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RESEARCH ARTICLE

Genetic diversity and population structure of *Hemibagrus guttatus* (Bagridae, Siluriformes) in the larger subtropical Pearl River based on COI and Cyt *b* genes analysis

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Abstract – Understanding the genetic diversity and population structure of fish species is crucial for the sustainable use and protection of fish germplasm resources. *Hemibagrus guttatus* (Bagridae, Siluriformes) is widely distributed in the large subtropical Pearl River (China) and is commercially important. It's population have been declining. The genetic diversity of wild *H. guttatus* is not clear, despite its important ecological significance. In this paper, genes mitochondrial cytochrome c oxidase subunit I (COI) and cytochrome b (Cyt *b*) were used to analyze the genetic structure of *H. guttatus* population collected from six geographical populations in the main streams of the Pearl River. The results showed that the nucleotide diversity (π) and haplotype diversity (Hd) of wild *H. guttatus* was low ($\pi < 0.005$; Hd < 0.5). In addition, *H. guttatus* haplotypes did not cluster into clades according to geographical distribution, as revealed by neighbor-joining tree analysis. Analysis of molecular variance analysis (AMOVA) and F-statistics (F_{st}) values showed high homogeneity among wild *H. guttatus* populations. Our results suggest that there is degradation in germplasm resources of *H. guttatus* that could destabilize the sustainable use of this species and there was an urgent need for conservation of this species in South China.

Keywords: Pearl River / *Hemibagrus guttatus* / genetic diversity / COI gene / Cyt *b* gene

1 Introduction

The Pearl River is a large subtropical river in southern China, stretching some 2400 km. It originates from Maxiong Mountain of Yunnan province, and flows into the South China Sea. This river is not only a hot spot of global biodiversity research, but is also an important gene pool of aquatic biological resources, due to its high habitat heterogeneity (Shuai *et al.*, 2017). However, due to human disturbances, such as over-fishing and the construction of dams in recent years, fish migratory paths have been obstructed and spawning grounds have disappeared, which has resulted in a sharp degradation of fish germplasm resources. *Hemibagrus guttatus* belongs to the family Bagridae fishes, they are benthic and settled fishes with relatively weak migration capability (Chu *et al.*, 1999). This kind of lifestyle make the long distance migration hard to realize for them, therefore, *H. guttatus* is not a wildly distribute species and mainly found in the Pearl River basin. *H. guttatus* is a commercially important fish in southern China and has earned a reputation as the "king of freshwater fish" due to its high nutritional value (Kottelat, 2001). Nevertheless, in recent decades, *H. guttatus* population declined sharply due to over-fishing, water pollution, and hydraulic constructions and led to a degradation of their germplasm resources. Therefore, it is particularly important to study the genetic diversity of wild *H. guttatus* in the Pearl River (World Commission on Dams, 2000).

However, up to now, only a few studies on the genus *Hemibagrus* have been conducted, such as the study of diversity and evolutionary rate of bagrid catfish (including *Hemibagrus*, *Pseudobagrus*, *Pelteobagrus* and *Leiocassis*) (Peng *et al.*, 2002; Ku *et al.*, 2007), the research of the phylogeny and biogeography of *H. guttatus* (Yang and He, 2008) and the work of genetic diversity of *Hemibagrus macropterus* populations (Yang *et al.*, 2009). The current genetic diversity of *H. guttatus* in the river basins of southern China is still unknown, despite its important ecological significance and its significance for conservation of fishery resources.

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Table 1. Sampling information of *H. guttatus*.

Site	Population	Latitude and longitude	River name	Sample number	Group
S1	Lubuge	104°31′45″E, 24°44′46″N	Beipanjiang	24	1 (Upstream)
S2	Baiceng	105°47′23″E, 25°22′54″N	Nanpanjiang	24	
S3	Heshan	110°04′19″E, 23°24′16″N	Hongshuihe	9	2 (Middle stream)
S4	Guiping	110°53′6″E, 23°21′46″N	Xijiang	25	
S5	Tengxian	112°27′33″E, 23°4′54″N	Xijiang	29	3 (Downstream)
S6	Zhaoqing	110°04′20″E, 23°24′15″N	Xijiang	17	

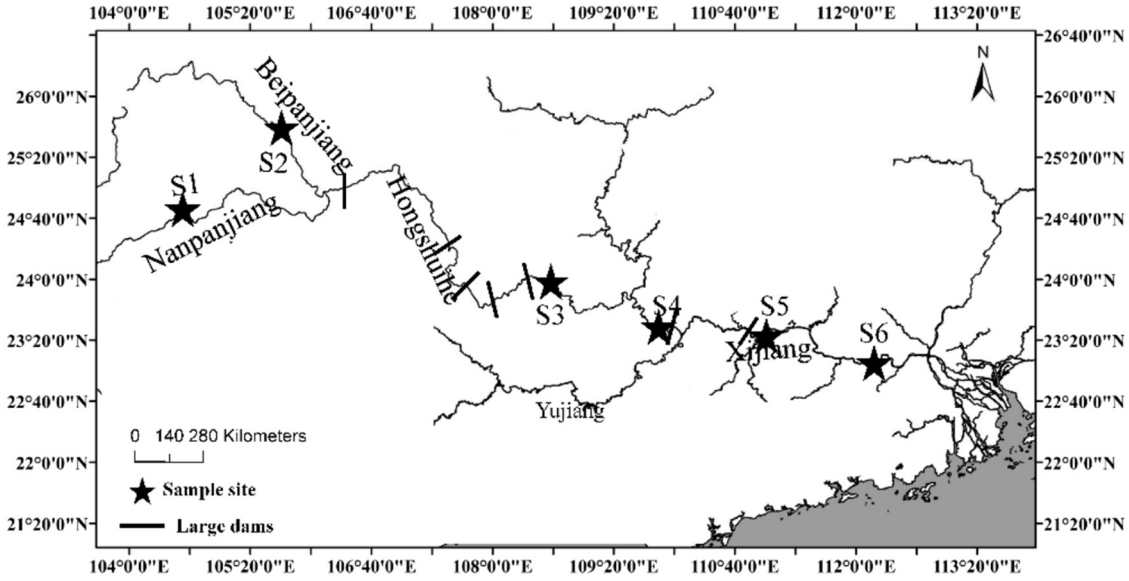

Fig. 1. Sampling locations of *H. guttatus*.

Mitochondrial genes are maternally inherited (Haye *et al.*, 2010). The evolutionary rates of mitochondrial cytochrome c oxidase subunit I (COI) and mitochondrial cytochrome b (Cyt *b*) genes are relatively moderate (Subramanian *et al.*, 2009), which makes them ideal molecular markers for the study of intraspecific genetic diversity in aquatic organisms (Beenaerts *et al.*, 2010; Haye *et al.*, 2010; Sun *et al.*, 2012). The monitoring of germplasm resources of the wildlife in the Pearl River basin should focus on the genetic level, instead of the number of individuals. Previous study have reported that the *H. guttatus* haplotypes identified base on Cyt *b* gene from the Wast river, North river and East River were very common and similar, indicated that *H. guttatus* from the Pearl River System was highly homogeneous (Yang *et al.*, 2008). In this study, we combined the two genes (COI gene and Cyt *b* gene), with longer Nucleotide base sequence, to improve the sensitivity to analysis the genetic diversity of *H. guttatus*.

The purpose of this study was to understand the genetic diversity and population structure of the *H. guttatus* in the Pearl River. Our results will provide a scientific basis for the conservation and management of commercial fish germplasm resources in the Pearl River.

2 Materials and methods

2.1 Sampling

In this study, a total of six sampling sites were set up, covering almost the entire mainstream of the Pearl River. The average interval between sampling sites was about 200 km. Detailed information and distribution of the sampling sites are shown in Table 1 and Figure 1. A total of 127 *H. guttatus* individuals were collected by hook fishing. The pectoral fins or tail fins were cut for COI and Cyt *b* genes analysis.

2.2 DNA extraction, amplification, and sequencing

Fin tissues (2 mg) were cut into fine pieces for total DNA extraction, using standard phenol/chloroform extraction method (Sambrook *et al.*, 2001). A 606 bp fragment at the 5' end of the mitochondrial COI gene was amplified using the universal primers:

FishF: 5'-TCAACCAACCACAAAGACATTGGCAC-3'; and

FishR: 5'-TAGACTTCTGGGTGGCCAAAGAATCA-3'.

A 1005 bp fragment at the 5' end of the mitochondrial Cyt *b* gene was amplified using the universal primers:

L14724: 5'-GACTTGAAAAACCACCGTTG-3'; and

H15915: 5'-CTCCGATCTCCGGATTACAAGAC-3' (Xiao *et al.*, 2001; Sun *et al.*, 2012).

The PCR reaction was prepared for a final volume of 40 uL, containing 4 uL of 10X buffer, 2 uL of dNTPs (10 mM), 1 uL each of upstream and downstream primers (20 uM), 2 uL of Taq polymerase (5 U), 2 uL of genomic DNA as template; ultrapure water was added to the total volume of 40 uL. The PCR program was as follows: pre-denaturation at 95 °C for

Table 2. Genetic diversity indexes in *H. guttatus* populations based on COI and Cyt *b* gene sequences.

Population	Sample number	Haplotype number	Unique haplotype number	Haplotype diversity (Hd)	Nucleotide number (π)	Nucleotide differences (k)
Lubuge	24	4	1	0.435 ± 0.119	0.00030 ± 0.00009	0.478
Baiceng	24	3	1	0.163 ± 0.099	0.00010 ± 0.00006	0.167
Heshan	8	4	2	0.643 ± 0.184	0.00109 ± 0.00055	1.750
Guiping	25	8	3	0.490 ± 0.123	0.00045 ± 0.00014	0.720
Tengxian	29	6	2	0.426 ± 0.113	0.00041 ± 0.00013	0.665
Zhaoqing	17	5	1	0.691 ± 0.075	0.00061 ± 0.00014	0.985
Total	127	15	10	0.465 ± 0.054	0.00042 ± 0.00007	0.680

3 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min, and then a final extension at 72 °C for 10 min. Amplification products were analyzed by 1% agarose gel electrophoresis and sent to Guangzhou IGE Biotechnology Ltd for sequencing. Sequencing primers were same as the amplification primers.

2.3 Data analysis

Seqman software was used to align the forward and reverse sequences. All the sequences were multiply aligned by Clustal X software (Thompson *et al.*, 1997). The sequences nucleotide composition was analyzed using MEGA 7.0 software (Tamura *et al.*, 2011). The genetic distance was calculated based on the Kimura two-parameter (K2P) model. Phylogenetic trees of individuals and haplotypes were constructed using the neighbor-joining (NJ) method (Nei and Kumar, 2000), whose confidence was tested by 1000 bootstrap replicates. In addition, a median-joining network of all haplotypes was constructed using Network 4.6 software (www. fluxus-engineering.com). Arlequin 3.5 software was used to estimate the genetic differentiation coefficient (F_{ST}) between populations based on a pairwise difference model (Garcia *et al.*, 2003; Excoffier and Lischer, 2010). Analysis of molecular variance (AMOVA) was performed on populations of *H. guttatus* at two different scales using Arlequin software. For one scale, the six populations of *H. guttatus* were combined into one group to verify if there were significant genetic differences among the six populations. For the other scale, the six populations were divided into three groups according to their geographical location (upstream, middle stream, and downstream), to verify whether there were significant genetic differences among the three groups.

3 Results

3.1 Information of base composition and variable sites

A total of 127 *H. guttatus* individuals from six geographical populations in the mainstream of the Pearl River were analyzed based on a 1611 bp sequences, which included a 606 bp fragment at the 5' end of the mitochondrial COI gene, and a 1005 bp fragment at the 5' end of mitochondrial Cyt *b* gene. The average content of A, T, G and C in the COI and the Cyt *b* gene sequence was 28.6%, 31.5%, 14.9% and 25.1%, respectively; which shows an AT bias (AT 60.1%, GC 39.9%). The haplotype number in all populations was 15. The haplotype diversity index was 0.465 ± 0.054. The nucleotide diversity index was 0.00042 ± 0.00007. The indexes of genetic diversity of each geographical population are shown in Table 2.

3.2 Haplotype distribution and haplotype analysis of COI and Cyt *b* genes sequence

No insertions or deletions were found in the COI and Cyt *b* gene sequences. There were 19 mutation sites, including 12 single mutation sites and seven parsimony-informative mutation sites. Mutation sites 544, 1591, 1366, and 1399 were at the first position of the codons, mutation sites 320 and 692 were at the second position of the codon, and the rest of the mutation sites were at the third position of the codons (Tab. 3).

Genetic structure analysis showed that the bootstrap values for most node branches in the NJ phylogenetic tree of haplotypes were higher than 50%. The haplotypes of populations were widely distributed in the NJ phylogenetic tree of haplotypes without a structure that reflected geographical distribution (Fig. 2).

Nineteen polymorphic sites, defined 15 haplotypes, were found on the COI and Cyt *b* gene sequences. Haplotype H1 occurred most frequently, accounting for 92.97% of all individuals; it was found in the six geographical populations. The second most common haplotype was H3, it was found in all the geographical populations, except in Heshan. Haplotypes, H2, H5–7, H9–11, and H13–15 were unique to single geographical populations. The distribution of each haplotype by geographical location and the network of the 15 haplotypes are shown in Figure 3 and Table 4.

3.3 Population differentiation

The pairwise F_{ST} values between populations ranged from −0.01410 to 0.20212, based on the COI and Cyt *b* gene sequences. The pairwise differences between the population of Zhaoqing and the other five populations were significant ($p < 0.05$). The pairwise differences between the rest of the geographical populations were not significant ($p > 0.05$), indicating that high genetic homogeneity existed among the opulations (Tab. 5). AMOVA analysis showed no obvious differentiation among the three groups ($p = 0.20821$) (Tab. 6).

Table 3. Haplotypes of COI and Cyt *b* gene sequences in *H. guttatus* populations.

Haplotypes	Mutation sites																		
	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
	1	3	3	4	5	5	5	6	6	6	7	9	0	0	1	3	3	5	5
	5	0	2	0	4	6	9	3	5	9	0	5	3	9	8	3	9	2	9
	6	9	0	2	4	4	7	3	4	2	2	7	6	5	2	5	9	1	1
H1	G	G	G	C	C	C	T	C	G	G	A	C	C	A	C	T	T	G	G
H2	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H3	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*
H4	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	A
H5	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*
H6	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	*
H7	*	A	*	*	*	T	*	*	*	*	*	*	T	G	T	*	*	*	*
H8	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	C	*	A	*
H9	A	*	*	*	*	*	*	*	*	*	G	*	*	*	*	*	*	*	*
H10	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*
H11	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H12	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*
H13	*	*	*	*	*	*	*	T	*	*	*	T	*	*	*	*	*	*	*
H14	*	*	*	*	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*
H15	*	*	*	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*

* Nucleotides are the same as the nucleotides of H1.

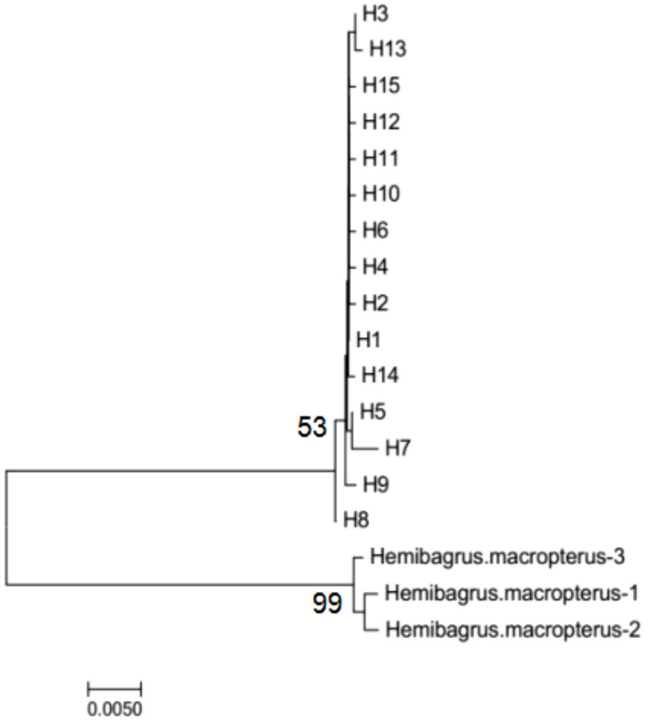

Fig. 2. Neighbor-joining tree of haplotypes based on COI and Cyt *b* gene sequences.

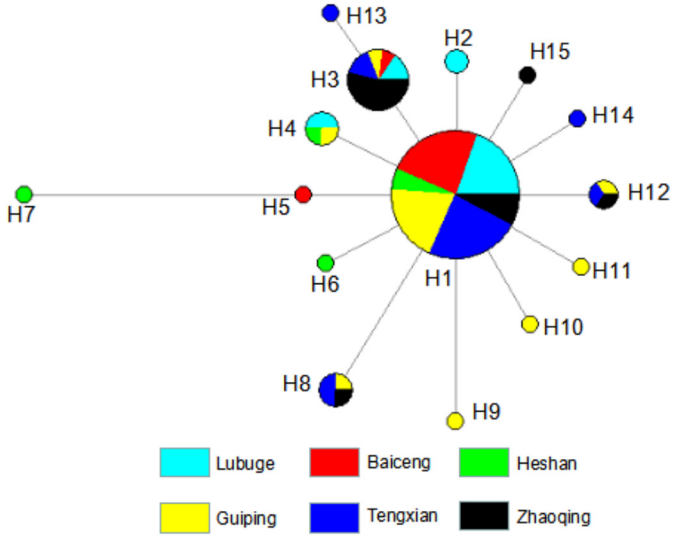

Fig. 3. Median-joining haplotypes network of *H.guttatus* based on COI and Cyt *b* gene sequences. Circle areas are proportional to haplotype frequencies, while colored portions represent the proportions of the same haplotype that occurs in each sampling region.

3.4 Populations' history

Tajima'*D* and Fu's *Fs* teat results are shown in Table 7. For Tajima'*D*, Baiceng, Heshan and Tengxian were significant *D* values ($p < 0.05$). For Fu's *Fs*, all of the populations were significant negative *Fs* values ($p < 0.05$) except Heshan and Zhaoqing. Overall, the neutral teat results showed not significant negative values. The results of the mismatch distribution analysis are shown in Figure 4. Combined with the results of the neutrality test, it is speculated that the population may not have had any population expansion in history.

4 Discussions

In this study, the genetic diversity and population structure of *H. guttatus* in the Pearl River was investigated based on

Table 4. Distribution of haplotypes in *H. guttatus* populations.

Haplotypes	Distribution of the haplotypes						Total
	Lubuge	Baiceng	Heshan	Guiping	Tengxian	Zhaoqing	
H1	0.75	0.92	0.63	0.72	0.76	0.41	92
H2	0.08						2
H3	0.08	0.04		0.04	0.07	0.41	13
H4	0.08		0.13	0.04			4
H5		0.04					1
H6			0.13				1
H7			0.13				1
H8				0.04	0.07	0.06	4
H9				0.04			1
H10				0.04			1
H11				0.04			1
H12				0.04	0.03	0.06	3
H13					0.03		1
H14					0.03		1
H15						0.06	1
Haplotypes number	4	3	4	8	6	5	127

Table 5. Genetic differentiation coefficient (F_{ST} Values) between wild *H. guttatus* populations based on COI and Cyt *b* sequences.

Population	Lubuge	Baiceng	Heshan	Guiping	Tengxian	Zhaoqing
Lubuge	0.00000					
Baiceng	0.01203	0.00000				
Heshan	0.05464	0.09725	0.00000			
Guiping	−0.00446	−0.00854	0.03562	0.00000		
Tengxian	0.00897	0.00423	0.06649	−0.01410	0.00000	
Zhaoqing	0.12842*	0.20212*	0.12926*	0.11742*	0.07705*	0.00000

* Significant F_{ST} Values ($p < 0.05$).

Table 6. AMOVA analysis of *H. guttatus* populations based on COI and Cyt *b* 15 sequences.

Source of variation	df	Sum of squares	Variance components	Percentage of variation	F_{ST}	*p*
Three groups						
Among groups	2	1.488	0.00034Va	0.10	0.00098	0.20821
Among populations within groups	3	1.988	0.01758Vb	5.12	0.05124	0.02151
Within populations	121	39.374	0.32541Vc	94.78	0.05217	0.00000
Total	126	42.850	0.34332			
One group						
Among populations	5	3.476	0.01785Va	5.20	0.05199	0.00098
Within populations	121	39.374	0.32541Vb	94.80		
Total	126	42.850	0.34325			

mitochondrial COI gene and Cyt *b* gene sequences. 15 haplotypes and 19 polymorphic sites were detected in a 1611 bp long segment of COI gene and Cyt *b* gene sequences from 127 individuals collected from six locations. The Hd and nucleotide diversity (π) were 0.465 and 0.00042 respectively. The haplotype diversity and the nucleotide diversity in our study were lower than in other studies on aquatic organisms, such as *Portunus sanguinolentus* ($h = 0.9576$, $\pi = 0.0051$) along south China Sea and east China Sea (Ren *et al.*, 2016); the bighead carp (*Aristichthys nobilis*) ($h = 0.9083$, $\pi = 0.0032$) in China (Li *et al.*, 2010); and *Penaeus monodon* ($h = 0.927$, $\pi = 0.0294$) in Thailand (Khamnamtong *et al.*, 2009). Such a low level of genetic diversity may be the result of human activities, such as the construction of dams. At present, 32 hydropower stations with capacities larger than 100 MW have been built in the Pearl River. These hydropower stations not

Table 7. Neutrality test of *H. guttatus* populations based on COI and Cyt *b* sequences.

Group	Population	Tajima's *D*		Fu's *Fs*	
		D	*p*	*Fs*	*p*
1	Lubuge	−1.01787	0.18300	−1.49901	0.03700*
	Baiceng	−1.51469	0.03800*	−2.07839	0.00600*
2	Heshan	−1.67405	0.01600*	−0.11377	0.44800
	Guiping	−2.26440	0.95843	−5.97152	0.00000**
3	Tengxian	−1.62518	0.02400*	−2.99003	0.00900*
	Zhaoqing	−1.07706	0.14400	−1.37521	0.11500
Total		−1.52888	0.06767	−2.33799	0.10250

* Significance at $p < 0.05$.
** Significance at $p < 0.001$.

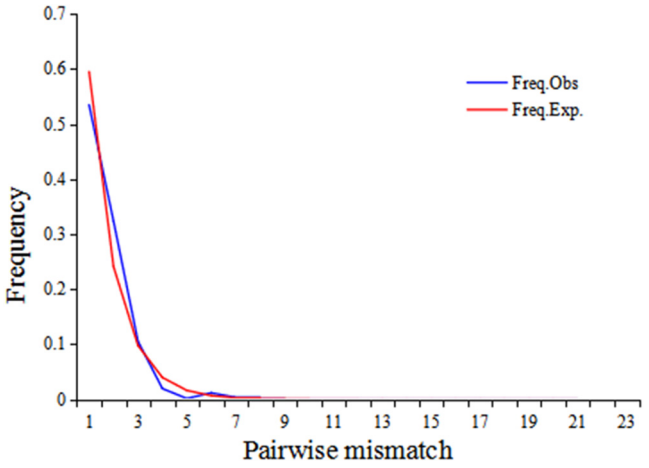

Fig. 4. Mismatch distribution of pairwise difference of COI and Cyt *b* gene sequences in *H. guttatus*.

only affected spawning grounds, but also reduced the exchange of genes between different populations, resulting in a sharp decrease in the population number and genetic diversity (Wang *et al.*, 2004; Tan *et al.*, 2010; Shuai *et al.*, 2017). In addition to hydropower stations, overfishing has led to a genetic bottleneck that also cannot be ignored (Khamnamtong *et al.*, 2009).

The evolutionary events, such as genetic drift, migration and natural selection, may also play a role in determining the patterns of genetic variation (Rubinoff, 2006; Yamaguchi *et al.*, 2010). The not significant F_{ST} values indicate a higher homogenization among populations. Male *H. guttatus* need six years to reach the sexual maturity while female fish need seven years. Longer *H. guttatus* generations lead a lower evolutionary rate (Wang *et al.*, 2017), which makes *H. guttatus* population differentiation slower when compared with carps.

Previous studies indicated that glacial cycles have a significant impact on the genetic diversity and distribution of many existing species. However, different species have different responses to glacial cycles (Hewitt, 2000, 2004; Provan and Bennett, 2008). In our study, neutral tests and mismatch analysis showed that *H. guttatus* populations in the Pearl River basin have maintained a relatively stable population size in the past, and have not experienced population expansion. This is mainly due to the fact that the Pearl River basin is located in the tropical and subtropical regions of southern China, which have mild climate (Weaver *et al.*, 1998; Ju *et al.*, 2007). Fish species can maintain a stable niche during a glacial period, which maintains a relatively stable population. In the recent years, the population size of *H. guttatus* have been declined massively because of the overfishing, which maybe was the main reason that lead the genetic diversity of the *H. guttatus* populations shown the relatively lower diversity in the Pearl River basin.

In conclusion, low levels of genetic variation in the populations of *H. guttatus* in the Pearl River and low sequence diversity among the six populations were revealed. Understanding current situation such as those outlined in this research is the basis of conservation of *H. guttatus* population diversity, which is a critical resource for successful sustainable fishery in the Pearl River.

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