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ORIGINAL ARTICLE

# Genetic diversity and population differentiation of the freshwater copepod *Sinocalanus tenellus* (Calanoida, Centropagidae) in China

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**ABSTRACT**

Copepods, present in a wide range of water bodies, are an important component of freshwater ecosystems and their biodiversity has been much studied in marine and freshwater ecosystems. However, no previous genetic data are available that allow an assessment of population-genetic diversity and differentiation of the copepod *Sinocalanus tenellus* from Chinese freshwaters. We analyzed DNA sequences of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene from eleven *S. tenellus* populations (individual lakes) from China, ten of which exhibited a high genetic diversity. Low to high population differentiation was detected among the populations. Interestingly, substantial genetic divergence was detected between WLS (Wuliangsu, in Inner Mongolia) and other locations, indicating the presence of two lineages of *S. tenellus* in East Asia. Moreover, we found that two distinct clades of *S. tenellus* were separated by the reference "*S. sinensis*" clade, suggesting they were parts of a complex of cryptic species of *S. tenellus*. This study will contribute to an understanding of the diversity and biogeography of copepods in freshwater ecosystem in China.

**Key words:** Copepods; genetic diversity; *COI* gene; population differentiations; China.

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## INTRODUCTION

Copepods are one of the largest crustacean taxa, with approximately 11,500 morphological species described (Humes, 1994). They are important components of aquatic ecosystems, having a significant role in food webs and in biogeochemical cycles (Roemmich and McGowan, 1995; Turner, 2004). Many studies have already shown that copepods are sensitive to climate change and might be useful indicators of natural and anthropogenic stressors (Beaugrand *et al.*, 2002; Chivers *et al.*, 2017; Hays *et al.*, 2005). Therefore, the biodiversity of copepods in marine and freshwater ecosystems has received a lot of attention (Blanco-Bercial *et al.*, 2011; Bradford-Grieve *et al.*, 2010; Saiz and Calbet, 2011).

In particular, molecular tools have successfully been employed to explore the genetic diversity and population structure of copepods (Baek *et al.*, 2016; Blanco-Bercial *et al.*, 2011; Blanco-Bercial and Bucklin, 2016; Bucklin *et al.*, 2000). For instance, a DNA barcoding approach was able to identify copepod species without relying on subtle morphological differences (reviewed in Bucklin *et al.*, 2011). Most recently, sequences of the mitochondrial cytochrome c oxidase subunit I (*COI*) gene were used to assess diversity of copepods in South Korea. The 133 individuals sequenced turned out to represent 94 species across six different orders (Baek *et al.*, 2016), indicating high lineage diversity of copepods in East Asia. Interestingly, at least some copepod species are believed to have limited dispersal ability. This is different from many other groups of freshwater invertebrates, which produce resting eggs (Green and Figuerola, 2005). Because of limited dispersal ability, the copepod populations in reservoirs or lakes are isolated and thus have low levels of gene flow. Indeed, a study of the diaptomid *Neodiaptomus schmackeri* populations from isolated reservoirs in Chinese Taiwan found negligible levels of gene exchange between waterbodies (Young *et al.*, 2013).

Copepods are widely distributed in Mainland China, ranging from inland waterbodies to marine waters (Chiang and Du, 1979; Zhu *et al.*, 2012). The calanoid copepod *Sinocalanus tenellus* (Kikuchi, 1928) is the predominant species in China's lakes, reservoirs and estuaries, and is also common in coastal waters (Chiang and Du, 1979; Li *et al.*, 2005; Zhu *et al.*, 2012). So far, however, no genetic data are available that would allow an assessment of genetic variation and diversity of this species from China, despite its wide use in aquatic ecotoxicology studies there (Jiang *et al.*, 2006; Xu and Liu, 2014).

In the present study, we analyzed planktonic copepod samples from twenty-three Chinese lakes, in eleven of which populations morphologically corresponding to *S. tenellus* were found. Then, we explored the genetic diversity and structure of the *S. tenellus* populations

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employing *COI* gene sequences as a genetic marker. We expected to detect high genetic diversity and substantial population genetic differentiation among the *S. tenellus* populations in China, as observed elsewhere in East Asia (Baek *et al.*, 2016; Young *et al.*, 2013). We also wanted to set our data in a phylogenetic framework to explore the phyletic diversity of *S. tenellus* and its relationship with a related species form the region, *S. sinensis* (Poppe, 1889).

## METHODS

### Sampling

*Sinocalanus tenellus* was recovered from eleven of twenty-three lakes sampled across China (Fig. 1 and Tab. S1). The species was identified based on morphological characters under a stereomicroscope (Chiang and Du, 1979). Seven of the waterbodies were located in the Eastern Plain, two on the Mongolian Plateau and the remaining two in the Northeast Plain (*i.e.,* northern China; Tab. 1). Samples were collected with a 125-µm plankton net hauled vertically through the whole water column at two or three different sites per lake. Samples from different locations in the same lake were pooled and preserved in 95% ethanol.

### DNA extraction

Individuals from eleven populations were processed for mtDNA sequencing (111 individuals in total, Tab. 1). The body of each individual *S. tenellus* was removed from the head, to avoid DNA contamination from other species in the gut, by using a sharp blade and a microscopic tweezer under the stereoscope. DNA was extracted from the head by proteinase K digestion as follows: each head was put in 30 µL H3-buffer (10 mm Tris-HCl, pH 8.3 at

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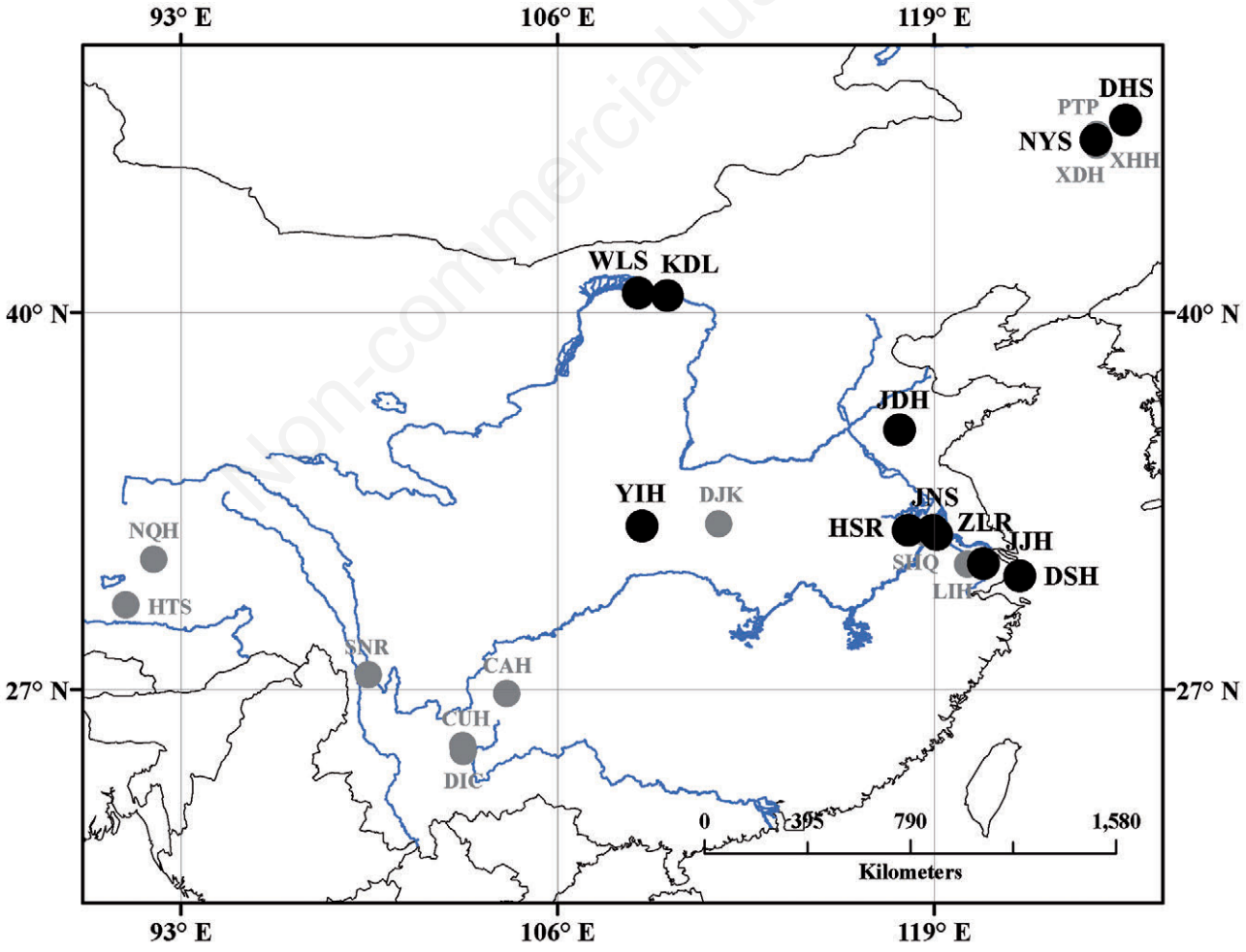


**Fig. 1.** Geographic locations of collection sites for *Sinocalanus tenellus* in China. Black dots: lakes inhabited by *S. tenellus*; Gray dots: sampled lakes with other copepod species.

25°C, 0.05 M potassium chloride, 0.005% Tween-20 and 0.005% NP-40; Replitherm Reaction Buffer, Biozym) and 15 µg proteinase K. Then samples were incubated overnight in a water bath at 55°C. Finally, the proteinase K was irreversibly denatured via a 10-min incubation at 95°C. The homogenate was stored at 4°C before being used in a PCR reaction.

### Amplification and sequencing

A 680-bp segment of the mitochondrial *COI* gene was amplified using standard primer pairs LCO1490 (5′GGTCAACAAATCATAAAGATATTGG-3′) and HCO 2918 (5′TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer *et al.*, 1994). The PCR reaction was carried out using a total volume of 20 µL, consisting of 2 µL 10 × PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 250 mM dNTPs, 350 nM of each primer, 2 units of *Taq* DNA polymerase (SuperTherm DNA polymerase, *Taq HS* from Takara Bio Inc., CA, USA) and 10–50 ng of genomic DNA. The reaction mixture was held for 1 min at 94°C, then subjected to 40 cycles of 1 min at 94°C, 1.5 min at 40°C and 1.5 min at 72°C. This was followed by a final incubation for 6 min at 72°C. PCR products were purified and sequenced in the forward direction on an ABI PRISM 3730 DNA capillary sequencer by Invitrogen Trading Co., Ltd. (Shanghai, China). The chromatograms were carefully checked for scoring errors. All newly obtained *COI* sequences were submitted to GenBank under accession numbers MH123837-MH123875.

### Phylogenetic analyses

Unique haplotypes were identified in DNASP 5.10 (Librado and Rozas, 2009), and then aligned together with 15 *COI S. tenellus* and 7 *COI S. sinensis* reference sequences (representing 6 haplotypes) obtained from GenBank (Tab. S2), using Clustal W (Thompson *et al.*, 1994) in MEGA 6 (Tamura *et al.*, 2013). A phylogenetic tree was constructed using a maximum likelihood (ML) analysis in RAxML (Stamatakis *et al.*, 2008). *Centropages tenuiremis*, a member of the Calanoida phylogenetically close to *S. tenellus*, was used as an outgroup. The mean genetic distance between distinct clades of *S. tenellus* based on the phylogenetic tree was calculated in MEGA 6 using the Kimura 2-parameter model of substitution (Kimura, 1980). To assess the intraspecific genetic variation and genetic relationships among populations, a network of *COI* haplotypes (including all 15 *S. tenellus* and 7 *S. sinensis* reference sequences from GenBank) was constructed using HAPLOVIEWER (Salzburger *et al.*, 2011).

### Population differentiation and diversity

To estimate genetic differentiation, the fixation index $F_{st}$ was calculated in Arlequin 3.11 (Excoffier *et al.*, 2005) with $10^4$ permutations, followed by sequential Bonferroni correction for the 55 pairwise population comparisons (Rice, 1989). Haplotype diversity ($h$) and nucleotide diversity ($\pi$) for the *COI* sequences were calculated in DNASP 5.10 (Librado and Rozas, 2009). The Tajima's D and Fu's Fs tests were implemented in DNASP 5.10, to test for departure from neutral evolution. Tajima's D test is performed based on the difference between segregating sites and the average of nucleotide differences (Tajima, 1989), and Fu's Fs test compares the observed number with the expected number of haplotypes in a random sample (Fu, 1997).

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**Tab.1.** Genetic diversity of eleven *Sinocalanus tenellus* populations from China based on a mitochondrial gene (*COI*).

| Locality (abbreviation) | Latitude, Longitude | Sampling season | $N_1$ | $N_2$ | $h$ | $\pi$ | Fu's Fs | Tajima's D |
|---|---|---|---|---|---|---|---|---|
| Donghu Reservoir (DHS) | 46.63N, 125.61E | Summer$^{2016}$ | 11 | 4 $^{a,b,c,g}$ | 0.745 | 0.00167 | -0.701 | -0.020 |
| Dishui Lake (DSH) | 30.90N, 121.96E | Summer$^{2016}$ | 10 | 7 $^{d,e}$ | 0.867 | 0.03894 | 2.972 | -1.894$^{*}$ |
| Huangshan Reservoir (HSR) | 32.48N, 118.09E | Spring$^{2013}$ | 5 | 4 $^{b,d,f}$ | 0.9 | 0.00417 | 5.666 | -0.079 |
| Jindou Lake (JDH) | 35.95N, 117.80E | Spring$^{2012}$ | 12 | 9 $^{b,c,d,e,g}$ | 0.909 | 0.00386 | -4.807 | -0.674 |
| Jinji Lake (JJH) | 31.32N, 120.70E | Spring$^{2016}$ | 11 | 10 $^{b,d,e,g,h}$ | 0.982 | 0.00813 | -4.654 | -1.755 |
| Jinniushan Reservoir (JNS) | 32.47N, 118.95E | Spring$^{2013}$ | 12 | 7 $^{d,e,g,h}$ | 0.833 | 0.00312 | -2.606 | -1.024 |
| Kundunlun River (KDL) | 40.57N, 109.77E | Spring$^{2013}$ | 10 | 8 $^{a,b,d,i}$ | 0.956 | 0.01132 | 1.502 | -1.818$^{*}$ |
| Nanyin Reservoir (NYS) | 45.95N, 124.58E | Summer$^{2016}$ | 13 | 7 $^{b,d,g,h,i}$ | 0.872 | 0.00686 | -0.246 | -1.388 |
| Wuliangsu Lake (WLS) | 40.66N, 108.77E | Summer$^{2013}$ | 11 | 1 | 0 | 0 | ns | ns |
| Ying Lake (YIH) | 32.62N, 108.90E | Autumn$^{2012}$ | 6 | 5 $^{b,d,f}$ | 0.933 | 0.00481 | -1.283 | -0.847 |
| Zaolin Reservoir (ZLR) | 32.33N, 119.07E | Spring$^{2013}$ | 10 | 7 $^{b,c}$ | 0.911 | 0.00379 | -5.049 | -1.244 |

*$N_1$, number of sequenced individuals; $N_2$, number of haplotypes; $^{a\text{-}i}$ID assigned to the 9 haplotypes found in more than one lake; π, nucleotide diversity;* h*, haplotype diversity; $^{*}P<0.05$; ns, not significant.*

## RESULTS

### Haplotypes and phylogeny

Sequences were successfully obtained from 111 *S. tenellus* individuals at the *COI* locus (575 bp in the aligned dataset); among these, 39 unique haplotypes were detected. In a phylogenetic tree, haplotypes fell into two very distinct clades, one including the reference *S. tenellus* sequences from China and the other the reference sequences from Korea (Fig. 2). Most haplotypes were clustered in one clade. Interestingly, the single haplotype from WLS (11 individuals sequenced), which is located on the Inner Mongolian Plateau (northern China), was very distant from all others and formed the second clade, along with the reference sequences from Korea (Figs. 2 and 3). Additionally, one haplotype from DSH fell into this clade and was identical to the Korea reference sequence KR3 (Figs. 2 and 3). The average genetic distance between these two distinct clades was 0.19. Nine haplotypes were found in two or more lakes (Tab. 1 and Fig. 3). The most abundant haplotype, d, was shared by

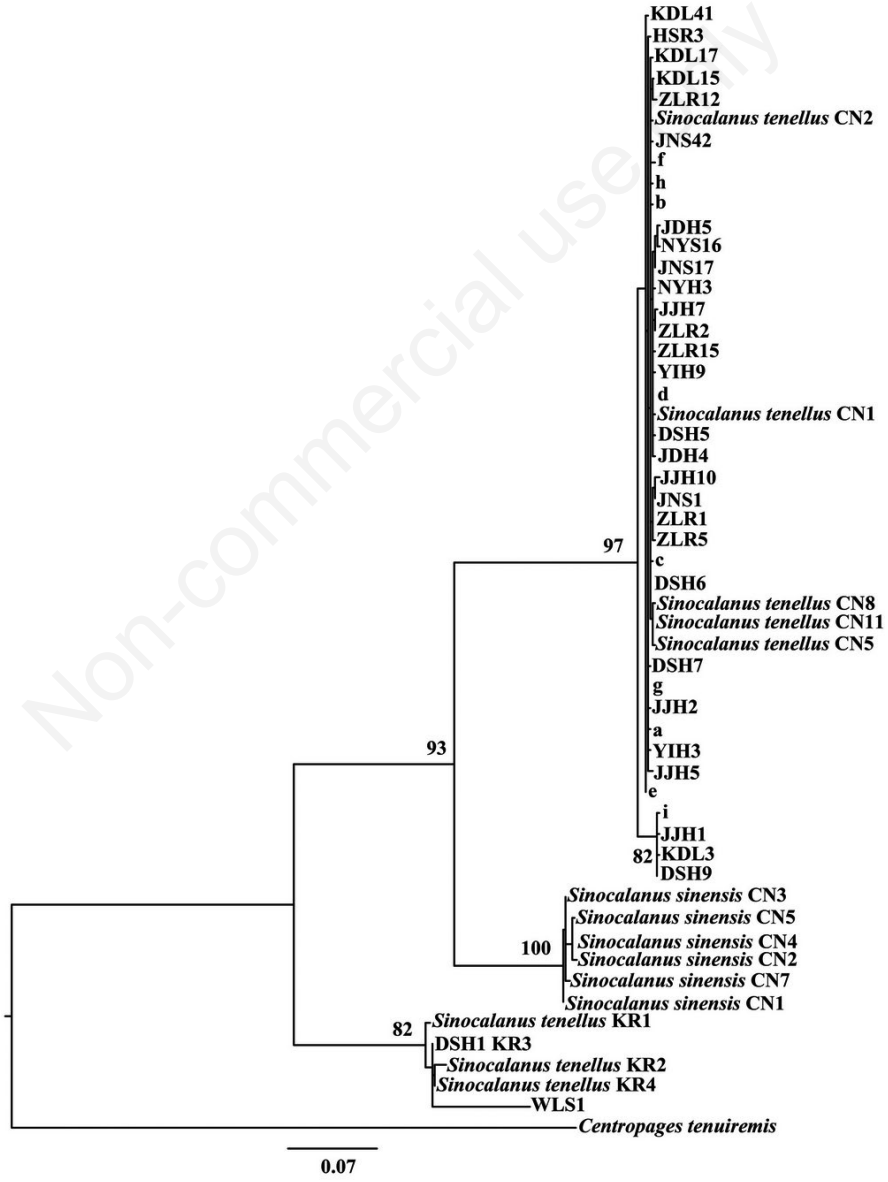


**Fig. 2.** Maximum likelihood (ML) phylogenetic tree inferred from *COI* haplotypes. Bootstrap support values <50% are not displayed. The IDs for shared haplotypes are listed in Tab. 1. The list of reference sequences is provided in Tab. S2. *Centropages tenuiremis* was used as an out-group.

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eight populations, including DSH, HSR, JDH, JJH, JNS, KDL, NYS, and YIH. Another abundant haplotype, b, was shared by DHS, HSR, JDH, JJH, KDL, NYS, YIH, and ZLR. The third abundant haplotype, g, was shared between five populations, i.e., DHS, JDH, JJH, JNS, and NYS (Tab. 1 and Fig. 3). Interestingly, it is quite clear that the clade containing reference sequences, which were purported to be from *S. sinensis*, render *S. tenellus* paraphyletic (Fig. 2). This pattern was maintained if all our new sequences were excluded (*data not shown*).

### Population differentiation and diversity

Pairwise *Fst* values ranged from 0 to 0.992 (between HSR and WSL), suggesting low to high population differentiation among populations. Thirteen pairwise comparisons were significant after the Bonferroni corrections (Tab. 2). The haplotype diversity (*h*) ranged from 0.86 to 0.93 (mean = 0.90), and the nucleotide diversity ($\pi$) ranged from 0.0038 to 0.0154 (mean = 0.0068; Tab. 1). Only two populations, DSH and KDL, showed a signal of historical expansion in both Tajima's D and Fu's Fs tests (Tab. 1). Values for Tajima's D were significantly negative in both these populations (Tab. 1), indicating each had experienced a demographical expansion under the neutral model.

## DISCUSSION

To our knowledge, this report is the first to survey *Sinocalanus tenellus* populations to assess the genetic diversity and the degree of genetic differentiation within the species in China. Using mtDNA *COI* sequences as a genetic marker, we detected high genetic diversity in ten out of eleven populations. Measures of pairwise population differentiation indicated limited to substantial gene flow among the populations. All individuals from WLS shared a haplotype not found elsewhere. As a result, WLS showed high population differentiation from all others, including the nearby KDL population.

High population genetic diversities here are consistent with previous studies on other copepod species using DNA barcoding (Baek *et al.*, 2016; Bucklin *et al.*, 1996; Young *et al.*, 2013). It was believed that the high diversity of *N. schmackeri* populations could result from recent founder effects (Young *et al.*, 2013). Nevertheless, the WLS population had very low nucleotide diversity: all eleven sequenced individuals shared an identical haplotype. This lake was located in Inner Mongolia at a medium-level altitude above sea level (1018 m). Low genetic diversity has also been observed in other zooplankton (*Daphnia*) populations from medium- and

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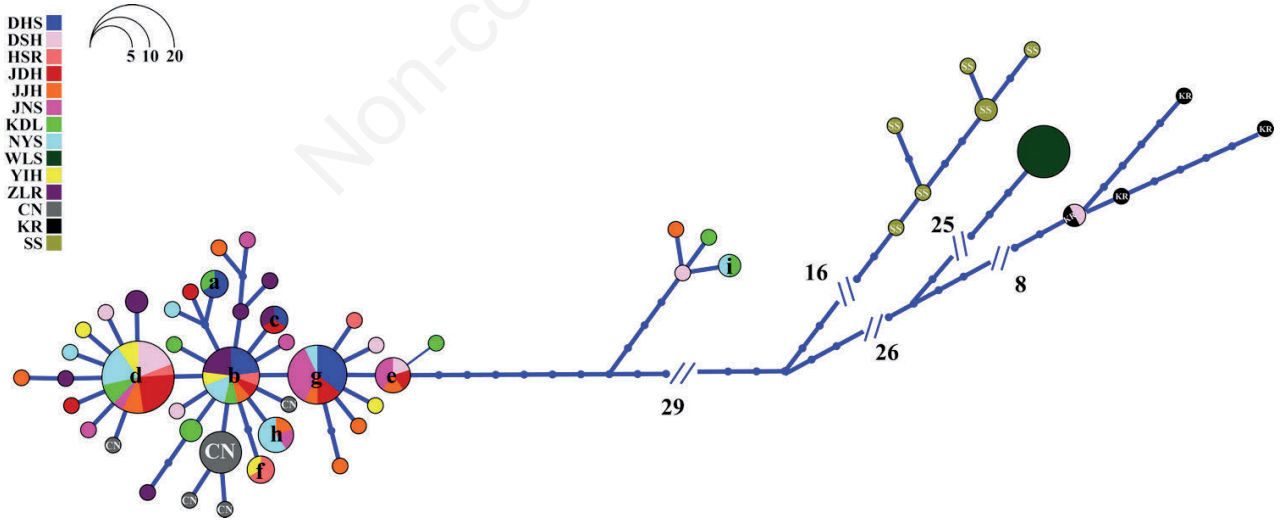


**Fig. 3.** Haplotype network of *Sinocalanus tenellus*, based on the mitochondrial *COI* gene (575 bp). Each circle represents a unique haplotype and its size reflects the number of individuals carrying that haplotype. Segment sizes within circles indicate the distribution of haplotypes among different populations. Color codes allow easy discrimination of populations in the network; the IDs for shared haplotypes are listed in Tab. 1. The number of marks on connecting lines indicates the number of mutations separating haplotypes; for lake abbreviations see Tab. 1. CN and KR refer to the previously deposited *S. tenellus* reference sequences from China and Korea, respectively; SS refer to the *S. sinensis* reference sequences from China (see Tab. S2).

high-elevation lakes, because of founder effects and extremely low effective migration rates (Ventura *et al.*, 2014). However, another population, KDL, which is also located in Inner Mongolia at a similar alitutude, had very high genetic diversity. Ecological differences among locations, for example parasite selection pressure, could affect the population genetic structure and diversity in zooplankton even when locations are close to each other (Jokela *et al.*, 2009; Little and Ebert, 1999; Wolinska and Spaak, 2009).

Lakes or ponds inhabited by freshwater invertebrates are not always connected to each other. However, many species of zooplankton occur over large geographical ranges because of efficient long-distance passive dispersal by waterfowl of sexually produced diapausing eggs (Bilton *et al.*, 2001; Figuerola *et al.*, 2005). This implies that there should be strong gene flow among populations (Baas-Becking, 1934). However, many freshwater zooplankton species capable of producing such eggs, including rotifers and cladocerans, have been shown to display strong population differentiation not only at a global scale, but even at regional levels (Gomez *et al.*, 2000; Penton *et al.*, 2004). Consistently, in this study, we observed substantial population differentiation among copepod populations. In particular, the $F_{st}$ value between KDL and WLS was extremely high (0.96) despite their small geographic separation (80.9 km). It is believed that some species of copepods have limited dispersal ability (Green and Figuerola, 2005). This could likewise result in the high population differentiation in species such as *S. tenellus*. Another explanation could be that the priority effect (De Meester *et al.*, 2002) results in restricted gene flow among the populations of *S. tenellus*, as observed in other freshwater invertebrates. Indeed, for freshwater calanoids, a combination of different factors has been proposed, such as founder effects and rapid local adaptation, that could be responsible for actual gene flow restriction (Marrone *et al.*, 2013; Previsic *et al.*, 2016).

Based on our phylogenetic tree, *S. tenellus* specimens are grouped in two genetically separate clades, all members of which are morphologically similar: a "southern clade" including most Chinese populations, and a "northern clade" including WLS from Inner Mongolia together with all four reference sequences from South Korea and one representative from Shanghai (DSH1). Interestingly, the reference *S. sinensis* clade was nested within *S. tenellus*, separating the two sub-clades of *S. tenellus*. This might be a result of morphological mis-identification of *S. sinensis* by the authors of the reference sequences, as the morphological taxonomy of copepods is difficult and controversial (Humes, 1994). However, the authors of these sequences have also deposited in GenBank reference sequences of *S. tenellus* that match our sequences, hence suggesting that morphological mis-identification is not the problem. An alternative explanation could be that *S. tenellus* actually is a complex of cryptic species, including "*S. sinensis*", and that a new un-named member of this complex was present among our specimens. That morphologically cryptic species can be paraphyletic in molecular trees has been observed in other crustacean species, *e.g. Triops granarius* morphospecies (Korn *et al.*, 2013) and *Hemidiaptomus ingens* s.l. (Marrone *et al.*, 2013).

## CONCLUSIONS

By sequencing a fragment of mtDNA *COI*, we detected a high genetic diversity of *S. tenellus* in China, together with substantial genetic differentiation among the populations. Overall, our findings provide the first preliminary exploration of the phylogeography and

**Tab. 2.** Pairwise genetic differentiation ($F_{st}$) among eleven *Sinocalanus tenellus* populations based on *COI* gene.

| | DHS | NYS | DSH | HSR | JDH | JJH | JNS | YIH | ZLR | KDL | WLS |
|---|---|---|---|---|---|---|---|---|---|---|---|
| DHS | | | | | | | | | | | |
| NYS | 0.126 | | | | | | | | | | |
| DSH | 0.053 | 0.013 | | | | | | | | | |
| HSR | 0.232 | 0.029 | -0.048 | | | | | | | | |
| JDH | 0.177 | -0.010 | 0.030 | 0.098 | | | | | | | |
| JJH | -0.001 | -0.028 | -0.008 | 0.005 | 0.012 | | | | | | |
| JNS | -0.019 | 0.046 | 0.036 | 0.117 | 0.061 | -0.039 | | | | | |
| YIH | 0.234* | -0.035 | -0.041 | -0.060 | -0.048 | -0.005 | 0.089 | | | | |
| ZLR | 0.241* | 0.018 | 0.031 | 0.060 | 0.035 | 0.039 | 0.137 | -0.001 | | | |
| KDL | 0.092 | -0.001 | -0.028 | 0.033 | 0.078 | -0.049 | 0.049 | 0.038 | 0.091 | | |
| WLS | 0.994* | 0.979* | 0.877* | 0.992* | 0.990* | 0.974* | 0.989* | 0.992* | 0.989* | 0.966* | |

**P*<0.00091 after the Bonferroni corrections.*

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lineage diversity of *S. tenellus* in China. Further studies are required to clarify whether *S. tenellus* constitutes a complex of morphologically cryptic species.

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