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Complete mitochondrial genomes throw light on budding speciation in three *Biston* species (Lepidoptera, Geometridae)

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Biston panterinaria, *Biston thibetaria* and *Biston perclara* are very closely related species in the genus *Biston*, judged on the basis of both morphological and molecular evidence. The distribution area and altitudes, and host plants of these three species also show both consistency and differences. However, the exact relationship between the three species is unclear. In this study, we used the 'distance-based method', the 'tree-based method' and Bayesian phylogenetics and phylogeography to elucidate the relationship between the three species. Phylogenetic trees based on mitogenomes, *COI+CYTB+16S* and three nuDNA genes were constructed. The results of the phylogenetic trees revealed that *B. thibetaria* and *B. perclara* were derived from *B. panterinaria* and render the latter paraphyletic. The budding process of speciation is therefore presumed to be the main factor causing a phylogenetic relationship of this pattern. A host shift from broad-leaved plants living at low altitudes to gymnosperms living at relatively high altitudes provides evidence of budding speciation in these three species. The divergence time suggests that the budding speciation occurred at approximately 1.38 Ma, which is consistent with the Kunlun-Yellow River Movement. Tectonic movements occurring around the Qinghai–Tibet Plateau may be the driver of the budding speciation.

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Introduction

The origin of species is a fundamental issue in evolution biology. Most biologists agree that speciation should be seen as the evolution of reproductive isolation between populations (Coyne 1994; Coyne & Orr 1998, 2004; Noor 2002). Many kinds of speciation have been described, including allopatric speciation, parapatric speciation, peripatric speciation and sympatric speciation (Mayr 1942, 1970; White 1978; Futuyma & Mayer 1980; Coyne & Orr 2004; Nosil 2012). Budding speciation is one specific type of peripatric speciation, which, as a concept, was first proposed by Mayr (1954): he envisaged an initially small colonizing population becoming reproductively isolated from the wider-ranging parental species. However, the proposal of this type of speciation did not at first draw attention. Later, Wiley & Mayden (1985), Harrison (1991, 1998), Frey (1993) and Rieseberg & Brouillet (1994) elaborated the concept of peripheral isolates. In particular, Harrison (1991) identified peripheral isolates based on molecular evidence, with species A embedding within paraphyletic species B. In 2003, Funk & Omland improved the concept of budding speciation and listed much evidence and interpretation, following which the concept of budding speciation began to be widely accepted. Funk & Omland defined budding speciation, or peripatric and peripheral isolates, as always implying a geographically restricted and monophyletic daughter species being embedded within a widely distributed and paraphyletic parental species. Subsequently, many examples of budding speciation were discovered, involving liverworts, kelps, flowers, insects, snails, frogs and shrews (Morse & Farrell 2005; Vanderpoorten & Long 2006; Chen *et al.* 2009, 2012; Tellier *et al.* 2009; Grossenbacher *et al.* 2014; Kruckenhauser *et al.* 2014).

Biston panterinaria (Bremer & Grey, 1853), *Biston thibetaria* (Oberthür, 1886) and *Biston perclara* (Warren, 1899) have since their original description been regarded as specifically valid members of the genus *Biston* of the family Geometridae, Lepidoptera, and are distributed in east Asia, south China and Taiwan, respectively. Importantly, these three species are closely related species forming Group III of the genus based on morphological characters (Jiang *et al.* 2011). In addition, the distributional areas and altitudes, and host plants of these three species show both consistency and differences. The host plants of *B. panterinaria* are mainly broad-leaved arbours (Yu 2001; Zhang & Li 2001; Cui 2004; Li *et al.* 2008) below 1500 m, while the host plants of *B. thibetaria* are mainly conifers belonging to the gymnosperms (Chen *et al.* 1982; Zheng & Yang 1991) from 1000 to 3000 m. However, the precise relationship between the three species is unclear.

During our work of phylogeographic analysis of *B. panterinaria*, we found that the relationships between *B. panterinaria*, *B. thibetaria* and *B. perclara* were unusual and that *B. panterinaria* seemed paraphyletic. However, we were not sure whether this phenomenon was authentic, because the sampling numbers were small. So, in this study, we used more samples, more data sets and more methods to explore the relationships of the three species in detail. Nowadays, complete mitochondrial genomes (mitogenomes) can produce more robust and stable phylogenetic reconstructions than those which rely on only part of the mtDNA, and have been increasingly applied to a variety of phylogenetic and phylogeographic studies in invertebrates (Ingman *et al.* 2001; Ma *et al.* 2012; Cameron 2014; Liu *et al.* 2015; Yang *et al.* 2015). So the phylogenetic relationships of *B. panterinaria*, *B. thibetaria* and *B. perclara* were reconstructed based on three data sets, involving mitogenomes, three mitochondrial DNA (mtDNA) loci and three nuclear DNA (nuDNA) loci, to test the paraphyly of *B. panterinaria* and its consistency with the hypothesis of budding speciation. If this result is deemed authentic, these three species will form a good case study for studying budding speciation in Lepidoptera.

Material and methods

Specimen sampling and DNA extraction

The sampling sites of *B. panterinaria*, *B. thibetaria* and *B. perclara* are shown in 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 Museum of IZCAS (the Institute of Zoology, Chinese Academy of Sciences, Beijing, China). Ten mitogenomes from four lineages of *B. panterinaria* (Cheng *et al.* 2015), three mitogenomes from *B. thibetaria* and one mitogenome from *B. perclara* were obtained. The mitogenomes were amplified as described in Yang *et al.* (2013). Three mitochondrial genes and three nuclear genes were obtained from total specimens from the above-mentioned three species and from ten other *Biston* species, including the cytochrome c oxidase subunit I (*COI*), cytochrome b (*CYTB*), lrRNA (*16S*), elongation factor 1a (*EF-1a*), wingless (*wg*) and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*), through polymerase chain reaction (PCR) amplification. PCR conditions were as follows: denaturation at 94 °C for 2 min, followed by 40 cycles at 93 °C for 1 min, annealing temperature (51 °C for *COI*, 47 °C for *CYTB*, 56 °C for *EF-1a*, 58 °C for *wg* and 55 °C for *GAPDH*) for 45 s, and 72 °C for 1 min, and a final 10 min at 72 °C. PCR conditions for *16S* was as follows: denaturation at 94 °C for 3 min, followed by 15 cycles at 94 °C for 30 s, annealing temperature at 55–60 °C for 30 s, 72 °C for 1 min, followed by 25 cycles at 94 °C for 35 s, annealing temperature at 50 °C for 30 s, 72 °C for 1 min, and a final 10 min at 72 °C. Multiple alignments were generated using CLUSTALX (Thompson *et al.* 1997). Sequences of all primers used in this study are listed in Appendix S1. Sequences were deposited in GenBank; the accession numbers are provided in Appendix S2.

Calculating interspecific sequence divergence and constructing the NJ tree

'Distance-based method' and 'tree-based method' were used to ascertain the relationship of the three *Biston* species. All *COI* sequences of the three species were used to calculate the interspecific sequence divergence and construct the neighbour-join (NJ) tree by MEGA 5.05 (Tamura *et al.* 2011) based on Kimura-2-parameter (K2P) distances. NJ analysis (Saitou & Nei 1987) was used to examine whether these three species were effective and authentic.

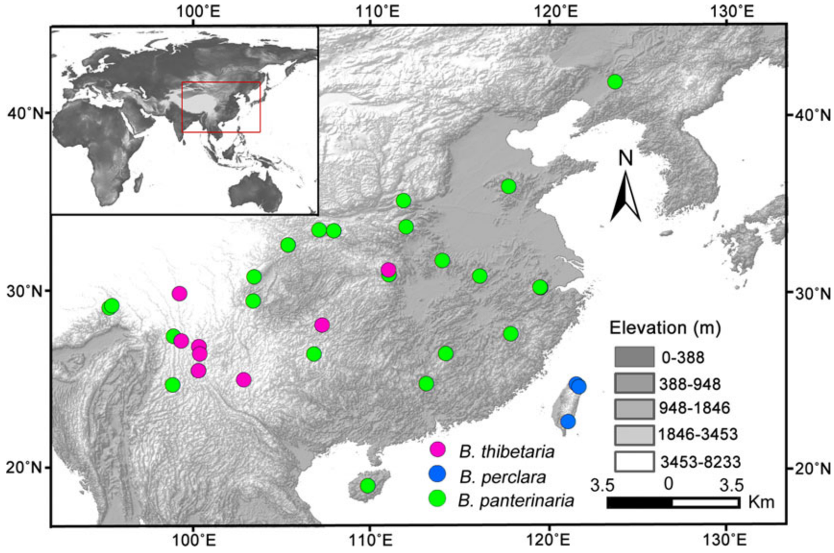

Fig. 1 Sampling sites of *B. perclara*, *B. thibetaria* and *B. panterinaria*. Colours represent different species.

Species tree estimation

A species tree of the three species was constructed using Bayesian phylogenetics and phylogeography (BPP v.2.0; Yang & Rannala 2010) with the full phased data set for the three mitochondrial loci. The model assumes no admixture following speciation, which is an assumption motivated by the biological species concept. The modes of rjMCMC species delimitation and NN1 over species/guide trees were chosen. Running the rjMCMC analyses for 500 000 generations (sampling interval of five) with a burn-in period of 10 000 produced consistent results across separate analyses initiated with different starting seeds. We ran each analysis ten times with different starting seeds to check convergence by examining agreement between runs. Ensuring adequate rjMCMC mixing involves specifying a reversible jump algorithm to achieve dimension matching between species delimitation models with different numbers of parameters, and we used algorithm 0 with the fine-tuning parameter $\varepsilon = 2$. Each species delimitation model was assigned equal prior probability. We evaluated the influence of the priors for population sizes (θ) and the root height of the guide tree (τ) on the posterior probability of species delimitation models by considering different combinations of priors.

Comparing the characters of female genitalia

In earlier taxonomic work on moths, the male genitalia were often used as a main basis for classification, a method which was accepted by almost all lepidopterists. As a result, little information about the female genitalia can be found in the literature. In our study, as the male genitalia of the three *Biston* species showed few differences, so we compared the female genitalia of the main geographical populations of the three species to obtain important characters. Morphological taxonomic methods were used to examine external features and genitalia preparations. The terminology of the genitalia follows that of Pierce (1914, reprint 1976), Klots (1970) and Nichols (1989). Photographs of the moths were taken with digital cameras. Composite images of genitalia were generated using Auto-Montage 5.03.0061 (Synoptics, Ltd, Cambridge, Cambridgeshire, UK). The plates were compiled using ADOBE PHOTOSHOP SOFTWARE (San Jose, CA, USA).

Phylogenetic analysis of Biston

To make sure of the close relationship of these three species according to the molecular evidence, we constructed a phylogenetic tree of Chinese *Biston* based on combined mtDNA and nuDNA genes. One or two specimens of every species were chosen. Two methods of Bayesian inference (BI) and maximum likelihood (ML) analysis were used.

For the BI analysis, the best-fit model of nucleotide substitution was selected using JMODELTEST 0.1.1 (Guindon & Gascuel 2003; Posada 2008) under the Bayesian information criterion (BIC) (Schwarz 1978). BI analysis was carried out in MRBAYES 3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003) under default priors, with each partition unlinked for parameter estimations. Four Markov chains (three heated and one cold) were run, starting from a random tree and proceeding for 1 000 000 Markov chain Monte Carlo (MCMC) generations, sampling the chains every 100 generations. Two concurrent runs were conducted to verify the results. A plot of sampled log-likelihood scores against generation time was used to determine the stationarity of the chains. The trees prior to stationarity were discarded. For all runs, the first 2500 trees were discarded as burn-in samples. The remaining trees were used to compute a majority-rule consensus tree with posterior probabilities (PP) (for the overcredibility of Bayesian phylogenetics, see Suzuki *et al.* 2002; Cummings *et al.* 2003; Simmons *et al.* 2004).

The maximum likelihood (ML) analysis was inferred in RAxML v7.2.6 (Stamatakis 2006; Stamatakis *et al.* 2008) with the GTRGAMMAI model 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).

Phylogenetic analysis of three Biston species

Phylogenetic analysis of *B. panterinaria*, *B. thibetaria* and *B. perclara* was constructed based on three data sets (mitogenomes, *COI*+*CYTB*+*16S* and each nuDNA gene). The mitogenome of *Biston suppressaria* (NC_027111) was used as an outgroup. Two methods of BI and ML were used according to the above-mentioned instructions. The data of mitogenomes were divided into 15 partitions (13 PCGs and 2 rRNA genes) and 41 partitions (13 PCGs partitioned by codon position and 2 rRNA genes partitioned by gene). Different models were used for different partitions, which were selected using the jModelTest. Poorly aligned nucleotide positions were omitted by the program GBLOCKS (Castresana 2000) with a more stringent selection criterion. The results were visualized using FIGTREE 1.3.1 (Rambaut 2009).

Estimate of divergence time

The maximum clade credibility tree from divergence-time-rooted phylogenetic analyses was estimated using BEAST 1.8.0 (Drummond & Rambaut 2007) based on *COI*+*CYTB*+*16S* data set. Although the use of a molecular clock as the only way for calibrating the divergence time of phylogenetic trees is controversial, it does provide a method for estimating approximate time when no other calibration information, such as fossil or geological evidence, is available (Maekawa *et al.* 2001; López-López *et al.* 2015). Thus, the widely accepted mutation rates for insect mitochondrial *COI* gene (0.0115–0.0177 per site per million years, Brower 1994; Papadopoulou *et al.* 2010) were adopted; other genes were scaled to the *COI* rate in BEAST. Finally, the already established divergence time of the northern and Yunnan–Tibet lineage of *B. panterinaria* (Cheng *et al.* 2015) could be used to calibrate the results. Each gene was assigned a separate unlinked relaxed clock model in the analysis. Default priors were used. Chains were analysed for 200 million generations, with sampling every 2000 generations. TRACER 1.5.0 was used to verify the posterior distribution and the effective sample sizes (ESSs) 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 that had been established. The tree and divergence times are displayed in FIGTREE 1.3.1.

Results

Interspecific sequence divergence and NJ tree of three Biston species

The results of interspecific divergence of these three species are listed in Appendix S3. The divergences between *B. panterinaria* and *B. thibetaria*, *B. panterinaria* and *B. perclara*, *B. perclara* and *B. thibetaria* are 3.9–5.2%, 3.7–5.6% and 3.6–4.3%, respectively. The results of the NJ analysis based on *COI* gene are summarized in Fig. 2. In the NJ tree, four lineages of *B. panterinaria* formed one distinct cluster. *B. perclara* and *B. thibetaria* formed another cluster.

Species tree

The results of BPP are shown in Fig. 3. The topology of the species tree is congruent with that of the NJ tree. Four lineages of *B. panterinaria* grouped into one clade, and *B. thibetaria* and *B. perclara* grouped into one clade, respectively.

The characters of female genitalia of three Biston species

The adults and female genitalia of the three species are shown in Fig. 4. Some characters, such as ostium bursae and signum, are very close among the three species. But they differ substantially in wing markings, and in the shape and degree of sclerotization of the lamella postvaginalis. The lamella postvaginalis is triangular, weakly to moderately sclerotized in *B. panterinaria*, whereas, it is tongue-like, and well sclerotized in *B. thibetaria* and *B. perclara*, and posteriorly narrower in *B. perclara*.

The close relationship of three Biston species

A total of thirteen species from three species groups (groups I, II and III) based on morphological evidence were used to reconstruct a phylogenetic analysis of Chinese *Biston*. Phylogenetic trees based on combined mtDNA and nuDNA genes showed that *Biston* is divided into two clades (Appendix S4), which is not consistent with the three-group system based on morphology. One clade included Group II (*B. falcata*, *B. brevipennata* and *B. quercii*) and some species of Group I (*B. regalis*, *B. thoracicaria* and *B. betularia*). The other clade included Group III (*B. perclara*, *B. thibetaria* and *B. panterinaria*) and some species of Group I (*B. bengaliaria*, *B. suppressaria*, *B. mediolata* and *B. contectaria*). The Group III of *B. perclara*,

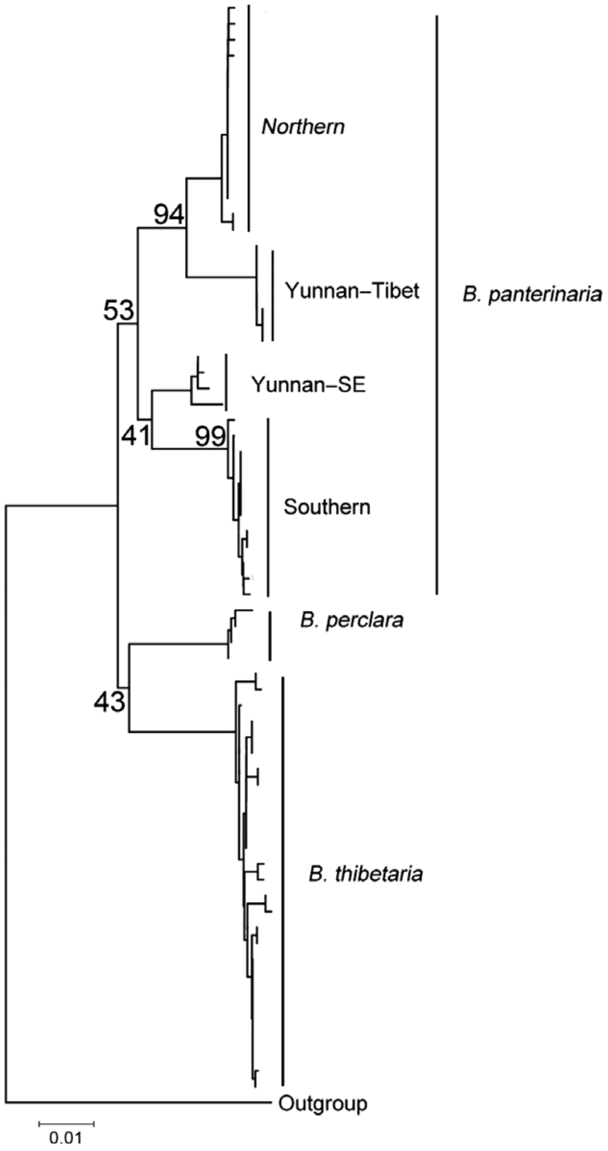

Fig. 2 NJ tree based on Kimura-2-parameter model for *COI* sequence from *B. perclara*, *B. thibetaria* and *B. panterinaria*. Nodal values of bootstrap support are shown.

B. thibetaria and *B. panterinaria* still grouped together, which confirmed the particularly close relationship of these three species.

Phylogenetic analysis of the three Biston species

The ML tree and BI tree based on mitogenomes and *COI+CYTB+16S* are essentially identical in topology, with the exception of the position of the southern and Yunnan–SE lineages of *B. panterinaria* (Figs 5 and 6 and Appendix S5). All phylogenetic trees suggested that *B. panterinaria* is not monophyletic and that *B. perclara* and *B. thibetaria* are embedded within *B. panterinaria*. The clade including *B. perclara* and *B. thibetaria* and another clade including the northern and Yunnan–Tibet lineages of *B. panterinaria* grouped together. The phylogenetic trees of each nuDNA gene (Appendix S6) showed an incomplete lineage sorting of the three *Biston* species, which did not form stable clusters.

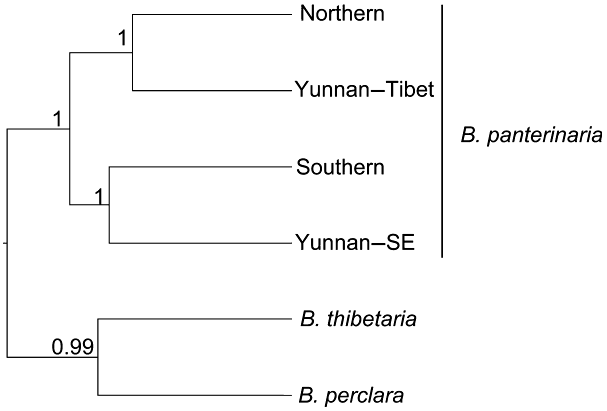

Fig. 3 Species tree estimated by BPP. Posterior probabilities of clade support are shown at nodes above branches.

Divergence-time estimation

The result of divergence-time analysis is shown in Fig. 7. The most recent common ancestor (TMRCA) of *B thibetaria* and *B. perclara* separated from lineage I (including northern and Yunnan–Tibet lineages) of *B. panterinaria* at approximately 1.38 Ma [(1.04–1.81 Ma, 95% highest posterior density (HPD)], which is within the era of the Kunlun-Yellow River Movement. The divergence time between *B thibetaria* and *B. perclara* is approximately 1.12 Ma (0.82–1.50 Ma, 95% HPD).

Discussion

The phylogenetic relationship of the Chinese Biston

The genus *Biston* in China includes 17 species and has been divided into three species groups based on morphological characters (Jiang *et al.* 2011). Group I comprises the 'typical' species of *Biston*. Group II comprises *B. brevipennata* Inoue, 1982, and the species which were placed in the subgenus *Eubyjodonta* of *Biston* by Wehrli (1941). Group III comprises *B. panterinaria*, *B. thibetaria* and *B. perclara*, which were considered somewhat different from the typical *Biston* species by Sato (1996). This division does not accord with the phylogenetic analysis based on molecular evidence. The result of phylogenetic analysis based on multiple loci divides *Biston* in China into two clades. One clade includes Group II and some species of Group I. The other clade includes Group III and some species of Group I. Importantly, *B. panterinaria*, *B. thibetaria and B. perclara* grouped together and consisted of one subclade of the second clade.

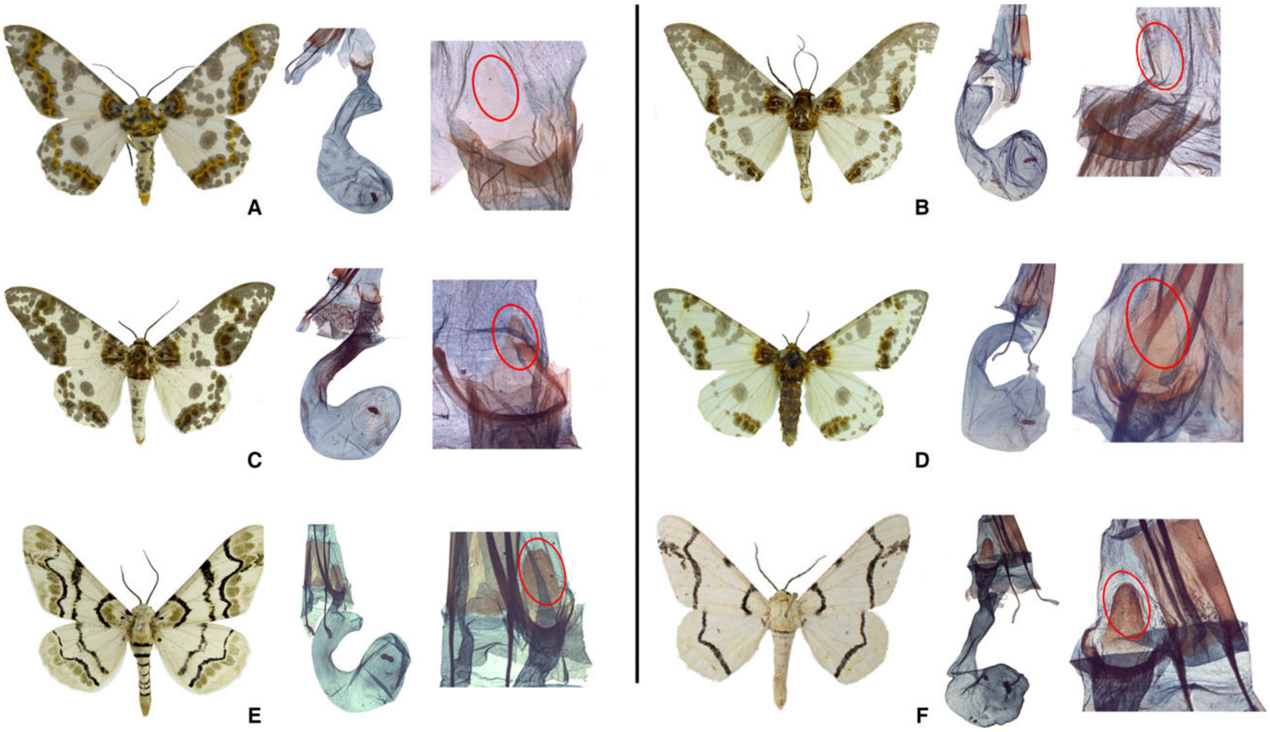

Fig. 4 Adults, female genitalia and enlarged lamella postvaginalis. A, B, C and D are northern, Yunnan–Tibet, southern and Yunnan–SE lineages of *B. panterinaria*, respectively; E: *B. thibetaria*; F: *B. perclara*.

This phylogenetic analysis confirmed the close relationship of *B. panterinaria*, *B. thibetaria and B. perclara*, which is congruent with the morphology. A close relationship of sister species is one foundation of studying budding speciation within the three species.

The relationship of the three Biston species

In our study, *B. panterinaria*, *B. thibetaria and B. perclara* are confirmed as closely related species or sister species in the genus *Biston* based on morphological and molecular evidence. They share many features in the genitalia, such as the bifurcated uncus, short and apically rounded gnathos, and very short ductus bursae (Sato 1996; Jiang *et al.* 2011). They cannot be distinguished by the male genitalia, but can be distinguished by wing pattern and venation, and especially by the shape and degree of sclerotization of the lamella postvaginalis in the female genitalia. In addition, the NJ tree and species tree also supported the existence of three good species, on the basis of four lineages of *B. panterinaria*, *B. thibetaria* and *B. perclara* grouped as one clade respectively.

Phylogenetic trees based on the mitogenomes and three mtDNA genes (*COI*+*CYTB*+*16S*) show a similar topology, with the exception of the relationship of the southern and Yunnan–SE lineages of *B. panterinaria* (Figs 5 and 6 and Appendix S5). In our previous study of *B. panterinaria*, four lineages were reciprocally monophyletic, and the southern and Yunnan–SE lineages and the northern and Yunnan–Tibet lineages grouped together, respectively (Cheng *et al.* 2015): which is different with the phylogenetic trees of three species. Among all phylogenetic trees of the three *Biston* species, *B. perclara*, *B. thibetaria* and the northern and Yunnan–Tibet lineages of *B. panterinaria* grouped into one stable clade. It seems that *B. panterinaria* is not a monophyletic species.

Based on our phylogenetic results, we consider that the most reasonable conclusion is that *B. perclara* + *B. thibetaria* are derived from within *B. panterinaria*, rendering the latter paraphyletic. Based on the concept of budding speciation, we think that the common ancestor of *B. perclara* and *B. thibetaria* is the result of budding speciation from their parent species (*B. panterinaria*) (Mayr 1954; Funk & Omland 2003; Morse & Farrell 2005; Vanderpoorten & Long 2006; Toussaint *et al.* 2013; Grossenbacher *et al.* 2014). The following evidence is relevant: (i) these three species are most closely related species or sister species, which is one important basis of budding speciation (Grossenbacher *et al.* 2014); (ii) *B. perclara* + *B. thibetaria* are embedded in *B. panterinaria* in the phylogenetic trees, which means *B. panterinaria* is a widely distributed and

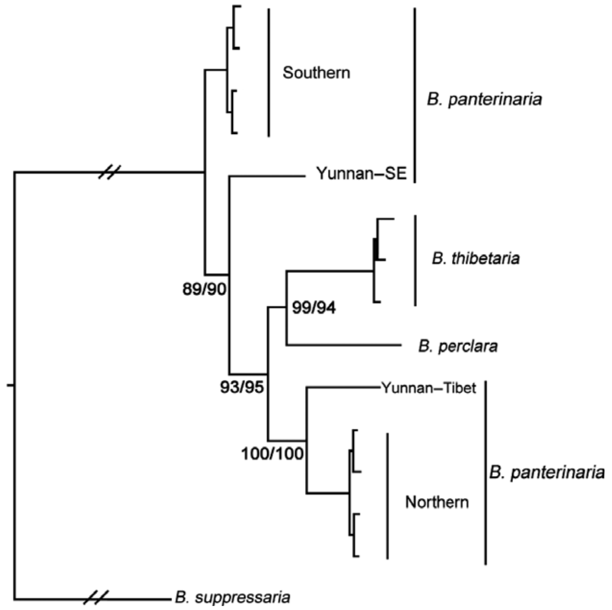

Fig. 5 Phylogenetic tree of *B. perclara*, *B. thibetaria* and *B. panterinaria* based on mitogenomes. Values on the left of nodes indicate bootstrap supports and posterior probabilities of ML/BI for major clades.

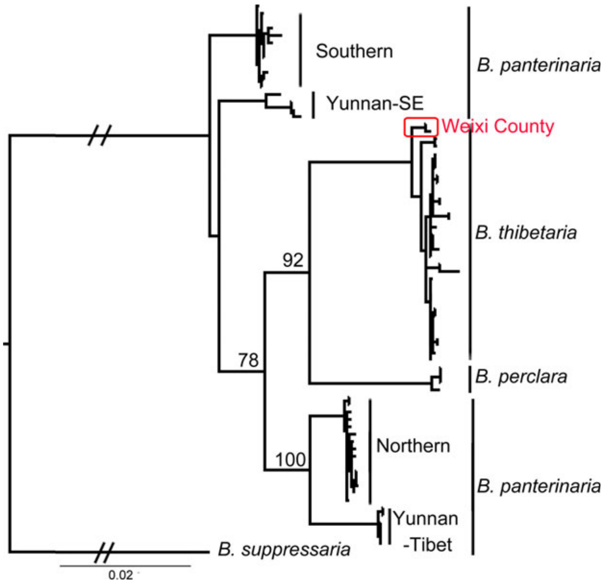

Fig. 6 Phylogenetic tree of *B. perclara*, *B. thibetaria* and *B. panterinaria* based on *COI*+*CYTB*+*16S* data set. Values on the left of nodes indicate bootstrap supports of ML for major clades.

paraphyletic parental species and *B. perclara* + *B. thibetaria* are geographically restricted monophyletic daughter species; (iii) the distribution of *B. perclara* + *B. thibetaria* overlaps with the distribution of *B. panterinaria*, which is congruent with the concept of peripatric speciation; (iv) the result of nuDNA analysis and male genitalia, and relatively low genetic distance showed the effect of incomplete lineage sorting; (v) different host plants and different altitudes provided little chance to do interspecific introgression (Funk & Omland 2003; McKay & Zink 2010).

However, an alternative conclusion from the molecular data might theoretically be that the four lineages of *B. panterinaria*, *B. thibetaria* and *B. perclara* actually comprise some four or five monophyletic taxa within the *B. panterinaria* complex, whether those taxa are considered to be valid at the specific or subspecific level, with the *B. panterinaria* populations at either end of the distribution evolving into separate taxa. According to Hausmann *et al.* (2011 and 2013) the average genetic distances between geometrid species are considerably higher, often between eight and 10%: in this case, the differences are clearly smaller. This conclusion would require the *B. panterinaria* clades on either side of the *B. thibetaria* +*B. perclara* clade to be treated as separate taxa. But we do not find this conclusion persuasive for a number of reasons: the distribution of *B. panterinaria* feeding on broad-leaved foliage is fairly continuous across its range, and there is no obvious mechanism which would interrupt gene flow between the various regions of distribution, whereas there are both altitudinal and food plant characteristics which provide a convincing isolation mechanism between *B. panterinaria* and the other two species, and in addition, there is geographical separation in the case of *B. perclara*. The existence of morphological differences in the female genitalia between *B. panterinaria* (from right across its range) on the one hand, and *B. thibetaria* and *B. perclara* on the other point is in the same direction. Moreover, the smaller genetic distances are exactly what would be expected where lineage sorting is incomplete. For these reasons, it seems much more likely that *B. panterinaria* represents a comparatively stable entity, albeit with genetic variations, across its range, while the other two species are isolated and diverging, but derived from within the *B. panterinaria* complex on the ‘budding speciation’ principle.

The process of budding speciation

In this study, the divergence time of the common ancestor of *B. perclara* + *B. thibetaria* and *B. panterinaria* is approximately 1.38 Ma (1.04–1.81 Ma, 95% HPD), which is consistent with the Kunlun-Yellow River Movement induced by the uplift of the Qinghai–Tibet Plateau (Cui *et al.* 1997, 1998). So it is likely that the time of budding speciation was during the period of the Kunlun-Yellow River Movement. In the phylogenetic tree, the common ancestor of *B. perclara* + *B. thibetaria* and the Yunnan–Tibet + northern lineages of *B. panterinaria* clustered as one clade (Figs 5 and 6). The distribution of the Yunnan–Tibet and

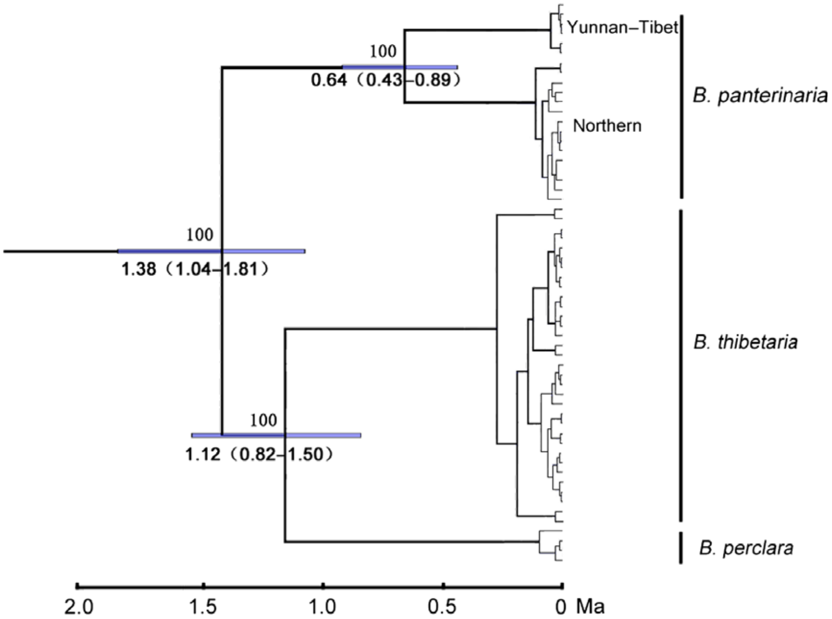

Fig. 7 Divergence-time-rooted phylogenetic analysis of *B. perclara*, *B. thibetaria* and northern and Yunnan–Tibet lineages of *B. panterinaria* based on *COI+CYTB+16S* data set. Posterior probabilities of clade support are shown at nodes above branches. Estimates of divergence time with 95% confidence intervals are shown at nodes as purple bars and numbers below branches.

northern lineages of *B. panterinaria* is on the east slope of the Gaoligong Mountains, south-eastern Tibet and north China, and the distribution of *B. perclara* + *B. thibetaria* is the east slope of the Gaoligong Mountains, south China and Taiwan Island. The common distribution is the east slope of the Gaoligong Mountains, which therefore seems to be the most likely region of budding speciation. In addition, the population of Weixi County (the possible westernmost distribution region of *B. perclara* + *B. thibetaria*) is located at the root of the phylogenetic tree. So, after budding speciation, the common ancestor of *B. perclara* and *B. thibetaria* may have dispersed from west to east and eventually colonized Taiwan Island.

In budding speciation, the newly formed small-range species should occupy a distinct realized niche when compared to their large-range sisters (Grossenbacher *et al.* 2014): it has been widely recognized that the majority of speciation events in phytophagous insects were accompanied by shifts in host plants (Ehrlich & Raven 1964; Mayr 1982; Mitter *et al.* 1988; Thompson 1994; Funk *et al.* 1995; Farrell 1998; Morse & Farrell 2005). Considering the big differences in diet between *B. perclara*, *B. thibetaria* and *B. panterinaria*, we think the likely sequence is that one population of *B. panterinaria* from the east slope of the Gaoligong Mountains, previously feeding on broad-leaved plants at low altitudes, adapted to feeding on gymnosperms at higher altitudes, following which reproductive isolation occurred, resulting in the appearance of the distinct common ancestor of *B. perclara* and *B. thibetaria* during the Kunlun-Yellow River Movement. If this is so, it seems that tectonic movements induced by the uplift of the Qinghai–Tibet Plateau were the main driver of this speciation process. The host shift from angiosperm to gymnosperm is also found in other insects (Sequeira *et al.* 2000; Farrell *et al.* 2001).

Budding speciation represents a situation where lineage sorting is incomplete (Funk & Omland 2003; Vanderpoorten & Long 2006; Enroth *et al.* 2009; Kruckenhauser *et al.* 2014), which implies that the paraphyletic situation will disappear and the genetic distance of the three species will increase, as soon as the lineage is completely sorted.

Driver of budding speciation

Although this form of speciation is still controversial, multiple reported examples, involving insects, snails, frogs and shrews (Morse & Farrell 2005; Chen *et al.* 2009, 2012; Kruckenhauser *et al.* 2014), have improved the credibility of the concept. Basing on the study of *Mimulus* (monkeyflowers), budding speciation seems to be very common in certain taxonomic groups and in certain regions (Kruckenhauser *et al.* 2014). In these studies, one important character of budding speciation is that the newly emerging species, compared to parental species, always occupies distinct and different niches, involving host plant divergence (Morse & Farrell 2005), and adaptation to different habitats and soils (Anacker & Strauss 2014; Kruckenhauser *et al.* 2014), colonizing other regions by long-distance dispersal (Wiley & Mayden 1985; Vanderpoorten & Long 2006). Budding speciation is driven by ecological opportunities offered by the emergence of largely unoccupied habitats with few competitors (Hughes & Eastwood 2006;

Hines 2008). There are two important factors resulting in new ecological opportunities: climatic fluctuations and tectonic movements. When climate changes (e.g. the presence of an ice age), new environmental conditions (e.g. temperature, humidity), along with new host plants create ecological opportunities (Morse & Farrell 2005; Vanderpoorten & Long 2006; Toussaint *et al.* 2013). In addition, tectonic movements create multiple new ecological opportunities (Chen *et al.* 2009). The uplift of the Qinghai–Tibet plateau provided ecological opportunities in the unoccupied habitats of higher altitudes (Cao *et al.* 1981). When organisms take full advantage of such opportunities, speciation and diversification can occur rapidly (Chen *et al.* 2009). The conclusion from our study, with the uplift of the Qinghai–Tibet plateau, is that one population of *B. panterinaria* from the east slope of Gaoligong Mountains occupied the feeding niche created by the proliferation of gymnosperms at the high altitudes, which promoted the budding process of the common ancestor of *B. thibetaria* +*B. perclara*. Our study is thus a good example of budding speciation driven by tectonic movements of the Qinghai–Tibet plateau.

The relationship of B. perclara and B. thibetaria

Based on morphology and molecular evidence, *B. perclara* and *B. thibetaria* are two sister species, which were isolated by the Taiwan Strait. This form of evolution is very common in almost all organisms, and the fauna of Taiwan Island has very close ties with that of the mainland of China (Ying & Hsu 2002; Huang *et al.* 2004; Yen 2013). Taiwan Island separated from the mainland at approximately 5 Ma for the first time (Sibuet & Hsu 1997, 2004) and then was rejoined through a land bridge during multiple glacial periods and completely separated after the last glacial maximum (Voris 2000). The divergence time between *B. perclara* and *B. thibetaria* is about 1.12 (0.82–1.50, 95% HPD) Ma (Fig. 7), which is close to the Xixiabangma glaciation (0.8–1.17 Ma, Shi 2002; Zheng *et al.* 2002). A reasonable conclusion would be that after the Xixiabangma glaciation, when the land bridge disappeared, and the Taiwan Strait again became a geographical barrier, gene flow between these two populations ceased and the two populations evolved independently into separate species.

The distribution range of phytophagous insects can be expected to have a close relationship with their host plants (Atkinson 1980; Lance & Barbosa 1982; Jermy 1984; Stoyenoff *et al.* 1994; Tikkanen *et al.* 1999; Ballabeni *et al.* 2001; Lopez-Vaamonde *et al.* 2003). During glaciation, conditions are created in which gymnosperms, which normally occupy high altitudes, can migrate to lower altitudes, replacing broad-leaved vegetation which is less suited to the changed climatic conditions: this is a common strategy for gymnosperms avoiding the influence of glaciation (Hewitt 2001; Qu *et al.* 2014; Lei *et al.* 2015). Such migration may well have provided a more continuous path, enabling the common ancestor of *B. perclara* and *B. thibetaria*, both gymnosperm feeders, to spread far enough across southern China to reach Taiwan across the land bridge. After glaciation, the gymnosperms will have retreated again to higher altitudes, so that the dispersal route was cut off, and the moth became restricted to rare high altitude areas of southern China, especially the Hengduan Mountains, which is quite distant from Taiwan. The moth then evolved through the isolation mechanism into two distinct species at the two ends of its former range. In summary, the discontinuity of host plants may be the reason for the current restricted distributions of *B. perclara* and *B. thibetaria*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Total primers used in this study.

Appendix S2. Sampling information and the GenBank accession numbers of all sequences of this study.

Appendix S3. Percentage of divergence in *COI* sequences between *B. panterinaria*, *B. thibetaria* and *B. perclara*.

Appendix S4. Phylogenetic trees of Chinese *Biston* based on combined mtDNA and nuDNA genes.

Appendix S5. Phylogenetic tree of *B. perclara*, *B. thibetaria* and *B. panterinaria* based on *COI+CYTB+16S* dataset.

Appendix S6. Phylogenetic trees of each nuDNA gene.