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Community Characteristics Analysis of Eukaryotic Microplankton via ITS Gene Metabarcoding Based on Environmental DNA in Lower Reaches of Qiantang River, China

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Abstract

Eukaryotic microplankton plays an important role in water biotic community and in maintaining the stability of water ecosystems. Environmental DNA metabarcoding provides the opportunity to integrate traditional and emerging approaches to discover more new species, and develop molecular biotic indices that can be more rapidly, frequently, and robustly used in water quality assessments. In order to examine assemblages of eukaryotic microplankton in lower reaches of Qiantang River, ITS gene metabarcoding technology based on environmental DNA was carried out. As a result, various species of phytoplankton, fungi and zooplankton were annotated on. More phylum, classes and specieses of eukaryotic phytoplankton and zooplankton were found after compared communities taxa based on metabarcoding with that obtained from morphological examination. Nevertheless, *Chlorophyceae* was the most common assemblage both identified by using these two methods, also *Mesocyclops leuckarti* and *Acanthocyclops bicuspidatus* were both found to be the dominant species of Cyclopoida in the river. Additionally, the reads proportions of phytoplankton and zooplankton at the three freshwater sampling sites (Tonglu, Fuyang and Wenyan) decreased as temperature drop. Meanwhile, twenty classes of fungi were annotated on, of which the community characteristic was first researched in the river. There were significant spatial differences in values of Chao1 index for eukaryotic microplankton. Cluster analysis and Non-metric multidimensional scaling ordination further

confirmed that the community composition of eukaryotic microplankton at class level for Jiashao-September sample had the most dissimilarity with the others.

Keywords

Microplankton, eDNA, Metabarcoding, ITS, Diversity, Qiantang River

1. Introduction

Eukaryotic microplankton is a group of plankton with particle sizes less than 20-μm [1]. It plays an important role in water biotic community and in maintaining the stability of water ecosystems due to its important link in energy flow and material circulation [1]. As the basis of the food chain, analyzing its metacommunity structure is very important to assess the status and development tendency of an ecosystem [2].

Traditional practices for biological surveys of inland waters usually center on a common set of ecological indicators or indices/measurements of biodiversity [3], requiring many preparations, such as morphological taxonomic expertise, intact specimens and adequate time [4] [5]. Environmental DNA (eDNA) metabarcoding provides the opportunity to integrate traditional and emerging approaches to discover more new species [6] [7], and develop molecular biotic indices that can be more rapidly, frequently, and robustly used in water quality assessments [6] [8] [9]. So far, metabarcoding technology has been widely used for biodiversity monitoring in biological and environmental samples [10] [11] [12] [13]. Despite some limitations (each marker region might import some biases and the blast sequence database is incomplete), the practices of metabarcoding-based analysis for estimating diversity and relative abundance of taxonomic groups in aquatic systems will likely increase as technology improved [14].

Now, marker genes used for the analysis of plankton communities via eDNA metabarcoding often focuses on ribosomal DNA (rDNA), ribulose bisphosphate carboxylase large subunit gene (rbc L) and cytochrome C oxidase subunit gene 1 (cox1) [8] [15] [16] [17] [18]. Among these, the internal transcribed spacer (ITS), as one gene fragment of rDNA, has been widely used due to its fast evolution and high specificity [17], and its relevant metabarcoding practices for the purpose of monitoring diversity of eukaryon communities mainly focused on soil, plant and marine systems [19] [20] [21] [22].

Here, an ITS metabarcoding assessing assemblages of eukaryotic microplankton were conducted. Specifically, ITS gene sequence analysis was performed on surface water samples collected from four sites in Qiantang River lower reaches (Zhejiang, China), an important freshwater fishing water used for drinking, electricity generation, flood control and recreation, in order to analyze the community diversity of eukaryotic microplankton in the section, to assess the

utility of this approach for monitoring diversity of freshwater eukaryotic microplankton community. As a whole, this study provided a theoretical basis for further study on the function of eukaryotic microplankton in freshwater ecosystems.

2. Materials and Methods

2.1. Water Sample Collection and Physic Chemical Analysis

A total of eight water samples were collected in September and November 2019, at four separate sampling sites in Qiantang River lower reaches, including Tonglu, Fuyang, Wenyan and Jiashao sites, hereafter referred to as TL, FY, WY and JS separately (Figure 1). All sampling, filtering, and other equipments were sterilized before use.

2 L of surface water was collected at a set time by boat from each site. Simultaneously, five environmental variables, such as transparency, salinity, water temperature (WT), pH and dissolved oxygen (DO), were measured in situ by using a secchi disk and a portable water quality detector (Hach, USA). For each

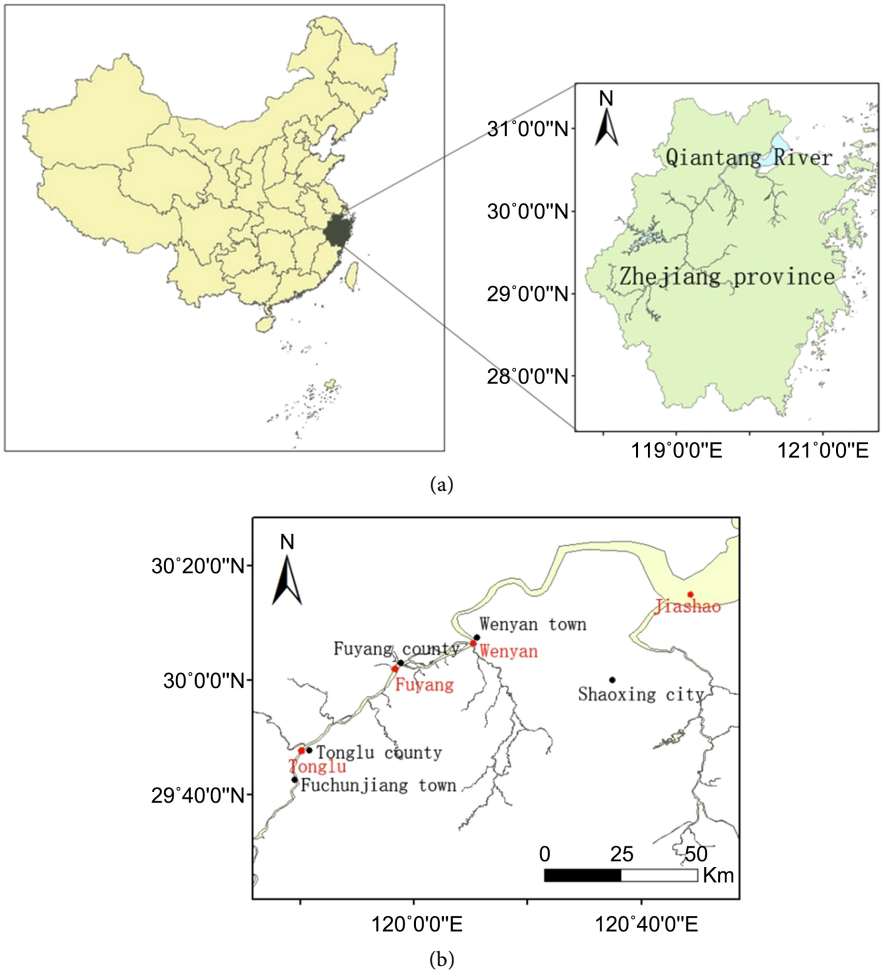


Figure 1. Map shows the study site. (a) The location of Qiantang River in Zhejiang province of China; (b) Sampling sites in Qiantang River lower reaches. The red dot represents the water sampling site, the black dot represents the local government seat.

water sample, a 1.5 L water subsample was used to test six environmental variables immediately after transported to the laboratory within 24 hours at 4˚C, including Chemical oxygen demand (COD), Chlorophyll a (Chla), total nitrogen (TN), total phosphorus (TP), ammonium (NH_4-N) and nitrate-nitrite (NO_3-N), the recording of which were following the scheme of [23]. All environmental variables were measured in triplicate.

2.2. DNA Sample Processing and High-Throughput Sequencing (HTS)

For each water sample, a residual 500-mL water subsample was then filtered through a 0.22-μm cellulose acetate filter paper using a peristaltic pump in the field. Then, each paper was placed inside a commercial sterile centrifuge tube and stored in a container filled with liquid nitrogen until subsequent manipulations were performed. DNA was extracted from filters using EZNA water DNA kit (Omega, USA) following the manufacturer's protocol. The concentration and purity of DNA were determined using NanoDrop 2000c spectrophotometer (Thermo, USA), then followed by multiplex PCR using the universal primers for ITS belonging to eukaryotic mitochondrial DNA fragments, ITS-F (5'-GTGA ATCATCGARTC-3), ITS-R (5'-TCCTCCGCTTATTGAT-3') [22]. Each eDNA sample was subjected to PCRs in triplicate [17.35 μL dH_2O, 2.5 μL 10 × PCR buffer, 0.15 μL 5 U/μL Thermo scientific Taq DNA polymerase, 2 μL dNTPs mix (2.5 mM), 1 μL of each primer (10 mM), and 1 μL sample eDNA in a total volume of 25 μL]. PCR cycling parameters were as follows: 96˚C for 3 min, followed by 35 cycles of 96˚C for 30 s, 50˚C for 30 s, and 72˚C for 90 s, with a final elongation step at 72˚C continued for 7 min. Negative control was conducted simultaneously. After subjecting the PCR products to 1% polyacrylamide gels (see **Figure S1**), the quantified, size-selected libraries were constructed and continuously diluted to a concentration suitable for sequencing. The libraries were finally sequenced on the Illumina MiSeq 2000 platform by following the manufacturer's protocols step by step.

2.3. Phytoplankton Samples Collection and Treatment

Phytoplankton samples were also simultaneously collected at the four sites. For phytoplankton counts, 1.0 L of water samples were sampled each time and preserved with 1% Lugol's iodine solution. Phytoplankton samples were concentrated to a final volume of 30 ml after sedimentation for 48 h. Thereafter, the taxa were verified and counted under 200× and 400× magnifications for at least 500 specimens [23]. The data were made to compare with that collected from ITS gene metabarcoding method.

2.4. Bioinformatics and Sequencing Data Upload

The raw sequencing FASTQ file was transformed to a FASTA file by the Fastx toolkit V0.0.1 [24]. Clean reads were gained after trimming the low quality se-

quences and PCR chimeras by using Fqtrim V0.9.4 (http://ccb.jhu.edu/software/fqtrim/) and Vsearch 2.3.4 [25]. Vsearch 2.3.4 software continued to be utilized to cluster Operational taxonomic units (OTUs) at 97% cutoff of sequence similarity. Representative sequences of OUTs were assign to taxonomic groups against the NCBI Genbank by using Blast+ 2.6.0 at 80% cutoff of identity thresholds with over 80% matches and expect values less than 1e−5. The Sequencing data have been uploaded to NCBI Sequence Read Archive database, with accession numbers vary from SRR10800795 to SRR10800802.

2.5. Date Analysis

Three α-diversity indices, including Chao1 estimators, Simpson index, and Shannon index, were calculated based on data obtained by metabarcoding monitoring. Additionally, Cluster analysis taken by group average method and Non-metric multidimensional scaling (NMDS) was employed to cluster samples in Primer 5.0 environment [26], of which species data were first transformed according to [27]. Additionally, basic data processing, drawing and statistical analyses (e.g. one-way ANOVA) were conducted using Excel 2007 and SPSS 16.0 software.

3. Results

3.1. Environmental Characterization

The results of environmental variables are showed in **Table 1**. WT showed significant differences between months. TN showed significant negative association with transparency at $p < 0.05$, with COD at $p < 0.01$, and significant positive association with TP at $p < 0.01$. In addition, NH4-N showed a significant positive association with TP and WT at $p < 0.05$. The values of TN: TP mass ratios were all higher than 7 in all sampling sites, indicating the research area was generally P-limited at the experimental period.

3.2. Sequencing Analysis

ITS gene metabarcoding yielded 67,469 - 129,150 raw reads, of which 55,687 - 112,832 clean reads were obtained after optimization, resulting in effective data rates varying from 64.1% to 88.8% (**Table 2**). The sequences clustered into a total of 5795 OTUs, varying from 706 to 1911 at an average of 1245 (**Table 2**). Meanwhile, the rarefaction curves of each sample all showed the observed species number flatted out as sequence increasing, indicating the amount of sequencing data at the 97% similarity threshold was sufficient to satisfy the assessment of species diversity.

3.3. Community Structure Composition

In total, Phytoplankton, fungi, zooplankton and other eukaryotes were annotated on after Blast. Five classes of eukaryotic phytoplankton were annotated on,

Table 1. Values of the eleven environmental variables from sampling sites.

Sample	In situ					In lab					
	DO (mg/L)	pH	WT(˚C)	Salinity	Transparency (cm)	Chl a (μg/L)	COD (mg/L)	TP (mg/L)	TN (mg/L)	NH_4-N (mg/L)	NO_3-N (mg/L)
TL-A	6.66	7.28	24.8	–	170	1.77	17.00	0.05	2.11	0.33	1.25
TL-B	7.21	7.38	18.0	–	300	0.40	14.00	0.06	1.73	0.11	1.51
FY-A	6.53	7.37	26.3	–	75	3.21	15.00	0.05	1.93	0.32	1.26
FY-B	7.83	7.35	18.3	–	250	0.95	14.00	0.06	1.51	0.12	1.32
WY-A	8.20	7.29	27.5	–	65	6.97	17.00	0.06	2.00	0.32	1.36
WY-B	8.25	7.48	18.7	–	110	5.52	8.00	0.04	1.88	0.42	1.28
JS-A	7.29	7.79	30.3	+	5	3.82	2.02	0.28	3.77	0.89	1.68
JS-B	9.33	7.95	18.5	+	10	2.94	2.76	0.29	3.46	0.12	2.89

A: the "September" sample; B: the "November" sample. Hereinafter inclusive; +: salinity measured; –: no salinity measured.

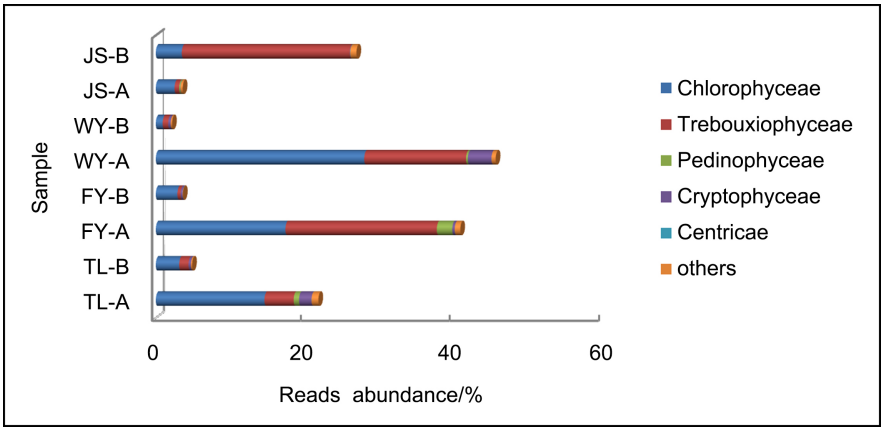
Table 2. Temporal and spatial variation of indices based on ITS gene metabarcoding data.

Description	Sep.	Nov.	Sep.	Nov.	Sep.	Nov.	Sep.	Nov.
	TL		FY		WY		JS	
Number of raw reads	86,859	87,871	72,481	83,729	67,469	73,525	68,724	129,150
Number of clean reads	55,687	75,178	60,425	73,516	55,784	65,278	59,221	112,832
effective data rate (%)	64.1	85.6	83.4	87.8	82.7	88.8	86.2	87.4
Number of OTUs	1911	1370	1485	978	1641	1130	706	744
Reads abundance for phytoplankton (%)	21.8	4.9	40.9	3.7	45.7	2.2	3.6	27.0
Reads abundance for zooplankton (%)	0.3	0.1	0.3	0.0	0.1	0.1	0.0	0.1
Reads abundance for fungi (%)	9.7	10.2	4.7	3.8	3.1	22.4	22.2	43.7
Reads abundance for other eukaryotes (%)	0.7	0.1	0.1	0.0	0.2	0.1	0.0	0.2
Reads abundance for unclassified (%)	89.3	89.6	94.9	96.2	96.6	77.4	77.8	56
Shannon index based on metabarcoding	7.7	5.1	7.0	4.6	7.6	6.0	3.3	6.1
Simpson index based on metabarcoding	1.0	0.9	1.0	0.8	1.0	0.9	0.7	1.0
Chao1 index based on metabarcoding	2063.2	1,494.1	1666.4	1077.2	1813.8	1288.3	810.0	684.9

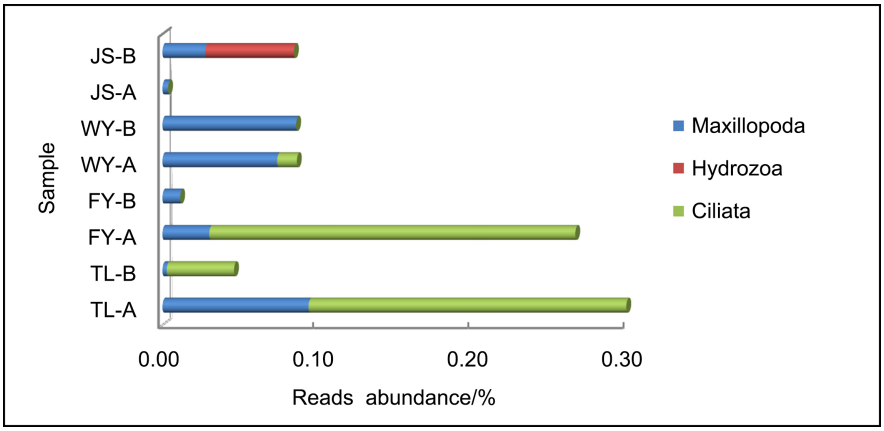
Sep.: September; Nov.: November.

in which Chlorophyceae, Trebouxiophyceae and Cryptophyceae had more reads abundance (Figure 2(a)). Genera *Chlamydomonas*, *Micractinium*, *Chlorella*, *Crucigenia*, *Cryptomonas*, *Actinastrum*, *Gonium*, *Dictyosphaerium* and *Compactochlorella* were the common phytoplankton, most of which belong to phylum Chlorophyta, except *Cryptomonas* which belongs to phylum Cryptophyta. Meanwhile, three classes of zooplankton, including Ciliatea (Protozoa), Maxillopoda (Arthropoda) and Hydrozoa (Cnidaria), were annotated on, and the first two classes were the common zooplankton assemblages (Figure 2(b)). Further-

more, twenty classes of fungi were annotated on, Dothideomycetes, Sordariomycetes, Eurotiomycetes and Tremellomycetes were the common classes (Figure 2(c)). However, the common classes of fungi in JS-A sample were others, such as Malasseziomycetes, Agaricostilbomycetes, Ustilaginomycetes and Wallemiomycetes.



(a)



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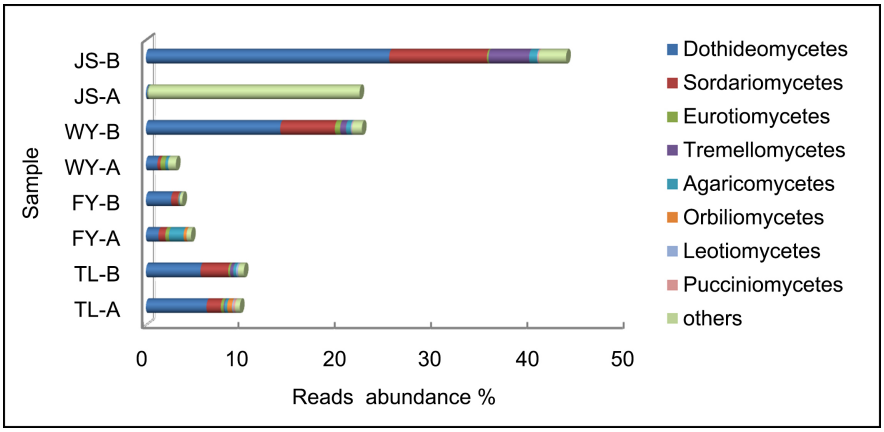

(c)

Figure 2. Reads abundance of eukaryotic microplankton (phytoplankton (a), zooplankton (b) and fungi (c)) based on ITS gene metabarcoding in eight samples at class level.

3.4. Seasonal Dynamics of Communities

Overall, the total reads of ITS annotated on eukaryotic microplankton in September was higher than that in November. The dominant assemblage in TL, FY and WY sites in September was phytoplankton, contributing 21.8%, 40.9% and 45.7% of the total reads respectively, followed by fungi (9.7%, 4.70% and 3.1%, respectively) and zooplankton (0.3%, 0.3% and 0.1%, respectively), however, the result in JS site was different, in which fungi was dominant (Table 2; Figure 3). Nevertheless, fungi changed to be the dominant in November in all four sampling sits with 10.2% of total reads in TL, 3.8% in FY, 22.4% in WY and 43.7% in JS site, and phytoplankton became to be the secondary assemblages simultaneously (Table 2; Figure 3). Combined with water temperature and salinity condition, the reads proportions of phytoplankton and zooplankton in the three freshwater sampling sites (TL, FY and WY) decreased as temperature drop, while fungi increased in majority of sites except FY. The reads abundance of phytoplankton and zooplankton at the estuarine site, JS, were fewer than the contemporaneous reads of three freshwater sites, and the reads of fungi were also opposite.

3.5. Diversity Analysis of Eukaryotic Microplankton

Significant spatial differences in values of Chao1 index were deduced ($p < 0.05$). However, Shannon and Simpson indexes showed no significant spatio-temporal differences. Generally, the three indexes in September at the freshwater sampling sites were all higher than that in November, which was a little different from that in JS sites (Table 2).

Eight samples were divided into two clusters at the 20% level, cluster for JS-A and cluster for the other seven samples, indicating that the microplankton class composition of JS-A had the least similar with that of the other samples (Figure 4(a)), which was also verified by using NMDS ordination method (Figure 4(b)).

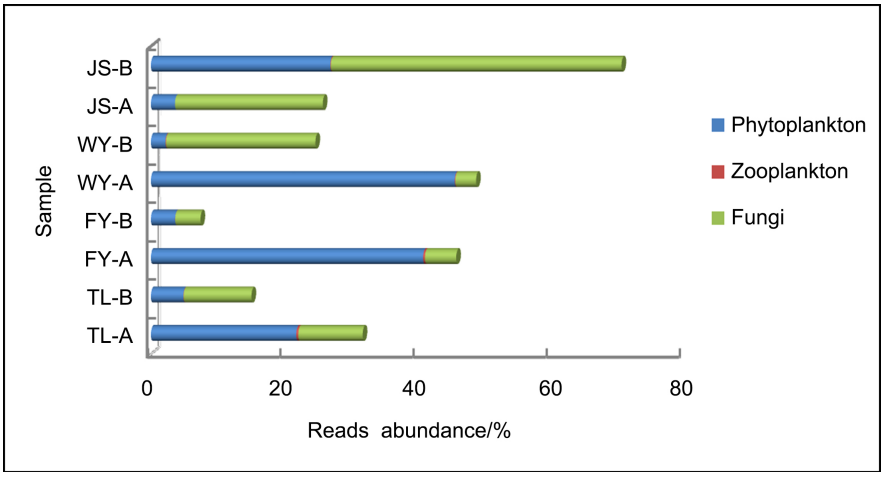

Figure 3. Reads abundance vibration of eukaryotic microplankton based on ITS gene metabarcoding in eight samples.

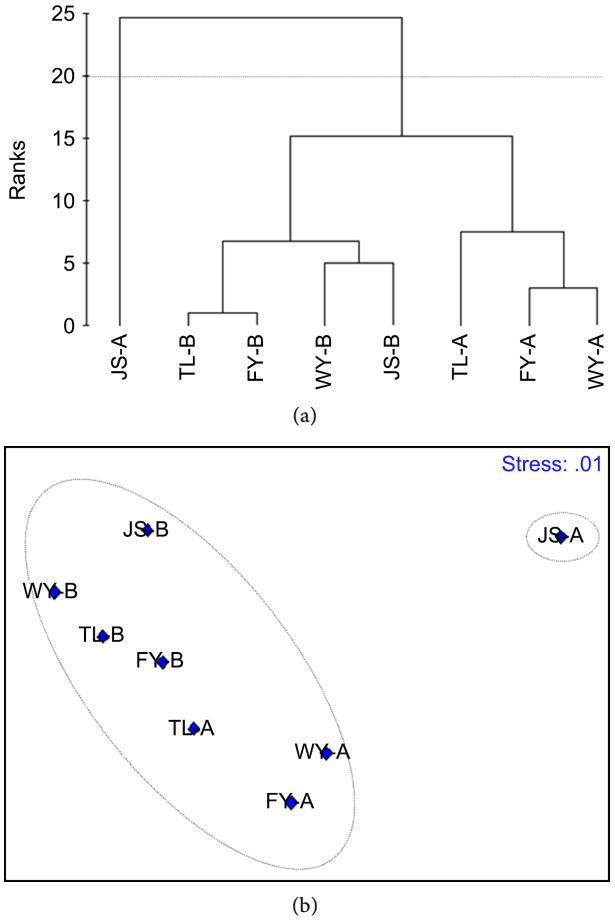

Figure 4. Cluster analysis of microplankton community on eight sampling sites based on metabarcoding monitoring. (a) Cluster analysis taken by group average method; (b) Non-metric multidimensional scaling ordination based on Bray-Curtis similarity of microplankton class composition.

3.6. Data of High-Throughput Sequencing (HTS) and Morphology Comparison

We compared the communities of phytoplankton and zooplankton taxa with results obtained from morphological analysis in order to determine potential biases of the primer set used in our study. Species of three phylum and five classes of eukaryotic phytoplankton were annotated by metabarcoding method, which was different from that identified by microscopic examination. Also, more than 104 phytoplankton species were identified by metabarcoding (193 species vs. 89 species) (**Table 3**). *Chlorophyceae* was the most common assemblage, which was consistent with the finding via morphology, accounting for 12.93% - 79.45% of the total eukaryotic phytoplankton reads, among which *Chlamydomonas reinhardtii* was dominant, contributing 1.22% of the total reads. Meanwhile, genera with higher reads proportion, such as *Chlamydomonas*, *Chlorella*, *Crucigenia*, *Cryptomonas*, *Actinastrum*, *Gonium* and *Dictyosphaerium*, were widespread in freshwater of Zhejiang province [27] [28] and were also dominant genera that identified via morphology (**Table S1**).

Table 3. Comparison analysis of eukaryotic phytoplankton data based on metabarcoding technology and morphological examination.

Description	Metabarcoding monitoring	Morphological monitoring
Number of phylum	3	5
Phylum list	Chlorophyta, Bacillariophyta and Cryptophyta	Chlorophyta, Bacillariophyta, Cryptophyta, Euglenophyta and Dinoflagellate
Number of classes	5	6
Classes list	Pedinophyceae, Chlorophyceae, Trebouxiophyceae, Centricae and Cryptophyceae	Chlorophyceae, Centricae, Pennatae, Cryptophyceae, uglenophyceae and Dinophyceae
Number of genus	99	50
Number of species	193	89

For the zooplankton, the comparisons were made with data reported by [29] and [30]. As a widely distributed taxa in Qiantang River, Cyclopoida was identified in this study, and the dominant species of Cyclopoida annotated on were *Mesocyclops leuckarti* and *Acanthocyclops bicuspidatus*, which was consistent with the discovery of [29]. However, as another dominant assemblage [30], rotifers were not annotated here.

4. Disscussion

4.1. The Feasibility of Microplankton Community Analysis Based on ITS Gene Metabarcoding

In this study, we selected ITS with fast evolution and high specificity as the amplicon to describe the community structure and its dynamics of eukaryotic microplankton in Qiantang River lower reaches. Here, the community characteristics of fungi in the Qiantang River were first researched, it's found that Dothideomycetes, Sordariomycetes, Eurotiomycetes and Tremellomycetes were the most common groups, which were different from that in the Yellow Sea concluded by using the same amplicon [22].

After compared the communities of phytoplankton and zooplankton taxa with results obtained from metabarcoding and morphological analysis, we revealed the same most common assemblage, and discovered 104 more species via metabarcoding. Compared with previous studies [28], it's speculated that the phytoplankton community had changed somewhat since then, but some dominant specieses maintained unchanged, genera *Chlamydomonas* and *Cryptophyllum* were still common dominant groups. Meanwhlie, Anthoathecata, a zooplankton taxa that hasn't been identified by microscopic examination, was annotated on JS samples. Anthoathecata is an order of class Hydrozoa and distributes on the east and south coast of China. Here, JS sites are located in the estuary area of Qiantang River, indicating a possibility that species of Anthoa-

thecata distribution. However, rotifers were not detected by metabarcoding. The predatory relationship between species may be a considerable reason. Generally, the DNA of the prey does not get separated out during the blast, especially if the prey items belong to the same genus as the predator. In addition, the preference of primer and differences in rRNA gene copy numbers may also explain some of these differences.

Overall, these findings demonstrated that metabarcoding could yield comparable results to conventional methods for several abundant eukaryotic taxa, but that each method has different limitations as far as accurately describing the eukaryotic composition in this river.

4.2. Community Diversity Characteristic of Eukaryotic Microplankton

Here, geographically, the sampling sites from top to bottom are, in order, Tonglu, Fuyang, Wenyan and Jiashao. There is no hydraulic dam between the four sampling sits, and all of them are in the tidal reach of the river, resulting in a relatively frequent water exchange caused by the flow of tide. Compared with other sites, JS is near estuarine region and more affected by seawater. Correlation analysis showed that there were significant spatial differences in values of Chao1 index ($p < 0.05$), indicating the species richness had obvious dissimilarity between sampling sites. Cluster analysis and NMDS ordination further confirmed the microplankton class composition of JS-A had the most dissimilarity with that of the other samples, the content of salinity might be one of the reasons [31]. Studies have shown that some spatial differences in eukaryotic plankton α-diversity is more the result of selection by local environmental conditions than dispersal [32], the feasibility of α-diversity based on ITS rRNA gene metabarcoding might be a useful indicator for discriminating ecological condition.

5. Conclusion

Our data were generated using a primer set that targets the ITS region of ribosomal RNA gene, a region that has been widely used in biodiversity assessments in phytoplankton, fungi, zooplankton, etc. As a result, various species of phytoplankton, fungi and zooplankton were annotated. We identified several groups of eukaryotic phytoplankton and zooplankton that were not described by morphological analysis, and increased research on fungi in Qiantang River that never had been studied before. *Chlorophyceae* was the most common assemblage both identified by using ITS gene metabarcoding and morphological examination methods, also *Mesocyclops leuckarti* and *Acanthocyclops bicuspidatus* were both found to be the dominant species of Cyclopoida in the river. The reads proportions of phytoplankton and zooplankton at the three freshwater sampling sites (Tonglu, Fuyang and Wenyan) decreased as temperature drop. In addition, there were significant spatial differences in values of Chao1 index for eukaryotic microplankton. Finally, it's confirmed that the Metabarcoding-based approach

herein described can be used in analyzing community characteristics of eukaryotic microplankton to some extent and will significantly be complete as technology improved.

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Conflict of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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Supplementary Information

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Additional file 1: Figure S1. Results of eight water DNA samples amplified by the ITS primer. Table S1. Species of eukaryotic phytoplankton indentified by using ITS gene metabarcoding technology and morphological examination.

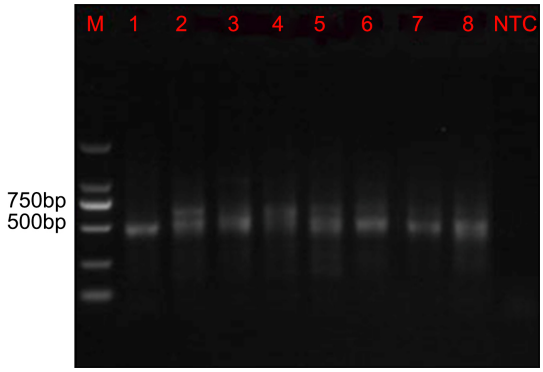

Figure S1. Results of eight water DNA samples amplified by the ITS primer. Lane M: Takara DL2000 DNA Maker; Lane 1 to 8: PCR products of JS-A, WY-A, FY-A, TL-A, JS-B, WY-B, FY-B and TL-B; Lane NTC: Negative control.

Table S1. Species of eukaryotic phytoplankton indentified by using ITS gene metabarcoding technology and morphological method.

Number	Metabarcoding monitoring		Morphological monitoring	
	Classes	Species	Classes	Species
1	**Chlorophyta**	*Pedinomonas* sp.	**Chlorophyta**	*Cosmarium* sp.
2		*Acutodesmus bajacalifornicus*		*Cosmarium abbreviatum*
3		*Acutodesmus deserticola*		*Cosmarium circulare*
4		*Acutodesmus* sp. KNUA038		*Cosmarium leave*
5		*Coelastrella rubescens*		*Cosmarium absoletum*
6		*Coelastrella vacuolata*		*Euastrum dubium*
7		*Coelastrella* sp.		*Penium Cruciferum*
8		*Coronastrum ellipsoideum*		*Closterium* sp.
9		*Nephrochlamys subsolitaria*		*Pleodorina* sp.
10		*Planktosphaeria gelatinosa*		*Tetraedron minimum*
11		*Desmodesmus armatus*		*Tetraspora* sp.
12		*Desmodesmus asymmetricus*		*Raphidonema* sp.
13		*Desmodesmus bicellularis*		*Chlamydomonas* sp.
14		*Desmodesmus brasiliensis*		*Platymonas elliptica*
15		*Desmodesmus communis*		*Coelastrum microporum*
16		*Desmodesmus costato-granulatus*		*Eudorina* sp.

Continued

17	*Desmodesmus denticulatus*		*Pandorina* sp.
18	*Desmodesmus insignis*		*Schroederia spiralis*
19	*Desmodesmus intermedius*		*Chlorella* sp.
20	*Desmodesmus maximus*		*Actinastrum* sp.
21	*Desmodesmus opoliensis*		*Ankistrodesmus angustus*
22	*Desmodesmus pannonicus*		*Ankistrodesmus convolutus*
23	*Desmodesmus perdix*		*Ankistrodesmus falcatus*
24	*Desmodesmus pirkollei*		*Ankistrodesmus falcatus var.mirabilis*
25	*Desmodesmus santosii*		*Oocystis lacustris*
26	*Desmodesmus* sp.		*Pediastrum simplex*
27	*Hariotina reticulata*		*Pediastrum tetras*
28	*Pectinodesmus pectinatus*		*Crucigenia quadrata*
29	*Pectinodesmus regularis*		*Crucigenia apiculata*
30	*Sphaeroplea robusta*		*Scenedesmus* sp.
31	*Rotundella rotunda*		*Scenedesmus bijuga*
32	*Monoraphidium griffithii*		*Scenedesmus quadricauda*
33	*Tetradesmus acuminatus*	**Bacillariophyta**	*Cyclotella* sp.
34	*Tetradesmus bernardii*		*Cyclotella meneghiniana*
35	*Tetradesmus distendus*		*Cyclotella aslerocastata*
36	*Tetradesmus incrassatulus*		*Melosira granulata*
37	*Tetradesmus nygaardii*		*Melosira granulate*
38	*Tetradesmus obliquus*		*Melosira granulata var. angustissima*
39	*Tetradesmus reginae*		*Melosira varians*
40	*Verrucodesmus parvus*		*Coscinodiscus* sp.
41	*Neochloris conjuncta*		*Coscinodiscus subtilis*
42	*Neochloris vigensis*		*Coscinodiscus oculusiridis*
43	*Neochloris* sp. AY2		*Skeletonema costatum*
44	*Stauridium privum*		*Synedra acus*
45	*Stauridium tetras*		*Synedra ulna*
46	*Lacunastrum gracillimum*		*Synedra ulna var. impressa*
47	*Monactinus sturmii*		*Fragilaria* sp.
48	*Hydrodictyon reticulatum*		*Fragilaria capucina*
49	*Pseudopediastrum* sp. KNUA039		*Fragilaria biceps*
50	*Chlamydomonas applanata*		*Gomphonema* sp.
51	*Chlamydomonas asymmetrica*		*Gomphonema subclavatum*

Continued

52	*Chlamydomonas bacca*		*Gomphonema simus*
53	*Chlamydomonas debaryana*		*Gomphonema parvulum*
54	*Chlamydomonas dorsoventralis*		*Cocconeis placentula*
55	*Chlamydomonas globosa*		*Nitzschia* sp.
56	*Chlamydomonas hedleyi*		*Nitzschia acula*
57	*Chlamydomonas inflexa*		*Nitzschia palea*
58	*Chlamydomonas leiostraca*		*Nitzschia acicularis*
59	*Chlamydomonas mexicana*		*Surirella ovata*
60	*Chlamydomonas proboscigera*		*Surirella robusta*
61	*Chlamydomonas rapa*		*Gyrosigma acuminatum*
62	*Chlamydomonas reinhardtii*		*Didymosphenia geminata*
63	*Chlamydomonas sphagnophila*		*Amphoraovalis*
64	*Chlamydomonas splendida*		*Frustulia vulgaris*
65	*Chlamydomonas sp.*		*Navicula* sp.
66	*Gloeomonas anomalipyrenoides*		*Navicula rhynchocephala*
67	*Lobochlamys segnis*		*Navicula cuspidata*
68	*Chloromonas perforata*		*Cymbella* sp.
69	*Carteria eugametos*		*Cymbellalanceolata*
70	*Carteria incisa*		*Cymbellaturgidula*
71	*Tetraselmis suecica*		*Diatoma vulgare*
72	*Vitreochlamys nekrassovii*		*Eunotia* sp.
73	*Colemanosphaera charkowiensis*		*Pinnularia* sp.
74	*Volvulina compacta*		*Cymatopleura* sp.
75	*Gonium pectorale*		*Cymatopleura elliptica*
76	*Coelastrum astroideum*		*Leptocylindrus danicus*
77	*Coelastrum microporum*		*Trioeratium favm*
78	*Coelastrum pseudomicroporum*	**Cryptophyta**	*Cryptomonas ovata*
79	*Eudorina cylindrica*		*Cryptomonas erosa*
80	*Eudorina elegans*		*Chroomonas caudata*
81	*Eudorina* sp. KMMCC 1278	**Euglenophyta**	*Euglena* sp.
82	*Eudorina unicocca*		*Euglena geniculata*
83	*Pandorina colemaniae*		*Trachelomonas curta*
84	*Pandorina morum*		*Strombomonas fluviatilis*
85	*Volvox carteri*	**Pyrrophyta**	*Peridinium* sp.
86	*Dunaliella parva*		*Peridinium pusillum*

Continued

87	*Dunaliella pseudosalina*	*Peridinium umbonatum*
88	*Characium oviforme*	*Ceratium hirundinella*
89	*Schroederia setigera*	*Gymnodinium* sp.
90	*Closteriopsis acicularis*	
91	*Didymogenes anomala*	
92	*Didymogenes palatina*	
93	*Kalenjinia gelatinosa*	
94	*Marasphaerium gattermannii*	
95	*Marvania geminata*	
96	*Masaia oloidia*	
97	*Meyerella planktonica*	
98	*Graesiella emersonii*	
99	*Pseudochlorella signiensis*	
100	*Nannochloris* sp. AICB 424	
101	*Compactochlorella dohrmannii*	
102	*Compactochlorella kochii*	
103	*Chlorella miniata*	
104	*Chlorella pulchelloides*	
105	*Chlorella sorokiniana*	
106	*Chlorella sp.*	
107	*Actinastrum hantzschii*	
108	*Selenastrum capricornutum*	
109	*Kirchneriella aperta*	
110	*Kirchneriella lunaris*	
111	*Ankistrodesmus falcatus*	
112	*Ankyra judayi*	
113	*Chlorococcum oleofaciens*	
114	*Chlorococcum* sp. CCAP 11/52	
115	*Micractinium belenophorum*	
116	*Micractinium inermum*	
117	*Micractinium reisseri*	
118	*Micractinium* sp.	
119	*Neospongiococcum* sp. SAG 2474	
120	*Coenochloris* sp. KR 2006/325	
121	*Franceia amphitricha*	

Continued

122	*Oocystella oogama*
123	*Oocystis* sp. KMMCC 251
124	*Pediastrum duplex*
125	*Pedinomonas minor*
126	*Crucigenia lauterbornii*
127	*Westella botryoides*
128	*Scenedesmus armatus*
129	*Scenedesmus bijugus*
130	*Scenedesmus quadricauda*
131	*Scenedesmus* sp.
132	*Dictyosphaerium ehrenbergianum*
133	*Dictyosphaerium lacustre*
134	*Dictyosphaerium libertatis*
135	*Dictyosphaerium* sp.
136	*Sorastrum pediastriforme*
137	*Oedogonium angustistomum*
138	*Oedogonium cardiacum*
139	*Oedogonium cylindrosporum*
140	*Oedogonium donnellii*
141	*Oedogonium subdissimile*
142	*Oedogonium tenerum*
143	*Oedogonium undulatum*
144	*Oedogonium* sp.
145	*Gloeotilopsis planctonica*
146	*Chamaetrichon basiliensis*
147	*Uronema* sp. AF-2012
148	*Uronema* sp. CCAP 334/1
149	*Uronema* sp. CCAP 335/1B
150	*Urospora neglecta*
151	*Chaetopeltis orbicularis*
152	*Chaetophora* sp. BEA 0173B
153	*Hormotilopsis gelatinosa*
154	*Hormotilopsis tetravacuolaris*
155	*Draparnaldia plumosa*
156	*Stigeoclonium helveticum*
157	*Schizomeris leibleinii*

Continued

158		*Aphanochaete magnum*
159		*Cladophora* sp. ZZ-2016
160		*Rhizoclonium pachydermum*
161		*Tetracystis excentrica*
162		*Tetracystis intermedia*
163		*Tetracystis pulchra*
164		*Tetracystis sarcinalis*
165		*Tetracystis* sp. 14601-7.1
166		*Tetracystis tetraspora*
167		*Tetracystis texensis*
168		*uncultured Chlorophyta*
169		*Pleurastrum* sp. CCCryo 194-04
170		*Deasonia* sp. 14615-7
171		*uncultured Desmodesmus*
172		*Spermatozopsis exsultans*
173		*Spermatozopsis similis*
174		*Chlamydocapsa ampla*
175		*Chlamydopodium starrii*
176		*Scherffelia dubia*
177		*Chlorosarcinopsis* sp. WJT16-VFNP5
178		*Desmochloris halophila*
179		*Dicloster acuatus*
180		*Hindakia fallax*
181		*Elliptochloris marina*
182		*uncultured Trebouxiophyceae*
183		*Heterochlorella luteoviridis*
184		*Chloroidium saccharophilum*
185		*Choricystis parasitica*
186		*Chlorophyta* sp. MCWWS13
187		*Chlorophyta* sp. SP2-3
188	**Bacillariophyta**	uncultured Thalassiosirales
189		*Cyclotella cf. scaldensis* G18W42
190		*Cyclotella meneghiniana*
191	**Cryptophyta**	*uncultured Cryptophyta*
192		*Rhinomonas nottbecki*
193		*Rhodomonas* sp. CCMP740