discoidal cell and a straight phallus. Finally, the third group comprises *Eulaceura*, *Herona*, *Helcyra*, and *Apaturopsis*; in this group, *Eulaceura* have a curved aedeagus, *Helcyra* have an aedeagus that is thicker than its saccus, and *Herona* and *Apaturopsis* lack a gnathos. However, members of the third group do not share an apomorphic character state, which implies this group may be either polyphyletic or paraphyletic. Furthermore, the monophyly of the first and second groups is also ambiguous because the polarity and parallel evolution of the character states for the discoidal cell (i.e., opened or closed) remain undetermined.

The Apaturinae show disjunct distributions; majority of members are distributed in Eurasia and South-East Asia except for *Apaturina* extending to Australasian region; *Thaleropis* and *Euapatura* are endemic to Central Asia; *Apaturopsis* is endemic to Africa, whereas *Doxocopa* and *Asterocampa* are mainly distributed in South America and North America, respectively. Similar disjunct distributions have been reported for many other insect taxa, and such distribution has been attributed to large dispersal and vicariance events across Eurasia and North America in association with global climatic changes during the Tertiary and Quaternary (Esaki, 1932; Linsley, 1963; Akimoto, 1985; Saigusa and Kato, 2002; Sinclair and Saigusa, 2002; Sano and Akimoto, 2005; Havill et al., 2006; Praz et al., 2008; Sota et al., 2008). For tropical or temperate biota, warm intervals in the Tertiary created intercontinental habitat on land bridges at high-latitudes, but subsequent glaciations interrupted this continuity (Wen, 1999; Sanmartìn et al., 2001). In this scenario, the divergence times of New World taxa should be consistent with the warm intervals. However, for insects, few studies have estimated divergence times based on molecular phylogenetic analysis to test the land-bridge hypothesis across Eurasia and North America (Sota et al., 2008).

Apaturine butterflies are associated mainly with hackberry trees (*Celtis*, Cannabaceae) and related families (Urticaceae and Fagaceae), as a larval food. However, larvae of the genus *Apatura* feed on willow and poplar trees (*Salix* and *Populus*, Salicaceae), which are distantly related to Cannabaceae (APGIII, 2009). Host shifting to a novel plant species decreases intraspecific competition for immigrants and provides enemy-free space for them (Denno et al., 1995). However, the length of time required from the origin of the host plant to the insect's adaptation to it is controversial (Wheat et al., 2007). Therefore, intensive phylogenetic work and divergence time estimation can provide insight into the insect–plant associations and biogeography of Apaturinae.

The first molecular phylogenetic study of Apaturinae was conducted by Zhang et al. (2007). This milestone included 13 genera and revealed that the genera, *Timelaea*, *Chitoria*, *Sephisa*, *Mimathyma*, and *Apatura*, are each monophyletic. Their results also indicated that Apaturinae are divided into two major lineages, with each lineage having two sub-clades, but the classification by Le Moult (1950) was not supported. However, because the analysis of Zhang et al. (2007) was based only on a single mitochondrial gene (COI), statistical support for the deeper nodes was weak. Therefore, the higher classification of Apaturinae and their biogeographical and host-association history remained ambiguous.

The present study aims to clarify the systematic relationships among apaturine genera and to estimate the history of colonization of the New World and of host shifting to Salicaceae. To resolve the deeper nodes of Apaturinae, we analyzed eight nuclear genes (EF1a, Wg, ArgK, CAD, GAPDH, IDH, MDH and RpS5) and seven mitochondrial genes (COI, COII, ATPase8, ATPase6, COIII, ND3 and ND5). We also aim to compare the performance of phylogenetic informativeness between mitochondrial and nuclear genes for Apaturinae.

2. Materials and methods

2.1. Taxon sampling

Twenty exemplar species representing all 20 apaturine genera were used for analyzing mitochondrial genes, but sequences could not be obtained from *Thaleropis* and *Euapatura* (Table 1). Here, the genera, *Apaturina* and *Herona* are used in molecular phylogeny for the first time. Nuclear-gene sequences of 12 exemplar species representing 12 genera (Table 2) were derived from Wahlberg et al. (2009a). Thus, we used the following four datasets for the present phylogenetic inferences: nuclear dataset comprising 12 ingroups (nuclear), mitochondrial dataset comprising 18 ingroups (mt18), mitochondrial dataset comprising 12 ingroups that are shared in the nuclear dataset (mt12), and combined dataset of mt12 and nuclear (mt12 + nuclear). For the mt12 + nuclear dataset, the same butterfly individual was used for determining each of mitochondrial and nuclear partitions in *Apaturopsis* and in *Timelaea*, but the two partitions were derived from different individuals in each of the remaining 10 genera. Terminal units of *Apatura*, *Sephisa*, *Hestina*, *Doxocopa* and *Asterocampa* comprised partitions representing different exemplar species (Tables 1 and 2). However, the monophyly of each of the five genera is well established both by morphology and molecular data (Le Moult, 1950; Friedlander, 1987; Zhang et al., 2007), thus these treatments should not hamper the conclusion of the present study. Two terminal units representing two genera, *Ariadne* and *Dichorragia*, from the two related subfamilies (Biblidinae and Pseudergolinae, respectively; Wahlberg et al., 2003, 2009a) were used as outgroups for all the four datasets. The sequence of *Ariadne* for the mt12 + nuclear dataset comprised partitions derived from the two species (Tables 1 and 2). Specimens in Table 1 were collected as fresh adults from the field using a hand net and then stored in 99.5% ethanol or dried.

2.2. DNA extraction for mitochondrial-gene analyses

Total DNA was extracted from one to three legs. The legs were squashed in 500 μL Proteinase K solution (10 mM Tris–HCl [pH 8.0], 10 mM EDTA, 0.5% SDS, and 0.5 mg/mL proteinase K [Boehringer Mannheim, Mannheim, Germany]), and incubated at 55 °C for 2 h. The resulting solution was extracted once with phenol saturated with TE buffer (10 mM Tris–HCl [pH 8.0] and 1 mM EDTA), twice with phenol/chloroform/isoamyl alcohol (26:25:1), and once with chloroform/isoamyl alcohol (25:1). The supernatant was transferred to a small centrifuge tube, and RNA was digested with 8 mg/L RNase A (Wako) at 37 °C for 30 min. The total DNA was precipitated by adding twice the volume of 99.5% ethanol and one tenth the volume of 3 M sodium acetate to the supernatant, washed with 70% ethanol, dried, and then dissolved in 200 μL TE buffer. DNA extracts of *Apaturopsis cleochares* and *Timelaea albescens* were kindly provided by Dr. N. Wahlberg (University of Turku, Finland).

2.3. Amplification and sequence determination of mitochondrial genes

ND5 and COI genes were amplified using previously described primers V1 (Yagi et al., 1999), k698, Ron, Pat, PatII (Caterino and Sperling, 1999) and newly designed primers KA1L, T1A1L, T2A1L