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CONTRIBUTIONS TO ZOOLOGY 94 (2025) 142–179

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Phylogeography and genetic diversity of some *Sinocalanus* species (Copepoda, Calanoida, Centropagidae) detected in inland and estuarine waters of China

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RECEIVED 6 SEPTEMBER 2024; ACCEPTED 24 NOVEMBER 2024;
PUBLISHED ONLINE 13 DECEMBER 2024; PUBLISHED IN ISSUE 31 MARCH 2025
EDITOR: RONALD VONK

Abstract

In spite of the important ecological roles of copepods, their phylogeny and taxonomy remain largely unexplored in China. Here, we investigated the taxonomy and diversity of some *Sinocalanus* species (Copepoda, Calanoida, Centropagidae) in China. We observed two morphospecies

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(*S. tenellus* and *S. doerrii*) from this genus in 42 of 636 Chinese waterbodies examined. Molecular phylogenies inferred from a mitochondrial (mitochondrial cytochrome *c* oxidase subunit I; COI) and a nuclear marker (the nuclear 18S ribosomal RNA gene; 18S) verified the identities of these species and allowed us to amend previous misidentifications. Pronounced ecological separation between the two *Sinocalanus* species detected was found: *S. doerrii* inhabited freshwaters and was widely distributed across China, whereas *S. tenellus* was mainly found in brackish estuaries. One clade of *S. doerrii* had been misidentified (based on molecular data only) as *S. tenellus* in previous works, with consequent mislabeling of the sequences uploaded to GenBank. Additionally, we detected a new mitochondrial lineage of *S. tenellus*, most frequently in Jiaozhou Bay, China, whereas its sibling lineage was distributed in estuaries along the coasts of the East China Sea. Our study contributes to understanding the taxonomy and genetic diversity of the genus *Sinocalanus* in Chinese freshwater and brackish-water ecosystems.

Keywords

biogeography – China – copepods – cryptic lineages – phylogenetics – *Sinocalanus*

Introduction

Planktonic copepods are among the most abundant and widely distributed taxa of metazoans (Boxshall, 2005; Boxshall & Defaye, 2008; Huys & Boxshall, 1991), representing the largest biomass of all animals on Earth (Mauchline et al., 1998). They play a vital role in the food web and biogeochemical cycling in both marine and freshwater aquatic ecosystems (Brandl et al., 2005; Chivers et al., 2017; Turner, 2004). Despite these ecological roles, their taxonomy remains largely underexplored, especially in Chinese freshwater ecosystems.

Copepods are an extremely ancient group (Bradford-Grieve, 2002; Selden et al., 2010). Approximately 14,000 described species have been recognized globally (Boxshall, 2005; Boxshall & Defaye, 2008). Misidentification of copepods often occurs due to the complex and unsettled nature of copepod morphological taxonomy (Blanco-Bercial et al., 2014; Gabrielsen et al., 2012). For example, a recent phylogenetic reconstruction of the genus *Acartia* using mitochondrial cytochrome *c* oxidase subunit I (mtCOI) gene sequences found many potential misidentifications of sequences deposited in GenBank (Figueroa et al., 2020). Indeed, traditional copepod taxonomy depending only on morphology is now known to be inadequate to reliably distinguish some of the copepod species, especially when the morphological differences are subtle (Choquet et al., 2018; Knowlton, 1993) or the rates of morphological evolution and speciation are incongruent (Lee & Frost, 2002; Marrone et al., 2013). Recently, molecular systematics has been successfully applied in taxonomy and phylogeny of copepods (Adamowicz et al., 2010; Baek et al., 2016; Blanco-Bercial et al., 2011; Bucklin et al., 1999).

Copepods are renowned for their cryptic diversity (Eyun et al., 2007; Goetze, 2003; Lee, 2000), which has been clearly demonstrated using molecular tools (Andrews et al., 2014; Chen & Hare, 2011; Marrone et al., 2013). For instance, *Eudiaptomus hadzici* (Copepoda, Calanoida) is comprised of four highly differentiated lineages with evidence of cryptic diversity in the Western Balkans (Previšić et al., 2016). Substantial genetic divergences have also been observed within the genus *Hemidiaptomus* (Copepoda, Calanoida), suggesting the existence of cryptic species and/or lineages (Marrone et al., 2010). A recent study detected a high level of lineage diversity in two *Tropodiaptomus* morphospecies, i.e., *T. vicinus* and *T.* cf. *lanaonus* (Copepoda, Calanoida), suggesting the presence of cryptic or pseudocryptic species in this genus (Saetang et al., 2022).

The genus *Sinocalanus* Burckhardt, 1913 (Copepoda, Calanoida, Centropagidae) is endemic to East Asia (Adamowicz et al., 2010). This genus is widely distributed in Asian estuarine and freshwater habitats, especially in China (Adamowicz et al., 2010; Baek et al., 2016; Sakaguchi et al., 2011; Shen & Song, 1979; Wang et al., 2018). Non-native *Sinocalanus* populations occur in America (Bollens et al., 2012; Orsi et al., 1983). Eight species are listed in both WoRMS (WoRMS Editorial Board, 2024) and the "World of Copepods" database (Walter, 2024): *Sinocalanus sinensis* (Poppe, 1889), *Sinocalanus doerrii* (Brehm, 1909), *Sinocalanus mystrophorus* Burchhardt, 1913, *Sinocalanus megalolimnetis* Burckhardt, 1913, *Sinocalanus schachti* Burckhardt, 1913, *Sinocalanus tenellus* (Kikuchi, 1928), *Sinocalanus solstitialis* Brehm, 1923 and *Sinocalanus laevidactylus* Shen & Tai, 1964. One of them, *S. mystrophorus*, is regarded as a junior synonym of *S. doerrii* according to Shen and Song (1979). Thus, the three subspecies of *S. mystrophorus* – *Sinocalanus mystrophorus supolites* Burckhardt, 1913, *Sinocalanus mystrophorus cyanopotamius* Burckhardt, 1913, and *Sinocalanus mystrophorus megalolimnetis* Burckhardt, 1913 – must also fall as synonyms of *S. doerrii s.s.*. Similarly, *S. tenellus* has four recognized subspecies, including *Sinocalanus tenellus continentalis* Smirnov, 1932, *Sinocalanus tenellus insulanus* Smirnov, 1932, *S. tenelleus tenelleus* (Kikuchi, 1928) and *Sinocalanus tenellus sachalinensis* Rylov, 1932. *Sinocalanus schachti* Burckhardt, 1913 is often not recognized as a valid species, but only as a variety of *S. sinensis* (Dussart & Defaye, 2002; Shen & Song, 1979). Therefore, the genus *Sinocalanus* includes five valid species: *S. sinensis* (Poppe, 1889), *S. doerrii* (Brehm, 1909), *S. tenellus* (Kikuchi, 1928), *S. solstitialis* Brehm, 1923 and *S. laevidactylus* Shen & Tai, 1964. All five species have been recorded in China based on their morphology (Shen & Song, 1979). The phylogenetic relationships within the genus *Sinocalanus* remain largely unexplored. A previous study explored the molecular phylogeny and biogeographic history of Centropagidae, showing that *Sinocalanus* was likely to have colonized Asian estuarine and freshwaters in the Pleistocene and subsequently diversified there (Adamowicz et al., 2010). Our previous study using partial mtCOI sequences claimed that *S. tenellus* fell into two well-separated clades and was rendered paraphyletic by the inclusion of a clade containing *S. sinensis* (Wang et al., 2018). In that study, one clade of "*S. tenellus*" was misidentified (now confirmed as belonging to *S. doerrii*), with consequent mislabeling of the sequences uploaded to GenBank (see supplementary table S1).

This study aims at an assessment of the species/lineage diversity of *Sinocalanus* in inland and estuarine waters from China. This was done by first identifying specimens based on morphology. Subsequently, sequences of two gene fragments (the mitochondrial cytochrome oxidase *c* subunit I (COI), and the nuclear 18S ribosomal RNA gene (18S)) were subjected to phylogenetic and other analyses. Our extensive sampling covered 636 waterbodies from the East China Plain, Inner Mongolia-Xinjiang Plateau and the Northeast China Plain, and *Sinocalanus* populations were found at 42 sampling sites. As frequently observed in copepods (Barrera-Moreno et al., 2015; Guinto et al., 2023; Lorenzo et al., 2023), we expected to detect a high lineage diversity of *Sinocalanus* in China, with the possible presence of morphologically cryptic lineages.

Materials and methods

Sampling

Zooplankton sampling was carried out in 636 localities across China from 2014 to 2023. Samples were collected using a plankton net (mesh size 64 μm) hauled vertically through the water column from three to four different sites per locality. Samples collected from the same locality were pooled and preserved in 95% ethanol at 4 °C in the laboratory for further analyses.

Morphological examination

Valid *Sinocalanus* species were identified based on morphological descriptions (Orsi et al., 1983; Shen & Song, 1979). Animals were randomly 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). In this way, we identified two *Sinocalanus* species in our samples: *S. doerrii* and *S. tenellus*. For each *Sinocalanus* morphospecies, approximately five adult males and five adult (egg-bearing) females were examined and the morphology of adults of each sex was drawn based on microphotographs taken by a camera connected to the optical microscope.

DNA extraction and sequencing

On average, eight *Sinocalanus* individuals (ranging from 1 to 24; based on preliminary morphological identification) per locality were randomly selected. DNA was extracted from each animal using the proteinase-K method (Schwenk et al., 1998). Specifically, each animal was mixed with H3 buffer with proteinase K (20 μL), containing final concentrations of 10 mM Tris-HCl, 50 mM KCl, 0.005% Tween 20, 0.005% NP-40 and 10 mg/mL proteinase K (MERCK, Germany). Then, the mixed solution was incubated in a 55 °C water-bath with mild shaking for 16–20 h. Finally, proteinase K was denatured through a 12 min incubation at 95 °C. DNA samples were centrifuged briefly and stored at 4 °C for genetic analyses.

A total of 346 individuals (~eight individuals per location; table 1) were selected for the sequencing of a fragment of mtCOI

(680 bp) gene. The amplification was carried out using the primer pair LCO1490 and HCO2918 (Folmer et al., 1994). The PCR was conducted in a total volume of 20 μL, comprising 10 μL 2 × Hieff™ PCR Master Mix (With Dye), 6 μL ddH_2O, 1 μL 10 μM solution of each primer and 2 μL DNA sample. The PCR conditions were as follows: incubation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 45 °C for 45 s and extension at 72 °C for 45 s; and a final extension at 72 °C for 7 min. Five individuals on average from each population (206 individuals in total; table 1) were also chosen for sequencing of a portion of the nuclear gene for 18S ribosomal RNA (18S; 1,805 bp). The PCR procedure was the same as for COI, except for the primers used: 18Sf (5′-TAC CTG GTT GAT CCT GCC AG-3′) and 18Sr (5′-TAA TGA TCC TTC CGC AGG TTC AC-3′). The PCR conditions were as follows: incubation at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min and extension at 72 °C for 1 min 30 seconds; and a final extension at 72 °C for 7 min. Cloning was performed following the protocol established in our previous studies (Ni et al., 2019; Wang et al., 2021) due to the potential for multiple heterozygous sites in 18S fragments. Ten clones were sequenced for each 18S PCR product, and only nucleotide sequences that were identical and obtained at least twice per PCR product were retained for further analysis. All COI PCR products were sequenced in the forward direction and all 18S PCR products were sequenced in both directions on an ABI PRISM 3730 DNA capillary sequencer using the PCR primers as sequencing primers (Sangon Biotech Co., Ltd, Shanghai, China). Subsequently, all the chromatograms of COI and 18S sequences underwent careful examination and manual correction for scoring errors in MEGA X (Kumar et al., 2018). All new sequences have been submitted to GenBank under accession numbers: COI: PP750470–750551 and 18S: PP755206–755333.

Genetic diversity and phylogeny

All of the obtained COI sequences were aligned using the Clustal W algorithm (Thompson et al., 1994) and subsequently translated into amino acids in MEGA X to identify premature stop codons that might indicate that nuclear pseudogenes of mitochondrial origin had been sequenced (Song et al., 2008). Then, unique haplotypes were identified using DNASP 6 (Librado & Rozas, 2009). All unique COI haplotypes (table 1), along with all 61 published sequences of *Sinocalanus* species retrieved from GenBank (supplementary table S1), were aligned in MEGA X. For each *Sinocalanus* species, the number of individuals for sequencing per location (N_1), the number of haplotypes per location (N_2), population haplotype diversity (H) and nucleotide diversity (π) were computed in DNASP 6 for the COI marker (table 1). In order to improve the confidence in the evolutionary relationships inferred, we concatenated the COI gene and the 18S gene sequences (COI+18S) in PhyloSuite v1.2.2 (Zhang et al., 2020). Similarly, the unique COI+18S haplotypes (table 1) were identified in DNASP 6 and then aligned in MEGA X. For each species, the number of individuals for COI+18S

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (name, abbreviation and geographical position) and genetic characterization of sequenced individuals. N_1, the number of individuals for COI sequencing per location; N_2, the number of COI haplotypes at that location; H, the population COI haplotype diversity; π, the population COI nucleotide diversity; N_3, the number of individuals for COI+18S sequencing per location; N_4, the number of concatenated COI+18S haplotypes per location; NA, not applicable; na, not available. See supplementary table S2 for additional information on each specimen sequenced.

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	**N_1**	**N_2**	**H**	**π**	**COI haplotype (number of individuals)**	**N_3**	**N_4**	**Concatenated haplotype (number of individuals)**
East China Plain										
Baoying Lake (BYH)	33.16 N, 119.24 E	*S. doerrii*	9	6	0.889	0.00301	C-AMT1 (2), C-AMT4 (1), C-AMT6 (3), C-BYH8 (1), C-BYH9 (1), C-BYH10 (1)	6	9	CS-BYH1 (1), CS-BYH3 (1), CS-BYH4A (1), CS-BYH4B (1), CS-BYH5A (1), CS-BYH5B (1), CS-BYH6 (1), CS-BYH7A (1), CS-BYH7B (1)
Chaohu Lake (CHH)	31.60 N, 117.86 E	*S. doerrii*	1	1	NA	NA	C-AMT1 (1)	0	0	na
Chunyanglu Pond (CYL)	36.31 N, 120.20 E	*S. tenellus*	14	4	0.495	0.00096	C-CYL1 (10), C-CYL9 (1),	12	6	CS-CYL1 (4), CS-CYL3 (1),

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					**Mitochondrial and nuclear maker (COI+18S)**		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	N_1	N_2	**H**	π	**COI haplotype (number of individuals)**	N_3	N_4	**Concatenated haplotype (number of individuals)**
							C-CYL10 (2), C-CYL25 (1)			CS-CYL10 (1), CS-CYL12 (4), CS-CYL25 (1), CS-CYL26 (1)
Dongfanghong Reservoir (DFH)	32.21 N, 119.00 E	*S. doerrii*	4	4	0.900	0.00336	C-AMT4 (1), C-AMT6 (1), C-DFH1 (1), C-DFH4 (1)	0	0	na
Daguantang Reservoir (DGT)	32.26 N, 118.47 E	*S. doerrii*	1	1	NA	NA	C-AMT6 (1)	0	0	na
Daming Lake (DMH)	32.25 N, 118.31 E	*S. doerrii*	7	4	0.810	0.00300	C-AMT1 (1), C-AMT6 (2), C-DMH3 (3), C-DMH4 (1)	0	0	na
Dishui Lake (DSH)	30.90 N, 121.96 E	*S. doerrii*	1	1	NA	NA	C-AMT6 (1)	1	1	CS-DSH13 (1)
		S. tenellus	1	1	NA	NA	C-DSH1 (1)	0	0	na
Dongtan-A (DTA)	31.61 N, 121.87 E	*S. tenellus*	5	4	0.900	0.00252	C-DSH1 (1), C-DTA6 (1), C-DTA7 (2), C-DTA10 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	Latitude, longitude	Taxon	N_1	N_2	H	π	COI haplotype (number of individuals)	N_3	N_4	Concatenated haplotype (number of individuals)
Dongzhang Reservoir (DZR)	25.70 N, 119.26 E	*S. doerrii*	24	13	0.902	0.00450	C-AMT1 (3), C-AMT4 (1), C-AMT6 (2), C-DMH3 (7), C-DZR2 (1), C-DZR3 (1), C-DZR4 (2), C-DZR11 (1), C-DZR24 (2), C-DZR26 (1), C-DZR29 (1), C-DZR31 (1), C-DZR34 (1)	9	15	CS-DZR21 (1), CS-DZR23 (1), CS-DZR24A (1), CS-DZR24B (1), CS-DZR25A (1), CS-DZR25B (1), CS-DZR26A (1), CS-DZR26B (1), CS-DZR27A (1), CS-DZR27B (1), CS-DZR28 (1), CS-DZR3A (1), CS-DZR3B (1), CS-DZR9A (1), CS-DZR9B (1)
Fuwan Reservoir (FWR)	32.26 N, 118.44 E	*S. doerrii*	17	10	0.904	0.00357	C-AMT1 (4), C-AMT4 (1), C-AMT6 (4), C-BYH8 (2), C-BYH9 (1),	9	15	CS-FWR22A (1), CS-FWR22B (1), CS-FWR24 (2), CS-FWR25B (1), CS-FWR26A (1),

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	Latitude, longitude	Taxon	N_1	N_2	H	π	COI haplotype (number of individuals)	N_3	N_4	Concatenated haplotype (number of individuals)
							C-DMH3 (1), C-FWR11 (1), C-FWR12 (1), C-FWR16 (1), C-FWR29 (1)			CS-FWR26B (1), CS-FWR27A (1), CS-FWR27B (1), CS-FWR28A (1), CS-FWR28B (1), CS-FWR29A (1), CS-FWR29B (1), CS-FWR30A (1), CS-FWR30B (1), CS-FWR31 (1)
Gaoyou Lake (GYH)	32.53 N, 119.15 E	*S. doerrii*	13	8	0.897	0.00448	C-AMT1 (2), C-AMT6 (4), C-BYH8 (2), C-BYH9 (1), C-GYH4 (1), C-GYH5 (1), C-GYH6 (1), C-GYH11 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					**Mitochondrial and nuclear maker (COI+18S)**		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	N_1	N_2	**H**	π	**COI haplotype (number of individuals)**	N_3	N_4	**Concatenated haplotype (number of individuals)**
Hewangba Reservoir (HWB)	32.53 N, 118.83 E	*S. doerrii*	11	5	0.855	0.00362	C-AMT1 (3), C-AMT4 (1), C-AMT6 (2), C-BYH8 (2), C-HWB4 (3)	0	0	na
Hongze Lake (HZH)	33.29 N, 118.72 E	*S. doerrii*	8	8	1.000	0.00618	C-AMT1 (1), C-BYH8 (1), C-HZH5 (1), C-HZH9 (1), C-HZH10 (1), C-HZH13 (1), C-HZH15 (1), C-HZH16 (1)	6	8	CS-HZH9 (1), CS-HZH10 (1), CS-HZH11A (1), CS-HZH11B (1), CS-HZH12 (1), CS-HZH13 (1), CS-HZH16A (1), CS-HZH16B (1)
Jiangbinzhilu River (JBZ)	29.97 N, 121.74 E	*S. tenellus*	7	1	0.000	0.00000	C-DTA10 (7)	7	1	CS-JBZ1 (7)
Jinniushan Reservoir (JNS)	32.47 N, 118.95 E	*S. doerrii*	6	5	0.933	0.00478	C-AMT1 (1), C-AMT6 (2), C-AMT7 (1), C-JNS21 (1), C-JNS22 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	**N_1**	**N_2**	**H**	**π**	**COI haplotype (number of individuals)**	**N_3**	**N_4**	**Concatenated haplotype (number of individuals)**
Jingtian Lake (JTH)	31.21 N, 121.55 E	*S. doerrii*	4	4	1.000	0.00787	C-AMT1 (1), C-JTH3 (1), C-JTH4 (1), C-JTH5 (1)	0	0	na
Lijiangxilu River (LJX)	35.94 N, 120.17 E	*S. tenellus*	1	1	NA	NA	C-CYL1 (1)	0	0	na
Luoma Lake (LMH)	34.10 N, 118.17 E	*S. doerrii*	9	4	0.750	0.00282	C-AMT1 (1), C-AMT4 (3), C-AMT6 (4), C-LMH15 (1)	4	5	CS-BYH1 (1), CS-LMH9 (1), CS-LMH11 (1), CS-LMH15A (1), CS-LMH15B (1)
Minjiang River (MJR)	26.06 N, 119.27 E	*S. doerrii*	3	3	1.000	0.00350	C-AMT4 (1), C-AMT6 (1), C-MJR7 (1)	0	0	na
Pingtao Highway Pond (PTH)	29.95 N, 120.72 E	*S. doerrii*	3	3	1.000	0.00466	C-AMT4 (1), C-BYH8 (1), C-JTH5 (1)	0	0	na
Shahequ Reservoir (SHQ)	32.20 N, 119.02 E	*S. doerrii*	6	3	0.600	0.00233	C-AMT1 (4), C-KDL11 (1), C-SHQ11 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	**N_1**	**N_2**	**H**	**π**	**COI haplotype (number of individuals)**	**N_3**	**N_4**	**Concatenated haplotype (number of individuals)**
Shanhu Reservoir (SHR)	32.43 N, 118.78 E	*S. doerrii*	10	6	0.889	0.00365	C-AMT4 (2), C-AMT6 (2), C-BYH8 (3), C-BYH9 (1), C-DMH3 (1), C-SHR13 (1)	8	8	CS-BYH1 (1), CS-BYH3 (1), CS-SHR3 (1), CS-SHR4 (1), CS-SHR6A (3), CS-SHR6B (1), CS-SHR13A (1), CS-SHR13B (1)
Shijiu Lake (SJH)	31.46 N, 118.86 E	*S. doerrii*	8	4	0.750	0.00206	C-AMT4 (4), C-AMT6 (2), C-SJH4 (1), C-SJH9 (1)	3	4	CS-BYH3 (1), CS-SJH12 (1), CS-SJH24A (1), CS-SJH24B (1)
Sanyou Reservoir (SYR)	32.21 N, 119.00 E	*S. doerrii*	13	4	0.679	0.00296	C-AMT1 (5), C-AMT6 (6), C-SYR2 (1), C-SYR9 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	**N_1**	**N_2**	**H**	**π**	**COI haplotype (number of individuals)**	**N_3**	**N_4**	**Concatenated haplotype (number of individuals)**
Tongxin Lake (TXH)	29.93 N, 121.62 E	*S. doerrii*	8	6	0.929	0.00805	C-AMT11 (2), C-SHH12 (1), C-TXH8 (1), C-TXH9 (1), C-TXH12 (2), C-TXH14 (1)	3	4	CS-TXH10 (1), CS-TXH11 (1), CS-TXH14A (1), CS-TXH14B (1)
Wusongpaotai (WSPT)	31.40 N, 121.51 E	*S. doerrii*	9	6	0.889	0.00719	C-AMT1 (3), C-AMT6 (2), C-AMT11 (1), C-AMT14 (1), C-BYH8 (1), C-DFH4 (1)	5	7	CS-WSPT1 (1), CS-WSPT4 (1), CS-WSPT11A (1), CS-WSPT11B (1), CS-WSPT21 (1), CS-WSPT27A (1), CS-WSPT27B (1)
Xiangmao River (XMH)	36.27 N, 120.29 E	*S. tenellus*	13	3	0.410	0.00103	C-CYL1 (10), C-CYL10 (2), C-XMH22 (1)	8	6	CS-CYL1 (1), CS-CYL3 (3), CS-CYL26 (1), CS-XMH3 (1), CS-XMH21 (1), CS-XMH22 (1)

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	Latitude, longitude	Taxon	N_1	N_2	H	π	COI haplotype (number of individuals)	N_3	N_4	Concatenated haplotype (number of individuals)
Yuetang Reservoir (YTR)	32.24 N, 119.05 E	*S. doerrii*	4	4	1.000	0.00291	C-AMT1 (1), C-AMT4 (1), C-AMT6 (1), C-BYH8 (1)	0	0	na
Yongjiangdadao (YJD)	29.90 N, 121.63 E	*S. doerrii*	10	4	0.711	0.00198	C-AMT11 (5), C-SHH12 (3), C-YJD9 (1), C-YJD26 (1)	2	2	CS-YJD7 (1), CS-YJD25 (1)
Yangmaogou River (YMG)	36.31 N, 120.26 E	*S. tenellus*	15	2	0.133	0.00023	C-CYL1 (14), C-YMG25 (1)	15	4	CS-CYL1 (2), CS-CYL3 (10), CS-CYL12 (2), CS-YMG25 (1)
Zaolin Reservoir (ZLR)	32.33 N, 119.07 E	*S. doerrii*	12	8	0.924	0.00373	C-AMT1 (3), C-AMT4 (2), C-AMT6 (1), C-BYH8 (2), C-DFH4 (1), C-DZR34 (1), C-ZLR4 (1), C-ZLR8 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	Latitude, longitude	Taxon	N_1	N_2	H	π	COI haplotype (number of individuals)	N_3	N_4	Concatenated haplotype (number of individuals)
Zhuangyuanao Pond (ZYA)	29.71 N, 121.28 E	*S. doerrii*	2	2	1.000	0.00350	C-ZYA1 (1), C-ZYA2 (1)	0	0	na
Zhuangyuanao Reservoir (ZYR)	29.71 N, 121.28 E	*S. doerrii*	6	3	0.733	0.00152	C-AMT11 (2), C-SHH12 (3), C-SHH21 (1)	0	0	CS-BYH1 (1), CS-BYH3 (1), CS-BYH4A (1), CS-BYH4B (1), CS-BYH5A (1), CS-BYH5B (1), CS-BYH6 (1), CS-BYH7A (1), CS-BYH7B (1)
Inner Mongolia-Xinjiang Plateau										
Kundulun lake (KDL)	40.57 N, 109.77 E	*S. doerrii*	9	3	0.556	0.00175	C-AMT6 (6), C-KDL6 (2), C-KDL11 (1)	7	10	CS-BYH1 (1), CS-KDL11A (1), CS-KDL11B (1), CS-KDL3A (1), CS-KDL3B (1), CS-KDL4 (1),

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	**N_1**	**N_2**	**H**	**π**	**COI haplotype (number of individuals)**	**N_3**	**N_4**	**Concatenated haplotype (number of individuals)**
										CS-KDL6 (1), CS-KDL7A (1), CS-KDL7B (1), CS-KDL9 (1)
Shahu Lake (SHH)	38.80 N, 106.36 E	*S. doerrii*	15	9	0.924	0.01022	C-AMT1 (2), C-AMT4 (3), C-AMT6 (3), C-DMH3 (1), C-HZH15 (1), C-SHH11 (1), C-SHH12 (2), C-SHH21 (1), C-SHH23 (1)	10	15	CS-BYH6 (1), CS-SHH9 (2), CS-SHH11A (1), CS-SHH11B (1), CS-SHH12 (1), CS-SHH13A (1), CS-SHH13B (1), CS-SHH14A (1), CS-SHH14B (1), CS-SHH15B (1), CS-SHH16 (1)
Wuliangsuhai (WLSH)	40.66 N, 108.77 E	*S. tenellus*	9	3	0.417	0.01355	C-WLSH1 (7), C-WLSH6 (1), C-WLSH8 (1)	7	2	CS-WLSH1 (5), CS-WLSH2 (2)

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					**Mitochondrial and nuclear maker (COI+18S)**		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	N_1	N_2	**H**	**π**	**COI haplotype (number of individuals)**	N_3	N_4	**Concatenated haplotype (number of individuals)**
Northeast China Plain										
Amuta Pao (AMT)	46.30 N, 124.06 E	*S. doerrii*	9	7	0.944	0.00796	C-AMT1 (2), C-AMT2 (1), C-AMT4 (1), C-AMT6 (2), C-AMT7 (1), C-AMT11 (1), C-AMT14 (1)	3	3	CS-AMT11 (1), CS-AMT14 (1), CS-AMT15 (1)
Donghu Reservoir (DHR)	46.63 N, 125.61 E	*S. doerrii*	10	3	0.711	0.00155	C-AMT1 (4), C-AMT4 (4), C-AMT6 (2)	7	11	CS-DHR9A (2), CS-DHR9B (2), CS-DHR10A (1), CS-DHR10B (1), CS-DHR12A (1), CS-DHR12B (1), CS-DHR13A (1), CS-DHR13B (1), CS-DHR14A (1), CS-DHR14B (1), CS-DHR15 (1)

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	**N_1**	**N_2**	**H**	**π**	**COI haplotype (number of individuals)**	**N_3**	**N_4**	**Concatenated haplotype (number of individuals)**
Hongqi Reservoir (HQR)	46.21 N, 125.08 E	*S. doerrii*	11	7	0.927	0.01030	C-AMT1 (2), C-AMT4 (1), C-AMT6 (2), C-AMT7 (1), C-AMT11 (2), C-DMH3 (1), C-DZR34 (2)	8	11	CS-HQR9 (1), CS-HQR10 (1), CS-HQR11A (1), CS-HQR11B (1), CS-HQR12 (1), CS-HQR13A (1), CS-HQR13B (1), CS-HQR14 (1), CS-HQR15 (1), CS-HQR16A (1), CS-HQR16B (1)
Huoshaoli Pao Lake (HSL)	46.66 N, 124.04 E	*S. doerrii*	2	2	1.000	0.02273	C-AMT4 (1), C-AMT11 (1)	0	0	na

TABLE 1 List of localities where *Sinocalanus* species were collected for this study (*cont.*)

			Mitochondrial marker (COI)					Mitochondrial and nuclear maker (COI+18S)		
Waterbody (abbreviation)	**Latitude, longitude**	**Taxon**	N_1	N_2	**H**	π	**COI haplotype (number of individuals)**	N_3	N_4	**Concatenated haplotype (number of individuals)**
Pond Nearby Lianhuan Lake (LHF)	46.49 N, 124.24 E	*S. doerrii*	8	4	0.750	0.00368	C-AMT1 (2), C-AMT4 (1), C-AMT6 (4), C-LHF9 (1)	8	11	CS-BYH1 (1), CS-FWR24 (1), CS-LHF1 (1), CS-LHF2A (1), CS-LHF2B (1), CS-LHF3 (1), CS-LHF7A (1), CS-LHF7B (1), CS-LHF9 (1), CS-LHF10B (1), CS-LHF11B (1)
Nanyin Reservoir (NYR)	45.95 N, 124.58 E	*S. doerrii*	8	6	0.929	0.00793	C-AMT1 (1), C-AMT4 (2), C-AMT6 (2), C-AMT11 (1), C-DMH3 (1), C-NYR16 (1)	0	0	na

sequencing per location (N_3) and the number of COI+18S haplotypes per location (N_4) were calculated in DNASP 6 (table 1). The codes of COI and "COI+18S" haplotypes corresponding to each of the specimens are listed in supplementary table S2.

The evaluation of potential substitution saturation, which could lead to the loss of phylogenetic signal in COI sequences, was conducted using DAMBE 5 (Xia, 2013). A COI Bayesian phylogenetic tree was constructed using the program in MrBayes v.3.2.7 (Ronquist et al., 2012). Two independent runs were performed for 20 million generations, with one cold and three heated chains, and with a tree being sampled every 2,000 generations. All published COI sequences of *Sinocalanus* (available for only three species of this genus: *S. tenellus*, *S. doerrii* and *S. sinensis*) were included in this Bayesian tree. A sequence of *Centropages tenuiremis* Thompson & Scott, 1903 (GenBank ID: KX620039), a member of the Calanoida phylogenetically close to *Sinocalanus* (Adamowicz et al., 2010), was used as an outgroup. The best substitution model (Hasegawa-Kishino-Yano model + Gamma + invariant sites, HKY + G + I) was determined through the corrected Akaike Information Criterion in jModeltest v. 2.1.7 (Darriba et al., 2012). Tracer v1.7 (Rambaut et al., 2018) was then used to confirm that adequate generations had been computed. A maximum parsimony (MP) tree was run based on COI dataset using PAUP* v.4.0a (Swofford, 2003). Heuristic searches used random sequence addition and Tree Bisection-Reconnection (TBR) branch swapping, with a maximum of 100 trees retained per search. Bootstrap support was calculated through 1000 replicates, and were then mapped onto the COI Bayesian consensus tree.

Similarly, a Bayesian phylogenetic tree based on the COI+18S datasets was constructed in MrBayes using the General Time Reversible Model + Gamma + invariant sites (GTR+G+I) substitution model, the best model found using jModeltest. COI and 18S sequences of *C. tenuiremis* (GenBank ID: KX620039 and GU969162) were concatenated in the same way and used as an outgroup. An MP tree based on COI+18S dataset was also constructed in PAUP* using the same method (see above), with heuristic searches and TBR branch swapping. Bootstrap support was calculated through 1,000 replicates, and then mapped onto the COI+18S Bayesian consensus tree.

Detection of new lineage and phylogeographic analyses

We employed three independent species-delimitation methods to investigate the number of species/lineages in *Sinocalanus* across China for both COI and COI+18S datasets. These methods were the general mixed Yule coalescent model (GMYC; Pons et al., 2006), a Bayesian implementation of Poisson Tree Processes model (bPTP; Zhang et al., 2013) and Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012). GMYC analysis was carried out with a single threshold (Fujisawa & Barraclough, 2013) using its webserver (https://species.h-its.org/gmyc/). bPTP calculations were conducted on the bPTP websever (http://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 using BEAST 1.8 (Bouckaert et al., 2014), with a tree recorded every 1,000 generations among 40,000,000 generations. The initial 25% of trees were discarded as burn-in, and the final 30,000 sampled trees summarized using TreeAnnotator. ABGD analysis was conducted on the online server (http://wwwabi.snv.jussieu.fr/public/abgd/) with the default settings. Finally, we constructed COI haplotype networks for *Sinocalanus* using the TCS method (Clement et al., 2000) in PopART 1.7 (Leigh & Bryant, 2015) in order to visualize genealogical relationships within species/lineages.

Environmental preferences

We employed factor analysis based on direct ordination to elucidate the relationship between *Sinocalanus* lineages and environmental factors using the "vegan" package (Oksanen et al., 2015) in R. The species data matrix was initially analyzed using Detrended Correspondence Analysis (DCA) to determine whether the distribution pattern of the species data was linear or unimodal. The explanatory variables for the sites included two predictors of geographical position: longitude and latitude, and six environmental descriptors: salinity (a categorical descriptor with two levels: freshwater and brackish water), surface area, average depth, eutrophication level (a categorical descriptor with two levels: eutrophic and mesotrophic), whether or not the site freezes in winter, and type (a categorical descriptor with two levels: natural and artificial). Categorical variables were converted to dummy variables, and the remaining numerical environmental variables were standardized. We performed Redundancy Analysis (RDA) to further explore the relationships between species and environmental variables. A one-way ANOVA was then applied with the presence/absence of each *Sinocalanus* lineage as a main factor (using only data relating to the waterbodies where *Sinocalanus* were found) to determine whether these lineages were nonrandomly distributed along each component of the RDA.

Results

Morphological examination

Representatives of the genus *Sinocalanus* were detected in 42 out of the 636 localities, covering the East China Plain (33 localities), Inner Mongolia-Xinjiang Plateau (3 localities) and the Northeast China Plain (6 localities; fig. 1 and table 1). We did not detect *Sinocalanus* from waterbodies on the Qinghai–Tibetan Plateau. The genus *Sinocalanus* (figs. 2 and 3) can be easily identified by six key characteristics: (1) cephalothorax narrow-oval in shape, with 5–6 segments, (2) last pediger bears small spines on both sides on posterior corner, (3) antenna I reaches middle of apical caudal setae, (4) exopod segment 2 of legs V in females bears a long spine extending inward, (5) exopod of right leg V in males 2-segmented with hooked end; and (6) endopods of legs V in males 3-segmented and longer than exopods.

In the present survey, we found only two *Sinocalanus* morphospecies across China: *S. doerrii* and *S. tenellus*.

Sinocalanus doerrii: Female body length 1.50–1.75 mm (fig. 2A), male body length

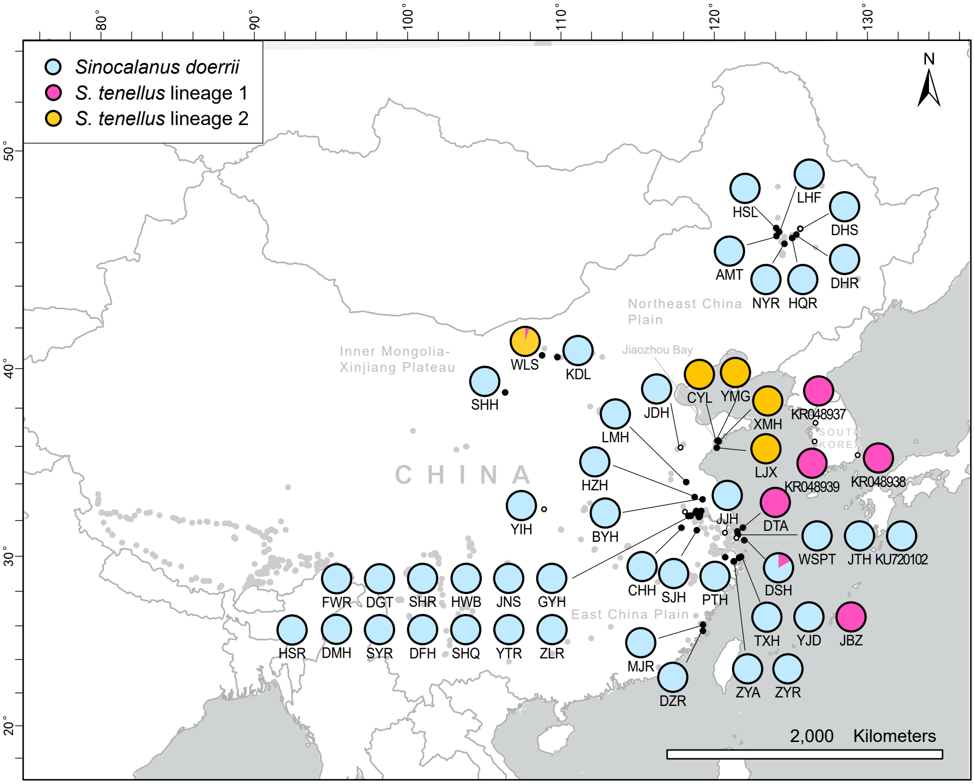

FIGURE 1 The known geographical distribution of *Sinocalanus* in China. Solid black dots indicate the locations for *Sinocalanus* recorded for the first time in this study, empty black dots indicate previously known locations for *Sinocalanus* and small solid grey dots indicate sampled localities at which *Sinocalanus* was not detected. Locality abbreviations on the map correspond to the abbreviations in table 1. The large circles with different color fillings represent mitochondrial species/lineages (IDs see fig. 4) found at each sampling locality. Segments of circles with different colors indicate the proportion of each lineage based on COI.

1.50–1.85 mm (fig. 2B). Cephalothorax of both sexes in six segments (fig. 2A,B). Female abdomen in 3–4 segments (fig. 2A), male abdomen in 5 segments (fig. 2B). Caudal ramus ~6 times longer than wide (fig. 2A,B). Legs I to IV all similar in structure, identical in both sexes (fig. 2E–H). Leg V differs between sexes (fig. 2C,D). Female legs V form a symmetrical pair with three-segmented exopod: segment 1 with semicircular protrusion on medial side of base corner and one short spine at outer end (fig. 2C); segment 2 with strong spine extending inward, bearing row of small spines above and below (fig. 2C); segment 3 bears 4 feathery bristles on inner side, one strong terminal spine and one spine on middle and end of outer side (fig. 2C); endopod three-segmented, with feathery bristle on medial end of segment 2; 6 feathery bristles on segment 3 (fig. 2C). Male legs V not a symmetrical pair. Male right leg V carries one finger-like process on medial basal corner of basipod segment 2, accompanied by multiple small spines below (fig. 2D);

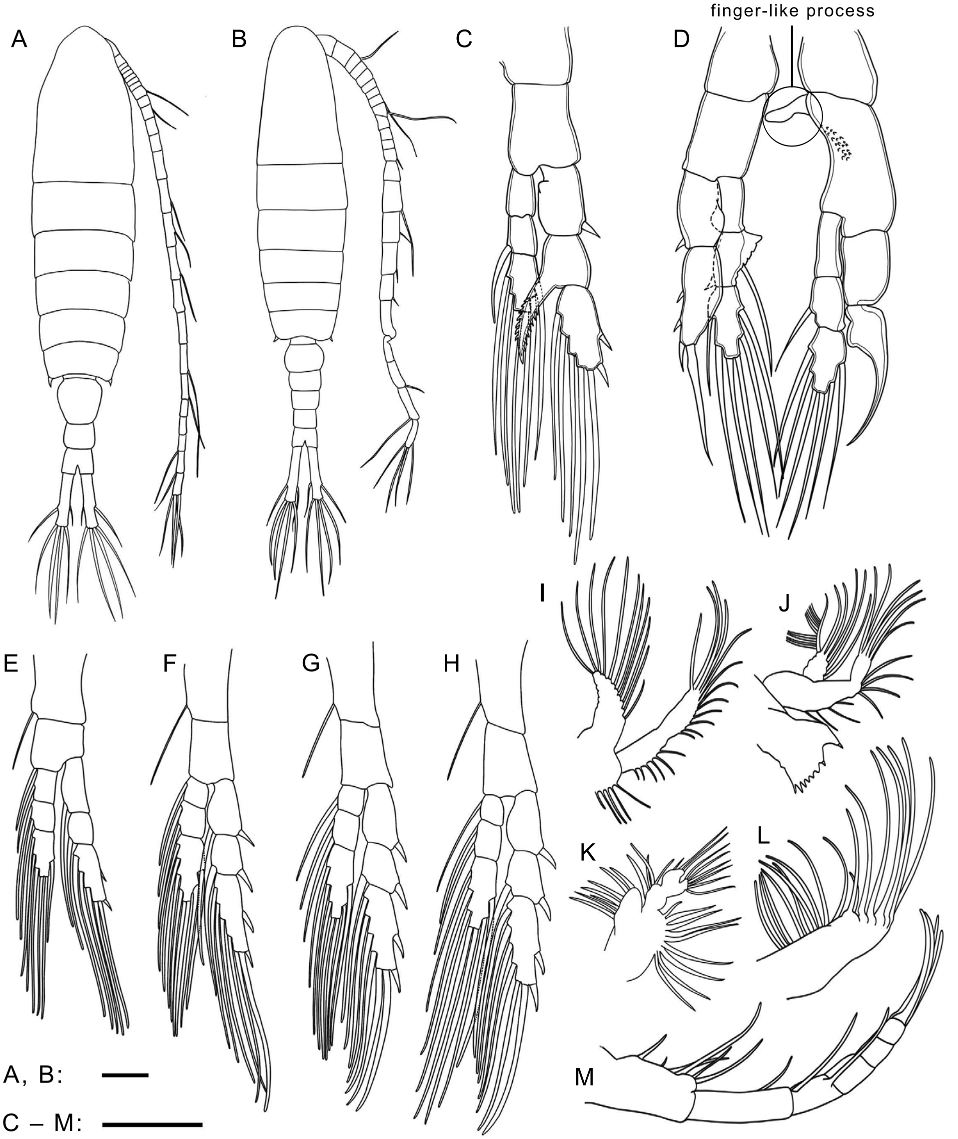

FIGURE 2 Morphology of *Sinocalanus doerrii* (Brehm, 1909) from Dongzhang Reservoir (DZR), China. A, adult female, dorsal. B, adult male, dorsal. C, female leg V, anterior. D, male legs V. E, leg I. F, leg II. G, leg III. H, leg IV. I, antenna II. J, mandible. K, maxilla I. L, maxilla II. M, maxilliped. Scale bars 0.1 mm.

exopod two-segmented with segment 2 bearing several small spines medially and terminal hook-like spine (fig. 2D); endopod three-segmented, a feathery bristle on medial end of segment 2 and six feathery bristles on segment 3 (fig. 2D). Male left leg V exopod two-segmented, which nearly as long as endopod; segment 1 bears one spine on outer basal corner and segment 2 bears three spines on outer edge and one

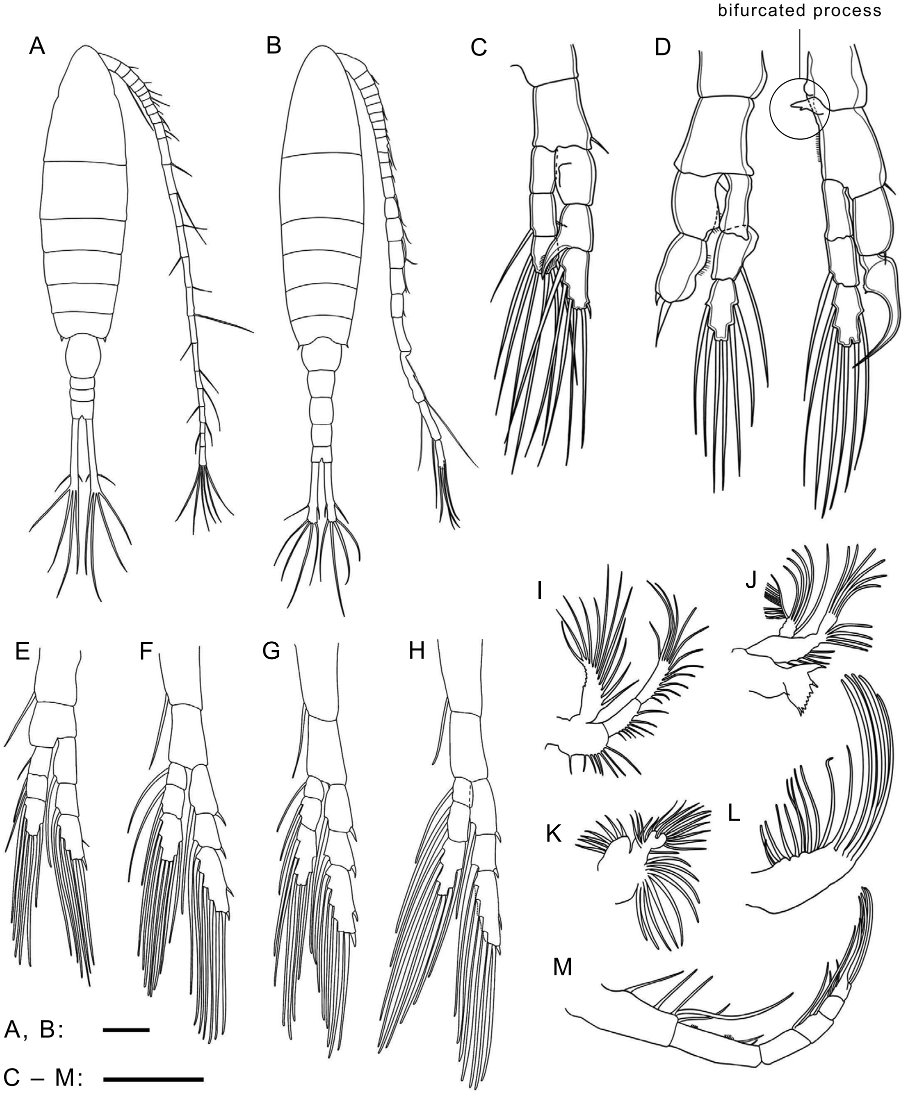

FIGURE 3 Morphology of *Sinocalanus tenellus* (Kikuchi, 1928) from Dongtan-A (DTA), China. A, adult female, dorsal. B, adult male, dorsal. C, female leg V, anterior. D, male legs V. E, leg I. F, leg II. G, leg III. H, leg IV. I, antenna II. J, mandible. K, maxilla I. L, maxilla II. M, maxilliped. Scale bars 0.1 mm.

long terminal spine, with a scalloped inner edge (fig. 2D); endopod bears prominent sharp protrusion on medial side of segment 2, a feathery bristle on medial end of segment 2 and six feathery bristles on segment 3 (fig. 2D).

Sinocalanus tenellus: Female body length 1.55–1.70 mm (fig. 3A), male body length 1.40–1.65 mm (fig. 3B). Cephalothorax of both sexes in six segments (fig. 3A,B). Female abdomen in 3–4 segments (fig. 3A), male abdomen in 5 segments (fig. 3B).

Caudal ramus ~8 times longer than wide (fig. 3A,B). Legs I to IV all similar in structure, identical in both sexes (fig. 3E–H). Leg V differs between sexes (fig. 3C,D). Female legs V form a symmetrical pair with three-segmented exopod: segment 1 with semicircular protrusion on medial side of base corner and one short spine at outer end (fig. 3C); segment 2 with strong spine extending inward, bearing row of small spines above and below (fig. 3C); segment 3 bears 4 feathery bristles on inner side, one strong terminal spine and one spine on end of outer side (fig. 3C); endopod three-segmented, with feathery bristle on medial end of segment 2; 6 feathery bristles on segment 3 (fig. 3C). Male legs V not a symmetrical pair. Male right leg V carries one bifurcated process on medial basal corner of basipod segment 2, accompanied by a row of short setae below (fig. 3D); exopod two-segmented with segment 2 bearing several small spines medially and terminal hook-like spine (fig. 3D); endopod three-segmented, a feathery bristle on medial end of segment 2 and six feathery bristles on segment 3 (fig. 3D). Male left leg V exopod two-segmented, which nearly as long as endopod; segment 1 is unarmed and segment 2 bears one small spine on outer edge and one long terminal spine, with a scalloped inner edge (fig. 3D); endopod bears a prominent protrusion on medial side of segment 2, a feathery bristle on medial end of segment 2 and six feathery bristles on segment 3 (fig. 3D).

Genetic diversity

In total, we successfully sequenced 346 *Sinocalanus* individuals at the COI locus (572 bp in the aligned dataset) and obtained 82 unique COI haplotypes (table 1). For each morphologically defined species, the population haplotype diversity (H) of COI ranged from 0.000 to 1.000, and the intra-population nucleotide diversity (π) of COI ranged from 0.00000 to 0.02273 (table 1). There were 42 COI variable sites in *S. doerrii* and 67 sites in *S. tenellus* (data not shown). No COI sequences showed characteristics of nuclear pseudogenes, as would be evidenced by frame shifts or premature stop codons. Of the 346 individuals sequenced for COI, 179 individuals were also successfully sequenced at the 18S locus (1,569 bp in the aligned dataset), 55 heterozygotes and 124 homozygotes were detected, resulting in a total of 234 sequences and 128 unique haplotypes (table 1). There were 67 variable sites among the 18S sequences of *S. doerrii* and 16 in *S. tenellus* (data not shown).

Phylogeny

Our COI Bayesian and MP trees (fig. 4) confirmed the presence of two *Sinocalanus* species (*S. doerrii* and *S. tenellus*) in our samples, in accordance with their morphological identifications (figs. 2 and 3). By choosing the most conservative estimate (i.e., ABGD and bPTP) of lineage diversity by the three implemented species-delimitation methods (i.e., GMYC, bPTP and ABGD), we suppose that all *S. doerrii* individuals belong to a single lineage, likely representing a single species, whereas the studied samples of *S. tenellus* fell into two mtDNA lineages (lineages 1 and 2, fig. 4). Previously published sequences of *S. sinensis* belong to a single lineage (fig. 4), and some sequences from *S. doerrii* were previously erroneously

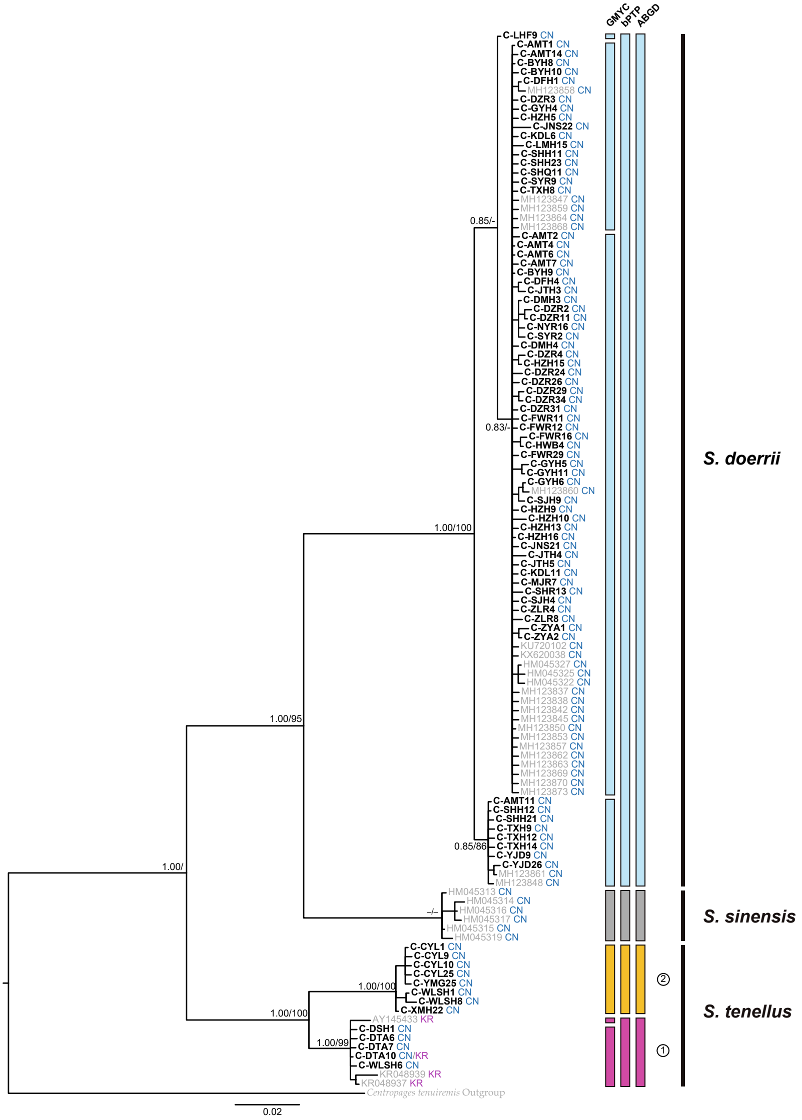

FIGURE 4 Bayesian phylogenetic tree of *Sinocalanus* spp. according to the mitochondrial COI gene (572 bp). A single representative of each haplotype (including all haplotypes represented in GenBank) was included in the tree. Bayesian posterior probabilities from MrBayes (left) higher than 0.75 and bootstrap values from the maximum parsimony analysis with PAUP* (right) higher than 75 are shown above each branch, and nodes with low support are indicated by dashes (–). Nodes for within-species relationships are not shown for very short branches. Lineage delimitation according to the bPTP, GMYC and ABGD methods are indicated, and the lineage IDs for *S. tenellus* are shown in circles at the right. Abbreviations of country names in which each haplotype was detected are, CN: China, KR: South Korea. COI haplotypes of *Sinocalanus* from this study are provided in table 1; for previously published sequence IDs see supplementary table S1 and for the samples and codes used in this study, see supplementary table S2.

identified as *S. tenellus* (Wang et al., 2018). Lineage 2 of *S. tenellus* had not been detected previously (fig. 4). By choosing the most conservative estimate (i.e., ABGD and GMYC) of lineage diversity by the three implemented species-delimitation methods, our COI+18S Bayesian and MP trees also supported the presence of two *Sinocalanus* species with three lineages among our samples (fig. 5). The COI+18S Bayesian and MP trees classified all individuals into species/lineages consistent with those in the COI Bayesian and MP trees.

Biogeography

Sinocalanus doerrii was the species most frequently found among our samples. It was detected in 35 out of 636 waterbodies, and was widely distributed across the East China Plain, Inner Mongolia-Xinjiang Plateau and the Northeast China Plain (table 1 and fig. 1). *Sinocalanus tenellus* was detected in 8 out of 636 waterbodies in China and was most frequent in coastal estuaries of the East China Plain (table 1 and fig. 1). Most *S. doerrii* populations (34 out of 35) were in freshwater, whereas all *S. tenellus* populations were found in brackish waters. Interestingly, both species co-occurred in a single waterbody, Dishui Lake (DSH in fig. 1), an artificial brackish lake with salinity values ranging from 0.9 to 4.8‰ (Zhao et al., 2020). Lineage 1 of *S. tenellus* was mainly found in estuaries along the coasts of East China Sea and South Korea, whereas lineage 2 of *S. tenellus* was mainly found in estuaries along the coasts of Jiaozhou Bay (Yellow Sea) in China (table 1 and fig. 1). Both *S. tenellus* lineages were found in a brackish lake Wuliangsuhai (WLSH) from Inner Mongolia-Xinjiang Plateau, the only site far inland where this species occurred (table 1 and fig. 1).

The haplotype networks for both species had some features in common (fig. 6). In both cases there were one or more common haplotypes surrounded by a number of rare haplotypes differing by one or a few mutational steps. In the case of *S. doerrii*, the three most common haplotypes (C-AMT1, C-AMT4 and C-AMT6) were present at multiple sampling sites, covering three regions of China: the East China Plain, Inner-Mongolia-Xinjiang Plateau and the Northeast China Plain (fig. 6). Specifically, the most abundant haplotype C-AMT6 was found in 25 waterbodies from all three regions (see above), haplotype C-AMT1 was shared by 22 waterbodies from the East China Plain and the Northeast China Plain, and haplotype C-AMT4 was shared by 20 waterbodies from all three regions. In the case of *S. tenellus*, the two lineages were separated by many mutational steps, but each exhibited one common haplotypes (C-DTA10 in lineage 1 and C-CYL1 in lineage 2 of *S. tenellus*; Fig. 6) and a few closely related rare haplotypes. C-DTA10 was found in both China and South Korea, shared by 2 waterbodies from the East China Plain and 1 waterbody from the coastal and oceanic areas around South Korea. C-CYL1 was found in 4 waterbodies of the East China Plain.

Environmental preferences

The DCA result shows that the maximum axis length was 2.27 standard deviations, suggesting that the relationship between *Sinocalanus* lineages and environmental variables is linear. Therefore, we performed the Redundancy Analysis (RDA). The first and second RDA axes explained 85.76% and 13.20% of the variability in

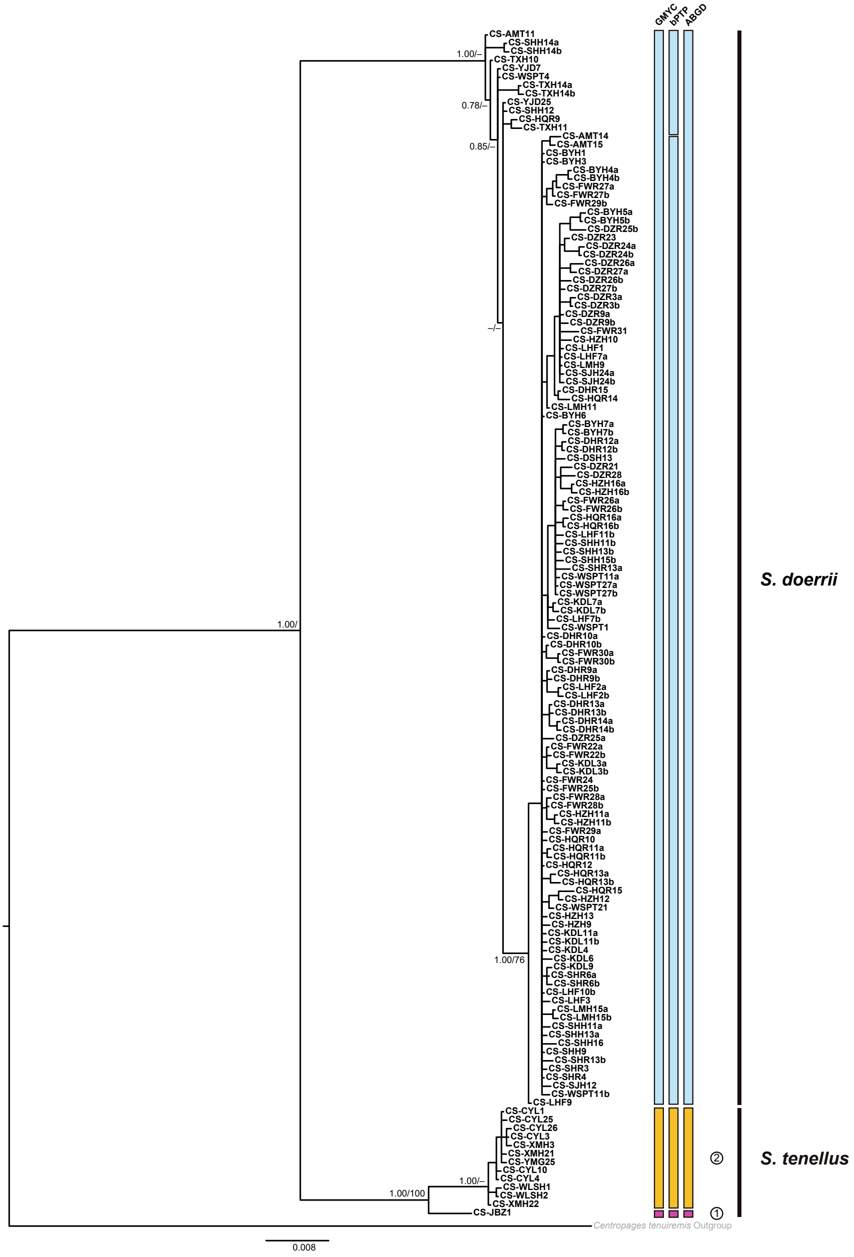

FIGURE 5 The Bayesian phylogenetic consensus tree of the COI region (572 bp) and 18S region (1,569 bp) of *Sinocalanus* spp. A single representative of each haplotype was included in the tree. Bayesian posterior probabilities from MrBayes (left) higher than 0.75 and bootstrap values from the maximum parsimony analysis with PAUP* (right) higher than 75 are shown above each branch, and nodes with low support were indicated by dashes (–). Nodes for within-species relationships are not shown for very short branches. Lineage delimitation is shown according to the bPTP, GMYC and ABGD methods. The IDs of *Sinocalanus* "COI+18S" haplotypes from this study are provided in table 1 and for the samples and codes used in this study, see supplementary table S2.

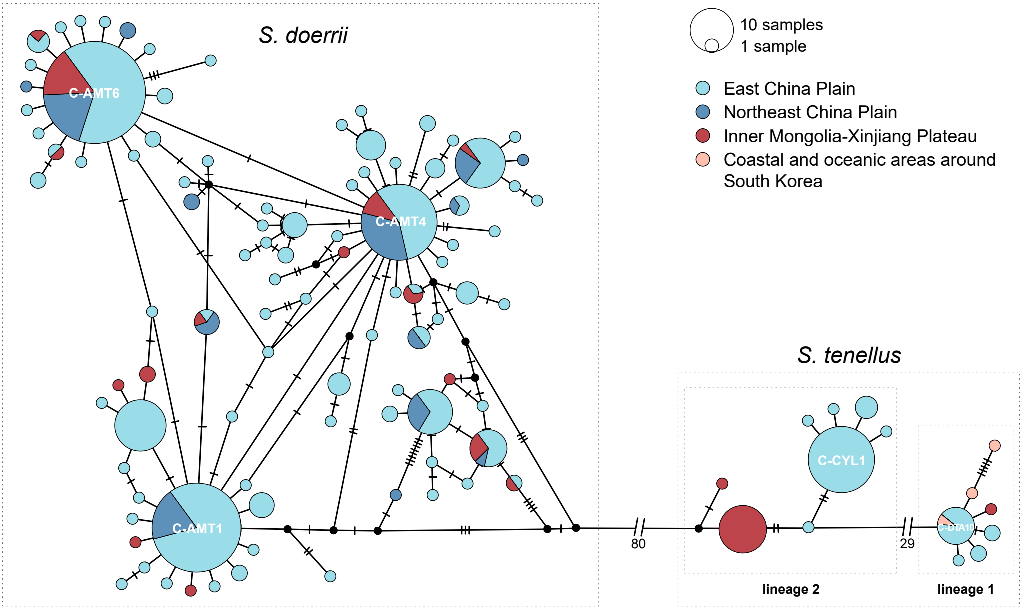

FIGURE 6 Haplotype network (based on COI sequences; 572 bp) of *Sinocalanus* spp. in the present study. Each circle indicates a unique haplotype and its size reflects the number of individuals carrying that haplotype. The colors of circles represent four regions from China and one region from South Korea. The number of tick marks on connecting lines denotes the number of mutations separating haplotypes.

the data, respectively. Cumulatively, RDA1 and RDA2 accounted for 98.96% of the total variation (fig. 7). The first component was directly related to salinity. Overall, waterbodies inhabited by different *Sinocalanus* species/lineages were characterized by opposite component loading on the first RDA axis. Thus, waterbodies inhabited by *S. doerrii* were usually freshwater and *S. tenellus* (two lineages) were usually brackish water (df = 1, F = 24.58, p < 0.001). In contrast, neither latitude (df = 1, F = 0.47, p = 0.63), longitude (df = 1, F = 0.55, p = 0.55), lake origin (df = 1, F = 0.28, p = 0.82), average depth (df = 1, F = 0.56, p = 0.42), surface area (df = 1, F = 0.29, p = 0.63), eutrophication level (df = 1, F = 0.15, p = 1.00) nor tendency to freeze in winter (df = 1, F = 0.083, p = 1.00) had any influence on the presence of particular *Sinocalanus* species or lineages.

Discussion

In the present study, we identified two morphospecies of *Sinocalanus* (*S. doerrii* and *S. tenellus*), and both lineages of *S. tenellus* show an identical morphology. We did not find the other species previously reported from China, including *S. sinensis*, *S. laevidactylus* and *S. solstitialis*, likely because of our limited sampling efforts in estuarine habitats. *Sinocalanus sinensis* is a brackish water species, and *S. laevidactylus* and *S. solstitialis* occur in freshwater and brackish waters. Our identification of *S. doerrii* and *S. tenellus* based on morphology is

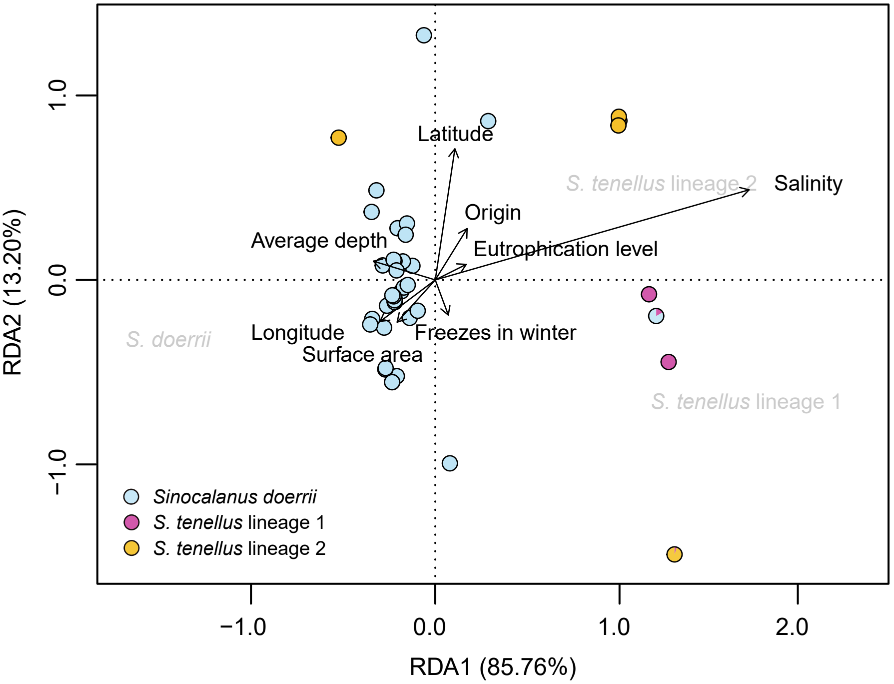

FIGURE 7 The RCA plot of the *Sinocalanus* species, showing the relationship between the distribution of *S. doerrii* and *S. tenellus* mtDNA lineages and geographical and environmental parameters. Blue dots represent lakes inhabited by *S. doerrii*, carmine dots represent lakes inhabited by *S. tenellus* lineage 1, and yellow dots represent lakes inhabited by *S. tenellus* lineage 2.

further supported by molecular phylogenetic analysis. Some of the COI sequences currently available in Genbank (specifically HM045320–HM045321, reported in Wang et al., 2011) and labelled as *S. tenellus* actually represent *S. doerrii*, as shown in our COI Bayesian and MP trees. Several subsequent genetic studies, without morphological corroboration, accepted this incorrect identification (Dong et al., 2020; Jiang et al., 2017; Wang et al., 2018). Thus, for example, Wang et al. (2018) erroneously labelled one clade of *S. doerrii* as *S. tenellus*. All erroneously identified sequences are included in supplementary table S1. This illustrates that, the use of integrative taxonomy (combining molecular and morphological methods) is mandatory when dealing with microcrustacean taxonomy.

A clear ecological separation between the two *Sinocalanus* species was found: *S. doerrii* occurred only in freshwaters across China, whereas *S. tenellus* was mainly present in brackish-water coastal estuaries of the East China Plain. This was further supported by the environmental preference analysis: salinity emerged as a significant factor influencing the separation of these two species, as evidenced by its strong contribution on the first RDA axis and its high significance in explaining variation in the species data. Similar ecological separations of congeners have frequently been found in zooplankton taxa (Alonso et al., 2021; Lowe et al., 2007; Ni et al., 2019; Wang et al., 2023). For example, the cladoceran *Daphnia galeata* was the only species in the *D. longispina* species complex in eutrophic lakes from the East China Plain, whereas its sibling species, *D. dentifera*, dominated in oligotrophic lakes on the Tibetan Plateau (Ma et al., 2015, 2019).

We detected a wide distribution of *S. doerrii* in freshwaters from the East China Plain, Inner Mongolia-Xinjiang Plateau and the Northeast China Plain. This is consistent with a previous nationwide survey carried out in the 70s that found *S. doerrii* to be widespread in the subtropical and temperate regions of China and especially abundant in the East China Plain (Shen & Song, 1979). This species is believed to be native to China (Orsi et al., 1983; Shen & Song, 1979). However, *S. doerrii* has recently spread to estuaries on the Californian coast of the United States, presumably introduced in ships' ballast water (Bollens et al., 2012; Orsi et al., 1983), thus demonstrating the considerable dispersal ability of this species. Consistent with this, we detected three very similar haplotypes of *S. doerrii* (C-AMT1, C-AMT4 and C-AMT6) widely distributed among freshwaters across China, suggesting relatively recent colonization and/or dispersal events. This phenomenon has been commonly observed in copepods (Guinto et al., 2023; Maturana et al., 2022; Ni et al., 2020) and other freshwater zooplankton, such as cladocerans (Yin et al., 2018) and rotifers (Deng et al., 2022). *Sinocalanus* individuals and their resting eggs (Dahms, 1995; Hansen, 2019; Holm et al., 2018) can achieve inter-continental dispersal by human activities such as live fish transport (Reid, 2007; Taylor et al., 1993), by biological vectors such as birds (Frisch et al., 2007; Proctor & Malone, 1965) and by abiotic vectors such as wind (Brendonck & Riddoch, 1999; Vanschoenwinkel et al., 2009).

We detected *S. tenellus* from brackish waters in inland lakes as well as in coastal estuaries of China. This finding is consistent with previous studies that *S. tenellus* has a wide geographical distribution in brackish waters. It has been detected in estuaries along the coasts of China (Shen & Song, 1979), Japan and Korea (Sakaguchi et al., 2011) and Sakhalin, Russia (Rylov, 1932). Furthermore, we found two mitochondrial lineages of *S. tenellus* across China, one of which is newly reported, indicating cryptic lineage diversity within this species. Cryptic lineages are frequently found in zooplankton taxa when molecular tools have been applied (Cornils et al., 2017; Deng et al., 2021; Gómez et al., 2002). For example, the copepod *Haloptilus longicornis* is comprised of a pair of globally distributed and often sympatric cryptic species (Andrews et al., 2014) and a widespread marine copepod, *Oithona similis* s.l., has seven distinct mitochondrial lineages, representing most likely cryptic or pseudocryptic species (Cornils et al., 2017).

Our results suggest a geographical separation between the two *S. tenellus* lineages: *S. tenellus* lineage 1 mostly occurs in estuaries of the Yangtze River and in coastal Korea, and lineage 2 occurs in one lake on the Inner-Mongolia Plateau and in estuaries of Jiaozhou Bay on the western side of the Yellow Sea. However, caution is needed in drawing any general conclusions because we only found eight populations of *S. tenellus*, so that the gap currently observed between its two clades might be an artifact due to the undersampling of the actual genetic diversity of the species. Spatial separation of sibling (mitochondrial) lineages within a single species or species complex has often been observed in freshwater zooplankton taxa in China. For instance, this was reported for the rotifer *Brachionus asplanchnoidis* by Deng (2022) and for the cladoceran *Chydorus sphaericus* by Wang et al. (2021). Such patterns of allopatric distribution between lineages may result from founder effects, genetic drift, and/or local adaptation (De Gelas & De Meester, 2005; De Meester et al., 1999; De Meester et al., 2002; Gómez et al., 2000). Additionally, we found both of the *S. tenellus* lineages in a single lake, Wuliangsuhai (WLSH in fig. 1), on the Inner-Mongolia Plateau, providing the possibility of gene exchange among lineages. It is most likely that this species was anthropogenically introduced by human activities such as live fish transport (Reid, 2007; Taylor et al., 1993). Gene exchange among lineages has frequently been observed in cladocerans (Hebert, 1985; Ma et al., 2019; Makino & Tanabe, 2009; Schwenk et al., 1998; Wen et al., 2016). For example, gene exchange was found between two mtCOI lineages of the calanoid copepod *Acanthodiaptomus pacificus* in their contact zone in Japan (Makino & Tanabe, 2009).

In conclusion, we have sampled inland and estuarine waters of China for species of *Sinocalanus*. Both morphological and molecular data demonstrated the presence of two species (*S. doerrii* and *S. tenellus*), which were ecologically separated. We detected a new mitochondrial clade within *S. tenellus* which mostly occurs in estuaries of Jiaozhou Bay (western Yellow Sea). A broader sampling from brackish-water lakes and estuaries in China and a genomic approach will be needed to better explore the ecological adaptation of the genus *Sinocalanus*.

Acknowledgments

This research was funded by the National Natural Science Foundation of China (32271690) to MY. We thank the anonymous reviewer for useful comments on the earlier version of this article.

Author's contributions

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

Conflict of interest

The authors declare that they have no competing interests.

Data availability

All new sequences have been submitted to GenBank under accession numbers: COI: PP750470–750551 and 18S: PP755206–755333.

Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.27925017

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