



**Full Length Article**

# Evolutionary Tendency of Clearhead Icefish *Protosalanx hyalocranius* Inferring Mitochondrial DNA Variation Analyses in Amur (Heilongjiang) River Catchment, China

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## Abstract

Clearhead icefish (*Protosalanx hyalocranius* Abbott) is a small commercial fish, which is widely transplanted in lakes and reservoirs in northern China. To detect the genetic evolution of introduced *P. hyalocranius* in Amur River basin, 209 individuals were sampled from Amur River (HLJ), Songhua River (SHJ), Lianhuan Lake (LHH), Xingkai Lake (XKH), and aboriginal Taihu Lake (TH), and the Cytb genes of mitochondrial DNA were sequenced. There were 35 haplotypes of gene Cytb in these populations *in toto*. Ten haplotypes were detected in both Amur and Songhua River, haplotype number from Lianhuan Lake is the largest (14 haplotypes), and only 5 haplotypes were detected from the Xingkai Lake population. A total of 14 haplotypes were detected from the aboriginal Taihu Lake population. Compared with the native population of Taihu Lake, new haplotypes variation accounted for 39.3–56.1% of the total haplotypes in Amur waters. Haplotype diversity was between (0.667 + 0.040) (XKH) and (0.876 ± 0.038) (SHJ), and nucleotide diversity was between (0.00141 ± 0.00010) (LHH) and (0.00263 ± 0.00020) (TH), which exhibited the characteristics of low nucleotide and high haplotype diversity, while the haplotype diversity in Xingkai Lake just over the boundary value of 0.5. These data indicated that all these populations had experienced a genetic bottleneck, which was then overcome through rapid growth of the population. The genetic distances between populations showed that there was obvious genetic differentiation between introduced populations in Amur basin and indigenous population in Taihu Lake. This research indicated that the transplantation of *P. hyalocranius* in the northern area of China has significantly increased the genetic diversity of this species. © 2018 Friends Science Publishers

**Keywords:** Amur river catchment; Cytb gene; Evolution; Genetic diversity; *Protosalanx hyalocranius*

## Introduction

As we all know, the invasion of alien species will lead to the extinction of native species and loss of biodiversity, and produce an irreversible ecological impact (Barel *et al.*, 1958). However, the introduction of species does not always merely have a negative influence. Most introductions of freshwater fishes have produced great economic benefits rather than negative ecological effects (Gozlan, 2008; Silva *et al.*, 2016). Studies on biological invasion mechanism have shown that the genetic variation of new exotic species is conducive to their successful colonization and invasion (Sakai *et al.*, 2001; Peter and Young, 2008). Furthermore, researches in the conservation biology field have also shown that a high genetic diversity is critical to the sustainability of a species (Ellegren and Sheldon, 2008).

The indigenous *Protosalanx hyalocranius* is mainly distributed in Chinese Qiantang River estuaries, the Yangtze River estuaries and the estuaries of the lakes of lower

reaches of the Yangtze River, the Yellow Sea and Bohai Sea coastal estuary (Xie and Xie, 1997; Wang *et al.*, 2002). *P. hyalocranius* is a small commercial fish, which is widely introduced into lakes and reservoirs in northern China, and have produced great economic benefits (Hu, 1995; Hu *et al.*, 2011). The introduction of *P. hyalocranius* in the Amur basin began in 1995, and the species were directly or indirectly introduced from Taihu Lake (Li *et al.*, 2002; Kong *et al.*, 2007). At present, in some water bodies such as the Lianhuan Lake, the *P. hyalocranius* keeps a higher yield, but is decreasing gradually in some water such as Xingkai Lake. *P. hyalocranius* was already distributed in the Heilongjiang (Amur) River, and has become a new species in this basin (Tang *et al.*, 2011, 2015). But the indigenous lakes, such as Taihu lake, are facing the problem of recessionary resources, and the protection of *P. hyalocranius* has been paid much attention. *P. hyalocranius* has been widely introduced for over 40 years (Li *et al.*, 2002), but since its genetic variation

in new water bodies has not been studied, the status of genetic resources of *P. hyalocranius* is very unclear at present.

*P. hyalocranius* is a very popular fish to eat in eastern Asia countries especially Japan, China and Malaysia, for it is boneless, translucent and neat. It can be fried, made into soup or used in barbecue. The price of this fish is about 35000 CNY per ton in China, and it can be exported to another country in a higher price. Stocking this fish into lakes and reservoirs can be very beneficial and make a great fortune for the investment is relatively rather low.

Studies on genetic diversity of the *P. hyalocranius* have shown that the diversity of *P. hyalocranius* RAPD markers and isozyme markers is lower than *Neosalanx taihuensis* and *N. oligodontis* (Xia *et al.*, 1999; Zhang *et al.*, 2005). A comparative study by Gao *et al.* (2004) on *P. hyalocranius* and *Salanx microdon* mitochondrial cytochrome b (Cyt b) gene and 16S rRNA gene has revealed that mitochondrial Cyt b gene evolution rate is about 4 times that of evolution of 16S rRNA gene. A study by Zhang *et al.* (2010) showed that the 18S rRNA gene of the *N. tangkahkeii* and *P. hyalocranius* has no difference. Xiao *et al.* (2016) pointed out that the mitochondrial cytochrome b gene diversity of Huaihe *P. hyalocranius* was also high. Researchers have tried to develop microsatellite marker of higher polymorphism. Zhao *et al.* (2010) also screened *P. hyalocranius* six microsatellite loci of polymorphism by magnetic bead enrichment, we have developed the *P. hyalocranius* microsatellite polymorphism in the whole genome via second generation sequencing technology, but still low (unpublished). The objective of this study was to apply Cyt b gene to find out genetic variation in *P. hyalocranius* after its introduction in Amur River catchment.

## Materials and Methods

### Experimental Materials

In this study 171 individuals of 4 populations of *P. hyalocranius* in Amur River catchment, were used among which, 32 fishes were from Heilongjiang Fuyuan section (HLJ), 28 fishes from Harbin section of Songhua River (SHJ), 70 fishes from Lianhuan lake (LHH), 41 fishes from Xingkai Lake (XKH) including 26 (XKB) from piscivorous group, and 15 fishes (XKS) from non-piscivorous group (group classification reference, Tang *et al.*, 2013). Because the *P. hyalocranius* of Heilongjiang River are directly or indirectly derived from Taihu Lake, so this research collected 35 samples from Taihu Lake (THP), and downloaded 3 Taihu sequences (THP, NCBI accession number: DQ191117~DQ191119) from GenBank, which were applied to compare the genetic variation of the *P.*

*hyalocranius* in Heilongjiang river system. All collected samples were preserved in 75% ethanol.

### Sequence Amplification and Sequencing of Cyt b Gene

The Genomic DNA was extracted by phenol-chloroform extraction (Sambrook and Russell, 2002). The primers for the amplification of gene Cyt b were L14321 and L15634 (Zhang *et al.*, 2007). The sequence of primer L14321 was: 5'-CCAGTGACTTGAAAAACCACCG-3'; and that of H15634 was: CTTAGCTTTGGGAGTTAAGGGT-3', which were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China).

PCR amplified method was referenced and improved according to Zhang *et al.* (2007), Xiao *et al.* (2013). A 25  $\mu$ L PCR reaction system was established, including 18  $\mu$ L buffers (10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200  $\mu$ M each dNTP), upstream and downstream primers (10  $\mu$ mol/L) of each 0.5  $\mu$ L, template DNA (50 ng/ $\mu$ L) 2  $\mu$ L, *Taq* DNA polymerase 1 U. The following reaction procedures were performed with ABI 9700 PCR Thermal Cycler (Applied Biosystems, Foster City, CA, USA): 5 min of pre-degeneration in 94°C, 94°C 30 s, 56°C 45 s, 72°C 1 min, totally 30 cycles; extension 7 min. After electrophoresis of 2% agarose gel, the PCR product was purified and sequenced by Shanghai Sangon Biotech Engineering Technology Service Co., Ltd.

### Sequence Analysis

The sequence analysis process comprised, comparing the Cytb gene of *P. hyalocranius* sequences obtained by sequencing with those from Genbank database to identify homology of Cyt b gene sequence from *P. hyalocranius*, and putting in the sequence to Clustal X2.0 software (Thompson *et al.*, 1997) for sequence alignment, and proofread, truncating the partial sequences of the same length for genetic analysis; calculating the variation sites; parsimony informative sites; haplotype and nucleotide polymorphism with DnaSP v. 5 software (Rozas *et al.*, 2003), calculating the genetic differentiation among populations with AMOVA of Arlequin 3.11 software; analyzing the base compositions and percentage difference, the number of mutation sites, the number of parsimony informative sites and the transition / transversion values with MEGA 7 software (Kumar *et al.*, 2016). The genetic distance (D) among different populations based on the Cyt b gene was calculated by MEGA 7.0 software according to the Kimura two parameter model (Kimura, 1980). Neighbor-Joining (NJ) relation was built for clustering tree among populations and finding genetic distance between populations with MEGA 7 software. Bootstrap (repeat number =1 000) was used to test confidence degree of branches of the molecular

phylogenetic tree. Sequence of three Cytb gene of Taihu Lake *P. hyalocranius* was obtained from the GenBank database for comparison purpose.

## Results

### Sequence Characteristics of Gene Cyt b of *P. hyalocranius* Populations

The result of Blast operation of 1141 bp in GenBank confirmed that the sequences were the mitochondria gene Cyt b sequence of *P. hyalocranius*. Among the gene Cyt b sequences of 209 individuals, there were 1111 conserved sites and 30 mutant sites, including 9 single mutant sites and 21 parsimony informative sites. A, T, C and G proportion of all the sequences were 21.73, 29.26, 32.35 and 16.66%, respectively. The A+T (50.99%) proportion was higher than the proportion of G+C (49.01%) and the ratio of transit/transversion was 12.043.

### Haplotype and Nucleotide Diversity

There were 35 haplotypes in the gene Cytb of 209 individuals of 5 *P. hyalocranius* populations. The proportion of Hap4, Hap1 and Hap3 were larger (Table 1). Ten haplotypes were detected in both Heilongjiang and Songhua River. Haplotype number from Lianhuan Lake was the largest (14 haplotypes), and only 5 haplotypes were detected from Xingkai (Khanka) Lake population. One special haplotype was detected in either piscivorous group or non-piscivorous group, the other 3 were the haplotypes existing in both groups. A total of 14 haplotypes were detected from the aboriginal Taihu Lake population. Hap1, Hap2, Hap3, Hap4, Hap5 were existent in Heilongjiang River, Songhua River and lake Lianhuan Lake.

There were 2 unique haplotypes from Xingkai Lake, which accounted for 66.7% of the total. There were nine unique haplotypes from LianHuan Lake, which accounted for 64.3% of the total. Likewise, there were nine unique haplotypes from Taihu Lake, accounting for 64.3% of the total. There were four unique haplotypes from Songhua River, making up to 40% of the total; Three unique haplotypes from Heilongjiang, accounted for 30% of the total. Compared with the native population of Taihu Lake, there were four new variation haplotypes found from Xingkai Lake, individual variation accounted for 56.1% of the total number of individuals. There were 11 new variation haplotypes found from Lianhuan lake, which accounted for 50% of the total number of individuals. Seven new haplotypes were found from Heilongjiang River, accounted for 43.7% of the total number of individuals. Six new variation haplotypes were found from Songhua River, accounting for 39.3% of the total number of individuals. Although the diversity from Xingkai Lake was the lowest, the proportion of individual new haplotypes was the highest. Diversity and new haplotype proportion of individuals from

**Table 1:** Haplotype distribution of *P. hyalocranius* Cyt b gene in Heilongjiang River catchment and Taihu Lake

Haplotype	Quantity	HLJ	SHJ	LHH	XKH		THP
					XKB	XKS	
Hap1	43	2	4	22	9	6	-
Hap2	12	7	3	2	-	-	-
Hap3	26	8	4	12	-	-	2
Hap4	50	9	8	14	11	7	1
Hap5	16	1	3	9	-	-	3
Hap6	2	1	1	-	-	-	-
Hap7	1	1	-	-	-	-	-
Hap8	2	1	-	-	-	-	1
Hap9	1	1	-	-	-	-	-
Hap10	1	1	-	-	-	-	-
Hap11	1	-	1	-	-	-	-
Hap12	5	-	2	-	-	-	3
Hap13	1	-	1	-	-	-	-
Hap14	1	-	1	-	-	-	-
Hap15	6	-	-	-	5	1	-
Hap16	1	-	-	-	1	-	-
Hap17	1	-	-	-	-	1	-
Hap18	2	-	-	2	-	-	-
Hap19	2	-	-	2	-	-	-
Hap20	1	-	-	1	-	-	-
Hap21	1	-	-	1	-	-	-
Hap22	1	-	-	1	-	-	-
Hap23	1	-	-	1	-	-	-
Hap24	1	-	-	1	-	-	-
Hap25	1	-	-	1	-	-	-
Hap26	1	-	-	1	-	-	-
Hap27	1	-	-	-	-	-	1
Hap28	1	-	-	-	-	-	1
Hap29	5	-	-	-	-	-	5
Hap30	1	-	-	-	-	-	1
Hap31	12	-	-	-	-	-	12
Hap32	1	-	-	-	-	-	1
Hap33	4	-	-	-	-	-	4
Hap34	1	-	-	-	-	-	1
Hap35	2	-	-	-	-	-	2

Lianhuan Lake were high; the individual haplotype number from Heilongjiang and Songhua River were less than the individual haplotype of indigenous individual numbers.

The haplotype diversity of 5 populations of *P. hyalocranius* was (Hd)  $0.873 \pm 0.013$ . The nucleotide diversity (Pi) was  $0.00205 \pm 0.00010$ , and the average nucleotide difference (k) was 2.339. There were 5~14 haplotypes detected in these populations, haplotype diversity was between  $(0.667 + 0.040)$  (XKH) and  $(0.876 \pm 0.038)$  (SHJ), and nucleotide diversity was between  $(0.00141 \pm 0.00010)$  (LHH) and  $(0.00263 \pm 0.00020)$  (TH). The neutral detected value of Tajima's D in 5 populations varied from -1.11219~0.41600 to neutral. The results of neutral test showed no significant ( $P > 0.1$ ) difference, as seen in neutral mutation (Table 2).

### Genetic Differentiation of Populations

Cyt b Gene was applied to compare the genetic differentiation among 5 populations. AMOVA results showed that the genetic difference was mainly from internal

**Table 2:** Haplotype and nucleotide polymorphism of Cyt b gene of *P. hyalocranius*

Sampling site	Quality	Haplotype quality	Qty of mutation sites	Qty of Single informative sites	Qty of mutation sites	Single nucleotide polymorphism (Pi)	Number of average nucleotide differences	Tajima's D	Probability
HLJ	32	10	9	4	0.827±0.038	0.00202±0.00028	2.302	-1.11219	<i>P</i> >0.10
SHJ	28	10	2	8	0.876±0.038	0.00187±0.00023	2.130	-0.55090	<i>P</i> >0.10
LHH	70	14	3	8	0.823±0.026	0.00141±0.00010	1.605	-0.81674	<i>P</i> >0.10
XKH	41	5	2	6	0.667±0.041	0.00188±0.00027	2.144	0.41600	<i>P</i> >0.10
TH	38	14	4	10	0.872±0.040	0.00263±0.00020	2.999	-0.31885	<i>P</i> >0.10

**Table 3:** AMOVA analysis results of gene Cyt b

Variation source	Degree of freedom	quadratic sum	variance components	Percentage of total variation
Inter-population	4	25.055	0.12829 Va	10.71
Intra-population	204	218.175	1.06949 Vb	89.29
Total	208	243.230	1.19777	

Fst=0.10711

**Table 4:** Genetic differentiation between populations

Population	HLJ	SHJ	LHH	XKH	TH
HLJ		0.52252±0.0446	0.07207±0.0227	0.09009±0.0192	0.00000±0.0000**
SHJ	-0.00806		0.17117±0.0252	0.09910±0.0252	0.00000±0.0000**
LHH	0.02360	0.01401		0.00000±0.0000**	0.00000±0.0000**
XKH	0.03101	0.02769	0.05387**		0.00000±0.0000**
TH	0.15823**	0.16500**	0.22369**	0.19811**	

\*\*\*\* Significance Level, *P* < 0.0500

of population (89.29%), and few of them came from among the population (10.71%; Table 3). Genetic differentiation coefficient (Fst) is an important parameter to reflect the degree of genetic differentiation among the populations. The genetic differentiation coefficient (Fst) among the populations was 0.10711, which indicated a moderate genetic differentiation (Table 3).

The genetic distances between the populations showed clear genetic differentiation between various populations in Heilongjiang River basin and indigenous population in Taihu Lake. The genetic difference between Xingkai Lake population and Lianhuan Lake population in Heilongjiang River basin was significant (Table 4).

#### Genetic Distances and Construction of Phylogenetic Tree

The results showed that the genetic distance between different populations was 0.001659~0.002820 (Table 5). The genetic distance between population in Xingkai Lake and indigenous population in Taihu Xingkai Lake was the largest of 0.002820; the genetic distance between populations in Lianhuan Lake and the population in the Songhua River was 0.001659, the nearest of all. The genetic distances between the population in Taihu Lake and various populations in Heilongjiang River basin were more than 0.002550. The genetic distances between various populations in Heilongjiang River basin were relatively closer; only the genetic distance between population in Xingkai Lake and populations in Heilongjiang River was greater than 0.002000, The distance between populations in Songhua

River and Heilongjiang River or the population in Xingkai Lake were 0.001900.

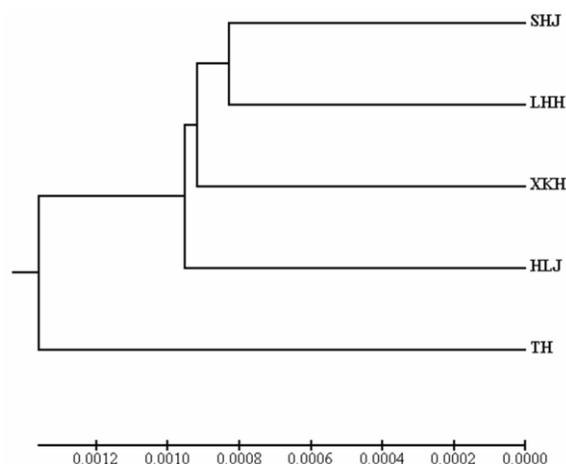
UPGMA phylogenetic tree shows that the affinity between the population in Songhua River and in Lianhuan Lake was the closest. The affinity between the population in Xingkai Lake and in Heilongjiang River population took second place, the indigenous population in Taihu Lake was the most remote relative of others (Fig. 1).

#### Discussion

To find out haplotypes in *P. hyalocranius*, Grant and Bowen (1998) Hd=0.5 and Pi=0.005 was applied as the boundary value for measuring haplotype diversity and nucleotide diversity degree. All five populations had the characteristics of low nucleotide diversity and high haplotype diversity, while the haplotype diversity in Xingkai Lake was just over the boundary value of 0.5. This indicated that these populations had experienced a genetic bottleneck, and then overcame the genetic bottleneck through rapid growth, not only in the transplanted population but also in the indigenous population in Taihu Lake. Xingkai Lake population is still in the process of overcoming genetic bottleneck. As a result of extensive fishing in 2010 for the purpose of population control, *P. hyalocranius* population is decreasing year by year, and the largest population of an individual has become smaller. Taihu Lake native population had experienced a genetic bottleneck in the 1980s, because the *P. hyalocranius* population declined due to overfishing and environmental pollution. Then there was a rapid population growth, which should have mutated

**Table 5:** Genetic distance between populations of *P. hyalocranius*

Population	HLJ	SHJ	LHH	XKH
HLJ				
SHJ	0.001935			
LHH	0.001752	0.001659		
XKH	0.002018	0.001934	0.001737	
TH	0.002781	0.002721	0.002551	0.002820

**Fig. 1:** UPGMA evolutionary tree of various populations of *P. hyalocranius*

individuals to overcome the genetic bottleneck after environmental improvement. To overcome the genetic bottleneck of *P. hyalocranius* transplantation, managers of Lianhuan Lake have adopted the method of exchanging roe with different waters during stocking every year. This study showed that the high genetic diversity in *P. hyalocranius* Lianhuan Lake, mean that their approach is effective.

As regards reason for diversity in *P. hyalocranius*, this study showed that the affinity between *P. hyalocranius* population in Songhua River and the population in Lianhuan Lake was the closest which is related to the fact that the Lianhuan Lake interlinks with the Songhua River. Since 1995 the *P. hyalocranius* firstly introduced into the Lianhuan Lake in Heilongjiang River basin, *P. hyalocranius* had been introduced into almost all large and medium-sized reservoirs and lakes in this basin. The fisheries manager of Lianhuan lake introduced recruitments from other *P. hyalocranius* stocking waters of China every year, which contributed to the high genetic diversity of population in Lianhuan lake. Lianhuan lake interlinks with Nenjiang and Songhua River, *P. hyalocranius* has been found in Nenjiang after the great floods in 1998, and was first discovered in 2011 in Harbin section of the Songhua River (Tang *et al.*, 2013). After that *P. hyalocranius* population was also found in Heilongjiang River (Tang *et al.*, 2015). *P. hyalocranius* population in Xingkai Lake was first introduced in the satellite lake of Xingkai Lake in 2000, and then went into Xingkai Lake with flood and became the largest population

in 2010 (Tang *et al.*, 2011); but reduced year by year since then. This study showed that although the Xingkai Lake sinks into Heilongjiang River via Wusuli River, Songhua River also sinks into Heilongjiang, but *P. hyalocranius* population in Heilongjiang River was not formed by diffusion of the population in Xingkai Lake or Songhua River. It was said that the *P. hyalocranius* population in Heilongjiang River was formed from the population introduced in Dalijia Lake with drainage. But as the Songhua River population growth and expansion, and larval drifting, the population may gradually spread to Heilongjiang River; and Xingkai Lake population is living in a relatively stable condition, where the possibility of outward diffusion is relatively smaller.

## Conclusion

Since the *P. hyalocranius* is mainly distributed in China, it is of worldwide significance to protect this fish resource. As it is widely introduced in northern China, we can infer that the evolution of *P. hyalocranius* in has led to its wide genetic variation in Heilongjiang River basin, and thus playing an important role in the sustainability of this species.

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