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Detection of endangered fish in Dianchi Lake, Kunming, China, using eDNA and traditional fishing

By

Kai Zhang

A Thesis
Submitted to the Faculty of Graduate Studies
through the Faculty of Science
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the Degree of Master of Science
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DECLARATION OF ORIGINALITY

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ABSTRACT

Dianchi Lake, Yunnan, China is a eutrophic plateau lake. Many native fish species in the lake are currently endangered or extinct due to a variety of stressors. Community surveys are important for ecological management and protection, though efforts are hampered by low abundance of some species. The goal of this thesis was to understand the community structure and distribution of fish in Dianchi Lake and to detect *Sinocyclocheilus grahami*, a critically endangered species endemic to the lake, using both traditional netting methods and environmental DNA. Using traditional netting and trapping, I found that the fish community of Dianchi Lake has been almost completely converted to nonnative species (~97% of abundance). Loss of native fishes was also very serious, with ~77% of species not detected. I designed specific primers for the COI region mitochondrial gene of *Sinocyclocheilus grahami*. While only two individuals of *Sinocyclocheilus grahami* were caught using traditional methods during the survey, results of eDNA analysis revealed that this species is likely present across the central and southern region of the lake. This study highlights the sensitivity of environmental DNA and its utility in non-destructively detecting presence of very rare species.

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CHAPTER 1. General Introduction

The management of rare species (e.g., endangered species) is an important aspect of biodiversity conservation (Jerde et al. 2011; Dejean et al. 2011; Lynch et al. 2016). Endangered fish protection is a problem and conservation goal worldwide, but a lack of effective and standardized detection methods may lead to mismanagement and a waste of resources (Thompson 2004). Traditional fish collection methods include fishing or electrofishing, though their capture probabilities can be very low (Dejean et al. 2011), thus these methods are more suitable when species abundance is moderate-to-high (i.e., species is not endangered) (McDonald 2004; Piggott et al. 2020). Consequently, these methods are not conducive to the detection of rare fishes (Harvey et al. 2009), as they produce type II errors otherwise known as false negatives (i.e., a false absence; Gu et al. 2004; Zhan and MacIsaac 2015).

In addition to the difference in detection sensitivity, traditional fish survey methods also face problems. Methods such as fishing nets and traps with bait are biased towards a specific target to be caught (size, hunger, mobility, etc.). Others like electric shocking and gillnets can harm or even kill targets and non-target fish (Lintermans, 2015), and may also result in habitat damage (Auster et al. 1998). Therefore, these methods have certain and well-defined limitations and disadvantages when used to detect endangered fish (Costello et al. 2016).

Underwater visual detection (camera and snorkel) is a fish survey method that can be superior to traditional methods (King et al. 2018). It is a widely used non-harmful detection method, which is conducive to the protection of endangered species (Struthers et al. 2015). Studies have shown that its ability to detect rare fish in rivers is great (Castañeda et al. 2020). However, it is less effective in large or turbid systems, because it is restricted by the visible distance and clarity (Boussarie et al. 2018).

Environmental DNA detection

Over the past 20 years, technology development has allowed analysis of DNA in the environment; this technique has been named *environmental DNA* or *eDNA* (Taberlet et al. 2012; Wang et al. 2019). eDNA is shed by organisms as they carry out everyday activities, allowing researchers to collect and analyze its signal and composition. The application of eDNA technology has greatly improved sensitivity (the detection limitation) of rare species detection. For example, eDNA is 44% more effective than other methods in marine species detection (Boussarie et al. 2018). eDNA-based detection can prevent damage to target species since the organism itself need not be collected (Beja-Pereira et al. 2009; Piggott et al. 2020). eDNA has progressed as a high-speed, high-sensitivity and high-accuracy technology (Harper et al. 2019) and is widely used for species detection and community structure estimation in freshwater ecosystems (Bohmann et al. 2014; Sepulveda et al. 2020). However, eDNA detection is affected by DNA sources, mismatches, etc., and false positives cannot be completely ruled out (Evans et al. 2017).

Two main amplification methods exist for eDNA analysis: conventional PCR (cPCR) and real-time quantitative PCR (qPCR hereafter). cPCR technique is a mature technology with a long history of application which has been widely used to detect the presence and distribution of single species in the environment (e.g. Taberlet et al. 2012; Piggott et al. 2020). A key problem with cPCR is that sensitivity may vary widely depending on gene and primer pair selected (Xiong et al. 2016; Xia et al. 2018) and may have a relatively high Type II error rate. On the other hand, qPCR has higher sensitivity when compared with cPCR (Biggs et al. 2015; Xia et al. 2018), is more cost-effective (Jerde et al. 2011), and thus has virtually displaced cPCR

(Ricciardi et al. 2017; Matter et al. 2018; Xia et al. 2018). A third method, droplet digital PCR (ddPCR), is the newest approach, with a stronger ability to overcome PCR inhibitors and higher sensitivity (Harper et al. 2019), though it is not commonly used yet (Xia et al. 2021). SYBR Green and TaqMan are the two main qPCR methods, SYBR Green is a fluorescent dye, while TaqMan qPCR has a specific probe, making it more specific (reduces false positives) but more expensive and difficult primer design (Tajadini et al. 2014).

Metabarcoding can detect multiple species in combination with the next-generation sequencing (NGS) technology (Bohmann et al. 2014). Metabarcoding is invaluable in profiling community complements as it allows both qualitative and quantitative detection for multiple species simultaneously from a single sample using robust universal primers (Pinol et al. 2019). It has been used in species detection and biodiversity studies (Seymour, 2019; Pukk et al. 2021). The method is highly efficient and has a comparatively low cost per sample (Sales et al. 2020). However, environmental DNA metabarcoding faces many challenges (Deiner et al. 2017), such as incomplete reference database (lack of gene sequence species), primer design, primer sensitivity, and interference (amplification inhibition and bias)(Bailey et al. 2020; Sakata et al. 2020; Wang et al. 2021). The large data load generated from metabarcoding greatly increases the difficulty of data analysis (Scott et al. 2018) and the sequencing requires complicated personnel training (Shendure et al. 2008). As well, different data handling and filtering approaches are needed depending on whether the researcher is focused on quantifying the complement of species in a community versus attempting to identify rare - often introduced - species (Scott et al. 2018).

Researchers also use nested primer technology to for species detection, which amplifies the sample several times and employs different primers after the previous PCR (Taberlet et al. 1996). Each new primer is used to amplify the products from the previous PCR reaction until it achieves its detection purpose (Keskin et al. 2014; Dejean et al. 2011). This approach is more sensitive and efficient than using single PCR primers, but also a higher potential to detect contaminations and generate false positives (Clusa et al. 2017).

Gene chip (or DNA microarrays) is another method for multiple species detection, which encompasses a solid surface attached with a collection of microscopic DNA spots (Taub et al. 1983), allowing for detection of multiple species in a single run. It has been used in aquatic and soil eDNA research (Wakelin et al. 2016; Holman et al. 2019; LeKang et al. 2020), though it has a high and hard-to-quantify false negative rate (Kochzius et al. 2010), which needs further optimization (Bohmann et al. 2012).

The main difference between qPCR and metabarcoding lies in the difference of detection objects. Metabarcoding is mainly used to detect a certain type of target in the environment, which can understand the structure of the community and even discover unknown species (passive detection). Through the application of multi-species-specific primers (active detection), qPCR can detect a single target species with higher sensitivity and accuracy (Simmons et al. 20015).

With the high sensitivity of qPCR and species-specific primer amplification to avoid possible interference between primers (Rees et al. 2009), many studies have used optimized qPCR technology to detect rare fishes (Weltz et al. 2017; Tang et al. 2019; Riaz et al. 2020), thus this technology is a powerful tool for endangered species detection.

Study area and target species

Sinocyclocheilus grahami (golden-line barbel) is an endangered species endemic to Dianchi Lake, southwest China (Chen et al. 2008). The Dianchi Lake watershed is the only known distribution area for this species. *Sinocyclocheilus grahami* grows slowly (Pan et al. 2009) and has high nutritional value and thus was once a common local commercially fished species (Zhang et al. 2021). It usually lives in slow-moving areas, preferring pebblestones and large aquatic plants. The spawning period is from January to April, water flow is required for reproduction, eggs are laid on hard surfaces (sand, stones; Pan et al. 2009).

The diverse topography and climate of Yunnan Province has made it a well-known biodiversity hotspot in China and the world (Yang et al. 2004). High-altitude plants and vertebrates in the region account for 51.6% and 54.8% of China's total, respectively, the proportion of protected animals is as high as 72.5%, of which 15% are unique to Yunnan (Yang et al. 2004). However, in the past 60 years, overfishing, species invasion, damming, habitat destruction, climate change, metals contamination, and cultural eutrophication have dealt a heavy hit to Yunnan's aquatic organisms (Wang et al. 2021). Its fish species are especially hard hit, with the most serious affected system being Dianchi Lake, where 90% of endemic fish species are considered extinct (Wang et al. 2013).

The situation faced by Dianchi Lake is very similar to Lake Victoria. The impact of invasive species, pollution and overfishing has resulted in a substantial decrease in the original native species (Sayer et al. 2018; Breitinger, 2022). A critical issue in Lake Victoria was invasion of predatory Nile perch. Conversely, habitat loss in South-East Asia has more pronounced impacts on biodiversity (Tian et al. 2022).

Thesis Objectives

The primary goals of this thesis were to: 1) understand the community structure of fish in Dianchi Lake, and 2) to develop qPCR markers and test their sensitivity to detect whether *Sinocyclocheilus grahami* occurs in the lake, and, if so, locations of occurrence. This information will be of direct benefit to fisheries managers and conservation biologists who are attempting to conserve this endangered species (Chen et al. 2008).

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CHAPTER 2. Field Fish Survey and eDNA detection

Introduction

Fish species are an important part of aquatic ecosystems. With the rapid development of human society since the Industrial Revolution, the demand for natural resources and the destruction of the natural environment, including lakes and rivers, have become more intensive. Thirty years ago, researchers summarized the extinction of fish species in the Great Lakes region of North America owing primarily to habitat loss, introduced species, pollution, hybridization and overfishing (Miller et al. 1989). A more recent analysis of problems in the Great Lakes highlighted introduced mussels, excessive N and P nutrients, charter boat fishing and PCBs as main concerns (Allan et al. 2012). Marine fish are also facing an unprecedented crisis typically due to overfishing (Worm et al. 2006; Mitcheson et al. 2020). The protection of endangered species and the causal mechanism(s) of species extinction have become a globally-hot research topic (Lintermans et al. 2020; Montgomery et al. 2019; Ulman et al. 2020).

Many of the problems that have occurred in the Great Lakes and in coastal marine systems globally also occur in China. For example, with rapid urbanization in the upstream western city of Kunming, cultural eutrophication of Dianchi Lake began to accelerate in 1960 and became very extensive by the 1990s (Lu et al. 2015). Beginning in the 1960s, the Chinese carp family (i.e. silver carp *Hypophthalmictuthys molitrix*, bighead carp *Aristichthys mobilis*, grass carp *Ctenopharyngodon idellus*, and black carp *Mylopharyngodon piceus*) and other commercial fishes were introduced to Dianchi Lake (Wang et al. 2013). The proportion of introduced species kept increasing in the following decades, gradually resulting in the replacement of native species (Ye. et al. 2015). The shrinkage of the littoral habitats due to development,

changes in watershed use and man-made lakeshores also contributed to the massive loss of native fish habitat in coastal areas (Wang et al. 2013). A current problem for managers is detection of species whose abundances are very low and distributions very restricted.

eDNA is shed by organisms as they carry out everyday activities, allowing researchers to collect and analyze its signal and composition (Hebert et al. 2003; Taberlet et al. 2012). eDNA technology has greatly improved sensitivity (i.e. reduced type II errors) of rare species detection. For example, eDNA is deemed to be 44% more effective than other methods in marine species detection (Boussarie et al. 2018). eDNA-based detection is nondestructive, meaning only the DNA and not the target species is collected (Beja-Pereira et al. 2009; Piggott et al. 2020). eDNA has progressed as a high-speed, high-sensitivity and high-accuracy technology (Harper et al. 2019) and is widely used for species detection and community structure estimation in freshwater ecosystems (Bohmann et al. 2014; Sepulveda et al. 2020; Xia et al. 2021).

Study area

Yunnan province is located in southwest China and accounts for 50% of terrestrial biodiversity of the country (Yang et al., 2004). Dianchi Lake sits adjacent to the major city of Kunming (population ~7,000,000), which is the provincial capital city. Dianchi Lake is a source lake that is part of the upstream Jinsha River drainage within the basin of the Yangtze River (Jinsha is the name for the upstream part of the Yangtze River), the largest and the third-largest river in China, respectively (Yang et al., 2020).

With recent and sustained socio-economic development, lakes of Yunnan Province – the so-called Plateau Lakes - have experienced severe threats including

overfishing, species invasion, damming, habitat destruction, climate change, metals contamination, and cultural eutrophication (e.g. Luo et al., 2006; He et al., 2015; Ma et al., 2015; Zhang et al., 2017). These stressors collectively led to a large number of native fish species becoming endangered (Tang et al., 2013; Fu et al., 2019) or extinct (Zhou, 2011). The endangered fishes have high biodiversity value, as some are endemic to this region or even a single lake (Chen, 2013). Dianchi Lake is also rich in fish resources and is an important commercial fishing system (Table. 1). Therefore, Dianchi Lake has very high biodiversity, commercial and social value. Many comparative studies have been conducted recently on water quality (Yang et al., 2020; Yu et al., 2020), proliferation and effects of cyanobacteria blooms (Zi et al., 2018; Mu et al., 2019), fish community composition (Chen, 2013) and fish introductions (Shi et al., 2015; Ye et al., 2015) in Dianchi Lake. It has been subject to comprehensive ecological and environmental research, though there exists a paucity of studies focused on its endangered fish species. The golden-line barbell (*Sinocyclocheilus grahami*) is endemic to Dianchi Lake. It was once very common, though it experienced rapid population decline beginning in the 1960s due to a combination of introduced species, high levels of nutrients and attendant cultural eutrophication (Chen et al. 2008). This fish grows slowly but is delicious and nutritious, resulting in its heavy overexploitation in the lake (Zhang et al. 2020). During the 1990s, scientific surveys found that the species largely disappeared from the lake, with only fragmented subpopulations remaining in some tributaries and springs (Chen. et al. 2008). In 2007, artificial breeding of this species was successfully initiated (Yang. et al. 2007), and it has been continuously re-introduced into Dianchi Lake since 2009. Despite this, there exist no records on the current distribution of wild populations.

The objective of this thesis is to determine the community structure and distribution of fish in Dianchi Lake using capture-based methodology. Past studies showed that the number of local fish in Dianchi Lake was declining, and the proportion of introduced species was increasing (Wang et al., 2013). Therefore, I hypothesize that the fish community in Dianchi Lake is almost entirely composed of introduced species. I also sought to determine the distribution of *Sinocyclocheilus grahami* in Dianchi Lake. Past studies showed the species is regionally extinct in the lake (Chen et al. 2008), but in recent years local agencies have successfully bred and reintroduced the species (Yang et al. 2007). Therefore, I hypothesize that *Sinocyclocheilus grahami* distribution in Dianchi Lake is recovering.

Methods

Fish and water samples collection

Dianchi Lake (24.40 ~ 25.02°N, 102.37 ~ 102.48°E) is a large shallow plateau lake (1887m altitude; mean and maximum water depth are 4.4 m and 11.0 m, respectively), with a surface area of around 300 km^2 (Fan et al., 2017). Sampling sites were established using eight existing national water quality control sites of China (each site was marked with a buoy) and eight other sites recommended by experts from the Kunming Institute of Zoology (Chinese Academy of Sciences). Site selection was based on the existing Kunming Institute of Zoology fish survey sites in Dianchi Lake (Figure 1).

Many fishes in Dianchi Lake are small-sized species, including many benthic species (Chen, 2013). According to local fishermens' experiences, gillnets and fish traps are effective and are widely used in local fishing. In the present study, fish samples were collected from eight sites (Figure 1 and Table 2) in January 2018 by

local fishermen due to local regulations. Specifically, three different mesh–size gillnets (10, 6, and 1.5 cm, respectively; 2m in height and 16m in length) and fish traps (mesh–size 0.5 cm with a frame of 35cm wide and 25cm high and 10m long) were used. Gillnets and fish traps were set close to the lake bottom at each site. The fishing nets were set at dusk and were retrieved the next morning, with each deployment lasting for ~12 h.

Fishes captured were identified to species level (Cheng, 1987), counted, and intact individuals were preserved in 95% ethanol and on ice and transported to laboratory within 12h of collection.

One water sample for measuring chemical parameters (1 L, HDPE bottles) was collected from the subsurface (~ 20cm) at each site The samples were placed on ice in a cooler and transported to the laboratory within 12h of collection. Bottles filled with DD water from the lab were bought to the field, opened and then back to the lab, which then undergo the same sample processing process and used as blanks.

To avoid cross contamination, gloves were used in sample collection, and all bottles and caps were cleaned using DD water and rinsed three times with sample water before being filled with the sample. Water samples were stored in different coolers and separated from fish samples throughout the entire sampling process.

Chemical parameters measurement

Total nitrogen (TN) was measured by taking 10ml of the well-mixed water sample, 5ml of alkaline potassium persulfate were added to a 25ml colorimetric tube and placed in an autoclave for digestion for 30 min at 120°C. Two blank controls with ultra-pure water (Mili-Q, resistivity of >18 2MΩ-cm, a conductivity of <0.056 μS/cm and <50 ppb of Total Organic Carbon (TOC)) was included in analysis. After being

cooled to room temperature, 1ml (1+9) of hydrochloric acid solution was added, and ultra-pure water was added to make up to 25ml. The samples were then analyzed using Automatic Discrete Analyzer Cleverchem 380 (DeChem-Tech. Hamburg, Germany). Final concentrations of total nitrogen were calculated by subtract the blank control, and then multiplying the value by the dilution factor (2.5 times).

Total phosphorus (TP) was measured by taking 25ml of well-mixed water sample in a 50ml colorimetric tube, then 4ml potassium persulfate solution was added, following which samples were shaken and covered with a stopper. All samples were placed in an autoclave at 120°C and a pressure of 1.1~1.4 kg/cm^2 for 30 minutes. After cooled to room temperature, the samples were diluted to 50ml with pure water. Two blank controls were also made with ultra-pure water. Samples were analyzed using Automatic Discrete Analyzer Cleverchem 380 (DeChem-Tech. Hamburg, Germany). Concentration of TP was calculated by first subtracting the blank control and then multiplying it by the dilution factor (2 times). All methods followed the handbook of Automatic Discrete Analyzer Cleverchem 380 (DeChem-Tech. Hamburg, Germany).

Genetic Analyses

Primer design

No specific genetic markers for *Sinocyclocheilus grahami* have been published in existing studies, thus newly-designed markers were needed. I designed species-specific primers using the mitochondrial COI and D-loop regions based on complete mitochondrial genome of *Sinocyclocheilus grahami* (NCBI Reference Sequence: NC_013189.1). Oligo 7 and Primer Premier 5 were used to design primers and test for mismatches and dimers. A total of 104 pairs of primers were designed and

then tested online for non-specific amplification using the Primer-blast (NCBI) tool (i.e., *in silico* specificity check). Primers were sorted in descending order of the scores detected by Primer Premier 5 software, and then using Primer-BLAST online tool (NCBI) to blast with the NR database and fish database (tax id 7898) one by one.

Primer test

Fourteen primer pairs passed the online specificity check were synthesized by Sangon Biotech (Shanghai, China), following which I developed an optimal annealing temperature test for each. I then conducted an *in vitro* specificity test for non-specific amplification for each primer pair against all fish species in the lake with available tissue samples from my sampled fishes and local fish market (tissue samples were stored in 95% ethanol). Tissue DNA was tested with NanoDrop™ 2000/2000c Spectrophotometers (Thermo Scientific™) for concentration, all diluted to 1 ng/μl before use, and then subjected to conventional PCR amplification under the same conditions, and then was checked by running on a 2% agarose gel. Three tissue DNA samples (1 ng/μl) from target species were used as positive control.

To estimate the limit of detection (LoD; the smallest concentration of analyte in the test sample that can be reliably detected but not necessarily quantitated) and limit of quantification (LoQ; the lowest amount of analyte in a sample which can be suitably quantified), all primer pairs that passed the specificity check were tested by serial dilution. Samples were measured by a 3x dilution experiment using qPCR for LoD (Figure 5. Agersnap et al. 2017). Three replicates and five replicates were used for each concentration, respectively. Standard curves were measured by running a 10x serial dilution tissue DNA with each plate of environmental samples.

I conducted dilution gradient experiments to reduce amplification inhibition (Huggett et al. 2013). I then prepared three replicates of 2, 4, 8, and 16 times diluted eDNA samples. A 20-cycle conventional PCR amplification was performed under the same thermal condition, and the quality and brightness of each amplification product was examined by gel electrophoresis to determine the optimal dilution factor (Schabacker et al. 2020).

Water sampling

I collected water samples from January to February 2019. Surface water samples were collected at all 16 sampling points (Figure 1). All instruments were cleaned with 20% bleach and washed three times with sample site water before collection (Huggett et al. 2013). I collected four 300ml water samples (HDPE bottle) at each sampling site (Figure 1). During collection, bottles were rinsed with the lake water three times, and then the surface water was taken. Gloves were used and changed between two sample sites through sample collection. An extra bottle filled with ddH_2O was exposed in air and then sealed and stored with water samples as a blank control.

Sample filtration and DNA extraction

Due to a large amount of particulate matter present in the water samples, I conducted a pilot extraction experiment comparing a pre-filtering and non-pre-filtered protocol. Specifically, water samples were pre-filtered using a 45μm metal mesh before filtered with 0.45μm acetate filter membrane (diameter: 50 mm, Tianjin Keyilong Lab Equipment Co., Ltd., Tianjin, China). A Nano Drop test (Desjardins et al. 2010) demonstrated that the quality of extracted DNA from the pre-filtered

samples was much better than unfiltered ones. As a result, all Dianchi Lake samples were pre-filtered thereafter. All water samples were filtered within 24h of collection, and all filters were stored at -20°C and half of the filter was used for extraction while the other half was stored for future use.

A PCI extraction method was used for all DNA extractions (Balasingham et al. 2017). Tissue DNA of the target species and other captured fish species was extracted from fin samples for the *in vitro* specificity test. Blank (negative) controls and tissue samples (positive control) were used for each batch of extractions. I assessed both DNA quality and estimated amount (ng/µl) using a NanoDrop™ 2000/2000c spectrophotometers (Thermo Scientific™).

Quantitative PCR and Sanger sequencing

Primers that passed the above tests were used for real-time quantitative PCR (qPCR) examination of water samples. PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, MA, USA) was used for all qPCR reactions. Each 20µl reaction system contained 10µl master mix, 0.6µl forward primer, 0.6µl reverse primer, 2µl diluted (8X diluted) eDNA sample and 6.8µl of ultrapure water. No-template controls and standard curve (also serving as positive controls) using DNA extracted from *Sinocyclocheilus grahami* fin clips were also included in triplicate for each qPCR in a 96-well plate. A 2 min 50°C heat start was set at the beginning of each run following prescribed instructions of the used master mix (PowerUp™ SYBR™ Green Master Mix USER GUIDE). Thermal cycler conditions were set to an initial 95 °C denaturation for 3 min, followed by 50 cycles of 95 °C denaturation for 30s, then annealing at 58 °C for 30s and 70 °C for 60s, a default melt curve stage was performed for each run. All qPCR data was analyzed on the QuantStudio™ 7 Flex

Real-Time PCR System (Thermo Fisher Scientific, MA, USA). The qPCR productions were sent to Sangon Biotech (Shanghai, China) for Sanger sequencing to determine the species identity of the amplified product. I used Basic Local Alignment Search Tool (BLAST) to align the resulting sequences against GenBank nucleotide database sequences for *Sinocyclocheilus grahami* to verify amplification of the target species. Samples with a similarity higher than 97% were considered to be *Sinocyclocheilus grahami.* All lab work was completed in Dr. Qinjun Li lab, Yunnan University, Kunming. China.

PCR efficiency

To calculate the actual DNA concentration, a linear regression was used to determine the best fit for empirical data. I established a standard curve according following the method of Yun et al. (2006). Standard curves were also used to calculate the LoQ for the primer pair used in this experiment. LoD was tested using a 10-fold dilution series of PCR template DNA. One positive qPCR replicate is sufficient to be considered as a positive detection (Goldberg et al. 2016).

Data analysis

Spatial variation in fish composition was analyzed and visualized using Principal Component Analysis (PCA) on fish absolute abundance data across the six sampling sites (site PLJ is a river control, site CH's data was incomplete, Figure 2). Relationships between fish species (fish count) and measured environmental factors were analyzed by calculating the Kendall correlation coefficient (Myers et al., 2013). Data was Hellinger-transformed (Legendre and Legendre, 2012) to reduce weights to variables with low fish counts and zeros. All statistical analyses were conducted using

R (version 4.0.3). The PCA based on the abundance of the 13 fish species across the six sites was used to visualize the spatial characteristics of the fish community. “Ggplot” was used to plot the data (Wickhan, 2016). Spatial variation in target species DNA detections and other fish abundances were analyzed and visualized using Principal Component Analysis (PCA) across the six sampling sites (Figure 4). A binary logistic regression model was developed between the target species DNA detections and measured environmental factors.

Results

Physical and chemical parameters

The concentrations of nutrients in Dianchi Lake overall were much higher than the OECD standard (total phosphorus 0.035 mg/L. OECD, 1982). Total phosphorus (TP) and total nitrogen (TN) concentrations were measured at all 16 sample sites. TP ranged from 0.105 mg/L (site XH) to 0.384 mg/L (site GYSX), with an average of 0.272±0.096 (±SD) mg/L. The northeast part of the lake - closest to downtown Kunming - had the highest TP concentration. On the other hand, TP concentration was low in the west bank and in the downstream, southwest area (Table 4).

TN ranged from 1.568 mg/L (site DCN1) to 3.058 mg/L (site PLJ), averaged at 2.116±0.375 (±SD) mg/L. TN concentration was generally higher in the east and middle parts of the lake (Figure 1).

The dissolved oxygen concentration (DO), water temperature (site WT) and Oxidation-Reduction Potential (ORP) of 15 samples were measured on site (site CH was not measured due to equipment failure). Average DO was ~8.97±1.02 (±SD) mg/L and ranged from 7.30 mg/L (site PLJ) to 11.45 mg/L (site HWZ). Water

temperature was highly variable between sites, ranging from 12.2°C (site HK and HKX) to 15.6°C (site HWZ), with an average of 14.0±1.1°C (±SD). ORP ranged from 198.4 mV (site HD) to 121.3 mV (site HK and HKX), with an average of 165±22mV (±SD).

Fish Community

A total of 478 individual fish were collected from eight sample sites in Dianchi Lake (Table 3). Introduced species numerically dominated (~97%) in the fish community in Dianchi Lake. Among them, a total of 463 individuals belonging to 11 introduced species and only 15 individuals of three native species were collected (Table 3). Four introduced species were identified as numerically dominant (>7%): *Neosalanx taihuensis* (~36%), *Rhinogobius giurinus* (~19%), *Cultrichthys erythropterus* (~13%), and *Pseudorasbora parva* (~9%).

The distribution of native species was very limited. *Sinocyclocheilus grahami* was only found at one site (GD) in Dianchi Lake and site PLJ in Panlong River (Figure 1), while *Misgurnus anguillicaudatus* was distributed near the estuary. *Carassius auratus* was mainly distributed at site CH (Caohai area) (Figure 1). On the contrary, most invasive species were widely distributed throughout the lake, especially the dominant ones. *Neosalanx taihuensis*, *Cultrichthys erythropterus* and *Pseudorasbora parva* were all widely distributed and dominant in the whole lake, while *Rhinogobius giurinus* was distributed in all areas except the densely populated southeast coast of the lake.

The first two principal components accounted for 38.1% and 26.5% of total variation in fish abundance, respectively (Figure 2, Table 5). *Sinocyclocheilus graham*, *Rhinogobius cliffordpopei*, *Cyprinus carpio* and *Hemiramphus sajori* had a

similar contribution rate to axis one (~19.5%), the distribution of the four species was mainly concentrated at site GD (an artificial wetland). The introduced *Rhinogobius giurinus*, *Micropercops swinhonis*, *Hypomesus olidus*, *Abbottina rivularis* and *Pseudorasbora parva* had the highest contributions to the second axis (the contribution rate is higher than 10% each).

Rhinogobius giurinus, *Pseudorasbora parva*, *Misgurnus anguillicaudatus*, *Hemiculter leucisculus* and *Cultrichthys Erythropterus* were dominantly distributed in site DCN2, XH and HK (west and south part of the lake, Figure 2). *Micropercops swinhonis* were more concentrated in the southern lake basin (Figure 2), and *Neosalanx taihuensis* did not show strong spatial distribution difference (Figure 2). *Cyprinus carpio*, *Sinocyclocheilus grahami*, *Rhinogobius cliffordpopei* and Hemiramphus sajori were more concentrated at site GD, and *Abbottina rivularis* and *Hypomesus olidus* were most abundant at site GD and WL (the northeast part of the lake, Figure 2).

I used the Kendall correlation coefficient matrix to visualize the interaction of the 13 fish species and five environmental parameters across the six sites (Figure 3). The native species *Sinocyclocheilus grahami* was positively correlated with the introduced species *Rhinogobius cliffordpopei* and the native species *Cyprinus carpio,* but considering that the actual number was small, it may not be representative. *Pseudorasbora parva* and *Misgurnus anguillicaudatus* were positively correlated, while *Hypomesus olidus* was positively correlated with *Hemiramphus sajori*. The distribution of *Misgurnus anguillicaudatus* was negatively correlated with water temperature. *Micropercops swinhonis* was negatively correlated with *Neosalanx taihuensis* and with dissolved oxygen concentration. *Pseudorasbora parva* was negatively correlated with the potential of oxidation−reduction potential.

Environmental DNA

The species-specific primer set SGCOI-1f (5' CTACACTGATTTCCCCTACTAACC 3') and SGCOI-1r (5' TCCGATGGATAACACCGTG 3') was selected for environmental sample amplification of *Sinocyclocheilus grahami* DNA sequences. A 10-fold dilution series of PCR template DNA was performed, with the LoD of this primer set being 1 x 10^{-8} ng/µL. Two standard curves were established on the same 96 well-plate as per environmental water samples, with the LoQ being 38 and 42 cycles.

Real time qPCR for environmental samples

Dilution prior to qPCR amplification can minimize inhibitory effects and reduce errors between samples (McKee et al. 2015). I performed a 2, 5, 10, 15 and 20x dilution experiment with environmental samples and compared their amplification products by 2% gel electrophoresis. Ten-fold diluted samples had the brightest and clearest bands, so all samples and controls were diluted 10x before qPCR amplification.

I used negative controls using the extraction product of the sampling blank as templates. Among all negative controls (n=3), two had positive results but their melting temperature was 73.05°C and 72.18°C, while that of the positive control was 82.18°C, suggesting no contamination. PCR products were later demonstrated to be valid by Sanger sequencing.

Three water sample replicates and three intra-sample replicates provided a total of nine replicates for each sample site. All positive data was corrected according

to the standard curve. Many sites which had higher cycle number than the LoQ were also calibrated with the standard curve for further analysis and comparison.

Ten of the 16 sampling sites (62.5%) tested positive for *Sinocyclocheilus grahami* DNA. Since many DNA samples have CT values below the LoQ, I converted them to positive/negative detection. DNA was mainly distributed in the southwest, west, and deep area of the northern lake basin, sites GD, DCN1 and GYSX.

Principal Component Analysis (Figure 4) was performed with DNA detections of *Sinocyclocheilus grahami* (DNA) and other fish individual abundance measures across the six sampling sites in Dianchi Lake. The first two principal components accounted for 31.02% and 27.07% of the total variation, respectively (Figure 4, Table 6). Fish species with the highest contribution rate to the first PC axis were: *Hemiramphus sajori* (~20.70%), *Rhinogobius cliffordpopei* (~19.70%) and *Cyprinus carpio* (~19.70%). *Micropercops swinhonis* (~18.25%), *Rhinogobius giurinus* (~14.71%) and *Abbottina rivularis* (~12.53%) had the highest contribution rate to PC axis two.

Binary logistic regression model used to assess the relationship between DNA detections of the target species and measured environmental factors. The developed model was $\log(p/(1-p)) = 3178.1 - 852.6TP - 127.2\,DO - 126.4WT$. TP, DO and WT were negatively correlated with the DNA distribution of the target species, the beta coefficient rates were -852.6 (TP), -127.2 (DO) and -126.4 (WT), however, the P value showed no significant correlations ($P>0.05$).

Discussion

My research show that introduced species numerically dominate the fish community in Dianchi Lake (Table 3), as the numerical abundance of native species was less than 5%. Several species with smaller size and shorter life cycles were widely distributed throughout the lake and were dominant species. These findings are largely similar to previous fish surveys (e.g. Ye et al. 2015). At one time, there were 37 native fish species in Dianchi Lake, but only three were caught in this survey, suggesting that biodiversity may have dropped considerably. However, my results may also be a result of type II error owing to low sampling effort.

Water quality and the ecological environment of Dianchi Lake have been greatly improved in recent years, especially regarding input of treated sewage (He et al. 2020) and the construction of artificial wetlands (Niu et al. 2017). But the large human population in Kunming and fast-growing economy in the region still have a great impact on the environment (Yang et al. 2020). Other recent studies have identified that sediments were an important source of pollutants in the lake (He et al. 2020; Qian et al. 2020), as was climate change (Chen et al. 2020).

According to the results of PCA analysis (Figure 2), the local species of Dianchi Lake were concentrated in the east-southeast part of the system. Individuals of the critically endangered species *Sinocyclocheilus grahami* were captured only at mid-lake site GD. In addition, apart from the widely-distributed dominant species *Neosalanx taihuensis*, most introduced species had a higher density in the northeastern part of the lake. This provides indirect evidence that adaptability to adverse conditions (pollution tolerance, stronger reproductive ability, etc.) is stronger in many introduced fishes relative to native ones. Further research should be carried out to verify this.

Results of the correlation analysis (Figure 3) showed that the only two species negatively correlated in distribution were *Hemiculter leucisculus* and *Neosalanx taihuensis*. Studies demonstrated that the original distribution of the former species was strongly inhibited by the latter species, especially with regard to reproduction (Wang et al. 2013).

Dissolved oxygen content has played a certain role in limiting benthic fish such as *Micropercops swinhonis* (Figure 3). The distribution of *Pseudorasbora prva* and *Misgurnus anguillicaudatus* were affected by water temperature. In addition to the parameters measured in this study, disease (Minamoto et al. 2015), organic pollutants (Fan et al. 2017) and antibiotics (Wei et al. 2014) may also affect the distribution of fish in the lake.

In general, the fish community in Dianchi Lake was almost entirely dominated by introduced species, with minor roles played by native species. Habitat change was proposed as the main reason for fish community changes (Wang et al. 2013), followed by introduced species, through specific studies need to verify this in future. At the same time, frequent algal blooms caused by eutrophication and climate warming are also important influencing factors (Ye et al. 2015; Zi et al. 2018).

The native fish community (Table 1) of Dianchi Lake has been largely replaced by invasive and commercial species (Table 3). Commercial fish species are very similar to other freshwater lakes in China (Zhao et al. 2016). This is a typical Biotic Homogenization phenomenon (different communities become more similar over time (Petsch, 2016)). This survey found only ~23% of recorded species, and the loss of biodiversity is very severe. This can lead to fragility of the ecosystem and a potential threat to the economic and human activities of the region (Schmeller et al. 2020).

Distribution of Sinocyclocheilus grahami

Sinocyclocheilus grahami is a critically endangered fish species endemic to the Dianchi Lake Basin in Yunnan, China. According to existing field surveys, the wild population disappeared from Dianchi Lake Basin more than 10 years ago (Chen et al. 2008). In my research, a very small number of individuals were caught at site GD with fish traps, but this site was stocked with artificially bred fry about two months before sampling. Captured individuals may have been newly stocked individuals that failed to disperse. However, eDNA provides a more sensitive detection method compared to net and trap fishing. The eDNA signal of *Sinocyclocheilus grahami* was detected at 10 of the 16 sites, and thus the species could have a much broader distribution than revealed by traditional fish collection methods. The target eDNA was mainly distributed in the southern and western regions of the lake, as well as the Panlong River (site PLJ) and its estuary (site HD). Although no individuals of the target species have been captured in these areas, it has been reported that eDNA signals are more sensitive than traditional methods (Piggott et al. 2020) and can be used as a strong basis for management and protection (Jerde, 2019). Based on this, it is likely that *Sinocyclocheilus grahami* has restored its distribution in at least some regions in Dianchi Lake.

Factors affecting the distribution of Sinocyclocheilus grahami

According to the PCA results (Figure 4), the eDNA signal distribution of *Sinocyclocheilus grahami* was negatively correlated with two invasive fish: *Abbottina rivularis* and *Hypomesus olidus*, both of which are small and omnivorous (Chen, 2008). They may compete with each other and suppress the population of *Sinocyclocheilus grahami*.

Studies have concluded that protein intake is essential for the growth of *Sinocyclocheilus grahami* (Deng et al. 2014), but the protein and fat requirements of the species are similar to carp and that a higher level of protein intake is not beneficial to it (Wang et al. 2018). Therefore, it is highly likely that interactions with other fish (predation, competition, etc.) is not the main factor affecting the distribution of *Sinocyclocheilus grahami*. However, further research is needed to support this, as DNA concentrations may not accurately represent species abundance and the data used in this study do not account for possible seasonal variation.

The distribution of *Sinocyclocheilus grahami* was negatively correlated with oxidation-reduction potential and total phosphorus. The reason for this phenomenon is that the cyanobacterium *Microcystis* is dominant in Dianchi Lake, and mainly floats on the surface layer during the day (Ostrovsky et al. 2020). The wind direction across Dianchi Lake is primarily from the southwest for most of the year (Yu et al. 2020;), thus *Microcystis* distribution was likely affected by the wind and concentrated in the central and northern part of the lake (Zhang et al. 2020). *Microcystis* has a significant inhibitory effect on the heart and other organs of *Sinocyclocheilus grahami* (Zi et al. 2017). This was highly consistent with the distribution of eDNA signals, so it is possible that harmful cyanobacteria blooms could be important in affecting the distribution of *Sinocyclocheilus grahami* in Dianchi Lake. The species' preference for lower water temperature and sensitivity to water quality as a cave fish (Yang et al. 2016). The northern and eastern lakeshores are densely-populated, mostly urban areas, so they may also be affected by habitat changes that preclude presence of the species.

Since 2013, the local government has introduced water from the Niulan River into Dianchi Lake to control pollution, which not only dilutes pollutants but also

promotes their diffusion (Zhang et al. 2020). This may explain why the distribution of pollutants did not correlate significantly with the distribution of *Sinocyclocheilus grahami* in this study.

It should be noted that site GD is a constructed wetland, and a local institution released the target species' fry at the site six weeks before the study. Therefore, it is not overly surprising that I observed some fishes at this site. COI mitochondrial DNA hits across the central and southern portions of the lakes cannot distinguish whether the source individuals were wild or artificially introduced.

False positives and false negatives

The primer design steps utilized ensure high species-specificity, and Sanger sequencing (Table 7) results of qPCR products were all found to be specific for my target species. In my study, the main cause of false positive detection (type I error; DNA exists when the fish does not) could be due to eDNA signals from external sources. Many rivers enter Dianchi Lake are natural distribution areas of *Sinocyclocheilus grahami*, so the target eDNA of site HD may have originated from upstream rivers (Figure 1).

It is possible that either temperature changes or microbial activities increased the rate of DNA degradation, resulting in false negatives (target species present but no DNA detected) (Zulkefli et al. 2019). A third possibility is related to eDNA extraction. Dianchi Lake is a typical shallow, eutrophic lake, with massive quantities of algae and suspended solids that greatly increased difficulty of eDNA collection and extraction (Raemy et al. 2018). For this reason, I utilized a pre-filtration method to reduce interference and improve the DNA extraction rate (Turner et al. 2014). Although pre-filtration may cause eDNA loss, the extraction method used seems

appropriate to obtain as much DNA as possible with as few possible inhibitors present as possible (Hunter et al. 2019). PCR inhibition may also result from presence of organic pollutants in Dianchi Lake (Fan et al. 2017), or from presence of DNA of close relatives (Lance et al. 2020). The dilution method used in this study appears effective to address these possible types of PCR inhibition (Mckee et al. 2015). I recommend that future researchers use droplet digital PCR (ddPCR) or use multiple primers simultaneously to further reduce the possibility of false negatives and increase the LoD (Harper et al. 2019).

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Tables

Table 1. Historical fish list of Dianchi Lake.

common name	Scientific name	Native (N) / Introduced (I)
Rainbow trout	*Oncorhynchus mykiss*	I
Pond smelt	*Hypomesus olidus*	I
Taihu Silver Fish	*Neosalanx taihuensis*	I
Black carp	*Mylopharyngodon piceus*	I
longspiky-head carp	*Luciobrama macrocephalus*	I
Grass Carp	*Ctenopharyngodon idella*	I
Barbel chub	*Squaliobarbus curriculus*	I
Snake-head	*Ochetobius elongatus*	I
Yellow-cheek	*Elopichthys bambusa*	I
Tench	*Tinca tinca*	I
Spiny white fish	*Anabarilius polylepis*	N
Dianchi Silvery minnow	*Anabarilius alburnops*	N
Sharpbelly	*Hemiculter leucisculus*	I
-	*Toxabramis swinhonis*	I
White amur bream	*Parabramis pekinensis*	I
Wuchang bream	*Megalobrama amblycephala*	I
redfin culter	*Cultrichthys erythropterus*	I
Kunming nase	*Xenocypris yunnanensis*	N
Bighead carp	*Hypophthalmichthys nobilis*	I
Silver carp	*Hypophthalmichthys molitrix*	I
Rainbow gudgeon	*Sarcocheilichthys nigripinnis*	I
Chinese false gudgeon	*Abbottina rivularis*	I
Stone moroko	*Pseudorasbora parva*	I
Rosy bitterling	*Rhodeus ocellatus*	I
-	*Acheilognathus macropterus*	I
long-body acheilognathin	*Acheilognathus elongatus*	N
Khanka spiny bitterling	*Acanthorhodeus chankaensis*	I
Golden-line barbel	*Sinocyclocheilus grahami*	N
-	*Acrossocheilus yunnanensis*	N
-	*Spinibarbus sinensis*	N
-	*Discogobio yunnanensis*	N
-	*Schizothorax grahami*	N
-	*Cyprinus micristius*	N
-	*Cyprinus chilia*	N
Common carp	*Cyprinus carpio*	I
Goldfish	*Carassius auratus*	N

-	*Yunnanilus nigromaculatus*	N
-	*Yunnanilus plenrotaenia*	N
-	*Yunnanilus discoloris*	N
-	*Homatula variegata*	N
-	*Triplophysa grahami*	N
-	*Sphaerophysa Dianchiensis*	N
Pond loach	*Misgurnus anguillicaudatus*	N
-	*Paramisgurnus dabryanus*	I
Kunming catfish	*Silurus mento*	N
Brown bullhead	*Ameiurus nebulosus*	I
Suckermouth catfish	*Hypostomus plecostomus*	I
-	*Pseudobagrus medianalis*	N
-	*Liobagrus kingi*	N
-	*Liobagrus nigricauda*	N
	Oryzias sinensis	N
Mosquitofish	*Gambusia affinis*	I
Asian pencil halfbeak	*Hyporhamphus intermedius*	I
Asian swamp eel	*Monopterus albus*	N
Chinese perch	*Siniperca chuatsi*	I
-	*Micropercops swinhonis*	I
Barcheek Goby	*Rhinogobius giurinus*	I
Chiangmai stream goby	*Rhinogobius cliffordpopei*	I
Snakehead	*Channa argus*	N

All Latin names are consistent with historical records (Chen. 2013).

Table 2. Sample collection information.

Site	GYSX	DCN1	GYSZ	HKX	HD	GD	DCN2	HK	PLJ*	XH	HWZ	LJY	GYSD	BYK	CH	WL
Date (MM/DD)	01/24	01/24	01/24	01/24	01/20	01/12	01/14	01/25	01/08	01/20	01/24	01/24	01/24	01/24	01/11	01/21
Water Samples collected	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Fish Samples collection	No	No	No	No	Yes	Yes	Yes	Yes	Yes	Yes	No	No	No	No	Yes	Yes

PLJ* is a river control site, it is a known natural distribution area of *Sinocyclocheilus grahami.*

Table 3. Fish individuals collected from Dianchi Lake.

Sample site / Species name	Invasive Species	DCN2	GD	HD	CH	WL	PLJ	XH	HK	Total
Sinocyclocheilus grahami[1]		0	2	0	0	0	3	0	0	5
Misgurnus anguillicaudatus[1]		2	0	0	0	0	0	0	2	4
Carassius auratus[1]		0	0	0	6	0	0	0	0	6
Rhinogobius cliffordpopei[2]	+	0	2	0	0	0	1	0	0	3
Rhinogobius giurinus[2]	+	25	14	0	0	0	2	35	14	90
Neosalanx taihuensis[2]	+	21	30	20	2	30	0	40	26	169
Cultrichthys erythropterus[2]	+	17	6	8	0	15	0	15	2	63
Hemiculter leucisculus[2]	+	17	2	4	0	0	0	2	0	25
Micropercops swinhonis[2]	+	3	3	0	0	0	0	0	0	6
Cyprinus carpio[2]	+	0	5	0	4	0	0	0	0	9
Hypomesus olidus[2]	+	0	1	10	0	0	0	1	0	12
Hemiramphus sajori[2]	+	0	25	4	0	0	0	0	0	29
Abbottina rivularis[2]	+	0	0	8	5	0	0	0	0	13
Pseudorasbora parva[2]	+	10	2	4	11	0	2	7	8	44
Total		95	92	58	28	45	8	100	52	478

All fish were preserved in 95% ethanol and stored for later analysis.

Table 4. Physical and chemical parameters in Dianchi Lake.

	GYS X	DCN 1	GYS Z	HKX	HD	GD	DCN 2	HK	PLJ	XH	HWZ	LJY	GYS D	BYK	WL	CH*
TP (mg/l)	0.384	0.232	0.348	0.34	0.256	0.224	0.148	0.288	0.348	0.105	0.376	0.372	0.284	0.412	0.172	0.204
TN (mg/l)	1.805	1.568	1.968	1.888	2.683	1.928	2.106	1.926	3.058	2.255	2.478	2.089	1.959	2.448	1.768	1.752
DO (mg/l)	7.71	8.5	8.37	8.9	9.68	8.43	8.56	8.9	7.3	9.5	11.45	10.22	8.8	9.58	8.6	NA
WT (℃)	12.9	13.6	14.2	12.2	14.9	14.4	13.3	12.2	13.9	14.7	15.6	14.5	14.6	12.9	15.5	NA
ORP (mV)	168.8	174.8	167.7	121.3	198.4	131.3	176	121.3	188	174.8	180.9	167.6	171.4	168.8	164	NA

TP: total phosphorus, TN: total nitrogen, DO: dissolved oxygen WT: water temperature, ORP: oxidation−reduction potential. (part of CH data was not measured due to equipment damage).

Table 5. Contribution (%) of each species to the principal axis of principal component analysis (PCA) on the abundances of the 13 fish species across the six sampling sites in Dianchi Lake.

	SG	MA	RC	RG	NT	CE	HL	MS	CC	HO	HS	AR	PP
PC 1	19.47	4.12	19.47	0.44	0.79	3.02	2.04	4.97	19.47	0.09	19.31	0.42	6.39
PC 2	0.69	9.41	0.69	17.44	1.08	3.00	9.23	12.30	0.69	17.30	0.03	17.72	10.41

SG: *Sinocyclocheilus grahami*; MA: *Misgurnus anguillicaudatus*; RC: *Rhinogobius cliffordpopei*; RG: *Rhinogobius giurinus*; NT: *Neosalanx taihuensis*; CE: *Cultrichthys Erythropterus*; HL: *Hemiculter leucisculus*; MS: *Micropercops swinhonis*; CC: *Cyprinus carpio*; HO: *Hypomesus olidus*; HS: *Hemiramphus sajori*; AR: *Abbottina rivularis*; PP: *Pseudorasbora parva*.

Table 6. Contribution (%) of each species to the Principal Component Analysis (PCA) on the DNA concentration of *Sinocyclocheilus grahami* (DNA) and other fish species across the six sampling sites in Dianchi Lake.

	DNA	MA	RC	RG	NT	CE	HL	MS	CC	HO	HS	AR	PP
Dim.1	0.12	9.45	19.70	3.01	0.96	4.60	6.19	1.78	19.70	0.25	20.70	0.02	13.53
Dim.2	6.44	6.39	5.54	14.71	0.49	0.27	7.53	18.25	5.54	11.29	3.33	12.53	7.68

DNA: positive detection of *Sinocyclocheilus graham* DNA; MA: *Misgurnus anguillicaudatus*; RC: *Rhinogobius cliffordpopei*; RG: *Rhinogobius giurinus*; NT: *Neosalanx taihuensis*; CE: *Cultrichthys Erythropterus*; HL: *Hemiculter leucisculus*; MS: *Micropercops swinhonis*; CC: *Cyprinus carpio*; HO: *Hypomesus olidus*; HS: *Hemiramphus sajori*; AR: *Abbottina rivularis*; PP: *Pseudorasbora parva*.

Table 7. Sanger sequencing and BLAST results.

Sample name	blast	Sample site
(GL-SC8) _[GL-COI-1F]	*Sinocyclocheilus grahami* (97.40%)	DCN2
(GL-SC8) _[GL-COI-1R}	*Sinocyclocheilus grahami* (98.73%)	
(GL-SA10) _[GL-COI-1F]	*Sinocyclocheilus grahami* (98.05%)	HD
(GL-SA10) _[GL-COI-1R}	*Sinocyclocheilus grahami* (99.35%)	
(GL-SD5) _[GL-COI-1F]	*Sinocyclocheilus grahami* (99.35%)	HK
(GL-SD5) _[GL-COI-1R}	*Sinocyclocheilus grahami* (99.36%)	
(GL-SD9) _[GL-COI-1F]	*Sinocyclocheilus grahami* (100%)	PLJ
(GL-SD9) _[GL-COI-1R}	*Sinocyclocheilus grahami* (98.73%)	
(GL-SD7) _[GL-COI-1F]	*Sinocyclocheilus grahami* (99.35%)	PLJ
(GL-SD7) _[GL-COI-1R}	*Sinocyclocheilus grahami* (99.36%)	
(GL-SC6) _[GL-COI-1F]	*Sinocyclocheilus grahami* (98.73%)	GD
(GL-SC6) _[GL-COI-1R}	*Sinocyclocheilus grahami* (98.73%)	
(GL-SB10) _[GL-COI-1F]	*Sinocyclocheilus grahami* (99.35%)	HD
(GL-SB10) _[GL-COI-1R}	*Sinocyclocheilus grahami* (99.36%)	
(GL-SE11) _[GL-COI-1F]	*Sinocyclocheilus grahami* (100%)	XH
(GL-SE11) _[GL-COI-1R}	*Sinocyclocheilus grahami* (98.73%)	
(GL-SE12) _[GL-COI-1F]	*Sinocyclocheilus grahami* (98.70%)	XH
(GL-SE12) _[GL-COI-1R}	*Sinocyclocheilus grahami* (100%)	
(GL-SF4) _[GL-COI-1F]	*Sinocyclocheilus grahami* (98.70%)	HK
(GL-SF4) _[GL-COI-1R}	*Sinocyclocheilus grahami* (98.09%)	
(GL-IB5) _[GL-COI-1F]	*Sinocyclocheilus grahami* (98.10%)	GYSX
(GL-IB5) _[GL-COI-1R]	*Sinocyclocheilus grahami* (98.17%)	
(GL-IF5) _[GL-COI-1F]	*Sinocyclocheilus grahami* (98.10%)	DCN1
(GL-IF5) _[GL-COI-1R]	*Sinocyclocheilus grahami* (98.17%)	
(GL-IC5) _[GL-COI-1F]	*Sinocyclocheilus grahami* (99.35%)	GYSX
(GL-IC5) _[GL-COI-1R]	*Sinocyclocheilus grahami* (98.73%)	
(GL-IF9) _[GL-COI-1F]	*Sinocyclocheilus grahami* (97.40%)	GYSZ
(GL-IF9) _[GL-COI-1R]	*Sinocyclocheilus grahami* (98.73%)	
(GL-IH5) _[GL-COI-1F]	*Sinocyclocheilus grahami* (99.35%)	HKX
(GL-IH5) _[GL-COI-1R]	*Sinocyclocheilus grahami* (98.73%)	

Figures

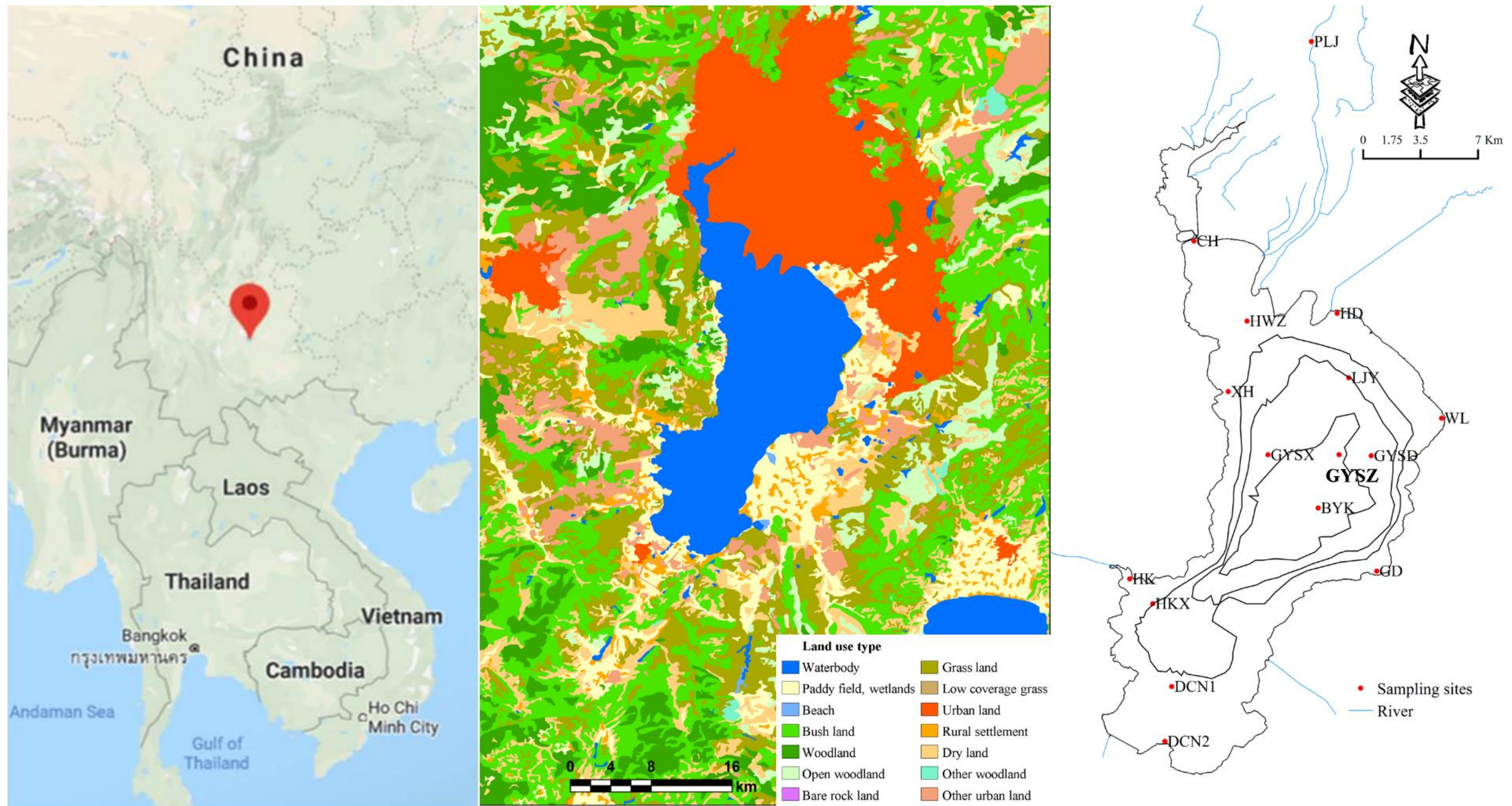

Figure 1. Location, land use and sampling sites (red dots; on right) within Dianchi Lake, China.

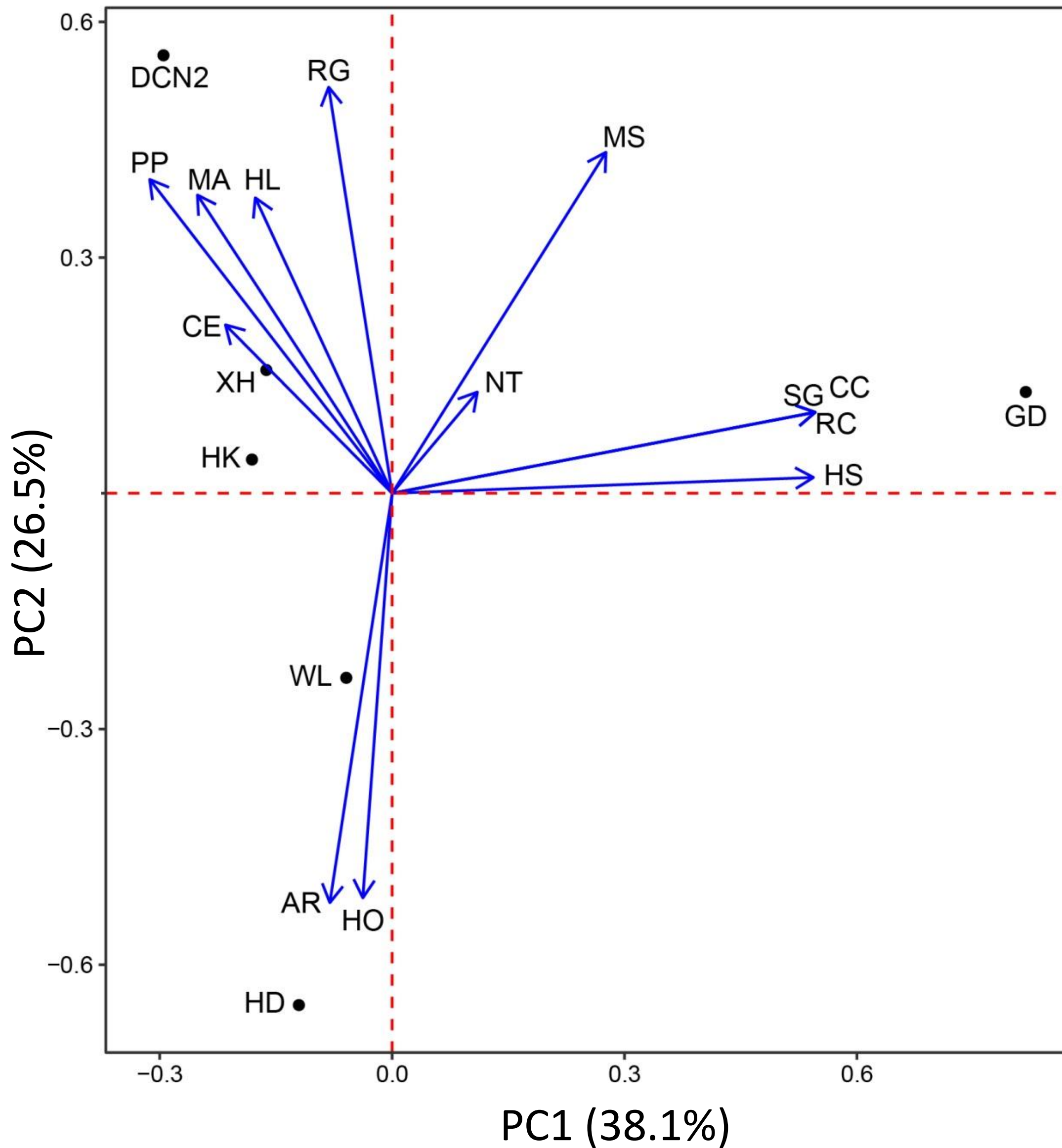

Figure 2. Principal Component Analysis (PCA) on the abundances of the 13 fish species across the six sampling sites in Dianchi Lake. Axes 1 and 2 described 38.1% and 26.5% of the total fish variation, respectively. SG: *Sinocyclocheilus grahami*; MA: *Misgurnus anguillicaudatus*; RC: *Rhinogobius cliffordpopei*; RG: *Rhinogobius giurinus*; NT: *Neosalanx taihuensis*; CE: *Cultrichthys Erythropterus*; HL: *Hemiculter leucisculus*; MS: *Micropercops swinhonis*; CC: *Cyprinus carpio*; HO: *Hypomesus olidus*; HS: *Hemiramphus sajori*; AR: *Abbottina rivularis*; PP: *Pseudorasbora parva*.

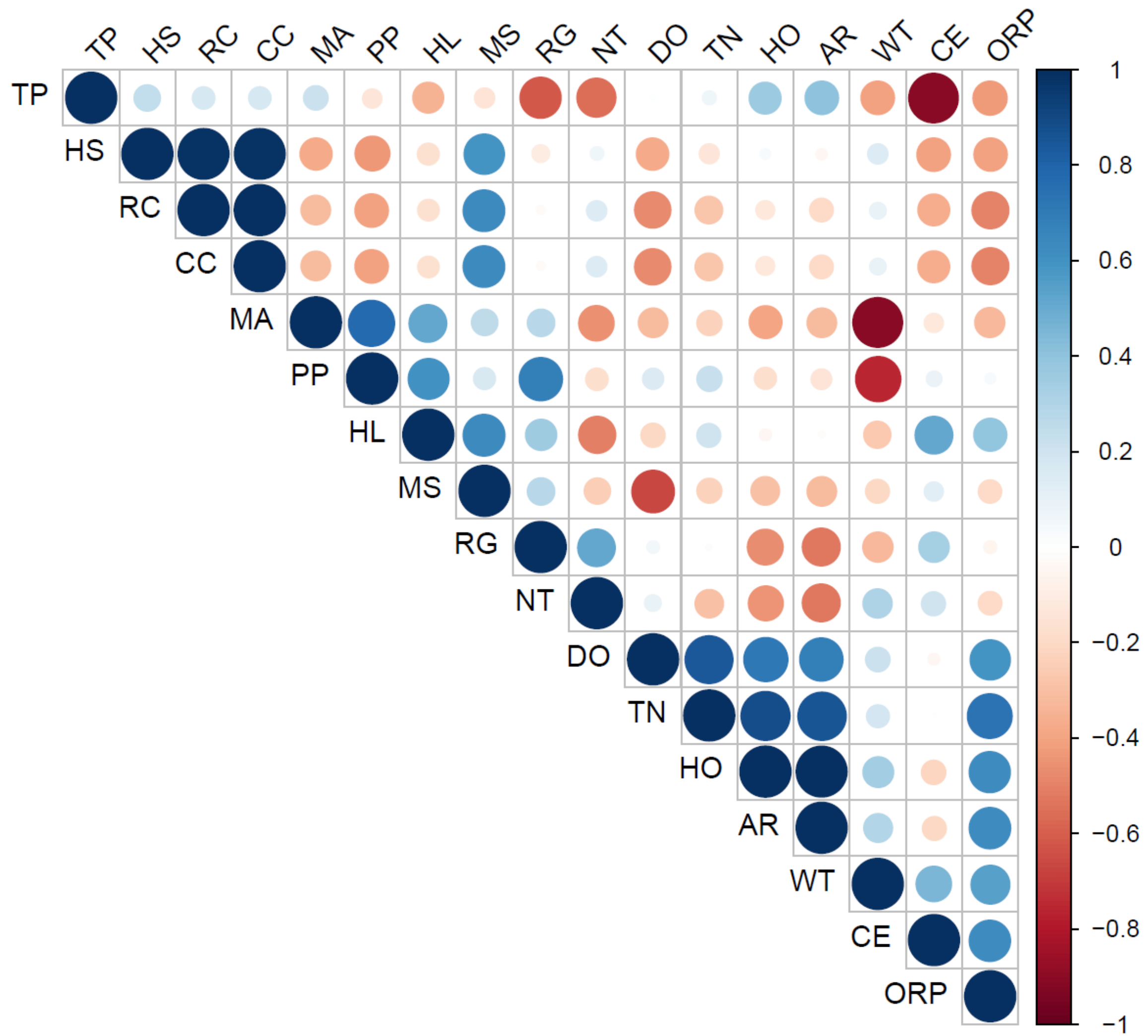

Figure 3. Kendall correlation coefficient on the relationships between the 13 fish species and the five environmental factors across the six sampling sites in Dianchi Lake. Refer to Table 2 for environmental factor abbreviations (blue) and Figure 2 for species name abbreviations.

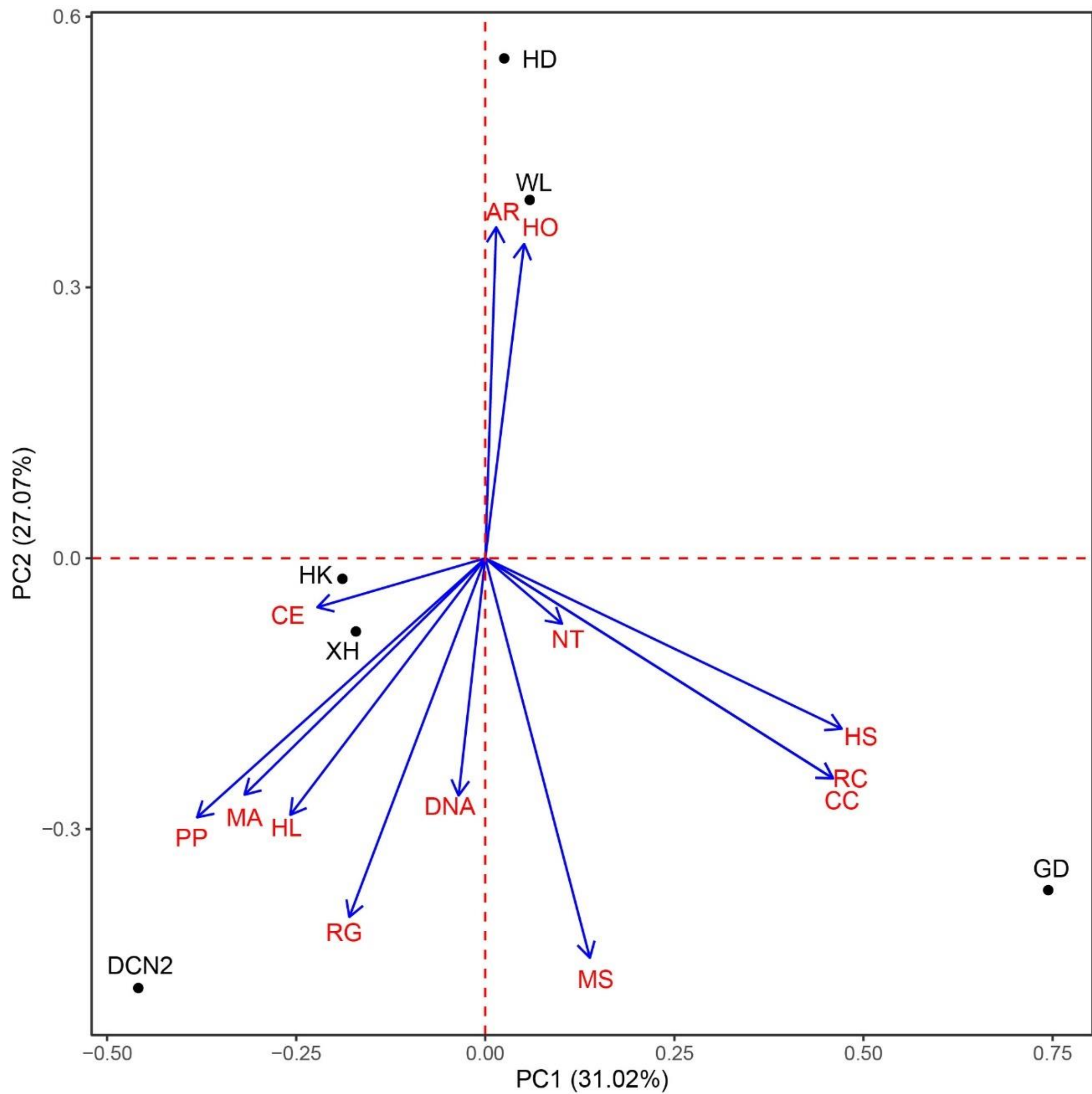

Figure 4. Principal Component Analysis (PCA) on the DNA concentration of *Sinocyclocheilus grahami* (DNA) and other fish species across the six sampling sites in Dianchi Lake. Axes 1 and 2 described 31.02 % and 27.07 % of the total fish variation, respectively. Refer to Table 3 for environmental factor abbreviations.

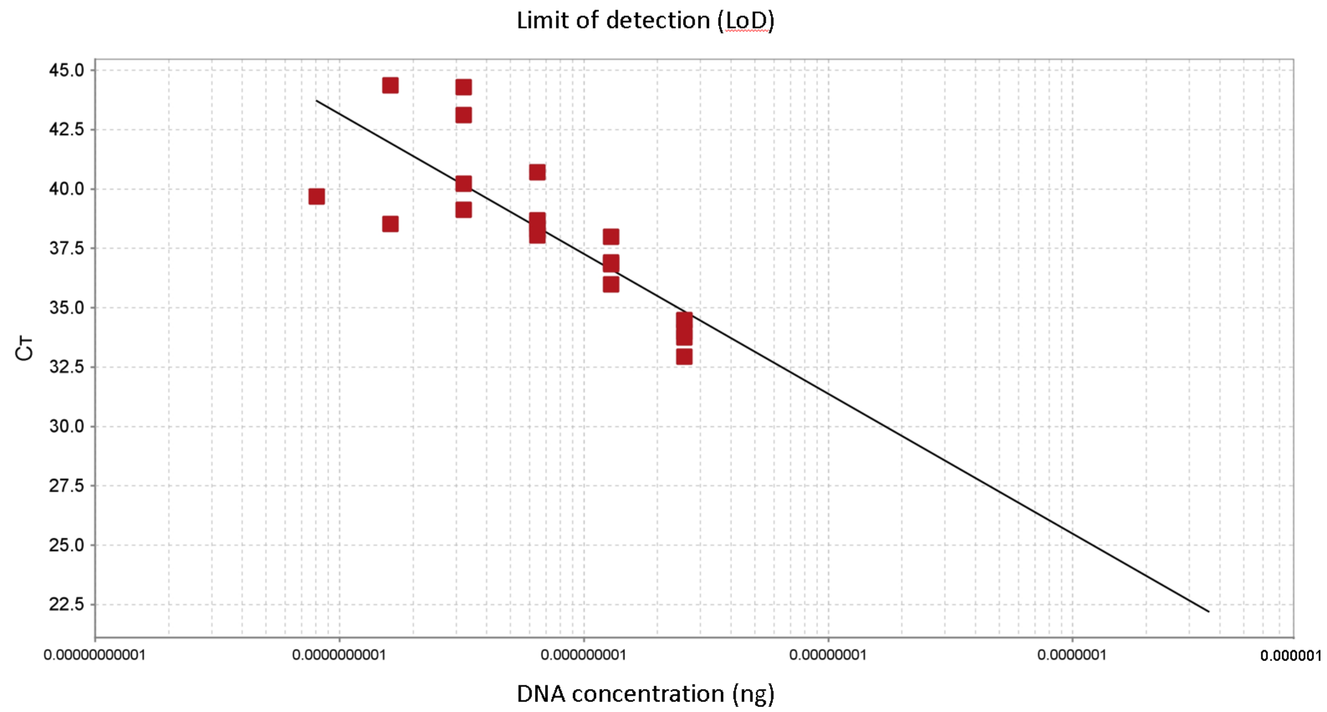

Figure 5. Limit of detection test based on 3-fold PCR template DNA dilution series using the new designed species-specific primer set. Limit of detection (LoD) is 1x 10^{-10} ng / µL.

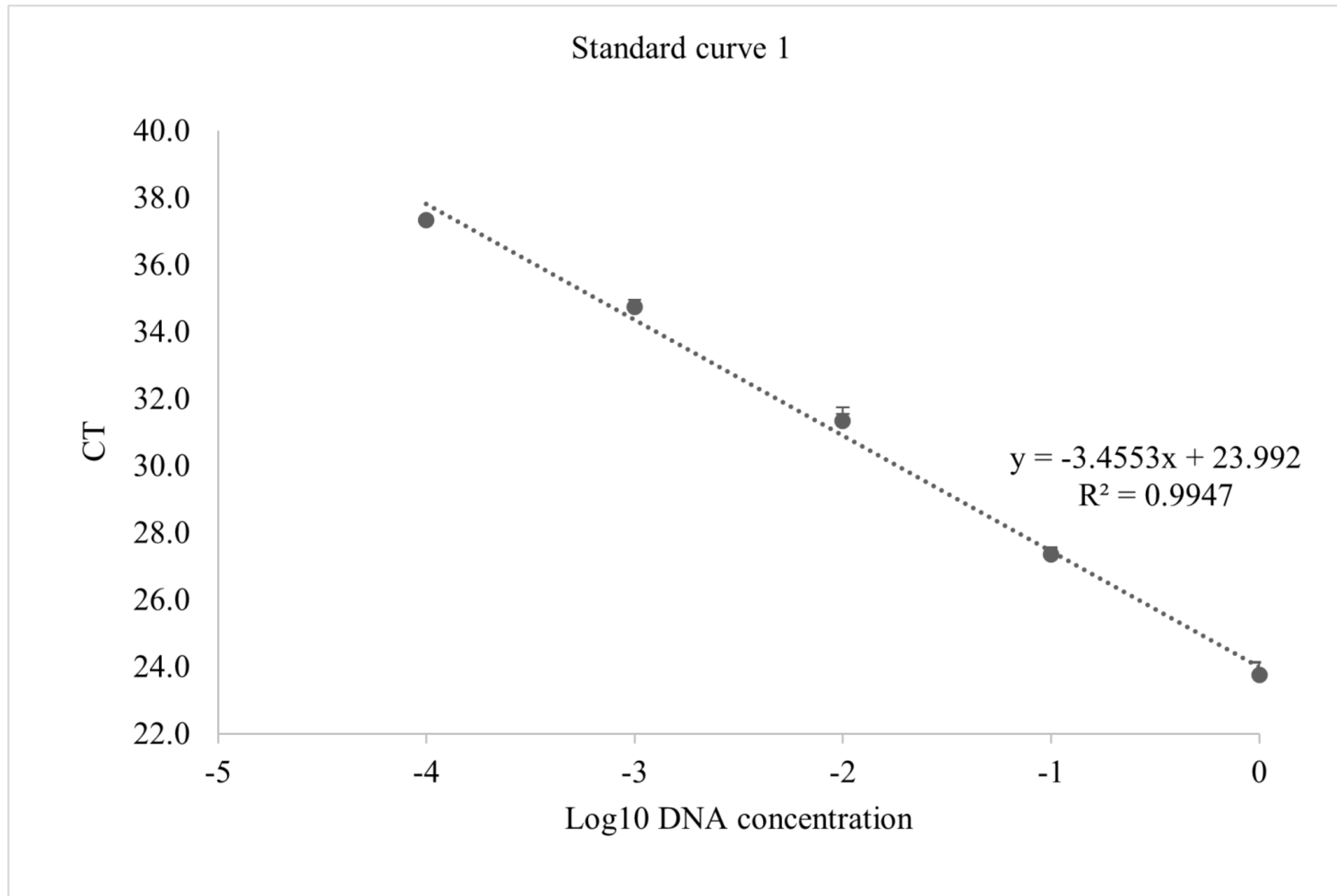

Figure 6. Standard curve 1based on 10-fold PCR template DNA dilution series using the new designed species-specific primer set. Limit of quantification (LoQ) is 38 cycles.

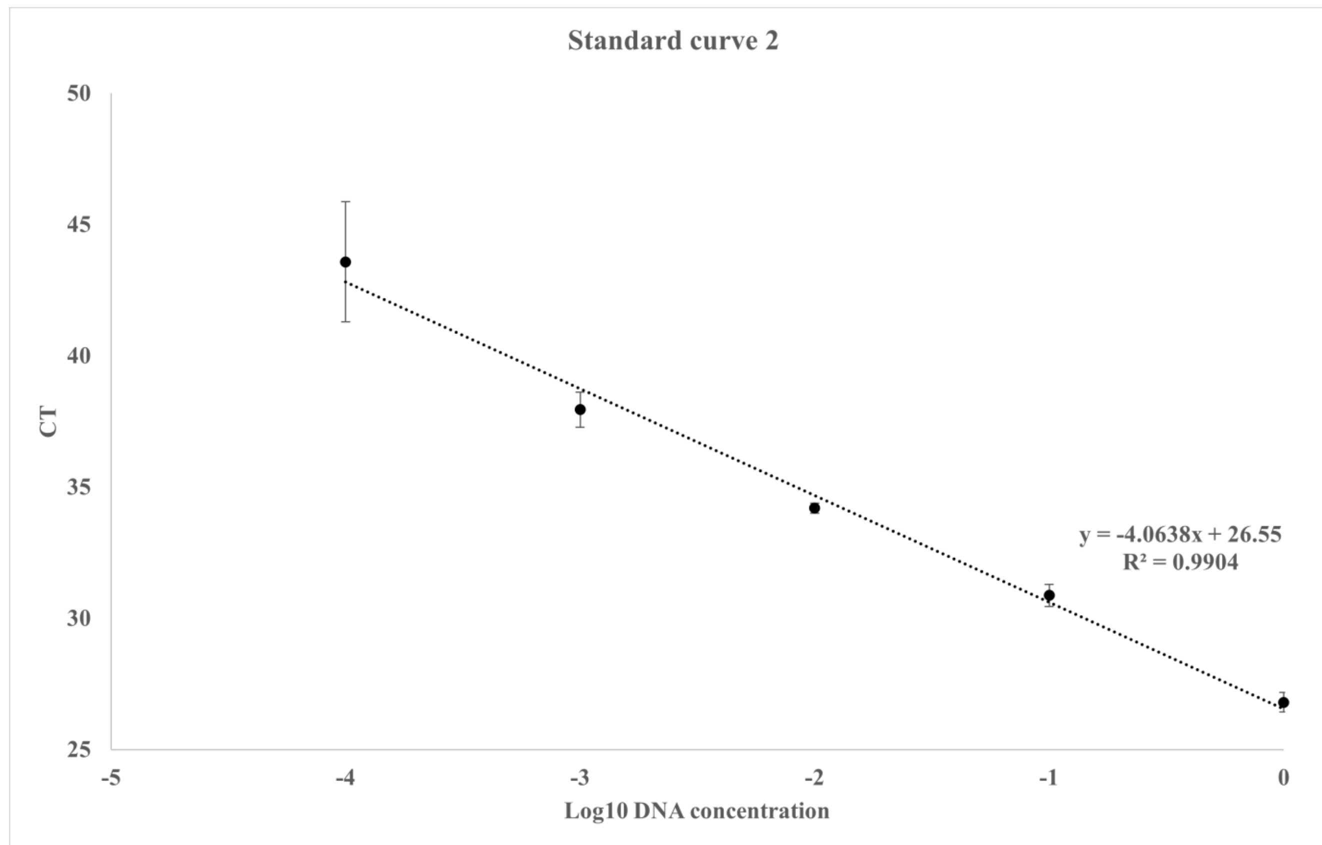

Figure 7. Standard curve 2 based on 10-fold PCR template DNA dilution series using the new designed species-specific primer set. Limit of quantification (LoQ) is 43.5 cycles.

CHAPTER 3. General Conclusions

The distribution of fish in Dianchi Lake is affected by multiple stressors such as habitat loss, eutrophication, pollution, predation and competition of introduced species (Wang et al. 2018; Gao et al. 2020). The importance of these factors has changed over time. Eutrophication and pollution have been controlled to a certain extent, but the nutrient load is still very large (Wu et al. 2018).

My study found that invasive and commercial species numerically dominate the Dianchi fish community (~97%), and the biodiversity of Dianchi lake has declined severely (over 70% native species loss). The protection of habitats and the interactions between species still need to be studied. At the same time, attention should be paid to the ecosystem consequences associated with the loss of biodiversity.

My research has demonstrated that *Sinocyclocheilus grahami*, once thought to be extinct, was detected at low abundance in the central and southern parts of the lake. This may be related to lower algal concentrations and lower intensity of human activity in these regions. It may also be due to the effectiveness of wetland protection in the south, especially the southeast coast. The specific primers designed for *Sinocyclocheilus grahami* in this study can be used in future studies, providing a new method for species identification and field conservation of this species that far exceeds traditional sampling methods. It is also possible that environmental DNA will contribute to fishery management and biodiversity conservation in the region.

It remains unclear if detected *Sinocyclocheilus grahami* are naturally occurring (established and reproducing) or the result of on-going stocking efforts. However, since the lake's fish community is so heavily biased toward introduced fish species, and the distribution of *Sinocyclocheilus grahami* is limited to the southern half of the lake,

continued restoration efforts for both critical habitats and populations of this fish must continue.

Local institutions and researchers - especially the team of Professor Junxing Yang - have done plenty of research on conservation of *Sinocyclocheilus grahami* and other local fish. This study demonstrates the seeming effectiveness these extensive captive breeding and population restoration programs for some species (Yang et al. 2007). Their breeding improvement program also provided a new way to commercialize *Sinocyclocheilus grahami*, a species of high economic value, thereby easing the conflict between protecting its wild population and exploiting its economic value.

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