Species diversity of drifting fish eggs in the Yangtze River using molecular identification

~~Liu~~ Mingdian Liu; ~~Wang~~ Dengqiang Wang; ~~Gao~~ Lei Gao; ~~Tian~~ Huiwu Tian; ~~Liu~~ Shaoping Liu; ~~Chen~~ Daqing Chen; ~~Duan~~ Xinbin Duan

(Yangtze River Fisheries Research Institute of Chinese Academy of Fishery Science~~, Scientific Observing and Experimental Station of Fishery and Environment in the Upper and Middle Reaches of the Yangtze River, Ministry of Agriculture~~, Wuhan, Hubei 430223, China)

ABSTRACT

Approximately 30 species of fish lay drifting eggs in the Yangtze River. However, recent dam construction threatens the viability of drifting eggs and hence, the reproductive behavior of these species. In this study, molecular methods were used to identify specific species ~~in order~~ to analyze the constitution of drifting fish eggs. A total of 8,983 fish eggs were collected from nine stations from 2014 to 2016, 7,933 of which were sequenced successfully. Thirty-nine fish species were identified~~y~~, and were assigned~~belonging~~ to four families and two orders. Approximately 64% of the species identified, and 67% of the eggs, were classified in the Family Cyprinidae. Abundance of eggs, as well as species richness, were higher in the main river than in tributaries of the river. However,~~ the~~ tributaries~~y rivers~~ may be important spawning grounds for some fish species. The~~ Mantel test between species, the~~ Jaccard's similarity index~~,~~ and river-way distances among sampled stations were negatively correlated suggesting strong~~showed strongly~~ native relationships. ~~These~~ Our data suggest that the environment shapes species composition in the sampled spawning grounds.

~~KEYWORDS~~

~~Species diversity; Fish egg; Drifting egg; Molecular Identification; Yangtze River~~

CORRESPONDING AUTHOR

~~Duan~~ Xinbin Duan, duan@yfi.ac.cn

INTRODUCTION

Monitoring fish spawning grounds in natural rivers provides useful information concerning fish habitat, spawning activity, and the effect of anthropogenic stressors. Data regarding spawning species, scale, and time are important for improving management and policy decisions.

The Yangtze River is the third largest river in the word. The section of the river above Yichang is generally called the "upper reaches" and the section from Yichang to Hukou is called the "middle reaches." In recent years, more dams have been constructed in the river, which seriously affect the spawning environment for fish. Such drastic ecological and environmental changes particularly affect fish that lay drifting eggs. There are approximately four hundred species of fish in the Yangtze River, and of these, approximately thirty species lay drifting eggs. Some of these fish are of commercial importance in China, such as grass carp (*Ctenopharyngodon idella*), black carp (*Mylopharyngodon piceus*), silver carp (*Hypophthalmichthys molitrix*), and bighead carp (*Hypophthalmichthys*, *nobilis*); these are referred to as the four domestic Chinese carps (FDC) in China. The FDC spawning activities are controlled by temperature and hydrographs (Stanley 1978; ~~Liu et al.1992;~~ Li et al. 2013), which shape more than 40 spawning grounds within mainstream regions of the Yangtze River. The mean annual egg production was over 100 billion in the 1960's (Yi~~h~~ and Liang 1964). The construction of the Gezhouba dam in the 1980s, and then the Three Gorges dam in the early 2000s have greatly changed the hydrologic conditions in the middle Yangtze River (Yi et al. 2010). These dam constructions significantly affected the spawning activities of indigenous river fish. The abundance of FDC larvae in the middle Yangtze River had decreased to 105 million in 2005 (Duan et al. 2009), and the spawning time was also delayed by approximately one month (Zhang et al. 2012). In addition, there are some species of fish that prefer certain river locations for spawning. For example, the largemouth bronze gudgeon, *Coreius guichenoti*, primarily spawns in the Lower Jinsha River, which is the main tributary of the Yangtze River (Cao et al. 2007). Impoundment of the Xiangjiaba dam in 2012 has destroyed the spawning ground of this species, and as a result no eggs or larvae have been found since then (Gao et al. 2015). If this species cannot find another suitable spawning ground, it is in danger of extinction (Cheng et al. 2015). Many tributaries of the Yangtze River are also distributed spawning grounds of fish that lay drifting eggs, and some of these fishes are endemic to one or several river sections (Xie et al. 2014; Cai et al. 2011; Wu et al. 2010).

Fishery resources in the Yangtze River have been undergoing a serious recession over the past 30 years (Chen et al. 2009; Huang & Li, 2016). In an attempt to remedy the situation, the government has implemented measures to restore fish resources and the ecosystem. Several parameters include setting up protected regions, closed fishing seasons, releasing artificial fish into the river, and restricting fishing instruments (Chen et al. 2009). Monitoring spawning grounds could assess whether these measures have been successful. During the spawning season there may be more than a dozen or more species of fish spawning in the same location (Xie et al. 2014; Cai et al. 2011; Wu et al. 2010). Different species are associated with different nursery habitats and dispersal during early life history stages, and also have different protection requirements. Thus, quantifying and classifying fish eggs is crucial for conservation, management, and assessment of environmental effects. It is challenging to determine the species of the drifting eggs because most of the fish with this reproductive pattern have similar morphological characters in the early egg stages. In the past, several researchers tried to identify eggs species by size, color, and oil droplets (Gao et al. 2010; Cao et al. 2007; Yi et al. 1988), but they~~ and ~~ ~~couldn't~~were not able to obtain enough diagnosable information at species-level, because the egg size was variable and overlapping among most fishes, and the color was not so clear. Others tried to hatch eggs to identify distinguishable characteristics (Xu et al. 2015; Liu et al. 2014), however, it proved difficult to hatch eggs in the field.

DNA–based species identification is a rapid method to distinguish different species ~~from ~~among various samples~~,~~ and has recently been used as an effective tool for species identification of fish eggs and larvae (Baldwin et al. 2011; Wibowo et al. ~~2015~~2017; Harada et al. 2015). In comparison to morphological diagnosis, DNA identification has many advantages, including sample preservation, finding cryptic and rare species, and large-scale surveys (Lewis et al. 2016; Harada et al. 2015; Gleason ~~& ~~and Burton, 2012). ~~In the recent~~Recently, molecular methods have~~s~~ been used ~~to~~for the delimitation of various taxonomic groups of fishes (Hubert et al.2010; Costa et al. 2012; Landi et al.2014; Frantine-Silva et al. 2015). It also has been successfully applied ~~for~~during the identification of fish eggs and larvae ~~study on ~~biodiversity and spawning activities'studies~~y~~ in habitat (Harada et al. 2015; Lewis et al.2016; Ardura et al. 2016; Thompson, 2016). In this study, we have applied DNA techniques for identification of drifting fish eggs sampled from several spawning grounds in the Middle and Upper Yangtze River. The objective was to i) test identification efficiency for drifting eggs in the Yangtze River, and ii) to investigate species composition and distribution in different spawning grounds.

MATERIALS AND METHODS

Sampling location and egg collection

May to July of each year is the spawning season for most fish in the Yangtze River. During this period in 2016, nine stations were set up to collect drifting fish eggs. These were the Yangtze River Panzhihua section (YPZ), the Yangtze River Jiangjin section (YJJ), the Yangtze River Yidou section (YYD)~~-~~, the Yangtze River Jingzhou section (YJZ), the Yangtze River Sanzhou section (YSZ), the Yangtze River Yanwo section (YYW), the Minjiang River Yibin section (MYB), the Chishui River Chishui section (CCS) and the Xiangjiang River Yingtian section (XYT) (Fig.1). Among these stations, the four stations of YPZ, YJJ, CCS and MYB were located in the upper reaches and the five stations of YYD, YJZ, YSZ, YYW and XYT in the middle reaches of the Yangtze River. Three stations, MYB, CCS and XYT were located in tributary rivers, and the other six stations were located in the main stream of the Yangtze River. Eggs were collected from YJJ in 2014 and 2015, and from YSZ and YYW in 2015. These collections were also used in this study.

Collecting nets and methods were described as Duan et al. (2009). Trap nets were put down in water at 6:00 to 9:30 and 16:00 to 19:00 every day during spawning ~~season, and~~season and retrieved every 15 minutes. Each egg was recorded for morphological traits, such as developmental stage and size, and then preserved in a 2 mL EP tube with absolute ethanol.

The sampling work has been approved by the local related management departments named fishing administration supervision and administration station. This study is fully complied with the relevant laws and ethics of the country.

DNA extraction, PCR

Ethanol was decanted off specimens and eggs were soaked in 1.5 mL ddH_2O for 1 h. Water was then removed with a pipette, and egg genomic DNA was extracted using an easy-DNA Kit (Omega, Connecticut, USA).

Mitochondrial (mt) cyt *b* gene was used as a molecular marker for species identification. The forward primer was L14322:5′-GAC TTG AAG AAC CAC CGT TGT TAT TCA AC-3′and the reverse primer H15576: 5′-GCG CTA GGG AGG AAT TTA ACC TCC-3′. PCR reactions had a final volume of 25 µl and contained 2.5 µl 10 x PCR Buffer (TaKaRa, Dalian, China), 0.2 µl of 10 mmol/L dNTPs, 1 µL of each 10 µmol/L primers, 0.5 u Taq enzyme (TaKaRa, Dalian, China), 1 µl of template DNA. ~~L14322:5′-GACTTGAAGAACCACCGTTGTTAT TCAAC-3′and the reverse primer was H15576: 5′-GCGCTAGGGAGGAATTTAAC CTCC-3′. PCR reactions had a final volume of 50 µl and contained 5µl 10 x PCR Buffer (TaKaRa, Dalian, China) , 0.4 µl of 10 mmol/L dNTPs, 2 µL of each 10 umol/L primers, 1.5 µl Taq enzyme (TaKaRa, Dalian, China), 1 µl of template DNA.~~ The PCR reaction cycles were as follows: Pre-denaturation at 94 ℃ for 4 min, 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 54 ℃ for 30 s, extension at 72 ℃ for 90 s, and a final extension at 72 ℃ for 8 min.

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PCR products were checked via 1.5% agarose gel electrophoresis and visualized with ethidium bromide to verify successful amplification. PCR DNA bands with expected size were purified using Cycle-Pure Kit (Omega, Connecticut, USA) and sequenced with the same primers as PCR (Tianyi biotech. Co. Ltd, Wuhan, China).

Data analysis

All sequences were aligned and trimmed to the same length using Muscle (Edgar,2004) in MEGA X (Kumar et al. 2018)~~Clustal X (Thompson et al., 1994)~~. Sequence properties of indels, frameshift mutations, in-frame stop codons were careful examined to eliminate presence of pseudogene or nonfunctional sequences. The genetic distance based on Kimura 2-parameter (Kimura, 1980) between the individuals were calculated using MEGA X –(Kumar et al. 2018)~~MEGA v6.0 (Tamura et al. 2013)~~ and a neighbor-joining tree (NJ tree) (Saitou and Nei, 1987) was constructed with 1000 bootstrap replicates (Felsenstein, 1985). Sequences with 2% or lower of genetic distance and clustered as a monophyletic group in the NJ tree will be assigned to an operational taxonomic unit (OTU) and considered as a species. One or two sequences from each OTU were selected to compare with nucleotide sequences from GenBank (National Center for Biotechnology Information, NCBI) with the standard nucleotide BLAST (Basic Local Alignment Search Tool). Eggs were associated with a given species only if the similarity values were 98% or greater.

Species diversity of spawning ground was characterized using Shannon-Wiener diversity index (Schoener 1968). The Shannon-Wiener index is calculated as:

$$H' = -\Sigma\, Pi \ln Pi$$

Where pi is the proportion of species in total samples (egg numbers).

Similarities of species component between spawning grounds were evaluated by Jaccard's similarity index (JI) (Real and Vargas, 1996) using the formula JI = a/(a+b+c)~~-~~, where 'a' is the number of species shared by two spawning ground, and 'b' and 'c' are the numbers of species specific~~private species~~ in each spawning ground. Correlation between JI and the river-way distance was determined by the Mantel test with 1000 random permutations performed in Arle~~i~~quin v3.1 (Excoffier & Lischer 2010).

RESULTS

Species identification and diversity

Overall, 8,983 drifting eggs were recovered from the nine stations, of which 7933 were sequenced successfully. All sequences were trimmed to the same length with 605 bp. Comparing with sequences of adults and GenBank data, a total of 39 species were identified, representing 28 genera, 4 families, and 2 orders (Table 1). Approximately 64% of the species and 67% of eggs belonged to the Family Cyprinidae. In addition, 26% of the species and 28% of eggs were from the Family Cobitidae. The most commonly distributed species were the silver carp (*H. molitrix*), grass carp (*C. idellus*), silver gudgeon (*S. argentatus*) and Yichang gudgeon (*G. filifer*~~-~~). No fish species were found in all sampled stations.

The spawning ground with the ~~most~~ highest species diversity was YJJ with 27 species identified, ~~there~~ from 2014–2016. The next were YYD and YJZ, with 20 species found in both. The Shannon-Wiener (H) diversity index of these three spawning grounds were greater than 2.0, and the other five were under 1.9. Stations with the lowest~~least~~ species diversity were YPZ, having 8 species.

Species similarity among sites

Pairwise Jaccard's similarity index and river-way distances among sampled stations are listed in Table 2. There are only one or two identical species among 19 to 28 species between YPZ and the four stations in the Middle Yangtze River (YYD, YJZ, YSZ, and YYW) and XYT station in the Xiangjiang River, which obtained the least JI values, ranging from 0 to 0.0952. However, JI values among the four stations in the Middle Yangtze River had the highest values, ranging from 0.5652 to 0.7391.

A Strongly negative correlation was found between the JI and river-way distance (Mantel test, R=-0.9003).~~The Mantel test between the JI and river-way distance showed a correlation coefficient of -0.9003, indicating strongly negative correlation between them.~~

DISCUSSION

In previous studies, Cao et al. (2007~~8~~) and Li et al. (2013) ~~presented~~ have shown that approximately 25 species of fish ~~that~~ lay drifting eggs in the Yangtze River. Here we identified 39 species from captured drifting fish eggs in seven sampled stations using the mitochondrial Cyt *b* gene sequences. All obtained sequences had 98% identity with sequences in GenBank, indicating that reference data of the fish are available. Thus, fish eggs can successfully be identified to the species level. Intraspecific similarity of Cyt *b* gene sequence was greater than 98%, and interspecific distance higher than 2% for all these fish species. These studies indicate that mitochondrial sequence data~~molecular methods~~ are a power tool of species identification of drifting eggs in the Yangtze River, and ~~also~~ may also contribute to identify~~ing~~ rare or endangered species of fish. For example, some fish such as *Saurogobio gymnocheilus*, *Botia reevesae*, *Paracobitis potanini,* and *Jinshaia abbreviata*, have had a low catch rate in recent years, but were detected in one or two spawning grounds in this study (Table 1). This observation may suggest that there are also smaller populations living in the river. However, two common fish species that lay drifting eggs in the Upper Yangtze River, *C. guichenoti* and *Rhinogobio ventralis* (Xiong et al. 2014; Liu et al. 2010; Xiong et al. 2016~~-~~), were not found in this study. This may indicate that their spawning grounds were not in our monitoring stations. In addition, two recorded Leuciscin carps laying drifting eggs, *Ochetobius elongatus* (still found in Pearl River) and *Luciobrama macrocephalus* in the middle Yangtze River, were also not found from analysis of eggs. Since they have been not ~~been~~ captured in recent years it is possible that these species are ~~possibly~~ endangered to extinction in the Yangtze River.

Several species from the *Culters* genus were found in the collected eggs, such as *Culter alburnus*, *Culter mongolicus,* and *Cultrichthys erythropterus*. Interestingly, these are not typical fish that lay drifting eggs. They generally spawn weakly viscous eggs among water plants or in river gravel~~,~~ and can also spawn in still water (Cao et al. ~~2008~~2007). They were detected in the YJJ station due to falling off from plants or gravels by river torrents. *Glyptothorax sinensis* which is distributed in the Yangtze River and its tributaries and lakes, also produces viscous eggs not drifting, and their eggs usually adhere to stones in the water~~*Glyptothorax sinensis* also produces viscous but not drifting eggs that adhering to stones in the river distributed in the Yangtze River and its tributaries and lakes~~ (Ding, 1994; Chu et al. 1999). Two eggs of *G. sinensis* were found in the YPZ station, where the current velocity could be greater than 2 m/s (Xu et al. 2013).

The negative correlation between species similarity and river way distance reflected the diversity of ecological conditions in the Yangtze River. The nearer river stations have similar ecological characteristics, such as temperature, current velocity, and water flow, which attract more of the same fish to spawn. There are distinct differences in hydrology between the middle and upper reaches of the Yangtze River. The river is narrow, swift, and with a steep fall in elevation in the upper reaches compared with the wider, slower, and gradual fall in the middle reaches. Ecological differences shape the specificity of fish in the spawn ground. Six species, including *Squaliobarbus curriculus*, *Elopichthys bambusa*, *Parabramis pekinensis*, *Pseudobrama simoni*, *Xenocypris argentea* and *Parabotia banarescui* are only found

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in the YYD, YJZ and/or YSZ, and YYW stations, consistent with their endemic presence in the middle Yangtze River. The upper Yangtze River involved fourteen species, including *S. gymnocheilus*, *Hemiculterella sauvagei*, *Xenophysogobio boulengeri*, *Xenoph~~l~~ysogobio nudicorpa*, *Leptobotia rubrilabris*, *Leptobotia microphthalm~~rn~~a*, *Leptobotia elongata~~-~~*, *B. reevesae*, *Botia superciliaris*, *P. potanini*, *Jinshaia sinensis*, and *J. abbreviate*. Adults of *L. elongate*, the largest fish in the Family Cobitidae, were also found in the middle Yangtze River, but no eggs were detected in stations there. Larvae of *L. elongate* born upstream should have crossed the Three Gorges Dam to the middle reaches of the river.

Species diversity of the YJJ station was shown to be the richest, and it harbored not only some importantly economic fish, such as FDC, also some unique fish, such as *S. gymnocheilus* (Table 1). This river reach is a very important spawning ground for fish laying drifting eggs. The numbers of species in the tributary river is less comparing with the Main River, but the rivers could be important spawning grounds for ~~special~~ particular species. For example, the number of the small eye loach, *L. microphthalm~~rn~~a* accounted for more than 86% of the total eggs in the MYB station, where in down reaches of Minjiang River. In the CCS station, located in the Chishui River, the species and egg numbers of species in the Family Cobitidae accounted for 50% and 36% of the total captures, respectively.

Though spawning and egg development of most of the fish identified in this study rely on running water, dependency levels were different. For example, FCD are apt to spawn in sudden floods (Duan et al. 2009, Li et al. 2011). However, spawning activity of *G. filifer* is negatively correlated with water discharge and water velocity (Tian et al. 2017). Understanding the composition of fish species at each spawning ground helps to assess the impact of environmental changes on fish, and molecular methods could be a useful tool for conducting this task effectively~~the work~~.

Our study presented a highly effective approach for rapid species identification of drifting eggs in Yangtze River. Our success ~~, and it~~ should be due to a great availability of reference sequences ~~data obtained~~ in Genbank, which allowed the identification of a great proportion of the fish species in the current study. Only a ~~A~~ small percentage of collected eggs were not able to be sequenced (approximately 11%)~~-~~, which can be attributed to several errors along the path from collection to sequencing, such as improper egg preservation, DNA extraction and PCR failure. For a ~~large scale~~large-scale survey, the lab time as well as amplification and sequencing costs cannot ~~to~~ be ignored. Using high-throughput pipelines as an automatization of egg sorting, and a 96-well system for DNA extraction and for PCR amplification (Hofmann et al. 2017) and/or multiplex arrays (Gleason and Burton, 2012) is recommended. Another problem using a single gene of mtDNA for species identification is presence of heteroplasmy or incomplete lineage sorting within or among some species, which lead to aborted identification at species-level (Hubert et al.2008; April et al. 2011; Shen et al. 2016~~-~~). Using more loci, for example COI, control region and ITS of rDNA, can improve the success rate of identification.

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CONCLUSIONS

Mitochondrial DNA sequence is a powerful and effective tool for identifying species of drifting fish egg in Yangtze River. Species diversity in main river is higher than that in tributaries~~y~~, however some fish species prefer to spawn in tributaries.

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~~The sampling work has been approved by the local related management~~

~~departments named fishing administration supervision and administration station. This study is fully complied with the relevant laws and ethics of the country.~~

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Fig.1 Location of nine sample stations (YPZ, MYB, CCS, YJJ, YYD, YJZ, YSZ, YYW, XYT) and species composition in Family

1-Gezhouba Dam, 2- Three Gorges Dam, 3- Xiangjiaba Dam