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Research Paper

Epiphytic zooplankton community profiles in a typical urban wetland as revealed by DNA metabarcoding*

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Abstract Zooplankton, a crucial component of urban wetland, are one of the effective bioindicators for monitoring the feeding stocks of organisms at higher trophic levels and assessing the ecological quality of ecosystems. However, information about the characteristics of epiphytic zooplankton community structure resulted from traditional methods is limited and hindered by the large amount of detritus and sludge attached to the macrophytes. We investigated the epiphytic zooplankton communities associated with macrophytes (*Vallisneria*, *Nymphaea*, and *Thalia dealbata*) in a subtropical wetland using as DNA markers of the 18S rRNA gene and the mitochondrial cytochrome c oxidase subunit I (COI) gene. A total of 241 OTUs of zooplankton were obtained from COI amplicons, including 194 OTUs of Rotifera, 22 of Cladocera, and 25 of Copepoda, while only 62 OTUs of zooplankton were obtained from 18S rDNA amplicons including 34 OTUs of Rotifera and 28 of Copepoda. The zooplankton communities associated with the three macrophytes were similar, but they differed significantly from those in the open waters. However, there were no significant temporal differences among the zooplankton communities. Epiphytic zooplankton communities were dominated by littoral zooplankton such as *Testudinella*, *Lecane*, and *Philodina*. Microzooplankton, especially littoral species, utilize macrophytes as food sources and as refuges against predation. This further led to an increase in α and β diversity of zooplankton communities in urban wetlands. Our result suggests that the joint use of multiple molecular markers could improve the taxonomic resolution and generate a comprehensive biodiversity profile of zooplankton.

Keyword: environmental DNA; metabarcoding; diversity; macrophyte; cytochrome c oxidase subunit I (COI); 18S rRNA

1 INTRODUCTION

Zooplankton, small but essential components of urban wetlands, connect primary producers and secondary consumers, playing an important role not only in a trophic cascade but also in the recycling microbial food web (Liu et al., 2018). Monitoring of zooplankton communities is an efficient way to quantify the feeding resources of higher trophic levels and to assess the ecological quality of ecosystems, such as trophic status and intrusion of invasive non-indigenous species (Ershova et al., 2021). Anthropogenic disturbance has greatly affected the biodiversity of urban ecosystems. Macrophytes in urban wetlands play an important role in improving the water quality and increasing aquatic biodiversity at the same time (Ding et al., 2019). However, numerous studies of zooplankton community structure have focused on their distribution patterns in the open water area, while fewer studies compared the epiphytic zooplankton communities associated with different types of macrophytes.

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Investigations about the effects of macrophytes on zooplankton began earlier in temperate wetlands, and it was found that there were many rotifers on the surface of macrophytes (Kuczyńska-Kippen and Nagengast, 2003). Some researches indicated that microhabitat structure, created by macrophytes, is an important factor in determining the community structure of zooplankton (Choi et al., 2014a). The different types of macrophyte bed as well as their percentages of shading support different functional groups (pelagic and littoral) or trophic-associated rotifers (e.g., eutrophic and mesotrophic groups) (Choi et al., 2015; Kuczyńska-Kippen, 2018). Epiphytic microzooplankton prefer elodeid and pleustophyte species over other plant types (Choi et al., 2014b; Kuczyńska-Kippen et al., 2021). However, the coupling relationship between macrophytes and epiphytic zooplankton is still unclear. Deep understanding about the characteristics of epiphytic zooplankton community structure is limited due to methodological inadequacy (Choi et al., 2014b). This is partly due to the practical difficulty associated with morphological methods, which are hindered by the large amounts of detritus and sludge attached to the macrophytes.

DNA-based high-throughput sequencing makes DNA metabarcoding a powerful approach in biomonitoring, because of its noninvasiveness, high sensitivity, and inexpensiveness (Ji et al., 2022). Unlike morphological methods, metabarcoding can potentially detect all species within a habitat, regardless of developmental stage or preservation of distinguishing features (Ershova et al., 2021). Metabarcoding can improve taxonomic resolution of rare species that are unlikely to be captured. It can also detect intact organisms or debris that are hidden behind the macrophyte beds. Thus, it is a promising method for achieving a complete assessment of epiphytic zooplankton communities.

Several gene markers have been applied in assessing zooplankton community structure, including regions of the nuclear 18S rDNA (Albaina et al., 2016), 28S rDNA, the mitochondrial 16S rRNA (Bucklin et al., 2016) and cytochromec oxidase subunit I (COI) genes (Leray et al., 2013). Moreover, 18S rDNA and COI have been the most widely used molecular markers for zooplankton diversity assessment. Many studies demonstrated that COI was an ideal candidate marker for zooplankton DNA metabarcoding, due to its universal coverage for invertebrates and its advantages in the identification of closely related species or subspecies (Yang et al., 2017; Ershova et al., 2021). On the other hand, 18S rRNA gene has a broader taxonomic coverage in the database and is suitable for studying communities with distantly-related species (Albaina et al., 2016). No matter which primers are used for amplification, a certain degree of data bias is often generated. The varying binding affinities of a given set of primers for different DNA templates, which affects the subsequent PCR amplification efficiency for different species (Kelly et al., 2019). In addition, the taxonomic coverage of sequence reference databases for most universal primers are still insufficient (Yao et al., 2022). Performing multiple primers may reduce amplification bias associated with individual primers and generate a more complete biodiversity profile (Zhang et al., 2022).

Here, we sampled bimonthly and analyzed the samples using the hypervariable regions of 18S ribosome DNA and the mitochondrial COI partial genes. Since the macrophytes withered and were removed during winter, we only collected samples of the zooplankton assemblages from five different habitats in Haizhu National Wetland Park during the growth period of macrophytes from June to October, 2021. The current study addressed the following questions: (1) how effective is DNA metabarcoding in assessing epiphytic zooplankton biodiversity? (2) what are the distribution patterns of zooplankton communities associated with different macrophytes' habitats? (3) how do macrophytes maintain a higher biodiversity of zooplankton in urban wetlands?

2 MATERIAL AND METHOD

2.1 Sample collection and environmental factor measurements

A total of 15 samples, including nine samples of epiphytic zooplankton communities on three types of macrophytes and six zooplankton communities in the pelagic zone were collected within the connected water system of Haizhu National Wetland Park (Guangzhou, China) from June to October, 2021 (Fig.1; Supplementary Table S1). The sample names of *Vallisneria* (Val), *Nymphaea* (Nym), and *Thalia dealbata* (Tha) represented the epiphytic zooplankton communities from elodeids, nymphaeids, and helophytes, respectively. For quantification, a 50-mL volume of each macrophyte sample (determined by drainage method) was cut using small branch shears and eluted into a measuring

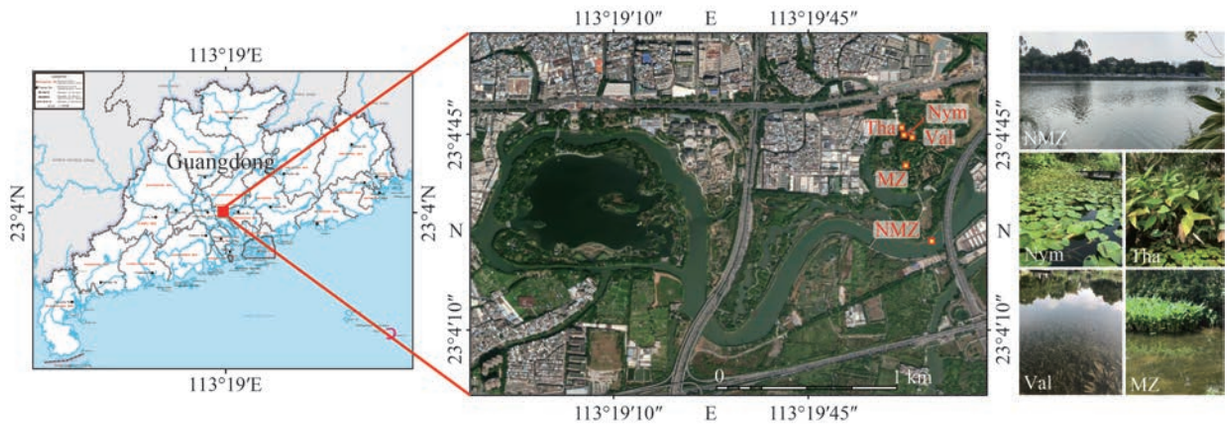

Fig.1 Location of the sampling sites in the connected water system of Haizhu National Wetland Park

Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone. Map review No. YueS(2022)314.

cup. After stirring and elution, all liquid was filtered with a plankton net (mesh size 30 μm) and preserved in a 50-mL centrifuge tube. The sample names of MZ and NMZ represented the pelagic zooplankton communities from macrophyte zone and non-macrophyte zone, respectively. For quantification, 10-L water (surface and subsurface) were concentrated by a plankton net with a mesh size of 30 μm.

Duplicate samples were collected at each sampling site and fixed immediately in neutral Lugol's solution at 2% final concentration. The sample for metabarcoding analysis was stored at -80 ℃ , while the sample for morphological methods (traditional approach) was stored at room temperature. Morphological methods were conducted as in the previous studies (Liang et al., 2023). The inferred biomass of zooplankton was roughly estimated by the abundance and body length (biomass=abundance×bodylength^3 (Yang et al., 2017)).

Measurements of environmental factors, including water temperature (Temp), dissolved oxygen (DO), pH, salinity, water transparency (SD), chlorophyll *a* (Chl *a*), chemical oxygen demand (COD_{Mn}), total phosphorus (TP), ammonium nitrogen (NH_4-N), and total nitrogen (TN) were carried out as described in a previous study (Liang et al., 2019).

2.2 DNA extraction, amplification, and sequencing

Samples were filtered immediately on a 5-μm polycarbonate membrane (EMD Millipore TMTP04700, USA) before total DNA extraction. Total DNA in each sample was extracted from the polycarbonate membrane using DNA Lysis buffer+ proteinase K+CTAB+Clean & Concentrator kit (Zymo Research, USA). The DNA concentrations and purities were determined with a NanoDrop 2000 Spectrophotometer.

Both the V4 region of the 18S ribosomal RNA gene (18S rDNA) and the partial mitochondrial COI were amplified for DNA metabarcoding analysis. The 18S rDNA was amplified using the universal primers 528F (5′-GCGGTAATTCCAGCTCCAA-3′) and 706R (5′-AATCCRAGAATTTCACCTCT-3′) (Cheung et al., 2010). The 18S rDNA was amplified by PCR: 3 min of denaturation at 94 °C, followed by 27 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and a final extension at 72 °C for 5 min. While the COI gene was amplified by use of the mlCOIintF (5′-GGWACWGGWTGAACWGTWTAY CCYCC-3′) and jgHCO2198 (5′-TANACYTCNGGR TGNCCRAA RAAYCA-3′) (Leray et al., 2013). The PCR of COI was conducted for 16 initial cycles as follows: denaturation for 10 s at 95 °C, annealing for 30 s at 62 °C (-1 °C per cycle), and extension for 60 s at 72 °C, followed by 25 cycles at an annealing temperature of 46 °C. The final extension was performed at 72 °C for 10 min. PCR reactions were performed in triplicate 20-μL mixture containing 4 μL of 5×FastPfu Buffer, 2 μL of 2.5-mmol/L dNTPs, 0.8 μL of each primer (5 mmol/L), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. A negative control reaction with no DNA template was included in all experiments. The PCR products were extracted, quantified and purified according to the methods described in a previous study (Liang et al., 2022). Library construction and sequencing were performed by the Shanghai Meiji Sequencing Centre, using 2×300 bp paired-end sequencing on a MiSeq PE300 System (Illumina, San Diego, USA).

2.3 Bioinformatic processing and statistical analysis

The Quantitative Insights into Microbial Ecology (QIIME) V. 1.9.1 platform was used to filter the sequencing data. Raw FASTQ files were demultiplexed, quality-filtered by fastp and merged by FLASH to minimize the effects of random sequencing errors (Magoč and Salzberg, 2011). Sequences were discarded if they contained ambiguous base calls, or their lengths were shorter than 240 bp or the average quality score was <20. After sequence screening, the remaining high-quality sequences were clustered with a 97% similarity cutoff using UPARSE (Version 7.1, http://drive5.com/uparse/) to yield operational taxonomic units (OTUs) and chimeric sequences were identified and removed using UCHIME (Edgar, 2013). The taxonomy of each OTU representative sequence was assigned using RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the NCBI nucleotide sequence database (NT) using confidence threshold of 0.7. Finally, the corresponding species information of each OTU was obtained. Raw sequence data were deposited in the NCBI Sequence Read Archive (SRA, https://www.ncbi.nlm.nih.gov/sra) under BioProject No. PRJNA906342.

As this study aims at analyzing zooplankton that can be divided into three major subgroups (rotifers, cladocerans, and copepods), it is necessary to subtract the other sequences (e.g., phytoplankton and Mollusca) from samples before relative reads abundance (RRA) is calculated. Rarefaction curves were plotted on OTU level using Mothur for each sample (Schloss et al., 2009).

Other statistical analyses were conducted with R (Version 4.1.1, http://www.r-project.org/) using "vegan", "picante", "graphics", "maptools", "stats", and "ggplot2" packages. Heatmaps were generated using Bray-Curtis distance and automatically organized by hierarchical clustering. Nonmetric multidimensional scaling (NMDS, based on Bray-Curtis distance) was applied to characterize the spatial-temporal pattern of zooplankton communities. To determine whether there were significant differences in zooplankton communities among the five habitats, dissimilarity tests were performed by the analysis of similarities (ANOSIM). The Kruskal-Wallis Analysis was executed to compare the variation of α diversity of zooplankton communities among the five habitats and compare differences in the data among COI amplicon, 18S rDNA amplicon and morphological methods. The average relative reads abundance (RAA) differences and the significance levels of species differences among different habitats were compared using Wilcoxon ranksum test. The calculated *P*-value was calibrated through false discovery rate (FDR) correction, taking FDR ≤0.05 as a threshold. Canonical correspondence analysis (CCA) or Redundancy analysis (RDA) was used to identify the effects of environmental factors on the zooplankton communities within the five habitats. Whether a CCA or RDA model was selected was determined based on the community composition by Detrended Correspondence Analysis (DCA). If the longest gradient was >3, CCA was applied. If that value was <3, RDA was a better choice. Only the environmental variables with varying inflation factors less than 10 (VIF<10) were included in the analysis (ter Braak and Šmilauer, 2002).

3 RESULT

3.1 Composition of zooplankton communities among different habitats

Rarefaction curves for all the samples were nearly saturated (Supplementary Fig.S1), suggesting that our sequencing depth was enough to recover most diversity. For COI amplicons, a total of 451 901 clean reads were obtained from all 15 samples. After BLASTN analysis against NCBI database, all eukaryotic sequences were assigned to 919 OTUs. Approximately 26% (241 in 919) of the total assigned OTUs were classified as zooplankton, including 194 OTUs of Rotifera, 22 of Cladocera, and 25 of Copepoda. Additionally, 31 OTUs did not match any reference nucleotide sequence or matched to the "invertebrate environmental sample". The highest number of OTUs of zooplankton occurred in Nym (118) and the lowest occurred in Tha (71). The number of unique OTUs in each habitat was high, ranging from 9 to 44, while only 10 OTUs were shared among the five habitats (Fig.2a).

For 18S rDNA amplicons, a total of 874 647 clean reads were obtained from all 15 samples. After BLASTN analysis against NCBI database, all eukaryotic sequences were assigned to 1 075 OTUs. Approximately 6% (62 in 1 075) of the total assigned OTUs were classified as zooplankton, including 34 OTUs of Rotifera and 28 of Copepoda. Besides, 291 OTUs did not match any reference nucleotide sequence or matched to the "uncultured eukaryote". The highest number of zooplankton 18S

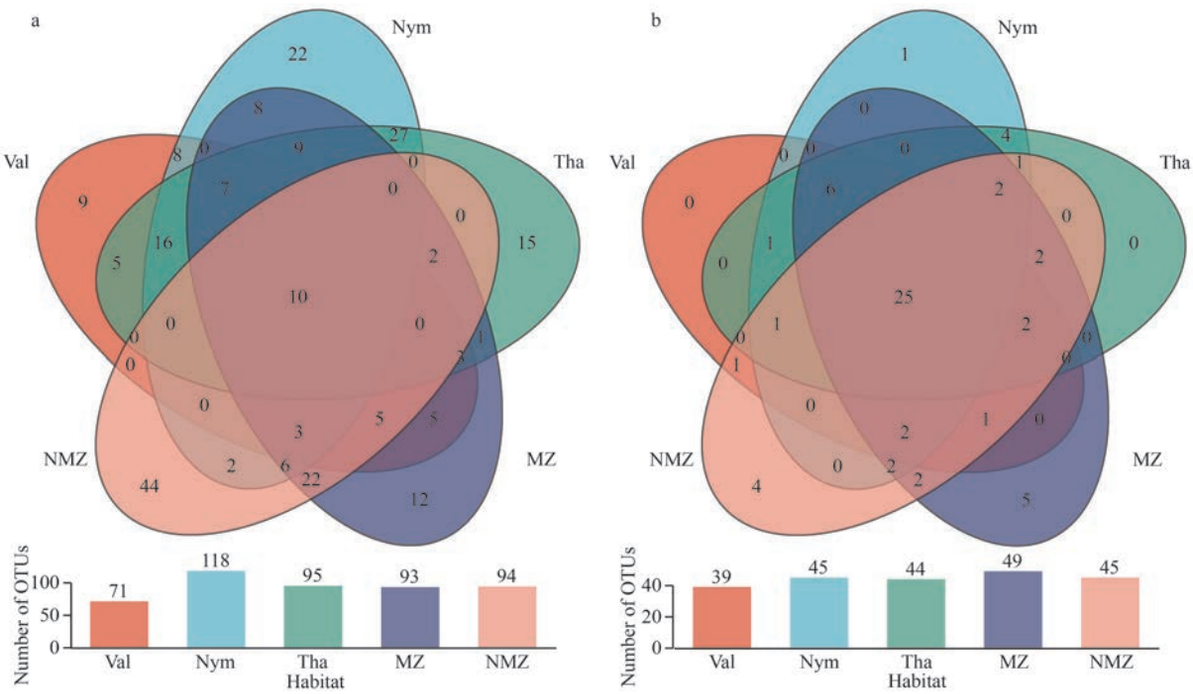

Fig.2 Venn diagram showing the numbers of unique and shared OTUs among five different habitats

a. COI amplicons; b. 18S rDNA amplicons. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone.

rDNA OTUs occurred in MZ (49) and the lowest occurred in Val (39). The number of shared OTUs among the five different habitats was as high as 25. In contrast, the number of unique OTUs in each habitat was low, ranging from 0 to 5 (Fig.2b).

The zooplankton compositions varied with habitats. The results of COI amplicons showed that the most abundant genus (or family) of zooplankton community in Val was Brachionidae (36.8%), followed by Notommatidae (21.3%) and *Alona* (4.4%). Nym was dominated by *Testudinella* (55.2%), Brachionidae (8.2%), and *Polyarthra* (5.0%). Tha was dominated by *Testudinella* (26.0%), *Alona* (13.8%), and Brachionidae (8.1%). On the other hand, MZ and NMZ were dominated by *Mesocyclops* (67.6% and 3.0%, respectively), *Polyarthra* (19.5% and 10.5%, respectively), and *Pseudodiaptomus* (3.0% and 66.6%, respectively) (Fig.3a).

For 18S rDNA amplicons, the most abundant genus (or class) of zooplankton community in Val was *Eucyclops* (16.6%), followed by *Philodina* (16.1%) and *Microcyclops* (4.7%). Nym was dominated by *Eucyclops* (33.4%), *Philodina* (30.7%), and *Microcyclops* (2.6%). Tha was dominated by *Philodina* (14.6%), *Microcyclops* (10.3%), and *Eucyclops* (10.1%). In MZ, *Microcyclops* (31.3%) was the most abundant taxon, followed by *Keratella* (16.7%) and *Eucyclops* (9.1%). In NMZ, *Pseudodiaptomus* (61.9%) was the most abundant taxon, followed by *Microcyclops* (7.6%) and *Eucyclops* (1.3%) (Fig.3b).

3.2 Comparison of zooplankton communities among different habitats

NMDS showed that the samples collected from macrophytes (Val, Nym, and Tha) were mostly together and distributed on the left of the plots, while samples from non-macrophyte zone (NMZ) tended to be on the right but separated from the macrophyte zone (MZ) (Fig.4). The community similarity analysis (ANOSIM) of COI and 18S rDNA amplicons showed that there were significant differences of zooplankton communities among the five habitats (COI: R=0.69, P<0.01; 18S rDNA: R=0.62, P<0.01). The zooplankton communities associated with macrophytes (Val, Nym, and Tha) were similar (COI: R=0.15, P<0.01; 18S rDNA: R=0.10, P<0.01), but they differed significantly from those in the open waters (NMZ) (COI: R=0.97, P<0.01; 18S rDNA: R=0.92, P<0.01). On the contrary, there was no significant temporal differences among the zooplankton communities (COI: R=0.09, P<0.01; 18S rDNA: R=0.07, P<0.01) (Supplementary Fig.S2).

The results of DNA metabarcoding were consistent with those of the morphological method

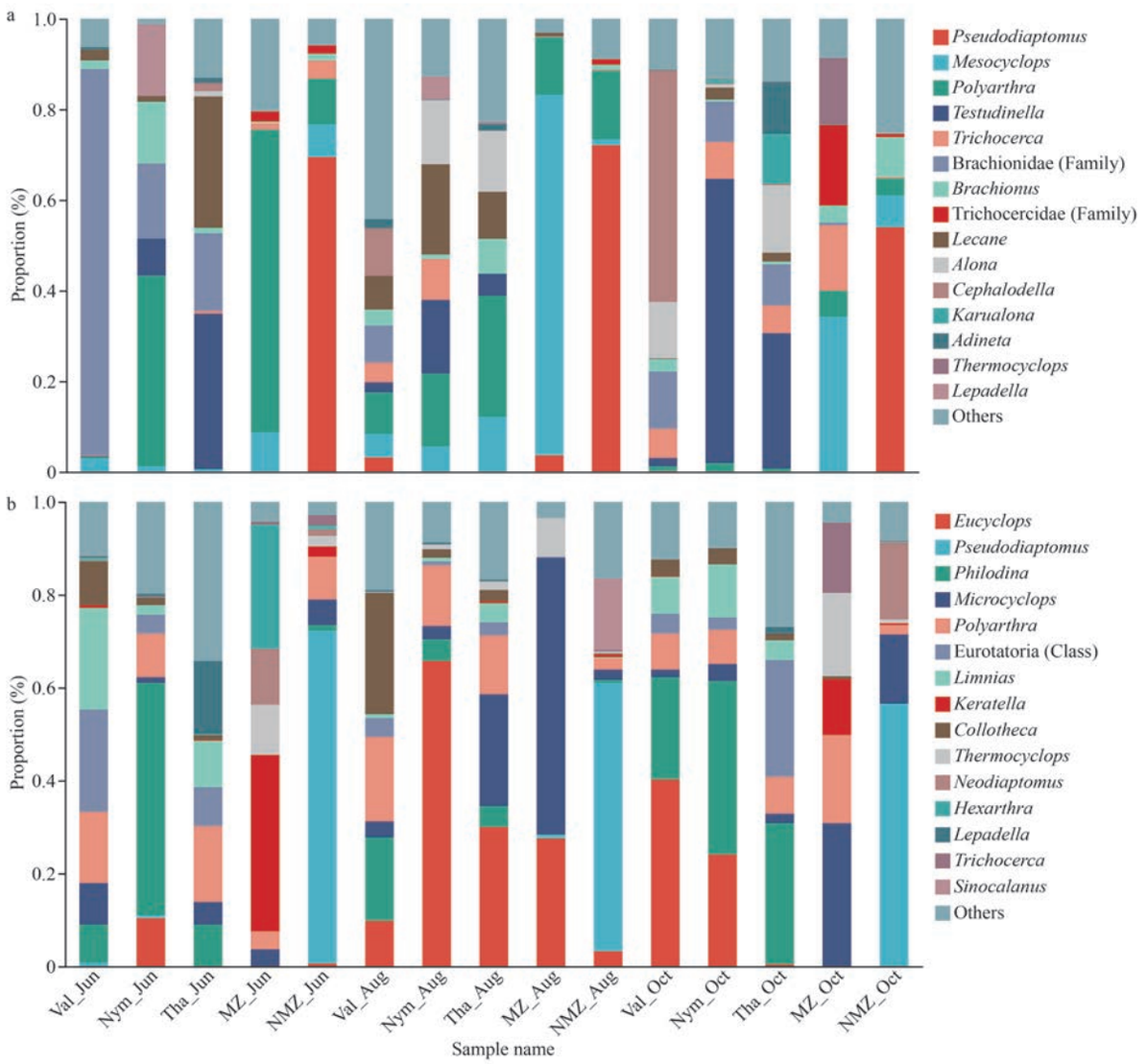

Fig.3 Zooplankton compositions (based on reads abundance) of the samples from five different habitats (top 15 on genus level)
a. COI amplicons; b. 18S rDNA amplicons. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone.

(Fig.4). The distribution patterns (based on the inferred biomass) of the main species detected by morphology were closer to the result of COI metabarcoding than that of 18S rDNA (Supplementary Fig.S3). The results of Mantel test (Spearman's, number of permutations: 999) showed that the distribution characteristics of the main species from COI metabarcoding data exhibited good agreement (R=0.67, P<0.01) with the morphological data (the inferred biomass of zooplankton from the morphological method), followed by 18S rDNA metabarcoding versus COI metabarcoding data (R=0.57, P<0.01). The lowest was 18S rDNA metabarcoding data versus morphological data (R=0.55, P<0.01).

The hierarchical heatmaps were classified into two groups and showed that the samples from Val, Nym, and Tha were positioned together on the left side of the plot, while the samples collected from pelagic water zone (MZ and NMZ) were clustered on the right side (Fig.5). Results of both COI and 18S rDNA amplicons show that the littoral zooplankton (e.g., *Testudinella patina*, *Notommata* sp., *Mytilina ventralis*, *Lecane bulla*, *Philodina megalotrocha*, *Collotheca tenuilobata*, *Lepadella triptera*, *Eothinia elongata*, and *Trichotria tetractis*) tended to prevail in the epiphytic communities. The pelagic species (e.g., *Pseudodiaptomus forbesi*, *Mesocyclops pehpeiensis*, *Cyclopoida* sp., *Trichocerca* sp., *Hexarthra* sp., *Polyarthra* sp., *Thermocyclops crassus*, and *Sinocalanus sinensis*) dominated in MZ and NMZ. Furthermore, in samples from Val, Nym,

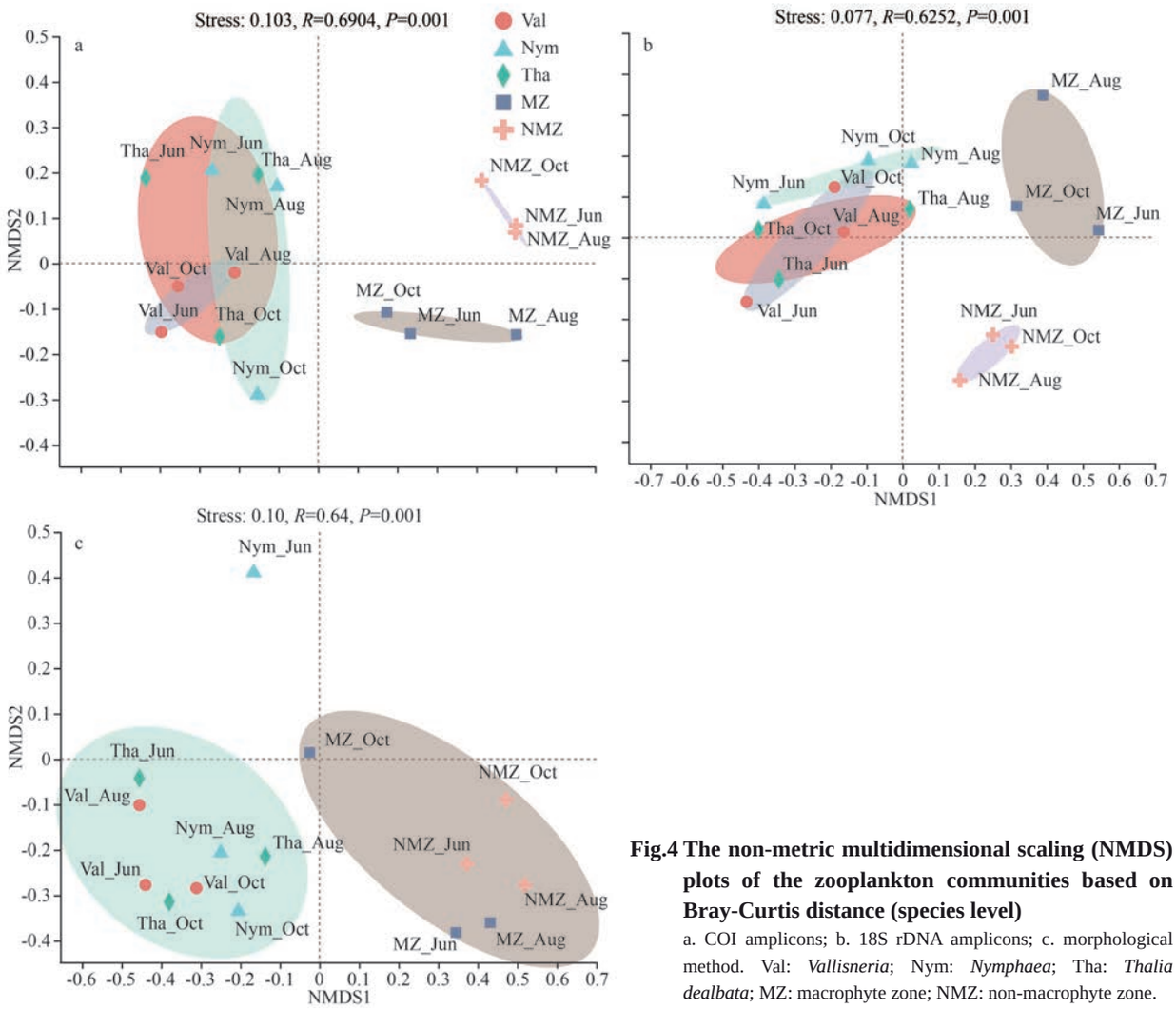

Fig.4 The non-metric multidimensional scaling (NMDS) plots of the zooplankton communities based on Bray-Curtis distance (species level)

a. COI amplicons; b. 18S rDNA amplicons; c. morphological method. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone.

and Tha, most species of zooplankton were rotifers, which have relatively smaller bodies compared to copepods and cladocerans. In contrast, the proportion of copepods (more planktonic and competitive) was greater in MZ and NMZ and littoral rotifers were less common (Fig.5).

The Wilcoxon rank-sum test was used to analyze the significant differences in the average RRA of the dominant species among the five habitats (Supplementary Fig.S4). For COI amplicons, the top nine dominant zooplankton including *Pseudodiaptomus forbesi*, *Testudinella patina*, *Polyarthra* sp., *Lepadella* sp., *Trichocerca* sp., *Adianta vaga*, *Lecane inermis*, *Cyclopoida* sp., and *Mesocyclops* sp., made a great contribution to the variation of zooplankton communities among different habitats. For 18S rDNA amplicons, the dominant zooplankton including *Philodina megalotrocha*, *Bdelloidea* sp., *Pompholyx sulcate*, *Limnias melicerta*, *Collotheca tenuilobata*, *Lepadella triptera*, *Floscularia armata*, *Sinantherina socialis*, *Trichotria tetractys*, and *Mesocyclops pehpeiensis* greatly contributed to the variation of zooplankton communities among different habitats.

3.3 Diversity index among different habitats

Analyzing the alpha diversity index between specific habitats, significant differences (P<0.05) were found in the case of community evenness (Shannon evenness index) and community diversity (Shannon-Wiener index) (Fig.6). For COI amplicons, both Shannon-Wiener and Shannon evenness indices of the zooplankton community from the Tha habitat were significantly higher than those from the NMZ habitat (P<0.05). For 18S rDNA amplicons, The Shannon evenness indices were markedly higher (P<0.05) among epiphytic sites (Val, Nym, and Tha) compared to the open water area (NMZ). On the other hand, the Shannon-Weiner indices of the zooplankton communities

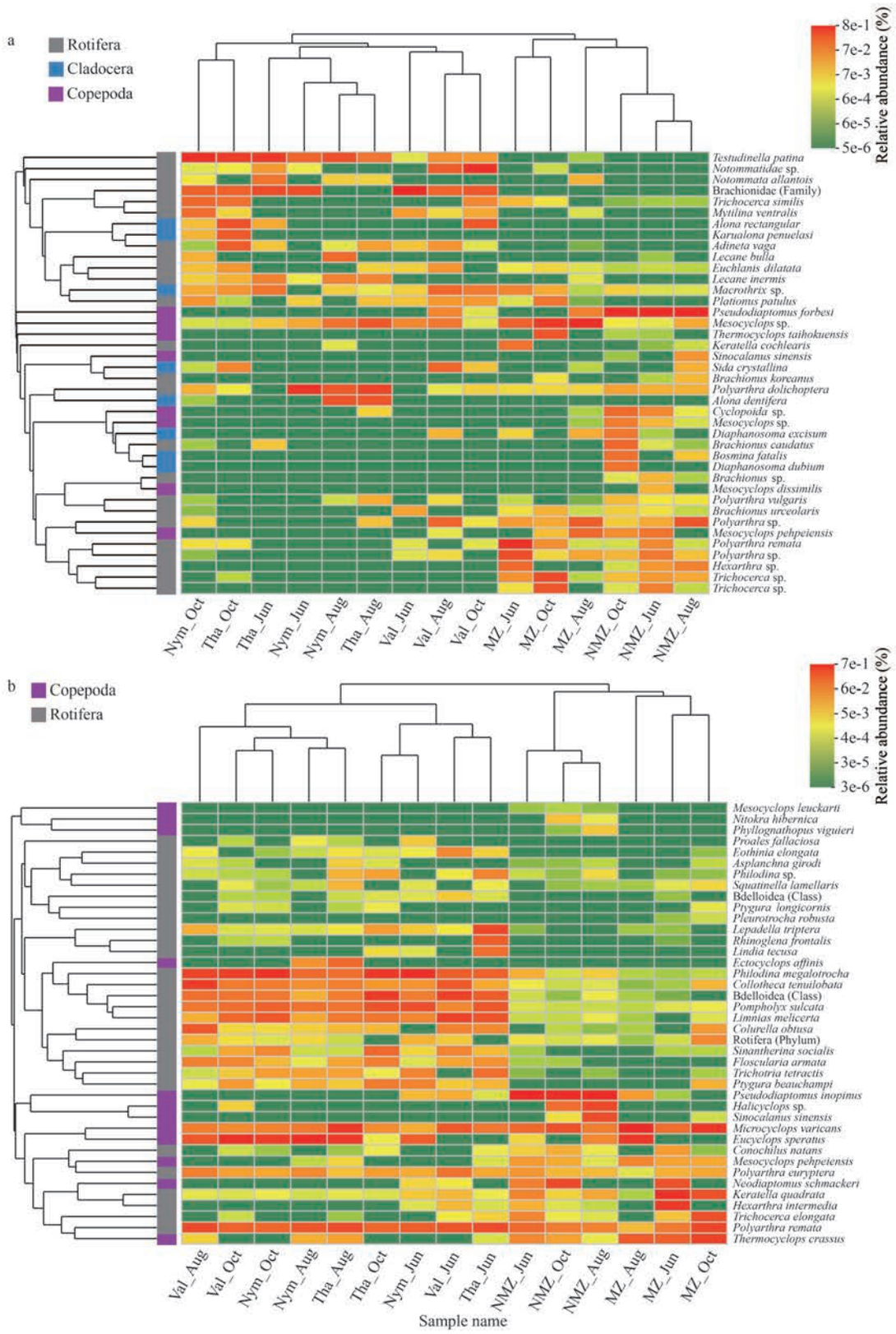

Fig.5 Heatmaps of the zooplankton compositions from five different habitats based on Bray-Curtis distance (top 40 abundant OTUs on species level)

a. COI amplicons; b. 18S rDNA amplicons. Samples and taxa are automatically organized by hierarchical clustering. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone.

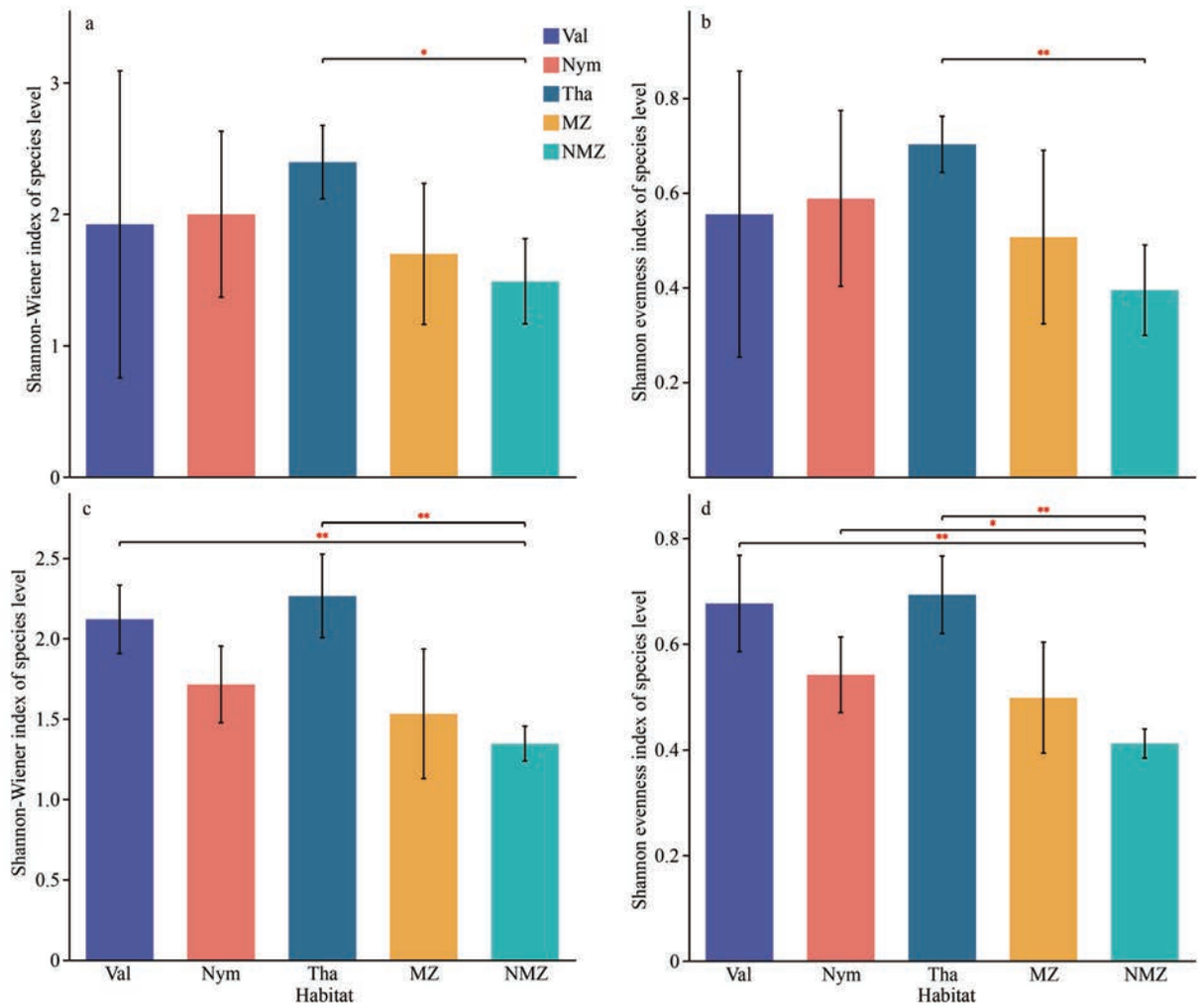

Fig.6 The *α* diversity of zooplankton communities in five different habitats

a. the Shannon-Wiener index of COI amplicons; b. the Shannon evenness index of COI amplicons; c. the Shannon-Wiener index of 18S rDNA amplicons; d. the Shannon evenness index of 18S rDNA amplicons. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone. Kruskal-Wallis test; *: $P<0.05$; **: $P<0.01$; error bars: standard deviation.

from Val and Tha were significantly higher than those from NMZ ($P<0.05$).

3.4 Relationship between the zooplankton communities and environmental variables

As the longest gradients of DCA were >3 (5.1 for COI amplicons and 3.9 for 18S rDNA amplicons), CCA models were used to estimate the relationships between zooplankton communities and environmental variables. For COI amplicons, the first two ordinate axes explained 31% of the species-environment variability in the ordination of environmental variables (Fig.7a; Supplementary Table S2). After forward selection, only SD, pH, DO, TP, TN, COD, and NH_4-N were significant contributors to the variation of the zooplankton communities (Supplementary Table S3). Furthermore, pelagic copepods including *Mesocyclops pehpeiensis* and *Pseudodiaptomus forbesi* were related to higher NH_4-N, TP, and TN content. Littoral rotifers such as *Testudinella patina* were associated with DO.

For 18S rDNA amplicons, the first two ordinate axes explained 45% of the eukaryotes-environment variability in the ordination of environmental variables (Fig.7b; Supplementary Table S4). After forward selection, only SD, pH, DO, Chl *a*, TP, and NH_4-N were significant environmental factors to the variation (Supplementary Table S3). Additionally, *Pseudodiaptomus forbesi* was also related to higher Chl *a* and TP content. *Keratella quadrata*, the planktonic rotifer, were associated with higher SD. However, littoral copepods as *Microcyclops varicans* and *Eucyclops speratus* were correlated to higher DO.

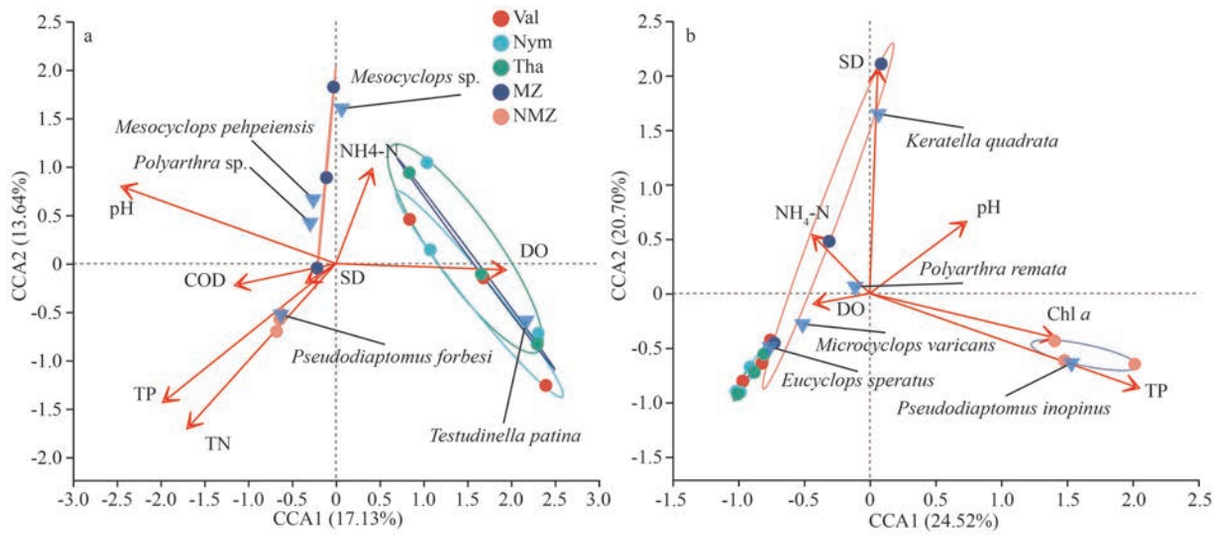

Fig.7 CCA of the relationship between the zooplankton communities and environmental factors

a. COI amplicons; b. 18S rDNA amplicons; blue triangle: top 5 species based on RRA; red arrows: environmental variables; circle: samples from different habitats. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone. COD: chemical oxygen demand; SD: water transparency; DO: dissolved oxygen; TP: total phosphorus; TN: total nitrogen; NH_4-N: ammonium nitrogen; Chl *a*: chlorophyll *a*.

3.5 Comparison of metabarcoding data with monitoring data based on morphology

A total of 165 zooplankton species were identified in all sampling sites with the three methods. There were 56 unique species in COI amplicons, and most of them belonged to rare taxa. Additionally, COI amplicons detected more species than other two methods. Venn diagram analysis indicated that the highest number of species (39) were shared between COI amplicons and the morphological method (Fig.8a). Compared to the morphological method, the number of species per site was notably higher for DNA metabarcoding including COI amplicons and 18S rDNA amplicons (P<0.05; Fig.8b). In addition, COI amplicons detected significantly more rotifer species at the per site than other two methods. Since no Cladocera was identified by 18S rDNA amplicons, the number of species in Cladocera identified by 18S rDNA amplicons was significantly lower than those by other two methods (Fig.8c). Moreover, there were no significant differences in the relative abundance of rotifers and copepods among the three methods except Cladocera (Fig.8d).

4 DISCUSSION

4.1 Effectiveness of DNA metabarcoding in assessing epiphytic zooplankton biodiversity

Zooplankton samples collected from macrophytes tend to contain more sludge and detritus than those collected in open waters. Thus, the complete biodiversity of epiphytic zooplankton on macrophytes is underestimated by morphological method. Our results show that the identification of rotifer and copepod species was significantly improved by DNA metabarcoding. Specifically, it increased the taxonomic resolution and detectability of epiphytic rotifers, particularly for rare species. Moreover, the large number of unique taxa detected by COI and 18S rDNA amplicons in this study supports the idea that performing multiple primers can reduce amplification bias and generate a more complete biodiversity profile (Zhang et al., 2022). DNA metabarcoding, especially when using high-throughput sequencing, is a method that not only detects intact organisms, but also can quantify the presence of rare species by their extracellular molecules, organelles, cellular debris, and tissues (Yao et al., 2022). Additionally, owing to the obvious advantages, including high detection efficiency, accuracy, and sensitivity, high-throughput sequencing-based methods are expected to serve as robust and powerful tools for rare species detection and the warning of newly introduced non-indigenous species in management programs (Xiong et al., 2016).

Another benefit is that DNA metabarcoding is powerful at resolving taxon diversity in copepods that are impossible to distinguish in their larval stages using a microscope (the traditional morphological method assigns these to “copepod

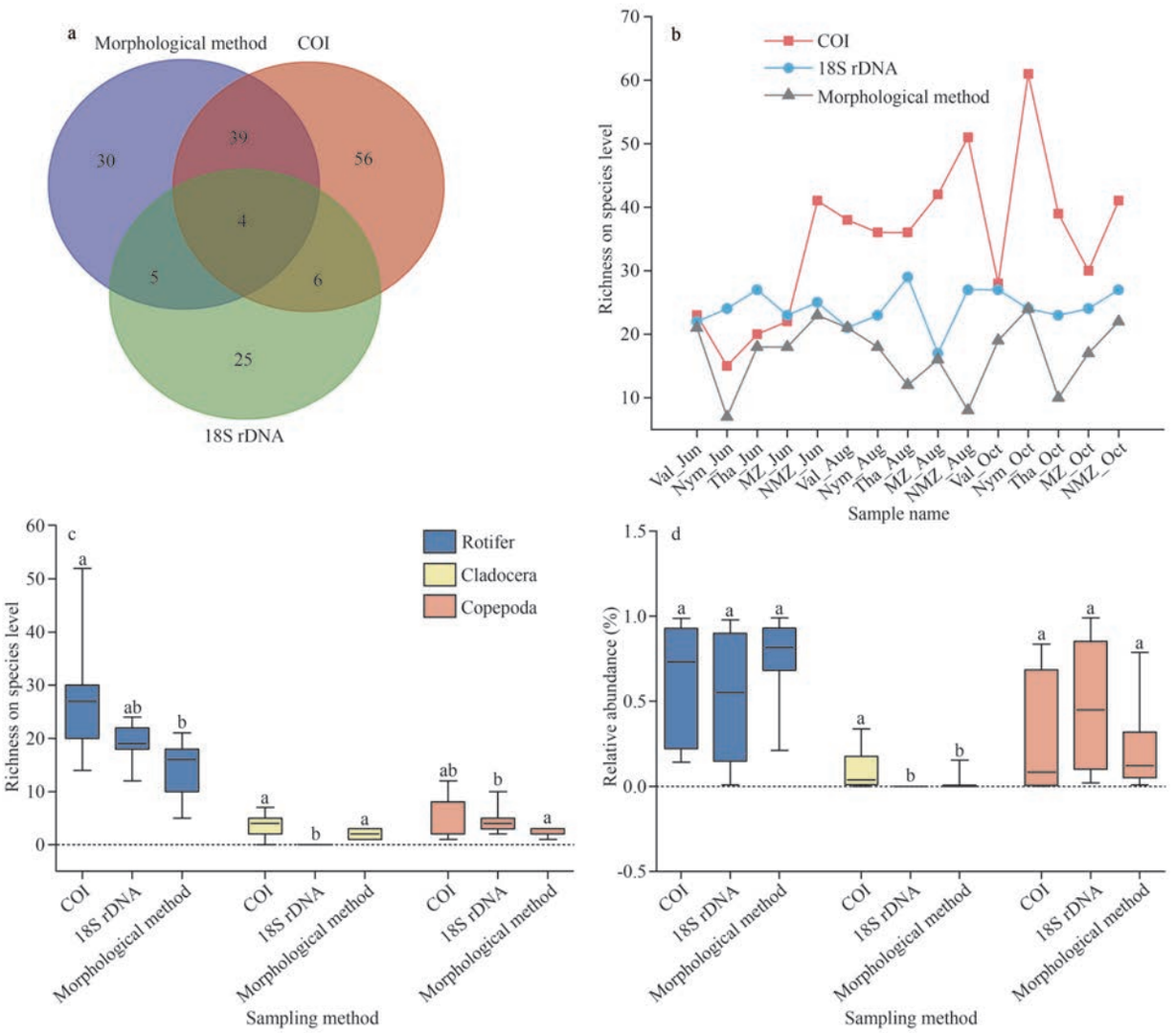

Fig.8 Comparison of metabarcoding data with morphological data on zooplankton species monitoring

a. Venn diagram showing the numbers of unique and shared species detected by three different methods; b. the number of zooplankton species detected by three different methods in each sample; c. the total number of zooplankton species detected by three different methods; d. the relative abundance of zooplankton species detected by three different methods. Val: *Vallisneria*; Nym: *Nymphaea*; Tha: *Thalia dealbata*; MZ: macrophyte zone; NMZ: non-macrophyte zone. Kruskal-Wallis test. Different letters indicate sample means that are significantly different among groups. Error bars: standard deviation.

larvae") (Yang et al., 2017). According to our previous study by traditional methods, nauplii and copepodites of Copepoda were found to be relatively high in both MZ and NMZ, with an average relative abundance from 12% to 21% (Liang et al., 2023). As most of the copepods are in larvae stages, copepod diversity within the urban wetland ecosystem may be underestimated. Here, taxonomy based on DNA barcoding makes it possible to identify the species of copepods larvae, which improves the comprehensive understanding of zooplankton diversity.

In addition, DNA metabarcoding methods may unveil cryptic species that have eluded traditional surveys (Zhang et al., 2022). Cryptic species are difficult or even impossible to distinguish by their morphology and are common not only in the study of zooplankton (Ershova et al., 2021), but also in fishes (Zhang et al., 2022) and insects (Ma et al., 2019). Within our study, examples included cryptic species or complexes of the rotifer *Polyarthra dolichoptera*, *Brachionus calyciflorus*, *Euchlanis dilatata*, *Lecane bulla*, *Lepadella patella*, *Adineta vaga*, etc. Since several closely related species (e.g., *Brachionus calyciflorus* var. *amphiceros* and *Brachionus calyciflorus* var. *borgerti*), share a very similar morphology, they may coexist in the water bodies, with few studies of zooplankton community

attempting to distinguish between them. The advantage of metabarcoding using hypervariable markers such as COI is that it provides intra-species information (cryptic diversity) for multiple species simultaneously, which can be applied to detect subtle changes of zooplankton community structure (Turon et al., 2020). In the present study, the greater numbers of rotifer species presented in the COI amplicons can be partly attributed to their high cryptic diversity.

Significant correlations have been observed between biomass and the number of sequences in recent studies of zooplanktons (Yang et al., 2017), benthic invertebrates (Klunder et al., 2022) and fishes (Stoeckle et al., 2021). Our Mantel test also showed moderate correlation between DNA metabarcoding data and the inferred biomass data detected by morphological method in our previous study (Liang et al., 2023). Results of both COI amplicons and 18S rDNA amplicons show that zooplankton communities in macrophyte (Val, Nym, and Tha) habitats were significantly different from those in non-macrophyte (NMZ) habitat. Although the two methods have consistent results in reflecting the distribution patterns of zooplankton communities, DNA metabarcoding does not require morphological experience, which greatly saves labor and time costs (Yang et al., 2017).

4.2 Microhabitat support and key environmental factors for epiphytic zooplankton community

The "top-down forces" is one of the important factors regulating the community structure of zooplankton (Liu et al., 2018). For example, rotifer abundance can be reduced when mesozooplankton are abundant, because rotifers suffer predation by and competition (both interference and exploitative) with mesozooplankton such as copepods and cladocerans (Yoshida et al., 2003). High plant abundance can interfere with the swimming and feeding behavior of fishes and copepods (Choi et al., 2014b). Macrophytes are utilized as a refuge for predator avoidance (Choi et al., 2014a). Due to the absence of mesozooplankton and the lack of their suppressing effect, rotifers may thrive in macrophyte-dominated areas (Kuczyńska-Kippen et al., 2021). Our result supports this idea with the evidence that copepods such as *Pseudodiaptomus forbesi* and *Mesocyclops pehpeiensis* dominated in the non-macrophytes water zone, while littoral rotifers dominated in the epiphytic zooplankton communities. As pelagic rotifers have evolved a variety of antipredator strategies and move faster than littoral rotifers, they can easily develop in the open water area (Yin et al., 2017).

Different types of macrophytes improve the structural heterogeneity of microhabitats in wetlands, which would strongly affect zooplankton distributions, particularly in epiphytic species (Choi et al., 2015). Our results of COI amplicons showed that the number of unique OTUs in each habitat was high, ranging from 9 to 44. This implies that the complicated plant beds may create different microhabitats and it can be expected that they will be chosen by distinct zooplankton species. Several previous studies indicated that zooplankton assemblages in the water bodies covered by helophytes, pleustophytes, nymphaeids, and elodeids were significantly different (Choi et al., 2014b; Kuczyńska-Kippen et al., 2021). In temperate wetlands, rotifers exhibited a high preference for nymphaeids habitats. Elodeids mainly supported rotifers, such as *Filinia* and *Synchaeta*, copepods, such as *Eucyclops*, and cladocerans, such as *Alona* (Choi et al., 2014a). These were consistent with our study in the subtropical wetland.

There were no significant temporal differences between zooplankton communities, which may result from the lack of temperature changes in subtropical water bodies during June–October (Liang et al., 2019). In most macrophyte-dominated waters, the key factors for zooplankton communities are trophic status and plant biometric features, rather than temperature (Kuczyńska-Kippen and Joniak, 2016; Kuczyńska-Kippen, 2018). Littoral and pelagic zooplankton showed opposite responses to food sources. The CCA results indicate that pelagic zooplankton such as *Pseudodiaptomus forbesi* was favored by an increase in Chl *a*, TN, and TP contents, while littoral zooplankton (e.g., *Testudinella patina* and *Microcyclops varicans*) was correlated to higher DO. Generally, pelagic zooplankton with its eutrophic fraction was positively correlated with Chl-*a* content, while littoral zooplankton as well as the mesotrophic fraction was associated with dissolved organic matter (DOM) (Kuczyńska-Kippen et al., 2021). Bacterioplankton, epiphytic algae, and macrophyte detritus are potential food sources for zooplankton (Wolters et al., 2019). They provide carbon subsidies to them in the form of particulate organic carbon and dissolved organic carbon (de Kluijver et al., 2015). Decaying macrophytes also serve as food source for the omnivorous epiphytic

zooplankton. Therefore, even though the concentration of Chl *a* decreased, the abundance and the proportion of littoral rotifers increased (Liang et al., 2023).

4.3 Effect of macrophytes on maintaining aquatic ecosystem health

Our results indicated that the α diversity of zooplankton communities on macrophyte surfaces was significantly higher than that in the non-macrophyte water zone. The mixture of various macrophyte species increased the heterogeneity of microhabitats in wetlands, which further led to an increase in α and β diversity of zooplankton communities. It has been reported that zooplankton diversity is higher in lentic waters at a medium trophic level but lower under extreme oligotrophic or hyper-eutrophic levels (Qian et al., 2007). However, in macrophyte-dominated areas, zooplankton diversity in eutrophic water is significantly higher than that in other trophic water bodies. Additionally, Chl *a* and phosphorus concentrations have negative effects on the increase of littoral zooplankton abundance (Kuczyńska-Kippen, 2018). This is in accordance with the CCA results in the present study.

Healthy aquatic ecosystems include high-quality water environment, diversity of species and complex food webs. On the one hand, macrophytes in water bodies leads to the improvement of water quality (Schernewski et al., 2023). On the other hand, they create complex habitats for littoral rotifers as well as the mesotrophic fraction of the pelagic zooplankton, which can maintain a high biodiversity in water bodies under eutrophic and mesotrophic states. At the same time, the presence of elodeids improves the ecological status of wetlands by increasing the proportion of littoral rotifers and reducing the abundant eutrophic fraction of zooplankton (Wolters et al., 2019; Kuczyńska-Kippen et al., 2021). The exception is that adverse environmental conditions (e.g., decline in transparency, overgrowth of filamentous algae) in hypereutrophic waters could cause the elimination of macrophyte. As a result, zooplankton diversity is reduced by the loss of refuges (Kuczyńska-Kippen and Joniak, 2016). Thereby the presence of macrophytes increases zooplankton diversity by enhancing habitat heterogeneity and improves water quality at the same time.

5 CONCLUSION

Owing to the advantages including high detection efficiency and sensitivity, DNA metabarcoding is a robust approach for biomonitoring and helping to fully understand the potential biodiversity of epiphytic zooplankton in urban wetlands. The joint use of multiple molecular markers improves the taxonomic resolution and detectability of epiphytic rotifers, particularly for rare species, resulting in a more comprehensive biodiversity profile. The zooplankton communities associated with macrophytes (Val, Nym, and Tha) were similar, but they differed significantly from those in the open waters (NMZ). However, there were no significant temporal differences among the zooplankton communities, which may result from the lack of temperature changes. Microzooplankton, especially littoral species, utilizes macrophytes as food resources and as refuges against predation, which further leads to an increase in α and β diversity of zooplankton communities. Macrophytes can improve water quality in wetland ecosystems and maintain a high biodiversity under eutrophic and oligotrophic states simultaneously.

6 DATA AVAILABILITY STATEMENT

Raw sequencing data of this study have been deposited in the NCBI database under BioProject No. PRJNA906342.

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Electronic supplementary material

Supplementary material (Supplementary Tables S1–S4 and Figs.S1–S4) is available in the online version of this article at https://doi.org/10.1007/s00343-024-3148-3.