

Species reassessment congruent with the phylogeographical study of the *Biston falcata* species group

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Abstract. The Himalaya, Hengduan, and Qinling mountains are very important mountain systems in China. Because they are geographically adjacent, species distributed in these three mountains usually have close relationships, but species/populations distributed in the first two mountain systems have higher genetic diversity and more complex phylogenetic structures than those found in the Qinling Mountains. In this study, we used molecular and morphological methods to explore the phylogeographical structure, species differentiation, and evolutionary history of the *Biston falcata* species group, which is widely distributed in the Himalaya, Hengduan, and Qinling mountains. Five species were identified; *B. quercii* is distributed in the Qinling Mountains and its adjacent areas, and the other four species are mainly distributed in the Himalaya and Hengduan Mountains. Four gradual species differentiation events occurred at approximately 3.07, 1.75, 1.50, and 1.30 Ma, corresponding to the stage with strong geological movements of the Qinghai-Tibet Plateau. The low land between the Qinling and the northern Hengduan Mountains, the Sichuan Basin, the low land between the eastern and central Himalaya Mountains and the Mekong-Salween Divide are four geographical barriers that resulted in multiple speciations. In addition, long-range dispersal as another mechanism for speciation is non-negligible. In spite of failing to confirm the ancestral area, our study provided direct evidence of the speciation induced by the geographical barriers and adaptation to the plateau.

Introduction

In recent years, phylogeographical investigation has burgeoned in mainland China; in particular, species in the Qinghai-Tibet Plateau (QTP) [including the plateau platform and the Himalaya and Hengduan Mountains (HHM)] have received considerable attention from vertebrate, plant and insect phylogeographers exploring the evolutionary history and dispersal route of species (Qiu *et al.*, 2011; Liu *et al.*, 2012; Lei *et al.*, 2014). The central aim of phylogeography is to infer how historical events have shaped genetic differentiation and demographic processes of

species over time and space (Comes & Kadereit, 1998; Hewitt, 2000; Avise, 2009). Beyond biological features, historical events, including geological changes and climate oscillations associated with Pleistocene glaciation, are considered another important driver for the genetic structure and evolutionary process of species (Hewitt, 2000; Lei *et al.*, 2015; Cheng *et al.*, 2016a; Luo *et al.*, 2017). Geographical changes, such as the uplift of mountain systems, result in habitat fragmentation and form barriers to gene flow, which accelerates the genetic diversification of local species (Antonelli *et al.*, 2009; Qu *et al.*, 2014; Li *et al.*, 2017). Repeated glacial–interglacial cycles during the Quaternary also profoundly affected the current distribution patterns of many temperate insect species in East Asia and many other regions of the northern hemisphere (Hewitt, 1999, 2000; Lei *et al.*, 2014).

The HHM, located at the southern and southeastern boundary of the QTP, are recognized as the most important mountain

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system in East Asia (Li, 1988; Zhao *et al.*, 2007; Lei *et al.*, 2015). As the global biodiversity hotspot, these two mountain ranges have high levels of endemism and species richness (Myers *et al.*, 2000; Huang *et al.*, 2008). Complex topographical heterogeneity, such as large altitudinal differences that often exceed 2000 m (asl), layered vegetation distribution (Liu *et al.*, 1985; Zheng *et al.*, 1986; Yao *et al.*, 2010) and ecological stability (Carnaval *et al.*, 2009) may be able to explain the high biodiversity. One phenomenon that has been observed is that species or populations within the HHM always have higher genetic diversity and more complex phylogenetic structure than in central and east China (Qu *et al.*, 2014; Cheng *et al.*, 2016a). Among multiple geographical barriers, the Ward Line–Mekong–Salween Divide (MSD) is a classic barrier that plays an important role as the biogeographical boundary between the eastern Himalaya and Hengduan regions, although mainly for plants and vertebrates (Ward, 1921; Gao *et al.*, 2007; Li *et al.*, 2011; Päckert *et al.*, 2012; Luo *et al.*, 2017). In addition, the east–west Qinling Mountains border the Hengduan Mountains in Gansu Province. Species distributed on these three mountains always have close relationships. However, making sure which population is the ancestral one for a species distributed in these mountains is very significant and interesting.

In this study, we focus on the *B. falcata* species group as our research material, which is widely distributed in China and part of the regions of India and Nepal, especially in the Qinling, Hengduan and Himalaya Mountains. This species group can be easily distinguished by special and normal speckles on the wings, legs and abdomens, waved forewing outer margins and several brown terminal spots on the ends of veins (Jiang *et al.*, 2011). At present, the group contains three currently recognized species: *B. brevipennata* Inoue, *B. quercii* (Oberthür) and *B. falcata* (Warren). *Biston falcata* contains two subspecies: *B. falcata falcata* (Warren) and *B. falcata satura* (Wehrli). A special phenomenon has been found that the species within this group show obvious sexual dimorphism: the female adults have larger body size and wings and simpler speckles, such as fewer dots and lighter lines. The similarities in morphology, sexual dimorphism and overlapping distribution increase the difficulty of taxonomic classification. Except for *B. quercii* which is mainly distributed in the Qinling Mountains and in adjacent regions, the other species are widely distributed in the HHM. According to the accessible literature and our observations during the collection of specimens, the host plants of the *B. falcata* species group are gymnosperms that live at altitudes > 2500 m (but 1000–1500 m on Qinling Mountain) (Wu *et al.*, 2007). Such a distribution pattern has biological implications that suggest this species group will be an interesting case study of species origins and diversification.

Among morphological structures, the male genitalia in animals with internal fertilization are considered as one of the most rapidly evolving and divergent characteristics (Eberhard, 1985, 2010; Arnqvist, 1997; Hosken & Stockley, 2004). However, in some Lepidopteran taxa, wing speckles seem to have evolved more rapidly than male genitalia (Jiang *et al.*, 2011; Cheng *et al.*, 2017). In this study, the *B. falcata* species group

has obvious sexual dimorphism and is an ideal candidate for the examination of speckle evolution, because the speckles show some striking diversification and particularity within the group. Independent mapping of the divergent morphological characteristics onto molecular phylogenies is a widely used method (Richmond *et al.*, 2012; Wojcieszek & Simmons, 2012; Yassin & Orgogozo, 2013; Bennik *et al.*, 2016; Mitter *et al.*, 2017). This method can allow ancestral states to be reconstructed and the likelihood of independent or convergent evolution of specific morphological characters to be assessed (Barmina & Kopp, 2007; Schärer *et al.*, 2011; Mitter *et al.*, 2017; Ospina-Garcés & De Luna, 2017).

In this paper, we examine the phylogeny and evolutionary history of the *B. falcata* species group based on mitochondrial DNA (mtDNA) and nuclear DNA (ncDNA) data. The primary aim of this study is to explore the species delimitation, phylogeography structure and origin of the species group and to test the relationship of species distributed on different mountains. In addition, the second aim is to compare the speckles of different body parts, including wings, legs and abdomens, as the basis of the phylogenetic tree, which will help us to find the trends in evolution and understand the morphological evolution.

Materials and methods

Sampling and sequence data

A total of 331 specimens of the *B. falcata* species group were collected from 56 sampling sites throughout most of the distribution range of the species group. We pooled the 56 sampling sites into 28 geographical locations (see Appendix S1; Fig. 1). Samples for DNA extraction were preserved in 100% ethanol and stored at -20°C . DNA was extracted using the DNeasy Tissue Kit (Qiagen, Beijing, China), and vouchers were deposited at the Institute of Zoology, Chinese Academy of Sciences, Beijing, China. Four mitochondrial genes and two nuclear genes were obtained, including the cytochrome *c* oxidase subunit I (*COI*), cytochrome *b* (*CYTB*), cytochrome *c* oxidase subunit II (*COII*), rRNA (*16S*), elongation factor 1 α (*EF-1 α*) and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), through PCR amplification. The PCR conditions have been described in Caterino & Sperling (1999) and Cheng *et al.* (2016a,b). Sequences of all primers used in this study are listed in Appendix S2. Sequences were deposited in the GenBank database; the accession numbers are provided in Appendix S1.

Phylogenetic analyses

Two congeneric species, *B. panyerinararia* and *B. suppressaria*, with published molecular data and distribution range (Cheng *et al.*, 2016a,b) were used as outgroup. Phylogenetic relationships were reconstructed based on three datasets (mtDNA genes, ncDNA genes and all genes combined) and inferred with Bayesian inference (BI) using MRBAYES 3.1.2

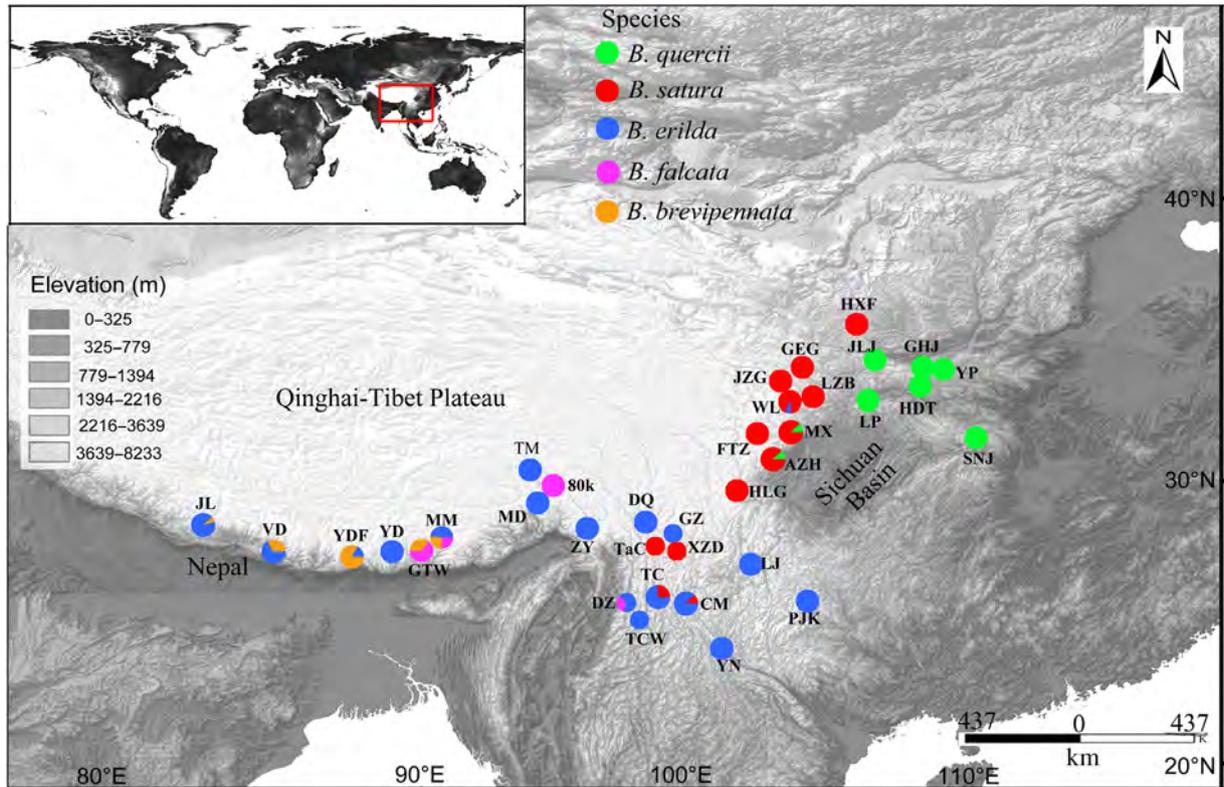


Fig. 1. Sampling sites for the *Biston falcata* species group used in this study. Different species are shown in different colours. [Colour figure can be viewed at wileyonlinelibrary.com].

(Huelsenbeck & Ronquist, 2001) and maximum likelihood (ML) analyses using RAXML v.7.2.6 (Stamatakis, 2006; Stamatakis *et al.*, 2008). For BI analysis, the best-fit model of nucleotide substitution was selected for each gene using JMODELTEST 0.1.1 (Posada, 2008). Two independent parallel runs of four incrementally heated Metropolis-coupled Monte Carlo Markov chains (MCMCs) were run for 50 million or more generations, with trees sampled every 1000 generations, until the average standard deviation of the split frequencies was < 0.01 . The first 10% of generations was discarded as burn-in when summarizing tree results. Convergence of the MCMC results was verified in TRACER 1.6 (Rambaut *et al.*, 2014). For ML analyses, the GTR+GAMMA+I model was used for each partition. All model parameters were estimated during the ML analysis. A rapid bootstrapping algorithm with a random seed value of 12345 (command `-f a -x 12345`) was applied with 1000 replicates (Siddall, 2010).

We constructed haplotype networks for combined mtDNA sequence datasets and each ncDNA sequence to better visualize nonbifurcating (multifurcations and reticulations) relationships (Posada & Crandall, 2001). A maximum parsimony method, TCS v.1.23 (Clement *et al.*, 2000), was used to draw an unrooted network to evaluate the haplotype relationships for the mtDNA and the nuDNA sequences, with 95% parsimoniously plausible branch connections.

Constructing the species tree and estimate of divergence time

For estimates of divergence time, we used BEAST 1.8.0 (Drummond & Rambaut, 2007), and we used *BEAST for species trees based on the *COI*+*CYTB*+*COII* dataset. For individual datasets for each locus, BEAUTI 1.8.0 (in the BEAST 1.8.0 package) was used to create the XML-formatted input files for *BEAST. The appropriate evolutionary model for each dataset was identified by JMODELTEST. Species were indicated for each sequence under the 'traits' tab, and the evolutionary model was specified for each locus. All mitochondrial loci were analysed under an HKY model in the *BEAST analyses due to low convergence when more complex models were used. A molecular clock was used, because there was no other calibration information, such as fossil or geological evidence (Maekawa *et al.*, 2001; López-López *et al.*, 2015). However, the divergence time of population differentiation within two outgroups (*B. panyerinaria* and *B. suppressaria*) is treated as the reference time. Thus, the widely accepted mutation rates for insect mitochondrial *COI* genes (0.0115–0.0177 per site/Ma; Brower, 1994; Papadopoulou *et al.*, 2010) were adopted; other genes were scaled to the *COI* rate in BEAST. We ran the analyses with Yule tree priors. Both a strict molecular clock model and an uncorrelated lognormal relaxed molecular clock were tried, and the model comparison of Bayes factors by the Akaike information content was carried out in TRACER 1.6. The MCMC chains

were analysed for 200 million generations, with sampling every 2000 generations. TRACER 1.6 was used to verify the posterior distribution and the effective sample sizes from the MCMC output. We used TREEANNOTATOR in the BEAST package to summarize tree data with 'mean height' and discarded the first 25% of trees as the 'burn-in' period, which ended well after the stationarity of the chain likelihood values had been established. The tree and divergence times were displayed in FIGTREE 1.3.1.

Morphological identification and comparison of speckle characteristics

For the *B. falcata* species group, conventional taxonomic methods were used to examine external features and genitalia preparations. Photographs of the adults, wings, legs and genitalia were taken with digital cameras. Composite images of middle legs were generated using AUTO-MONTAGE 5.03.0061 (Synoptics Ltd, U.K.). The plates were compiled using Adobe PHOTOSHOP software. In this study, we compared the speckles of five species from the wings, middle legs and abdomens of all samples. By combining them with the phylogenetic tree, we could trace trends in morphological evolution.

Population genetic analyses

To assess how genetic diversity varied across geographical populations, we calculated the following summary statistics based on combined mtDNA sequence datasets: haplotype diversity (h), nucleotide diversity (p) and the mean number of pairwise differences were calculated to estimate DNA polymorphism using DNASP 5.10.01 (Librado & Rozas, 2009). Analysis of molecular variance (AMOVA) and F-statistic (F_{ST}) calculations were performed using ARLEQUIN 3.5 (Excoffier & Lischer, 2010) with 10 000 permutations, based only on populations that contained more than three individuals.

Demographic history

Signatures of population demographic changes were tested for four lineages based on combined mtDNA genes. Tajima's D (Tajima, 1989) and Fu's F_s statistics (Fu, 1997) were used to assess whether nucleotide polymorphisms deviated from expectations under neutral theory in ARLEQUIN. Bayesian skyline plots (BSPs) implemented in BEAST were used to estimate population size changes through time. For each BSP, the substitution model was selected using JMODELTEST. Samples were drawn every 1000 steps for 50 million steps under an uncorrelated lognormal relaxed clock model. The mutation rate was set to 0.0115–0.0177 per site/Ma. Demographic plots were visualized in TRACER 1.5, with a burn-in of 20%.

Gene flow analysis

To provide direct evidence for the dispersal pattern, effective population size (θ) and effective migration rates (M) between

species were calculated using MIGRATE v.3.6.11 in Windows (Beerli, 2009). Only subdivisions ($n \geq 4$) were available to avoid pooling populations and to prevent over-parameterization of the analysis. MIGRATE uses BI with long chains (500 000 steps samples, 5000 steps recorded) and 1000 burn-ins per chain. The mutation scaled M entering and leaving each population per generation and the mutation scaled θ was estimated by applying the Bayesian search strategy to determine if there was asymmetrical gene flow between populations. N_{em} (effective migration rate) was calculated by multiplying M and θ .

Ancestral area reconstruction

Statistical dispersal-variance analysis (S-DIVA) and Bayesian binary MCMC (BBM) were implemented in RASP 3.02 (Yu *et al.*, 2015) and were used to reconstruct the possible ancestral distribution areas of the *B. falcata* species group. We used the mitochondrial ultrametric tree inferred by BEAST as input to the program. According to the distribution range of five phylogroups, the distribution range of the species group was divided into five main areas: A, Qinling Mountains and adjacent regions; B, northern Hengduan mountains; C, southern Hengduan mountains; D, eastern Himalaya; and E, central Himalaya. These two analyses used the DIVA method in a statistical context, calculating the probability of ancestral areas over a Bayesian posterior distribution of tree topologies. The estimation of ancestral area marginal probabilities, considering phylogenetic uncertainty, has been suggested to reduce uncertainty in a biogeographical reconstruction (Nylander *et al.*, 2008).

Results

Genetic diversity

We obtained 3442 bp mitochondrial genes and 1617 bp nuclear genes for the *B. falcata* species group from China, including *COI* (660 bp), *CYTB* (1001 bp), *COII* (719 bp), *16S* (1062 bp), *EF-1 α* (942 bp) and *GAPDH* (675 bp). All sequences were deposited in GenBank (Table S1). In the entire mtDNA alignment, 466 sites were variable and 370 were parsimony-informative. Values for haplotype (h) and nucleotide diversity (π) of *B. erilda* were the highest (h , 0.866; π , 0.0137), followed in turn by *B. satura*, *B. falcata*, *B. brevipennata* and *B. quercii* (Table 1). The AMOVA analyses based on the combined mtDNA alignment revealed significant genetic differentiation among all species ($F_{ST} = 0.70$). The average F_{ST} values for *B. erilda*, *B. satura*, *B. quercii*, *B. falcata* and *B. brevipennata* were 0.32, 0.63, 0.03, –0.05 and 0.93, respectively. Most variation was accounted for by variation among all five species (56.46%), followed by variation within geographical populations (29.88%) and variation between different populations (13.67%).

Phylogenetic relationships and species tree

The phylogenetic analyses of the mtDNA and combined DNA (four mtDNA and two nuDNA genes) (Fig. 2) revealed

Table 1. Summary of genetic diversity including sample size, the number of haplotypes, nucleotide diversity, haplotype diversity, average number of nucleotide differences, Fu's F_s and Tajima's D . We have only included the sampling sites with fresh tissue samples in this table.

Species	Sampling size	No. of haplotypes	Nucleotide diversity	Haplotype diversity	Fu's F_s	Tajima's D
<i>B. erilda</i>	110	23	0.0137	0.866	0.332	1.645
<i>B. satura</i>	116	19	0.00355	0.765	*-6.749	-1.589
<i>B. quercii</i>	31	2	0.00082	0.516	1.724	2.637
<i>B. falcata</i>	32	10	0.00169	0.675	-1.545	-1.68767
<i>B. brevipennata</i>	33	3	0.01079	0.606	**26.176	2.82142

** $P < 0.01$.

distinct phylogenetic structure in the *B. falcata* species group. Samples were grouped into five reciprocally monophyletic lineages; here, we only show the tree based on the combined dataset. Combining the morphological character and the species tree, we reconfirmed the taxonomy status of the five lineages. Polymorphic sites of the mtDNA datasets defined 46 haplotypes, 19 of which were each found in single individuals. The most abundant haplotype was shared by 29 individuals distributed in Sichuan. The median-joining network analysis corroborated the split of five species in phylogenetic trees (Fig. 2). No haplotype was found to be shared by any two species. In contrast to the other three mtDNA genes, the single gene ncDNA datasets and the *16S* dataset failed to distinguish four species, except for *B. quercii* (Appendix S3). The result of the species tree is consistent with the phylogenetic tree (Fig. 3B). The five-species system received high support values, except for the value for *B. falcata* and *B. brevipennata*.

Species information and morphological identification

According to the species tree and morphological characteristics (Fig. 3; Appendix S4), five species were supported and included in the species group: *B. falcata*, *B. quercii*, *B. brevipennata*, *B. satura* (Wehrli, 1941) **stat.n.** and *B. erilda* (Oberthür, 1910) **stat.n.** Although there is low support value for *B. falcata* and *B. brevipennata*, the considerable morphological difference still supported the validity of these two species. Among the five species, the body size of *B. brevipennata* was smaller than that of the other four species. The detailed species description accompanied by photographs is given in Appendix S4. The distribution of the five species roughly corresponds to the different geographical regions (Fig. 1). *Biston quercii* is mainly distributed in the Qinling Mountains and adjacent areas. *Biston satura* is distributed in the northern Hengduan Mountains and part of the southern Hengduan Mountains. *Biston erilda* includes two lineages (Hengduan lineage and Himalaya lineage) that are distributed in the Hengduan Mountains and the Himalaya Mountains, respectively. *Biston falcata* and *B. brevipennata* are distributed in the Himalaya Mountains, where the latter is more western.

Three body parts (wing, middle leg and abdomen) with speckles from representative samples of four species in the HHM (except *B. quercii*) are shown in Fig. 3. From left to right, the black speckles on the wings gradually increased, and the reddish brown colour on the edge of the wing turned black in

B. brevipennata. For the inner middle legs, the white colour decreased, and the black colour gradually increased. For the abdomen, a similar tendency was found where the black speckle gradually increased.

Divergence time and demographic dynamics

Our results show that an uncorrelated lognormal relaxed molecular clock performed better than a strict molecular clock model. The Bayes factors for model comparison (lognormal relaxed vs strict) were 49.99 for the combined dataset. A lognormal relaxed molecular clock could not be rejected. Therefore, we applied a lognormal relaxed clock model for subsequent analyses. Divergence time dating (Fig. 4) indicated that four speciation events occurred among species in the *B. falcata* species group during 1.30–3.07 Ma; *B. quercii*, *B. satura*, *B. erilda*, *B. falcata* and *B. brevipennata* were separated. Within *B. erilda*, two lineages diverged at approximately 0.98 Ma [0.71–1.28 Ma; 95% highest posterior density (HPD)]. Within *B. brevipennata*, two populations diverged at approximately 0.96 Ma (0.64–1.31 Ma, 95% HPD).

Demographic history

Biston erilda and *B. quercii* had positive Tajima's D and Fu's F_s values, and *B. satura* and *B. falcata* had negative but not significant values (Table 1). *Biston brevipennata* had positive significant Fu's F_s and not significant Tajima's D . The results of the BSPs suggest that the five species had different responses to glaciation (Fig. 5). The BSP analyses rejected population stability in five species, except for the Hengduan lineage of *B. erilda*. Surprisingly, *B. brevipennata* went through a reduction of population size after the last glacial maximum (LGM). Respective expansion within *B. satura* and *B. erilda* occurred before the LGM, whereas it occurred within *B. falcata* after the LGM.

Gene flow analysis

The potential gene flow between the five species is shown in Fig. 6. Except for the gene flow between *B. falcata* and *B. satura*, no obvious gene flow was found ($N_{em} < 4$). Between the parapatric or sympatric species, the gene flow size from early- to later-diverged species was larger than that from later- to early-diverged species, e.g. *B. quercii* to *B. satura* > *B. satura* to *B. quercii*.

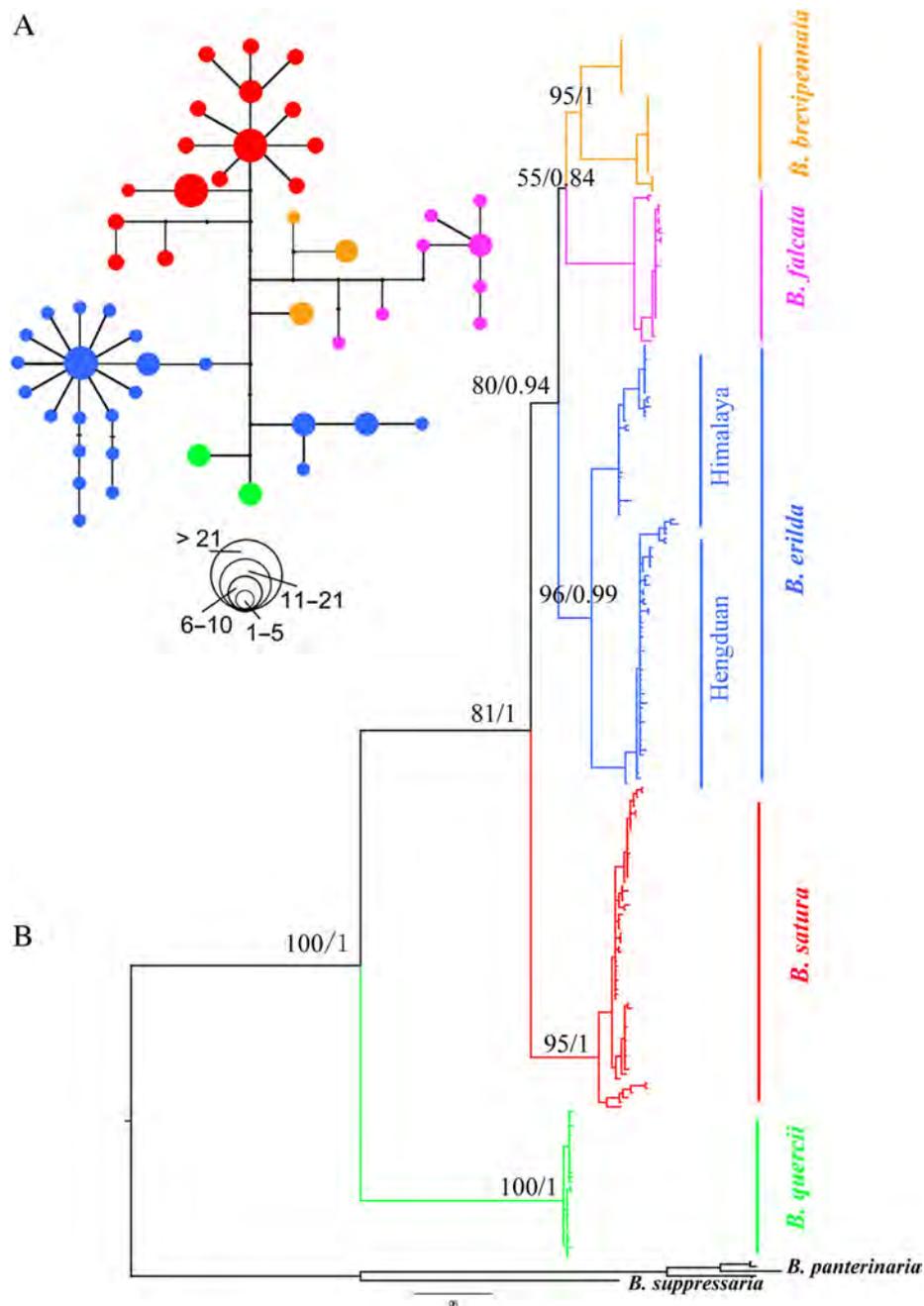


Fig. 2. (a) Median joining network based on mitochondrial DNA for the *Biston falcata* species group. Each circle represents a haplotype, and the size of the circle is proportional to that haplotype's frequency. Dots represent unsampled haplotypes and dashes represent the corresponding mutational steps. Colours denote species membership and are the same as in Fig. 1. (b) Bayesian tree based on combined genes for the *Biston falcata* species group. Values on the left of the nodes indicate posterior probabilities and bootstrap supports of Bayesian inference/maximum likelihood for major clades. [Colour figure can be viewed at wileyonlinelibrary.com].

Ancestral area reconstruction

The results of the two methods, S-DIVA and BBM, are shown in Fig. 4. The two methods revealed different origins of the *B. falcata* species group. The former inferred that the origin was

I + II + IV (Qinling + southern Hengduan + eastern Himalaya region), whereas the latter inferred that the origin was I (the Qinling region). However, both methods gave support to the eastern Himalaya being the area of origin of the two youngest species, *B. falcata* and *B. brevipennata*.

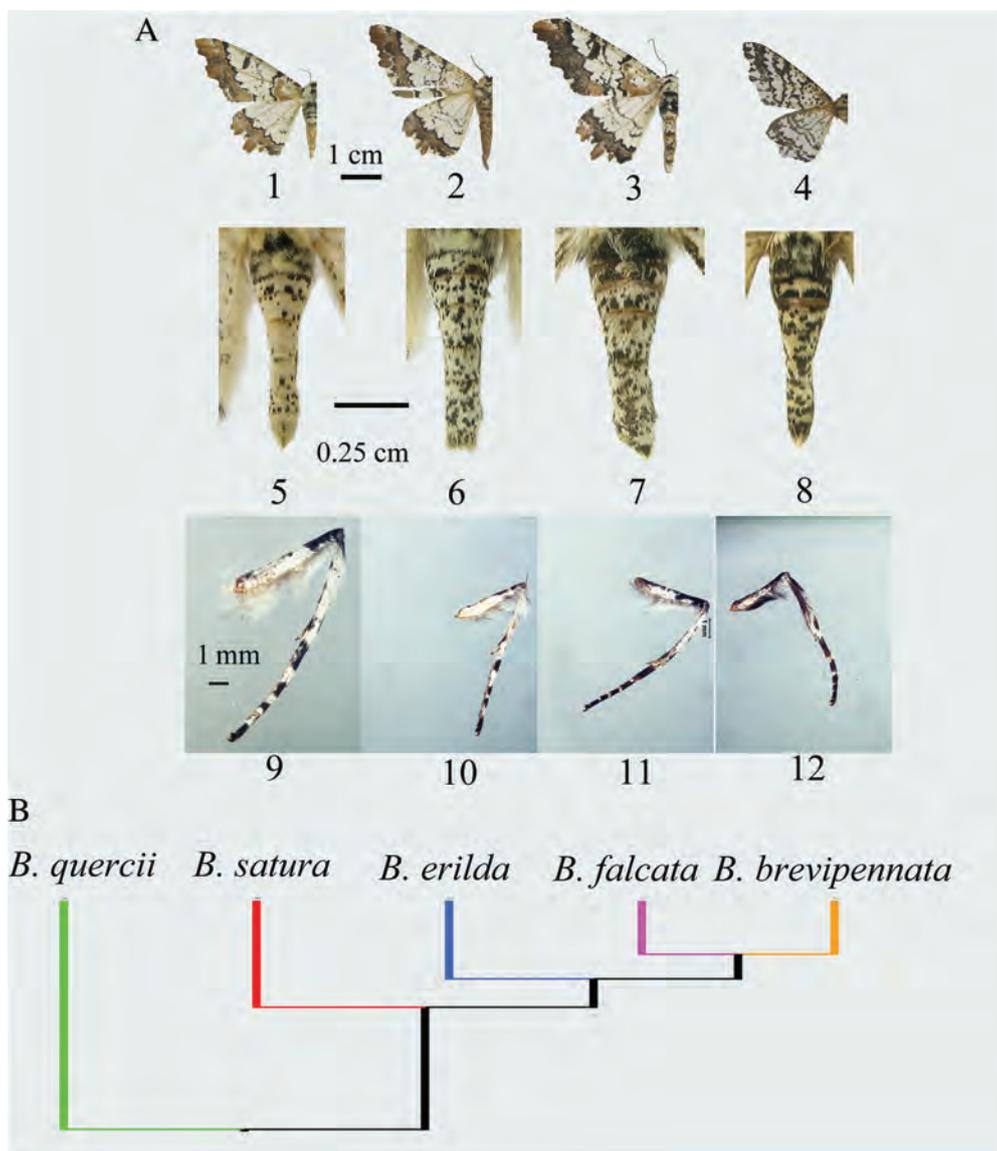


Fig. 3. (A) Morphological features from three organs (wing, abdomen and middle leg) of four species distributed in the Himalaya and Hengduan Mountains. Each column relates to each of the four species. (B) Species tree of the *Biston falcata* species group constructed with the mitochondrial DNA gene. [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

Species status and distribution information within the Biston falcata species group

The species in the *B. falcata* species group share several morphological features, including the waved forewing outer margin, brown terminal spots on wings and sexual dimorphism (Inoue, 1982; Jiang *et al.*, 2011). There are many incompatible and unresolved problems in the previous taxonomy, and insufficient sampling is one undoubted reason. Therefore, our team collected samples from different altitudes and different locations through the total distribution during 2009–2017, especially

in the HHM. Importantly, we combined the molecular and morphological methods to improve new and authentic taxonomy (Fig. 2; Appendix S3). For the *B. falcata* species group, the phylogenetic analyses and species tree supported reciprocal monophyly for five species, *B. quercii*, *B. falcata*, *B. brevipennata*, *B. satura* and *B. erilda*, which include one subspecific elevation and one restoration. *Biston erilda* consists of two lineages (Himalaya and Hengduan lineages). All species and lineages were supported by molecular data and morphological evidence (Appendix S4).

Except for *B. quercii*, which is distributed in the Qinling Mountains and adjacent areas, the other species in the group are mainly distributed in the HHM (Fig. 1). *Biston satura*

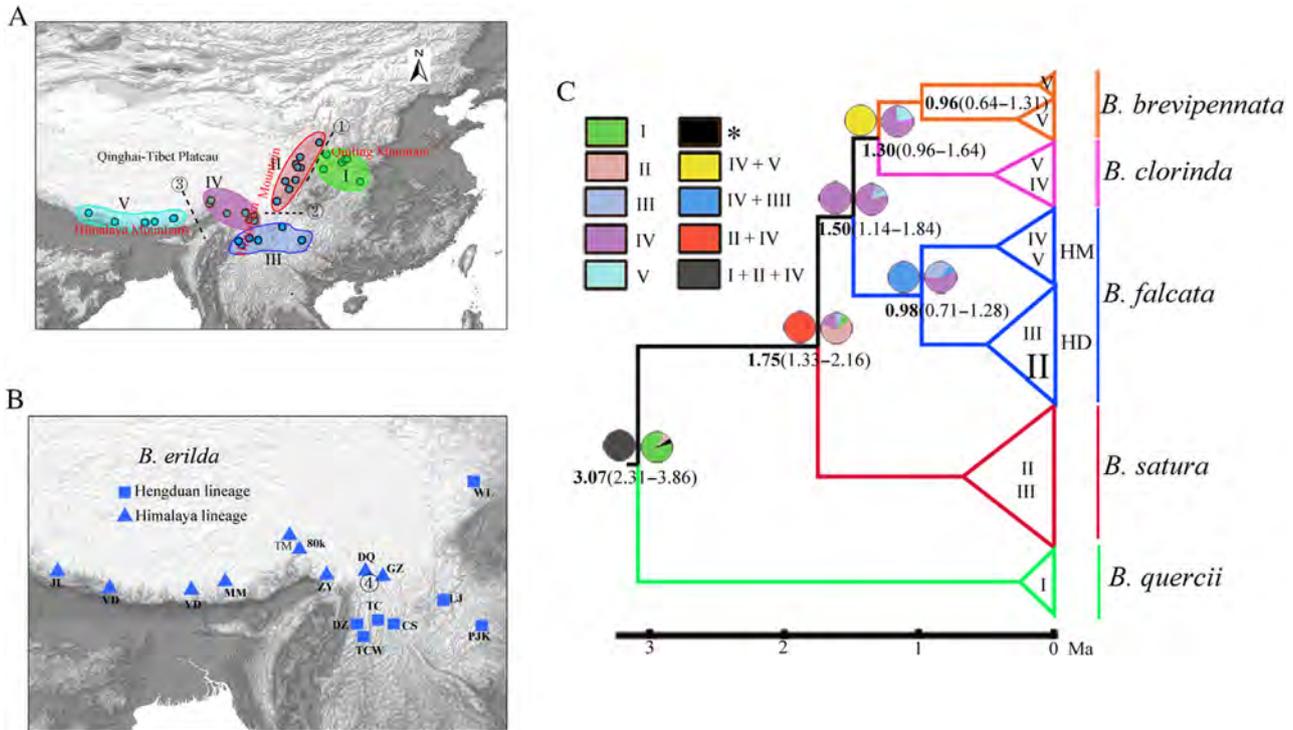


Fig. 4. (A, B) Four regions for ancestral area reconstruction (I–V) and four geographical barriers ①–④. (B) Distribution map of two lineages of *Biston erilda*. (c) The maximum clade credibility tree from divergence-time-rooted phylogenetic analysis of the *Biston falcata* species group and RASP results. Circles on the left and right nodes relate to the two methods used (statistical dispersal-vicariance analysis and Bayesian binary Monte Carlo Markov chain, respectively). I–V are congruent with that of Fig. 4A–B. HM, Himalaya; HD, Hengduan [Colour figure can be viewed at wileyonlinelibrary.com].

is distributed in the northern Hengduan Mountains and part of the southern Hengduan Mountains. Two lineages of *B. erilda* are distributed in the southern Hengduan Mountains and the Himalaya Mountains. *Biston brevipennata* and *B. falcata* are distributed in the Himalaya Mountains, whereas the former is more western. Most neighbouring species pairs have overlapped distribution ranges (Fig. 1). According to the phylogenetic structure and low gene flow between different species at any one location, overlapping distribution induced by re-dispersal after speciation seems plausible. After the first differentiation event between *B. quercii* and the other four species, ancestral species living in and out of the HHM began to experience different evolutionary processes. The rest of the speciation occurred in the HHM.

Origin region of the species group

In this study, two RASP approaches revealed two origin scenarios (Fig. 4), isolation after dispersal and dispersal after isolation; how to distinguish between them is both essential and difficult (Hewitt, 1988; Barton & Hewitt, 1989; Prentis *et al.*, 2008). If the Qinling Mountains are the only ancestral area, the *B. falcata* species group originated there, then dispersed to the Hengduan Mountains and finally colonized the eastern Himalayas. The other scenario supported the Qinling, Hengduan

and eastern Himalaya region as the ancestral area. Under this scenario, the species group dispersed across the area before species differentiation occurred, and then *B. quercii* and *B. satura* diverged from the ancestral species induced by the geographical events of the QTP. The distribution became fragmented following this divergence. Because the speciation events in our study are related to the geographical changes, the discussion of the ancestral area should consider the age of the uplift of the QTP. The time of the first divergence between *B. quercii* and the other four species is approximately 3.07 Ma, which is near (but after) the time of strong uplift of the QTP (3.4 Ma; Chen, 1992; Li & Fang, 1998; Shi *et al.*, 1999). Based on this point, both origin scenarios seem possible. However, Renner (2016) suggests that the QTP has been 4–5 km high since the mid-Eocene (approximately 40 Ma). Based on this point and during the period 3.07–40 Ma, the QTP had passed through multiple geographical events and the current topography had been generated, whereas the species group did not experience any divergence induced by the plateau uplifting. Therefore, an origin in the Qinling, Hengduan and Himalaya regions seemed impossible because species were inevitably impacted by the geographical environments. In addition, the gene flow within the species group is unbalanced. Based on the results of the gene flow (Fig. 6), the size of gene flow within sister species from early- to late-diverged species is larger than in the opposite direction, which is congruent with

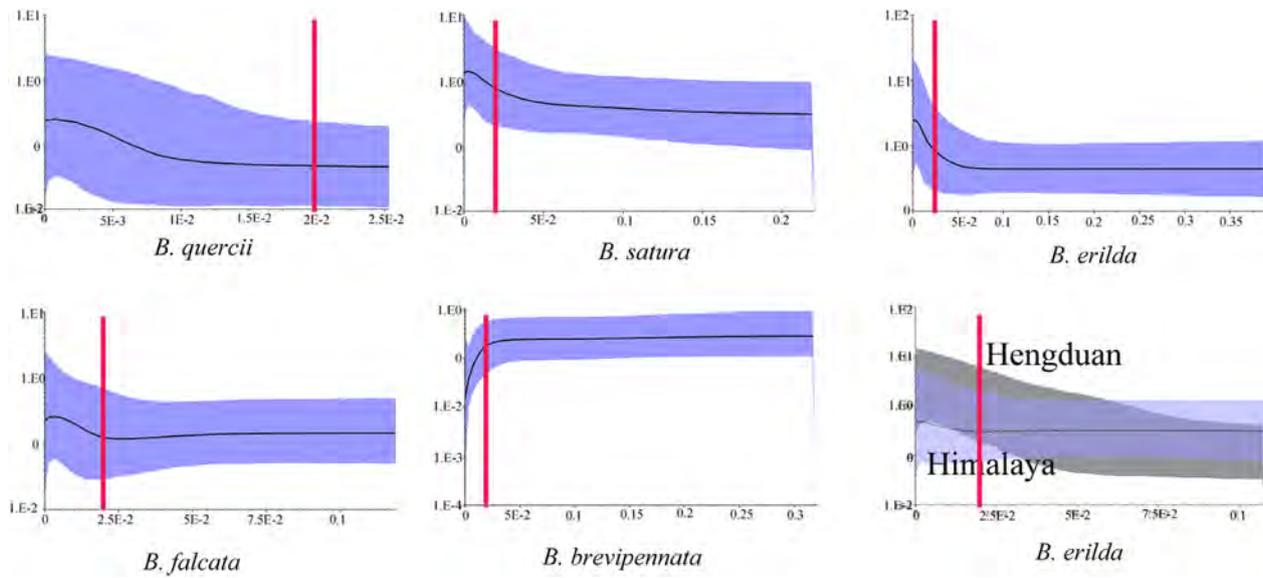


Fig. 5. Bayesian skyline plots for five species and two lineages of *Biston erilda*. The x-axis is in units of Ma. The y-axis is equal to the effective population size. Estimates of means are connected by a solid line, whereas the upper and lower lines delineate the 95% highest posterior density limits. E is a natural constant 2.718. LGM, last glacial maximum. [Colour figure can be viewed at wileyonlinelibrary.com].

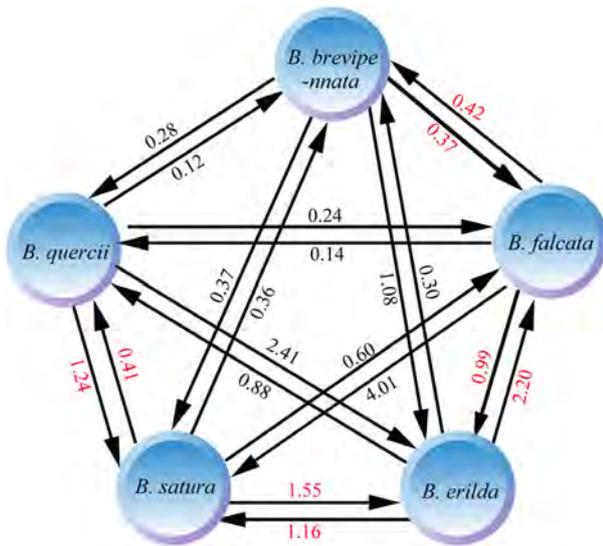


Fig. 6. Results of gene flow between each pair of species conducted in MIGRATE. Species pairs labelled with red numbers have adjacent distributions. [Colour figure can be viewed at wileyonlinelibrary.com].

one point of view that gene flow always occurs from ancestral to daughter populations/species along the migration direction. Given such an asymmetric gene flow, a single origin in the Qinling Mountains is credible. Identifying the real ancestral area still needs more geographical results and new methods. However, the two methods supported the eastern Himalayas as the origin area of the two youngest species, *B. falcata* and *B. brevipennata*.

Species differentiation and evolutionary history of the Biston falcata species group

The phylogenetic analyses of the *B. falcata* species group based on the combined dataset (mtDNA + ncDNA) supported reciprocal monophyly for five species. The mtDNA (*16S*) and two ncDNA (*EF-1α* and *GAPDH*) genes failed to distinguish the other four species except for *B. quercii* (Appendix S3), which may be consistent with the less-variable sites and a slower evolutionary rate than the other mtDNA gene (Miyata *et al.*, 1982; Sunnucks, 2000). Given the low gene flow, incomplete lineage sorting seems the most likely reason for this pattern.

In this study, four gradual species differentiation events were demonstrated in the evolution of the *B. falcata* species group, and all differentiation events occurred near the Himalaya–Hengduan region. The four differentiation events occurred at *c.* 3.07, 1.75, 1.50 and 1.30 Ma. All of these events occurred during a period with strong geological movements (Li *et al.*, 1979; Li & Fang, 1998; Li, 1999; Shi *et al.*, 1999). During these movements, the QTP experienced strong uplift, the temperature decreased, and the climate changed considerably. During these periods, multiple differentiation events of endemic species occurred, including birds, plants, mammals and insects (Jing *et al.*, 2007; Jin *et al.*, 2008; Ci *et al.*, 2009; Fan *et al.*, 2012; Li *et al.*, 2012; Tan *et al.*, 2012; Cheng *et al.*, 2016a,b). However, that one ancestral species evolved into five species is very rare in such a small range and over such a short period of time.

Biston quercii mainly lives at altitudes < 2000 m in the Qinling Mountains, and the other four species live at altitudes > 2000 m. Previous studies have shown that Pleistocene glaciations were restricted to high altitudes (> 2000 m) near the HHM,

unlike the situation in nearby central China, which was heavily glaciated (Li *et al.*, 1991; Liu *et al.*, 2002). For species living at high altitudes, vertical migration to low altitudes during the glaciations and returning to high altitudes after the glaciation is one common way to survive the glaciers in the HHM (Hewitt, 2000; Lei *et al.*, 2014, 2015; Cheng *et al.*, 2017). Therefore, compared with congruent adaptation models for climatic fluctuations of low-altitude populations or species (Lei *et al.*, 2014, 2015; Cheng *et al.*, 2016a,b), the four species that live at high altitudes represent a diversified pattern (Fig. 5). Except for *B. brevipennata*, the effective population size of the other four species increased before (*B. satura* and *B. erilda*) or after the LGM (*B. falcata*). Expansion after the LGM has been demonstrated in multiple organisms living at high altitudes in this region, including birds and insects (Zhao *et al.*, 2012; Xun *et al.*, 2016), but expansions before LGM are also found in birds (Dai *et al.*, 2011; Wang *et al.*, 2013; Ye *et al.*, 2014). Therefore, we speculate that vertical migration along altitudes did not necessarily have an impact on population size. Moreover, species with different biological features, such as cold resistance, show different adaptation modes; previous studies also proved this point (Hewitt, 2000; Provan & Bennett, 2008; Bisconti *et al.*, 2011).

Multiple speciations induced by geographical barriers

Four of five species in the *B. falcata* species group are endemic to the Himalaya and Hengduan region, and four gradual species differentiation events occurred around this region. The species group is distributed at altitudes near to or > 2000 m, which promotes low land as an isolation barrier. Four important barriers were confirmed in the study (Fig. 4). The low land between the Qinling Mountains and the northern Hengduan Mountains is the barrier of the first differentiation (speciation of *B. quercii*) and is also an important barrier for other organisms (Zhang, 1999; Xie *et al.*, 2004). The Sichuan Basin between the northern and southern Hengduan Mountains is the barrier of the second differentiation (speciation of *B. satura*), and it resulted in differentiations of birds (Song *et al.*, 2009). The low land between the eastern and central Himalaya Mountains is the barrier of the third differentiation (speciation of *B. erilda*), and it also resulted in differentiations of one bird (Qu *et al.*, 2014). The cause of the last differentiation between *B. falcata* and *B. brevipennata* cannot be determined because they have similar host plants, altitudes and distribution ranges.

In addition, there is one barrier between the Himalaya and Hengduan lineage of *B. erilda*, and this barrier roughly coincides with the border of the Himalaya and Southwest Subregion (Zhang, 1999). This region also included one famous divide, the MSD (Ward, 1921). As one typical barrier that consists of mountains and valleys with over 4000 m elevation, this divide resulted in multiple population or species isolation, especially in plants and vertebrates (Gao *et al.*, 2007; Li *et al.*, 2011; Päckert *et al.*, 2012; Luo *et al.*, 2017). According to previous studies by our team, this border or divide has profound effects on multiple population divergences of several moths (Cheng

et al., 2016a,b). Based on large-scale drainage patterns, Clark *et al.* (2004) inferred that the formation time of the MSD was probably at least 4 Ma; however, this time is earlier than the divergence time of two lineages of *B. erilda* on the basis of evolutionary rate. Thus, long-range dispersal as another mechanism for speciation is non-negligible and the dispersal across the MSD took place more recently after it was formed. After speciation, *B. erilda* may be restricted at the Hengduan Mountains, and then dispersed into the Himalaya range by corridors or other routes bypassing the MSD. Further studies of the MSD are required in future.

Supporting Information

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

Appendix S1. Sampling information, the GenBank accession numbers of the *Biston falcata* species group used in the study.

Appendix S2. Total primers used in this study.

Appendix S3. Phylogenetic tree of three ncDNA genes.

Appendix S4. Taxonomy information and related photos of the *Biston falcata* species group.

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