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Phylogenetic analyses of the leafhopper tribe Chiasmini Distant, 1908 and delimination of species of the genus *Exitianus* Ball, 1929 (Hemiptera: Cicadellidae: Deltocephalinae: Chiasmini) in China based on molecular data

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Abstract. Previous phylogenetic analyses of the grass-specialist leafhopper tribe Chiasmini have resolved relationships among genera but have included few representatives of individual genera. Here the phylogeny of 20 Chinese species belonging to 8 chiasmine genera was investigated by combining DNA sequence data from two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*H3*, *28S*). In both maximum likelihood (ML) and Bayesian inference (BI) analyses, relationships among genera were largely consistent with prior analyses, with most members of the tribe placed into two sister clades: (*Exitianus* + *Nephotettix*) and the remaining five sampled genera. To examine morphology-based species definitions in the taxonomically difficult genus *Exitianus* Ball, 1929, one mitochondrial gene (*COI*) and one nuclear gene (*ITS2*) were used to infer the phylogenetic relationships and status of two common and widespread species and compare the performance of different molecular species-delimitation methods. These analyses divide the included populations into two well-supported clades corresponding to current morphological species concepts but some inconsistencies occurred under the jMOTU, ABGD and bPTP methods depending on the which gene and analytical parameter values were selected. Considering the variable results yielded by methods employing single loci, the BPP method, which combines data from multiple loci, may be more reliable in *Exitianus*.

Keywords. jMOTU, ABGD, bPTP, BPP, molecular phylogeny.

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Introduction

The grass-specialist leafhopper tribe Chiasmini Distant, 1908 (Hemiptera: Cicadellidae: Deltocephalinae) includes 324 species in 21 genera worldwide. Some of these species are important agricultural pests because of their ability to suck plant sap and spread plant pathogens. Adults and nymphs feed on phloem sap, resulting in inhibition of plant growth and development, yellowing of leaves and withering of the whole plant. Some species of *Nephotettix* Matsumura, 1902 transmit rice common dwarf disease, verticillium wilt disease and yellow dwarf disease, which can cause substantial economic and yield losses (Ruan 1985). Species of *Exitianus* Ball, 1929 are important vectors of corn stunt (Carloni *et al*. 2011), Napier grass stunt disease (Arocha *et al*. 2009), and Bermuda grass white leaf phytoplasma (Salehi *et al*. 2009).

The taxonomic status and scope of Chiasmini has changed substantially since the tribe was established by Distant (1908) as Chiasmusaria Distant, 1908, one of the eleven tribes of Jassidae Amyot & Audinet-Serville, 1843. Emeljanov (1962) established Doraturini Emeljanov, 1962, which was assigned to Deltocephalinae Fieber, 1869. Hamilton (1975) treated Chiasmusaria as a synonym of Eupelicini Sahlberg, 1871 (Eupelecinae Sahlberg, 1871) and assigned Doraturina Sahlberg, 1871 as a subtribe of Aphrodini Haupt, 1927 (Aphrodinae Haupt, 1927). Oman, Knight & Nielson (1990) included both Chiasmusini Distant, 1908 and Doraturini Emeljanov, 1962 in Deltocephalinae. Zahniser & Hicks (2007) considered Chiasmusini and Doraturini to be synonyms, with Chiasmini having priority. Zahniser (2008, 2010) accurately defined the scope of the tribe using phylogenetic criteria and identified morphological synapomorphies uniting the group.

Kuoh (1966) was the first to study this tribe in China, recording one genus and three species. Zhang (1990) recorded five species in two genera. Duan *et al*. (2009) reviewed the subgenus *Leofa* (*Prasutagus*) Distant, 1918 and described two new species from China. Duan & Zhang (2012a) recorded four species of *Doratura* Sahlberg, 1871 from China for the first time. Duan & Zhang (2012b) redescribed the genus *Doraturopsis* Lindberg,1935 and described the new genus *Zahniserius* Duan & Zhang, 2012 and species *Zahniserius cylindricus* Duan & Zhang, 2012 from China. Duan & Zhang (2012c) reviewed six species of the genus *Aconurella* Ribaut, 1948 from China including two new records and two new species. Duan & Zhang (2012d) reviewed the genus *Gurawa* Distant, 1908 and described a new species *Gurawa truncata* Duan & Zhang, 2012. At the same time, the genus *Chiasmus* Mulsant & Rey, 1855 was also recorded from China for the first time. Duan & Zhang (2013a) established the first records for the genus *Aconura* Lethierry, 1876 and species *Aconura ochrargentea* Emeljanov, 1972 from China. Duan & Zhang (2013b) reviewed the genus *Exitianus* from China. Duan & Zhang (2014) reviewed the species of *Nephotettix* from the Chinese mainland, describing and illustrating variation in structure of the male pygofer spines and aedeagus. At present, a total of 11 genera and 39 species of Chiasmini have been recorded in China, but 13 species records are doubtful due to lack of specimens (see checklist in Appendix 1).

The traditional classification of genera and species of Chiasmini was mainly based on external morphological characteristics. Singh-Pruthi (1925) first showed that the morphology of the male genitalia is useful for classification, thus, the identification of species has increasingly become dependent on

examination of the male terminalia. More recently, combinations of morphological and molecular data have been used to estimate phylogenetic relationships and delimit species. Ross (1968) published a phylogeny of *Exitianus* based on an intuitive assessment of morphological variation in this genus.

Fang *et al*. (1993) used *16S rDNA* gene sequences to study the phylogeny of New World *Deltocephalus*-like leafhopper genera, including *Exitianus exitiosus* Uhler, 1880 of Chiasmini. Kamitani (1999) included several genera now placed in Chiasmini in his phylogeny of Deltocephalini Fieber, 1869 and Paralimnini Distant, 1908. Comprehensive phylogenetic analyses of Deltocephalinae based on morphology (Zahniser & Dietrich 2008) and combined molecular and morphological data (Zahniser & Dietrich 2010, 2013) grouped Chiasmini and most other grass-specializing tribes of Deltocephalinae into a single large clade, and supported the monophyly of Chiasmini. However, a subsequent, more detailed analysis of relationships within the tribe based on DNA sequences placed the genera of Chiasmini in two large lineages but failed to support the monophyly of the tribe as a whole (Zahniser & Dietrich 2015). A few subsequent phylogenetic analyses have included very few species of Chiasmini (Song *et al*. 2017; Gao *et al*. 2021;Wu *et al*. 2022; Yan *et al*. 2022). Most recently, Cao *et al*. (2022) analyzed a dataset comprising 730 terminal taxa and > 160 000 nucleotide positions obtained through anchored hybrid enrichment to produce detailed, comprehensive phylogenetic estimates for Deltocephalinae. This analysis strongly supported Chiasmini as monophyletic, in contrast to previous analyses in which the two main lineages of this group (one comprising the macropterous genera *Nephotettix* and *Exitianus*, the other comprising the remaining, mostly brachypterous genera) did not consistently group together. Stenometopiini Baker, 1923 was recovered as sister group of Chiasmini in the coalescent gene tree analysis but concatenated maximum likelihood analyses suggested that the sister group of Chiasmini includes Stenometopiini plus three other grass-specialist tribes.

Relationships among major lineages of Chiasmini are well understood, thanks to these prior analyses, but relationships among species within single genera remain poorly explored. The species-level taxonomy continues to be based on very few characters of the male genitalia and many species have been recognized based on relatively minor differences. Thus, more detailed phylogenetic studies, including multiple species within genera and multiple populations within species, are needed to elucidate relationships among species and test the stability of morphological characters traditionally used to distinguish species. Few Chinese species of Chiasmini have been incorporated into prior phylogenetic analyses. Phylogenetic analyses, coupled with molecular-based species delimitation methods, are needed to provide a more reliable species-level classification of this group (Fujisawa *et al*. 2013; Huang *et al*. 2013; Zhang *et al*. 2013; Solís-Lemus *et al*. 2015; Yang 2015).

Among the most widespread and abundant tropical and temperate species of grassland leafhoppers are the moderately large fully winged forms comprising the genus *Exitianus*. *Exitianus* was described by Ball (1929) for *Cicadula obscurinervis* Stål. Ross (1968) published a phylogeny of *Exitianus* based on an intuitive assessment of morphological variation in this genus and revised the genus. Fang *et al.* (1993) used 16S rDNA gene sequences to study the phylogeny of New World *Deltocephalus*-like leafhopper genera, including *Exitianus exitiosus* Uhler, 1880. Subsequently, Emeljanov (1999) moved *Exitianus* from Athysanini Van Duzee, 1892 into Doraturini. Duan & Zhang (2013b) reviewed for the first time the species of *Exitianus* from China, and showed that the two widespread Chinese species are more variable in the male genitalia than suggested by Ross. Worldwide, this genus contains 43 species of which 6 species occur in Asia but only a few species appear to be relatively widespread.

In this study, the phylogenetic relationships of 8 genera and 20 species of Chiasmini collected in China are inferred based on two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*H3*, *28S*). In addition, two widespread and morphologically similar species, *Exitianus indicus* Distant, 1908 and *E. nanus* (Distant, 1908), were selected for more detailed study using molecular species delimitation methods to

determine whether the current morphology-based definitions of these species is reliable. Based on one mitochondrial gene (*COI*) and one nuclear gene (*ITS2*), three single-gene species delimitation methods (jMOTU, ABGD, bPTP) and one multi-gene species division method (BPP) were tested to determine whether the inferred species composition matches current morphological species concepts. The goals of this study are: (i) analyse the phylogenetic relationships of Chinese genera and species of this tribe; (ii) evaluate the validity of the current classification; and (iii) use molecular methods to test the validity of current species concepts.

Material and methods

Taxa sampling and species identification

Leafhopper samples included in this study were collected between 2010 and 2019 from various locations across China. 20 representative species belonging to 8 genera of Chiasmini were selected for the phylogenetic study of this tribe. 41 specimens of two species of *Exitianus* sampled from across their known range in China were selected for DNA barcoding and molecular delimitation of species. Specimens were collected directly into 95% or 100% ethanol and stored at -80°C prior to study. Identification of each individual was based on examination of external morphology and male genitalia of the adult using an Olympus SZX10 stereoscopic microscope (Olympus Corporation, Tokyo, Japan) using keys provided by Duan & Zhang (2014). All specimens used in this study are deposited at Northwest A&F University, Yangling, China. The taxa included in the phylogenetic analyses listed in Table 1, and maps of collecting localities are shown in Fig. 1. All *Exitianus* specimens used for molecular identification

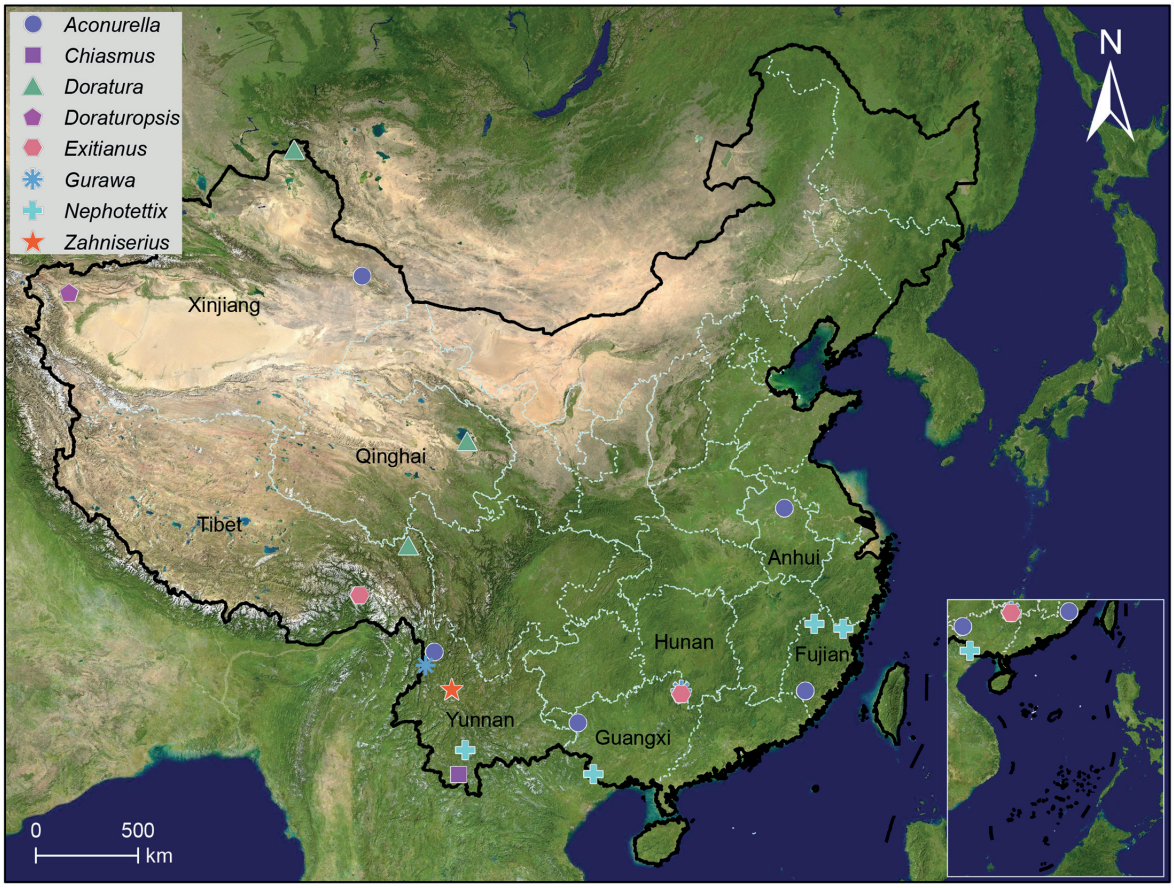

Fig. 1. Distribution of sampled specimens of Chiasmini Distant, 1908 in China. Map generated by ArcMap (https://desktop.arcgis.com/zh-cn/desktop/).

Table 1. Specimens of Chiasmini Distant, 1908 used in this study (all from China), with morphological identifications, collecting localities and GenBank accession numbers.

tribe	species	voucher number	locality	GenBank accession number				reference
				COI	*16S*	*H3*	*28S*	
Chiasmini Distant, 1908	*Aconurella diplachnis* Emeljanov, 1964	Hm086373	China: Xinjiang	OL985767	OL985682	OL989954	OL985748	this study
	Aconurella furcata Duan & Zhang, 2012	Hm084045	China: Yunnan	OL985768	OL985683	OL989955	OL985749	this study
	Aconurella montana (Distant, 1908)	Hm087348	China: Guangxi	OL985769	OL985684	–	OL985750	this study
	Aconurella prolixa (Lethierry, 1885)	Hm080617	China: Fujian	OL985770	OL985686	OL989956	OL985751	this study
	Aconurella sibirica Lethierry, 1888)	Hm083152	China: Anhui	OL985771	OL985685	OL989957	OL985752	this study
	Chiasmus sp.	Hm088752	China: Yunnan	OL985772	OL985692	OL989963	OL985758	this study
	Doratura homophyla (Flor, 1861)	Hm086296	China: Xinjiang	OL985775	OL985690	OL989961	OL985756	this study
	Doratura gravis Emeljanov, 1966	Hm086974	China: Qinghai	OL985774	OL985689	OL989960	OL985755	this study
	Doratura stylata (Boheman, 1847)	Hm087339	China: Tibet	–	OL985691	OL989962	OL985757	this study
	Doraturopsis (*Doraturopsis*) *heros* (Melichar, 1902)	Hm082652	China: Xinjiang	OL985794	OL985693	OL989967	OL985759	this study
	Exitianus indicus (Distant, 1908)	Hm087218	China:Hunan	OL985776	OL985687	OL989958	OL985753	this study
	Exitianus nanus (Distant, 1908)	Hm068610	China: Tibet	OL985777	OL985688	OL989959	OL985754	this study
	Gurawa minorcephala Singh-Pruthi, 1930	Hm086936	China: Guangxi	OL985792	OL985695	OL989966	OL985760	this study
	Gurawa truncata Duan & Zhang, 2012	Hm088748	China: Yunnan	OL985788	OL985697	OL989965	OL985761	this study
	Nephotettix cincticeps (Uhler, 1896)	Hm085943	China: Fujian	OL985783	OL985678	OL989950	OL985744	this study
	Nephotettix malayanus Ishihara & Kawase, 1968	Hm080580	China: Fujian	OL985784	OL985677	OL989949	OL985743	this study
	Nephotettix nigropictus (Stål, 1870)	Hm087152	China: Guangxi	OL985785	OL985679	OL989951	OL985745	this study
	Nephotettix parvus Ishihara & Kawase, 1968	Hm086554	China: Yunnan	OL985786	OL985681	OL989953	OL985747	this study
	Nephotettix virescens (Distant, 1908)	Hm082984	China: Yunnan	OL985787	OL985680	OL989952	OL985746	this study
	Zahniserius cylindricus Duan & Zhang, 2012	Hm080183	China: Yunnan	OL985779	OL985698	OL989964	OL985762	this study
Stenometopiini Baker, 1923	*Doratulina* (*Doratulina*) *dmitrievi* Zahniser & Dietrich, 2013	DEL136	Zambia: Northwestern	–	–	KR230257	KR230124	Zahniser & Dietrich (2015)
	Doratulina undescr. sp.	DEL137	Zambia: Northwestern	–	–	KR230258	KR230125	Zahniser & Dietrich (2015)
	Stirellus bicolor (Van Duzee, 1892)	DEL008	Mexico: Jalisco	–	–	KR230301	KR230147	Zahniser & Dietrich (2015)
	Stirellus sagittarius (Naudé, 1926)	DEL134	South Africa: KZN	–	–	KR230299	KR230145	Zahniser & Dietrich (2015)

Table 2. Specimens of *Exitianus* Ball, 1929 used in this study (all from China), with morphological identifications, collecting localities and GenBank accession numbers.

species	code	locality	GenBank accession number		reference
			COI	*ITS2*	
Exitianus indicus (Distant, 1908)	Hm086015	China: Jiangxi	–	OL989173	this study
	Hm086016	China: Jiangxi	–	OL989174	this study
	Hm086972	China: Yunnan	–	OL989175	this study
	Hm081999	China: Yunnan	–	OL989176	this study
	Hm082135	China: Yunnan	–	OL989177	this study
	Hm068694	China: Yunnan	–	OL989178	this study
	Hm081908	China: Yunnan	–	OL989179	this study
	Hm080722	China: Guangdong	OL958655	OL989180	this study
	Hm086995	China: Guangxi	OL958657	OL989181	this study
	Hm086197	China: Guizhou	–	OL989182	this study
	Hm081350	China: Hainan	–	OL989183	this study
	Hm086829	China: Hainan	–	OL989184	this study
	Hm081394	China: Hainan	–	OL989185	this study
	Hm081273	China: Hainan	–	OL989186	this study
	Hm087218	China: Hunan	OL958656	OL989187	this study
	Hm082247	China: Shanxi	–	OL989188	this study
	Hm080811	China: Fujian	OL958681	OL989189	this study
	Hm086943	China: Guangdong	OL958658	OL989190	this study
Exitianus nanus (Distant, 1908)	Hm068726	China: Hainan	–	OL989191	this study
	Hm068728	China: Hainan	OL958659	OL989192	this study
	Hm082784	China: Yunnan	OL958660	OL989193	this study
	Hm068682	China: Yunnan	OL958661	OL989194	this study
	Hm083115	China: Yunnan	OL958662	OL989195	this study
	Hm083130	China: Yunnan	OL958663	–	this study
	Hm082161	China: Yunnan	–	OL989196	this study
	Hm080328	China: Fujian	OL958664	OL989197	this study
	Hm086933	China: Guangxi	OL958665	OL989198	this study
	Hm081349	China: Hainan	OL958666	–	this study
	Hm086837	China: Hainan	–	OL989199	this study
	Hm081409	China: Hainan	OL958667	OL989200	this study
	Hm068688	China: Hainan	OL958668	OL989201	this study
	Hm081783	China: Hainan	OL958669	OL989202	this study
	Hm081814	China: Hainan	OL958670	–	this study
	Hm068612	China: Tibet	OL958671	OL989203	this study
	Hm068830	China: Yunnan	OL958672	–	this study
	Hm068684	China: Yunnan	OL958673	OL989204	this study
	Hm081980	China: Yunnan	OL958674	OL989205	this study
	Hm081766	China: Yunnan	OL958675	–	this study
	Hm080701	China: Yunnan	OL958676	–	this study
	Hm082190	China: Yunnan	OL958677	–	this study
	Hm068611	China: Tibet	OL958678	–	this study
Nephotettix nigropictus (Stål, 1870)	Hm080627	China: Fujian	MW429123	MW439111	Gao *et al.* (2021)
Nephotettix virescens (Distant, 1908)	Hm081320	China: Yunnan	MW429150	MW439145	Gao *et al.* (2021)

and phylogenetic analyses are listed in Table 2 and geographical distributions are illustrated in Fig. 2. Whenever possible, specimens representing different intraspecific morphological variants were selected. Because the most reliable morphological characters for distinguishing species are found in males, only male specimens were selected. Voucher specimens are deposited in the insect collection of the Anhui Agricultural University.

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from abdominal muscle of individual, non-parasitized adult male specimens using an EasyPure Genomic DNA Kit (EE101; Transgen, Beijing, China) following the manufacturer's standard protocol, except that 20 µl proteinase K was mixed with 100 µl buffer for overnight lysis at 56°C and the final elution volume was 60 µl due to small specimen size. After DNA extraction, the DNA solution was stored at -20°C for subsequent molecular experiments. The abdominal exoskeleton of each extracted individual was stored in glycerin in a micro vial as a morphological voucher specimen.

Standard PCR methods were used to amplify partial sequences of two mitochondrial genes (*COI*, *16S*) and three nuclear genes (*H3*, *ITS2*, *28S*). Primer sequences are shown in Table 3. The amount of template DNA was adjusted according to the DNA concentration and varied between 2 and 3 µl (Folmer *et al.* 1994; Simon *et al.* 1994; Colgan *et al.* 1998; Ji *et al.* 2003; Dietrich *et al.* 2001), combined with 12.5 µl

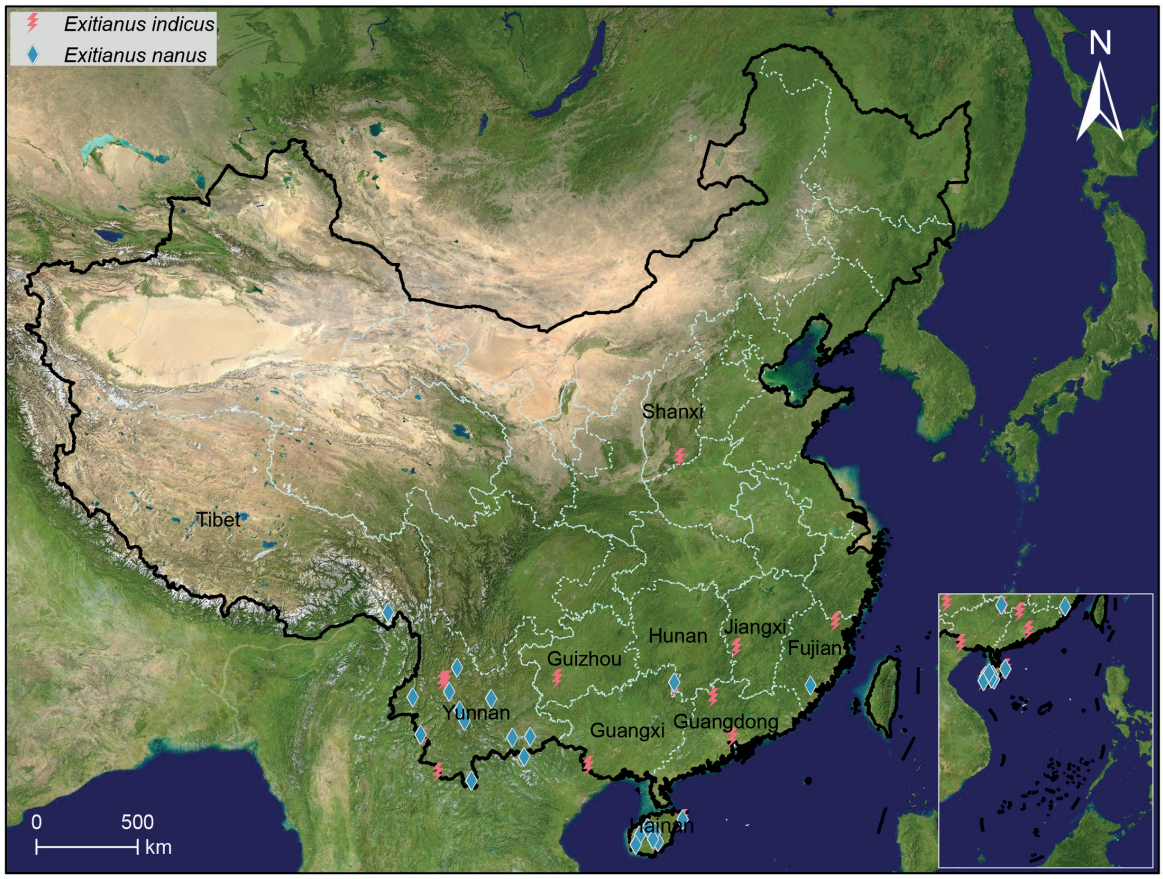

Fig. 2. Distribution of sampled specimens of *Exitianus* Ball, 1929 in China. Map generated by ArcMap (https://desktop.arcgis.com/zh-cn/desktop/).

Table 3. Primer sequences for PCR amplification and sequencing.

gene segment	primer name	primer sequence (5'-3')	reference
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.* (1994)
COI	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.* (1994)
16S	LR-J-12887	CCGGTCTGAACTCAGATCACGT	Simon *et al.* (1994)
16S	LR-N-13398	CGCCTGTTTAACAAAAACAT	Simon *et al.* (1994)
H3	H3AF	ATGGCTCGTACCAAGCAGACGGC	Colgan *et al.* (1998)
H3	H3AR	ATATCCTTGGGCATGATGGTGAC	Colgan *et al.* (1998)
ITS2	ITS2-F	TGAACATCGACATTTYGAACGCACAT	Ji *et al.* (2003)
ITS2	ITS2-R	TTCTTTTCCTCCSCTTAYTRATATGCTTAA	Ji *et al.* (2003)
28S D2	28SD2F	AGTCGKGTTGCTTGAKAGTGCAG	Dietrich *et al.* (2001)
28S D2	28SD2R	TTCGGGTCCCAACGTGTACG	Dietrich *et al.* (2001)

of 2 Taq MasterMix, 1μl each of forward and reverse primer, and ddH_2O added to make a total volume of 25 μl for each reaction.

The PCR conditions differed according to the gene and the specific primers, especially the annealing temperature, which was the most critical factor influencing product quality. Thermal cycling conditions for each gene were as follows: an initial denaturing step at 94°C for 5 min; 35 cycles of denaturing at 94°C for 1 min; annealing at 52, 54, 55, 56 and 52°C for 1 min for *COI*, *16S*, *H3*, *ITS2* and *28S* respectively; an extension at 72°C for 1 min; and a final extension step of 72°C for 10 min, and ending with incubation at 12°C. The PCR products were examined using 1% agarose gel electrophoresis with ethidium bromide stain to ensure the products were the target size. DNA products were subsequently sequenced in both directions by Qingke Biotech (Xi'an) Co., Ltd, using the original PCR primers.

Data analyses

To obtain single consensus sequences, chromatograms, including sense and antisense strands, were analyzed and assembled using Seqman software (Swindell & Plasterer 1997). In order to ensure that the correct target gene fragment was obtained, the Basic Local Alignment Search Tool (BLAST) was used to check all sequences against the NCBI database (Altschul *et al.* 1990). Concurrently, for the encoding gene fragments, MEGA 7 was used to translate the assembled contigs into amino acids to ensure that stop codons and pseudogenes did not exist (Kumar *et al.* 2016). Conserved sites (C), variable sites (V), parsimony-informative sites (PI), and the average nucleotide composition for each region were calculated by MEGA 7 (Kumar *et al.* 2016). Genetic diversity parameters including the haplotype number (H), haplotype diversity (Hd), and nucleotide diversity (Pi) were calculated by DNASP ver. 5.0 for our subsequent experiments (Librado & Rozas 2009). Before combining multiple genes to build trees, scatter plots of transitions/transversions of each gene were made by using DAMBE ver. 5.0 software (Xia 2013) to test for substitution saturation. A heuristic search in PAUP ver. 4.0b10 (Swofford 2002) was used to test for partition homogeneity of the six gene sequences, prior to combining them for analyses.

Phylogenetic analyses

Phylogenetic analyses of Chiasmini Distant, 1908

Based on the analysis of Zahniser & Dietrich (2015), four species from Stenometopiini (*Doratulina dmitrievi* Zahniser & Dietrich, 2013, *Doratulina* sp., *Stirellus bicolor* Van Duzee, 1892 and *Stirellus sagittarius* Naudé, 1926 were obtained from GenBank as outgroups (Table 1). Two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*H3*, *28S*) were combined to reconstruct phylogenetic relationships

within the tribe. Each of the two coding protein gene fragments (*COI* and *H3*) was aligned separately using the MAFFT plugin and G-INS-I algorithm in PhyloSuite, with gaps and ambiguous sites removed using Gblocks under default settings in PhyloSuite ver. 1.2.2 (Zhang *et al.* 2020). The ribosomal gene fragment (*16S* and *28S*) was aligned using the Q-INS-I method on the MAFFT ver. 7 alignment server (Katoh & Standley 2013). Alignments of individual genes were concatenated to generate one 24-taxa data set using PhyloSuite (Zhang *et al.* 2020). Phylogenetic reconstruction was conducted using Maximum likelihood (ML) and Bayesian inference (BI). The most suitable substitution models and partition scheme were determined for the combined dataset using PartitionFinder ver. 2.1.1 (Lanfear *et al.* 2017). The best-fitting model was selected for each partition with the model search 'all' and 'mrbayes' for ML and BI analyses, 'greedy' search algorithm, 'linked' to estimate branch lengths and using the Bayesian information criterion (BIC) (Table 4).

Maximum likelihood analysis was conducted using the IQ-TREE plugin in PhyloSuite ver. 1.2.2 (Zhang *et al.* 2020). Ultra-fast Boot (UFB) algorithm with 1000 repetitions (Minh *et al.* 2013) and the SH-aLRT test was used to assess branch support. Bayesian inference analysis was performed using the MrBayes plugin in PhyloSuite (Zhang *et al.* 2020). Two simultaneous runs of 5 000 000 generations were conducted for the matrix and trees were sampled every 1000 generations. Convergence and stability were evaluated in Tracer ver. 1.7 ensuring effective sample size > 200 for all parameters (Rambaut *et al.* 2018). With the first 25% of trees discarded as burn-in, Bayesian posterior probabilities were calculated for a 50% majority rule consensus tree of the remaining trees. Trees were visualized using FigTree ver. 1.4.3 (Rambaut 2016).

Phylogenetic analyses of *Exitianus* Ball, 1929

Based on the sister-group relationship of *Exitianus* and *Nephotettix* recovered by the analysis of Zahniser & Dietrich (2015), *Nephotettix virescens* Distant, 1908 and *Nephotettix nigropictus* Stål, 1870 were selected as the outgroup for analysis of Chinese populations of *Exitianus* (Table 2). The *Exitianus* phylogenetic tree was reconstructed by combining one mitochondrial gene (*COI*) and one nuclear gene (*ITS2*). Phylogenetic reconstruction was conducted using BI analysis and ML analysis. The analyses were run the same as described above for the broader analysis if the tribe, except that two simultaneous runs of 1 000 000 generations were conducted for the matrix in Bayesian inference analysis. The most suitable substitution models and partition scheme for the combined dataset are listed in Table 5.

Species delimitation

Three independent single loci species delimitation methods without a priori taxonomic information were performed: jMOTU (Jones *et al.* 2011), ABGD (Puillandre *et al.* 2012), and bPTP (Zhang *et al.* 2013). In addition, a species delimitation method that requires priori taxonomic information was used, and multiple loci were used to verify the above results using Bayesian coalescent method in the software BPP (Yang 2015).

jMOTU

According to the differences in genetic distance of sequences, the jMOTU divides sequences into different molecular operational taxonomic units (MOTUs). Whenever the genetic distance difference of the sequence is less than the specified threshold, it will be assigned into the same taxon. Before running jMOTU, we configured JAVA and set the paths of formatdb and megablast. The FASTA sequence was loaded into the software. Then based on the empirical sequence cut off values (1–30), the shortest sequence matching percentage (95%), and the lowest BLAST value (97%) were used for cluster analysis. The number of MOTUs was plotted against the threshold values to determine the number of species inferred from each value of the different distance thresholds (Jones *et al.* 2011).

Table 4. Best partitioning scheme and models for four genes selected to construct the phylogenetic tree of Chiasmini Distant, 1908 by the PartitionFinder.

subset partitions	models for IQ-TREE	models for MrBayes
P1: *COI*_pos1	TVM+I	F81+I
P2: *COI*_pos2	TRNEF+G	SYM+G
P3: *COI*_pos3	TVM+G	HKY+G
P4: *16S*	TVM+I+G	GTR+I+G
P5: *H3*_pos1	TVM+G	GTR+G
P6: *H3*_pos2	JC+I	JC+I
P7: *H3*_pos3, *28S*	TRN+I	GTR+I

Table 5. Best partitioning scheme and models for two genes selected to construct the phylogenetic tree of *Exitianus* Ball, 1929 by the PartitionFinder.

subset partitions	models for IQ-TREE	models for MrBayes
P1: *COI*_pos1	F81	F81
P2: *COI*_pos2, *ITS2*	TRNEF+G	SYM+G
P3: *H3*_pos3	K81UF+G	HKY+G

ABGD

Based on the assumption of a barcoding gap (i.e., intraspecific divergences are smaller than interspecific divergences), the ABGD procedure first sorts the data into hypothetical species, and then computes recursively based on previous groupings to obtain an optimal number of partitions. The graphical web version was used (http://www.abi.snv.jussieu.fr/public/abgd/abgdweb.html). The FASTA sequences were uploaded and the analysis used the following parameters: prior intraspecific divergence values (Pmin = 0.001 and Pmax = 0.1), relative gap width (X = 1.0) and the Kimura 2-P (K80) distance model.

bPTP

For the bPTP analysis, branch lengths represent the number of substitutions, not time, eliminating the problems associated with requiring a calibrated tree when a priori information on divergence time is not available. An ML tree for each gene was reconstructed using the IQ-TREE plugin in PhyloSuite ver. 1.2.2 (Zhang *et al.* 2020). The bPTP analysis was carried out on the bPTP server (https://species.h-its.org/) with the ML tree as input, specifying an outgroup, considering 100 000 MCMC generations, a thinning value of 100, and a burn-in of 10%.

BPP

For the BPP analysis, the BI tree constructed from the above two gene datasets (*COI*, *ITS2*) was selected as the guide tree for BPP analysis. The data for *N. virescens* and *N. nigropictus* for the BPP analysis was included because the statistical power of BPP can be increased when closely related outgroups are included (Rannala & Yang 2013). The program BPP 3.3. was used to validate the delimitation results generated by the above methods (Yang 2015). The analysis was run for 1 000 000 generations, with a sample frequency of 1000. The first 25% of trees were removed as burn-in. The convergence and stability were evaluated in Tracer ver. 1.7 ensuring an effective sample size > 200 for all parameters (Rambaut *et al.* 2018).

Table 6. C, V, PI, average nucleotide composition, haplotype number, Hd, and Pi of four genes of Chiasmini Distant, 1908 and two genes of *Exitianus* Ball, 1929.

gene	length (bp)	C	V	PI	S	T (%)	C (%)	A (%)	G (%)	A+T (%)	C+G (%)	H	Hd	Pi
COI	612	359	253	221	52	32.2	15.3	33.9	18.6	66.1	33.9	19	1.000	0.17258
16S	487	274	203	155	46	41.0	9.2	33.1	16.7	74.1	25.9	20	1.000	0.14246
H3	276	212	64	42	22	20.8	28.5	16.3	34.4	37.1	72.9	20	0.988	0.06736
28S	625	555	70	52	18	20.2	32.7	19.8	27.3	40.0	60.0	17	0.967	0.03167
COI	615	447	168	135	33	31.3	15.8	33.8	19.1	65.1	34.9	10	0.650	0.07450
ITS2	517	422	96	84	10	21.3	27.8	23.6	27.3	44.9	55.1	9	0.820	0.05859

Species delimitation in BPP requires a priori estimation of two evolutionarily significant parameters: ancestral population size (θ) and degree of divergence among species (τ). According to Yang's procedure, we used the following combinations: 1. Θ: G (2:1000), τ: G (2:2000); 2. Θ: G (2:100), τ: G (2:200); 3. Θ: G (2:100), τ: G (2:2000); 4. Θ: G (2:1000), and τ: G (2:200) (Yang 2015). All BPP analyses were run for 500 000 generations with sampling every five generations after discarding an initial burn-in of 20 000 generations. For verification, every analysis was run twice to check for convergence between runs and agreement on the posterior probability of the species delimitation models.

Results

Data analyses

Trimmed alignments of four genes (*COI*, *16S*, *H3*, *28S*) used for phylogenetic analysis of the Chinese Chiasmini were 612 bp, 487 bp, 276 bp, and 625 bp, respectively. For the analysis of *Exitianus*, one mitochondrial gene (*COI*) and one nuclear gene (*ITS2*) were used. Trimmed alignments for two genes were 615 bp and 517 bp, respectively. C, V, PI, average nucleotide composition, haplotype number, Hd, and Pi are listed in Table 6. Both included species are represented by multiple haplotypes for at least one locus.

Tests for substitutional saturation indicated that none of four genes have reached saturation and are appropriate for use in Chiasmini phylogenetic analysis. The ILD tests indicated that the different loci have phylogenetic signal sufficiently homogeneous to allow four gene fragments to be combined into a single concatenated alignment for analyses. Similar results were obtained for the two genes (*COI* and *ITS2*) used for analysis of *Exitianus*.

Phylogenetic analyses

Phylogenetic analyses of Chiasmini Distant, 1908

The phylogenies obtained from ML and BI analyses (Figs 3–4) are highly similar, differing only in the relationship among three species of *Doratura* that received low branch support in both analyses; most other branches had moderate to strong support by ML bootstrap and Bayesian posterior probabilities. All genera for which more than one representative was included are recovered as monophyletic and the ingroup topology is as follows: *Doraturopsis* + ((*Exitianus* + *Nephotettix*) + ((*Zahniserius* + (*Chiasmus* + *Gurawa*)) + (*Doratura* + *Aconurella*))). Within Chiasmini, the genus *Doraturopsis* was consistently sister to all other genera included Chiasmini. The ML and BI analyses grouped the remaining genera into two sister clades. In clade I, *Exitianus* and *Nephotettix* are sister groups with strong support (BS = 100, PP = 1) and relationships among species within each genus are well resolved with moderate to strong support (BS > 84, PP > 0.88). Clade II comprising the remaining genera also has strong support (BS = 92, PP = 1). The clade (*Zahniserius* + (*Chiasmus* + *Gurawa*)) is sister to *Doratura* and *Aconurella*.

The monophyly of *Aconurella*, *Doratura* and *Gurawa* are well supported but relationships among these genera received only low branch support. Relationships among *Aconurella* are: (*Aconurella prolixa* + ((*Aconurella sibirica* + *Aconurella diplachnis*) + (*Aconurella montana* + *Aconurella furcata*))). *Nephotettix* relationships are as follows: ((*Nephotettix virescens* + *Nephotettix nigropictus*) + (*Nephotettix malayanus* + (*Nephotettix parvus* + *Nephotettix cincticeps*))).

Molecular species delimitation analyses of *Exitianus* Ball, 1929

The ML and BI analysis grouped the ingroup taxa into two identical monophyletic clades with maximum support (BS = 100, PP = 1). All analyses consistently support the monophyly of the two species *E. indicus* and *E. nanus* (Fig. 5).

Species delimitation

jMOTU

For the *COI* dataset, sequence cut off values of 2–24, yielded three MOTUs with all individuals of *E. nanus* included in one MOTU, and *E. indicus* divided into two MOTUs. When the sequence cut off values are 25–30, only two MOTUs are recognized, corresponding to the morphological definitions of the two species. For the *ITS2* dataset, the MOTUs are identical to the morphological species when the sequence cut off values are 3–30 (Figs 6–7).

ABGD

For *COI*, the distance-based method ABGD recovers two molecular operational taxonomic units (MOTUs) corresponding to the morphologically defined species when the prior intraspecific divergence (P value) is 0.001624 to 0.100000. For *ITS2*, when the prior intraspecific divergence ranges from 0.001274 to 0.002069, seven MOTUs are recognized, including two for *E. indicus* and five for *E. nanus*. When the prior intraspecific divergence is 0.002069, the *ITS2* dataset places all individuals of the two species into a single MOTU. This indicates that this gene cannot distinguish the species defined according to traditional morphological criteria using this method (Figs 6–7).

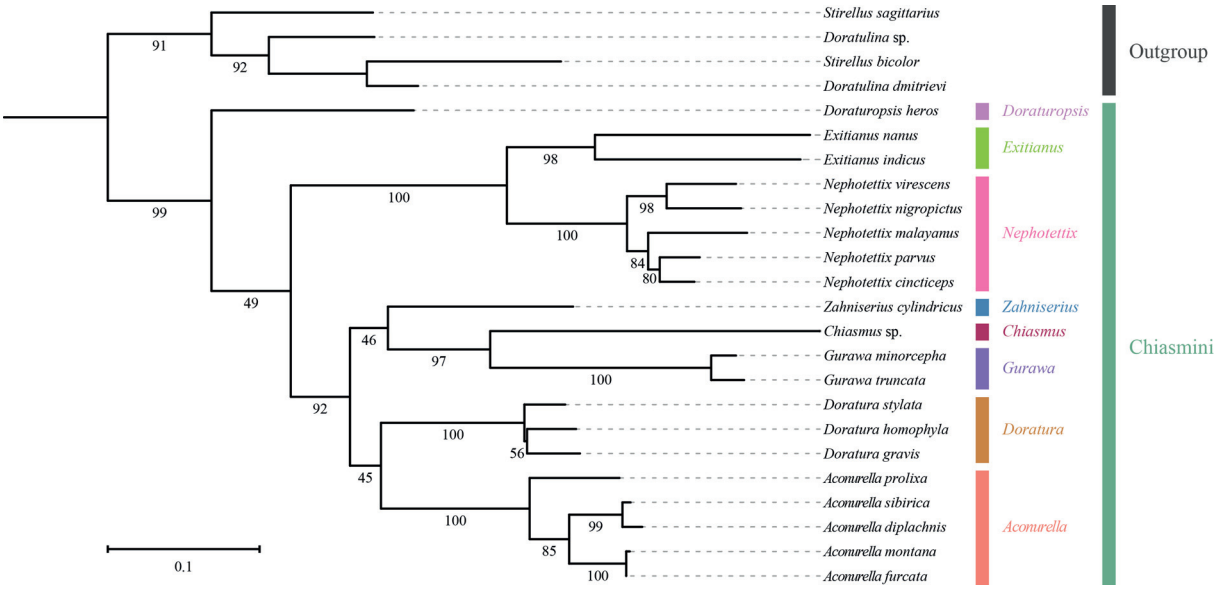

Fig. 3. ML bootstrap consensus phylogenetic tree for Chiasmini Distant, 1908 based on concatenated sequence data from two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*H3*, *28S*). Bootstrap support of nodes is indicated below the branches. Note the proposed taxonomic arrangements shown by vertical coloured lines.

bPTP

The bPTP analysis gives two results based on Bayesian and ML support, respectively. The ML method result corresponds to the PTP analysis result, and the Bayesian method corresponds to the bPTP analysis result. Both methods give the same results for the *COI* gene recognizing two MOTUs corresponding to the two morphologically defined species. *ITS2* also yields two MOTUs based on the ML method, consistent with the morphological species. Based on Bayesian method, *ITS2* generated excessive partitions. The ML results are shown for bPTP (Figs 6–7).

BPP

Phylogenetic trees from two-gene (*COI*, *ITS2*) analysis were used as inputs to create the BPP guide tree. The analysis was run twice. All results recognize two species with strong support (Table 7, Fig. 5).

Discussion

Aside from recent phylogenomic analyses that used data from large numbers of genes, most molecular phylogenetic studies of insects, including those focused on leafhoppers and other Auchenorrhyncha have been based on sequence fragments of the *16S rDNA*, *ND1*, *H3*, *28S rDNA* and *COII* genes. In general, these analyses have yielded phylogenies that agree well overall with both morphological taxon concepts and phylogenomic results (Fang *et al.* 1993; Zahniser & Dietrich 2010, 2013, 2015; Cao *et al.* 2022). The nuclear gene regions evolve more slowly and are, therefore, more helpful in solving the relationships among genera and tribes, while the mitochondrial genes evolve more quickly and better reflect the relationships between species.

In this study, combining data from four genes consistently resolved relationships among genera and species of Chiasmini. For the genera *Aconurella* and *Nephotettix*, represented by multiple species, the interspecific relationships are well resolved. Nevertheless, the relationships among some genera within the tribe are inconsistent with those of previous studies (Zahniser & Dietrich 2015; Cao *et al.* 2022), with the main area of disagreement confined to the relationship of *Doratura* +*Aconurella* and

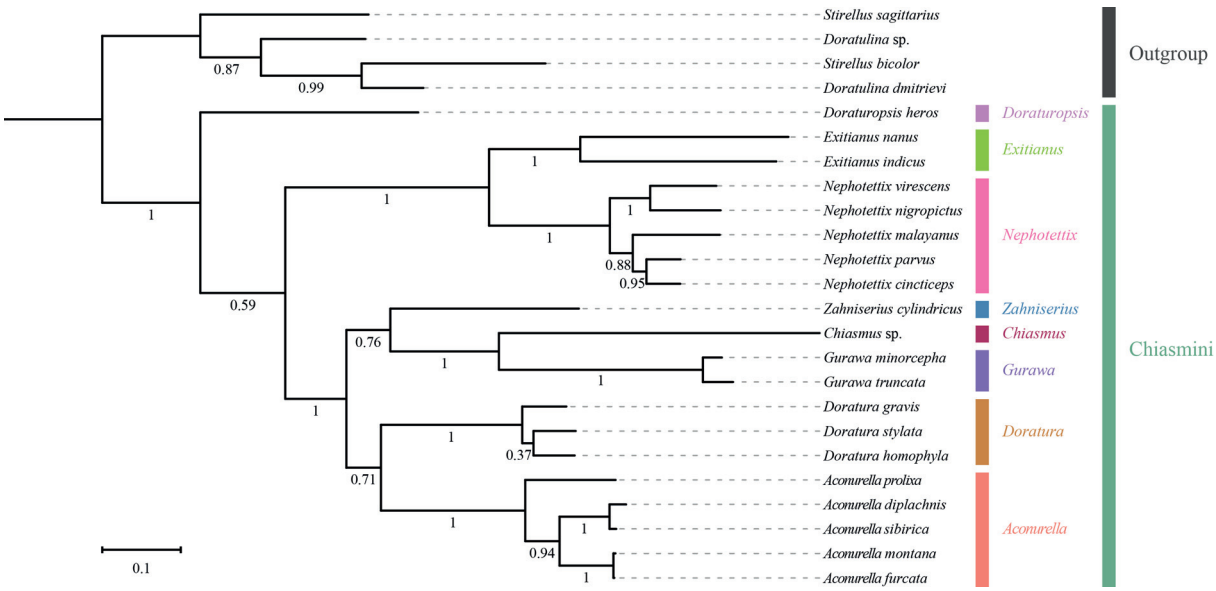

Fig. 4. BI consensus phylogenetic tree for Chiasmini Distant, 1908 based on concatenated sequence data from two mitochondrial genes (*COI*, *16S*) and two nuclear genes (*H3*, *28S*). Posterior probabilities of nodes are indicated below the branches. Note the proposed taxonomic arrangements shown by vertical coloured lines.

Gurawa+*Chiasmus*, and the position of *Doraturopsis*. Because the present phylogenetic analysis included species of Chiasmini primarily from China, further analyses with a broader sample of taxa from other geographic regions, and data from additional gene regions, are needed to resolve relationships among genera and species and confirm previous morphological species concepts.

Owing to high intraspecific variation in morphological characters traditionally used for species identification and interspecific similarity, delimitation of *Exitianus* species using morphology alone has been challenging. Previous taxonomic studies of *Exitianus* focused only on morphology-based classification, without assessing species boundaries using other types of data (Duan & Zhang 2013b). In our study, populations of two common and widespread species of this genus showed very low genetic

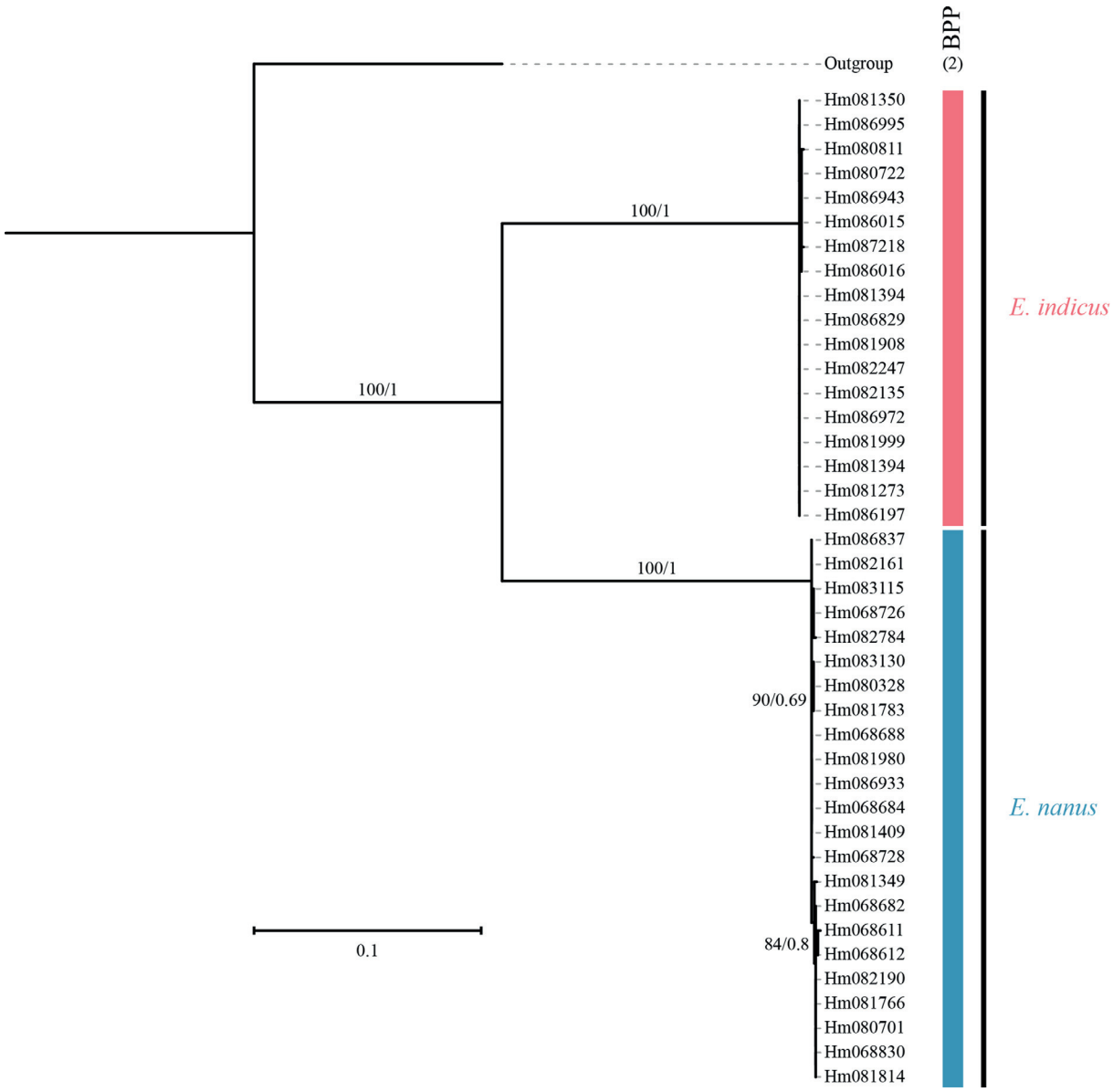

Fig. 5. ML bootstrap and BI consensus phylogenetic tree for *Exitianus* Ball, 1929 based on two-gene data set (*COI* and *ITS2*). Bootstrap support and posterior probabilities of nodes are indicated above the branches. The right vertical bars indicate the putative species using BPP. Morphological species are uniquely coloured.

Table 7. Posterior probabilities for the number of delimited species using different priors for model parameters in Bayesian phylogenetics and phylogeography on concatenated data sets of mitochondria markers and all genetic markers.

prior	posterior probability for the number of delimited species (mitochondrial gene)	posterior probability for the number of delimited species (all genes)
Θ: G (2:1000), τ: G (2:2000)	P 4 = 0.97950	P 4 = 0.97950
Θ: G (2:100), τ: G (2:200)	P 4 = 0.96589	P 4 = 0.96589
Θ: G (2:100), τ: G (2:2000)	P 4 = 0.96594	P 4 = 0.96594
Θ: G (2:1000), τ: G (2:200)	P 4 = 1.00000	P 4 = 1.00000

variability, suggesting that gene flow among Chinese populations is high. This is not surprising given that *Exitianus* species are fully winged and disperse readily. The different molecular species delimitation methods yielded results mostly consistent with current morphology-based species definitions. These

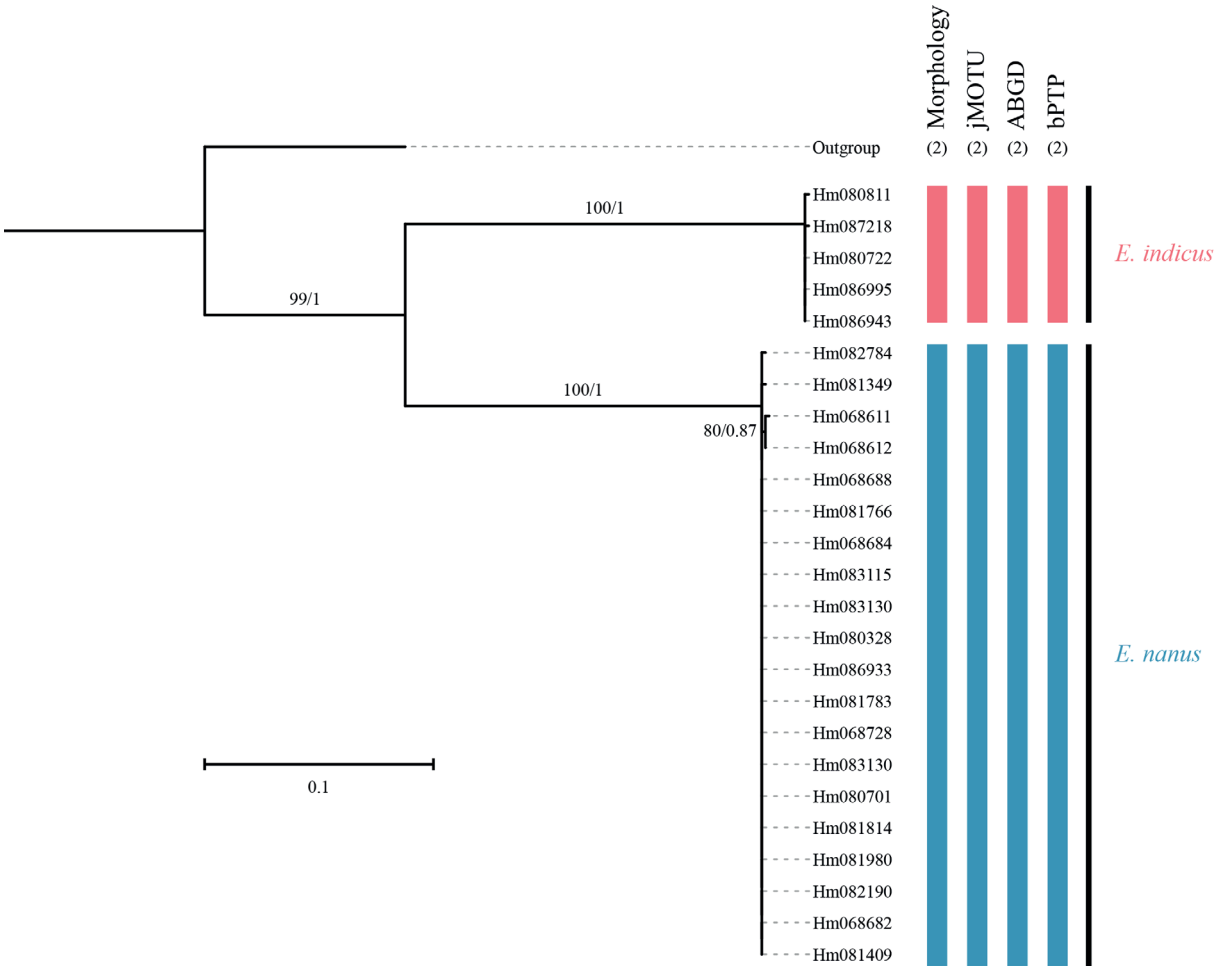

Fig. 6. Phylogenetic tree for *Exitianus* Ball, 1929 based on mitochondrial *COI*. Bootstrap support and posterior probabilities of nodes are indicated above the branches. The right vertical bars indicate the number of putative species using various methods as indicated at the top. Morphological species are uniquely coloured.

results are consistent with those of our previous studies on the chiasmine genera *Nephotettix* and *Aconurella* (Gao *et al.* 2021; Yan *et al.* 2022). The validity of morphology-based species concepts in Chiasmini was also confirmed for several Palearctic species of *Doratura* using data from courtship calls (Tishechkin 2011).

In general, we found that the different molecular species delimitation methods yield similar results, but differences sometimes occur depending on the particular parameter settings selected. For methods such as jMOTU incorporating a user-specified cut-off for genetic divergence, more stringent cut-offs yield fewer MOTUs. Results can also vary depending on the particular gene region selected, as we observed between *COI* and *ITS2* for some methods. This accentuates the need to consider multiple sources of evidence when attempting to delimit species. Our results demonstrate that species delimitation analyses based on multiple loci give a more credible and consistent result than methods using a single locus and are more congruent with morphological differences (Gao *et al.* 2021; Yan *et al.* 2022).

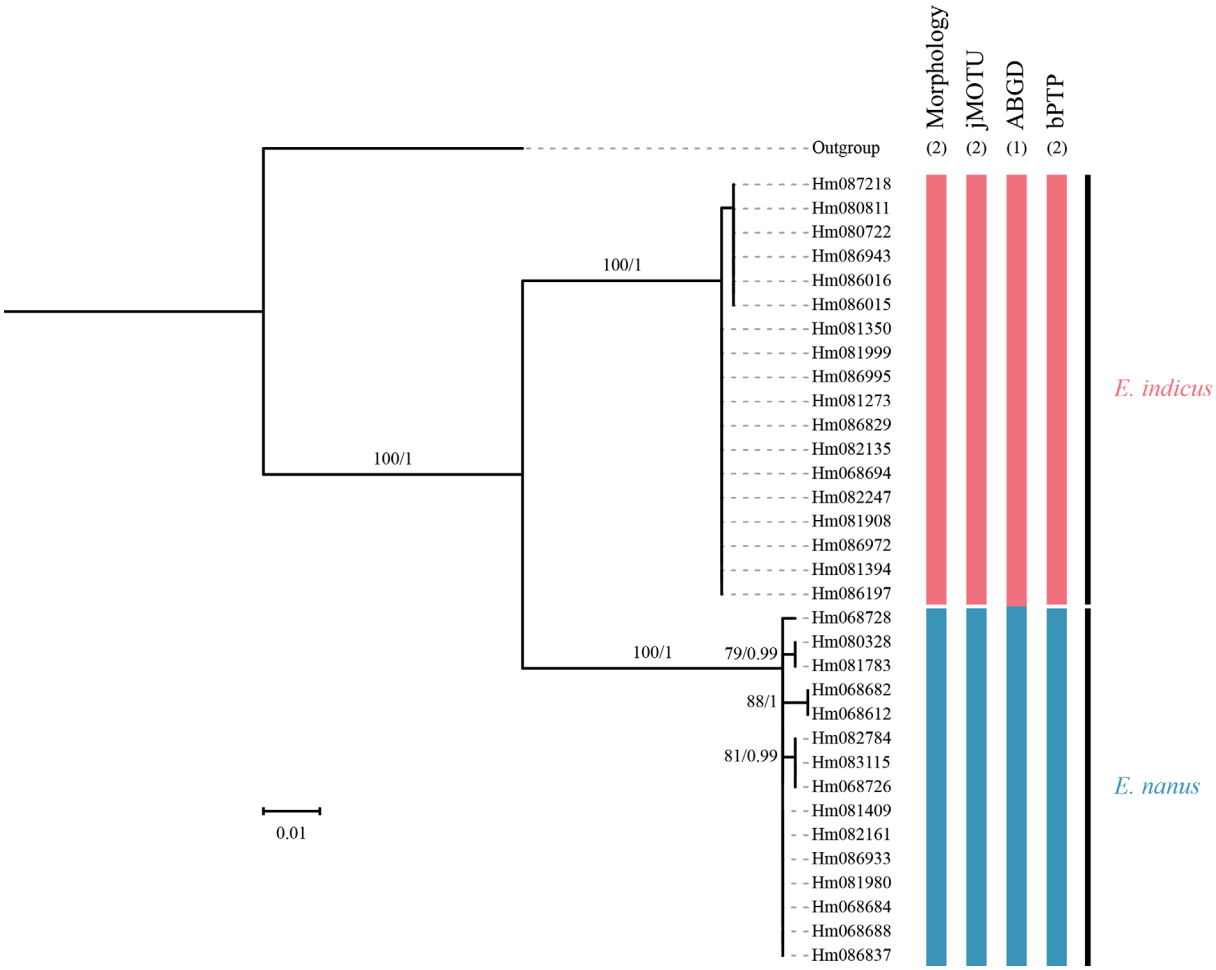

Fig. 7. Phylogenetic tree for *Exitianus* Ball, 1929 based on ribosome *ITS2*. Bootstrap support and posterior probabilities of nodes are indicated above the branches. The right vertical bars indicate the number of putative species using various methods as indicated at the top. Morphological species are uniquely coloured.

Conclusions

For the ML and BI trees constructed from 8 genera and 20 species of Chinese Chiasmini combined with four gene fragments (*COI*, *16S*, *H3*, *28S*), a phylogenetic relationship of (*Doraturopsis* + ((*Exitianus* + *Nephotettix*) + ((*Zahniserius* + (*Chiasmus* + *Gurawa*)) + (*Doratura* + *Aconurella*))) was obtained. For the molecular species delimitation analyses of Chinese *Exitianus*, the ML result and BI result both show that *E. indicus* and *E. nanus* are well-supported independent clades that are highly genetically divergent from each other but with low intraspecific genetic variability.
Based on the *COI* gene, the single-locus species delimitation methods jMOTU, ABGD and bPTP divided the included *Exitianus* populations into two species, consistent with their definition as morphological species. For *ITS2* gene, jMOTU recognized a two species result while ABGD grouped populations into a single species. The multi-locus BPP method consistently delimited the two species as previously defined based on morphology and appears more robust. However, this needs to be further tested by including a broader sample of *Exitianus* populations occurring outside of China.

Data availability statement

The sequence data that support the findings of this study are openly available in GenBank of NCBI at (https://www.ncbi.nlm.nih.gov/) and are listed in Tables 1–2.

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Appendix 1

Checklist of Chinese Chiasmini Distant, 1908

1. *Aconura* (*Aconura*) *jakowlefi* Lethierry, 1876 (recorded only by foreign scholars)
2. *Aconura* (*Platyacina*) *depressa* Emeljanov, 1964 (recorded only by foreign scholars)
3. *Aconura brevis* Zhang, 2014 (synonym)
4. *Aconura ochrargentea* Emeljanov, 1972
5. *Aconurella diplachnis* Emeljanov, 1964
6. *Aconurella furcata* Duan & Zhang, 2012
7. *Aconurella koreana* (Matsumura, 1915) (synonym)
8. *Aconurella montana* (Distant, 1908)
9. *Aconurella paradiplachnis* Duan & Zhang, 2012
10. *Aconurella prolixa* (Lethierry, 1885)
11. *Aconurella sibirica* (Lethierry, 1888)
12. *Baileyus brachynotus* Singh-Pruthi, 1936 (recorded only by foreign scholars)
13. *Baileyus brunneus* Singh-Pruthi, 1930 (recorded only by foreign scholars)
14. *Chiasmus alatus* Singh-Pruthi, 1930 (species cannot be accurately identified in this genus)
15. *Chiasmus mustelinus* (Distant, 1908) (species cannot be accurately identified in this genus)
16. *Doratura concors* Horváth, 1903
17. *Doratura gravis* Emeljanov, 1966
18. *Doratura homophyla* (Flor, 1861)
19. *Doratura stylata* (Boheman, 1847)
20. *Doraturopsis* (*Doraturopsis*) *heros* (Melichar, 1902)
21. *Doraturopsis* (*Eprepusa*) *microcephala* (Kusnezov, 1938)
22. *Doraturopsis bimaculata* Zhang, 2014 (synonym)
23. *Exitianus fusconervosus* (Motschulsky, 1863) (misidentification)
24. *Exitianus indicus* (Distant, 1908)
25. *Exitianus nanus* (Distant, 1908)
26. *Gurawa intermediate* Singh-Pruthi, 1936 (recorded only by foreign scholars)
27. *Gurawa minorcephala* Singh-Pruthi, 1930
28. *Gurawa truncate* Duan & Zhang, 2012
29. *Gurawa vexillum* Distant, 1908 (misidentification)
30. *Leofa* (*Prasutagus*) *forcipata* Duan & Zhang, 2009
31. *Leofa* (*Prasutagus*) *pulchella* (Distant, 1918)
32. *Leofa* (*Prasutagus*) *yangae* Duan & Zhang, 2009
33. *Nephotettix apicalis* (Motschulsky, 1859) (misidentification)
34. *Nephotettix cincticeps* (Uhler, 1896)
35. *Nephotettix malayanus* Ishihara & Kawase, 1968
36. *Nephotettix nigropictus* (Stål, 1870)
37. *Nephotettix parvus* Ishihara & Kawase, 1968
38. *Nephotettix virescens* (Distant, 1908)
39. *Zahniserius cylindricus* Duan & Zhang, 2012