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Hybridization in Selected Species and Genera of Diaptomid Copepods in China

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Abstract: To better understand the fauna of freshwater calanoid copepods of China, including the occurrence of intra- and intergeneric hybridization, we studied five species, distributed across the whole of China or in South China. We sequenced a mitochondrial (COI) and the nuclear ribosome 18S operon (ITS) to reconstruct the phylogenetic trees by using a Bayesian and maximum likelihood (ML) approach with 161 individuals. The phylogeny tree revealed five clades and two geographically separated subclades in both *S. ferus* and *P. tunguidus*. We found, for the first time, that the hybrid specimens occurred in Diaptomidae, but low hybridization suggested effective barriers to hybridization and introgression. One hypothesis, that hybridization is recent and was initiated by invasions via canals built between the Yangtze and Pearl rivers c. 2000 years ago, is not supported by K2P genetic distances of the order of 20%. Furthermore, COI analysis of different populations of *S. ferus* and *P. tunguidus* revealed two geographical clades in each species, with genetic distances commensurate with cryptic speciation. Both clades occupy subranges maintained without visible barriers to mixis.

Keywords: hybridization; molecular phylogeny; distribution; China; *Neodiaptomus schmackeri*; *Phyllodiaptomus tunguidus*; *Sinodiaptomus sarsi*; *Sinodiaptomus ferus*; *Sinodiaptomus cavernicolax*

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1. Introduction

Formation of new species or merging of species by hybridization are interesting phenomena [1,2]. In freshwater Zooplankton, natural hybridization is common in the Cladocera [3–5], while Parent et al. (2012) found hybrids between *Calanus finmarchicus* and *C. glacialis* (Copepoda, Calanoida) in the Arctic and Northwest Atlantic [1]. In freshwater, the family Diaptomidae is the dominant taxon of the calanoida. Its taxonomy, both at the genus and the species level, relies heavily on the morphology of the fifth thoracic limb (P5) in males [6,7]. Historically, hybrid status of specimens was inferred from morphological analysis and ecological context, for example, in specimens morphologically intermediate and found in the zone of contact between the parental species [8]. However, for zooplankton species, it may be hard to find intermediacy in tiny characters. In fact, it should be shown that putative hybrids have genetic information of the two parental species. Hybridization has recently been suggested, using appropriate molecular techniques, between subspecies of *Eodiaptomus* endemic to the Malili lakes of Sulawesi [9].

The internal transcribed spacers (ITS) of the 18S nuclear ribosomal DNA have been considered a useful molecular marker for hybrids [10,11]. Mitochondrial DNA is a circular molecule, with parental inheritance, a rapid evolutionary rate, and a large number of copies. It has been widely used for analyses of metazoan phylogenetic relationships at various taxonomic levels [12–15].

The freshwater Diaptomidae of China comprise 16 genera, with about 50 species [16]. Diaptomidae tend to have restricted ranges that may encompass several river basins, a single river basin, or part of a river basin. One of the species discussed here, *Sinodiaptomus cavernicolax*, is even restricted to a short underground river in Longyan, a cave which is part of the Star Lakes complex of Zhaoqing city, Guangdong province. *Sinodiaptomus ferus* is found in the Star lakes and in some reservoirs in the same general area. It also penetrates Longyan cave and meets *S. cavernicolax* there [17]. Females of the two can only be distinguished by body size. *Sinodiaptomus sarsi*, a third Chinese species of the genus, is widespread over China but with limited penetration in the west. *Phyllodiaptomus tunguidus* is widely distributed in the Yangtze but especially the Pearl River basin, including Star Lakes, where it is syntopic with the two aforementioned *Sinodiaptomus* species but has never been recorded in the cave. *Neodiaptomus schmackeri* also often coexists with *P. tunguidus* in South China. Coexistence of species and genera makes it theoretically possible for hybridization to occur. To evaluate this expectation, we used a combination of mitochondrial and nuclear DNA markers.

2. Materials and Methods

Thirteen sites were sampled from 6 provinces in China between 2011 and 2016 (Figure 1 and Table 1). Special attention was paid to Star Lakes (23°6′N 112°23′, Zhaoqing City, China), a complex composed of five shallow lakes (Bohai, Zhongxin, Qinglian, Fairy and Li). Longyan cave contains a shallow lake, a few hundreds of meters long and ending at the entrance of the cave. The distance between lake Li and the cave is not more than 10 m [17]. Five samplings in Star Lakes were carried out: in November 2011, July and November 2012, and again in October and November 2013, and May 2015. Zooplankton was collected with a plankton net of 120 µm mesh size, towed from the bottom to the surface. All samples were preserved in ethanol 95% at 4 °C.

We studied the Internal Transcribed Spacers (ITS) and the cytochrome c oxidase subunit I barcoding fragment (COI) according to the DNA extraction HOTSHOT technique [18]. We also downloaded four COI sequences of *S. sarsi* from GenBank, numbers AB699197.1, AB454129.1, AB454128.1, KU720101.1 and KR048945.1.

Complete ITS sequences (including ITS1, 5.8SrRNA and ITS2) of 151 specimens were amplified from total genomic DNA using polymerase chain reaction (PCR). Primers used were F2F (AGCAAAAGTCGTAACAAGGT) and V2R (TTTCACTCGCCGTTACTAAGGGAATC). The PCR conditions for amplification were: 35 cycles set at 30 s at 95 °C (denaturation), 30 s at 54 °C (annealing), and 60 s at 72 °C (extension), followed by 7 min at 72 °C (final-extension). For the cocktail, each 30 µL consisted of 19.5 µL dd H_2O, 3 µL PCR buffer, 1.2 µL dNTP, 0.5 µL of each primer, 5 µL of DNA template and 0.3 µL of Taq DNA polymerase (TaKaRa Taq^{TM} Hot Star Version, Qiagen, Hilden, Germany) [19]. The COI sequences of 159 specimens were amplified. Primers used were ZplankF1 (TGTAAAACGACGGCCAGTTCTASWAATCATAARGATATTGG) and ZplankR1 (CAGGAAACAGCTATGACTTCAGGRTGRCCRAARAATCA) [20], as well as M13F (TGTAAACGACGGCCAGT) and M13R (CAGGAAACAGCTATGAC). The PCR conditions of COI for amplification were: 34 cycles set at 30 s at 98 °C (denaturation), 30 s at 51 °C (annealing) and 60 s at 68 °C (extension), followed by 5 min at 68 °C (final-extension) on a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). For the cocktail, each 30 µL consisted of 4.9 µL dd H_2O, 15 µL 2 × PCR buffer, 0.8 µL solution of each primer, 8 µL of DNA template, and 0.5 µL of MightyAmp DNA Polymerase (TaKaRa Taq^{TM} Ver.2). PCR products were sequenced on an ABI 3130XL automatic sequencer.

The authenticity of COI and ITS sequences were verified by a BLAST search in GenBank. The sequences were edited in Mega 7.0 (Institute for Genomics and Evolutionary Medicine, Temple University, USA) and Finch TV 1.5.0 (Geospiza Research Team, USA). The total length of the sequence segments after alignment were 555 bp for COI and 652 bp for ITS. We used *Acanthodiaptomus pacificus* as the outgroup species.

We used MRMODELTEST v.2.3 (Evolutionary Biology Centre, Uppsala University, Sweden) [21] to select the best-fit model of nucleotide substitution under the Akaike information criterion (AIC) [22]. Analyses were performed under Maximum likelihood (ML) and Bayesian inference. Maximum likelihood analysis was performed in PAUP* 4.0 beta 10 (Natural HistorySuvey, Champagne Urbana) [23], Bayesian analysis was performed using MRBAYES v3.1.2 (University of Rochester, Rochester, USA) [24,25]. Majority rule consensus trees were constructed after discarding a burn-in of 500 and displayed with FigTree v1.4.2 (Figtree HR Consultancy, The Hague, Netherlands). Genetic distances between COI sequences were calculated using the Kimura two-parameter (K2P) model in MEGA 7.0 [26].

Table 1. The summary of thirteen sampling locations in this investigation.

Species	Code	Province	City	Site
Neodiaptomus schmackeri	China	Guangdong (GD)	Qingyuan	Feilaixia Reservoir
	China	Guangdong (GD)	Kaiping	Dashahe Reservoir
	China	Hubei (HB)	Chibi	Lushui lake
	China	Yunnan (YN)	Kunming	Small pond
	China	Yunnan (YN)	Kunming	Sanwan
Phyllodiaptomus tunguidus	China	Hainan (HN)	Danzhou	Songtao Reservoir
	China	Heilongjiang (HLJ)	Haerbin	Hanxiao road
	China	Yunnan (YN)	Lijiang	Chahe
	China	Guangxi (GX)	Baise	Zhexian river
	China	Guangdong (GD)	Shaoguan	Tiantangshan
	China	Guangdong (GD)	Conghua	Lixihe Resrevoir
	China	Guangdong (GD)	Maoming	Luokeng Reservoir
	China	Guangdong (GD)	Zhaoqing	Star Lakes
	China	Guangdong (GD)	Zhuhai	Zhuyin Reservoir
	China	Guangxi (GX)	Baise	Yangxu
	China	Yunnan (YN)	Kunming	Xiaozhulong
	China	Yunnan (YN)	Kunming	Sanwan
Sinodiaptomus ferus	China	Guangdong (GD)	Zhaoqing	Star Lakes
	China	Guangdong (GD)	zhuhai	Zhuyin Reservoir
Sinodiaptomus cavernicolax	China	Guangdong (GD)	Zhaoqing	Star Lakes
Acanthodiaptomus pacificus	China	Heilongjiang (HLJ)	Haerbin	Hanxiao road

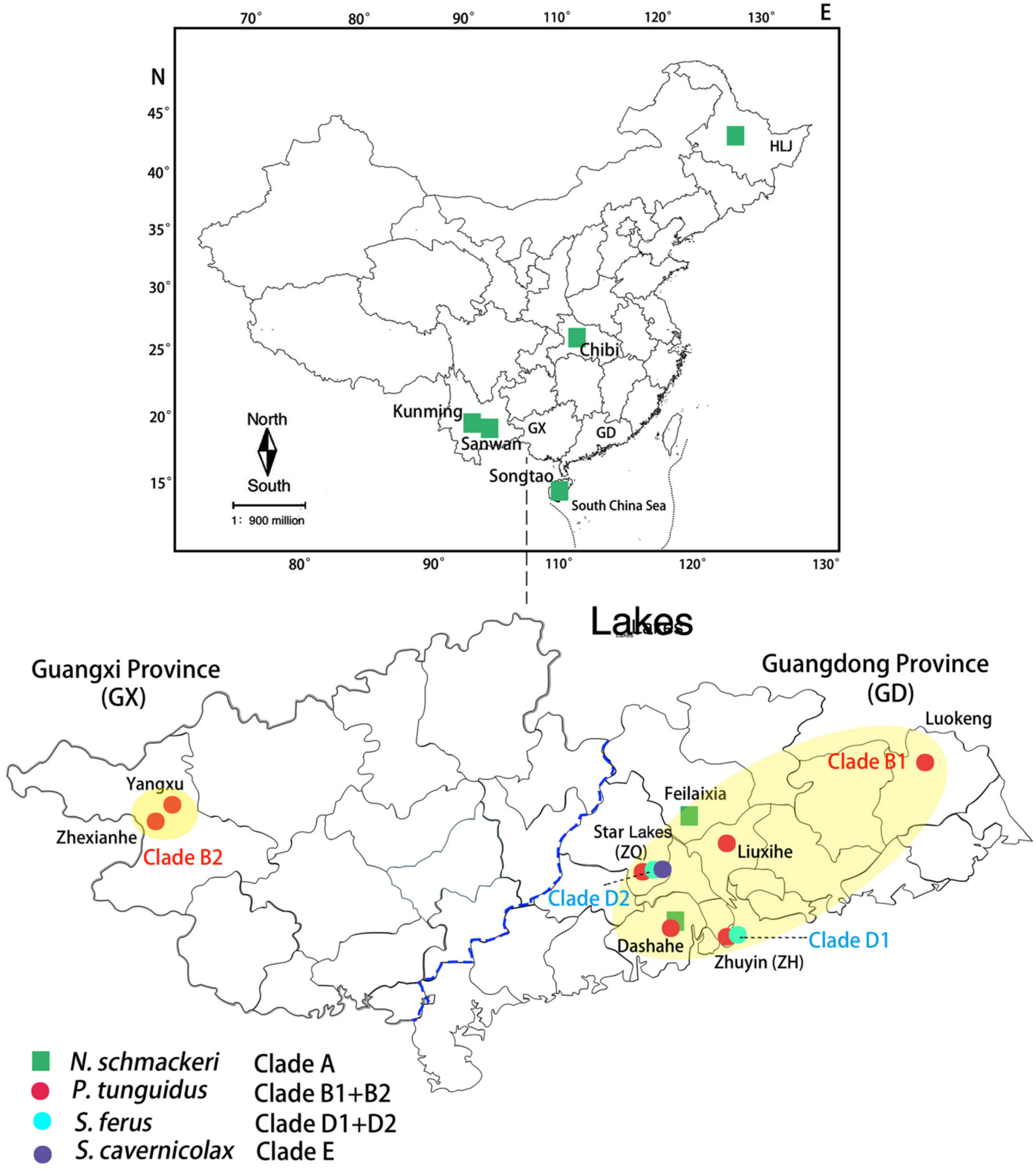

Figure 1. Thirteen sampling sites in China (Clade B1: *P. tunguidus* populations in Guangdong province (GD); Clade B2: *P. tunguidus* populations in Guangxi province (GX); Clade D1: *S. ferus* population in Zhuyin Reservoir, Zhuhai City, Guangdong province (ZH); Clade D2: *S. ferus* population in Star Lakes, Zhaoqing City, Guangdong province (ZQ)).

3. Results

3.1. Picking Hybrids from A Phylogenetic Tree

We obtained 159 sequences of the COI gene fragment from 29, 54, 14, 5 and 44 individuals of *N. schmackeri*, *P. tunguidus*, *S. sarsi*, *S. cavernicolax*, *S. ferus* and 13 female specimens of *Sinodiaptomus*, respectively. We also obtained 151 sequences of ITS from 32, 49, 16, 22 and 21 individuals of *N. schmackeri*, *P. tunguidus*, *S. sarsi*, *S. cavernicolax*, *S. ferus* and 11 female specimens of *Sinodiaptomus*, respectively. The final sequences are available in NCBI under accession numbers MW820030-820084, MW819873-819901, MW820091-820159, and MN852697-852848.

The best-fitting model selected by MRMODELTEST 2.3 for the ITS dataset was of GTR+G with a relative AIC weight of 0.5110 and gamma distribution shape parameter 0.4060. The best-fitting model for the COI dataset was GTR+I+G with a relative AIC weight of 0.7905 and gamma distribution shape parameter 0.7180.

Bayesian inference and ML for both genes revealed the same five clades: *N. schmackeri* (A), *P. tunguidus* (B), *S. sarsi* (C), *S. ferus* (D), *S. cavernicolax* (E) (Figures 2 and 3). However, while in the COI tree, all specimens were recovered consistent with their morphological taxonomy (Figure 2A,B); in the ITS tree, some specimens end up in a "wrong" cluster (Figure 3A,B). The COI gene sequence always recovers the mother; if the ITS gene finds another species, the specimen in question is a hybrid. In Clade A of the ITS tree, No.9 *S. ferus*, for example, is a hybrid with *N. schmackeri*. Clade B contains one hybrid of *S.* × *cavernicolax.* Clade D contains two well-supported sublineages from Zhuyin reservoir, Zhuhai city (D1) and Star Lakes, Zhaoqing city (D2). In this clade, there are four putative hybrid specimens of *S.* × *cavernicolax* and one *P.* × *tunguidus*. Clade E contains three hybrids of *S.* × *ferus*. The COI tree reveals the same five well-supported main clades as the ITS tree. Five of ten hybrids form a clade of their own, with strong bootstrap support. In the COI tree, Clade B and D both contain two well-supported sublineages from the Guangdong province population (B1) and Guangxi province population (B2), Zhuyin reservoir, Zhuhai city (D1) and Star Lakes, Zhaoqing city (D2), both in Guangdong province.

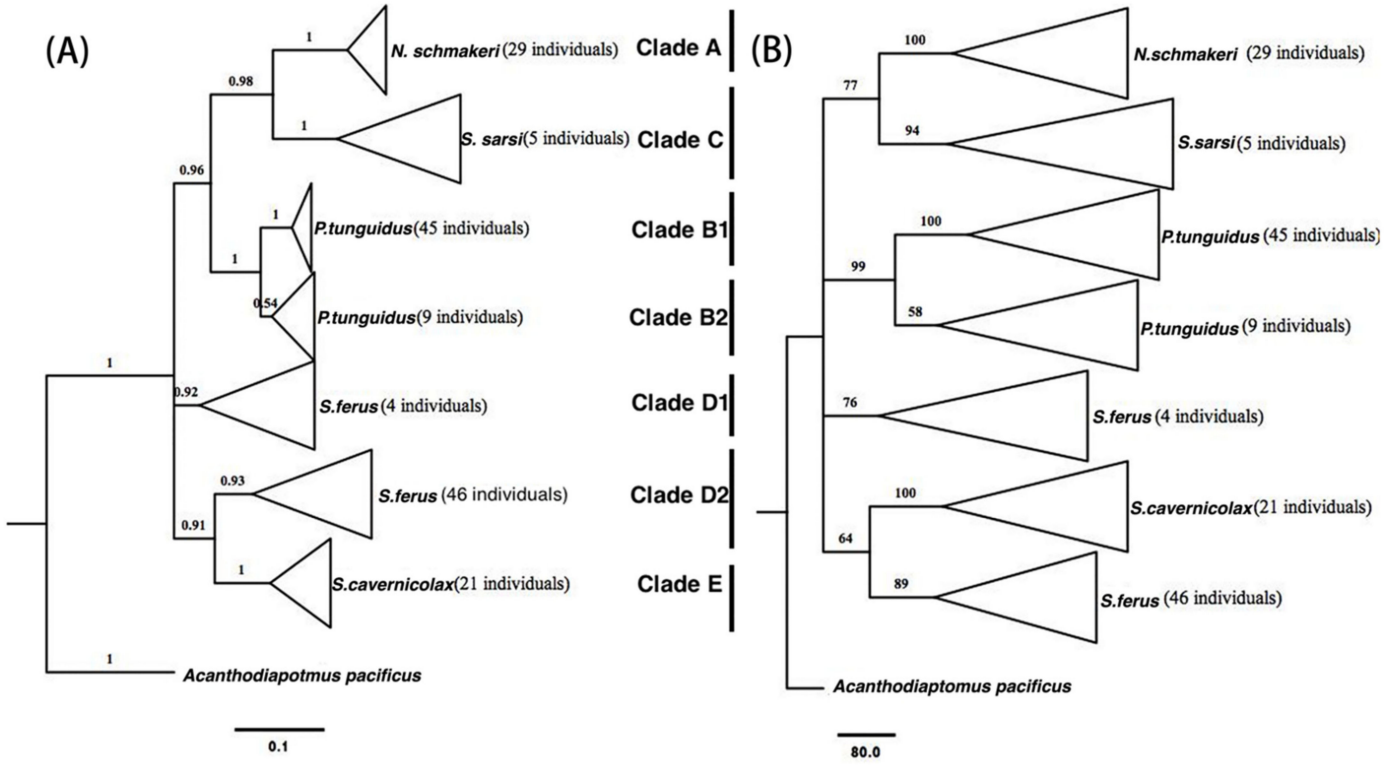

Figure 2. Phylogenetic tree of investigated species based on the sequences of their COI genes by Bayesian inference (**A**) and maximum likelihood method (**B**).

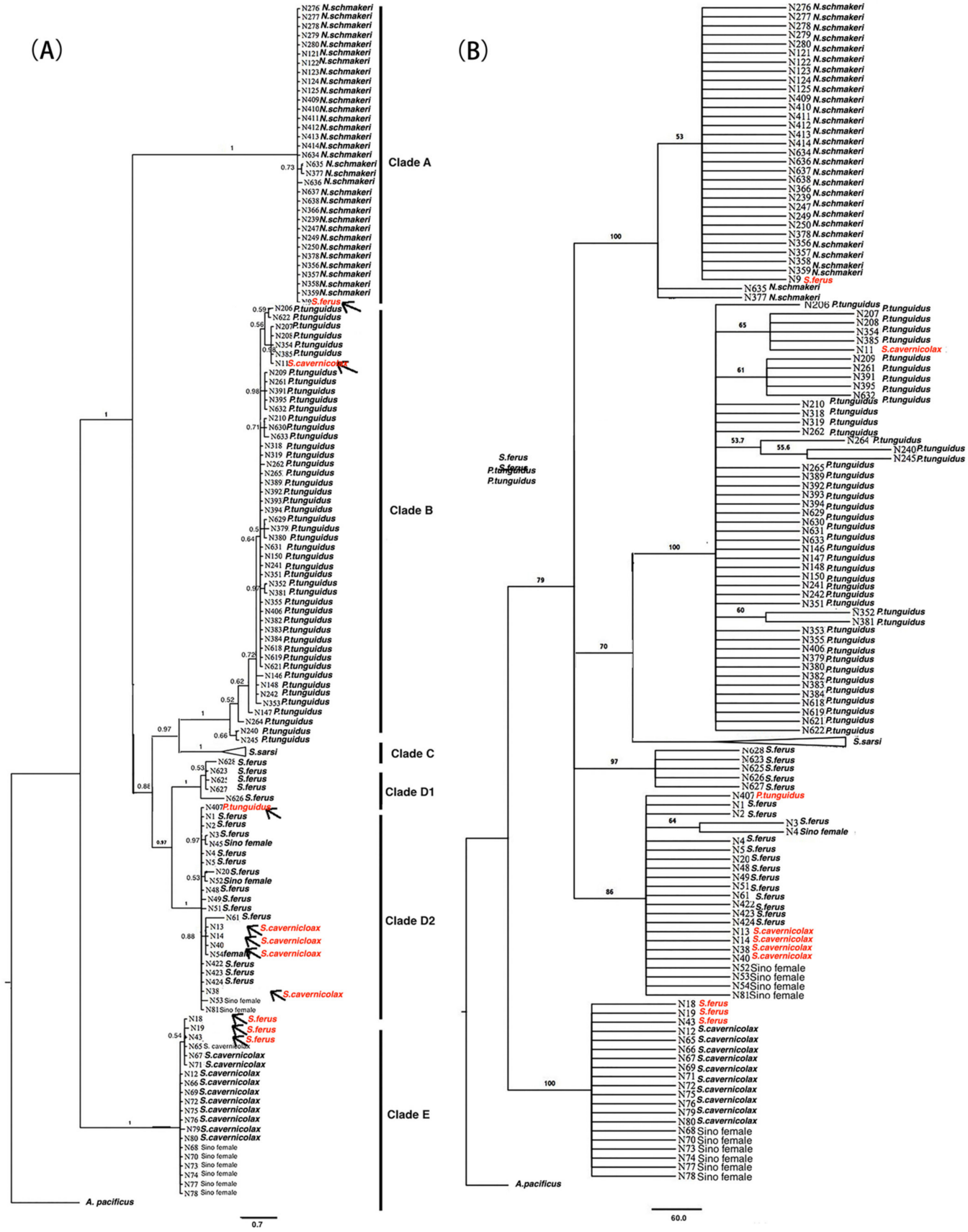

Figure 3. Phylogenetic tree of investigated species based on the sequences of their ITS genes by Bayesian inference (**A**) and maximum likelihood method (**B**).

3.2. COI Divergence

The K2P pairwise distances derived from COI varied between 11% and 32.6%. The highest distance was between clade C (*S. sarsi*) and clade D1 (*S. ferus*); the lowest was between clade B1 (*P. tunguidus*, Guangdong population) and B2 (Guangxi population). The second lowest genetic distance, 15.1%, occurred between clade D2 (*S. ferus*) and clade E (*S. cavernicolax*). The distance between clades D1 and D2 reached 17.8% even though they were same morphospecies, higher than between clade E and D2, and also higher than the average intra-specific variation in Crustacea [27]. The average genetic distance between genera was 21.1%; within genera it was 21.8% (Table 2).

Table 2. Range of maximum likelihood genetic distance (Kimura two-parameter model) between genera and lower-order clades, average ± SD.

Clade	Species	A	B1	B2	E	D2	D1
A	*N. schmackeri*						
B1	*P. tunguidus* GD	0.23 ± 0.025					
B2	*P. tunguidus* GX	0.242 ± 0.026	0.11 ± 0.015				
E	*S. cavernicolax*	0.23 ± 0.023	0.181 ± 0.019	0.206 ± 0.022			
D2	*S. ferus* ZQ	0.254 ± 0.025	0.179 ± 0.019	0.183 ± 0.020	0.151 ± 0.016		
D1	*S. ferus* ZH	0.243 ± 0.023	0.208 ± 0.019	0.202 ± 0.020	0.189 ± 0.018	0.178 ± 0.017	
C	*S. sarsi*	0.25 ± 0.025	0.266 ± 0.027	0.285 ± 0.028	0.303 ± 0.029	0.259 ± 0.025	0.326 ± 0.028

4. Discussion

Hybrids in freshwater microcrustaceans are hard to identify by morphology, despite a high genetic divergence, which surely contributes to their apparent rarity. However, our results show that *N. schmackeri*, *P. tunguidus*, *S. ferus* and *S. cavernicolax* do hybridize, but hybridization rates are low, of an order of 5.4% between species and of 2.3% between genera. These results suggest the operation of rather effective pre- or post-mating barriers, and introgression is unlikely. At this stage, it is not possible to ascertain whether the hybrids are fertile or not.

S. cavernicolax is not, as its describers claimed (Shen and Tai, 1965), a true stygobiont, because it is not depigmented and has eyes. However, it has never been found outside Longyan cave. Hybrids between the congeners *S. ferus* and *S. cavernicolax* are therefore by necessity limited to the cave where the two coexist. *S. ferus* has a fairly restricted range but extends outside of the Star Lakes to parts of the west branch of the Pearl River.

P. tunguidus has, to date, never been found in the cave. The pond at the mouth of the cave is separated from Star Lakes by a narrow land bar. Currently, the levels of the lake as well as the cave river are controlled by pumps. Historically, floods of the Pearl River may have overflown, bringing lake and cave waters into direct contact. Dry episodes long enough to allow allopatric speciation to proceed are equally possible. That the two *Sinodiaptomus* share a common ancestor is beyond a reasonable doubt: they are morphologically extremely close and can only be distinguished structurally in males. Females can be distinguished by size: females of *ferus* are larger than of *cavernicolax*, but there is a 'grey zone' between them.

P. tunguidus is considered to be endemic to South China [28]. *N. schmackeri* is widely distributed in the whole of China except the cold, mountainous northwest [29]. Reproductive isolation is supposed to become stronger as genetic distance increases; therefore, barriers should become more hermetic as one moves from the species to the genus level. That is what we seemed to observe, with intergeneric hybrids an order of magnitude more rare than interspecies hybrids.

Could it be that the hybrids are an artifact of human disturbance of the river basins? China is reputed to have constructed some of the first and longest canals in the world, such as the Jing Hang Canal, the 1800 km long Grand Canal linking the Yellow River to the Yangtze basin, built between 486 BC and 610 AD, and the Ling Qu canal in Guangxi Province, between the Yangtze and the Pearl River, built around 214 BC and which is 36 km

long. In *S. sarsi* and the couple *S ferus-cavernicolax*, another phenomenon seems to occur: here, the ranges are complementary, with possibly a hiatus between the two groups.

The species status of *S. cavernicolax,* finally, is not in doubt, and neither is its derivation from a common ancestor with *S. ferus*. Its genetic distance with *S. ferus* is consistent with a good species, and it appears to have evolved locally in Longyan cave.

5. Conclusions

In this study, we reconstructed the phylogenetic tree by Bayesian inference and Maximum likelihood method by using COI and ITS sequences from 161 specimens of *N. schmackeri*, *P. tunguidus*, *S. sarsi*, *S. ferus and S. cavernicolax*. The molecular analysis results provide evidence that there are certain cryptic species in Diaptomidae which have not yet been discovered, and we also find inter-specific and intra-specific hybridization in Diaptomidae with a low hybridization rate. This is the first time that hybrid Diaptomidae species have been found in freshwater.

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Institutional Review Board Statement: This study did not require ethical approval.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data available in a publicly accessible repository. The data presented in this study are openly available in GenBank, numbers AB699197.1, AB454129.1, AB454128.1, KU720101.1 and KR048945.1.

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Conflicts of Interest: The authors declare no conflict of interest.

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