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Impacts of hatchery release on genetic structure of rock carp *Procypris rabaudi* in the upper Yangtze River, China

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Abstract Rock carp *Procypris rabaudi* is a vulnerable endemic fish in the upper Yangtze River. Hatchery release has been carried out as a major stock enhancement strategy for this species. Ten microsatellite loci were chosen to compare genetic variation between one wild population and two hatchery groups to evaluate the potential impacts of hatchery release on the genetic structure of the wild population. Two different models indicated strong evidence of recent bottlenecks in all groups. The hatchery groups were lower in the mean number of alleles per locus, allelic richness, and allelic diversity compared with the wild population. The 80% membership coefficient indicated that 14% of the wild fish could be assigned as hybrids of wild and hatchery fish. Our results suggested that hatchery release will further reduce the natural genetic diversity in the wild population, change the genetic structure of the rock carp population, and may not benefit restoration of this vulnerable fish species.

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Introduction

Hatchery release is one of the major techniques for fish stock enhancement, and has been successfully applied in conservation of threatened species and restoration of some commercial species [1]. Hatchery release may have potential adverse effects on gene pools of wild populations [2], which may result in reduced offspring fitness and decreased effective population size of wild populations [3]. An investigation of the genetic background of both the wild population and hatchery fish is fundamental to evaluate effects of hatchery release on the wild population and the efficiency of hatchery release for conservation goals [4].

The fish fauna of the upper Yangtze Basin is highly diversified with more than 260 species recorded, including 112 endemic species [5]. However, this diversity is highly threatened, with several species dramatically declining in abundance and even becoming extinct [6]. Dam construction, pollution, and overexploitation have been suggested as primary agents of fish resource decline in the upper Yangtze River [5]. Habitat protection and formation of aquatic sanctuaries have been applied in some tributaries, but the protected habitats and targeted species are very limited [7]. Mass-scale hatchery release has been adopted and practiced as a major strategy for stock enhancement for both commercial and threatened endemic fishes [8]. Nevertheless, not much attention has been focused on the impact of hatchery releases on the genetic make-up of wild populations in the Yangtze Basin. As many of the threatened endemic species in the upper Yangtze Basin are

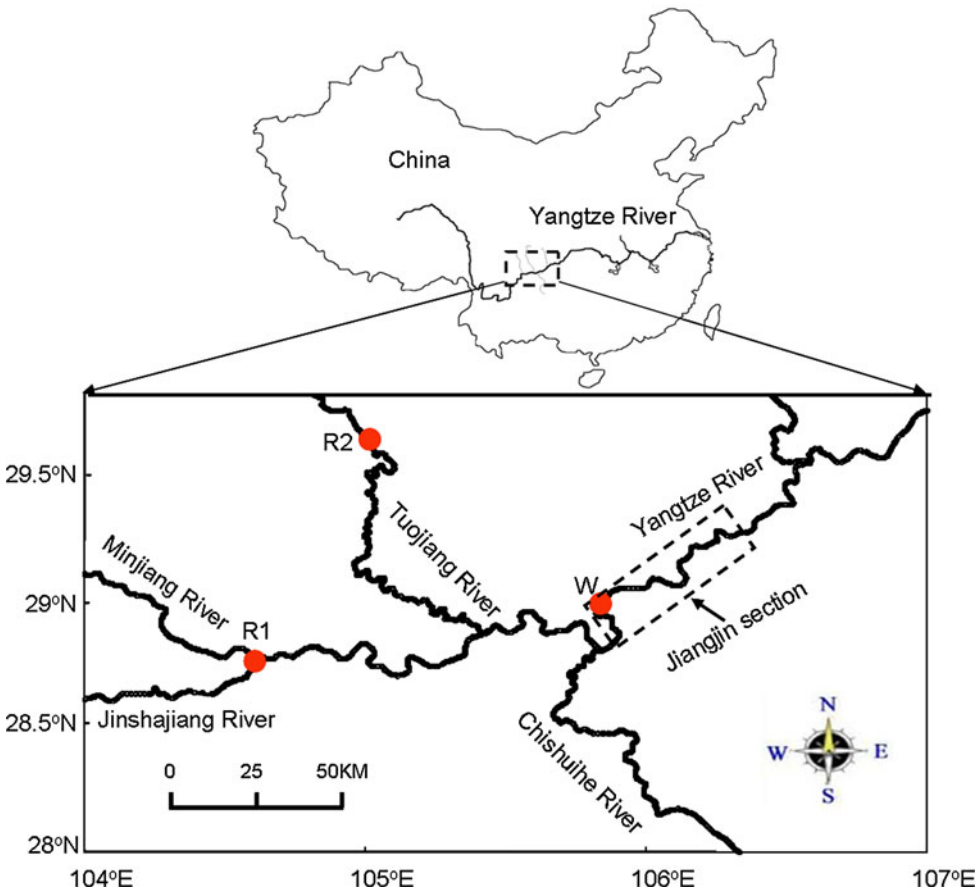

Fig. 1 Major distribution area and sampling locations of rock carp *Procypris rabaudi* in the upper Yangtze River. *W* sampling site of wild juveniles at Jiangjin, upper Yangtze River, *R1* location of the hatchery of Yibin Ancient and Rare Animal Institute, *R2* location of the hatchery of Neijiang Special Aquatic Animal Farm

limited in abundance and distribution area, investigations of the impacts of stocking practices on the genetic structure of the targeted populations, and evaluation of the efficiency of hatchery release for their conservation, are imperative.

The rock carp *Procypris rabaudi* is an endemic species in the upper Yangtze River and its tributaries, mainly Jinshajiang, Minjiang, Chishuihe, and Tuojiang [9]. It is a demersal fish, inhabits mainly slow-flowing deep water with rocky substrate, and overwinters in rocky holes in deep pools [10]. The fish matures at the age of 4 years. Fecundity ranges from 2.5×10^4 to 1.5×10^5 eggs per female, showing variation with body size [9]. Spawning mostly occurs during February and April when the water temperature is between 18°C and 26°C. During the spawning season, the adults migrate upward to the tributaries, and spawn under rocky shelters in rushing streams [11]. The eggs are adhesive, attaching to the gravel substrate at the bottom [12]. Rock carp is an omnivorous species, with an assortment of plants, phytoplankton, aquatic insect larvae, and small fishes as food. The wild population of rock carp has dramatically declined since the 1970s, and it is currently listed as a vulnerable species in China [10]. Associated with the resource decline, its distribution area has been getting narrow. The Three Gorges Reservoir further induced the decline of rock carp, directly eliminating its major habitat in the impounded area [13]. Hatchery release of artificially bred juveniles has been a major strategy for conservation and stock enhancement of rock carp since 2005. Release is carried out in May each year. The released juveniles are either hatched in the previous year or in February through April in the year of release. Size of the released juvenile is required to be larger than 20 mm standard length (SL). About 2×10^5 hatchery juveniles of rock carp are released at the upper section of Yangtze River each year.

The objectives of the present study are to compare the genetic make-up of the wild and hatchery rock carp, and to assess the effects of hatchery release on the genetics of the wild population. Results could provide essential information for successful conservation of this vulnerable endemic fish.

Materials and methods

Sampling design and specimen collection

A total of 146 juvenile rock carp were sampled from the wild population and two hatchery groups in 2009. The wild juveniles (W, $n = 50$) were collected at Jiangjin (29°03′N, 105°51′E) from the Yangtze River (Fig. 1). Specimens

Table 1 Information and amplification conditions of loci and amplification results of wild rock carp sampled at the Jiangjin section of the upper Yangtze River in 2009 ($n = 50$)

Locus	GenBank accession	Amplification conditions		Amplification results	
		Annealing temperature (°C)	$[Mg^{2+}]$ (mM)	No. of alleles	PIC
Pra02	EU541506	61	1.25	5	0.68
Pra18	EU541507	62	1.0	9	0.78
Pra20	EF570127	58	0.75	12	0.85
Pra23	EF570128	59	1.0	6	0.61
Pra26	EF570129	60	1.0	7	0.74
Pra27	EF570123	58	1.0	5	0.72
Pra72	EU541511	64	1.0	9	0.83
Pra77	EF570130	58	0.75	11	0.85
Pra86	EF570124	58	1.5	8	0.83
Pra117	EU541515	60	2.0	9	0.84

PIC polymorphism information content

were collected using a set net (3-mm mesh size). Being a vulnerable species, sampling for rock carp is restricted. Only young of the year (YOY) juveniles with SL smaller than 20 mm were collected and analyzed in this study. As the hatchery juveniles released have SL larger than 20 mm, the wild-collected juveniles with SL smaller than 20 mm were all wild-born fish.

Hatchery juveniles were collected from the hatchery of the Yibin Ancient and Rare Animal Institute (28°46′N, 104°37′E; R1, $n = 51$), and the hatchery of the Neijiang Special Aquatic Animal Farm (29°34′N, 105°04′E; R2, $n = 45$) (Fig. 1). These two hatcheries produce the majority of juvenile rock carp released into the Yangtze River. The original rock carp broodstocks of both hatcheries were collected at the Jiangjin section of the Yangtze River in the 1990s. Artificial propagation was successful in 2001. However, the information about broodstocks and breeding protocol at both hatcheries is very limited. We also did not get permission for any adult sampling from the hatcheries, due to potential damage to the fish. All specimens were preserved in 95% ethanol and stored at 4°C for DNA extraction.

Microsatellite genotyping

Total DNA was extracted from fin and muscle tissues using the Genomic DNA kit (Tiangen, China), detected by gelose gels, and quantified using a spectrophotometer. DNA concentration was calculated and diluted to create a standardized concentration of 2.5 ng/μl of each sample for further polymerase chain reaction (PCR) amplification.

Microsatellite primers of rock carp were screened [14, 15], and 10 highly polymorphic primers were selected to be amplified for all specimens (Table 1). Amplification was performed using a thermal cycler (GeneAmp PCR system 9400, USA) with an initial denaturing period of 5 min at 95°C followed by 30 cycles of 94°C for 30 s, primer-specific annealing temperature (Table 1) for 30 s, and 72°C for 45 s, followed by a final extension of 7 min at 72°C. Total volume of amplification was 20 μl, containing a final concentration of 1× PCR buffer, 1 U Taq polymerase (DBI, USA), 0.2 mM primer, 0.2 mM dNTP, and 10 ng DNA. Concentration of Mg^{2+} was adjusted depending on individual locus (Table 1). To verify the reliability of the reaction, all amplifications were conducted with negative controls.

Products of PCR (4 μl) were separated on 8% nondenaturing polyacrylamide gel for allele detection. Electrophoresis was conducted using the Liuyi electrophoresis system (Liuyi, Beijing, China). Time and voltage of electrophoresis depended on the size of amplified DNA fragment. The DNA fragments were visualized using silver staining protocol. Image data were scored using Alphalmager version 6.0 software (Alpha Innotech, California, USA), and the genotype was determined for each specimen.

Data analyses

Polymorphism information content (PIC) of each locus for the wild juveniles was calculated to evaluate the discrimination effectiveness of the microsatellite. The number of alleles per locus (A), observed and expected heterozygosities (H_o and H_e), and Hardy–Weinberg equilibrium (HWE) were calculated for W, R1, and R2, respectively, using ARLEQUIN version 3.1 (available from http://cmpg.unibe.ch/software/arlequin3). Allelic richness (A_r) was estimated for each group using FSTAT version 2.9.3.2 (available from http://www.unil.ch/izea/softwares/fstat.html). For A_r estimation, the number of fish in each group was standardized to 45 (the minimum number of individuals among groups). Differences in A, A_r, H_o, and H_e among groups were tested for significance using Wilcoxon's signed-rank tests or Friedman's analysis of variance (ANOVA) [16], carried out using STATISTICA 6.0.

Table 2 Allelic variability at 10 microsatellite loci of rock carp

Locus		Pra02	Pra18	Pra20	Pra23	Pra26	Pra27	Pra72	Pra77	Pra86	Pra117	Mean (SE)
S	W	260–296	352–424	158–236	234–266	236–274	250–282	172–238	174–266	244–306	268–334	–
	R1	260–296	372–424	164–236	238–266	236–274	250–282	172–238	180–266	244–290	276–334	–
	R2	266–296	372–424	158–236	238–266	236–274	250–282	172–238	204–248	260–306	284–334	–
A	W	5	9	12	6	7	5	9	11	8	9	8.1 (0.75)
	R1	5	7	10	5	7	5	9	10	7	7	7.2 (0.61)
	R2	4	5	10	5	7	5	8	6	7	7	6.4 (0.56)
A_r	W	5.00	8.79	11.9	5.99	6.90	5.00	8.90	10.9	7.99	8.89	8.03 (0.73)
	R1	5.00	6.99	9.65	4.88	7.00	5.00	8.88	9.75	7.00	6.88	7.1 (0.58)
	R2	4.00	5.00	10.0	5.00	7.00	5.00	8.00	6.00	7.00	7.00	6.4 (0.56)
H_o	W	0.40	0.50	0.46	0.50	0.30	0.40	0.50	0.64	0.40	0.34	0.44 (0.03)
	R1	0.20	0.55	0.51	0.37	0.63	0.27	0.64	0.57	0.59	0.41	0.47 (0.05)
	R2	0.36	0.40	0.33	0.53	0.42	0.22	0.42	0.69	0.27	0.44	0.41 (0.04)
H_e	W	0.72	0.8	0.86	0.67	0.77	0.76	0.85	0.87	0.85	0.85	0.8 (0.02)
	R1	0.62	0.74	0.77	0.73	0.83	0.78	0.84	0.84	0.82	0.80	0.78 (0.02)
	R2	0.64	0.63	0.83	0.67	0.82	0.77	0.73	0.80	0.76	0.82	0.75 (0.02)

W wild population, *R1* stock from the hatchery of the Yibin Ancient and Rare Animal Institute, *R2* stock from the hatchery of the Neijiang Special Aquatic Animal Farm, *S* size range in bp, *A* number of alleles per locus, total 81, 72, and 64 alleles in W, R1, and R2, respectively, *Ar* allelic richness, *Ho* observed heterozygosity, *He* expected heterozygosity

Evidence of recent bottleneck was tested using BOTTLENECK version 1.2.02 (available from http://www.ensam.inra.fr/URLB). Two mutation models were selected: the infinite alleles model (IAM) and the two-phase model (TPM). For the TPM, parameter values were chose as 90% for the percentage of the stepwise mutation model (SMM), and variance of 10 [17].

Hierarchical analysis of molecular variance (AMOVA) was applied to determine genetic differentiation (F_{st}) among groups using ARLEQUIN version 3.1. Statistics was tested by 1×10^3 permutations. The significance level for pairwise genetic differentiation (pairwise F_{st}) comparisons was adjusted to $p = 0.05/3$, where 3 is the number of comparisons made, based on Bonferroni correction [18].

The Bayesian assignment test was applied to evaluate the number of inferred genetic population clusters and to test the proportion of genetic admixture using STRUCTURE version 2.3 (available from http://pritch.bsd.uchicago.edu/structure.html). The admixture model was chosen and run with values of population clusters (K) from 1 to 10 to identify the most probable number of clusters. The ideal K was selected using the posterior probability of the data as suggested by Garnier et al. [19]. For each run, a Markov chain Monte Carlo (MCMC) method was run under conditions of 5×10^5 burn-in periods and 5×10^5 replications. Individuals were assigned to clusters with minimum membership of $q \geq 0.80$ or jointly to two or more clusters if the minimum sum of $q_i + q_j + \ldots q_n \geq 0.80$ for admixed individuals [20].

Results

Polymorphism information content of the selected loci ranged from 0.61 (Pra23) to 0.85 (Pra20 and Pra77) with mean ± standard error (SE) of 0.77 ± 0.03 (Table 1). In total, 81, 72, and 64 alleles were detected from the 10 loci in W, R1, and R2, respectively. Compared with W, there were 9 alleles (11%) that had disappeared in R1 and 17 alleles (21%) in R2 (Table 2). The allele size range was wider at 6 loci (Pra18, Pra20, Pra23, Pra77, Pra86, and Pra117) in W than in R1 and R2; on the other hand, there were no loci having narrower width of allele size range in W than in R1 and R2 (Table 2). *A* ranged between 5 (Pra02, Pra27) and 12 (Pra20) in W. Among the ten loci, W had higher *A* than R1 and R2 at six loci (Pra18, Pra20, Pra23, Pra27, Pra77, and Pra86); conversely, there were no loci having lower *A* in W than in R1 and R2 (Friedman's ANOVAs, $p < 0.01$) (Table 2). A_r was significantly lower

Table 3 One-tailed *p* values for a Wilcoxon test of heterozygote excess under two mutation models

Group	IAM	TPM
W	0.0005	0.0015
R1	0.0005	0.0161
R2	0.0005	0.0093

W wild population, *R1* stock from the hatchery of the Yibin Ancient and Rare Animal Institute, *R2* stock from the hatchery of Neijiang Special Aquatic Animal Farm, *IAM* infinite alleles model, *TPM* two-phase model

Table 4 Hierarchical analysis of molecular variance (AMOVA) for three groups

Source of variation	*df*	Sum of squares	Variance components	Percentage of variation	*p* value
Among population	2	36.25	0.13	3.16	0.00
Among individuals within populations	143	808.65	1.72	42.26	0.00
Within individuals	146	324.00	1.22	54.59	0.00
Total	291	1168.91	4.07	100	0.00

Table 5 Pairwise genetic differentiation (F_{st}) among the hatchery stocks and the wild population

W	W	R1
R1	0.03798*	–
R2	0.04735*	0.02193*

W wild population, *R1* stock from the hatchery of the Yibin Ancient and Rare Animal Institute, *R2* stock from the hatchery of the Neijiang Special Aquatic Animal Farm

* Significant differentiation after Bonferroni correction ($p = 0.05/3$)

in R1 and R2 than in W (Wilcoxon's signed-rank tests, $p < 0.05$). H_o (0.44, 0.47, and 0.41 in W, R1, and R2, respectively) and H_e (0.8, 0.78, and 0.75 in W, R1, and R2, respectively) were not significantly different among the groups (Friedman's ANOVAs: $p < 0.67$ for H_o, $p < 0.13$ for H_e) (Table 2), and H_o was significantly lower than H_e in every case (Friedman's ANOVAs, $p < 0.05$). Nine loci deviated from HWE in rock carp ($p < 0.05$) with the exceptions of Pra23.

Significant heterozygote excess was detected under IAM and TPM for all groups in the BOTTLENECK results ($p < 0.05$) (Table 3).

The overall F_{st} was significant among the three groups ($F_{st} = 0.036$, $p < 0.05$). However, it accounted for only 3.16% of the genetic variation among groups (Table 4). Pairwise F_{st} comparisons indicated that the hatchery groups were significantly differentiated from W and from each other ($p < 0.017$ after Bonferroni correction) (Table 5).

The results of Bayesian assignment test indicated two ($K = 2$) or three ($K = 3$) distinct clusters within the specimens. The $K = 2$ analysis distinguished two clusters representing two distinct genetic groups, i.e., the wild and hatchery genotypes (Fig. 2). To investigate the genetic structure of the wild population and hybrids of wild and hatchery fish in wild population, further analysis of structure and admixing of the wild population was performed using $K = 2$. The q of wild individuals was ranked from lowest to highest, where an individual with $q = 1$ would be a purely wild fish and $q = 0$ would be a purely domestic one. The 80% probability limits for q indicated that at least 7 individuals (14%) in W should be assigned as hybrids of wild and hatchery fish (Fig. 3).

Discussion

The wild juvenile rock carp analyzed in this study were collected at Jiangjin section. This area is currently near the downstream end of the rock carp distribution in the upper Yangtze River, and is the major nursery of rock carp [13]. Considering the narrow distribution area and the upward migration for spawning of the rock carp [11], we suggest that the collected juveniles should be an admixture of the offspring born in a variety of spawning grounds, and should primarily reflect the genetic background of the wild population. The original broodstocks of rock carp in both hatcheries sampled in this study were also collected from the Jiangjin section of the Yangtze River. Thus, any genetic difference between the wild and hatchery groups should reflect the genetic changes in the hatchery stocks induced by culture activities. The 10 microsatellite loci (PIC ranging from 0.61 to 0.85) investigated in this study were proved to have sufficient allelic information for effectively detecting the genetic background of rock carp and to fully compare genetic variations between hatchery and wild groups [21].

It is clear that stocking of hatchery-raised rock carp has reduced the genetic fitness of the wild population in the Yangtze River. The mean A and H_o detected in rock carp (8.1 and 0.45, respectively; Table 2) in the wild population were lower than in most freshwater species (mean 9.1 and 0.54, respectively) [22], indicating low genetic diversity of the wild carp rock. This low genetic diversity has also been observed in rock carp in the Hejiang section, adjacent to Jiangjin [23]. The tests using IAM and TPM found strong evidence of recent bottleneck in the wild rock carp (Table 3). Inbreeding depression is often exacerbated in populations suffering significant bottleneck effects, which will lead to further decline in genetic diversity [24].

A and A_r in both hatchery groups were lower than in wild population of rock carp (Table 2). Reduced allelic diversity was also observed in the hatchery fish compared with the wild population (Table 2). As the original broodstocks of released rock carp in both hatcheries originated from the same geographic area as the wild samples, results of the present study indicate reduced genetic diversity of the hatchery fish. Such reduction of genetic variability in hatchery fish has been reported in many other

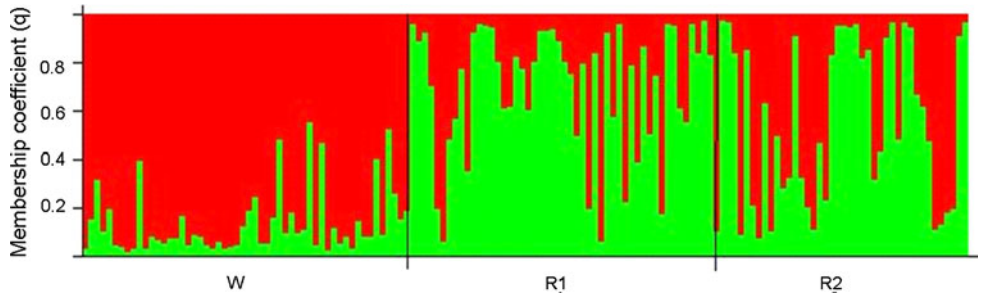

Fig. 2 Admixture analysis of rock carp genotypes sampled in the wild and two hatcheries in the upper Yangtze River in 2009. The analysis was performed for $K = 2$ with each *vertical bar* representing the membership coefficient (q) of an individual. The individuals were grouped by sampling locations. *W* wild juveniles, *R1* juveniles from the hatchery of Yibin Ancient and Rare Animal Institute, *R2* juveniles from the hatchery of Neijiang Special Aquatic Animal Farm. The analysis differentiated between wild and hatchery genotypes; length of vertical bar proportional to the estimated q in the 2 clusters. The membership q indicated to which cluster an individual should be assigned (threshold value of $q = 0.80$)

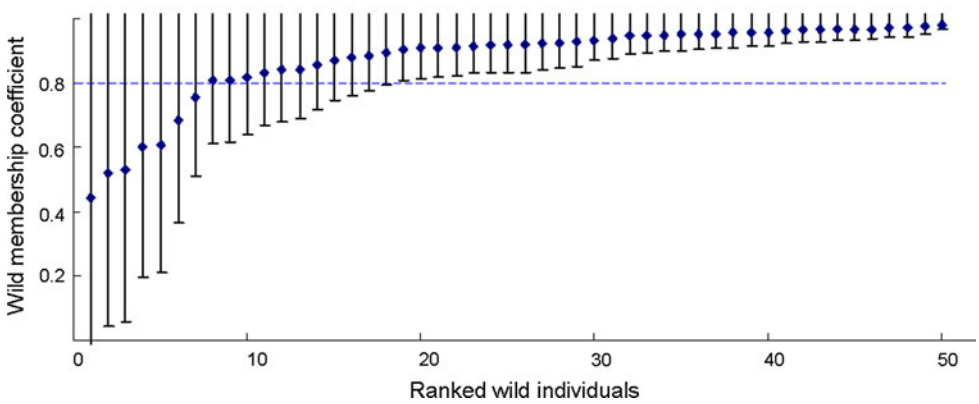

Fig. 3 Membership coefficients (q) ranked in increasing order for individuals of wild rock carp. An individual was considered as a wild rock carp when $q \geq 0.80$, and as a hybrid one when $q < 0.80$

species [25]. Release of these lower-diversity hatchery fish could directly reduce the genetic diversity of the population, particularly when the size of the wild population is small [4].

Significant genetic differentiation was observed between wild and hatchery groups of rock carp ($F_{st} = 0.036$, $p < 0.05$). Hybrids (accounting for 14% of the wild population) of wild and hatchery fish were detected among the wild rock carp examined in this study (Fig. 3). Hybridization between genetically distinct hatchery and wild fishes could change the genetic structure of the wild population and increase the frequencies of deleterious recessive alleles [26]. Such hybridization has been shown to adversely affect conservation efforts for many species such as Atlantic salmon *Salmo salar* [27] and steelhead trout *Oncorhynchus mykiss* [28].

Genetic diversity and population size are generally correlated with population fitness [29]. Abundances of rock carp have dramatically declined since the 1970s [10]. Our results indicated low genetic diversity and recent bottleneck in the wild population. This low genetic diversity will decrease the capacity of wild individuals for environmental adaptation, thus increasing the risk of extinction. Genetic differentiation and lower genetic diversity of the hatchery fish studied here (compared with the wild fish), together with the detection of hybridization between hatchery and wild fish, suggest that continued hatchery releases will further reduce genetic diversity and change the genetic structure of the wild population, thus making effective conservation and restoration of rock carp more difficult. Hatchery release has been proposed as a major conservation strategy for more than 20 vulnerable or endangered species in China [30]. It is imperative to develop proper genetic management protocols for conservation hatcheries to avoid the risk of reducing genetic diversity and causing deleterious genetic drift in wild fish populations that receive stockings of hatchery-raised fish. Simultaneously, other critical conservation strategies (e.g., habitat protection) should be emphasized for successful conservation and restoration of these species in the Yangtze River Basin.

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