Investigating the Fish Diversity in the Upper Reaches of Yangtze River Based by Environment DNA Metabarcoding

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Abstract: In recent years, environmental DNA metabarcoding (eDNA metabarcoding) has been widely utilized in fish diversity assessments due to its cost-effective and non-invasive strategies with increased sensitivity. Environmental DNA (eDNA) refers to DNA fragments that organisms leave behind in their surrounding environment (such as soil, sediment and water). eDNA technology sequences these DNA fragments and can provide information on aquatic species. As a novel method for surveying aquatic organisms, eDNA techniques have been widely used in biodiversity assessments of aquatic organisms, including investigating the fish diversity and species distribution patterns of species. To investigate species diversity and community patterns of freshwater fish and explore new methods for monitoring and protecting fish diversity in the upper reaches of Yangtze River. This study first used eDNA metabarcoding to detect fish diversity in the Upper Reaches of Yangtze River. This study used a standardized process of eDNA metabarcoding analysis, including water collection, water filtration, eDNA extraction, genetic marker amplification, sequencing and bioinformatic analyses. A total of 65 fish species were detected from 46 sampling sites. Although eDNA metabarcoding cannot completely replace traditional methods, it can be used as a supplementary tool to efficiently assess and monitor fish diversity and species distribution patterns in Yangtze River. The various indexes of Alpha diversity of fish at various points are relatively uniform, indicating that the ecological structure of fish in the reserve is relatively balanced and stable. In summary, this study showed that although environmental DNA metabarcoding cannot completely replace traditional fish resource monitoring methods, it is a good strategy to combine them to quickly investigate the diversity composition and distribution of fish species in the Upper Reaches of Yangtze River.

Keywords: the Upper Reaches of Yangtze River; eDNA metabarcoding; Non-invasive sampling; Fish diversity

1 INTRODUCTION

The decline of global biodiversity is one of the most serious challenges of the 21st century[1,2]. In this context, comprehensive and accurate biodiversity monitoring is of great significance for biodiversity conservation. Traditional biodiversity monitoring is mainly based on methods such as field visits, which require a lot of manpower, material and financial resources[3,4], and also cause certain damage to the research objects and sampling habitats, and rely on researchers with taxonomic expertise.

In recent years, environmental DNA (eDNA) technology has received widespread attention as a new, non-invasive and cost-effective monitoring method[5], which can replace or assist traditional monitoring methods and greatly improve the efficiency of biodiversity surveys and research [6, 7]. eDNA refers to the sum of DNA fragments extracted from environmental samples (e.g., water, soil, sediment, etc.), including intracellular DNA released by organisms into epidermal cells in the environment through skin, urine, feces, and mucus, as well as extracellular DNA released into the environment after cell death [8, 9]. Monitoring of all biomes can be achieved by PCR amplification and high-throughput sequencing of these eDNA fragments or genomes, and by comparing and annotating them against existing DNA databases.

At present, the technology has been widely used in the investigation of water biodiversity, such as: (1) detection of the existence of the target species. It has wide applications in the detection of invasive, endangered and other rare species, such as monitoring the invasion of Asian carps[10], and migratory routes of Acipenser sinensis[11]; (2) Conduct biomass estimation. Studies have shown that the amount of eDNA in water is positively correlated with biomass, and the population density of organisms in water can be inferred[12, 13]. (3) Biodiversity assessment [8, 14, 15] et al. For example, estuarine fish diversity was assessed using eDNA macro barcodes [16].

The eDNA experimental process generally includes several major steps including water sample collection and preservation, eDNA capture and extraction, PCR amplification and sequencing, and bioinformatics analysis [17] . Choosing different eDNA capture and extraction methods significantly affects the DNA concentration or yield obtained in the sample [18,19] and further affects the results of biomass estimation and biodiversity analysis. In aqueous ecosystem studies, the capture of eDNA is usually carried out using a filtration method. For example, Eichmiller et al. [20] studied the precipitation, centrifugation and filtration methods in the eDNA capture method, and the results showed that the filtration method obtained the largest eDNA yield. However, for different sample sources and different research groups, eDNA capture and experimental methods have their own characteristics, and there is a lack of unified standards, which reduces the comparability between different studies [21].

2 MATERIALS AND METHODS

2.1 The setting of the sample point and the sample time

The sampling began on August 2021, and a total of 24 sampling points were set up in the Upper Reaches of the Yangtze River from Yibin to Lijiatuo, in order: Yibin (NS-1),Guagongshan (NS-2), Lizhuang (NS-3), Huangjiaba (NS-4), Heishibao (NS-5), Jinyaer (NS-6), Diaoyuzui(NS-7), Jinjiwei(NS-8), Jinyuhao (NS-9), Hongdengqi (NS-10), Yeluxi (NS-11), Huoyanqi (NS-12), Shawan (NS-13), Maliu wan (NS-14),Lujiaotuo (NS-15), Zaijiao (NS-16), Jipoqi (NS-17), Jiuyanqi (NS-18), Jiziguai (NS-19), Wenzhongba (NS-20), Shatuo (NS-21), Baisha (NS-22), Dingjiatuo (NS-23), Baiyangtan (NS-24), the specific distribution of each sampling point is shown in Figure 1.

2.2 Water sample collection and preservation

Under the background of the current "ten-year fishing ban" in the Yangtze River, this sampling was conducted in strict accordance with the requirements of the relevant competent authorities for the fishing license and the approval of the relevant authorities before the water sample collection work. After arriving at each sampling point, record the water temperature, flow rate and geographic coordinate position at each water sample collection location, and after the engine of the survey ship is turned off for a few minutes, collect water samples from the surface water body (0.3-0.6 m below the water surface), and place them in a new 1 L sterile water sample collection bag. The water sampling device needs to be disinfected with sodium hypochlorite solution before use, rinsed with purified water and dried; the sampling personnel wear disposable gloves and replace it after sampling. [22]. Water samples are pumped onto a mixed cellulose membrane with a pore size of 0.45 μm within 24 h using a vacuum pump. Before and after each sample extraction, the extraction equipment is sterilized to remove DNA from the last time the device remains to avoid cross-contamination between samples [23-24]. To assess for exogenous DNA contamination, set up a negative control for each filter. Finally, place the membrane in a sterile 2 mL plastic centrifuge tube for immediate cryopreservation until DNA extraction.

2.3 Total DNA capture and extraction in water samples

Refer to Zhang et al. (2020b) method, using the DNA extraction kit DNeasy Blood & TissueKit (Qiagen, 69506) on the membrane for extraction, using NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA) to detect the concentration and mass of DNA, the ambient DNA solution obtained from each sample was rapidly placed at -20 °C for cryopreservation, Until the next PCR amplification [25].

2.4 PCR amplification and high-throughput sequencing

In this study, the mitochondrial gene 12S ribosomal RNA (rRNA), the most widely used molecular marker for fish diversity monitoring, was used as the target, and the fish-specific primer "Teleo" (F:GTYGGTAAAWCTCGTGCCAGC R:CATAGTGGGTATCTAATCCYAGTTTG) developed by Valentini was selected for amplification, and samples were added. Specific Barcode sequences. TransStart Fastpfu DNAPolymerase was used for PCR, and the amplification was made into a 20uL reaction system: 4uL 5xFastPfu Buffer, 2uL dNTPs, 0.4μL Fast Pfu Polymerase, 2-5uL template DNA (10ng/μL) and 0.8uL (10μmol/L) of upstream and downstream primers, and finally ddH2O Make up the system to 20uL. PCR reaction program: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30s, annealing at 55°C for 30s, extension at 72°C for 45s, final extension at 72°C for 10 min, and storage at 10°C (30 cycles of denaturation-extension-annealing). To assess contamination in PCR amplification, a PCR-negative control using a ddH2O template is required. When performing PCR amplification, perform

3 replicates per sample and mix the PCR products. PCR products were detected by 2% agarose gel electrophoresis. In this study, 72 samples were obtained with detectable PCR products. The PCR product glue is high-throughput sequencing by Shanghai Personalbio Technology Co.,Ltd.

2.5 Bioinformatics and statistical analyses

In this study, the Illumina platform was used to perform paired-end sequencing of the community DNA fragments. Sequencing raw data was saved in FASTQ format. Preliminary screening is carried out according to the sequence quality; retesting and supplementary testing are carried out on the problem samples. The main methods of sequence processing are Vsearch (v2.13.4_linux_x86_64) and cutadapt (v2.3) software processing. The Vsearch method mainly includes steps such as de-priming, splicing, quality filtering, de-duplication, de-chimera, and clustering. High-quality sequences are clustered at the 97% similarity level, and the representative sequences and OUT (Operational Taxonomic Unit) tables are output respectively. Finally, singletons OTUs (ie, OTUs with abundance of 1 in all samples, default operation) and their representative sequences were removed from the OTU table. The representative OTU sequences were aligned with the reference sequence database NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and crop taxonomic annotation was performed. The number of taxa contained in each sample at different taxonomic levels was counted, and the dominant species were further analyzed at the species level. The existence of various species in the environment can be obtained through the analysis of multiple barcodes; after obtaining the OUT abundance table, follow-up analysis can be carried out for the specific content of the study, such as α-diversity analysis, species classification and species abundance analysis, etc [26] .

Statistical analysis In this study, based on the results of OTU cluster analysis, species composition analysis and Alpha diversity (including 3 genera that were not identified to the species level) were carried out. (1) Species composition analysis. After removing the data information of non-fish (such as bacteria, amphibians, birds and mammals, etc.), screen out the OTUs that are compared to fish and have an identity value ≥97% E-value ≤10, and then compare them. OTUs to the same species were pooled. If there are OTUs that cannot be compared to the species level, statistics will be carried out to the upper level such as genus, family, etc. The proportion of the number of valid sequences of each fish in each sample was counted in Excel, and the fish taxonomic information was improved with reference to the Fishbase database and "Sichuan Fish Records". Finally, the histogram of fish composition of each sampling point is drawn by R language. (2) Alpha diversity analysis, alpha diversity represents species within the habitat, and alpha diversity refers to the richness, diversity and evenness of species in a locally uniform habitat. , also known as within-habitat diversity. In order to comprehensively evaluate the alpha diversity of microbial communities, this paper uses Chao1[27] (Chao, 1984), Shannon[28] (Shannon, 1948a, b) and Simpson[29] (Simpson, 1949) indices to characterize diversity , and the coverage is represented by the Good's coverage[30] (Good, 1953) index.

3 RESULTS

3.1 Fish species composition

A total of 5784 OUT sequences were obtained from 24 sampling points, and the difference in the number of OTUs at different sampling points of OTU is shown in Figure 2. It can be seen from Figure 2 that 24 sampling points have 22 common OTUs. Among them, NS-4 has the largest number of OTUs, a total of 1265; NS-19 has the least number, only 292. After annotating the OTU, a total of fish species were detected in this survey (excluding 3 genera that have not been identified to the species level (Table 1), belonging to 6 orders, 17 families, 49 genera and 65 species, and 5 species of fish endemic to the upper reaches of the Yangtze River: national level 2 species of key protected fish. Cypriniformes had the most cyprinids, with 30 species in 23 genera, accounting for 46.15% of the total fish species; while Acipenseriformes and Cyprinodotiformes both contained only one species. According to the sequence alignment results, the genus Acrossocheilus in this investigation was not identified at the species level.

3.2 Alpha diversity analysis

In this study, the Alpha diversity analysis of fish communities in the Yibin section to the Baiyangtan section of the upper Reaches of the Yangtze River was carried out by calculating the Chao1(a)[27] index, Shannon(b)[28] index, Simpson[29](c) diversity index and coverage Coverage[30](d), and the alpha diversity index of each sample was shown in Table 2. Shannon and Simpson diversity indices are

relatively uniform; the Coverage values of each sample range from 0.9923-0.9979, indicating that the sequencing basically covers all the OUT data and can reflect the true situation of the samples.

(a.) Chao1: is an index used to estimate the number of OTU contained in a sample using the chao1 algorithm, chao1 is commonly used in ecology to estimate the total number of species, first proposed by Chao (1984).

$$S_{chao1} = S_{obs} + \frac{n_1(n_1-1)}{2(n_2+1)} \quad \text{①}$$

where Schao1 = estimated OUT;

Sobs = number of OUT observed;

n1 = the number of OUTs with only one sequence (e.g. singletons);

n2 = the number of OUTs for only two sequences (e.g. doubletons).

The chao index is used to assess the number of OTU in a sample, and the larger the chao index, the greater the number of OTU, indicating that the number of species in the sample is relatively large.

(b.) Shannon- the Shannon index

Shannon: One of the microbial diversity indices used to estimate the sample, the larger the Shannon value, the higher the community diversity.

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} \ln \frac{n_i}{N} \quad \text{②}$$

where Sobs = the number of OUT observed;

ni = number of outs containing i-bar sequences;

N = the number of all sequences

(c.) Simpson: One of the microbial diversity indices used to estimate samples, proposed by Edward Hugh Hugh Simpson (1949), is commonly used in ecology to quantitatively describe the biodiversity of an area.

$$D_{simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i(n_i-1)}{N(N-1)} \quad \text{③}$$

$$D_{simpson} = 1 - \frac{\sum_{i=1}^{S_{obs}} n_i(n_i-1)}{N(N-1)} \quad \text{④}$$

where, in equation (3), the larger the Simpson exponential value, the lower the community diversity;

In Formula (4), the smaller the Simpson index value, the lower the community diversity;

Sobs = number of OUT observed;

ni = number of outs containing i-bar sequences;

N = the number of all sequences.

(d.) Coverage: Refers to the coverage of each sample (clone) library.

$$C = 1 - \frac{n_1}{N} \quad \text{⑤}$$

where n_1 = the number of OUTs with only one sequence;

N = the total number of sequences that appear in the sample.

The higher the Coverage value, the higher the probability that the sequence in the sample will be measured and the lower the probability that it will not be measured. The index reflects whether the sequencing results represent the true picture of the microorganisms in the sample.

3.3 Tables

Tab.1 Alpha diversity index of each sample

Sample	Chao1	Coverage	Shannon	Simpson
NS_1	995.592	0.993994	5.98151	0.935546
NS_2	475.402	0.996535	2.7696	0.532512
NS_3	468.253	0.996526	3.97911	0.876934
NS_4	1140.85	0.992373	5.548	0.909015
NS_5	462.061	0.99648	3.03244	0.771909
NS_6	400.059	0.997001	4.10226	0.904475

NS_7	391.171	0.996958	2.91637	0.741461
NS_9	413.662	0.996964	3.99948	0.897433
NS_10	340.576	0.997603	2.99759	0.7668
NS_11	378.173	0.99741	3.72581	0.858734
NS_12	824.487	0.994749	4.78716	0.849844
NS_13	527.83	0.996961	4.754	0.883501
NS_14	299.801	0.997992	3.20698	0.737177
NS_15	436.742	0.997713	5.57606	0.957363
NS_16	441.302	0.997315	4.06207	0.852495
NS_17	341.803	0.997857	3.37063	0.816086
NS_18	433.316	0.997261	3.86685	0.835658
NS_19	295.541	0.997946	3.40857	0.836273
NS_20	413.169	0.997629	4.21898	0.883328
NS_21	411.168	0.997511	4.01436	0.858973
NS_22	427.106	0.997445	3.54141	0.817803
NS_23	408.842	0.997526	4.34624	0.906151
NS_24	553.868	0.996581	4.41483	0.847724

3.4 Figures or illustrations

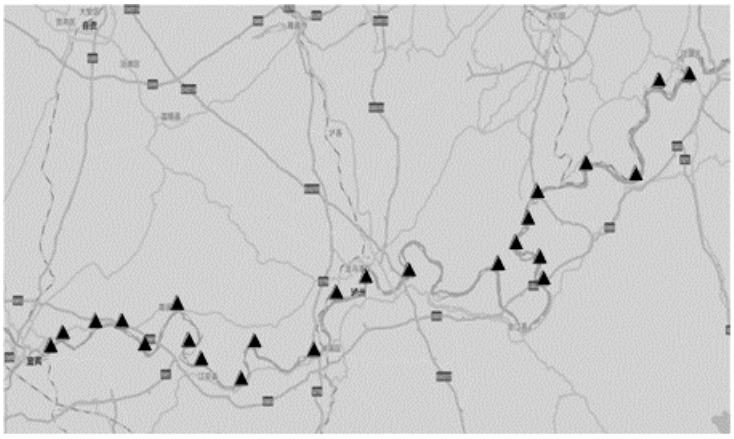

Fig.1 Information of sampling locations

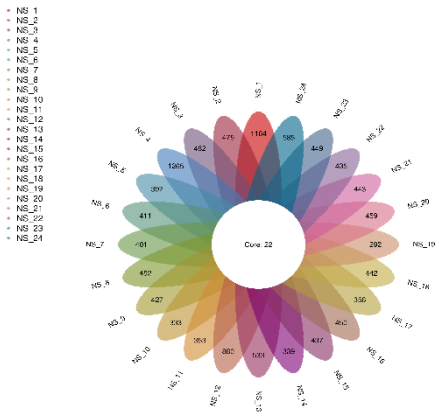

Fig.2 The number of OTU's contained in each sample alone Petal map of OUT shared by each sampling point

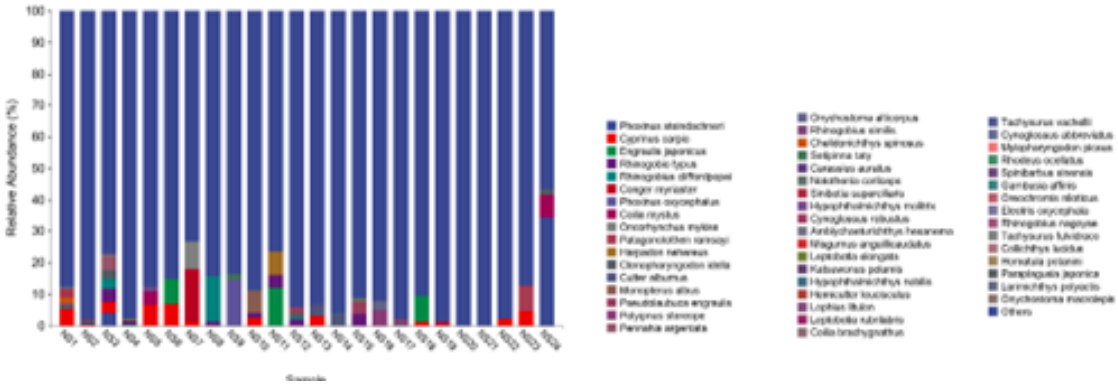

Fig.3 The composition of fish species at each sampling site

4 DISCUSSION

4.1 Analysis of fish composition in the upper reaches of the Yangtze River based on eDNA

In this study, the eDNA technique was used for the first time to analyze the fish diversity in the upper reaches of the Yangtze River to detect a total of 65 species of fish (excluding 2 genera that have not been identified at the species level), including Crucian carp (Carassius), tilapia (Oreochromi) and unidentified species levels. The genus Oreo-chromi was not identified because there was no corresponding reference sequence in the database; Only the genus Carassius and Acipenser can be identified because they are compared to different species but have the same mass. This may have something to do with the shorter amplification fragments and the incomplete species information contained in the database.

According to relevant records, in recent years, 103 species of catches have been detected through traditional methods such as gillnets and electric thorns (Table 3), of which Rhi nogobio, Coreius, Pelteoba-grus and Saurogobio are the main dominant populations. In this survey, the abundance and abundance of Cyprinus, Crucian carp (Carassius) and grass carp (Ctenopharyngodon) accounted for more than 50%, which may have a large relationship with the time and location of sampling. To improve the success rate of environmental DNA testing.

In addition, this study also detected three alien species, including Nile tilapia, Pike perch, etc., which may be that the fishery farms around the basin lack the necessary species isolation facilities and anti-escape means, resulting in the escape of alien species into the Yangtze River. Studies have shown that the introduction of alien species can bring great disasters to indigenous fish and ecosystems. Therefore,

species isolation and escape prevention facilities should be built for the farms around the section from Yibin to Baiyangtan to prevent the invasion of alien species from causing certain ecological stresses to the indigenous fish resources in the upper reaches of the Yangtze River.

4.2 Analysis of fish diversity in the upper reaches of the Yangtze River based on eDNA

Table 3 reflects the Alpha diversity index of fish community abundance, and the Chaol index in this study ranges from 295-1140, of which the NS-4 sample point value is the largest, indicating that the sample point has the highest community richness: the NS--19 sample point index is the smallest. Indicates the lowest community richness. This may be closely related to the geographical location and environment of each sample point. The complexity of NS-4's waters at the confluence of tributaries (Huangjiaba) and main streams (Yangtze River) may make the community richness at this location the highest, while the NS-19 sampling site is located near Jizijiao. The disturbance of the inflow and outlet of chickens and the whirlpool of rapids may lead to a relatively small distribution of fish to some extent, resulting in low community richness. The Chao1 index of other samples was more uniform, indicating that the ecological structure of fish in the reserve was relatively stable. Both the Shannon and Simpson indices reveal levels of diversity in fish. In this study, the Shannon and Simpson indices indicate higher diversity in NS-1 and NS-15 fish, which may be related to higher sequence abundances in species such as Rhinogobio typus and Pseudolaubuca. NS-2 fish have the lowest diversity. This may be related to the low concentration of DNA extraction at this sample point. The focus of different indexes on assessing fish diversity is somewhat different, and the community richness index does not fully measure the level of community diversity [38]. For example, the Simpson diversity index of the NS-2 site is low but the community richness is high, which may be related to the excessive abundance of a small number of fish species sequences in the sample.

4.3 Factors influencing the accuracy of eDNA monitoring

Due to the complexity of the environment in the Yangtze River Basin, the production and degradation rate of eDNA is susceptible to many environmental factors such as water temperature, pH, flow rate, ultraviolet rays, and water substrate [44,45]. A total of 24 sampling points and 72 samples were set up in this survey. Among them, the PCR amplification results of only THE DJT sampling points were not satisfactory, and in the 3 parallel samples, the concentration of pcR products in one sample was low, and the destination band of pcR products in the remaining 2 samples was too weak or undetected. The PCR amplification results of the remaining 5 sampling points reached A. That is, the band size of the product destination was correct, and the concentration was suitable to meet the sequencing requirements. This study suggests that this difference may be closely related to the environment at the sampling point. In addition to the amount of biological material contained in the water sample itself, the eDNA yield is also affected by a variety of factors such as the volume of single membrane extraction, the sediment content of the water sample, and the extraction operation. The NS-23 sampling site, located near Dingjiatuo in the Chongqing section of the reserve, is a site with turbid water samples and high sediment content, which leads to slow and time-consuming extraction during eDNA extraction. This may cause some DNA fragments to degrade during extraction, which affects the extraction result and concentration of DNA. In addition, sediment may adsorb some shedding free DNA, which may also have a certain impact on the efficiency of DNA extraction. Therefore, when sampling, you should try to select an area with less sediment, or use disposable medical gauze for coarse filtration before filtering the water sample. In addition, a membrane with a large pore size can be pre-filtered and then re-filtered onto a membrane with a smaller pore size.

5 CONCLUSION

Traditional fish resource surveys are time-consuming and laborious, but eDNA technology is superior to traditional methods in terms of sensitivity, standardization and species identification, and the operation is simple, which has great application prospects in the monitoring and protection of fish diversity. In this study, eDNA technology was used for the first time to investigate the diversity of fish in the upper reaches of the Yangtze River, and a total of 65 species of fish (excluding 1 genus that were not identified at the species level) were detected, which successfully verified the effectiveness and feasibility of eDNA technology in detecting the species composition of fish species in the Yangtze

River. The analysis results of the fish Alpha diversity at various points were small, indicating that the ecological structure of the fish in the reserve was relatively stable. In the current stage of Yangtze River fish resource monitoring, although eDNA technology cannot completely replace the traditional fish monitoring method, the advantages of this technology are also irreplaceable by traditional methods.

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