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Application of COI Primers 30F/885R in Rotifers to Regional Species Diversity in (Sub)Tropical China

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Abstract: Rotifers are the most diverse group in freshwater zooplankton and play an important role in food webs and ecosystems. DNA barcoding has become a useful approach to investigate species diversity at local and regional scales, but its application is still limited by efficient primers for the group. To test a pair of primers 30F/885R recently designed for rotifers, we applied them to investigating regional species diversity in the freshwater of South China. We sequenced the cytochrome c oxidase subunit I (COI) gene of rotifers collected from the investigated 23 reservoirs in a large river basin and obtained 145 COI sequences from 33 species in 14 genera. The mean PCR success rate for all tested species was 50%. The 145 sequenced mtCOI in this study covered 33 of 64 identified morphological taxa, including most of the common species in the basin. The intraspecific genetic distance was calculated with a K2P model for 24 rotifer species occurring in the quantitative samples, in which 15 rotifers, such as *Keratella cochlearis* and *Brachionus calyciflorus*, had a genetic distance higher than 5%. The high intraspecific genetic differentiation indicates that cryptic species are probably common in (sub)tropical China.

Keywords: rotifers; cryptic species; freshwater zooplankton; reservoir; species diversity; tropics

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1. Introduction

Rotifers are a group of zooplankton with high species richness in freshwater ecosystems [1]. There are more than 2000 species of rotifers described worldwide, including 1571 species from Monogononta and 461 species from Bdelloidea [2,3]. This group plays a critical role in the flow of energy and the cycling of matter in freshwater ecosystems [4]. Most species in the group graze or feed mainly on algae or bacteria and serve as food for small invertebrates and fish [5,6]. Due to a short lifespan and high reproduction, rotifers are highly dynamic in natural waters and sensitive to environmental change. Knowing their species richness and species composition is of great significance for understanding ecosystem functions and environmental monitoring [2,7].

The morphological taxonomy of rotifers is based on external shapes and internal structures. Their ciliated corona and lorica are important to species identification [8]. However, their small body size and complicated morphology make morphological identification difficult. In addition, environmental conditions, such as temperature and food concentration, can induce morphological changes in many species [9], due to the phenotypic plasticity, especially in monogononts [10]. Relying only on morphological features may lead to faulty identification, especially for species with high phenotypic plasticity. Up to now, more than 40 species complexes have been discovered in *Keratella cochlearis* (Gosse, 1851), *B. calyciflorus*, *Philodina flaviceps* Bryce, 1906, and *Lecane bulla*, (Gosse, 1886) [11–15]. High genetic variation can occur within local populations despite insignificant morphological differentiation [11,16]. Molecular classification has already been extensively applied to

rotifers [12]. Earlier studies relied on allozyme electrophoresis [17–19], but today, DNA barcoding has become an essential technique to identify species in monogonont and bdelloid rotifers [15,20–22].

Molecular classification provides critical supplementary information for morphological taxonomy. The mitochondrial cytochrome oxidase subunit I (COI) gene is the most widely used sequence segment in DNA barcode classification and has proved to help detect cryptic species, intraspecific variation, and phylogeographical patterns [17,23]. At present, Folmer's universal primers are commonly used for amplifying COI [24,25]. Meyer et al. [26] modified the Folmer primers to obtain the primers dgLCO/dgHCO and amplified the COI sequence of the *B. plicatilis* complex [24,27]. Wilts et al. [28] developed primers COI-F/COI-R to amplify the COI of *Proales daphnicola* (Thompson, 1892) [25,27,29]. Elías-Gutiérrez et al. [30] used the Zplank primers to amply rotifer COI and obtained 11 BINs (Barcode Index Numbers) of rotifers, with a sequencing success rate of 100%. Recently, Zhang et al. [31] used a metagenomics method to assemble nine mitochondrial genomes from *Brachionus* and *Keratella*, with which they designed a new pair of primers just for rotifer COI: 30F/885R. The pair of primers performed efficiently (86%), much higher than dgLCO/dgHCO (32%) and Folmer primers (59%). Despite that, the newly designed primers need further testing in rotifers from different water bodies.

Here, we test the primers 30F/885R and apply them to investigating rotifer species diversity and composition of rotifers at a regional scale. Hanjiang River Basin is located in Guangdong Province, South China, where rotifers dominate zooplankton in most drinking water [32,33]. We conducted both morphological identification and COI sequence amplification on rotifers from the 23 investigated reservoirs in the basin and tested further the primers 30F/885R and their usefulness in the assessment of species diversity in (sub)tropical regions.

2. Materials and Methods

2.1. Collection, Identification, and Counting of Rotifers

Rotifers were sampled from 23 reservoirs (Figure 1) in the Hanjiang River Basin, Guangdong Province, in southern China from November 2019 to January 2020. For quantitative samples used for the assessment of species diversity in rotifer communities, a 5 L water sampler was used to vertically collect 50 L water from the surface to the bottom evenly. The sample was filtered and concentrated with a plankton net with a mesh size of 38 μm and fixed with 5% formalin. For the qualitative samples used for DNA extraction of rotifers, a plankton net with a mesh size of 64 μm was trawled horizontally and vertically. The obtained zooplankton was immediately fixed with BBI's DNA-EZ Reagents F DNA-Be-Locked A and stored in a refrigerator at 4 °C in the field.

All rotifer species in our samples were first identified based on external shape and internal structure [8]. For rotifer species that could not be easily identified by morphological characteristics, individuals were picked out to check the shape of their lorica. After adding 10% glycerol and 5–10% sodium hypochlorite, the shape of the lorica was further observed under a microscope (400×) for morphological identification. All species were identified, measured, and counted under a microscope (Olympus BX41, Tokyo, Japan). Their individual body volume was calculated with approximate geometric volume formulae, and the density of 1 g/cm^3 was set to estimate the bodyweight [34,35]. If a species contributes at least 2% of the total abundance, it is considered dominant in that reservoir (Table S1) [36].

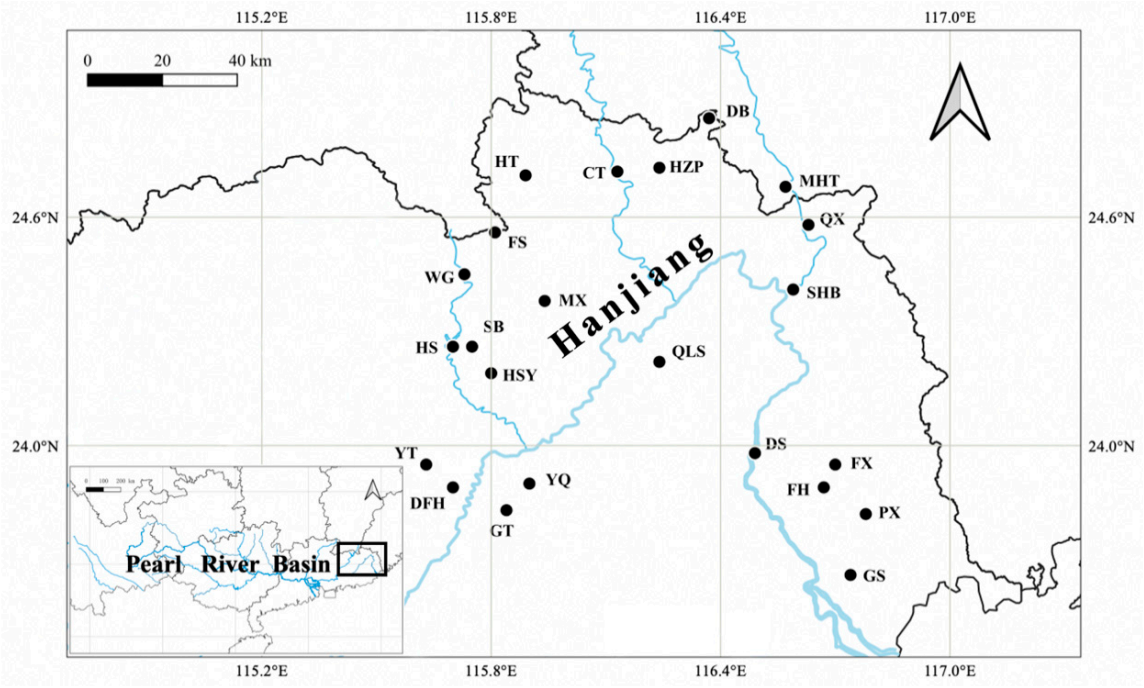

Figure 1. Localities of 23 investigated reservoirs in the Hanjiang River Basin, South China (Abbreviations of reservoir names are listed in Table S2.

2.2. DNA Extraction, PCR Amplification, and Sequencing

Before DNA extraction, rotifer specimens were washed with MilliQ water, and three or four individuals from one species were put into a 0.2 mL tube. Three microliters Proteinase K and 30 µL Chelex resin (BioRad, Hemel Hempstead, UK) were added into the tube for DNA extraction. The tube was centrifuged at 8000 rpm for 1 min and finally put into the PCR instrument. The DNA samples were incubated at 56 °C for 60 min at 99 °C for 10 min and stored at 12 °C. All DNA samples were stored at 4 °C, and/or at −20 °C for long-term storage. Finally, the samples were centrifuged at 10,000 rpm for 2 min, and the supernatant was directly used in each PCR reaction.

DNA from a single species was used as a template. A 760-bp segment of COI was amplified using the primers 30F and 885R [31]. The total amplification volume of primers 30F/885R was 30 µL, including 15 µL 2 × HieffTM PCR Master Mix (With Dye), 11 µL ddH_2O, 0.5 µL of forward and reverse primers (100 µM), 3 µL DNA, respectively. The amplification started with initial denaturation 2 min at 98 °C, then six cycles of (95 °C for 30 s, 54 °C for 40 s (−0.5 °C/each cycle), 72 °C for 30 s), and 36 cycles of (95 °C for 30 s, 51 °C for 40 s, 72 °C for 30 s) and final extension of 72 °C for 2 min [31].

The PCR products were detected in 1.0% agarose gel. The amplified products with clear and bright target bands were selected and sent to Tianyi Huiyuan Gene Technology Company for purification and sequencing. All DNA samples were paired-end sequenced. After that, all the chromatograms of forward and reserve sequences were checked with Finch TV1.5.0, and poor-quality sequences and repeated sequences were discarded (Geospiza Inc. https://www.digitalworldbiology.com/FinchTV (accessed on 1 December 2019)). The forward and reverse sequences from each sample were assembled into one sequence with Geneious v10.22, and all sequences were aligned with MAFFT and MACSE [37–39]. Poor-quality flanking regions of the sequences were discarded. We calculated the coverage of DNA barcodes for rotifers in the 23 reservoirs. The coverage of DNA barcodes is defined as the percentage of species with successfully obtained COI to the number of species identified morphologically in the quantitative sample.

2.3. Species Identification and Analysis Based on Molecular Methods

Each amplified sequence was submitted to NCBI for BLAST [40]. We obtained sequences for 33 species, among which six species, including *Pompholyx sulcata* (Hudson, 1885), *Ploesoma truncatum* (Levander, 1894), *Filinia opoliensis* (Zacharias, 1898), *Filinia camasecla cambodgensis* (Bērzinš, 1973), *Trichotria pocillum* (Müller, 1776), *Trichotria tetractis similis* (Stenroos, 1898), had no COI sequences deposited to NCBI. The interspecific distance, intraspecific genetic difference (K2P), and a NJ tree were calculated or constructed in MEGA 10.1.8 [41].

3. Results

A total of 64 rotifer species were identified from both quantitative samples and qualitative samples. Forty-seven species (Table S3) were morphologically identified from the quantitative samples that were fixed with formaldehyde. The dominant species included *Keratella tropica* (Apstein, 1907), *Keratella tect* (Gosse, 1851), *Trichocerca similis* (Wierzejski, 1893), *Synchaeta stylata* (Wierzejski, 1893), *Anuraeopsis fissa* (Gosse, 1851), *Polyarthra dolichoptera* (Idelson, 1925). There were 14 rare species, including *Brachionus quadridentatus* (Hermann, 1783), *Anuraeopsis coelata* (de Beauchamp, 1932), *T. tetractis similis*, *Lecane lunaris crenata* (Harring, 1913), *Lecane flexilis* (Gosse, 1886), *Lecane galeata* (Bryce, 1892), *Lecane pyriformis* (Daday, 1905), *Lecane arcuata* (Bryce, 1891), *Lecane thailandensis* (Segers et Sanoamuang, 1994), *Gastropus stylifer* (Imhof, 1891), *Trichocerca longiseta* (Schrank, 1802) *Trichocerca vargai* (Wulfert, 1961), *Filinia saltator* (Gosse, 1886), and *Filinia terminalis* (Plate, 1886).

In the qualitative samples fixed with DNA-EZ Reagents F DNA-Be-Locked A, 45 species were picked and sequenced (Table S4). A total of 145 COI sequences were obtained from 33 species in 14 genera (Table 1). The remaining 12 species failed to be amplified and sequenced. Seven of these are rare species in the samples. To test the 30F/885R primers, we amplified more than two times for the remaining five common species = (i.e., *Conochilus unicornis* (Rousselet, 1892) was done for nine times).

Among 47 species in the quantitative samples fixed with formaldehyde, only 23 species were covered at least by a COI sequence from the qualitative samples (Figure 2). Among the remaining 24 morphological species without COI sequences, 12 rare and 11 common species had too low abundance for PCR amplification and sequencing (i.e., <3 individuals), while one dominant species (*A. fissa*) failed to be amplified and sequenced. With morphological identification, the identified species number was between 6 and 20 for a single investigated reservoir, while with the molecular classification of COI sequences, the identified species number was between 0 and 13. The barcode recovery rate for a single reservoir was between 0% and 67%, with an average of 29% (Figure 3).

Among 33 species with COI sequences, we calculated the intraspecific K2P (Kimura two-parameter) genetic distance for 24 species that had at least two sequences. The average intraspecific genetic distance was from 0.00 to 0.32 (Table 2), with an averaged distance of 0.08. Many rotifers had high intraspecific genetic distances at the regional scale (Figure 4). Fifteen species had intraspecific genetic distance above 0.05: *K. cochlearis*, *Keratella tecta* (Gosse, 1851), *K. tropica*, *Brachionus leydigi* (Cohn, 1862), *B. calyciflorus*, *Plationus patulus* (Müller, 1786), *Asplanchna brightwelli* (Gosse, 1850), *Polyarthra vulgaris* (Carlin, 1943), *P. dolichoptera*, *T. similis*, *Ascomorpha ovalis* (Bergendahl, 1892), *Trichocerca dixonnuttalli* (Jennings, 1903), *Synchaeta oblonga* (Ehrenberg, 1831), and *P. sulcata*, *Hexarthra mira* (Hudson, 1871).

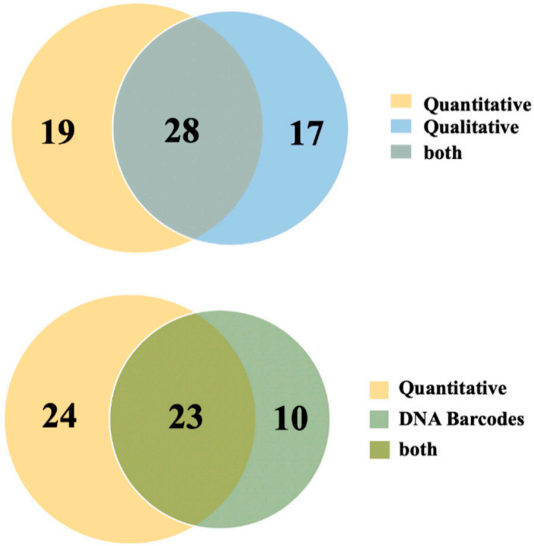

Figure 2. Venn diagrams, "Quantitative": the numbers of species identified with quantitative samples; "Qualitative": the numbers of species identified with qualitative samples; "DNA Barcodes": the number of species obtained mtCOI barcodes.

Table 1. PCR success rates for species that had at least one successful sequence.

Species	Number of Specimens	Number of Sequences	PCR Success Rate (%)
Keratella cochlearis	24	17	71
Keratella tropica	15	8	53
Keratella tecta	24	10	42
Brachionus diversicornis	8	6	67
Brachionus calyciflorus	5	4	80
Brachionus caudatus	2	1	50
Brachionus urceolaris	2	1	50
Brachionus angularis	3	2	67
Brachionus quadridentatus	3	1	33
Brachionus forficula	3	2	67
Brachionus leydigi	2	2	100
Brachionus budapestinensis	1	1	100
Asplanchna priodonta	9	6	67
Asplanchna brightwelli	2	2	100
Polyarthra vulgaris	14	4	29
Polyarthra dolichoptera	9	9	100
Ploesoma hudsoni	7	5	71
Ploesoma truncatum	1	1	100
Pompholyx sulcata	5	4	80
Trichocerca dixonnuttalli	4	4	100
Trichocerca capucina	23	11	48
Trichocerca cylindrica	18	4	22
Trichocerca similis	16	10	63
Filinia opoliensis	1	1	100
Filinia camaseclacambodgensis	2	2	100
Trichotria pocillum	1	1	100
Trichotria tetractis similis	3	1	33
Synchaeta oblonga	3	2	67
Synchaeta stylata	10	9	90
Lecane bulla	3	1	33
Ascomorpha ovalis	11	9	82
Hxarthra mira	8	2	25
Plationus patulus	3	2	67

Note: PCR success rate for a given species denotes the percentage of the obtained mtCOI sequences in the total number of tested specimens.

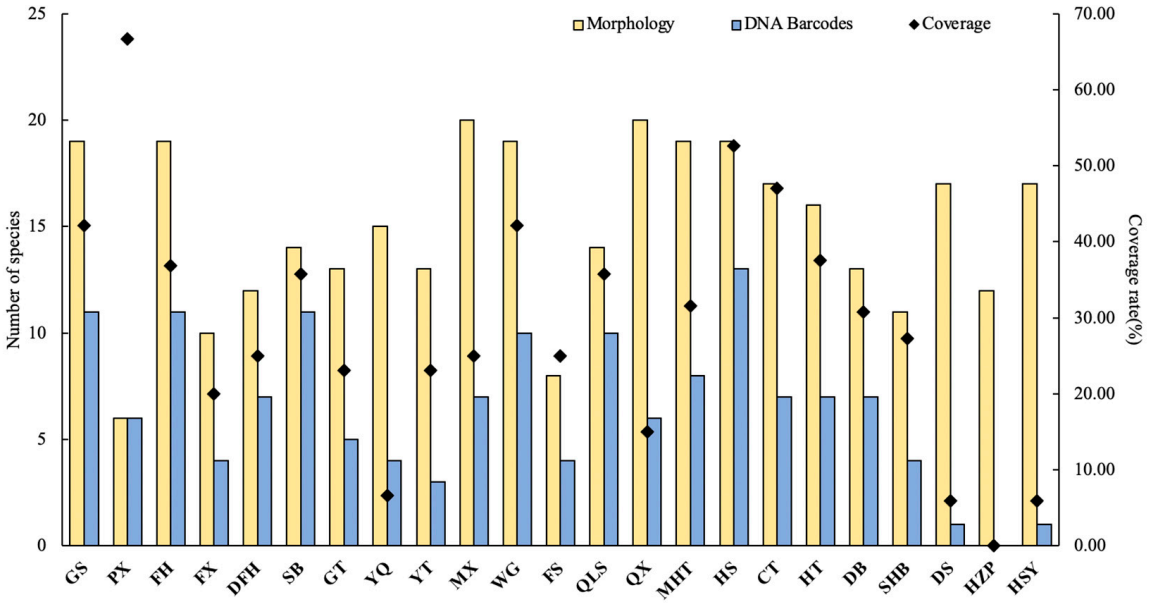

Figure 3. The number of rotifer species identified morphologically in the quantitative samples and number of species identified with COI barcodes from in the 23 reservoirs, and the coverage rate (%) of COI barcodes in the 23 investigated reservoirs (The coverage of DNA barcodes is the percentage of the number of species successfully obtained COI to the number of species identified morphologically in the quantitative sample.).

Table 2. Intraspecific genetic distance (K2P) of 24 rotifer species.

Species	Numbers of Sequences	Min Distance	Max Distance	Mean Distance
Keratella cochlearis	17	0.00	0.19	0.11
Keratella tecta	10	0.00	0.18	0.04
Keratella tropica	8	0.00	0.12	0.06
Brachionus diversicornis	6	0.00	0.00	0.00
Brachionus leydigi	2	N/A	N/A	0.15
Brachionus forficula	4	0.00	0.01	0.01
Brachionus angularis	2	N/A	N/A	0.01
Brachionus calyciflorus	4	0.04	0.18	0.14
Plationus patulus	2	N/A	N/A	0.10
Asplanchna priodonta	7	0.00	0.03	0.02
Asplanchna brightwelli	2	N/A	N/A	0.31
Polyarthra vulgaris	4	0.06	0.25	0.07
Polyarthra dolichoptera	9	0.00	0.25	0.19
Ascomorpha ovalis	11	0.00	0.14	0.08
Trichocercasimilis	10	0.00	0.32	0.17
Trichocerca cylindrica	5	0.00	0.07	0.04
Trichocerca dixon-nuttalli	4	0.00	0.18	0.09
Trichocerca capucina	13	0.00	0.01	0.00
Synchaeta stylata	9	0.00	0.06	0.02
Synchaeta oblonga	2	N/A	N/A	0.13
Ploesoma hudsoni	5	0.02	0.00	0.01
Pompholyx sulcata	4	0.00	0.12	0.06
Filiniacamasecla cambodgensis	2	N/A	N/A	0.00
Hexarthramira	2	N/A	N/A	0.07

Note: "N/A" means missing value.

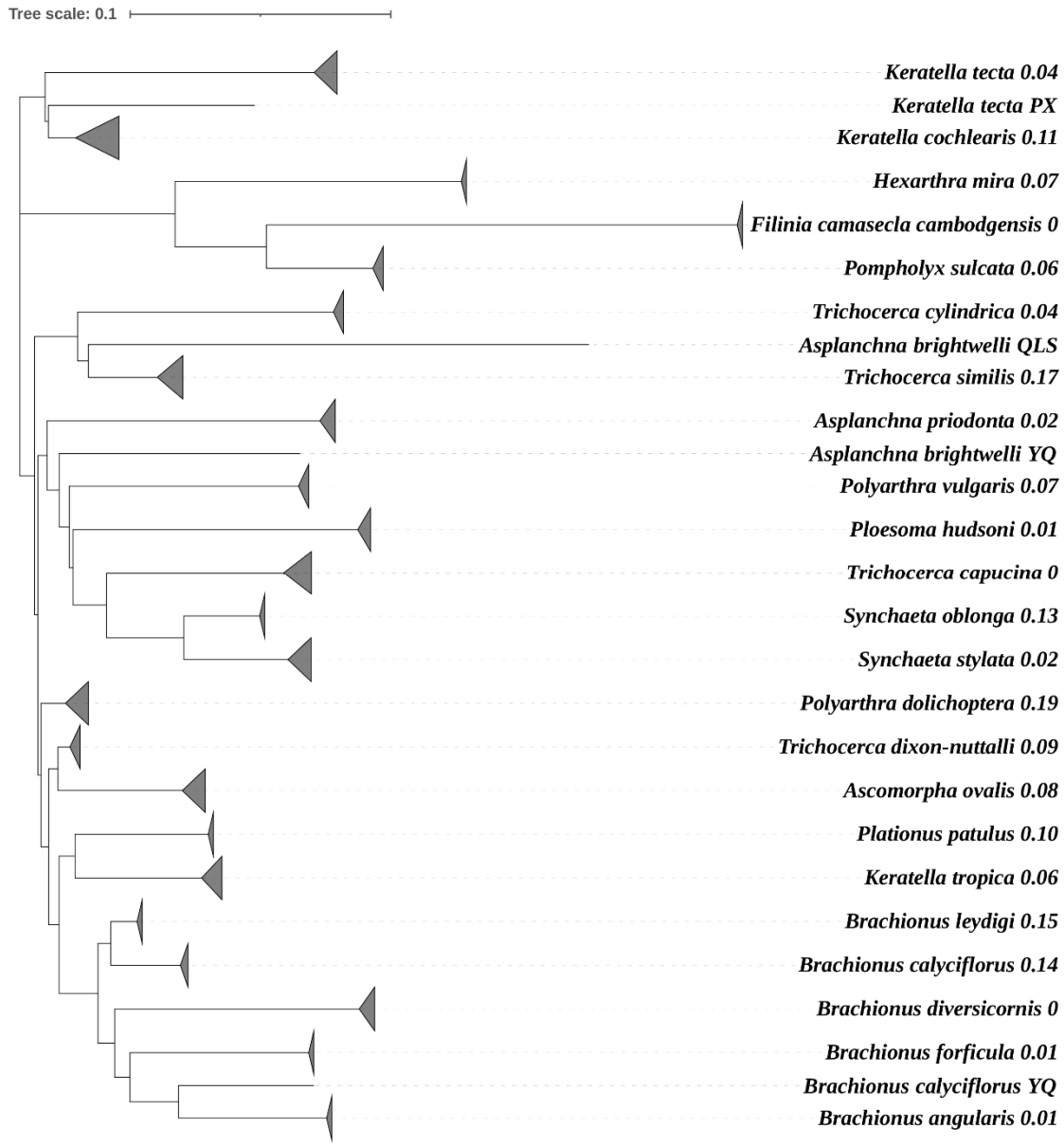

Figure 4. A simplified NJ tree with K2P distance for 24 morphological taxa. The triangle size represents the number of sequences, and the number after the species name is the mean K2P distance. The abbreviations after a species with a single sequence is the acronym of the reservoir name.

4. Discussion

The present study expanded the testing of a pair of recently reported DNA barcoding primers (30F/885R) and assessed the species diversity of rotifers at a regional scale. The average amplification success rate for all tested specimens (245) in 45 species was up to 50%. Among 45 species from our qualitative samples fixed for DNA sequencing, 12 species failed to be amplified and sequenced. Seven of 12 species are rare in this basin. The abundances for seven of 12 species might be too low for PCR amplification and sequencing. More individuals need to be collected to test these rare species for the amplification of COI. Surprisingly, *A. fissa* is one of the remaining five common species of the 12 failed species. It is dominant in the basin but failed to be sequenced. Rotifer species vary largely in body weight; the minimum weight of *A. fissa* is only 0.009 µg, while the individual weight of *Asplanchna girodi* (de Guerne, 1888) is up to 31.85 µg (Figure S1). In general, body weight determines the DNA amount for extraction, particularly mtDNA, which might affect subsequent amplification and sequencing [42]. However, smaller species,

such as *P. sulcata*, *K. tecta*, and *K. cochlearis*, were successfully amplified and sequenced. Having a small body size might not be the main reason for the failure of amplification and sequencing for *A. fissa*. More likely, the COI sequences among different species in rotifers varied greatly [24]. We suspect that the primer incompatibility might be the main reason for the failure of amplification and sequencing of the five common species.

The DNA barcoding in the present study covered most species (33) of the rotifers in the investigated basin, and the obtained barcode library (145 COI sequences) will benefit the future survey of rotifers in similar regions. First, they can provide references for validating species identification. DNA barcode libraries can be used as a standard for species identification and improve the accuracy of rotifer morphological classification. Second, the barcoding is suitable for all life stages of rotifers, including resting eggs [43,44]. In addition, the barcode library constructed in this basin could be used as a reference in high-throughput-based monitoring techniques, such as eDNA metabarcoding and mitochondrial metagenomics [45–49].

As previously reported, using COI sequences can efficiently identify most species in rotifers, with the divergence among conspecific individuals being less than 1% [24]. *K. cochlearis* is a widely distributed species with phenotypic diversity. The COI nucleotide sequence divergence of 4.4% was detected between spined and unspined forms. As a result, the species was split into different species [50]. In the present study, the intraspecific genetic distance for *K. cochlearis* was between 0 and 0.19 for pairs of 17 individuals from all the reservoirs, with an average equal to 0.11, indicating a high hidden diversity. *B. calyciflorus* is a widely distributed species that shows a significant morphological difference with multiple subspecies and varieties [51]. Xiang et al. [52] collected eight geographical groups of *B. calyciflorus* from eastern China and concluded that this complex was composed of three cryptic species. In the present study, *B. calyciflorus* was found in four reservoirs, and the average intraspecific genetic distance was high up to 0.14, indicating high genetic diversity. As reported in other studies [24,47], high genetic distance also occurred within *B. leydigi*, *A. ovalis*, and *S. oblonga*, and based on the intraspecific genetic distances estimated here, species complexes, such as *K. cochlearis*, *B. calyciflorus*, *B. leydigi*, *A. ovalis*, and *S. oblonga*, might co-exist in the Hanjiang River Basin. Therefore, further investigation of cryptic species in this basin is recommended.

In conclusion, our study showed that the COI primers (30F/885R) utilized in this study can be used to investigate the regional diversity of rotifers and that the 145 mtCOI sequences obtained will be helpful to uncover rotifer species diversity in South China. Intraspecific genetic variation is high in some species in our study, especially within some "cosmopolitan" species or species complexes, such as *B. calyciflorus*. Therefore, detailed sampling and in-depth analysis for detecting cryptic species are necessary for uncovering the full regional diversity of rotifers.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/d13080390/s1, Figure S1: Average body weight of individual rotifers of 44 species, Table S1: The abundances of rotifers in 23 reservoirs; Table S2: The information of 23 investigated reservoirs in the Hanjiang River Basin; Table S3: Morphological identification of rotifers from both quantitative and qualitative samples in the 23 investigated reservoirs and species identified by COI sequencing; Table S4: Amplification of COI sequence in 23 reservoir rotifers.

Author Contributions: Conceptualization, Y.-N.Z. and S.-L.X.; methodology, Y.-N.Z. and S.-L.X.; software, Q.H. and P.L.; validation, Y.-N.Z., S.-L.X., Q.H., P.L., and B.-P.H.; formal analysis, Y.-N.Z., S.-L.X., and B-P.H.; investigation, Y.-N.Z.; resources, Y.-N.Z., S.-L.X., Q.H., P.L., and B.-P.H.; data curation, Y.-N.Z.; writing—original draft preparation, Y.-N.Z. and B.-P.H.; writing—review and editing, Y.-N.Z., S.-L.X., Q.H., P.L., and B.-P.H.; visualization, Y.-N.Z. and S.-L.X.; supervision, Y.-N.Z. and B.-P.H.; project administration, S.-L.X., Q.H., and P.L.; funding acquisition, B.-P.H. All authors have read and agreed to the published version of the manuscript.

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