



Article

Genetic Differentiation of an Endangered *Megalobrama terminalis* Population in the Heilong River within the Genus *Megalobrama*

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Abstract: *Megalobrama terminalis*, which inhabits the Sino-Russian Heilong-Amur River Basin, has decreased critically since the 1960s. It has been listed in the Red Book of Endangered Fish Species by the Russian Federation in 2004. To guide the utilization and conservation programs of *M. terminalis* in the Heilong River (MTH), 3.1 kb of mitochondrial DNA (mtDNA) concatenated sequences and sequence-related amplified polymorphism (SRAP) markers (15 primer combinations) were applied to explore the genetic divergence and population differentiation of MTH within the genus *Megalobrama*. Clear genetic divergence between MTH and six other populations of the genus *Megalobrama* was found by haplotype network (mtDNA) and principal component (SRAP) analyses. Moreover, the STRUCTURE analysis based on SRAP data showed that MTH could be assigned to a particular cluster, whereas conspecific *M. terminalis* in the Qiantang River and Jinsha River Reservoir belonged to the same cluster. Analysis of molecular variance (AMOVA) and *F*_{st} statistics for the mtDNA and SRAP data revealed significant genetic variance and differentiation among all detected populations. Taken together, the results suggest that MTH has a strong genetic differentiation from other populations within the genus *Megalobrama*, which contributes to effective utilization in artificial cultivation and breeding of MTH. Furthermore, these results also provide a scientific basis for the management of MTH as a separate conservation unit.

Keywords: *Megalobrama terminalis*; genetic differentiation; population structure; mitochondrial DNA; SRAP

1. Introduction

The genus *Megalobrama* is a member of Cultrinae in Cyprinidae fish. This genus is a kind of middle-large, economically valuable fish and is famous for its tasty meat quality [1]. The genus *Megalobrama* in China is mainly composed of *M. amblycephala*, *M. terminalis*, *M. pellegrini* and *M. hoffmanni* [2,3]. Among these species, *M. amblycephala* and *M. terminalis* formed a certain aquaculture scale. The *M. amblycephala* has two new selective breeding varieties (Pujiang No. 1 and Huahai No. 1), and its production reached 700,000 tons in 2014 [4]. In contrast, no new breed of *M. terminalis* has been systematically selected, and germplasm resources for aquaculture originated from the Qiantang River in Zhejiang Province and the Jinsha River Reservoir of Hongan County in Hubei Province [5,6]. According to statistics from 2016, the aquaculture area of *M. terminalis* in the Qiantang River (MTQ) has

exceeded 600 ha in the areas of Hangzhou, Jinhua and other places in Zhejiang Province [1]. Since the success of artificial reproduction in 1988, *M. terminalis* in the Jinsha River Reservoir (MTJ) has been extended to the Hubei, Henan, Anhui, Sichuan, Guangdong and Fujian Provinces [6].

Remarkably, there is another native wild population of *M. terminalis* in the Heilong River (the Amur River in Russia), which is the northernmost, highest-latitude river in China. Although *M. terminalis* was historically widespread in bodies of water in the Heilong River Basin, such as the Heilong River, the Nen River, the Songhua River, Jingpo Lake and Khanka Lake [7] (Supplementary Materials Figure S1), its habitats have been shrinking year by year since the 1960s with the effects of overfishing and environmental changes [8]. Currently, only dozens of individuals can be captured per year in the Fuyuan section (134°28' E, 48°37' N) of the Heilong River (Figure S1). *M. terminalis* in the Amur River has been included in the Red Book of endangered species of the Russian Federation [9]. *M. terminalis* in the Heilong River (MTH) is highly popular among local consumers because of its large size and delicious taste. The potential value of these excellent properties determines that MTH will be an ideal commercially exploited species [3,7]. Due to the difficulty of wild fish collection and the high mortality in the course of temporary rearing and transport, however, systematic research on population genetics for MTH is highly limited. Recently, the phylogenetic relationships among seven populations from four species in the genus *Megalobrama*, including *M. terminalis* in the Heilong River (MTH), were clarified at the mitochondrial genome level [10]. Moreover, the artificial reproduction of MTH was achieved under pond domestication conditions [11]. However, the genetic divergence and differentiation at a population level between MTH and other populations from *M. terminalis* and other species within the genus *Megalobrama* have been not determined, which limits the effective utilization and protection of MTH.

Regarding fish species of the genus *Megalobrama*, genetic variance and differentiation have been well-evaluated in different natural and selected populations from *M. amblycephala* using morphometrics, isozymes, mitochondrial DNA (mtDNA), random amplified polymorphic DNA (RAPD) and sequence-related amplified polymorphism (SRAP) markers [12–15]. However, existing data only reflect the morphological differences between the Qiantang River and the Jinsha River reservoir, as well as between the Heilong River and the Liangzi Lake, in *M. terminalis* [8,16]. At the interspecies level, Li et al. completed a preliminary comparison of genetic variations among *M. terminalis* in the Qiantang River, *M. amblycephala* and *M. hoffmanni* using morphometrics, isozymes and RAPD markers [17]. It can be seen from existing reports that the investigations on intra- and interspecies relationships involved in *M. terminalis* are highly limited.

The amplified target site of SRAP is in the coding regions of the genes, and it is a highly reproducible and informative marker system. SRAP markers have been widely used to estimate the genetic diversity and structure, and varietal identification in plants [18,19] and their discriminating power is stronger than those of simple sequence repeats (SSR), inter-simple sequence repeats (ISSR) and RAPD [20]. In addition, this technique has been extended to studies of population genetics in other living organisms [21], including aquatic animals [15,22–28]. To understand the genetic relationships, especially the level of genetic differentiation of MTH with MTQ, MTJ and other representative geographic samples within the genus *Megalobrama*, containing *M. amblycephala* from the Liangzi Lake (MAL) and the Yi River (MAY) and *M. Pellegrini* from the Longxi River (MPL) and *M. hoffmanni* from the Xi River (MHX), we performed this survey based on mtDNA and SRAP markers.

2. Materials and Methods

2.1. Sampling and DNA Extraction

Two hundred and ten individuals, including 7 wild populations (30 per population) of 4 species in the genus *Megalobrama*, were sampled by means of drift-net fishing. Sampling sites are shown in Figure 1. The numbers of individuals used for mitochondrial and SRAP analysis are shown in Table S1. Genomic DNA from fin tissues was isolated using the phenol/chloroform method and preserved in 1×

TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The quality and quantity of the DNA were detected by agarose gel electrophoresis and absorbance at 260 nm and 280 nm. DNA samples were quantified 200 ng/ μ L and diluted to 50 ng/ μ L for PCR. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Heilongjiang River Fisheries Research Institute.

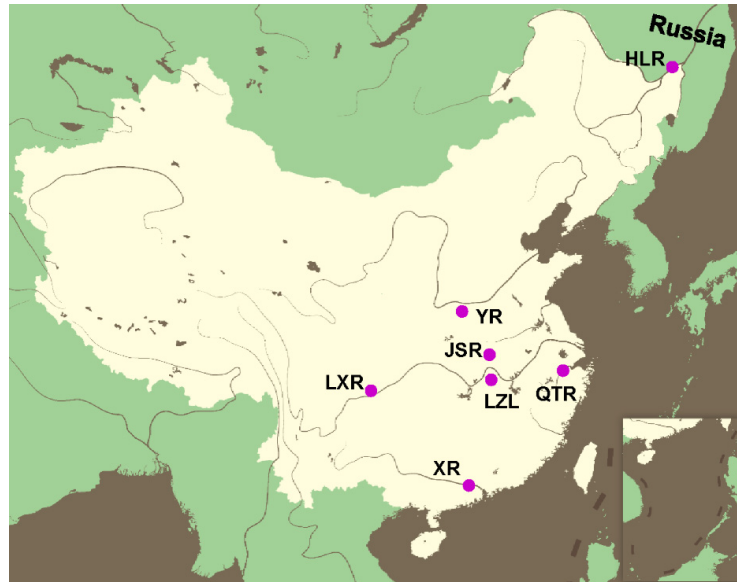


Figure 1. Sampling locations of fish in the genus *Megalobrama*. HLR, the Heilong River; QTR, the Qiantang River; JSR, the Jinshahe Reservoir; YR, the Yi River; LZL, the Liangzi Lake; LXR, the Longxi River and XR, the Xi River.

2.2. Mitochondrial Sequencing and Analysis

Based on our previous comparative analysis of mitochondrial genome sequences of 7 populations [10], three pairs of primers were designed using Primer 5.0 (Primer Biosoft International, Palo Alto, CA, USA) to amplify putative sequences with a total of 3845 bp, including a *D-loop* of 1115 bp, *ND2* of 1337 bp and *CYTB* of 1393 bp (Table S2). PCR products were amplified in a 20- μ L reaction system containing 2- μ L 10 \times PCR buffer, 1.6- μ L 1.5-mM $MgCl_2$, 0.6 μ L of each primer (10 μ M), 0.4- μ L dNTP Mix (10-mM each of dATP, dCTP, dGTP and dTTP), 0.1- μ L *Taq* DNA polymerase (5 U/ μ L), 13.7- μ L H_2O and 1- μ L genomic DNA (50 ng/ μ L). The amplification protocol was as follows: 94 $^\circ$ C for 5 min followed by 30 cycles at 94 $^\circ$ C for 30 s, 52 $^\circ$ C for 15 s and 72 $^\circ$ C for 70 s and an extension at 72 $^\circ$ C for 5 min. PCR products were purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA) and subcloned into pMD18-T vectors (TaKaRa, Shiga, Japan). Recombinant clones containing each target fragment were sequenced on an ABI 3700 sequencer (Applied Biosystems, PerkinElmer, Foster City, CA, USA).

All sequences amplified by PCR were edited and aligned using the CLUSTAL X program, as implemented in MEGA 7 (Temple University, Philadelphia, PA, USA) [29] and, subsequently, aligned manually. Analysis of population variation, such as the number of haplotypes, haplotype diversity (h) and nucleotide diversity (π), were calculated by DNAsp5.0 software (Universitat de Barcelona, Barcelona, Spain) [30]. A haplotype network was constructed using the median-joining method in NETWORK 5.0.1.1 software (Fluxus Technology Ltd., Clare, Suffolk, UK) to analyze and visualize the relationships among DNA sequences. The software ARLEQUIN v3.5.2 (University of Berne, Berne, Switzerland) [31] was used to complete the analysis of molecular (AMOVA) for detecting genetic variation levels among populations and calculate pairwise F_{st} values for estimating the extent of genetic differentiation among populations. Significance was tested using 1000 permutations. All accession numbers of the mtDNA fragments are MN958398-MN958523.

2.3. SRAP Primer, PCR Amplification and Analysis

The primers required for SRAP refer to the primer sequences published by Li et al. [21]; 15 primer combinations (Table S3) with clear amplification bands, strong reaction stability and high reproducibility were identified from 240 combinations. All 210 individuals from 7 populations (30 per population) were genotyped using 15 primer combinations selected. The reaction system of 20 μ L contained 7- μ L H₂O, 1- μ L 10 \times PCR buffer, 0.8- μ L 1.5-mmol/L MgCl₂, 0.2- μ L 10-mmol/L dNTPs, 0.5 μ L of each primer (10 μ M) and 0.5 μ L of 50-ng/ μ L genomic DNA. The PCR conditions were as follows: pre-denaturation for 5 min at 94 °C followed by the first cycle for 5 cycles of 94 °C for 1 min, 35 °C for 1 min and 72 °C for 1 min, followed by the second cycle for 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 5 min, followed by an extension of 10 min at 72 °C. The PCR product was detected by 1.5% agarose gel electrophoresis and was further loaded to a 10% polyacrylamide gel electrophoresis with reference to the pBR 322/*Msp* I marker (TIANGEN, Beijing, China). The silver staining method [32] was used to display the bands. The electropherogram was analyzed by Gel-pro analyzer 4.5 software (Media Cybernetics, Inc., Rockville, MD, USA). In most of the gels, in addition to the markers to indicate the size of the bands, we also clicked a sample from a certain population used for the marker or control between gels. Moreover, the samples were from several populations in the same gel, which were contrasted with each other to reduce the influence of differences between gels on the result judgment (Figure S2). The principle for determining bands was the stability of amplification. The judgment of weak bands needed to take into account the overall (multiple populations) amplification situations. If some bands were weak but others were clear in the same pair of primers, it should be caused by the different amplification efficiency among samples; such bands were recorded in the statistics. Whereas bands in different populations were weak, indicating unstable amplification; such bands were not included. Undecidable bands were regarded as missing data. Different and reproducible SRAP bands were scored as present (1) or absent (0), and ambiguous bands or missing data were recorded with number "9".

The polymorphism information content (*PIC*) of each SRAP marker was calculated by the following formula: $PIC = 1 - (f^2 + (1 - f)^2)$, where *f* is the frequency at which the band appeared in all samples [33]. Principal component analysis (PCoA) was performed based on genetic distance using the GenAIEx 6.5 package (Australian National University, Canberra, Australia) [34] to examine the positional relationship of each group on the two-dimensional clustering map of the main coordinates. Genetic structure analysis was carried out based on the Bayesian model using Structure 2.3.4 software (Stanford University, Palo Alto, CA, USA) [35,36]. The number of subgroups was set to 1~7 with 10 repetitions. The length of the burn period for each run was set to 10,000, and the number of iterations for Markov chain Monte Carlo (MCMC) after burning was set to 100,000. The results of the calculations were analyzed online using Structure Harvester program (University of California, Santa Cruz, CA, USA), and the most likely *K* values were determined based on Ln*P*(*K*) or ΔK values. The analysis of molecular variance (AMOVA) and the pairwise *F*_{st} values with 1000 permutations were performed using ARLEQUIN v3.5.2 software [31].

3. Results

3.1. Genetic Variability

The effective sequences of the *D-loop*, *ND2* and *CYTB* from each specimen were aligned and processed separately. The concatenated sequence length for haplotype analysis was 3116 bp in samples from *M. terminalis* and *M. pellegrini*, including a *D-loop* of 930 bp, *ND2* of 1045 bp and *CYTB* of 1141 bp (Table S2). The length in the *CYTB* gene is 1137 bp in all samples in *M. amblycephala*, and the length for the haplotype study in the *D-loop* is 927 bp, 929 bp or 931 bp in different samples in *M. hoffmanni*. Concatenated mtDNA sequences were obtained from 135 individuals across seven populations, and 42 haplotypes were defined. In MTH, all seven haplotypes (hap 26 to hap 32) were unique. Haplotype 26 was the most frequently observed (14 individuals), followed by hap 27 (five individuals). The haplotype

diversity (H_d) was lowest in MPL (0.189), highest in MHX (0.995) and moderate in other populations (0.567–0.784). The nucleotide diversity (π) was very low in MTH (0.00045), MAL (0.00023), MAY (0.0002) and MPL (0.00073) and relatively high in MTQ (0.0033), MTJ (0.00363) and MHX (0.00437). The H_d and π for seven populations are summarized in Table 1.

Table 1. Estimates of the molecular genetic diversity from combined mitochondrial DNA (mtDNA) sequences (3116 bp) of 7 populations in the genus *Megalobrama*.

Population	Sample Size	No. of Haplotypes	No. of Variable Sites	Percent Variable Sites (%)	Haplotype Diversity (H_d) (\pm SD)	Nucleotide Diversity (π) (\pm SD)
MTH	26	7	8	0.26	0.683 \pm 0.087	0.00045 \pm 0.00009
MTQ	20	8	57	1.83	0.784 \pm 0.084	0.00330 \pm 0.00104
MTJ	15	3	25	0.8	0.686 \pm 0.061	0.00363 \pm 0.00039
MPL	20	2	12	0.39	0.189 \pm 0.108	0.00073 \pm 0.00042
MAL	18	3	2	0.06	0.601 \pm 0.080	0.00023 \pm 0.00004
MAY	16	3	2	0.06	0.567 \pm 0.109	0.00020 \pm 0.00005
MHX	20	19	61	1.96	0.995 \pm 0.018	0.00437 \pm 0.00033
Total	135	42	167			

MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake; MAY, *M. amblycephala* in the Yi River and MHX, *M. hoffmanni* in the Xi River.

Fifteen SRAP primer combinations generated 221 stable and reproducible bands, of which 214 (97%) were polymorphic (Table 2). The amplification profiles of primer combinations 1f5r, 2f9r, 4f20r and 7f20r were shown in Figure S2. The most polymorphism was from the 7f20r combination, which produced 22 polymorphic bands. The PIC of each combination ranged from 0.262 to 0.412, with an average of 0.311 (Table 2). Based on the detection values of four SRAP diversity parameters, seven populations can be divided into two distinct groups in which data for different parameters were similar within each group. Three *M. terminalis* populations (MTH, MTQ and MTJ) showed relatively high levels of variability (number of polymorphic bands (NPB) = 112–115, percent polymorphic bands (PPB) = 50.68–52.04%, Nei's gene diversity (h) = 0.168–0.177, Shannon's Information Index (I) = (0.252–0.263) (Table 3). In contrast, the other four populations (MAL, MAY, MPL and MHX) exhibited relatively low variation (NPB = 76–83, PPB = 34.39–37.56, h = 0.109–0.128, I = 0.169–0.192) (Table 3).

Table 2. Amplification results of 7 populations in the genus *Megalobrama* at 15 sequence-related amplified polymorphism (SRAP) loci. PIC: polymorphism information content.

Primer Pair ID	Total Bands	Polymorphic Bands	Percent Polymorphic Bands (%)	PIC Values
1f2r	14	13	93	0.320
1f5r	11	10	91	0.352
1f17r	14	14	100	0.412
2f5r	12	12	100	0.309
2f9r	11	11	100	0.349
4f20r	17	17	100	0.278
7f8r	13	13	100	0.340
7f19r	21	20	95	0.262
7f20r	22	22	100	0.265
10f13r	13	12	100	0.298
10f18r	11	11	85	0.293
10f20r	15	15	100	0.282
12f3r	20	17	100	0.352
12f4r	17	17	97	0.286
12f5r	10	10	97	0.273
Total	221	214	\	\
Mean	14.73	14.27	97	0.311

Table 3. Genetic diversity of 7 populations in the genus *Megalobrama* at 15 SRAP loci.

Population	Number of Polymorphic Bands (NPB)	Percent of Polymorphic Bands (PPB, %)	Nei's Gene Diversity (<i>h</i>) (\pm SD)	Shannon's Information Index (<i>I</i>) (\pm SD)
MTH	113	51.13	0.177 \pm 0.205	0.263 \pm 0.291
MTQ	115	52.04	0.173 \pm 0.196	0.261 \pm 0.281
MTJ	112	50.68	0.168 \pm 0.198	0.252 \pm 0.283
MPL	76	34.39	0.125 \pm 0.191	0.185 \pm 0.274
MAL	82	37.10	0.109 \pm 0.167	0.169 \pm 0.246
MAY	83	37.56	0.128 \pm 0.184	0.192 \pm 0.267
MHX	80	36.20	0.120 \pm 0.182	0.181 \pm 0.263

MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake; MAY, *M. amblycephala* in the Yi River and MHX, *M. hoffmanni* in the Xi River.

3.2. Population Genetic Structure and Differentiation

Median-joining network analysis showed that MTH formed a single haplogroup, whereas there were only three haplotypes in MTJ, and one of them was clustered with the haplogroup of MTQ. Two populations for MAL and MAY constituted a haplogroup in which there were two shared haplotypes (Figure 2).

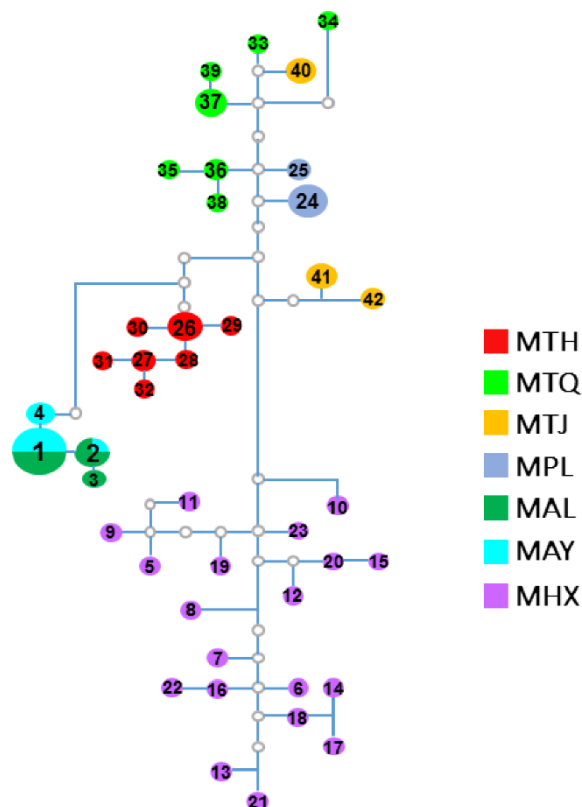


Figure 2. Median-joining network of *Megalobrama* fish mitochondria DNA (mtDNA) haplotypes based on 3.1 k mtDNA sequences. The area of the oval circle is approximately proportional to the haplotype frequency. Small gray circles on the lines connecting oval circles of haplotypes indicate substitutions. MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake; MAY, *M. amblycephala* in the Yi River and MHX, *M. hoffmanni* in the Xi River.

In the principal component analysis (PCoA), the first and second axes explained 30.79% and 47.80% of the variation, respectively. The single scattered point clusters corresponding to MTH, MPL and MHX had almost no overlap with other populations, while the points corresponding to MTQ and MTJ and between MAL and MAY had a certain degree of overlap (Figure 3).

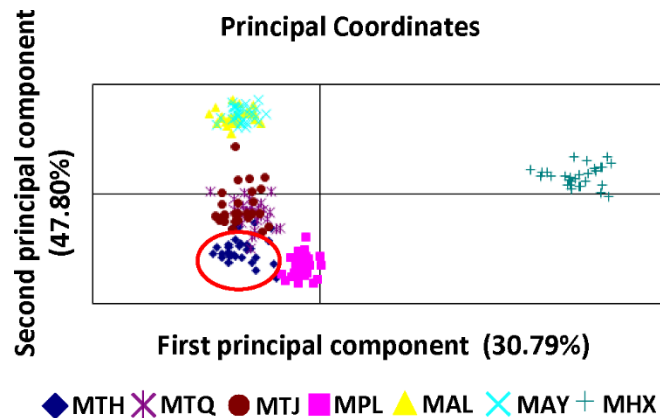


Figure 3. Two-dimensional scatterplot of the principal coordinate analysis (PCoA) of *Megalobrama* fish based on sequence-related amplified polymorphism (SRAP) markers. MTH is marked by red circle. MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake; MAY, *M. amblycephala* in the Yi River and MHX, *M. hoffmanni* in the Xi River.

Moreover, the best K value was determined online using Structure Harvester. Based on the method described by Evanno et al. [37], a clear peak ($\Delta K = 148.76$) was shown at $K = 5$ (Figure S3). Estimated subpopulations for the 210 individuals (seven populations) are shown in Figure 4. Any individual with a membership probability (Q) less than 0.8 was treated as admixed. All of the individuals from MTH were assigned irrefutably to the red subset ($Q > 0.98$). Individuals from MTQ and MTJ were placed in the green subset ($Q > 0.96$), except four individuals from MTJ, who were treated as admixed. The purple subset only consisted of individuals from MAL and MAY ($Q > 0.98$), while the blue ($Q > 0.94$) and yellow ($Q > 0.98$) subsets contained individuals from MPL and MHX, respectively.

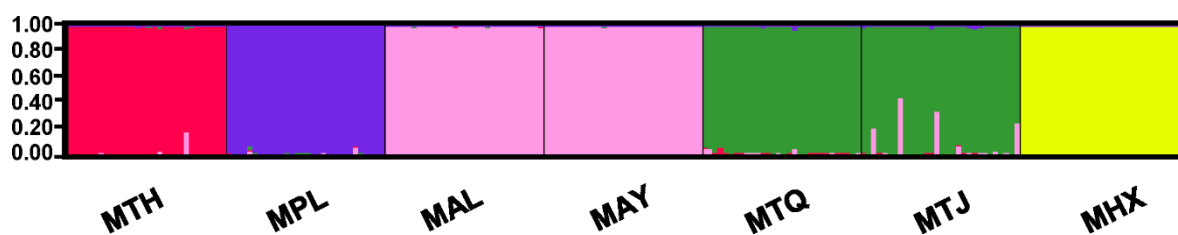


Figure 4. Structure analysis of 7 populations from the genus *Megalobrama* based on sequence-related amplified polymorphism (SRAP) markers. Each color represents a special cluster, and each individual is represented by a vertical line. MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake; MAY, *M. amblycephala* in the Yi River and MHX, *M. hoffmanni* in the Xi River.

To characterize the genetic variance among populations in four species of the genus *Megalobrama*, four groups were considered based on each species and compared on the basis of hierarchical AMOVA with both mtDNA and SRAP markers (Table 4). Significant genetic differentiation was detected among the four groups ($p < 0.01$), among the seven populations within the groups ($p < 0.01$) and within the populations ($p < 0.01$). In addition, each of the seven populations showed highly significant genetic

differentiation ($p < 0.01$), except between MAL and MAY ($p < 0.05$), by pairwise F_{st} based on mtDNA and SRAP genotypes (Figure 5).

Table 4. Analysis of molecular variance (AMOVA) of 7 populations in the genus *Megalobrama* based on combined mtDNA sequences (3116 bp) and 15 SRAP loci.

Source of Variation	Degrees of Freedom	Sum of Squares	Percentage Variation (%)	p -Value
Based on mtDNA sequences				
Among groups	3	3646.435	88.48	< 0.01
Among populations within groups	6	129.304	5.00	< 0.01
Within populations	128	356.276	6.52	< 0.01
Total	134	4132.015	100	\
Based on SRAP loci				
Among groups	3	170.656	8.00	< 0.01
Among populations within groups	6	83.906	9.78	< 0.01
Within populations	203	1242.700	82.22	< 0.01
Total	209	1497.262	100	\

p -value = significance level for AMOVA analysis (significance is $p < 0.01$).

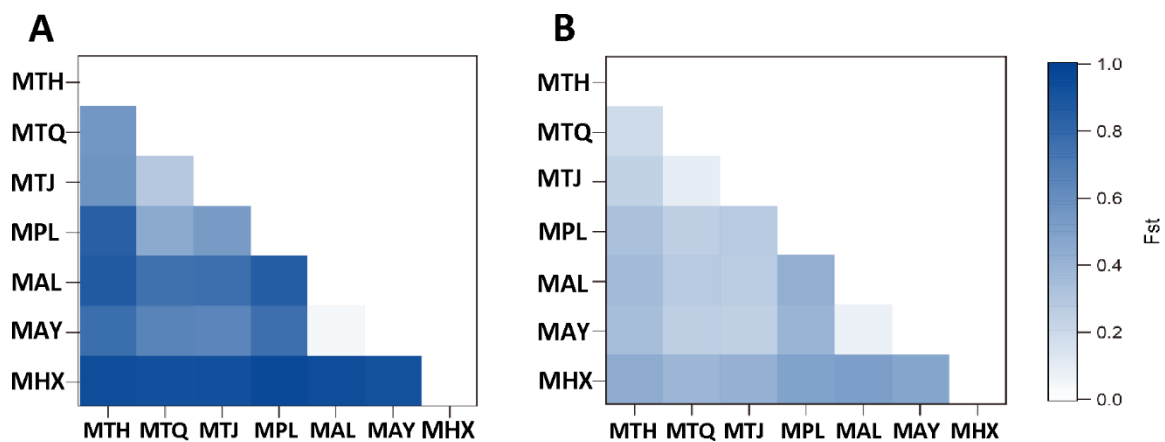


Figure 5. Heat map of pairwise F_{st} comparing between 7 populations of *Megalobrama* fish according to mtDNA (A) and SRAP (B) marker data. All estimates were significantly different from zero at the $p < 0.05$ level. MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake; MAY, *M. amblycephala* in the Yi River and MHX, *M. hoffmanni* in the Xi River.

4. Discussion

Our recent research clarified the characterization of the mitochondrial genome of MTH in the genus *Megalobrama* [10]. Based on the analysis of the genomic-level variation sites, the concatenated fragment of *D-loop*, *ND2* and *CYTB* was selected for the present study. MTH displayed a low level of genetic variability in the mtDNA sequences ($\pi = 0.00045$) compared with the other two *M. terminalis* populations (MTQ, $\pi = 0.0033$ and MTJ, $\pi = 0.0036$), while MTH was at the same level as MPL ($\pi = 0.00073$), MAL ($\pi = 0.00023$) and MAY ($\pi = 0.0002$). This level of variation of MPL was highly consistent with a previous report ($\pi = 0.00077$) based on the *D-loop* fragment [38], providing evidence of the reliability of this data. MTH need at least five years to reach sexual maturity, and it is easy to catch on account of body shape [7,9]. These characteristics of MTH makes it more susceptible to environmental and anthropogenic factors; we therefore speculate that the low diversity of MTH may be due to the bottleneck effect. Since samples were not collected before the population declined, the exact mechanism needs further study.

Moreover, SRAP markers have also been used in genetic variability analyses at the nuclear genome level. Among these markers in the MAL, we obtained 82 polymorphic bands, which is similar to the

result (88 polymorphic bands) reported by Ji et al. based on 13 pairs of primers in *M. amblycephala* of the Liangzi Lake [15], indicating that SRAP has desirable repeatability and stability. On the other hand, based on results of the SRAP analysis, the genetic variability level of MTH was comparable to MTQ and MTJ, which was inconsistent with the low variation level of MTH obtained by the mtDNA fragments. In many species, including fish, discordant results between nuclear diversity and mtDNA diversity are common [39–42], which may be related to inheritance patterns, mutation rates and differences in marker characteristics between the mitochondrial and nuclear genomes. Another probable reason is that the effective population size of MTH is sufficiently small, so that the combined effects of genetic drift produced a greater impact on mtDNA diversity than on nuclear DNA diversity [43].

Although phylogenetic research clarified that MTH shared a common ancestor with MTQ, MTJ and MPL, and provided evidence that MPL should be regarded as one of the geographical populations of *M. terminalis*, the exact genetic differentiation between MTH and other populations in the genus *Megalobrama* remains to be determined at the population level. In this study, information from the haplotype (*D-loop*, *ND2* and *CYTB*) network and PCoA (SRAP markers) analysis suggested that there was a strong genetic divergence of MTH with other closely related populations. Furthermore, the STRUCTURE analysis showed that MTH could be assigned to a particular cluster, whereas MTQ and MTJ belonged to the same cluster. These data demonstrated that MTH underwent long-term reproductive isolation resulting from habitat fragmentation and barriers to gene flow. On the other hand, this finding also reflected that MTH maintains a pure gene pool without any gene pollution from farmed *M. terminalis* or *M. amblycephala*. Notably, the hierarchical AMOVA and pairwise *Fst* values results for the mtDNA and SRAP data revealed significant genetic variation and differentiation among all populations, and it showed higher pairwise *Fst* values between MTH and MPL than MTH with MTQ and MTJ. These findings indicate the genetic differentiation level of MTH from other populations, and further provide evidence to support the species taxonomy of MTH [10]. It is not difficult to conclude that MTH has generated a substantial population structure.

The Heilong River Basin is currently one of the most well-preserved bodies of waters for fish diversity in China [44]. Fish species distributed in the Heilong River usually have the characteristics of large size, strong stress resistance and good meat quality [45]. As the only naturally distributed fish species in the genus *Megalobrama* north of the Yellow River, MTH possesses notably high economic, scientific and ecological value. In particular, MTH has the potential to be an excellent hybrid parent, because long-term segregation in unique habitats may enable it to evolve key genes required for the breeding of fish species in the genus *Megalobrama*. To the best of our knowledge, this report is the first to describe the genetic divergence and population differentiation between MTH and other populations in the genus *Megalobrama*. These data provide an important scientific basis for the further artificial cultivation, breeding application and scientific protection of this endangered species in the future.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1424-2818/12/10/404/s1>: Figure S1: Historical distribution of *M. terminalis* in the Heilong River Basin. HLR, the Heilong River (Fuyuan); AR, the Amur River; SHR, the Songhua River; NR, the Nen River; JPL, Jingpo Lake and KL, Khanka Lake. A red dot represents that wild *M. terminalis* in the location has disappeared. A blue dot indicates that wild *M. terminalis* in the location is still present. Figure S2: SRAP marker profiles generated from partial samples of 6 populations in the genus *Megalobrama* with primer combinations 1f5r (A), 2f9r (B), 4f20r (C) and 7f20r (D). M: pBR 322/*Msp* I marker; Lanes with different color represent samples of corresponding populations (MTH, *M. terminalis* in the Heilong River; MTQ, *M. terminalis* in the Qiantang River; MTJ, *M. terminalis* in the Jinsha River Reservoir; MPL, *M. pellegrini* in the Longxi River; MAL, *M. amblycephala* in the Liangzi Lake and MAY, *M. amblycephala* in the Yi River). The red arrows point to polymorphic bands with amplification stability. Figure S3: Establishment of the *K* value in the STRUCTURE analysis. (A) Relationship between *K* and Delta *K*. (B) Table output following the method of Evanno et al. [37]. Yellow highlight indicates the largest value in the Delta *K* column. Table S1: Sampling sites and number of individuals used for mtDNA and SRAP analysis. Table S2: Information on fragments for amplification and haplotype analysis in the *D-loop*, *ND2* and *CYTB* genes. Reference mtDNA sequence of *Megalobrama terminalis* in the Heilong River (MTH) (accession no. MH289765). Table S3: Sequences of 15 SRAP primers.

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editing, X.H., B.M., C.L., Z.J., X.J., Y.G., J.T. and L.S.; project administration, X.H. and L.S. and funding acquisition, X.H., B.M. and L.S. All authors have read and agreed to the published version of the manuscript.

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