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The genetic diversity, individual relatedness and possible mating system of an isolated population of the Cyprinid species *Megalobrama pellgrini* in upper reaches of the Changjiang (Yangtze) River, China*

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Abstract *Megalobrama pellegrini* is a cyprinid fish endemic to upper reaches of the Changjiang (Yangtze) River, China, which is also an important economic species in the local area. In recent years, resources of this species have decreased sharply and its conservation has drawn great attention. In the present study, we collected 120 individuals from the Longxi River, a tributary isolated from the main channel of the Changjiang River, where *M. pellegrini* is still relatively abundant. Using two different molecular markers, mitochondrial cytochrome *b* (cyt *b*) gene, and nuclear microsatellite (simple sequence repeat, SSR), we analyzed the genetic diversity of this isolated population. The results show that sequence genetic diversity was low (H_d: 0.290 and P_i: 0.000 77 for cyt *b* gene), while the SSR genetic diversity was high (H_o: 0.824 4±0.147 2, H_e: 0.823 5±0.145 1). Analysis indicated that this population had experienced a bottleneck, with inbreeding and small effective population size (around 50). Based on SSR data, relatedness analyzing revealed that the 120 samples were grouped into 10 completely independent clusters. It was inferred that the mating system of *M. pellegrini* was polygamy. We suggested that the low genetic diversity could be induced by the overfishing and inbreeding depression. Therefore, we suggested that the urgent conservation measures should be taken to control the overfishing and give better conditions for the fish to grow and spawn, then to restore population size.

Keyword: isolated population; genetic diversity; individual relatedness; mating system; conservation

1 INTRODUCTION

One of the greatest contributions for the new Darwinism is the recognition of the idea that all the species exists by the form of the population. To maintain high viability, a population has to be large in number and exchange genetic diversity with other populations (Huxley, 1940; Mayr, 1963). Once a population was isolated, it might experience a bottleneck effect (Allendorf and Luikart, 2007), suffer from inbreeding depression (Madsen et al., 1996; Allendorf and Luikart, 2007). This can lead to increasing linkage disequilibrium and decreasing allelic diversity in the population (Hauser et al., 2002; Pardo et al., 2005; Bonnen et al., 2006; Service et al., 2006; Bendjilali et al., 2014), result in decreased fitness and viability, and then ultimately lead the population to the so-called extinction vortex (Gilpin, 1986; Westemeier et al., 1998; Lindenmayer and Peakall, 2000; Grayson et al., 2014). Therefore, great concerns have been given to the isolated populations, some indicators have been proposed to evaluate the

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status of the populations, and many measures have been discussed for their conservation.

Megalobrama pellegrini is an endemic cyprinid fish in the upper reaches of the Changjiang (Yangtze) River (Luo, 1990; Chen, 1998; Li et al., 2007a). It has a very deep body depth in a bream shape and is very important economic fish in the local area. This fish used to inhabit widely the main channel and tributaries of the upper Changjiang River (Luo, 1990; Chen, 1998). However, in the recent decades, due to the intensive anthropologic activities like damming, pollution, and overfishing, it has become very rare in the main channel. Contrary to the resource scarcity in the main channel, *M. pellegrini* is found abundant in a tributary of the upper Changjiang River, the Longxi River (Li et al., 2007b). This river is approximately 97 km long with a drainage area of approximately 521 km^2 and flows into the Changjiang River in Luzhou City, Sichuan Province. In lower reaches of this river, there is a natural cliff up to 35 m high, which forms a natural barrier isolating fish populations of this river from those of the main channel of the Changjiang River (Li et al., 2007b). The previous investigation showed that *M. pellegrini* population in the Longxi River has lower genetic diversity, and differentiated from the nearby population at the median level (Wang et al., 2014). Our recent investigations indicate that the *M. pellegrini* individuals in the Longxi River showed slower growth rate than it was 10 years ago (Li et al., 2007a), and the resources have also become diminished. Therefore, concerns have been raised on how the genetic status of this population is at, whether it has suffered from some genetic catastrophe, and how we should decide conservation measures for this population.

In the present study, we collected samples of *M. pellegrini* individuals from the Longxi River. Using two different molecular markers, mitochondrial cytochrome *b* gene and nuclear microsatellite (simple sequence repeat, SSR), we analyzed their genetic diversity with the purpose (1) to evaluate genetic status of this population and to reveal the probable causes, (2) to identify their mating system and individual relatedness, and (3) to give suitable conservation suggestions.

2 MATERIAL AND METHOD

2.1 Sampling

120 individuals of *M. Pellegrini* were collected from the Longxi River in Luzhou, Sichuan Province by bottom gillnets (mesh size 4.0 cm) from July 2011 to June 2013. The tissues of muscle and fin were preserved in 95% ethanol.

2.2 Laboratory experiments

In the laboratory, genomic DNA was extracted using salt-extraction method proposed by Aljanabi and Martinez (1997). Two different types of markers, cytochrome *b* (cyt *b*) gene of mitochondrial DNA (mtDNA), nuclear microsatellite (simple sequence repeat, SSR) were chosen and amplified by polymerase chain reaction (PCR), which were performed in volumes of 20 μL (SSR) or 30 μL (cyt *b* markers) containing 30–50 ng template DNA, 0.5 μL dNTP mixture (2.5 mmol/L each), 0.3 U Taq DNA Polymerase with $MgSO_4$ (2 mmol/L of Mg^{2+}), 2 μL 10×Taq Buffer, 0.5 μL each primer (10 μmol/L) and supplement by sterile ddH_2O. The PCR reaction conditions for cyt *b* were: 94°C for 4 min, then 35 cycles with 45 s at 94°C, 45 s at the annealing temperature and 1 min at 72°C, with a final elongation at 72°C for 10 min; and for SSR were: 94°C for 5 min, then 35 cycles with 30 s at 94°C, 40 s at the annealing temperature and 1 min at 72°C, with a final elongation at 72°C for 10 min. The information of the primers is shown in Table 1. After being amplified, the PCR products were detected through agarose gel electrophoresis (cyt *b*) or 8% nondenaturing polyacrylamide gels (SSR). Then the fragments (cyt *b*) were sequenced with the same primers as for PCR by Shanghai Sangon Company while the gels (SSR) were stained with Ethidium bromide and viewed under UV light with Genesys software (Syngene).

2.3 Data analysis

cyt *b*: The alignment of cyt *b* sequences were performed with ClustalX version 2.0 (Larkin et al., 2007) and revised manually with SEAVIEW (Galtier et al., 1996). The diversity of population was analyzed using DnaSP v5.10 (Librado and Rozas, 2009) including the number of haplotypes (h), nucleotide diversity (P_i), haplotype diversity (H_d) and the average number of nucleotide differences (k). Software MEGA6.0 (Tamura et al., 2013) was used to calculate the nucleotide composition, conserved sites (C), variable sites (V), parsimony-informative sites (P), and singleton sites (S).

SSR: Genotypes were checked for scoring errors attributable to stutter-products, large allele dropout, or null alleles using Micro-Checker v2.2.3 (van Oosterhout et al., 2004). The number of alleles, allele

Table 1 The primers' information

Gene	Primer sequence (5′→3′)		Repeat motif	*T* (°C)	Reference
	Name	Sequence			
cyt *b*	L14724	GACTTGAAAAACCACCGTTG		56	Xiao et al., 2001
	H15915	CTCCGATCTCCGGATTACAAGAC			
SSR	Mp009F	TGAGTTCGCACCAGAAAGTG	(TG)15	55	KF523864
	Mp009R	ACTCACGACAGGGACAGGAG			
	Mp011F	TGTCATACCCATGCCATTATACA	(AC)27	55	KF523865
	Mp011R	TGGAACAATCAACCACAGATG			
	Mp012F	CCCGTAGAGGGAGAGAGAGC	(GA)19	56	KF523866
	Mp012R	TCCTTCTCTTTGTCAGCACGTA			
	Mp013F	AAAGGCCCTTGAATCATCTG	(CA)9	53	KF523867
	Mp013R	TTATGCCTCCCCCTAAACAG			
	Mp017F	CCCCAGCAGCACATCTCTA	(ACC)4	55	KF523868
	Mp017R	AGGCCACATTCCTTTCCTTC			
	Mp030F	TGGAAAGTGATAGTCAGACAGACA	(AC)19(AGAT)19	55	KF523871
	Mp030R	TCCTGAGTAAGAATGTAGAATAAGGTT			
	Mp043F	TTACCGGTCAAACTGGGAGT	(GATA)12	55	KF523874
	Mp043R	CAAATGTCTCGATCAGACTGC			
	Mp048F	GCTCTTCATCGCTTCTCTGC	(TTTC)22(TC)6	56	KF523875
	Mp048R	TGAGTCCTGAGTAACATTACCATACA			
	Mp049F	ATGGACTGTGAGAGGGACCA	(TG)23	55	KF523876
	Mp049R	GTTTTATTCCCTGGGCCTGT			
	Mp052F	AGCATTGCAGAGGTCAGAGC	(TG)17	56	KF523877
	Mp052R	TCATGATGGTTTGGTACAGGTC			
	Mp055F	GCAGAAGTGCACAGAAAACG	(GT)16	54	KF523878
	Mp055R	TCACATTCACAAGTGGTTCACA			
	Mp061F	AAGTTATTTCTCTTTGCGCTTTT	(TAGA)4	51	KF523881
	Mp061R	CGATTGCATCGTTGAGAGG			
	Mp062F	AAGCTCTGTGAGATTCACCAAAT	(GT)55	54	KF523882
	Mp062R	GGGGATTCTGGATGATGTTG			
	Mp064F	CGAAGGTCCCTGATTGATTG	(TGAT)19(GGAC)4(AGAT)3	55	KF523883
	Mp064R	AATGGGGTCATCGGTCAAC			
	Mp068F	TGTTGGAGTGCGAAAATCAG	(TG)22	53	KF523884
	Mp068R	GGGGAGGGGAAAGTAAGAAA			
	Mp070F	ACACAGCGGTCTGGAAACAT	(AG)22	55	KF523885
	Mp070R	ACACGTTCCCTCTCATGGAC			
	Mp080F	AAATGCAATCTGCGGTCAC	(GT)27	53	KF523886
	Mp080R	TGGTGAAGAGCGTAATCCAA			
	Mp087F	TTGCCAGAATCAGTCAATCAA	(TCAA)8(TCAG)3(CAGT)4(CAAT)5 (AATC)4(TCAG)3(TCTA)3	51	KF523887
	Mp087R	TGAATGGCAAATGCATAGGA			
	Mp126F	TGCTGGAATGAAGCTGTGTG	(TG)35	55	KF523891
	Mp126R	CCCAGCTCTGTTCCTGGTTA			
	Mp128F	CCCTTCAGCCTTGTGAAAGT	(CA)20	53	KF523892
	Mp128R	TCCTCTGCTGCTTGGAATTT			

Table 2 nucleotide composition, diversity, and mutations of cyt *b* gene

No. of sample	Length (bp)	*T* (%)	*C* (%)	*A* (%)	*G* (%)	*A*+*T* (%)	*G*+*C* (%)
120	1 140	27.5	29.0	28.4	15.1	55.9	44.1
h	H_d	P_i	*k*	*C*	*V*	*P*	*S*
9	0.290	0.000 77	0.874	1129	11	6	5

h: the number of haplotypes; H_d: haplotype diversity; P_i: nucleotide diversity; *k*: the average number of nucleotide differences; *C*: conserved sites; *V*: variable sites; *P*: parsimony-informative sites; *S*: singleton sites.

size range, H_o (observed heterozygosity) and H_e (expected heterozygosity) were calculated using PopGene v1.32 (Yeh et al., 1997). Inbreeding coefficient (FIS) was estimated using Arlequin 3.5.1.2 (Excoffier and Lischer, 2010).

We estimated the effective population size (N_e) using the Linkage Disequilibrium Method, as implemented in NE-Estimator V2 (Do et al., 2014). The program BOTTLENECK v1.2.02 (Cornuet and Luikart, 1996) was used to test for evidence of recent bottleneck events based on theoretical expectations under the Stepwise Mutation Model (SMM) (Shriver et al., 1993; Valdes et al., 1993).

The pairwise distances between individuals were calculated by software Populations v1.2.32 (Langella, 2002) and the Neighbor-joining tree was built by MEGA6.0 (Tamura et al., 2013) based on the distance matrix. The relatedness between individuals was estimated by software ML-relate (Kalinowski et al., 2006) and COLONY version2.0.5.8 (Jones and Wang, 2010).

3 RESULT

3.1 Sequence genetic diversity

We got sequences of the complete cyt *b* gene (1 140 bp) for all the 120 samples. There were 9 haplotypes for cyt *b* gene. After alignment, there were no insertion and deletion.

The average nucleotide composition of cyt *b* gene were *T*=27.5%, *C*=29.0%, *A*=28.4%, and *G*=15.1% respectively (Table 2). The content of *G* was very low (15.1%), with the content of *A*+*T* (55.9%) much higher than that of *G*+*C* (44.1%). The H_d was 0.290 and P_i was 0.000 77 (Table 2). There were 11 variable sites (0.96%) including 6 parsimony-informative sites and 5 singleton sites (Table 2).

3.2 SSR genetic diversity

Null alleles were checked by Micro-Checker. There was no evidence of large allele dropout and scoring errors caused by stuttering, but through the 3 loci there existed null alleles (P<0.05).

The expected and observed heterozygosity per locus ranged from 0.282 4 to 0.941 7 (H_e) and 0.178 0 to 1.000 0 (H_o), respectively (Table 3). The average levels of expected and observed heterozygosity were 0.823 5±0.145 1 (H_e) and 0.824 4±0.147 2 (H_o), respectively (Table 3). There were 12 loci with negative FIS and 8 loci with positive FIS. Polymorphism information content (PIC) per locus were from 0.259 6 to 0.938 7 with a mean of 0.805 7±0.155 0 (Table 3). HWE-test showed that 11 loci deviated from Hardy-Weinberg equilibrium.

The Wilcoxon test revealed the null hypothesis of mutation-drift equilibrium under SMM was significantly rejected (P=0.000 05<0.05), and this indicated a recent bottleneck.

Effective population size (N_e) of the whole population was calculated as 35.9, 52.7, and 70.5 respectively at different lowest allele frequency (lowest allele frequency used were 0.05, 0.02, and 0.01 correspondingly).

3.3 Individual relatedness

Individual relatedness for all the 120 samples was analyzed with the software ML-relate (Kalinowski et al., 2006) and COLONY version 2.0.5.8 (Jones and Wang, 2010). Their results were the same and showed that all the individuals were grouped into 10 completely independent clusters (Fig.1). Among them, Cluster 3 and Cluster 4 had the most individuals and were relatively complex than the other ones (Fig.1), while clusters 8, 9 and 10 only had 2 individuals respectively. This relatedness indicated that both male and female *M. pellegrini* might mate with several individuals showing a polygamy system.

We also calculated the pairwise distances between individuals and then built a neighbor-joining tree based on the distance matrix (Fig.2). The tree showed similar relationships to their relatedness clustered independently in overall but with several mixed samples.

Table 3 Genic diversity and fixation indices in *M. pellegrini*

Locus	N_a	N_e	I	H_o	H_e	FIS	PIC
MP09	15	6.058 6	2.036 5	1.000 0	0.834 9	-0.269 5	0.815 3
MP11	20	6.711 2	2.320 8	1.000 0	0.851 0	-0.252 0	0.836 9
MP12	21	12.208 7	2.700 8	1.000 0	0.918 1	-0.123 4	0.912 4
MP13	12	7.103 6	2.145 2	0.979 6	0.859 2	-0.185 9	0.844 3
MP17	7	3.749 6	1.545 0	0.178 0	0.733 3	0.731 9	0.697 1
MP30	22	10.909 9	2.613 6	0.714 3	0.908 3	0.166 5	0.901 3
MP43	23	8.523 0	2.523 7	0.756 3	0.882 7	0.102 5	0.873 1
MP48	27	16.219 8	2.983 7	0.834 9	0.938 3	0.109 9	0.935 0
MP49	25	12.471 7	2.768 4	1.000 0	0.919 8	-0.107 6	0.914 5
MP52	5	3.001 6	1.209 4	1.000 0	0.666 8	-0.507 6	0.606 9
MP55	20	7.612 9	2.348 9	1.000 0	0.868 6	-0.188 6	0.855 9
MP61	9	4.659 0	1.684 3	0.479 0	0.785 4	0.323 0	0.752 5
MP62	29	17.148 3	3.053 7	0.982 1	0.941 7	-0.071 0	0.938 7
MP64	12	5.562 6	1.955 4	0.534 5	0.820 2	0.286 0	0.798 9
MP68	23	9.892 7	2.599 2	0.788 1	0.898 9	0.057 0	0.891 5
MP70	12	6.810 1	2.074 3	1.000 0	0.853 2	-0.244 2	0.836 7
MP80	9	4.176 0	1.640 0	0.991 5	0.760 5	-0.410 0	0.725 8
MP87	5	1.393 5	0.566 7	0.258 3	0.282 4	0.080 4	0.259 6
MP126	28	13.121 3	2.844 5	0.991 1	0.923 8	-0.101 0	0.918 9
MP128	8	5.638 5	1.836 5	1.000 0	0.822 6	-0.312 4	0.799 1
Mean	16.6	8.148 6	2.172 5	0.824 4	0.823 5	-0.045 8	0.805 7
St.dev	8.055 1	4.346 0	0.635 9	0.147 2	0.145 1	0.287 6	0.155 0

N_a: number of alleles per locus; N_e: effective number of alleles; I: Shannon's information index; H_o: observed heterozygosity; H_e: expected heterozygosity (unbiased estimate); FIS: fixation index; PIC: polymorphism information content.

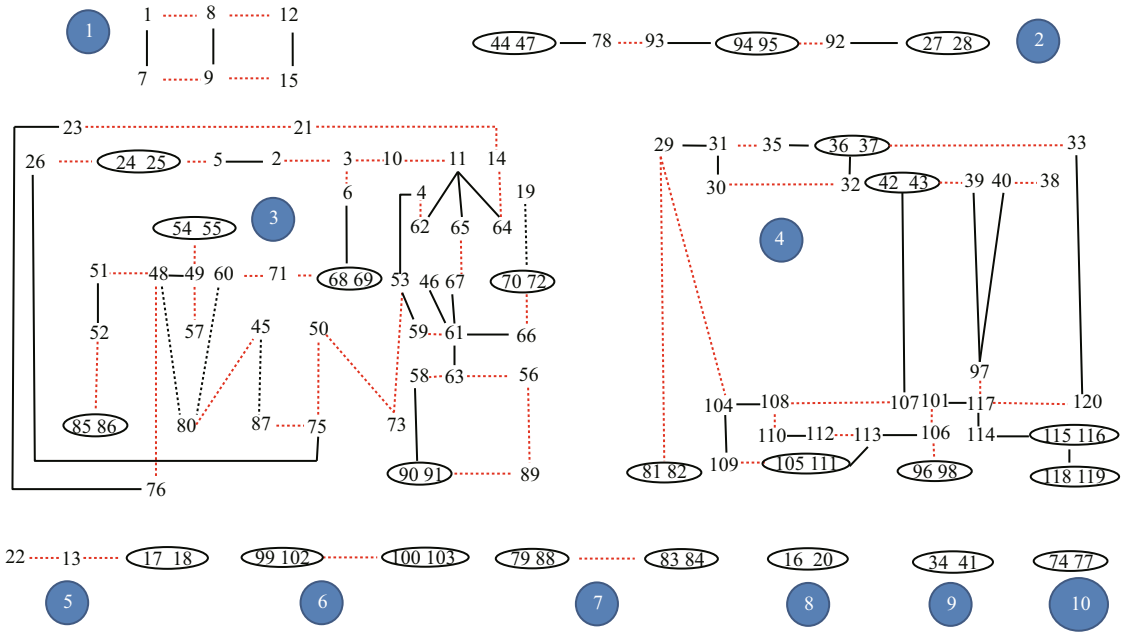


Fig.1 The relatedness between 120 individuals

Circles represent full-siblings, black line and red line represent the half-siblings with same father or same mother respectively; the numbers in blue circles represents the clusters they belong to.



Fig.2 The neighbor-joining tree based on the distance of DAS and Cp using 20 loci of microsatellite

4 DISCUSSION

4.1 Mating system

Normally animal-mating systems can be classified as two main types: mono-mating and poly-mating (Klug, 2011). It was suggested that the reproductive success of males is often limited by access to female mates, whereas the reproductive success of females is often limited by resource acquisition (Bateman, 1948). For the successful reproduction, males need females, and females need resources (Klug, 2011). Thus, the spatial distribution of resources may affect mating systems. It was hypothesized that single individuals may be difficult to monopolize more than one mate if critical resources are uniformly distributed in space. As a result, territoriality will occur, the potential for multiple mating will be low, and monogamy is likely to prevail (Emlen and Oring, 1977).

Alternatively, when critical resources are clumped, some proportion of individuals can potentially monopolize those resources, the potential for polygamy can be relatively high (Emlen and Oring, 1977). However, when the resources are highly clumped, it might become more difficult for individuals to monopolize resources due to the increased level of competition, and the potential for polygamy will possibly lessen (Klug, 2011).

Previous investigations show that *M. pellegrini* spawns adhesive eggs (Li et al., 2007b). In the present study, analysis to SSR data showed that *M. pellegrini* was in polygamy mode, indicating the spawning grounds of *M. pellegrini* were in clumped status.

4.2 Genetic diversity, bottleneck, and inbreeding

Similar to previous investigations, our present study showed that *M. pellegrini* population in the Longxi River was in low-level genetic diversity with cyt *b*. For mtDNA cyt *b* gene, the haplotype diversity was 0.290 and nucleotide diversity was 0.000 77, both were very low. Grant and Bowen (1998) suggested that this situation indicated a probable

recent bottleneck for the population. Conversely, for SSR, the diversity was relatively high, with the average H_o and H_e as 0.824 4±0.147 2 and 0.823 5±0.145 1 respectively. Again, this low-level sequence diversity and high-level SSR diversity suggest the extremely recent population bottleneck (Lee et al., 2006). Population bottlenecks can increase rates of inbreeding, loss of genetic variation and fixation of mildly deleterious alleles, and thereby reduce adaptive potential and increase the probability of extinction (Cornuet and Luikart, 1996; Luikart et al., 1998).

Besides, inbreeding was also found in this population. There were 12 loci with negative FIS, indicating heterozygote excess, and 8 loci with positive FIS meaning the population existing inbreeding. Inbreeding may depress the reproductive fitness and growth rate, reduce genetic variability, viability, and fecundity, and result in accumulations of new deleterious mutations. In addition, inbreeding is considered a fatal factor that will increase the risk of extinction of a wild population (Keller et al., 1994; Frankham and Ralls, 1998; Daniels et al., 2000; Brook et al., 2002).

In the present study, the effective population size was estimated anywhere from 35.9 to 70.5 under different allele frequency, close to 50. When N_e is low, populations can be hard to maintain a long-term survival. It was suggested that the effective population size should be no less than 50 for short time survival and 500 for long-term survival (Franklin, 1980). The N_e of our investigated *M. pellegrini* population was calculated at around 50, which is at the lower limit. We suggested that the population size should be increased.

4.3 Conservation suggestions

Analysis of the present study showed that *M. pellegrini* population in the Longxi River indeed have suffered from some genetic catastrophe, such as the extremely low genetic diversity, inbreeding, recent bottleneck and small effective population size. These results also indicated that the most urgent genetic catastrophe was not induced by the isolation, but overfishing and population miniaturization (Gao et al., 2009, Wang et al., 2014). As for the *M. pellegrini*, it lived in an isolating water environment and suffered from intensive artificial pressure, which may result in a small population size and increase the risk of extinction. Therefore, we suggested the urgent conservation measures should be taken to control fishing, so the fish can grow and reproduce in a normal mode, and then to restore the population size.

5 CONCLUSION

In conclusion, we selected two molecular markers to analyze the *M. pellegrini* population in the Longxi River, the results showed that this population had low genetic diversity, inbreeding, recent bottleneck and small effective population size because of isolation from the Changjiang River superadded human activities, it may face a threat if we do not take measures to protect the population.

6 DATA AVAILABILITY STATEMENT

All data generated and analyzed in this study are included in this manuscript.

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