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Molecular phylogeny of the scorpionflies Panorpidae (Insecta: Mecoptera) and chromosomal evolution

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Abstract

Panorpidae is the most species-rich family in Mecoptera with ca. 470 species in the Northern Hemisphere. However, the intergeneric phylogenetic relationships of Panorpidae remain unsatisfactorily resolved to date. Here, we used molecular and cytogenetic approaches to determine the phylogenetic relationships of Panorpidae in the evolutionary scenario of chromosomes, and estimated their divergence times using fossil-calibrated Bayesian analysis. In total, 89 species representing all seven genera of Panorpidae were used to reconstruct the phylogenetic trees using maximum parsimony, maximum likelihood and Bayesian inference based on the nuclear *28S* rRNA and mitochondrial *cox1* and *cox2* genes. The results reveal that Panorpidae is a well-supported monophyletic group that can be categorized into two major clades. Major Clade I comprises *Neopanorpa* and *Leptopanorpa*, and Major Clade II consists of all the other genera (*Cerapanorpa*, *Dicerapanorpa*, *Furcatopanorpa*, *Panorpa* and *Sinopanorpa*). *Neopanorpa* and *Cerapanorpa* are regarded as paraphyletic groups for the first time. BEAST analysis indicates that Panorpidae originated in the Lower Cretaceous approximately 122.5 Ma (96.8–149.3 Ma), and that most diversification occurred from the Selandian (59.8 Ma) to the Middle Pleistocene (0.6 Ma) in the Cenozoic. Cytogenetic data plotted on the cladogram show that the lineage differentiation of Panorpidae is closely related to the chromosomal evolution, especially the reduction of chromosome number. Our study suggests that a taxonomic revision of Panorpidae is urgently needed at the generic level.

Introduction

Chromosomes can provide uniquely important characters for phylogenetic analyses of eukaryotic organisms and may help reveal the evolutionary relationships of species or higher taxa due to their evolutionary conservation (Dyer, 1979; Appels et al., 1998; Dobigny et al., 2004; Gokhman and Kuznetsova, 2006). Chromosome numbers may provide substantial information related to phylogeny and karyotype evolution (White, 1974; Gokhman and Kuznetsova, 2006; Guerra, 2008). Although numerous approaches are available in chromosome analyses (Dyer, 1979; Appels et al., 1998; Czepulkowski, 2001; Gokhman and Kuznetsova, 2006), differential banding techniques are the most frequently used methods to identify the presence of numerous structural rearrangements that contribute to speciation (Ayala and Coluzzi, 2005; Butlin, 2005; Brown and O'Neill, 2010; Kirkpatrick, 2010). Such studies have been conducted on numerous insects, including butterflies (Kandul et al., 2007; Talavera et al., 2013; Šíchová et al., 2015), parasitic wasps (Gokhman, 2009), ants (Cristiano et al., 2013; Cardoso et al., 2014), bush crickets (Grzywacz et al., 2014) and coccids (Mills and Cook, 2014), but are still scarce in Panorpidae.

Panorpidae is the most species-rich family in Mecoptera and comprises approximately 470 species distributed mainly in the Northern Hemisphere (Penny and Byers, 1979; Byers and Thornhill, 1983; Bicha, 2010; Wang and Hua, 2017a). Panorpids are commonly known as scorpionflies due to the male genital bulb (the ninth abdominal segment) being enlarged and recurved upward,

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superficially resembling the stinger of scorpions. Panorpidae consists of seven genera to date (Penny and Byers, 1979; Wang and Hua, 2017a). The largest genus *Panorpa* Linnaeus, 1758 (ca. 260 species) is distributed widely in the Holarctic and northern Oriental regions. *Leptopanorpa* MacLachlan, 1875 (12 species) is exclusively distributed in Java, Indonesia (Chau and Byers, 1978). *Neopanorpa* Weele, 1909 is the second largest genus (ca. 160 species) in Panorpidae and occurs in the Oriental Region, especially abundant in southern China and Southeast Asia. *Sinopanorpa* Cai & Hua, 2008 (three species), *Furcatopanorpa* Ma and Hua, 2011 (one species) and *Dicerapanorpa* Zhong & Hua, 2013 (eight species) are endemic to China. *Cerapanorpa* Gao, Ma & Hua, 2016 comprises 22 species in the Oriental and eastern Palearctic regions.

Intergeneric phylogenetic relationships, however, remain unsatisfactorily resolved in Panorpidae to date. *Furcatopanorpa* formed the sister taxon to all other genera of Panorpidae based on a morphological analysis (Ma et al., 2012), but merely to *P. liui* based on molecular evidence (Hu et al., 2015). Previously, *Panorpa* was considered paraphyletic with *Neopanorpa* based on morphological and molecular data (Willmann, 1989; Misof et al., 2000; Whiting, 2002; Ma et al., 2012). However, through a recent molecular phylogenetic analysis of more robust sampling, *Neopanorpa* was confirmed as a sister taxon to all other genera of Panorpidae excluding *Leptopanorpa* (Hu et al., 2015).

Panorpidae is readily separated from other families of Mecoptera by several cytogenetic features, such as relatively high chromosome numbers and achiasmate male meiosis (Naville and Beaumont, 1934; Ullerich, 1961; Atchley and Jackson, 1970; Cooper, 1974; Xu et al., 2013; Miao et al., 2017). A recent cytotaxonomic investigation showed that cytogenetic data play an important role in the species delimitation and phylogenetic analyses of *Cerapanorpa* (Miao et al., 2017). However, only 14 species have been reported for their chromosomes in Panorpidae to date, accounting for 3% of the species described in this family.

In this paper, we present cytogenetic information for 46 species and add molecular data for 29 additional species in Panorpidae. The main aims of this study were: (i) to address the phylogenetic relationships by expanding the data set in Panorpidae and (ii) to infer the chromosomal evolution in Panorpidae, using new acquisitions of chromosome data together with previously reported chromosomal counts.

Materials and methods

Taxon sampling

The biological materials used for chromosome preparations were obtained from 46 species of Panorpidae, including one species of *Dicerapanorpa*, one of *Furcatopanorpa*, two of *Sinopanorpa*, three of *Cerapanorpa*, 14 of *Panorpa* and 25 of *Neopanorpa* (Table 1). Testes of fresh male specimens were dissected and submerged in fresh hypotonic KCl solution (0.045 M) for 30 min at room temperature. After a short fixation of 30–40 s in acetic ethanol (1 : 3, v/v), the testes were transferred to a drop of 45% acetic acid on slides, torn into small pieces and then air-dried for 24 h. Bodies were stored in 2-mL plastic vials with 100% ethanol for DNA analysis.

Cytogenetic analysis

The procedure for C-banding followed King (1980). The air-dried slides were placed in a saturated $Ba(OH)_2$ solution at 60 °C for 3 min, then incubated in Sørensen's phosphate buffer (pH 7.0) at 65 °C for 30 min, and finally stained with 5% Giemsa stain for 15 min.

Fluorescence staining was used for the C-banded spermatocytes that failed to stain with Giemsa and followed the protocol described by Rebagliati et al. (2003). The air-dried slides were stained with DAPI (4′-6-diamino-2-phenylindole) for 3−5 min at room temperature. Photographs were taken with a Nikon DS-Fil digital camera mounted on a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan). The fluorescence signals were observed with a UV filter (330–385 nm) for the fluorochrome DAPI.

DNA sequencing

Procedures for DNA extraction, amplification and sequencing followed Hu et al. (2015). Fragments of one nuclear gene, *28S* rRNA, and two mitochondrial genes, cytochrome *c* oxidase subunit I and II (*cox1* and *cox2*), were amplified using the primer pairs 28S rD1.2a, 28S rD3.2a and 28S Rd4.2b (Whiting, 2002); C1-J-1490 (Folmer et al., 1994), C1-J-1718 and C1-N-2329 (Pollmann et al., 2008); and COII-F-leu and COII-R-lys (Whiting, 2002). Sequences generated in this study together with collateral information of the specimens were deposited in the Barcode of Life Database (BOLD) and GenBank.

Phylogenetic analyses

In total, 279 DNA sequences of 93 species were used for phylogenetic reconstruction (Table S1). Four species of Panorpodidae, *Brachypanorpa oregonensis* (MacLachlan, 1881), *Br. sacajawea* Byers, 1990, *Panorpodes colei* Byers, 2005 and *Pd. kuandianensis* Zhong, Zhang & Hua, 2011, were chosen as outgroups based on the sister group relationship between Panorpodidae and Panorpidae (Willmann, 1989). The sequences

Table 1
Species of Panorpidae analysed by C-banding technique with Giemsa or fluorescence staining

Taxa	Chromosome number	Banding pattern	Source/references
Cerapanorpa Gao, Ma & Hua, 2016			
C. acutipennis (Hua, 1998)	$n = 23$	Undetected	Fig. S2B
C. brevicornis (Hua & Li, 2007)	$n = 22$	1 intermediate + 2 subterminal + 18 terminal	Miao et al. (2017)
C. byersi (Hua & Huang, 2007)	$n = 22$	4 intermediate + 17 terminal	Miao et al. (2017)
C. dubia (Chou & Wang, 1981)	$n = 22$	5 intermediate + 16 terminal	Miao et al. (2017)
C. liupanshana Gao, Ma & Hua, 2016	$n = 22$	Undetected	Fig. S2A
C. nanwutaina (Chou, 1981)	$n = 22$	4 intermediate + 1 heteromorphic + 16 terminal	Miao et al. (2017)
C. obtusa (Cheng, 1949)	$n = 22$	2 intermediate + 2 subterminal + 1 heteromorphic + 16 terminal	Miao et al. (2017)
C. protrudens Gao, Ma & Hua, 2016	$n = 22$	3 intermediate + 2 subterminal + 16 terminal	Miao et al. (2017)
C. reni (Chou, 1981)	$n = 22$	3 intermediate + 18 terminal	Fig. 1I
C. sinuata Gao, Ma & Hua, 2016	$n = 22$	3 intermediate + 2 subterminal + 16 terminal	Miao et al. (2017)
Dicerapanorpa Zhong & Hua, 2013			
D. magna (Chou, 1981)	$n = 23$	2 intermediate + 20 terminal	Fig. 1J
Furcatopanorpa Ma and Hua, 2011;			
F. longihypovalva (Hua & Cai, 2009)	$n = 21$	Large bands, 3 intermediate + 17 terminal	Fig. 1K
Neopanorpa Weele, 1909			
N. brevivalvae Chou & Wang, 1988	$n = 21$	2 intermediate + 1 large intermediate + 17 terminal	Fig. S1A
N. chelata Carpenter, 1938	$n = 21$	2 intermediate + 1 subterminal + 16 terminal + 1 no band	Fig. S1B
N. choui Cheng, 1949	$n = 21$	2 intermediate + 1 subterminal + 17 terminal	Fig. S1C
N. diancangshanensis Wang & Hua, 2018	$n = 21$	No data	–
N. dubis Chou & Wang, 1988	$n = 21$	2 intermediate + 18 terminal	Fig. S1D
N. hunanensis Huang & Hua, 2005	$n = 21$	2 intermediate + 18 terminal	Fig. S1E
N. lipingensis Cai & Hua, 2009	$n = 17$	6 intermediate + 10 terminal	Fig. 1E
N. longiprocessa Hua & Chou, 1997	$n = 21$	3 intermediate + 17 terminal	Fig. S1F
N. longistipitata Wang & Hua, 2018	$n = 21$	No data	Fig. S1P
N. lui Chou & Ran, 1981	$n = 21$	2 intermediate + 16 terminal + 2 heterochromatic	Xu et al. (2013); Fig. 1F
N. malaisei Byers, 1999	$n = 21$	4 intermediate + 16 terminal	Fig. 1H
N. mangshanensis Chou & Wang, 1988	$n = 21$	2 intermediate + 18 terminal	–
N. minuta Chou & Wang, 1988	$n = 21$	2 intermediate + 1 subterminal + 17 terminal	Fig. S1G
N. quadristigma Wang & Hua, 2018	$n = 21$	20 terminal	Fig. 1G
N. setigera Wang & Hua, 2018	$n = 21$	2 intermediate + 1 subterminal + 17 terminal	–
N. sheni Hua & Chou, 1997	$n = 17$	6 intermediate + 10 terminal	Fig. S1I
N. tienpingshana Chou & Wang, 1988	$n = 21$	2 intermediate + 18 terminal	Fig. S1J
N. tincta Wang & Hua, 2018	$n = 21$	2 intermediate + 1 subterminal + 17 terminal	–
N. validipennis Cheng, 1949	$n = 21$	2 intermediate + 1 subterminal + 14 terminal + 3 heterochromatic	Fig. S1K
Neopanorpa sp1	$n = 21$	2 intermediate + 1 large intermediate + 17 terminal	Fig. S1L
Neopanorpa sp2	$n = 21$	2 intermediate + 2 subterminal + 14 terminal + 2 heterochromatic	Fig. S1M
Neopanorpa sp3	$n = 21$	2 intermediate + 1 subterminal + 17 terminal	Fig. S1H
Neopanorpa sp4	$n = 21$	2 intermediate + 16 terminal + 2 heterochromatic	Fig. S1N

Table 1
(Continued)

Taxa	Chromosome number	Banding pattern	Source/references
Neopanorpa sp5	$n = 21$	2 intermediate + 1 subterminal + 17 terminal	–
Neopanorpa sp6	$n = 21$	3 intermediate + 17 terminal	Fig. S1O
Panorpa Linnaeus, 1758			
P. acuta Carpenter, 1931	$n = 23$	No data	Atchley and Jackson (1970)
P. alpina Rambur, 1842	$n = 23$	No data	Naville and de Beaumont (1934)
P. biclada Zhang & Hua, 2012	$n = 23$	22 terminal	Fig. 1C
P. bifasciata Chou & Wang, 1981	$n = 23$	1 intermediate + 2 subterminal + 19 terminal	Fig. S2C
P. changbaishana Hou & Hua, 2008	$n = 23$	3 intermediate + 19 terminal	Fig. S2D
P. chengi Chou, 1981	$n = 23$	3 intermediate + 19 terminal	Fig. S2E
P. cognata Rambur, 1842	$n = 22$	No data	Naville and de Beaumont (1934); Ullerich (1961)
P. communis Linnaeus, 1758	$n = 23$	No data	Naville and de Beaumont (1934); Ullerich (1961)
P. curva Carpenter, 1938	$n = 23$	3 intermediate + 19 terminal	Fig. S2F
P. decolorata Chou & Wang, 1981	$n = 22$	2 intermediate + 2 subterminal + 16 terminal + 1 no band	Fig. S2G
P. fulvastra Chou, 1981	$n = 23$ (+ 1B)	3 intermediate + 19 terminal (without B); small subterminal and intermediate bands (with B)	Figs 1B and S2H
P. germanica Linnaeus, 1758	$n = 21$	No data	Naville and de Beaumont (1934); Ullerich (1961)
P. japonica Thunberg, 1784	$n = 23$	No data	Cooper (1951)
P. kunmingensis Fu & Hua, 2009	$n = 24$	Intermediate small bands	Fig. 1D
P. liui Hua, 1997	$n = 23$	3 intermediate + 18 terminal + 1 no band	Fig. S2I
P. macrostyla Hua, 1998;	$n = 23$	Undetected	–
P. qinlingensis Chou & Ran, 1981	$n = 23$	2 intermediate + 20 terminal	Fig. S2J
P. semifasciata Cheng, 1949	$n = 23$	No data	–
P. sexspinosa Cheng, 1949	$n = 23$	3 intermediate + 19 terminal	Fig. S2K
Panorpa sp1	$n = 23$	No data	–
P. bashanicola Hua, Tao & Hua, 2018	$n = 18$	7 intermediate + 10 terminal	Fig. 1A
Sinopanorpa Cai & Hua, 2008			
S. nangongshana Cai & Hua, 2008	$n = 23$	3 intermediate + 19 terminal	Fig. 1L
S. tincta (Navás, 1931)	$n = 23$	3 intermediate + 19 terminal	Fig. S2L

Chromosome numbers, banding pattern and source showing the results are presented.

obtained were edited with BioEdit 7.0.9.0 (Hall, 1999) and aligned with MUSCLE 3.8.31 (Edgar, 2004) using default parameters. The three datasets were concatenated using SequenceMatrix 1.7.8 (Vaidya et al., 2011).

Maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference (BI) were performed using PAUP* v.4.0b10 (Swofford, 2003), RAxML (Stamatakis, 2014) and MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), respectively.

MP analysis was performed with heuristic searches, with 100 random sequence addition replicates conducted with tree–bisection–reconnection (TBR) branch-swapping. Support values were calculated with 1000 bootstrap replicates.

ML analysis was conducted in RAxML-VI-HPC (Stamatakis, 2014), using the graphical user interface raxmlGUI 1.3 (Silvestro and Michalak, 2012) with 1000 replications under the GTRGAMMA model.

For BI analysis, the best-fit partition schemes and models for the combined dataset were estimated using the Bayesian information criterion (BIC) implemented in PartitionFinder 2 (Lanfear et al., 2012). The models selected were GTR + Γ + I for the first codon position of *cox1* and *cox2*, HKY for the second codon position of *cox1*, HKY + Γ + I for the second codon position of *cox2*, HKY + Γ for the third codon position of *cox1* and *cox2*, and GTR + Γ + I for *28S*. We ran the analyses using two replicate runs with four chains for 10 million generations and sampled trees every 100 generations. The average standard deviation of split frequency was lower than 0.01, indicating that the runs reached stationarity. Following completion of each analysis, the first 25% of the total samples was discarded as "burn-in" and the remaining trees were used to generate a consensus phylogram and summarize statistics for the taxon bipartitions, clade credibility (posterior probability) values and branch lengths.

Divergence time estimation

Divergence time estimates were generated based on the combined dataset (*28S* rRNA, *cox1* and *cox2*) in BEAST 1.8.0 (Drummond et al., 2012). The BEAST analysis incorporated the GTR + G model for *28S* rRNA and SRD06 for *cox1* and *cox2*, a random starting tree, and a Yule speciation process. Fossil data available were used to calibrate the phylogenetic tree (Parham et al., 2012). Normal prior age distributions were used on two fossil-calibrated nodes as in Hu and Hua (2016). The node age of *Panorpodes* can be constrained to a normal distribution of 35.90 ± 1.20 Ma based on Priabonian fossils from Baltic amber (Perkovsky et al., 2007; Soszyńska-Maj and Krzemiński, 2013, 2015). The oldest confident panorpids from the Okanagan Highlands of Ypresian age (Archibald et al., 2013) were used to set a calibration of 52.90 ± 0.83 Ma (Archibald et al., 2010) for *Panorpa*.

The BEAST file was generated in BEAUti v.1.8.2 (Drummond et al., 2012), with the main lineages (*Panorpa* and *Panorpodes*) preset based on the results of Hu et al. (2015) and with *Panorpodes* as monophyletic (Hu and Hua, 2016). Two independent Markov chain Monte Carlo (MCMC) runs for 1000 million generations were implemented, sampling parameters every 100 generations. Stationarity and convergence were checked with Tracer 1.5 (Rambaut et al., 2009), and all parameter estimates had effective sample size (ESS) values > 200. The resulting trees were combined in LogCombiner 1.8.0 (Drummond et al., 2012) and summarized as a maximum clade credibility (MCC) tree using TreeAnnotator 1.8.0 (Drummond et al., 2012). The MCC tree was visualized with FigTree 1.4 (Rambaut, 2012).

Ancestral state reconstruction of chromosome numbers

From the phylogenetic topology and branch lengths, we inferred the chromosome evolution model and haploid chromosome number of the most recent common ancestor of Panorpidae with the software chromEvol 2.0 (Glick and Mayrose, 2014), using BI and maximum likelihood estimation (MLE). The program was executed with three plain text files: a control file, with the input/output files and various options to be used; a tree file, with a Newick format tree and branch length information; and a chromosome count file, with haploid chromosome numbers of species involved. Eight likelihood models were tested with 10 000 simulations, and the best-fit model was selected using the Akaike information criterion (AIC).

In addition, ancestral state reconstruction of chromosome numbers was performed in Mesquite 3.31 (Maddison and Maddison, 2017) across the phylogenetic trees. Haploid chromosome numbers were plotted on the trees as categorical data applying ancestral character reconstruction analysis under the MP and ML methods. Ancestral states were estimated across 10 000 random post burn-in trees, using the menu option "trace characters over trees". The results were visualized on the MCC tree from the BEAST analysis and summarized using the option "Count Trees with Uniquely Best States", which counted the only optimal state at a node.

Results

Chromosome counts

Chromosome numbers were counted from prophase spermatocytes for most species except four (*Neopanorpa*

diancangshanensis, *N. longistipitata*, *P. bashanicola* and *P. semifasciata*), the chromosomes of which were counted from the first metaphase spermatocytes (Figs 1, S1 and S2). The chromosome data are listed in Table 1.

Panorpa is remarkable for its extensive variation of haploid chromosome number, ranging from $n = 18$ in *P. bashanicola* to $n = 24$ in *P. kunmingensis*, with $n = 23$ being the most frequent number (Figs 1A–D and S2C–K). The chromosome number is $n = 21$ in *P. germanica*, but is $n = 22$ in its sister species *P. cognata*. Interestingly, intraspecific variation was observed in *P. fulvastra*, the standard chromosome number of which is $n = 23$ but with some having $n = 24$ (23 + B chromosome).

In *Neopanorpa*, most species show the chromosome number $n = 21$ (Figs 1F–H, S1), but there is a large reduction of chromosomes in *N. lipingensis* (Fig. 1E) and *N. sheni* (Fig. S1I), both of which exhibit an extraordinary chromosome number $n = 17$, the lowest found in Panorpidae so far.

The species of *Cerapanorpa* exhibit two different chromosome numbers. Nine species in central China show $n = 22$, but *C. acutipennis* in north-eastern Asia has $n = 23$ (Figs 1I and S2A–B).

Dicerapanorpa magna and two species of *Sinopanorpa* show $n = 23$ (Figs 1J,L and S2L), but *Furcatopanorpa longihypovalva* exhibits $n = 21$ (Fig. 1K).

Chromosome banding

Chromosome rearrangements and conspicuous heterochromatin are presented using the C-banding technique (Figs 1, S1 and S2). The banded bivalents reveal a predominance of constitutive heterochromatin, but the patterns vary among the taxa. Conspicuous heterochromatin at one bivalent terminal is the most frequent type of C-banding. The C-banding information is summarized in Table 1.

The C-banding patterns are simple in the species of *Panorpa*, *Sinopanorpa* and *Dicerapanorpa*, with only intermediate and terminal bands on bivalents (Figs 1A–D,L–J and S2C–L). The presence of a B chromosome in *P. fulvastra* is documented in Panorpidae for the first time. The B chromosome is completely heterochromatic (arrowhead in Fig. 1B). In the chromosome complement with a B chromosome, most bivalents exhibit small subterminal or intermediate bands (Fig. 1B). Without the B chromosome, however, the C-banding pattern of *P. fulvastra* is normal (Fig. S2H). In addition to these banding patterns, small dot-shaped blocks were observed on the bivalents of *P. kunmingensis* (Fig. 1D).

The C-banding patterns are complex in the species of *Neopanorpa* with heterochromatin in various positions (Figs 1E–H, S1). Completely heterochromatic bivalents were observed in *Neopanorpa* sp4, *N. lui* and *N. validipennis* (Figs 1F, S1K,N).

The species of *Cerapanorpa* also exhibit varied C-banding patterns (Figs 1I and S2A).

Furcatopanorpa longihypovalva is well characterized by large heterochromatic blocks occupying most of the chromosome length (Fig. 1K).

Phylogenetic analyses

Phylogenetic trees were reconstructed using an alignment of 1966 characters (564 for *cox1*, 552 for *cox2* and 850 for *28S* rRNA). The final matrix had 856 variable characters, among which 142 were uninformative and 714 were parsimony-informative. The parsimony analysis yielded 1316 most parsimonious trees (MPTs) of 5683 steps, with a consistency index (CI) of 0.23 and a retention index (RI) of 0.61.

The results of BI, ML and MP analyses produced trees of a similar general topology. Incongruence was found in the relationship of some *Neopanorpa* species, the positions of *C. dubia*, *C. protrudens*, *F. longihypovalva*, *P. biclada*, *P. bifasciata* and *P. rufostigma* (Figs 2 and 3). Moreover, the BI tree shows a high posterior probability supporting all the nodes. All panorpids join as a monophyletic group and can be categorized into two major clades (I and II) with MP bootstrap support (MPBS) = 100 (Fig. 2), ML bootstrap support (MLBS) = 99 and Bayesian posterior probability (PP) = 1 (Fig. 3).

Major Clade I comprises species of *Leptopanorpa* and *Neopanorpa* with two well-supported clades A and B (MPBS = 99, MLBS = 97, PP = 1). Clade A consists of *Leptopanorpa* and species of *Neopanorpa* with varied distributions (MPBS = 90, MLBS = 95, PP = 1), leading to the paraphyly of *Neopanorpa*. In contrast, Clade B comprises only the Himalaya-endemic species *N. chillcotti*.

Major Clade II is composed of *Cerapanorpa*, *Dicerapanorpa*, *Furcatopanorpa*, *Panorpa* and *Sinopanorpa* (MPBS = 46, MLBS = 100, PP = 1) and can be grouped into five clades (C–G). Clade C corresponds to *Dicerapanorpa* with high support values (MPBS = 100, MLBS = 100, PP = 1).

Clade D is split into two distinct groups (MPBS = 21, MLBS = 62, PP = 0.98). In the first group, the North American species of *Panorpa* constitute a monophyletic clade (MPBS = 49, MLBS = 66, PP = 0.82), including *P. acuta*, *P. maculosa*, *P. helena*, *P. nebulosa* and *P. speciosa*. In the second group, although the genus *Sinopanorpa* is monophyletic (MPBS = 100, MLBS = 100, PP = 1), the constituent species are nested within the species of *Panorpa*.

In Clade E, *Cerapanorpa* species from central China constitute a monophyletic group (MPBS = 47, MLBS = 82, PP = 1), which is a sister taxon to several

species of *Panorpa* with similar distributions. Strangely, two species of *Cerapanorpa sensu* Gao et al. (2016) (*C. acutipennis* and *C. fulvicaudaria*) from north-eastern Asia form a lineage, which is a sister taxon to species of *Panorpa* and other species of *Cerapanorpa*, indicating that *Cerapanorpa* is also a paraphyletic group.

Clade F comprises five European species (*P. rufostigma*, *P. communis*, *P. vulgaris*, *P. cognata* and *P. germanica*) and one north-eastern Asian species (*P. changbaishana*). It is noteworthy that this group is monophyletic from the ML and BI analyses (MLBS = 75, PP = 0.77), but paraphyletic from the MP analysis (Fig. 2 and 3).

Clade G is composed of the East Asian species (MPBS = 53, MLBS = 65, PP = 0.78). In this clade, *Furcatopanorpa longihypovalva* is a sister taxon to four south-western Chinese species of *Panorpa* from the MP analysis (MPBS = 52), but is the sister taxon to five north-eastern Asian species of *Panorpa* from the ML and BI analyses (MLBS = 65, PP = 0.74).

Divergence time

The divergence time chronogram (Fig. 4) placed the origin of Panorpidae at a mean age of 122.5 Ma (95% highest posterior density interval, HPD = 96.8–149.3 Ma). The estimated divergence time is ca.

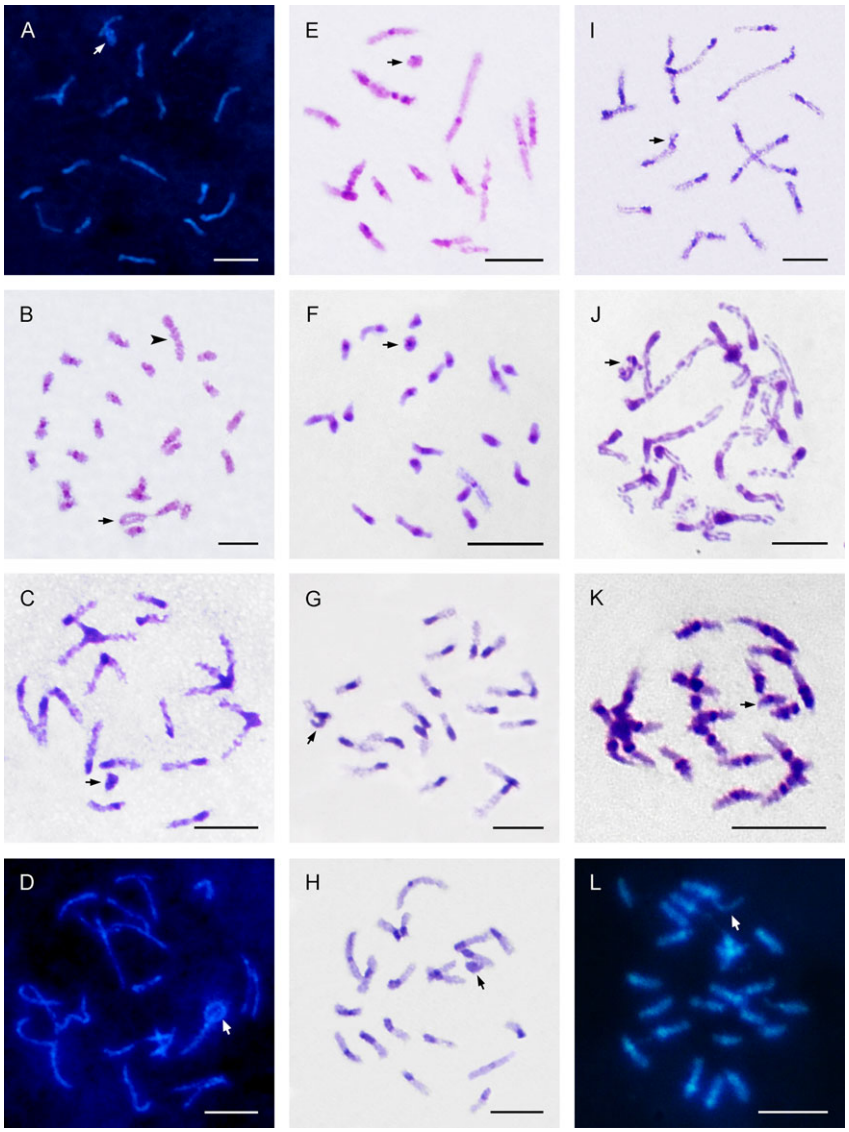

Fig. 1. Bivalent spreading of Panorpidae stained with Giemsa (B, C, E–K) and DAPI (A, D, L). A. *P. bashanicola* B. *P. fulvastra*. C. *P. biclada*. D. *P. kunmingensis*. E. *N. lipingensis*. F. *N. lui*. G. *N. quadristigma*. H. *N. malaisei*. I. *C. reni*. J. *D. magna*. K. *F. longihypovalva*. L. *S. nangongshana*. Arrows show the sex chromosome (X); arrowhead shows the B chromosome. Scale bars = 10 μm.

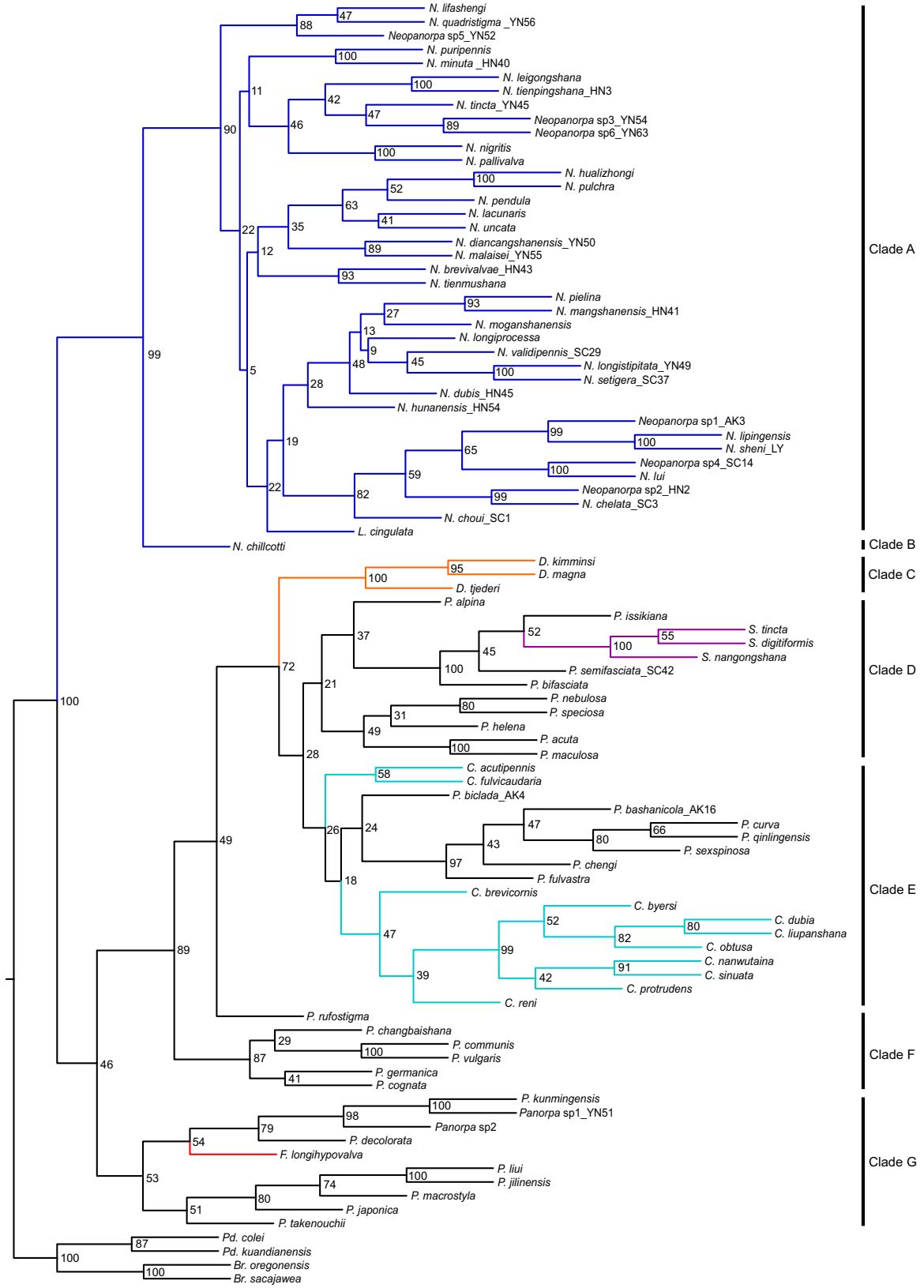

Fig. 2. Strict consensus tree of 1316 most parsimonious trees based on combined data of *cox1*, *cox2* and *28S* rRNA. Parsimony bootstrap values are given at internal nodes.

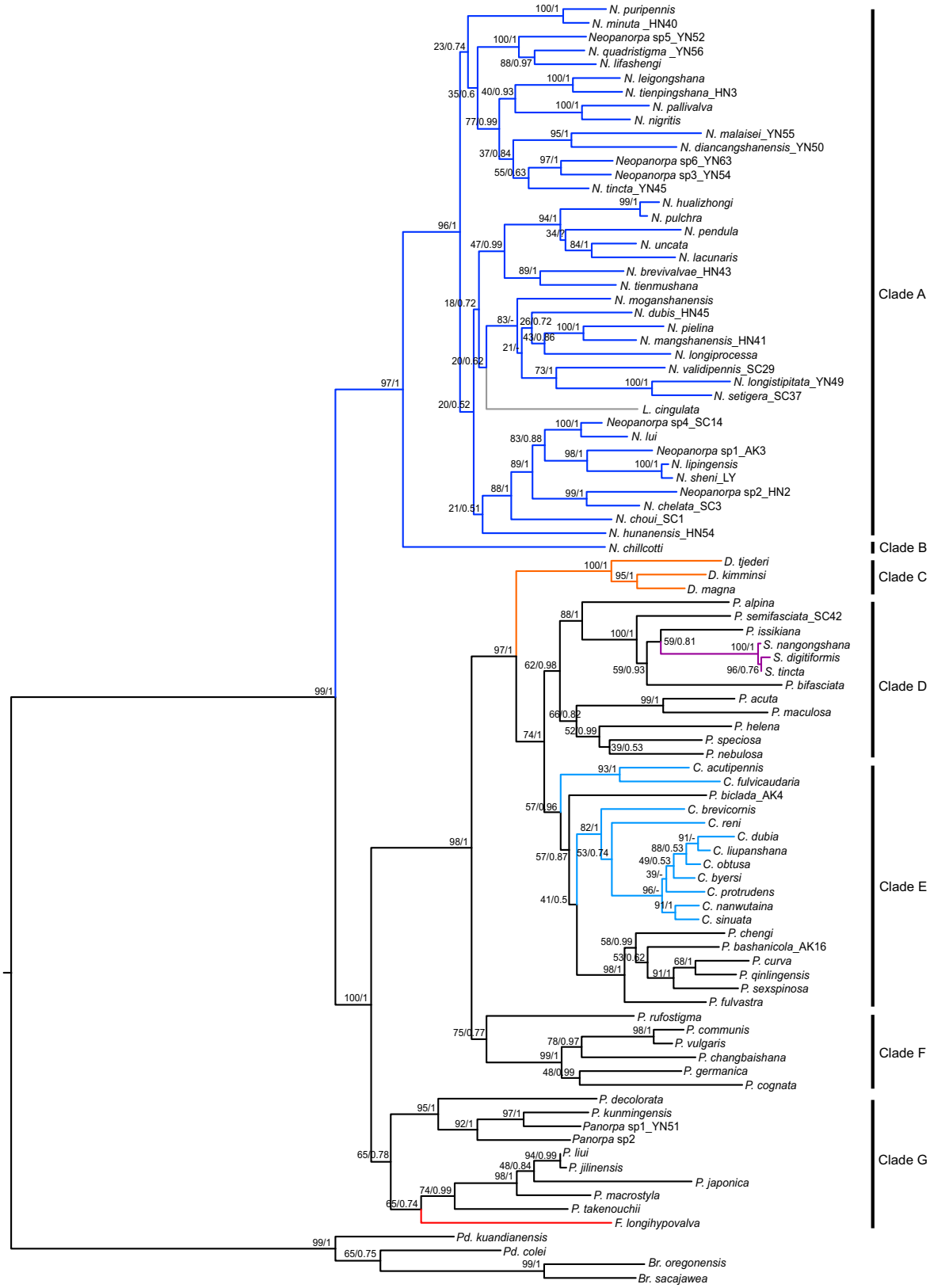

Fig. 3. Phylogenetic tree obtained from maximum likelihood (ML) analysis based on the combined dataset. ML bootstrap values and Bayesian posterior probabilities are indicated at internal nodes. "-" indicates that the positions of *N. mongshanensis*, *C. protrudens* and *C. dubia* in the Bayesian inference (BI) tree are slightly different from those in the ML tree; "?" indicates an unresolved node by BI.

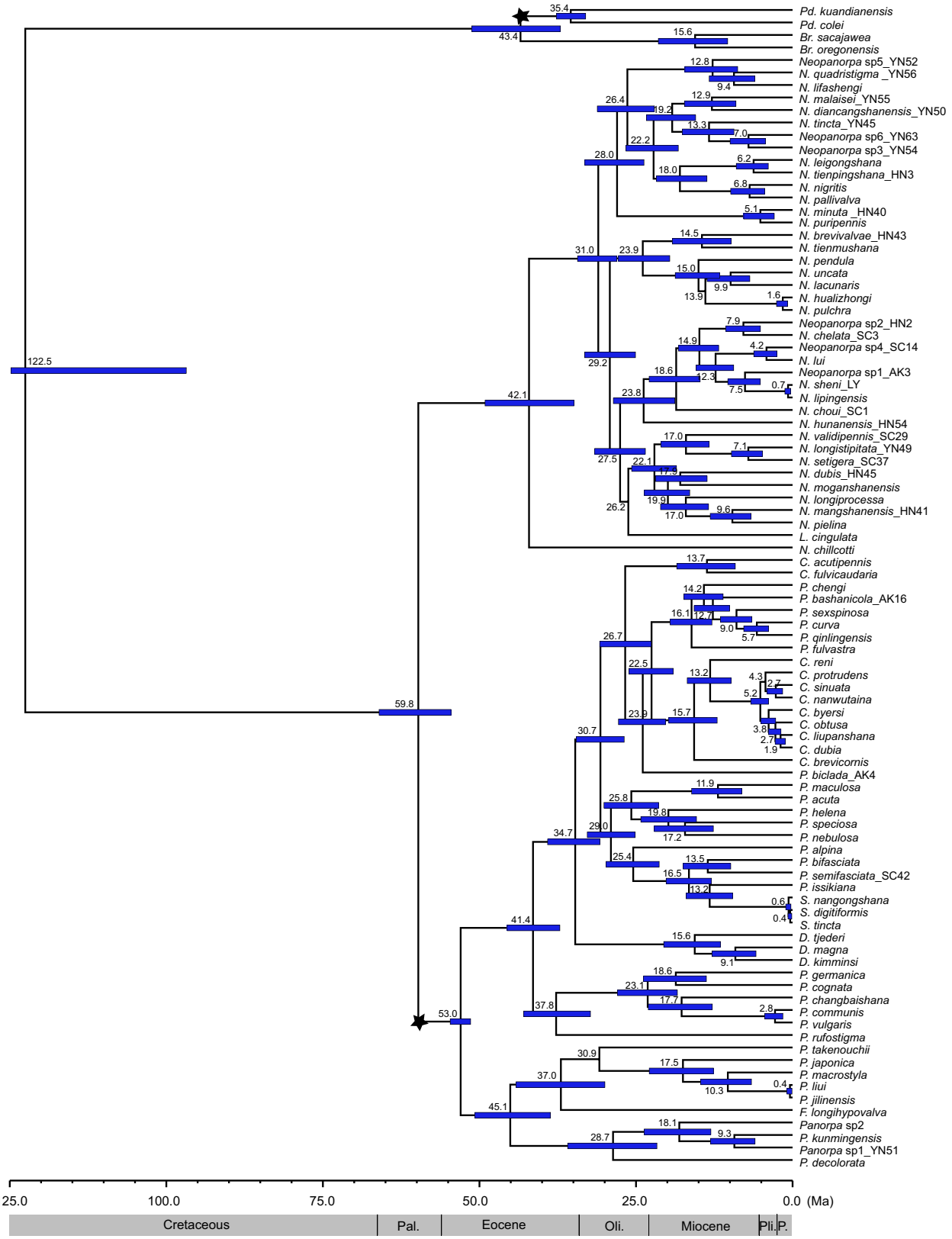

Fig. 4. Bayesian maximum clade credibility tree using a relaxed lognormal clock. Node numbers indicate the mean estimated divergence times of Panorpidae million years ago. Horizontal blue bars at nodes represent 95% highest posterior density date ranges. Black stars show the fossil calibration points. Pal., Palaeocene; Oli., Oligocene; Pli., Pliocene; P., Pleistocene.

59.8 Ma (95% HPD = 54.5–66.0 Ma) between the two major clades (I and II). In Clade I, *N. chillcotti* is the first species to diverge at 42.1 Ma (95% HPD = 34.9–49.1 Ma), while *N. lipingensis* and *N. sheni* are the most recently evolved species, which have the lowest number of chromosomes and diverged in the Middle Pleistocene at 0.7 Ma (95% HPD = 0.3–1.2 Ma).

Clade II began to diversify at 53.0 Ma (95% HPD = 51.4–54.7 Ma), consistent with the divergence time of *Panorpa*. In Clade II, *Furcatopanorpa* split from *Panorpa* at 37.0 Ma (95% HPD = 30.0–44.1 Ma). *Dicerapanorpa* separated from *Panorpa* at 34.7 Ma (95% HPD = 30.8–39.1 Ma). *Sinopanorpa* has a relatively recent origin at 13.2 Ma (95% HPD = 9.6–17.0 Ma). The north-eastern Asian lineage of *Cerapanorpa* diverged from *Panorpa* at 26.7 Ma (95% HPD = 22.6–30.8 Ma), slightly earlier than the origin of the central Chinese lineage.

Ancestral reconstruction of chromosome numbers

The chromosome numbers for the sampling species are listed in Table 1, in which the cytogenetic data of 45 species are documented for the first time. All the data are superimposed on the BI tree using the MP and ML analyses in Mesquite (Fig. 5A) and MLE and BI in chromEvol (Fig. 5B). Chromosome evolution was inferred from the BI consensus tree, because the support values in the BI tree were generally higher than those in the ML topology. Reconstruction of the optimal ancestral number of chromosomes through MLE is confirmed by other analyses (Fig. 5) except for some nodes that are equivocal under the ML model in Mesquite.

The best-supported model recovered in chromEvol, according to AIC, is Constant Gain and Loss without Duplication (Table S2). The rate parameters estimated are 4.90 for loss (δ) and 0.37 for gain (λ). The total inferred chromosome loss events are 20.59, gain 2.30, duplication 4.40×10^{-17} and demi-duplication 0. These results reveal that the main events inferred are fusion (loss) and fission (gain), and suggest that a stepwise increase or decrease in the original chromosome number is likely to be the principal mode during chromosome evolution of Panorpidae.

The ancestral chromosome number is $n = 23$ (ML probability, P = 0.59; Bayesian posterior probability, PP = 0.94) for Panorpidae under both methods, because it is the case for *Panorpa* (P = 0.98; PP = 0.98), *Sinopanorpa* (P = 1; PP = 0.99) and *Dicerapanorpa* (the ML analysis did not suggest any ancestral number at this point owing to a lack of data; PP = 0.98). The ancestral number remains $n = 23$ (P = 1; PP = 0.99) in the branch giving rise to the north-eastern Asian lineage of *Cerapanorpa* and then reduced to $n = 22$ (P = 0.99; PP = 0.99) by losing a chromosome in the central Chinese lineage.

Based on the MLE and BI analyses, the ancestral number becomes $n = 22$ (PP = 0.47) on the branch leading to Major Clade I comprising *Leptopanorpa* and *Neopanorpa*, then changes to $n = 21$ (P = 1; PP = 0.98) as the most common ancestor of the non-Himalayan species (Fig. 5B). Alternatively, a direct change from the ancestral $n = 23$ to $n = 21$ (P = 0.94) is feasible in this major clade from the ML analysis (Fig. 5A). Finally, there is an independent transition to $n = 17$ in the lineage of *N. lipingensis* + *N. sheni*.

Discussion

Chromosomal evolution

The species of Panorpidae show a series of chromosome numbers ranging progressively from $n = 17$ to $n = 24$, probably providing a strong phylogenetic signal in the evolution of Panorpidae. Based on our chromEvol analysis, $n = 23$ is inferred as the ancestral haploid number for Panorpidae. From this ancestral state, the increase or decrease in chromosome number progressed independently in the two major clades. In the first clade (*Leptopanorpa* + *Neopanorpa*), after a reduction to $n = 22$, the haploid number was further reduced to $n = 21$ probably prior to the diversification of this clade, and eventually to $n = 17$ (in *N. lipingensis* and *N. sheni*).

In the second major clade, loss events resulted in reductions from the ancestral $n = 23$ in *Dicerapanorpa*, *Panorpa* and *Sinopanorpa* to the derived $n = 22$ in *Cerapanorpa* and $n = 21$ in *Furcatopanorpa*. In *Panorpa*, from the most frequent number $n = 23$, losses of chromosomes progressed via $n = 22$ (in *P. cognata* and *P. decolorata*) to $n = 21$ (in *P. germanica*) and eventually to $n = 18$ (in *P. bashanicola*). By contrast, gain of a chromosome was responsible for the increase from the ancestral $n = 23$ to $n = 24$ in *P. kunmingensis*. Therefore, the extensive diversity of chromosome numbers in Panorpidae has arisen predominantly via loss events. The reduced chromosome numbers are characteristics of a monophyletic clade or a single species, suggesting that the decrease in chromosome number plays a significant role in the lineage differentiation seen in Panorpidae, as in the vascular plant *Phyteuma* (Schneeweiss et al., 2013), the frog *Pseudopaludicola* (Veiga-Menoncello et al., 2014) and the wood white butterfly *Leptidea* (Šíchová et al., 2015).

The stepwise decrease in chromosome numbers is a consequence of centric fusion by a Robertsonian translocation, insertional/nested fusion or complex mechanisms in many organisms (Guerra, 2008; Lysák and Schubert, 2013; Schneeweiss et al., 2013). Fusion

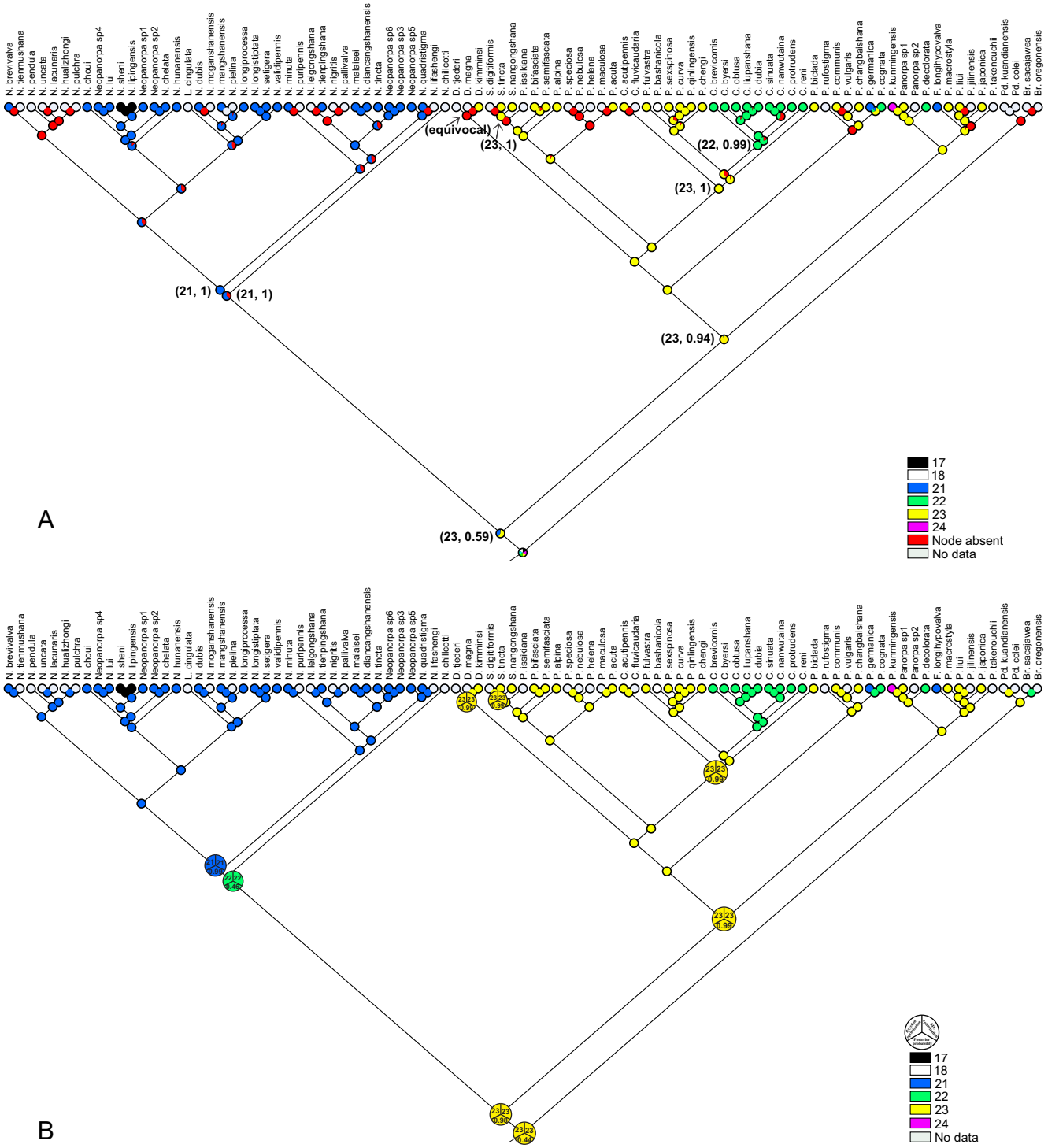

Fig. 5. Ancestral state reconstruction of chromosome numbers in Panorpidae, using Mesquite under the maximum parsimony (MP) and maximum likelihood (ML) models (A) and chromEvol under Bayesian and maximum likelihood estimation (MLE) optimizations (B). An absent node indicates that MP and ML model did not suggest any ancestral number at this node because of a lack of data. Numbers in parentheses at nodes indicate the inferred ancestral haploid chromosome number and the likelihood support values for each node. Pie charts at nodes show the inferred chromosome number in both approaches and the Bayesian posterior probabilities for each genus.

events alone could not explain the variation in chromosome numbers and banding patterns observed in the extant karyotypes of panorpids. The genera *Dicerapanorpa*, *Panorpa* and *Sinopanorpa* with $n = 23$ are characterized by gradient sizes of chromosomes and the uniform type of C-bands. The species of

Cerapanorpa ($n = 22$) also have chromosomes with gradually changed sizes, but exhibit varied C-banding patterns (Miao et al., 2017). *Furcatopanorpa* ($n = 21$) is characterized cytogenetically by large heterochromatic blocks, which may result from heterochromatin amplification following chromosome rearrangements and hybridization (Raskina et al., 2008). *Neopanorpa*, with $n = 21$ and 17, are represented by large bivalents with evident intermediate bands and complex C-banding patterns. These results suggest that there are a decrease in chromosome numbers and an increase in the variability of banding patterns during the karyological evolution of Panorpidae. The pronounced variations probably originated from a series of complex chromosome rearrangements, as suggested by Comings (1978) and Lönnig and Saedler (2002).

High frequencies of chromosome rearrangements, such as fusion, translocation and inversion, were detected in the species endemic to the Qinling–Bashan Mountains. These types of chromosome rearrangements have been proposed as important drivers of genetic differentiation through reproductive incompatibility or recombination suppression (Ayala and Coluzzi, 2005; Butlin, 2005; Brown and O'Neill, 2010; Faria and Navarro, 2010). Our BEAST analysis suggests that the extremely low chromosome number ($n = 17$ in *N. lipingensis* and *N. sheni*, and $n = 18$ in *P. bashanicola*) and the pronounced variation of banding patterns (in the closely related species of *Cerapanorpa*) occurred from the middle Miocene to the early Pleistocene (15.7−1.9 Ma) in the Cenozoic. During this era, these species experienced a rapid uplift of the Qinling–Bashan Mountains (Teng and Wang, 1996), a dramatic change in climate (Shi et al., 1998, 1999) and harsh environmental pressures, especially the succession of the Pleistocene glacial–interglacial cycles (Hewitt, 2000; Li et al., 2001; Provan and Bennett, 2008). This evidence suggests that the evolutionary divergence of these sibling species in Panorpidae is closely related to extreme changes in chromosome structure, which may interact with natural selection to promote speciation (Navarro and Barton, 2003; Ayala and Coluzzi, 2005; Butlin, 2005; Brown and O'Neill, 2010; Kirkpatrick, 2010).

Previous studies provided evidence for a correlation between karyotypic diversities and genetic differentiation among related species (Kandul et al., 2007; Talavera et al., 2013; Mills and Cook, 2014), demonstrating that chromosome rearrangements potentially contribute to lineage diversifications. Therefore, we considered that the remarkable variation in genome structure caused by elevated chromosome rearrangement frequencies may be critical for the Panorpidae to successfully adapt to their dramatically changing environments.

Phylogenetic relationships

The topology yielded in the present study is generally in accordance with the tree presented by Hu et al. (2015) except for some modifications, such as the interspecific relationship of *Cerapanorpa* and the position of *Furcatopanorpa*. The phylogenetic tree demonstrates that the species of Panorpidae fall into two distinct major clades: the first consists of *Leptopanorpa* and *Neopanorpa*, and the second comprises *Cerapanorpa*, *Dicerapanorpa*, *Furcatopanorpa*, *Panorpa* and *Sinopanorpa*.

The first clade is characterized cytogenetically by haploid chromosome numbers ≤ 21 and varied banding types, and morphologically by vein 1A joining the hind margin of wings before the origin of the radial sector (Rs) (Cheng, 1957). In contrast, the second clade is represented by haploid chromosome numbers ≥ 21 (with the exception of *P. bashanicola*), a uniform banding pattern and vein 1A joining the hind margin at or far beyond the origin of Rs (Cheng, 1957; Cai et al., 2008). The results provide cytological evidence to support the split of Panorpidae into two major clades (I and II), consistent with the previous result (Hu et al., 2015).

As far as we know, this is the first attempt to include a representative species of *Leptopanorpa* in the molecular phylogenetic analysis of Panorpidae. The Indonesian endemic *Leptopanorpa* is characterized by an extremely elongated abdomen and unique aedeagus in the males (Lieftinck, 1936; Byers, 1971). *Leptopanorpa* was considered to be closely related to *Neopanorpa* due to the pattern of wing venation, the presence of a notal organ and the structure of female genitalia (Lieftinck, 1936; Chau and Byers, 1978). Morphological analyses supported the close relationship of these two genera and suggested that *Neopanorpa* is paraphyletic with *Leptopanorpa* (Willmann, 1989; Ma et al., 2012). Our phylogenetic analysis found that *L. cingulata* is nested in *Neopanorpa*, indicating that *Neopanorpa* needs to be systematically revised.

Neopanorpa chillcotti, endemic to the Himalayan area, also bears a greatly elongated abdomen in the males (Wang and Hua, 2017b). Unlike the species of *Leptopanorpa*, *N. chillcotti* has a wide wing base and robust abdominal segments. Although being assigned to *Neopanorpa* (Byers, 1971), *N. chillcotti* is distinguished by its wing venation, male genitalia and female genital plates (Wang and Hua, 2017b). Our phylogenetic analyses provide strong support for the position of *N. chillcotti* as a basal sister lineage to *Neopanorpa* + *Leptopanorpa*, suggesting that *N. chillcotti* and related species are very likely to be genetically separated from *Neopanorpa*.

Based on our present phylogenetic analyses, the species of *Cerapanorpa* failed to form a monophyletic group on the trees. The north-eastern Asian species

(*C. fulvicaudaria* and *C. acutipennis*) form a monophyletic group, which is sister to the cluster of all the central Chinese species of *Cerapanorpa* and *Panorpa* rather than solely to the rest of *Cerapanorpa*, implying that the component species of *Cerapanorpa* need to be revised. The north-eastern Asian species of *Cerapanorpa* have their wings held roof-like over the abdomen at rest, and aedeagus with aedeagal hamulus and bilobed dorsal valves (Issiki, 1929; Hua, 1998), differing from the flat V-shaped wings and simple dorsal valves in the central Chinese species. Gao et al. (2016) transferred the north-eastern Asian species to *Cerapanorpa* based mainly on the presence of a single digitate anal horn on the posterior margin of tergum VI in the males. However, the chromosome number of the north-eastern Asian *C. acutipennis* is $n = 23$, different from $n = 22$ of its congeners from central China (Miao et al., 2017). Both the phylogenetic analysis and the karyological evidence suggest that the north-eastern Asian species are not suitable to be assigned to *Cerapanorpa*, and therefore should be removed back to *Panorpa*. Our analyses show that the central Chinese species of *Cerapanorpa* are clustered into a monophyletic clade, reconfirming the monophyly of this group as proposed by Ma et al. (2012) and Hu et al. (2015). A taxonomic revision and cytogenetic and phylogenetic analyses including additional Japanese and Korean taxa may help to clarify the relationship of these two lineages, and probably help to show that the several north-eastern Asian species assigned to *Cerapanorpa* by Gao et al. (2016) should be excluded from the genus.

In the previous phylogenetic analysis (Hu et al., 2015), the position of *F. longihypovalva* seemed strange forming a sister relationship merely with *P. liui*. In further analysis of DNA sequencing data, we found that a few odd results were in fact mistake made by confusing the *28S* rRNA gene sequences of *F. longihypovalva* and *P. liui* and misusing the *cox2* sequence of *F. longihypovalva* for *P. liui*. Having corrected this mistake, our present phylogenetic results indicate that *F. longihypovalva* is the sister taxon to the south-western Chinese species of *Panorpa* (the MP analysis) or the sister taxon to the north-eastern Asian species of *Panorpa* (the ML and BI analyses). The relationship of *F. longihypovalva* to the south-western Chinese species is supported by the non-constriction and non-elongation of abdominal segments VII and VIII in the males, and the wings held roof-like over the abdomen at rest (Issiki, 1933; Hua, 1998; Ma and Hua, 2011). *Furcatopanorpa* is characterized by a suite of autapomorphies, such as the bifurcate axis of the female medigynium, absence of the notal organ on male tergum III, the unique structure of male genitalia and atypical mating pattern (Ma and Hua, 2011; Zhong et al., 2015). Based on these characters, Ma et al. (2012) suggested a sister relationship between *Furcatopanorpa* and all other genera of Panorpidae. The derived cytogenetic characters of *Furcatopanorpa*, such as the low chromosome number and the specific banding pattern, suggest that multiplied chromosome rearrangements may contribute to the particular status of *Furcatopanorpa*.

Conclusions

In the present study, we used more representatives in all genera of Panorpidae to reconstruct the phylogeny and improved the resolution of the phylogram proposed by Hu et al. (2015). *Neopanorpa* is confirmed as paraphyletic with *Leptopanorpa* for the first time. *Cerapanorpa* is also paraphyletic and needs further revision. The particular status of *Furcatopanorpa* is attributed to frequent chromosome rearrangements. The fossil-calibrated molecular dating suggests that Panorpidae probably originated in the Lower Cretaceous, and most diversification in Panorpidae occurred in the Cenozoic. The reconstruction of ancestral chromosome numbers provides evidence for the significant role of changes in chromosome number in lineage differentiation and reveals that cytogenetic data can provide informative characteristics for the phylogenetic analyses of Panorpidae.

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Conflict of interest

The authors declare that they have no competing interests.

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Supporting Information

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

Fig. S1. Bivalent spreading of *Neopanorpa* stained with Giemsa (E, F, H, I, L, O) and DAPI (A–D, G, J, K, M, N, P). A. *N. brevivalvae*. B. *N. chelata*. C. *N. choui*. D. *N. dubis*. E. *N. hunanensis*. F. *N. longiprocessa*. G. *N. minuta*. H. *Neopanorpa* sp3. I. *N. sheni*. J. *N. tienpingshana*. K. *N. validipennis*. L. *Neopanorpa* sp1. M. *Neopanorpa* sp2. N. *Neopanorpa* sp4. O. *Neopanorpa* sp6. P. *N. longistipitata*. Scale bars = 10 μm.

Fig. S2. Bivalent spreading of *Cerapanorpa*, *Panorpa* and *Sinopanorpa* stained with Giemsa (A, C–E, H–K) and DAPI (B, F, G, L). A. *C. liupanshana*. B. *C. acutipennis*. C. *P. bifasciata*. D. *P. changbaishana*. E. *P. chengi*. F. *P. curva*. G. *P. decolorata*. H. *P. fulvastra*. I. *P. liui*. J. *P. qinlingensis*. K. *P. sexspinosa*. L. *S. tincta*. Scale bars = 10 μm.

Table S1. Species, locality, GenBank accession numbers, and source and/or references are presented only for the species with cytogenetic records. Families and genera are presented in bold for the sake of clarity.

Table S2. Log-likelihood and Akaike information criterion (AIC) score estimates for the dataset analysed by chromEvol.