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Ceriodaphnia (Cladocera: Daphniidae) in China: Lineage diversity, phylogeography and possible interspecific hybridization

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ABSTRACT

The distribution and species/lineage diversity of freshwater invertebrate zooplankton remains understudied in China. Here, we explored the species/lineage diversity and phylogeography of *Ceriodaphnia* species across China. The taxonomy of this genus is under-explored. Seven morphospecies of *Ceriodaphnia* (*C. cornuta*, *C. laticaudata*, *C. megops*, *C. pulchella*, *C. quadrangula*, *C. rotunda* and *C. spinata*) were identified across 45 of 422 water bodies examined. Rather little morphological variation was observed within any single morphospecies regardless of country of origin. Nevertheless, we recognized that some or all of these morphospecies might represent species complexes. To investigate this, phylogenetic relationships within and among these morphospecies were investigated based on mitochondrial (partial cytochrome *c* oxidase subunit I gene) and nuclear (partial 28S rRNA gene) markers. The mitochondrial marker placed these populations in nine lineages corresponding to the morphospecies: *C. laticaudata* and *C. pulchella* were each represented by two lineages, suggesting that both are species complexes. The remaining five morphospecies were each represented by a single mtDNA lineage. Three of the nine mitochondrial lineages (belonging to *C. pulchella*, *C. rotunda* and *C. megops*) are newly reported and exhibited a restricted distribution within China. The nuclear-DNA phylogeny also recognized seven *Ceriodaphnia* taxa within China. We detected occasional mito-nuclear discordances in *Ceriodaphnia* taxa across China, suggesting interspecific introgression and hybridization. Our study contributes to an understanding of the species/lineage diversity of *Ceriodaphnia*, a genus with understudied taxonomy.

1. Introduction

Because of their large population size and strong dispersal abilities, some small freshwater zooplankton species have often been regarded as "cosmopolitan" in their geographic distributions (Baas-Becking, 1934; Taylor et al., 1998). But many molecular studies have revealed that such "morphospecies" frequently consist of several cryptic species, despite their morphological similarities across broad geographical ranges (e.g. Andrews et al., 2014; Darling et al., 2007; Papakostas et al., 2016; Penton et al., 2004). For example, cryptic species have been observed in the freshwater copepod *Hemidiaptomus* Sars, 1903 (Marrone et al., 2013), rotifer *Brachionus calyciflorus* Pallas, 1766 species complex (Papakostas et al., 2016) and the cladoceran *Chydorus sphaericus* Müller, 1776 (Belyaeva and Taylor, 2009), the *Daphnia pulex* Leydig, 1860 species complex (Colbourne et al., 1998) and the *D. longispina* Mueller, 1785 species complex (Petrusek et al., 2012).

Members of the genus *Ceriodaphnia* Dana, 1853 (Cladocera: Daphniidae), a close relative of *Daphnia* Mueller, 1785 (Cornetti et al., 2019), are often key components of freshwater ecosystems as grazers of phytoplankton and as important prey for fish larvae. Despite the use of *Ceriodaphnia* species for eco-toxicological studies because of their high sensitivity and short generation time (e.g. Pakrashi et al., 2013; Versteeg et al., 1997), the taxonomy of the genus *Ceriodaphnia* is still poorly developed. Currently, 12 valid species and 21 "species inquirendae" are recognized (Kotov et al., 2013), together with one recently described species (Alonso et al., 2021). There have been some morphological investigations of *Ceriodaphnia* (e.g. Berner, 1987; Berner and Rakhmatullaeva, 2001; Kotov et al., 2018). However, this genus has been the focus of significantly fewer studies using molecular data (e.g. Alonso et al., 2021; deWaard et al., 2006; Sharma and Kotov, 2013) than have

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Table 1
List of localities inhabited by *Ceriodaphnia* (name, abbreviation and geographical position) and genetic characterization of sequenced individuals. N_1, the number of individuals for COI sequencing; N_2, the number of haplotypes.

Locality (Abbreviation)	Latitude	Lontitude	mtDNA Taxon	mtDNA Lineage	N_1	N_2	mtDNA Haplotype
Qinghai-Tibet Plateau							
Dongla Lake (DLL)	29.01 N	90.49 E	*C. laticaudata s.l.*	Cla01	10	7	DLL1, DLL2, DLL3, DLL4, DLL5, DLL6, CNH2
Dongla Pond (DLP)	29.00 N	90.51 E	*C. laticaudata s.l.*	Cla01	5	2	YJG1, DLP1
			C. cornuta s.l.	Cco01	2	2	QJP1, DLH1
Yajiageng #2 Lake (YJG)	29.92 N	101.99 E	*C. laticaudata s.l.*	Cla01	10	6	YJG1, YJG2, YJG3, YJG4, YJG5, YJG6
Cona Lake (CNH)	33.82 N	92.20 E	*C. laticaudata s.l.*	Cla01	7	6	CNH1, CNH2, CNH3, CNH4, CNH5, CNH6
Zheduoshan #2 Lake (ZDS)	30.08 N	101.84 E	*C. laticaudata s.l.*	Cla01	8	2	ZDS1, ZDS2
Caohaizi #1 Pond (CHZ)	29.59 N	102.03 E	*C. rotunda s.l.*	Cro01	1	1	CHZ1
Sidingcuo #2 Pond (SDC)	30.16 N	101.73 E	*C. quadrangula s.l.*	Cqu01	10	2	SDC1, SDC2
Gesangqiao nearby (GSQ)	29.65 N	91.12 E	*C. laticaudata s.l.*	Cla01	5	2	GSQ1, DLL5
			C. cornuta s.l.	Cco01	3	2	HYE2, HYE3
Zixin #1 Pond (ZXP)	28.47 N	90.28 E	*C. laticaudata s.l.*	Cla01	3	1	DLL1
Niulang Lake (NLL)	28.47 N	90.27 E	*C. laticaudata s.l.*	Cla01	1	1	CNH6
Tianyuan Pond (TYP)	28.48 N	90.50 E	*C. laticaudata s.l.*	Cla01	4	2	LLG1, DLL1
Gongbujiangda #5 Pond (G5B)	29.73 N	91.99 E	*C. laticaudata s.l.*	Cla01	1	1	G5B1
Gongbujiangda Pond (GBC)	29.80 N	91.83 E	*C. laticaudata s.l.*	Cla01	3	1	CNH2
	29.80 N	91.83 E	*C. pulchella s.l.*	Cpu02	1	1	GBC1
Lubian #4 Pond (L4B)	29.79 N	93.98 E	*C. laticaudata s.l.*	Cla01	7	2	L4B1, CNH6
Lubian #5 Pond (L5B)	29.74 N	94.11 E	*C. laticaudata s.l.*	Cla01	10	1	CNH6
Lubian #6 Pond (L6B)	29.74 N	94.11 E	*C. laticaudata s.l.*	Cla01	4	2	CNH6, L6B1
Yang Lake (YHZ)	28.94 N	90.05 E	*C. laticaudata s.l.*	Cla01	8	2	YHZ1, CNH2
Lalin Highway nearby (LLG)	29.70 N	91.36 E	*C. laticaudata s.l.*	Cla01	10	3	LLG1, LLG2, LLG3
Caicuo #2 Pond (CCP)	28.47 N	90.28 E	*C. laticaudata s.l.*	Cla01	1	1	DLL1
Yunnan-Guizhou Plateau							
Sangna Reservoir (SNR)	27.49 N	99.45 E	*C. laticaudata s.l.*	Cla01	10	1	YJG1
Er Hai (ERH)	25.81 N	100.22 E	*C. laticaudata s.l.*	Cla01	9	2	ERH1, ERH2
Nanhu Marsh (NHM)	25.12 N	98.56 E	*C. laticaudata s.l.*	Cla01	5	3	DLL1, DLL5, GSQ1
			C. cornuta s.l.	Cco01	4	3	NHM1, LIH1, HYE3
Shudu Lake (SDH)	27.54 N	99.56 E	*C. laticaudata s.l.*	Cla01	7	1	YJG1
			C. cornuta s.l.	Cco01	2	2	HYE1, HYE2
Inner Mongolia-Xinjiang Plateau							
Hasu Hai (HSH)	40.61 N	110.97 E	*C. pulchella s.l.*	Cpu01	3	1	HSH1
Wusutu Reservoir (WSR)	40.86 N	111.55 E	*C. spinata s.l.*	Csp01	5	3	WSR1, WSR2, WSR3
Eastern Plain							
Wanyao Reservoir nearby (WYR)	28.67 N	118.68 E	*C. megops s.l.*	Cme01	6	1	SJP1
Wanyao Reservoir (W2Y)	28.68 N	118.67 E	*C. cornuta s.l.*	Cco01	1	1	GEH1
			C. megops s.l.	Cme01	7	1	SJP1
Linping Pond (LPR)	30.43 N	120.03 E	*C. megops s.l.*	Cme01	10	4	LPR1, SJP1, DDR1, LPR2
Shuijing Park (SJP)	30.40 N	120.30 E	*C. megops s.l.*	Cme01	3	3	SJP1, HHU1, DDR1
Douding Reservoir (DDR)	26.15 N	119.30 E	*C. megops s.l.*	Cme01	10	3	DDR1, DDR2, DDR3
Luoma Lake (LMH)	34.10 N	118.17 E	*C. cornuta s.l.*	Cco01	10	5	GEH1, QJP2, LMH1, LIH4, LSH3
Li Lake (LIH)	31.53 N	120.24 E	*C. laticaudata s.l.*	Cla01	2	1	CNH2
			C. cornuta s.l.	Cco01	8	5	QJP1, QJP2, LIH1, LIH2, HYE2
Lushui Lake (LSH)	29.41 N	113.55 E	*C. cornuta s.l.*	Cco01	6	4	LSH1, LSH2, NHM1, LSH3
Baoying Lake (BYH)	33.10 N	119.14 E	*C. cornuta s.l.*	Cco01	2	2	BYH1, BYH2
Haiyanerzhong Pond (HYE)	30.11 N	120.00 E	*C. cornuta s.l.*	Cco01	3	3	HYE1, HYE2, HYE3
Ge Lake (GEH)	31.62 N	119.83 E	*C. cornuta s.l.*	Cco01	2	1	GEH1
Nanshui Reservoir (NSR)	30.68 N	120.96 E	*C. cornuta s.l.*	Cco01	1	1	NSR1
Gutianxi Reservoir (GTX)	26.59 N	118.80 E	*C. cornuta s.l.*	Cco01	10	2	GTX1, GTX2
Hehai university nearby (HHU)	31.82 N	119.99 E	*C. megops s.l.*	Cme01	1	1	HHU1
Northeast Plain							
Lianhuan Pond (LHH)	46.49 N	124.24 E	*C. laticaudata s.l.*	Cla01, Cla02	5	4	LHH1, LHH2, LHH3, LHH4
Dalonghu Pao (DLH)	46.48 N	124.19 E	*C. cornuta s.l.*	Cco01	10	3	QJP1, DLH1, DLH2
Talahong Pao (TLH)	46.46 N	124.13 E	*C. cornuta s.l.*	Cco01	4	2	QJP1, QJP2
Qijia Pao (QJP)	46.48 N	124.15 E	*C. cornuta s.l.*	Cco01	10	2	QJP1, QJP2
Amuta Pao (AMT)	46.30 N	124.06 E	*C. cornuta s.l.*	Cco01	10	2	DLH2, QJP1
Nanyin Reservoir (NYR)	45.57 N	124.34 E	*C. cornuta s.l.*	Cco01	9	5	NYR1, GEH1, DL2, QJP1, QJP2

some other major zooplanktonic cladoceran taxa, such as *Daphnia* (e.g. Ma et al., 2019a; Ma et al., 2019b; Zuykova et al., 2018) and *Moina* Baird, 1850 (e.g. Bekker et al., 2016; Deng et al., 2021; Ni et al., 2019).

When morphological descriptions are incomplete or inadequate, molecular data can help to clarify taxonomy, phylogeny and diversity of branchiopod crustaceans (deWaard et al., 2006). The first molecular study of the genus *Ceriodaphnia* revealed its evolutionary position among branchiopod crustaceans (deWaard et al., 2006). Then, Elias-Gutierrez et al. (2008) explored the diversity of Cladocera in Mexico and Guatemala based on DNA barcoding, and found an unnamed taxon of *Ceriodaphnia* and three distinct lineages morphologically close to *C. rigaudi* Richard, 1894. Later, Abreu et al. (2010) explored genetic differences between two morphologically similar species, *C. dubia* Richard, 1894 and *C. silvestrii* Daday, 1902, using PCR-RFLP. Sharma & Kotov (2013) used DNA sequences from two mitochondrial genes (COI and 16S) and one nuclear gene (28S) to investigate three potentially endemic sibling species within the *C. cornuta* complex in Australia. Their study, combined with later morphological work, established the existence of three *Ceriodaphnia* species in Australia: *C. dubia*, one reinstated species *C. spinata* Henry, 1919, and an unidentified species *C.* sp. 1 (Sharma, 2014). Most recently, a new species, *C. smirnovi* Alonso, Neretina & Ventura, 2021, has been described from Spain, using morphological and molecular phylogenetic evidence (Alonso et al., 2021). However, there have been no notable morphological or molecular taxonomy studies on *Ceriodaphnia* in China, where several important biogeographical hotspots occur (Myers et al., 2000).

Table 2
List of 28S alleles of *Ceriodaphnia*. Bold type indicates any mismatch assignment by COI versus 28S. N, the number of individuals.

28S Allele ID	28S Taxon	mtDNA Taxon	mtDNA Lineage	mtDNA Haplotype	N	Geographic Region
Clat_28S01	*C. laticaudata s.l.*	*C. laticaudata s.l.*	Cla01	YJG1, YJG2	2	Qinghai-Tibet Plateau
			Cla02	LHH4	1	Northeast Plain
Clat_28S02	*C. laticaudata s.l.*	*C. laticaudata s.l.*	Cla02	LHH3	1	Northeast Plain
Clat_28S03	***C. laticaudata s.l.***	***C. pulchella s.l.***	**Cpu01**	**HSH1**	**1**	**Inner Mongolia-Xinjiang Plateau**
Clat_28S04	*C. laticaudata s.l.*	*C. laticaudata s.l.*	Cla01	L6B1	1	Qinghai-Tibet Plateau
Clat_28S05	*C. laticaudata s.l.*	*C. laticaudata s.l.*	Cla01	ERH1	1	Yunnan-Guizhou Plateau
Clat_28S06	*C. laticaudata s.l.*	*C. laticaudata s.l.*	Cla01	CNH6, CNH4	2	Qinghai-Tibet Plateau
Crot_28S01	*C. rotunda s.l.*	*C. rotunda s.l.*	Cro01	CHZ1	1	Qinghai-Tibet Plateau
Cqua_28S01	*C. quadrangula s.l.*	*C. quadrangula s.l.*	Cqu01	SDC1, SDC2	5	Qinghai-Tibet Plateau
Cpul_28S01	*C. pulchella s.l.*	*C. pulchella s.l.*	Cpu01	HSH1	2	Inner Mongolia-Xinjiang Plateau
Cpul_28S02	*C. pulchella s.l.*	*C. pulchella s.l.*	Cpu02	GBC1	1	Qinghai-Tibet Plateau
Cmeg_28S01	*C. megops s.l.*	*C. megops s.l.*	Cme01	SJP1	1	Eastern Plain
Cmeg_28S02	*C. megops s.l.*	*C. megops s.l.*	Cme01	SJP1	1	Eastern Plain
Cmeg_28S03	*C. megops s.l.*	*C. megops s.l.*	Cme01	SJP1	2	Eastern Plain
Cmeg_28S04	*C. megops s.l.*	*C. megops s.l.*	Cme01	SJP1	1	Eastern Plain
Cmeg_28S05	*C. megops s.l.*	*C. megops s.l.*	Cme01	DDR3, SJP1	4	Eastern Plain
Cspi_28S01	***C. spinata s.l.***	***C. cornuta s.l.***	**Cco01**	**BYH1**	**1**	**Eastern Plain**
	C. spinata s.l.	*C. spinata s.l.*	Csp01	WSR2, WSR3	2	Inner Mongolia-Xinjiang Plateau
Cspi_28S02	*C. spinata s.l.*	*C. spinata s.l.*	Csp01	WSR1	1	Inner Mongolia-Xinjiang Plateau
Ccor_28S01	*C. cornuta s.l.*	*C. cornuta s.l.*	Cco01	QJP2, NYR1	2	Eastern Plain, Northeast Plain
Ccor_28S02	*C. cornuta s.l.*	*C. cornuta s.l.*	Cco01	DLH1	1	Northeast Plain

Similar to other zooplanktonic Cladocera, *Ceriodaphnia* utilizes cyclical parthenogenesis, in which several generations of parthenogenetically produced females (in suitable environments) alternate with a sexual generation (when environments become unfavorable) with males producing sperm and females producing haploid eggs (Balcer et al., 1984). These cyclical parthenogens have long been known to be prone to hybridization and introgression (Cuellar, 1977). This phenomenon has been well documented in cladocerans (Schwenk and Spaak, 1995) and rotifers (Papakostas et al., 2016). In particular, mito-nuclear discordance has been often observed in Cladocera, including species within *Daphnia* (Thielsch et al., 2017), *Moina* Baird, 1850 (Ni et al., 2019) and *Diaphanosoma* Fischer, 1850 (Liu et al., 2018). However, no introgressive hybridizations have ever been reported in *Ceriodaphnia*.

This study aims at an assessment of the species/lineage diversity of *Ceriodaphnia* in China, and investigation of potential hybridization, using sequences of two gene fragments: the mitochondrial cytochrome oxidase *c* subunit I (COI), and the nuclear 28S ribosomal RNA gene (28S). Using three independent methods of species delimitation, we expected to detect the existence of new lineages within *Ceriodaphnia* across China. Additionally, we tested our hypothesis that hybridization can occur between *Ceriodaphnia* species, as observed in other cladoceran genera (Hebert, 1985; Ni et al., 2019; Wang et al., 2021).

2. Materials and methods

2.1. Sampling

Zooplankton sampling was carried out in 422 localities across China. Samples were collected using a plankton net (mesh size 64 μm) hauled through the entire water column at three to four different sites per locality from a boat or from the shore if open water was accessible. Samples collected from same locality were pooled and preserved in 95% ethanol then stored at 4 °C in the laboratory for further analyses. Before the molecular analyses, Latin binomial names were assigned to morphospecies by comparing their morphology with those of named species (or species complexes) (e.g. Alonso et al., 2021; Hudec, 2010; Korovchinsky, 1995; Kotov et al., 2018; Sharma, 2014). It is usual to identify a species complex by "*sensu lato*" (or *s.l.*) following the name of the nominal species. This is to contrast with "*sensu stricto*" (*s.s.*), which is applied to a particular, named, member of a complex. In this paper, the use of a Latin name refers to a probable species complex based on that nominal species.

2.2. Morphological examination

For morphological examination, *Ceriodaphnia* individuals were selected from alcohol-preserved samples under a dissecting microscope, and then placed on slides and examined under a high-resolution optical microscope (ECLIPSE Ci-S, Nikon). For each *Ceriodaphnia* species complex (based on morphology), approximately ten adult parthenogenetic females were examined. Drawings of morphological features were based on microphotographs taken by a camera connected to the optical microscope and are here presented for the three most abundant and widespread *Ceriodaphnia* species complexes across China (i.e. *C. laticaudata*, *C. cornuta* and *C. megops*). Additionally, the morphology of males and ephippial females (if present) from each species complex were recorded.

2.3. DNA extraction and sequencing

On average, ten *Ceriodaphnia* individuals from each locality were randomly selected. Each individual was placed into a 0.2 mL tube. DNA was extracted from each animal following a proteinase-K method (Schwenk et al., 1998): each individual was mixed with 20 μL H3 buffer (containing final concentrations of 10 mM Tris-HCl, 50 mM KCl, 0.005% Tween 20, 0.005% NP-40) and 0.1 mg/mL proteinase K (MERCK, Germany). The mixed solution was then incubated for 16–20 h in a 55 °C water-bath with mild shaking. Finally, the proteinase K was denatured via a 12 min incubation at 95 °C. After a brief centrifugation, DNA samples were stored at 4 °C for genetic analyses.

A 680-bp fragment of mitochondrial cytochrome *c* oxidase subunit I (COI) gene was amplified using the primer pair LCO1490 and HCO2918 (Folmer et al., 1994). The PCR was performed in a total volume of 20 μL, which included 10 μL 2 × HieffTM PCR Master Mix (With Dye), 6 μL ddH_2O, 1 μL 10 μM solution of each primer and 2 μL DNA template. The PCR conditions were as follows: incubation 94 °C for 5 min, then 40 cycles of 45 s at 94 °C for denaturing, 45 s at 45 °C for annealing and 45 s at 72 °C for extending; and 7 min at 72 °C for the final extension. Additionally, an average of 5 individuals from each morphospecies (representing all mtDNA lineages and their geographic regions; Table 2) was chosen for sequencing a portion of the nuclear gene for 28S ribosomal RNA (28S). The PCR procedure was the same as for COI, except that the primers were 28 s1 and 28 s2 (Fontaneto et al., 2007) and the annealing temperature was 56 °C. As 28S fragments might contain multiple heterozygous sites, cloning was carried out for the individuals exhibiting heterozygosity using the protocol described in our previous

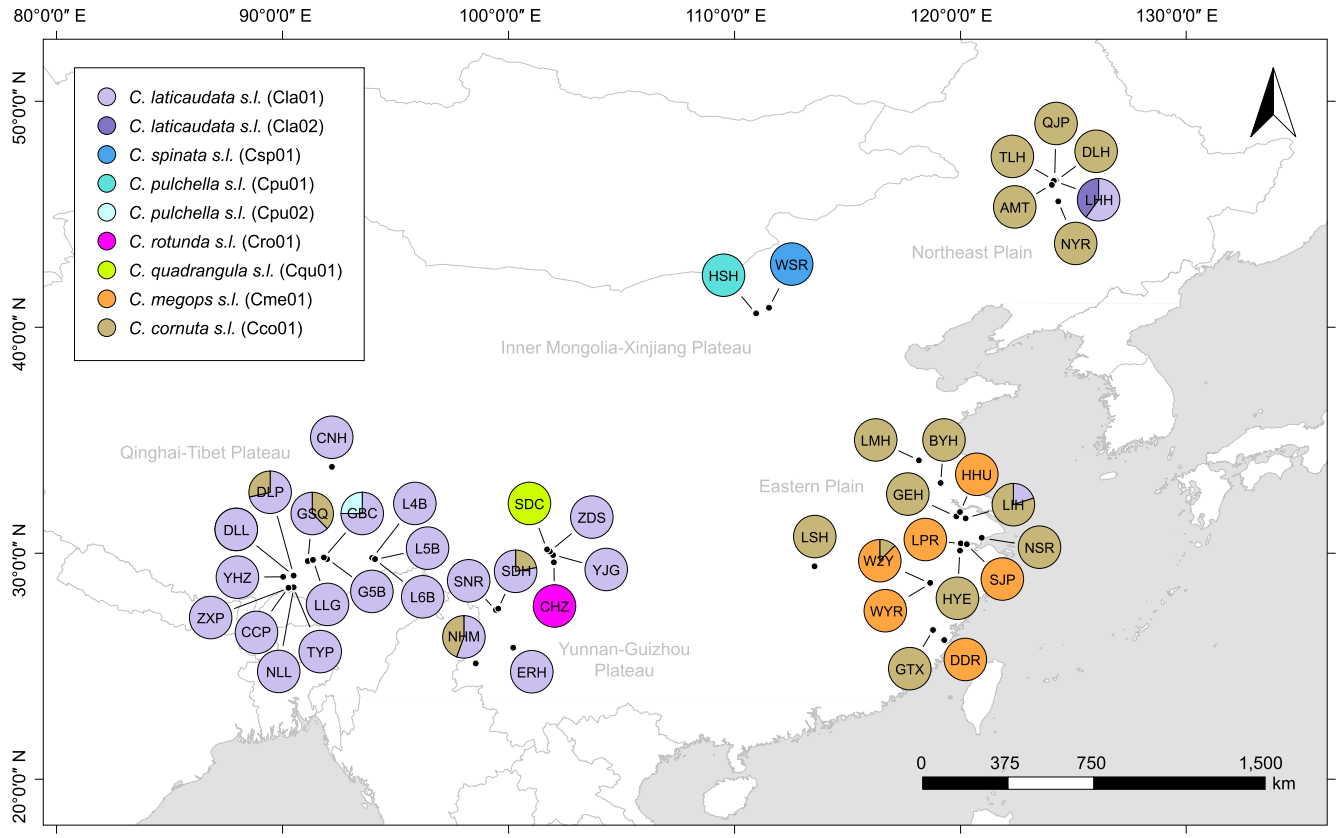

Fig. 1. The geographical distribution of *Ceriodaphnia* species in China based on the COI phylogeny. Solid black circles indicate localities with *Ceriodaphnia*, locality abbreviations on the map correspond to the abbreviations in Table 1. Each colored circle represents mtDNA lineages (Table 1) found at each sampling locality. Segments within circles of different colors indicate the proportions of each lineage.

studies (Ni et al., 2019; Wang et al., 2021): Fifteen clones were sequenced for each PCR product, and only identical sequences/alleles obtained at least twice per PCR product were retained for further analysis. All COI and 28S PCR products were sequenced using a forward primer on an ABI PRISM 3730 DNA capillary sequencer by Sangon Biotech Co., Ltd (Shanghai, China). All the chromatograms of COI and 28S sequences were carefully examined and manually corrected for scoring errors in MEGA X (Kumar et al., 2018). If chromatograms exhibited noise or double peaks, the PCR product was re-sequenced using the reverse primer. All new sequences have been submitted to GenBank under accession numbers: COI: OK561548-OK561618 and 28S: OK491060-OK491078.

2.4. Sequence alignment and genetic diversity

COI sequences were aligned using the Clustal W algorithm (Thompson et al., 1994) and subsequently translated into amino acids in MEGA X to check for the presence of stop codons. Then, unique haplotypes were verified in DNASP 6 (Librado and Rozas, 2009). All unique haplotypes were aligned together with all 170 publicly available sequences of *Ceriodaphnia* retrieved from GenBank (Table S1) in MEGA X. For the 28S sequences, unique alleles were detected in DNASP 6 and then aligned with all 12 publicly available sequences retrieved from GenBank (Table S2).

2.5. Phylogenetic analyses

The possibility of substitution saturation, which could result in loss of phylogenetic signal for COI sequences, was checked in DAMBE 5 (Xia, 2013). A COI Bayesian phylogenetic tree was constructed in BEAST 1.8 (Bouckaert et al., 2014), with a tree being recorded every 1,000 generations among 40,000,000, the first 25% were eliminated as burn-in, and the final 30,000 sampled trees summarized using TreeAnnotator. A sequence of *Simocephalus punctatus* (GenBank ID: MG936597), a putative sister taxon of *Ceriodaphnia* (Xu et al., 2021) was used as an outgroup. The best substitution model (HKY + I + G) was determined based on the corrected Akaike Information Criterion in jModeltest v. 2.1.7 (Darriba et al., 2012). Finally, Tracer v1.7 (Rambaut et al., 2018) was used to ensure that enough generations had been computed. Similarly, a Bayesian phylogenetic tree was constructed for the 28S alignment in BEAST 1.8 using the GTR + G substitution model. Finally, we constructed a Bayesian phylogenetic tree based on concatenated dataset with both COI and 28S sequences. The concatenated dataset was derived only from specimens for which both COI and 28S sequences were available to avoid a large amount of missing data. Our final dataset was completed with publicly available sequences of *Ceriodaphnia* specimens (when both COI and 28S genes were available) retrieved from GenBank (Table S3). Mitochondrial (COI) and nuclear sequences (28S) were concatenated and partitioned by gene. We then applied the incongruence length difference test in PAUP* v. 4.0a (Swofford, 2003) to test whether the mitochondrial and nuclear sequences could be combined for joint analyses. The best-fit models of nucleotide substitution for each partition were identified in jModeltest v. 2.1.7. The concatenated phylogenetic tree was constructed in BEAST 1.8 with the same general precautions and parameters as used for the single gene analyses (see above).

2.6. Detection of new lineages and phylogeographic analyses

Three independent species-delimitation methods, the general mixed Yule coalescent model (GMYC, Pons et al., 2006), Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012) and Poisson tree

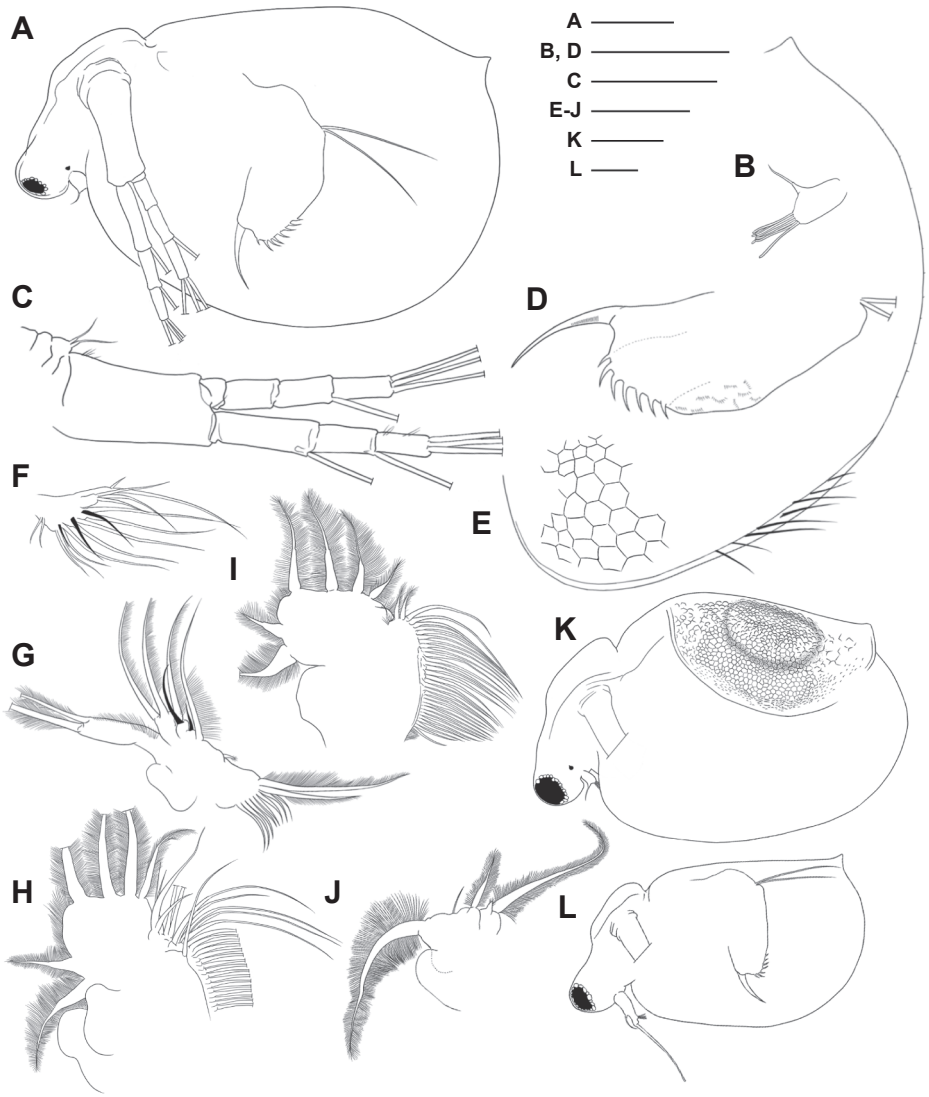

Fig. 2. *Ceriodaphnia laticaudata s.l.* P. E. Müller, 1867, parthenogenetic female (A-J), ephippial female (K) and adult male (L) from Lubian #4 Pond (L4B), the Qinghai-Tibetan Plateau of China. A, adult parthenogenetic female, lateral view. B, antenna I. C, antenna II. D, postabdomen. E, valve. F, limb I. G, limb II. H, limb III. I, limb IV. J, limb V. K, ephippial female, lateral view. L, male, lateral view. Scale bars 0.1 mm.

processes methods (PTP, Zhang et al., 2013), were used to explore the number of lineages in *Ceriodaphnia* for both COI and 28S markers. GMYC is a likelihood-based method for delimiting species/lineages by fitting within- and between-species branching models to reconstruct gene trees based on an ultrametric tree. GMYC analysis was carried out with a single threshold (Fujisawa and Barraclough, 2013) using its webserver (https://species.h-its.org/gmyc/). We performed the ABGD analysis to sort the sequences into hypothetical species that are based on the barcode gap using the online server (https://wwwabi.snv.jussieu.fr/public/abgd/) with the default settings. The PTP calculations were conducted on the bPTP websever (https://species.h-its.org/ptp/), with 100,000 MCMC generations, thinning set to 100 and burn-in at 25% and performing a Bayesian search. The input phylogenetic tree was generated with BEAST 1.8 (see above). To validate the outcomes of single-locus lineage delimitation, a Bayesian Phylogenetics and Phylogeography (BPP) analyses was applied to the multilocus dataset (i.e. COI + 28S) using BP&P v. 4.4.1 (Yang and Rannala, 2010). We used the A10 mode, which delimits lineages using a guide tree constructed in BEAST based on the concatenated dataset. The MCMC chain was run for 500,000 generations, sampling every 5 with a 10% burn-in, and the analyses were performed twice to confirm consistency. Finally, to visualize genealogical relationships among mitochondrial lineages, COI haplotype networks were constructed for the three most abundant taxa (i.e. *C. laticaudata*, *C. cornuta* and *C. megops*) using the TCS model (Clement et al., 2000) in PopART 1.7 (Leigh and Bryant, 2015).

3. Results

3.1. Morphological examination

Based on morphology, *Ceriodaphnia* was detected in 45 out of the 422 localities investigated in this study, covering the Eastern Plain (14 localities), Inner Mongolia-Xinjiang Plateau (2), Qinghai-Tibetan Plateau (19), Northeast Plain (6) and Yunnan-Guizhou Plateau (4; Fig. 1 and Table 1). We identified seven *Ceriodaphnia* morphospecies (*C. cornuta*, *C. laticaudata*, *C. megops*, *C. pulchella*, *C. quadrangula*, *C. rotunda* and *C. spinata*) in China (voucher specimens of all morphospecies are preserved in the Zooplankton Collection at Fudan University). Among them, *C. laticaudata* P. E. Müller, 1867 (Fig. 2), *C. cornuta* Sars, 1885 (Fig. 3) and *C. megops* Sars, 1862 (Fig. 4) were the three most abundant species. *Ceriodaphnia cornuta* could be easily distinguished by two key morphological features: its small body size (0.43–0.45 mm) and prominent pointed rostrum (Fig. 3A). In contrast, *C. megops* has the largest body size (0.67–0.81 mm) with a short, rounded rostrum (Fig. 4A and

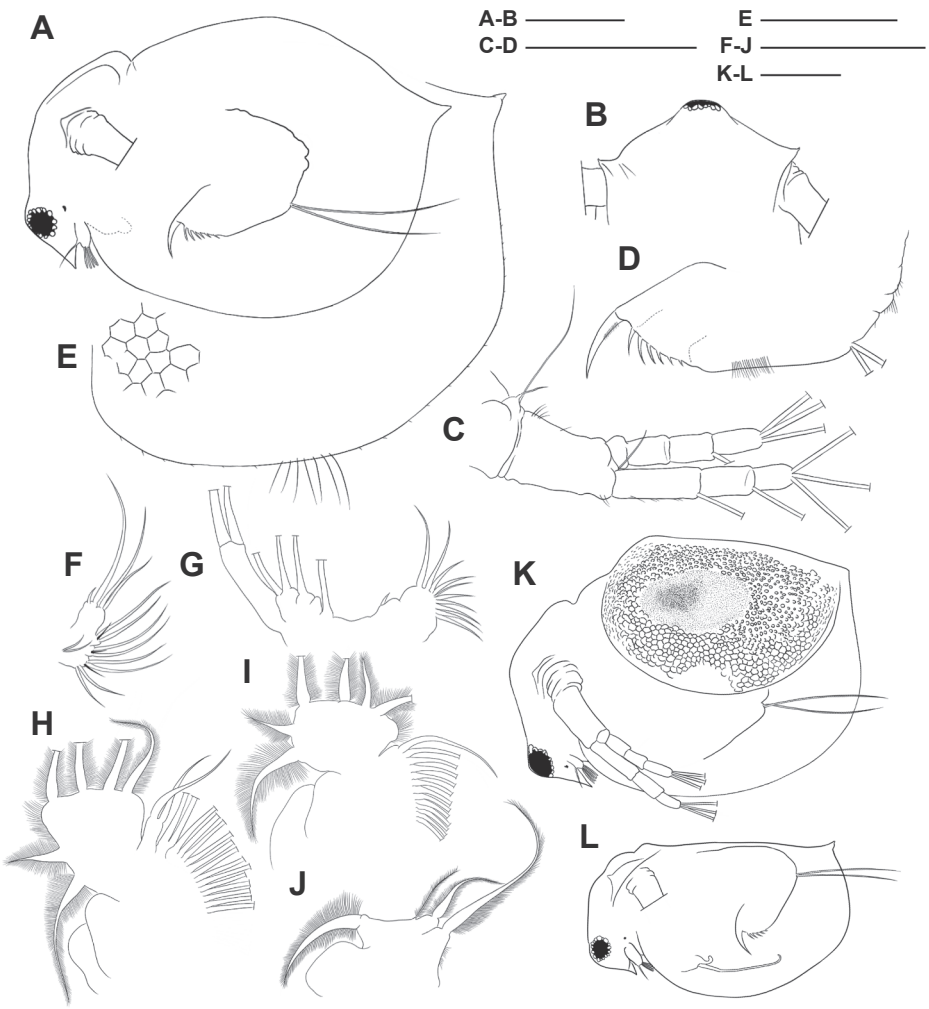

Fig. 3. *Ceriodaphnia cornuta s.l.* Sars, 1885, parthenogenetic female (A-J), ephippial female (K) and adult male (L) from Luoma Lake (LMH), the Eastern Plain of China. A, adult parthenogenetic female, lateral view. B, head, dorsal view. C, antenna II. D, postabdomen. E, valve. F, limb I. G, limb II. H, limb III. I, limb IV. J, limb V. K, ephippial female, lateral view. L, male, lateral view. Scale bars 0.1 mm.

B). The antenna II of *C. cornuta* bears two sensory setae of different lengths in the coxal part (Fig. 3C), but this is not the case for the two other common species (Fig. 2C and 4C). The postabdomen of *C. cornuta* is narrow and short, and the preanal and anal margins bears five to six pairs of sharp denticles (Fig. 3D), the postabdomen of *C. laticaudata* is elongated and with seven pairs of sharp denticles on the preanal and anal margins (Fig. 2D), whereas the postabdomen in *C. megops* is broad and securiform, with ten pairs of anal denticles (Fig. 4D). Males and ephippial females were only detected for *C. laticaudata* (Fig. 2K and L) and *C. cornuta* (Fig. 3K and L). The ephippium carried by *C. laticaudata* contains a sexual egg which is reticulated over its surface (Fig. 2K), and the ephippium of *C. cornuta* was covered by small and low projections (Fig. 3K). The male seta of *C. laticaudata* is robust and two times longer than the antennular body (Fig. 2L), whereas the male seta of *C. cornuta* is short and thin (Fig. 3L). We found rather little morphological variation within a single morphospecies of *Ceriodaphnia* regardless of country of origin (Table S4). For example, female *C. laticaudata* from China has a narrower postabdomen and fewer anal denticles than that from Denmark (Muller, 1867; Sharma, 2014), and Chinese *C. megops* has broader postadomen and more anal denticles when compared to *C. megops* from Europe (Błędzki and Rybak, 2016).

3.2. Genetic diversity

A total of 289 *Ceriodaphnia* individuals (an average of 6.4 individuals per population) were successfully sequenced at the COI locus (604 bp in the aligned dataset); among them, 71 unique COI haplotypes were found (Table 1). In total, 31 individuals were successfully sequenced at the locus 28S (3 heterozygotes and 28 homozygotes, resulting in a total of 34 sequences; 685 bp in the aligned dataset); among them, 19 unique 28S alleles were detected (Table 2).

3.3. Phylogeny and gene introgression

Our COI Bayesian phylogenetic tree revealed the presence of seven *Ceriodaphnia* species complexes across China, in agreement with the morphological observations. Three independent species-delimitation methods (i.e. ABGD, bPTP and GMYC) consistently indicated that Chinese *Ceriodaphnia* populations fell into seven species complexes, with nine distinct lineages. Additional lineages not represented in China were present in many species complexes. Both *C. laticaudata* and *C. pulchella* Sars, 1862, were represented by two lineages in China, the remaining five by a single mtDNA lineage each (Fig. 5). Interestingly, three lineages ("Cpu01" of *C. pulchella*, "Cro01" of *C. rotunda* (Straus, 1820) and "Cme01" of *C. megops*) were not represented by sequences from elsewhere in the world. Analysis using bPTP identified additional mt-lineages not recognized using the other two methods. But we have chosen the most conservative estimates of lineage diversity in our DNA taxonomy approach (i.e. ABGD and GMYC), to avoid dividing a species complex into lineages that could not be well supported.

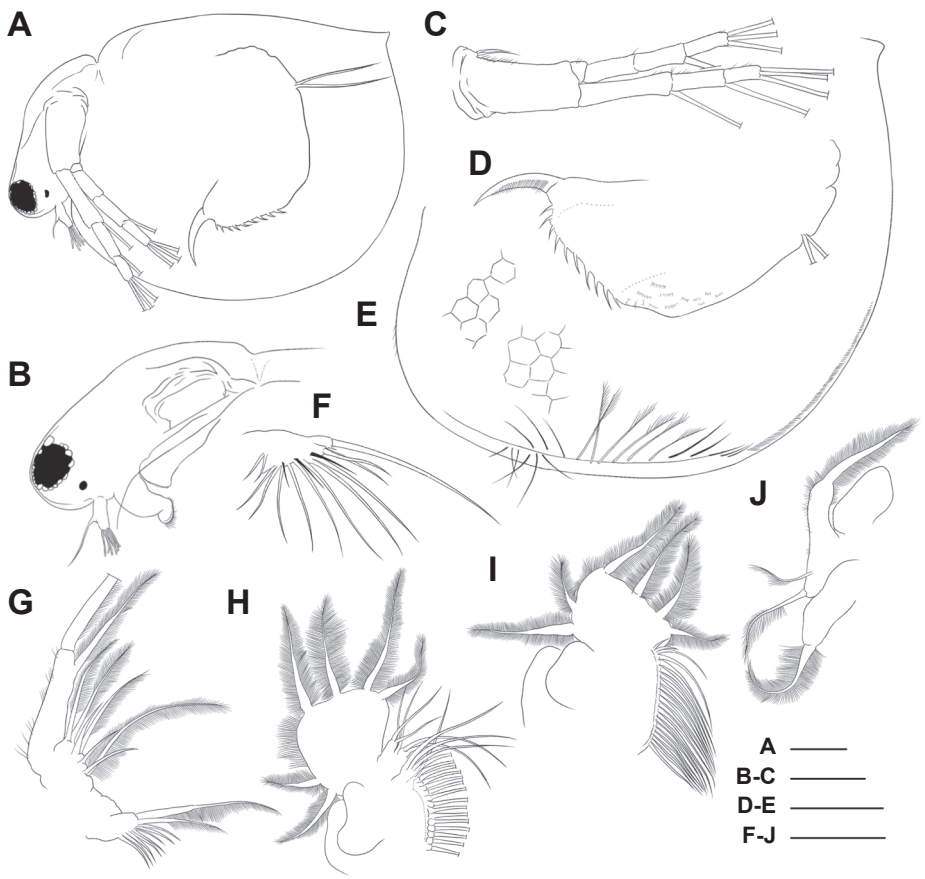

Fig. 4. *Ceriodaphnia megops s.l.* Sars, 1862, parthenogenetic female (A-J) from Linping Pond (LPR), the Eastern Plain of China. A, adult parthenogenetic female, lateral view. B, head, lateral view. C, antenna II. D, postabdomen. E, valve. F, limb I. G, limb II. H, limb III. I, limb IV. J, limb V. Scale bars 0.1 mm.

The nuclear 28S Bayesian tree also indicated the presence of seven species complexes in China (Fig. 6). Interestingly, one individual that had a 28S allele typical of *C. laticaudata* had mtDNA sequences typical of *C. pulchella*, and another individual with 28S alleles typical of *C. spinata* had mtDNA sequences typical of *C. cornuta* (Table 2 and Fig. 6). Moreover, two individuals from "Cla01" and one individual from mitochondrial lineage "Cla02" of *C. laticaudata* shared the same 28S allele (Clat_28S01) typical of *C. laticaudata* (Table 2 and Fig. 6).

The Bayesian phylogenetic tree inferred from concatenated COI and 28S sequences identified seven *Ceriodaphnia* taxa from China (Figure S1). Finally, the multilocus lineage delimitation method (i.e. BPP) based on the concatenated dataset recovered ten lineages from China with high posterior probability (all PP $>$ 0.95). This lineage delimitation agreed with that based on the COI locus alone, except that mitochondrial lineage "Cla01" was divided into two lineages based on BPP (Figs. 5 and S1).

3.4. Biogeography

Ceriodaphnia cornuta and *C. laticaudata* are the two most widely distributed species complexes of *Ceriodaphnia* worldwide: *C. cornuta* is present in Australian, Neotropical, Oriental and Palaearctic regions; and *C. laticaudata* occurs in Australian, Nearctic, Neotropical and Palaearctic regions (Fig. 5). In China, the *C. laticaudata* complex was the most abundant, detected in 23 out of 45 lakes (Fig. 1). The lineage "Cla01" of this species complex was dominant in high-altitude habitats (i.e. Qinghai-Tibetan Plateau and Yunnan-Guizhou Plateau), except for one occurrence in the Eastern Plain (LIH; Figs. 1 and 7A). Another genetically divergent lineage "Cla02" of *C. laticaudata* was found in a single location in the Northeast Plain (LHH; Figs. 1 and 7A). The second most abundant species in our dataset was *C. cornuta*, which was detected in 18 out of 45 lakes (Fig. 1). Most of them (14 out of 18) were located in lowland parts of China (i.e. the Eastern Plain and Northeast Plain; Figs. 1 and 7B). *Ceriodaphnia megops* was the third most abundant species (detected in 6 lakes) and was restricted in the Eastern Plain of China (Figs. 1 and 7C). Different *Ceriodaphnia* species complexes (and even mt-lineages) co-existing in the same lake were detected across China (Table 1 and Fig. 1). In particular, *C. cornuta* and *C. laticaudata* coexisted in five localities (Table 1 and Fig. 1). The coexistence of *C. laticaudata* with *C. pulchella* and of *C. cornuta* with *C. megops* was also detected, each in a single location. Sharing of lineages between China and other countries is frequently observed (Fig. 5). For example, lineage "Cla02" of *C. laticaudata* seems to occur in very different parts of the world, including Argentina, Australia, Canada, China, Mexico and the U.S.A (Fig. 5). A star-like COI haplotype network was observed in *C. megops* across China: the haplotype (SJP1) positioned in the center of this network, was found at 5 locations in the Eastern Plain (Fig. 7C). The occurrence of common haplotypes in multiple localities from China was also observed for *C. cornuta* (haplotype QJP1: shared by 7 locations from the Eastern Plain, Northeast Plain and Qinghai-Tibetan Plateau) and *C. laticaudata* (haplotype CNH2: shared by 5 locations from the Qinghai-Tibetan Plateau and Eastern Plain; see Table 1 and Fig. 7). We also found shared COI haplotypes of *C. cornuta* (LIH1 and DLH1) between China and Japan (Fig. 5).

4. Discussion

Ceriodaphnia, despite being a major and widely distributed genus, is one of the most taxonomically confusing among the Daphniidae (e.g. Alonso et al., 2021; Sharma and Kotov, 2013). Combining

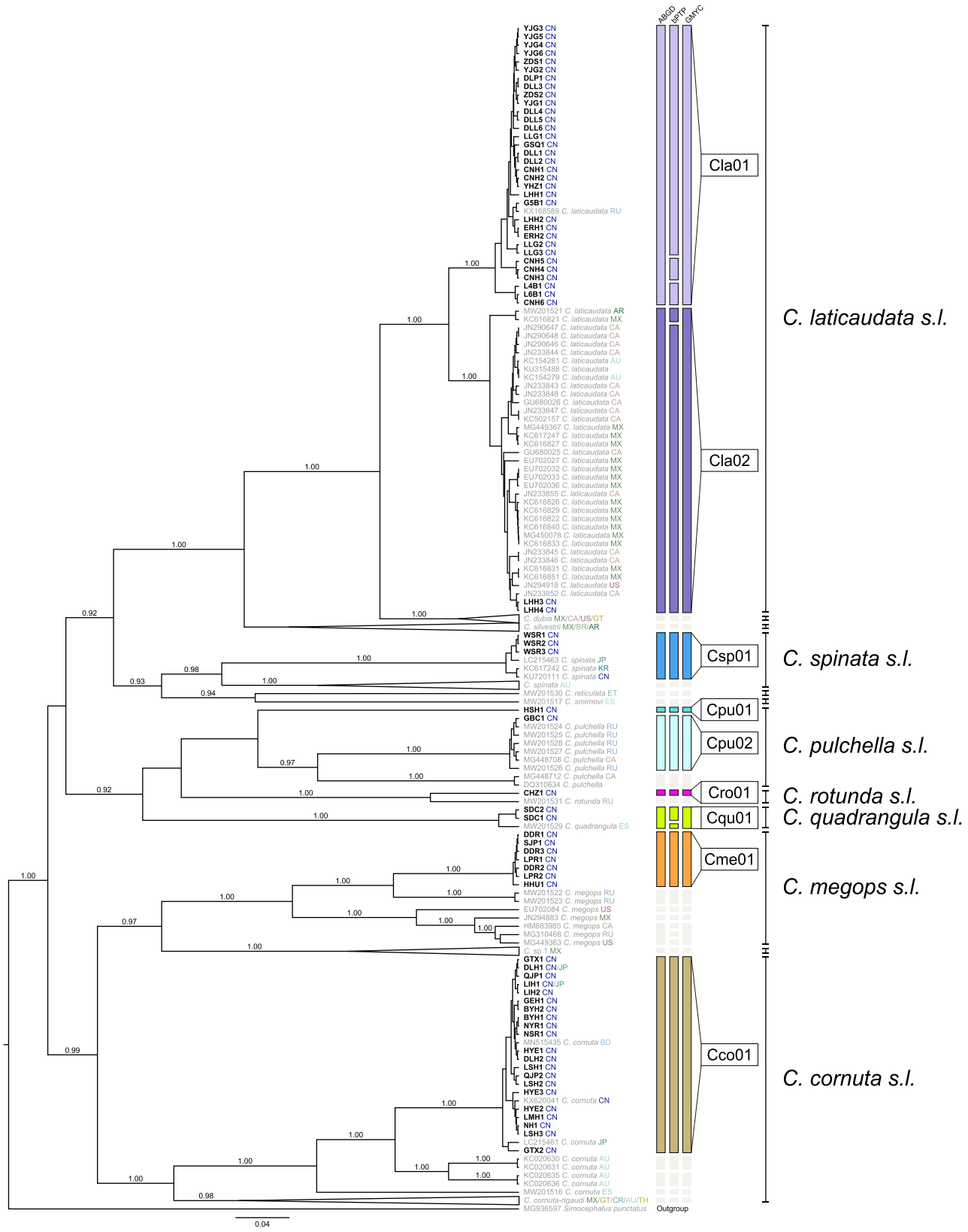

Fig. 5. Bayesian phylogenetic tree of the genus *Ceriodaphnia* according to the mitochondrial COI gene (604 bp). Labels for haplotypes of *Ceriodaphnia* from this study are provided in Table 1; for previously published sequence IDs see Table S1. Posterior probabilities higher than 0.90 are shown above each branch, and support values for within-species relationships are not shown for very short branches. Lineage delimitation according to the ABGD, bPTP and GMYC methods are indicated, and the lineage IDs are shown in boxes at the right. The lineage delimitation is not shown for collapsed portions of the tree. Abbreviations of country names are, AR: Argentina, AU: Australia, BD: Bangladesh, BR: Brazil, CA: Canada, CN: China, CR: Costa Rica, ES: Spain, ET: Ethiopia, GT: Guatemala, JP: Japan, KR: South Korea, TH: Thailand, MX: Mexico, RU: Russia and US: U.S.A.

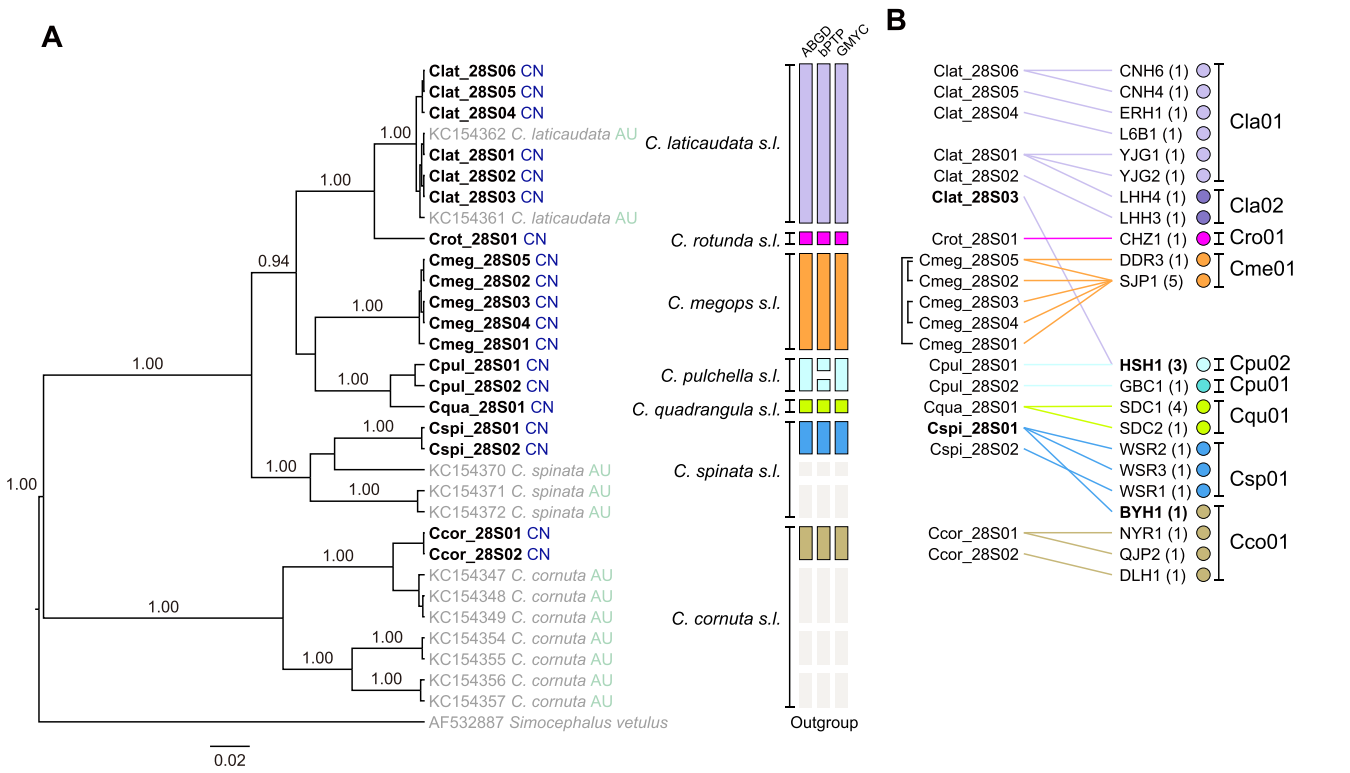

Fig. 6. (A) The Bayesian phylogenetic trees of the 28S region (685 bp) of *Ceriodaphnia*. Posterior probabilities higher than 0.90 are shown above each branch, and support values for within-species relationships are not shown for very short branches. Lineage delimitation was shown according to the ABGD, bPTP and GMYC methods. The list of 28S alleles is provided in Table 2. For abbreviations of country names see Figure 5. (B) Combinations of the variants (showing in lines) of two independently inherited genes for *Ceriodaphnia* specimens that were sequenced for both genes. For specimens with heterozygous 28S, combinations of 28S alleles are shown with vertical square brackets. Numbers in round brackets after the COI haplotype names indicate the number of analyzed specimens. The COI lineage IDs are shown the circles at the right. The mismatch assignment by COI and 28S is shown in bold type.

morphological and genetic analyses, we explored the diversity of *Ceriodaphnia* in China. We identified seven morphospecies (little morphological variation within any single morphospecies regardless of geographical origin), some or all of which might represent species complexes, as sequence data from other parts of the world, together with our new Chinese data, increasingly suggest. Five of these morphospecies were each represented in China by a single mitochondrial lineage and two by two lineages each. Three of the Chinese lineages are new: one within *C. pulchella*, one within *C. rotunda* and one within *C. megops*. Our results suggested an extensive cryptic lineage diversity within *Ceriodaphnia* in China indicative of the presence of species complexes.

Among the three lineage-delimitation methods based on the COI matrix alone, the highest number of lineages was identified by the bPTP analyses (here 13 lineages from China), whereas more conservative results were obtained from GMYC and ABGD (9 lineages from China). The delimitation algorithms based on coalescence (i.e. GMYC and bPTP) have already been shown to overestimate the number of lineages due to large effective population sizes (Klimov et al., 2019) and gene flow (Luo et al., 2018). Thus, our over-split of lineages in bPTP might have resulted from gene flow between different *Ceriodaphnia* lineages, of which mitonuclear discordances was evidence. The presence of nine *Ceriodaphnia* lineages (inferred by ABGD and GMYC) from China was also well supported by the multilocus lineage-delimitation method (BPP) based on the concatenated (COI + 28S) dataset. This method is expected to yield high posterior probabilities for correct species/lineage delimitation under a variety of conditions (Luo et al., 2018). However, the accuracy of the BPP analysis is negatively influenced by gene flow, and may identify intraspecific population structure rather than species boundaries (Sukumaran and Knowles, 2017). The validation of numbers of lineages in *Ceriodaphnia* calls for further studies using multiple datatypes such as phenotypic and ecological information.

Some of the morphospecies we identified have broad distributions. Specifically, *C. cornuta* has been reported from Australia (Sharma and Kotov, 2013), Europe (Alonso et al., 2021), South America (Elias-Gutierrez et al., 2008) and Asia (Makino et al., 2017). Consistent with this, our data showed a wide distribution of *C. cornuta* across China, and a single haplotype (QJP1) of this species complex was detected from the Eastern Plain and Northeast Plain in the east, to the Qinghai-Tibetan Plateau in the west. We also found shared lineages (and even haplotypes) between China and other countries. In particular, lineage "Cla02" of *C. laticaudata* is present in Australia, China and widely across the Americas from Canada to Argentina, suggesting a successful global dispersal of this species. These findings suggest long-distance dispersal and recent colonization events in *Ceriodaphnia*. Biological agents (e.g. birds and cattle) and abiotic agents (e.g. wind) could be important vectors for the passive dispersal of dormant eggs of aquatic zooplankton (Fontaneto, 2019; Incagnone et al., 2015). The worldwide dispersal of *Ceriodaphnia* could also have been mediated via human trade and movement in recent decades.

Interestingly, each of our three new lineages showed a localized distribution within China. This finding is in line with a previous observation from Australia, also based on COI and 28S sequences: three potentially Australian-endemic species from the *C. cornuta* complex were detected there (Sharma and Kotov, 2013). It is a common phenomenon in planktonic cladocerans that species whose distributions were originally assumed to be global are in fact mosaics of many lineages, some of which show local endemism (e.g. Adamowicz et al., 2009; Colbourne et al., 1998; Ni et al., 2019). For instance, several lineages of *Moina* exhibited localized distributions, with some of them restricted to the Qinghai-Tibet Plateau in China (Ni et al., 2019). In spite of the strong dispersal potential of zooplanktonic species, some lineages might have evolved in a small area and have not dispersed beyond that (Ma et al.,

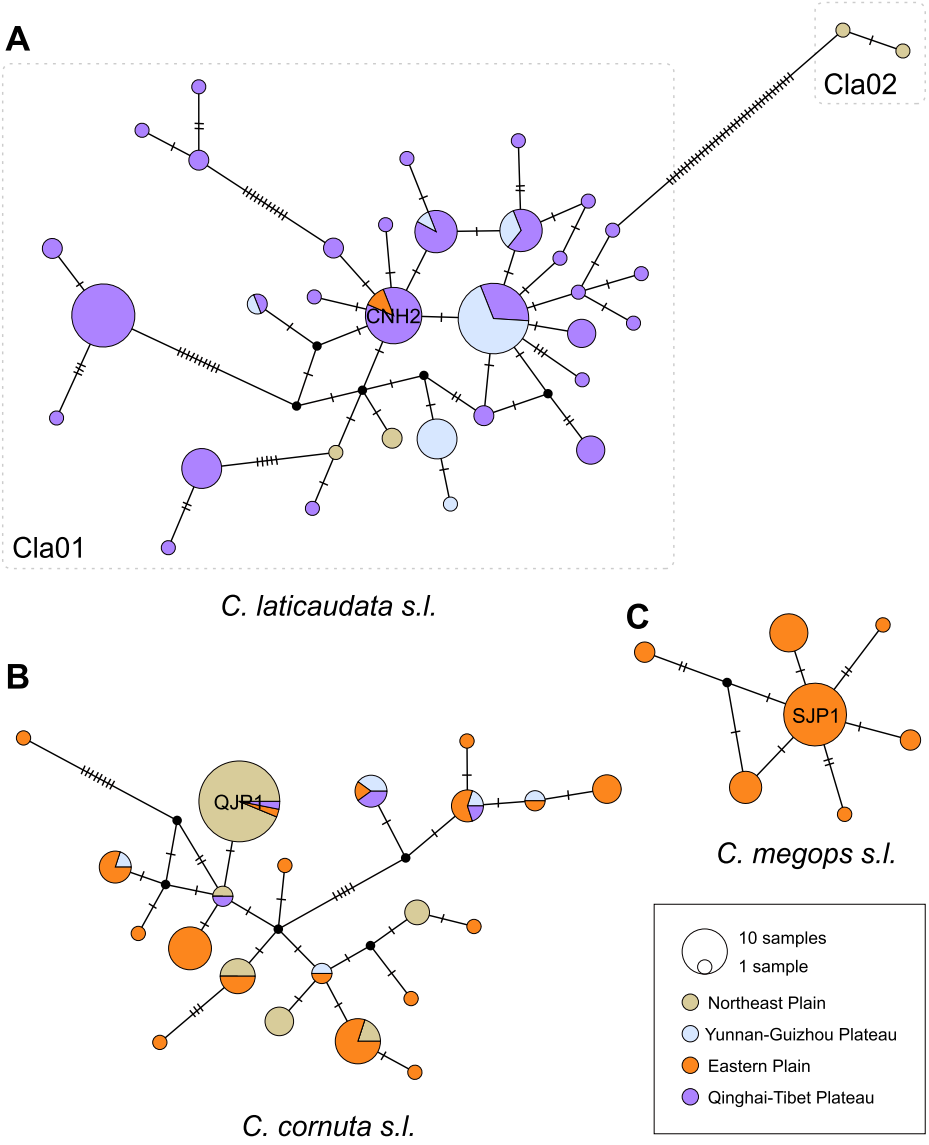

Fig. 7. Haplotype network of (A) *C. laticaudata s.l.*, (B) *C. cornuta s.l.* and (C) *C. megops s.l.* COI sequences (604 bp) found in the present study. Each circle indicates a unique haplotype and its size reflects the number of individuals carrying that haplotype. Color codes allow easy discrimination of four regions from China in the network. The number of tick marks on connecting lines denotes the number of mutations separating haplotypes.

2020; Ni et al., 2019).

Previous studies have often shown that multiple species of cladocerans can occur in a single habitat (e.g. Keller et al., 2008; Pichlova, 1997). For example, within the *D. longispina* species complex, parental species and their hybrids often coexist in the lakes of the European Alps (Keller et al., 2008). Similarly, six littoral species complexes of *Ceriodaphnia* (i.e. *C. affinis* Lilljeborg, 1900, *C. laticaudata*, *C. megops*, *C. reticulata* Jurine, 1820, *C. rotunda* and *C. setosa* Matile, 1890) coexisted in a small shallow fishpond in the Czech Republic (Pichlova, 1997). Consistent with this, we found that different morphospecies of *Ceriodaphnia* co-existed in the same lake, especially from the high-altitude regions of China. Given that *Ceriodaphnia* is most often associated with littoral environments but most of our samples were collected from the pelagic zone, the extent of coexistence of different *Ceriodaphnia* species complexes might be heavily underestimated in our study. Sympatry could provide a possibility for interspecific hybridization such as is frequently observed in cladocerans (Ni et al., 2019; Wang et al., 2021).

Interestingly, we detected discordances between mtDNA and nuclear 28S phylogenies of *Ceriodaphnia*. This phenomenon is indicative of interspecific introgression and hybridization among species/lineages and has been observed in many taxa of animals (Toews and Brelsford, 2012). Examples of mito-nuclear discordances have been often observed in Cladocera (e.g. Thielsch et al., 2017; Ni et al., 2019). Hybridization and subsequent introgression might be a key driver of the observed cyto-nuclear discordance (Gompert et al., 2008; Linnen and Farrell, 2007), as clearly demonstrated in *B. calyciflorus* (Papakostas et al., 2016). However, other explanations for mito-nuclear discordance exist, for example incomplete lineage sorting (Franco et al., 2015; Mckay and Zink, 2010), cannot be totally ruled out from our study. Relevant crossing experiments and/or application of high-resolution nuclear markers (such as SNPs and microsatellites) are needed to provide solid evidence for introgression/hybridization in *Ceriodaphnia*. We note that species can remain distinct despite occasional hybridization with members of other closely related species (Mallet, 2005; Scascitelli et al., 2010; Vonholdt et al., 2010).

The taxonomy of the genus *Ceriodaphnia* remains understudied. Here, using morphological and genetic analyses, we detected a high species/lineage diversity of *Ceriodaphnia* in water bodies in China. We also found cases of mito-nuclear discordance, indicative of interspecific/inter-lineage hybridization and introgression in this genus. *Ceriodaphnia*

has a global distribution, but samples for genetic studies have been taken from few localities: Australia, North America, South America and Europe (and the present study from East Asia). Therefore, much more geographical sampling is required to obtain a global picture of the distribution/diversity of this genus.

5. Data availability

The sequences have been deposited in GenBank: COI: OK561548-OK561618 and 28S: OK491060-OK491078.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author's contributions

MY designed the study, ZD and YY carried out the sampling and molecular work, ZD, YY, DB, WH and MY analyzed and interpreted genetic data. MY wrote the manuscript with the help of ZD. All authors read and approved the final version.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107586.

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