Genetic Diversity of *Daphnia pulex* in the Middle and Lower Reaches of the Yangtze River

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Abstract

Increased human activities and environmental changes may lead to genetic diversity variations of Cladocerans in water. *Daphnia pulex* are distributed throughout the world and often regarded as a model organism. The 16S rDNA, cytochrome c oxidase subunit I (COI), and 18S genes were used as molecular marks. The genetic diversity and phylogeny of *D. pulex* obtained from 10 water bodies in the middle and lower reaches of the Yangtze River were studied. For 16S rDNA, COI gene, and 18S gene, the A+T content (65.4%, 58.4%, and 54.6%) was significantly higher than the G+C content (34.6%, 41.6% and 45.4%). This result was consistent with higher A and T contents among invertebrates. Based on the genetic distances of 16S rDNA and COI genes, the genetic differences of *D. pulex* from 10 water bodies located in the middle and lower reaches of the Yangtze River in China was minimal (0%–0.8% for 16S rDNA and 0%–1.5% for COI gene). However, *D. pulex* evolved into two branches in the phylogenetic trees, which coincided with its geographical distribution. Compared with *D. pulex* from other countries, the average genetic distance of *D. pulex* obtained from 10 water bodies in the middle and lower reaches of the Yangtze River reached 9.1%–10.5%, thereby indicating that *D. pulex* may have evolved into different subspecies.

Introduction

Cladocerans are important components of the food chain in aquatic ecosystems [1]. First, these organisms can feed on algae and improve water quality [2–4]. Second, cladocerans are predated by fish as food. Cladocerans undergo parthenogenesis in suitable environments and form large populations. However, sexual reproduction of Cladocerans occurs under bad conditions and fertilized eggs are produced. *Daphnia pulex* is a cosmopolitan species that is widely distributed in inland fresh waters, particularly in eutrophic waters [5–6]. To date, frequent human activities have led to environmental differences among lakes, such as variations in nitrogen and phosphorus concentrations in the sediment in the middle and lower reaches of the Yangtze River [7]. Eutrophication and the structure of the fish population may also have affected the population dynamics of *D. pulex* in these lakes [8–10].
Multiple methods are available for species identification and phylogeny reconstruction of crustaceans [11–15]. The 16S rDNA and the cytochrome c oxidase subunit I (COI) and 18S genes are more popular among these methods [15–21]. The classification of 16S rRNA and COI gene sequences were more convincing in Daphnia. The mitochondrial divergences of different Daphnia species are below 5% between North and South America [22–24] and between North America and Europe [25].

John et al. (2011) reported the gene sequences of D. pulex [26]. A few functional genes of crustaceans were widely studied [16, 20, 26–28]. Benzie (2005) described the D. pulex complex, including D. pulex, D. pulicaria, and D. middendorffiana [6]. The different D. pulex complexes are distributed worldwide, and the species was studied as a model by many investigators [28–32]. Ceresa et al. (2012) investigated the intercontinental phylogeography of the D. pulex complex by analyzing the mitochondrial NADA dehydrogenase subunit 5 and the COI gene [29]. Some works in the literature showed that the genetic distance ranged from 5% to 14% for D. pulex complex [16, 29, 33]. Although the molecular phylogeny of D. pulex was extensively reported, the genetic differences of the D. pulex from China and comparison of species in China and those in other countries have not been reported.

In this study, the genetic difference among the D. pulex from 10 water bodies located in the middle and lower reaches of the Yangtze River and the genetic difference of the D. pulex between China and other countries were analyzed by amplifying and sequencing the 16S rDNA, as well as the COI and 18S genes. Our results could become an important evidence for the global phyletic evolution of D. pulex.

**Materials and Methods**

**Sampling, identification and culturing**

Field collection of Daphnia was carried out after obtaining permission from the Ministry of Environment, and the field studies did not include endangered or protected species.

The fertilized eggs of D. pulex were collected from the sediment of 10 water bodies located in the middle and lower reaches of the Yangtze River with a modified Peterson grab (Table 1). The eggs were hatched in an intelligent lighting incubator (Ningbo Saifu, China) at 25°C. D. pulex was identified morphologically (Fig 1) under the microscope (Olympus, Japan) according to the differences in the morphological features of the species.

### Table 1. Origin and number of D. pulex species in this study.

| Locality                      | Longitude and latitude | Collection catalog numbers |
|-------------------------------|------------------------|---------------------------|
| Donghu Lake, Hubei province   | N: 30°32'46.04" E: 114°22'31.20" | WD1WD2WD3 WD1WD2WD3 WD   |
| Guohe River, Anhui province  | N: 33°52'37.25" E: 115°47'27.00" | BZ1BZ2 BZ1BZ2 BZ         |
| Qianlong Lake, Anhui province | N: 33°54'21.49" E: 116°48'55.27" | QL QLQL2QL3 QL          |
| Pond in Anhui province        | N: 33°38'59.33" E: 116°57'35.21" | SZ1 SZ2 SZ               |
| Shengjin Lake, Anhui province | N: 30°21'0.10" E: 117°0'36.30"  | SJ1 SJ2 SJ               |
| Chaohu Lake, Anhui province   | N:31°33’28.74’ E: 117°0’36.30’  | CH CH CH1 CH2 CH3       |
| Nanyi Lake, Anhui province    | N:31°4’27.11’ E: 118°58’40.64’  | XC1XC2XC3XC4 XC1XC2XC3XC4 XC |
| Taihu Lake, Jiangsu province  | N: 31°29’9.29’ E: 120°11’43.70’  | TZ TZ TZ                |
| Hongze Lake, Jiangsu province | N: 33°17’48.74’ E: 118°39’44.37’  | HZ1HZ2 HZ               |
| Pond in Shanghai city         | N: 31°13’48.02’ E: 121°24’16.20’  | SH1 SH2 SH1 SH2 SH3 SH |

Note: Four different individuals were collected from each waterbody, but only one individual was chosen for each sequence. N indicates the North latitude, and E indicates East longitude.

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to the methods of Jiang and Du [5] and Benzie [6]. For each water body, four individuals of *D. pulex* were selected by hatching different fertilized eggs. Monoclonal organisms were cultured in an intelligent light incubator (Ningbo Saifu, China) with 12 h light:12 h dark illumination at
25°C. *Scenedesmus obliquus* was used as their food. The medium was aerated tap water over 48 h, and pH was approximately 7.

**DNA extraction, amplification, and sequencing**

Young *D. pulex* hatched from the fertilized eggs became adults and became pregnant after roughly 7 days at 25°C. An adult individual was selected. Genomic DNA of *D. pulex* was extracted by the TIANamp Micro DNA Kit (Tiangen, Beijing). Each *D. pulex* body was crushed with a sterile 10 μL tip before extraction because the chitin carapace of *D. pulex* could hinder the digestion of internal organs by proteinase K. The concentration of DNA extraction was measured by the Spectrophotometer (Biofuture, England). The concentrations of DNA were 65–85 ng/μL and the A280/A260 was 2.3.

The mitochondrial 16S rDNA was amplified with the L2510 (5'-CGCCTGTTTAACAACACAT-3') and H3059 (5'-CCGGTCTGAACCTCAGATGTGT-3') primers [34]. The mitochondrial COI gene was amplified with the COIF (5'-AYCAATCATAGGAYATTGAAC-3') and COIR (5'-KGTGATWCCNACHGCTAC-3') primers from Xu et al. [35]. The nuclear 18S gene was amplified with the 18S-F (5'-AACCTGGTTGATCCTGCCAGT-3') and 18S-R (5'-TGATCCTTTGCAGGTTTACCAC-3') primers from Huang et al. [19].

To validate the predicted sequences of chemosensory genes, the PCR (Eppendorff, Germany) products obtained from genomic DNA of adult *D. pulex* were purified using the AxyPrep TM PCR Cleanup Kit (Axygen) and sub-cloned into a T/A plasmid using the pEASY-T3 cloning vector system (TransGen, China) following the manufacturer’s instructions. The plasmid DNA was used to transform to Trans1-T1 competent cells. The positive clones were checked by PCR and sequenced by GenScript (Nanjing, China).

The 25 μL PCR reaction contained 1.0 μL of genomic DNA, 14.75 μL of double-distilled H2O, 2.5 μL of 10× LA-Taq Buffer II, 4.0 μL of dNTPs (2.5 mM) (Shanghai Shenggong, China), 0.5 μL of Mg²⁺ (25 mM), 1.0 μL of each primer (10 nM) (Shanghai Shenggong, China), and 0.25 μL of DNA polymerase TaKaRa-LA-Taq (5 U/μL) (Clontech, USA).

The conditions of the 16S rDNA amplification included an initial denaturing step of 3 min at 94°C, 35 cycles of 45 s at 94°C, 45 s at 50°C, and 55 s at 72°C, and a final extension of 72°C for 10 min. The conditions of the COI gene amplification included an initial denaturing step of 1 min at 94°C, 35 cycles of 40 s at 94°C, 45 s at 45°C, and 1 min at 72°C, and a final extension of 72°C for 10 min. The conditions of the 18S gene amplification included two cycles of 30 s at 94°C, 45 s at 60°C, and 45 s at 72°C, followed by five cycles of 30 s at 93°C, 45 s at 55°C, and 45 s at 72°C, and a final 35 cycles of 30 s at 93°C, 30 s at 50°C, and 3 min at 72°C.

**Analytical procedure**

According to the peak in SeqMan, the bidirectional sequencing of the nucleotide sequence was proofread by DNASTar to remove unreliable bases. The percentage of the detected sequence differences was obtained.

For each water body in the middle and lower reaches of Yangtze River, the sequences of the four *D. pulex* individuals were obtained. Unreliable bases were removed by SeqMan (DNASTar). A total of 517–539 valid bases for 16S rDNA, 522–527 valid bases for the COI gene, and 2335–2344 valid bases for the 18S genes were detected (Table 1). Other sequences that were used for analysis were downloaded from GenBank (Tables 2–4). In this study, the standard of the selected sequences was the similarity of the homologous sequence (over 80%) compared with the sequences from Genbank.

Multiple sequence alignment was performed with CLUSTALX (ref.). DNAspV5 (ref.) was used to analyze the variation of sites among the sequences. The conversion/transversion and the
genetic distance of interspecies were calculated with MEGA 6.0 (ref.). The genetic distances among sequences were calculated by the Kimura two-parameter model with 1,000 bootstraps. The maximum likelihood (ML) analysis, which used the GTR+G+I evolutionary model indicated by Modeltest version 3.7, was performed with MEGA 6.0 (ref.) and bootstrap resampled 1,000 times. In addition, we constructed phylogenetic trees via Bayesian inference in MrBayes 3.1.2 (ref.). This program was run for 10,000,000 generations, and sampling from the chain was performed every 10,000 generations. Initially, 25% of the trees were discarded as burn-in, and the 50% majority rule consensus tree was constructed from the remaining Bayesian trees after the posterior probability values for each node were calculated. To better reveal the genetic difference of *D. pulex*, the suitable outgroups were employed to construct phylogenetic trees. For 16S

| Species       | Code in the study | GenBank accession number | Collection location | Reference  |
|---------------|-------------------|--------------------------|---------------------|------------|
| *Daphnia pulex* | KF64              | KF993364                 | China               | Xu et al. [35] |
| *D. pulex*     | KF63              | KF993363                 | China               | Xu et al. [35] |
| *D. pulex*     | AF17              | AF117617                 | Canada              | Crease et al. [40] |
| *D. pulex*     | JN07              | JN874607                 | Russia              | Zuykova et al. [42] |
| *D. pulex*     | JN06              | JN874606                 | Russia              | Zuykova et al. [42] |
| *D. pulex*     | JN05              | JN876405                 | Russia              | Zuykova et al. [42] |
| *D. pulex*     | GQ75              | GQ343275                 | Canada              | Briski et al. [41] |
| *D. parvula*   | GQ64              | GQ343264                 | Canada              | Briski et al. [41] |
| *D. parvula*   | GQ65              | GQ343265                 | Canada              | Briski et al. [41] |
| *D. parvula*   | GQ66              | GQ343266                 | Canada              | Briski et al. [41] |
| *D. parvula*   | GQ67              | GQ343267                 | Canada              | Briski et al. [41] |
| *D. parvula*   | GQ71              | GQ343271                 | Canada              | Briski et al. [41] |
| *D. parvula*   | FJ73              | FJ427473                 | Canada              | Adamowicz et al. [33] |
| *D. cf. parvula sp.* | FJ74 | FJ427474                 | Canada              | Adamowicz et al. [33] |
| *D. obtusa group sp.* | FJ71 | FJ427471                 | Canada              | Adamowicz et al. [33] |
| *D. obtusa*    | FJ66              | FJ427466                 | Canada              | Adamowicz et al. [33] |
| *D. obtusa group sp.* | FJ70 | FJ427470                 | Canada              | Adamowicz et al. [33] |
| *D. obtusa group sp.* | FJ67 | FJ427467                 | Canada              | Adamowicz et al. [33] |
| *D. magna*     | D. magna          | AY921452                 | USA                 | Colbourne et al. [46] |
| *Bosmina sp.*  | Bosmina           | EU650743                 | USA                 | Kotov et al. [18] |

Note: *D. pulex* (GenBank accessions: KF993364 and KF993363) were obtained from Lake Chaohu in China.

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| Species       | Code in the study | GenBank accession | Collection location | Reference |
|---------------|-------------------|-------------------|---------------------|-----------|
| *Daphnia pulex* | KJ74              | KJ461674          | China               | Geng et al. [47] |
| *D. pulex*     | KF72              | KF993372          | China               | Xu et al. [35] |
| *D. pulex*     | KF71              | KF993371          | China               | Xu et al. [35] |
| *D. cf. pulex* | GU92              | GU595192          | Japan               | Kotov et al. [43] |
| *D. cf. pulex* | GU90              | GU595190          | Japan               | Kotov et al. [43] |
| *D. jollyi*    | *D. jollyi*       | AF308969          | Canada              | Hebert et al. (2000) |
| *Ceriodaphnia cf. reticulata* | C.cf.reticulata | KC617252          | Mexico              | Prosser et al. [48] |

Note: *D. pulex* (GenBank accession: KJ461674, KF993372, and KF993371) were obtained from Lake Chaohu in China.

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rDNA, *D. magna* (AY921452) and *Bosmina* sp. (EU650743) were used as outgroups. For COI gene, *D. jollyi* (AF308969) and *Ceriodaphnia cf. reticulata* (KC617252) were used as outgroups. For 18S gene, *D. magna* (AM490278) and *C. dubia* (AF144208) were used as outgroups. Analysis of molecular variance (AMOVA) test was conducted by using Arlequin 3.5 [36].

## Results

### Genetic diversity of *D. pulex* from the middle and lower reaches of Yangtze River based on 16S rDNA

The alignment of the 37 16S rDNA sequences identified 403 conserved sites, including 334 invariable sites, 69 variable sites, 9 single sites, and 60 parsimony-informative sites. Among the 16S rDNA sequences of the *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze River, the average A, T/U, C, and G content was 32.6%, 32.8%, 13.6%, and 21.0%, respectively. The A+T content (65.4%) was significantly higher than the G+C content (34.6%). The overall transition/transversion ratio was 1.09. The genetic distances between sequences were calculated by the Kimura 2-parameter distance (0%–9.8%) and maximum likelihood estimate (0%–11.5%). The phylogenetic trees produced highly congruent tree topologies (Fig 2). The main divergences in the ML tree were in accordance with

| Species      | Code in the study | GenBank accession | Collection location | Reference                  |
|--------------|-------------------|-------------------|---------------------|-----------------------------|
| *D. pulex*   | KJ027             | KJ775027          | China               | Huang et al. [19]           |
| *D. pulex*   | AF011             | AF014011          | Canada              | Crease et al. (1997)        |
| *D. obtusa*  | AY600             | AY887600          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY601             | AY887601          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY604             | AY887604          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY608             | AY887608          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY611             | AY887611          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY612             | AY887612          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY614             | AY887614          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY624             | AY887624          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY630             | AY887630          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY642             | AY887642          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY545             | AY887545          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY547             | AY887547          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY552             | AY887552          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY562             | AY887562          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY565             | AY887565          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY568             | AY887568          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY577             | AY887577          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY578             | AY887578          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY580             | AY887580          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY582             | AY887582          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY583             | AY887583          | Canada              | McTaggart et al. [49]       |
| *D. obtusa*  | AY598             | AY887598          | Canada              | McTaggart et al. [49]       |
| *D. magna*   | AM490278          | Belgium           | Van Damme et al. [50]|
| *Ceriodaphnia dubia* | C. dubia | AF144208         | USA                  | Spears et al. [51]         |

Note: *D. pulex* (GenBank accession: KJ775027) was obtained from Zhejiang province in China.

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Fig 2. The phylogeny of *D. pulex* inferred from 16S rDNA sequences as a consensus tree formed from trees constructed using maximum likelihood (ML), and neighbor-joining (NJ), Bayesian inference (BI) methods.

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those of the MrBayes and NJ trees (Fig 2). In the phylogenetic trees, the branches represented 99% support for the presumed biological species of *D. pulex*. *D. pulex* from 10 water bodies located in the middle and lower of the Yangtze River evolved into two branches in the NJ tree. One branch included *D. pulex* from Lake Donghu in Hubei Province, as well as Lake Shengjin, Lake Nanyi, Lake Chaohu, and Guohe River in Anhui Province. The other branch included *D. pulex* from a pond in Shanghai City, Lake Taihu, and Lake Hongze in Jiangsu Province, as well as Lake Qianlong, a pond, Lake Chaohu, and Lake Nanyi in Anhui Province. *D. pulex* from Lake Chaohu and Lake Nanyi was present in both branches, which coincided with its geographical locations. In addition, the *D. pulex* from 10 water bodies located in the middle and lower reaches of the Yangtze River and the *D. pulex* from abroad were clustered in two distant branches (Fig 2).

**Genetic diversity of *D. pulex* from the middle and lower reaches of Yangtze River based on the COI gene**

The alignment of 26 COI sequences identified 487 conserved sites, including 433 invariable sites, 54 variable sites, 9 single sites, and 45 parsimony-informative sites. Among the COI sequences of the *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze River, the average A, T/U, C, and G content was 23.5%, 34.9%, 20.1%, and 21.5%, respectively. The A+T content (58.4%) was significantly higher than the G+C content (41.6%). The overall transition/transversion ratio was eight. The genetic distances between sequences were calculated by the Kimura two-parameter distance (0%–11.3%) and maximum likelihood estimate (0%–11.4%). The main divergence in the ML tree was in accordance with that of the MrBayes tree and NJ tree (Fig 3). In the phylogenetic trees, the branches represented 100% support for the presumed biological species of *D. pulex*. The *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze River diverged into two branches in the phylogenetic trees, which was consistent with the results of 16S rDNA sequence analysis. In addition, the *D. pulex* (GU595190) from Japan and the *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze River were evidently different, with an average genetic distance of 10.5%.

**Genetic diversity of *D. pulex* from the middle and lower reaches of Yangtze River based on the 18S gene**

The alignment results of 36 18S gene sequences identified 1963 conserved sites, including 1932 invariable sites, 31 variable sites, 20 single sites, and 11 parsimony-informative sites. Among the sequences of the 18S gene for the *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze River, the average A, T/U, C, and G contents were 20.5%, 24.9%, 24.3%, and 30.3%, respectively. The A+T content (54.6%) was significantly higher than the G+C content (45.4%). The overall transition/transversion ratio was 2.5. The genetic distances between sequences were calculated by the Kimura two-parameter distance (0%–2.0%) and the maximum likelihood (0%–1.3%). The main divergence in the ML tree was in accordance with that of the MrBayes and NJ trees. The phylogenetic trees produced highly congruent tree topologies (Fig 4). In the phylogenetic trees, the branches represented 99% support for a presumed biological species of *D. pulex*. The *D. pulex* from Lake Chaohu in Anhui province (CH3), Lake Hongze in Jiangsu province, Hangzhou City in Zhejiang province (KJ775027), and Canada (AF014011) belonged to the same branch, whereas smaller differences were observed with the other *D. pulex* individuals. The average genetic distance was 0.45%–0.64%.
Fig 3. The phylogeny of *D. pulex* inferred from mitochondria cytochrome c oxidase subunit I (COI) sequences as a consensus tree formed from trees constructed using maximum likelihood (ML), and neighbor-joining (NJ), Bayesian inference (BI) methods.

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Fig 4. The phylogeny of *D. pulex* inferred from 18S gene sequences as a consensus tree formed from trees constructed using maximum likelihood (ML), and neighbor-joining (NJ), Bayesian inference (BI) methods.

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Tests on the genetic difference of *D. pulex* from the middle and lower reaches of Yangtze River

Based on the sequences of 16S rDNA, COI gene, and 18S gene, the genetic differences of *D. pulex* were analyzed within lakes and between lakes in the middle and lower reaches of Yangtze River. Mann-Whitney Test showed that the genetic differences of the *D. pulex* between within-lakes and between-lakes were significant (COI gene: \( Z = -3.172, P = 0.002; \) 16S rDNA: \( Z = -3.096, P = 0.002; \) 18S gene: \( Z = -3.378, P = 0.001 \)). Two-Sample Kolmogorov-Smirnov test showed the significant differences in both within-lakes and between-lakes (COI gene: \( Z = 1.789, P = 0.003; \) 16S rDNA: \( Z = 2.012, P = 0.001; \) 18S gene: \( Z = 2.012, P = 0.001 \)). The box diagram of *D. pulex* genetic diversity also demonstrated significant differences between within-lakes and between-lakes based on the sequences of 16S rDNA, COI gene, and 18S gene (Fig 5), which indicate that the genetic structure of *D. pulex* exhibiting differentiation among lakes.

Within lakes, the genetic difference of *D. pulex* from Lake Chaohu (CH) was bigger than that of other lakes based on the sequences of COI gene and 18S gene, and the genetic difference of *D. pulex* from Lake Nanyi (XC) was bigger than that of other lakes based on the sequences of COI gene and 16S rDNA (Fig 5).

Analysis of molecular variance

Based on the sequences of 16S rDNA, COI gene and 18S gene, the Molecular Variance of *D. pulex* were analyzed to calculate the population genetics for each site in the middle and lower reaches of Yangtze River. The result suggested that the pairwise differences were greater among populations than within populations based on the sequences of 16S rDNA, COI gene and 18S gene (Table 5). The AMOVA test showed that there were significant differences between two groups (middle reach and lower reach) based on 16S rDNA and COI gene (Table 6).

Discussion

For 16S rDNA and COI gene sequences of *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze river, the A+T content (65.4% and 58.4%, respectively) was significantly higher than the G+C content (34.6% and 41.6%, respectively). Those results were consistent with the higher A and T contents among invertebrates [37–38]. Moreover, the overall transition/transversion bias of *D. pulex* based on COI gene (8) was obviously higher than those based on the 16S rDNA (1.09) and 18S gene (2.5).

Based on the genetic variation of the 16S rDNA and COI genes, the *D. pulex* from 10 water bodies located in the middle and lower reaches of the Yangtze River evolved into two branches, as shown in the phylogenetic trees. One branch included the *D. pulex* from Lake Donghu in Hubei Province, as well as Lake Shengjin, Lake Nanyi, Lake Chaohu, and Guohe River in Anhui Province. The other branch included *D. pulex* from a pond in Shanghai City, Lake Taihu and Lake Hongze in Jiangsu Province, as well as Lake Qianlong, a pond, Lake Chaohu, and Lake Nanyi in Anhui Province. The *D. pulex* from Lake Chaohu and Lake Nanyi in Anhui province were present in both branches, which coincided with its geographical distribution in the middle and lower reaches of the Yangtze River. Based on the sequences of 16S rDNA, COI gene and 18S gene of *D. pulex*, the AMOVA test also showed that there all were greater genetic differences among lakes than within lakes in the middle and lower reaches of the Yangtze River. And significant genetic differences between two groups (middle reach and lower reach) were showed based on 16S rDNA and COI gene of *D. pulex*. Then the genetic distances of *D. pulex* from 10 water bodies located in the middle and lower reaches of Yangtze River showed minimal divergence based on 16S rDNA (0%–1.0%), COI gene (0%–1.7%), and 18S gene (0%–
0.9%), and all those differences were within the scope (<5%) of species [16, 33, 39]. These findings implied that the *D. pulex* from the lakes located in the middle and lower reaches of Yangtze River region should belong to the same species. In addition to further geographical distance, other environmental conditions, such as different climate, altitude, and fishery in the middle and lower reaches of the Yangtze River, may be important factors to the evolution of *D. pulex*.

Compared with the *D. pulex* from Canada (AF117817, GQ343275) [40, 41] and Russia (JN874605, JN874606, and JN874607) [42], the genetic distances of *D. pulex* from 10 water bodies located in the middle and lower reaches of the Yangtze River reached 9.1%–9.6% based
on 16S rDNA sequence. The genetic differences was obviously beyond the scope of a species (<5%) [16, 33, 39], and it indicated the presence of subspecies. Long-term geographic isolation may be the main reason for the evolution of the *D. pulex* in China and other countries. In addition, the average genetic distance between the *D. pulex* in Japan (GU595190) and the *D. pulex* in China reached 10.5% based on the COI gene sequence. The genetic distance was in the scope of the *Daphnia* complex (5%–14%). Thus, compared with the *D. pulex* (GU595190) in Japan, the *D. pulex* from China should belong to different subspecies or the *D. pulex* complexes [6, 16, 33, 43]. On the other hand, the genetic distance of the *D. pulex* (GU595192) in Japan and in China was below 5%. We speculated that the *D. pulex* had same ancestor and evolved to different directions by natural selection in Japan and China. Although the average genetic distances of the *D. pulex* from Canada (AF014011) and from China were small (0.45%–0.64%) based on Table 5. Analysis of molecular variance (AMOVA) based on the 16S rDNA, COI gene and 18S gene sequences of *D. pulex* in the middle and lower reaches of Yangtze River.

| Source of variation | Degrees of freedom | Sum of squares | Variance components | Variation (%) | p-value | FST  |
|---------------------|--------------------|----------------|---------------------|---------------|---------|------|
| 16S rDNA            | Among population   | 9              | 158.425             | 3.63403 Va    | 59.23   |      |
|                     | Within population  | 30             | 92.000              | 2.56667 Vb    | 40.77   |      |
|                     | Total              | 39             | 250.425             | 6.20069       | 0.00    | 0.59234 |
| COI gene            | Among population   | 9              | 124.850             | 2.90139 Va    | 56.14   |      |
|                     | Within population  | 30             | 68.000              | 2.26667 Vb    | 43.86   |      |
|                     | Total              | 39             | 192.850             | 5.16806       | 0.00    | 0.56141 |
| 18S gene            | Among population   | 9              | 300.275             | 8.12222 Va    | 90.27   |      |
|                     | Within population  | 30             | 26.250              | 0.87500 Vb    | 9.73    |      |
|                     | Total              | 39             | 326.525             | 8.99722       | 0.00    | 0.90275 |

Note: populations were evaluated as a single group. Each lake was as a population.

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Table 6. Analysis of molecular variance (AMOVA) based on the 16S rDNA and COI gene sequences of *D. pulex* about the two groups (middle reach vs. lower reach) in the Yangtze River.

| Source of variation | Degrees of freedom | Sum of squares | Variance components | Variation (%) | FSC/ FST | p-value |
|---------------------|--------------------|----------------|---------------------|---------------|----------|---------|
| 16S rDNA            | Among groups       | 1              | 34.240              | 1.29190 Va    | 20.22    |        |
|                     | Among populations within groups | 6 | 89.167              | 3.25434 Vb    | 50.93    |        |
|                     | Within populations | 24             | 44.250              | 1.84375 Vc    | 28.85    |        |
|                     | Total              | 31             | 167.656             | 6.38999       | 0.63834/ | 0.000/ |
|                     |                     |                |                     |               | 0.71146  | 0.000/ |
| COI gene            | Among groups       | 1              | 48.385              | 2.79421 Va    | 54.88    |        |
|                     | Among populations within groups | 6 | 38.833              | 1.39149 Vb    | 27.33    |        |
|                     | Within populations | 24             | 21.750              | 0.90625 Vc    | 17.80    |        |
|                     | Total              | 31             | 108.969             | 5.09196       | 0.60559/ | 0.000/ |
|                     |                     |                |                     |               | 0.82202  | 0.000/ |

Note: populations were evaluated as two groups (middle reach vs. lower reach) except Lake Chaohu and Lake Nanyi. Each lake was regarded as a population.

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the 18S gene sequence, the difference was evident. In general, the evolutionary divergences of the *D. pulex* among different lakes located in the middle and lower reaches of the Yangtze River were minimal. However, the evolutionary divergence was relatively high compared with that of other countries. The global molecular phylogeny of *D. pulex* needs to be further studied and discussed.

The intercontinental phylogeny of the *D. pulex* complex is extremely complicated. Based on the sequences of the mitochondrial dehydrogenase NADH 5 subunit and *COI* genes of 398 *D. pulex* individuals from five continents, Crease et al. (2012) concluded that 11 lineages of the *D. pulex* complex can be observed worldwide [29]. By studying the *D. pulex* complex from 12 Bolivian high-altitude lakes, the *D. pulicaria* group in North America was found to originate in South America, whereas these South American water fleas originated through reciprocal hybridization between different sexually reproducing parental lineages [44]. In the present study, based on the 16S rDNA sequence, the average genetic distances of the *D. pulex* from China and the *D. parvula* and the *D. obtusa* from Canada were 7.3% and 8.2%, respectively. Their differences belong to the scope of the *D. pulex* complex (5%-14%) [16, 29–33, 45]. Benzie (2005) hypothesized that the main factor that led to the formation of species complexes between the *D. pulex*, *D. pulicaria*, and *D. middendorffiana* was their long-term coexistence in the same habitat, which resulted in the occurrence of interspecies complexes [6,16]. In the middle and lower reaches of the Yangtze River in China, the coexistence of *D. pulex*, *D. galeata*, and *D. similoides* was common in some lakes (e.g. Lake Donghu, Lake Taihu, and Lake Chaohu) [8–10]. Thus, the existence of species complexes among *Daphnia* species in these Chinese lakes was possible, and further investigation is needed.

**Author Contributions**

Conceived and designed the experiments: W-PW D-GD KZ. Performed the experiments: W-PW KZ S-XP X-XX. Analyzed the data: W-PW D-GD Y-NZ. Contributed reagents/materials/analysis tools: W-PW D-GD KZ Y-NZ. Wrote the paper: W-PW D-GD Y-NZ KZ.

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