Genetics and Molecular Research

Microsatellite markers reveal genetic divergence among wild and cultured populations of Chinese sucker *Myxocyprinus asiaticus*

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ABSTRACT. Studies of genetic diversity and genetic population structure are critical for the conservation and management of endangered species. The Chinese sucker *Myxocyprinus asiaticus* is a vulnerable monotypic species in China, which is at a risk of decline owing to fluctuations in effective population size and other demographic and environmental factors. We screened 11 microsatellite loci in 214 individuals to assess genetic differentiation in both wild and cultured populations. The single extant wild population had a higher number of alleles (13) than the cultured populations (average 7.3). High levels of genetic diversity, expressed as observed and expected heterozygosity (H_O = 0.771, H_E = 0.748, respectively), were found in both wild and cultured populations. We also report significant differentiation among

wild and cultured populations (global F_{ST} = 0.023, P < 0.001). Both STRUCTURE analysis and neighbor-joining tree revealed three moderately divergent primary genetic clusters: the wild Yangtze population and the Sichuan population were each identified as an individual cluster, with the remaining populations clustered together. Twenty-two samples collected from the Yangtze River were assigned to the cultured population, demonstrating the efficacy of artificial propagation to avoid drastic reduction in the population size of *M. asiaticus*. These genetic data support the endangered status of the *M. asiaticus* and have implications for conservation management planning.

Key words: Chinese sucker; Microsatellite; Genetic differentiation; Population structure; Conservation

INTRODUCTION

The management of endangered species involves preserving extant populations and increasing their abundance and distribution (Primack, 1998). In addition to knowledge on the behavior and ecology of the species, it is useful to assess the genetic diversity of populations, which can affect their adaptive potential (Lande, 1988). Small or isolated populations present limited genetic diversity due to the loss of alleles, and are therefore at risk of extinction (Frankham, 2005). Genetic variation is essential for ensuring the evolutionary potential of a species (Allendorf et al., 2008), and determining the genetic structure and diversity of small and isolated populations may be critical for management and conservation planning (Palsbøll et al., 2007).

The Chinese sucker *Myxocyprinus asiaticus*, which is designated a class II state-protected animal and vulnerable species by the International Union for Conservation of Nature (IUCN) (Wang, 1998), is the only representative of the Catostomidae in Asia (Nelson, 1976; Harris and Mayden, 2001). Historically, *M. asiaticus* was mainly distributed in the Yangtze and Minjiang rivers, but currently, the only extant wild population is that of the Yangtze River, where it is mainly confined to the upper reaches (Wang, 1998). The population decline of this species has been attributed to over-fishing, water pollution, dam construction, especially the Gezhouba Dam in the 1980s and the Three Gorges Dam in the 1990s, and other anthropogenic effects (Wang, 1998; Zhang et al., 2000). Sun et al. (2002, 2004) analyzed the genetic structure of the Yangtze River *M. asiaticus* population using the mitochondrial d-loop region and a mitochondrial DNA marker. They observed a positive relationship between the aquatic distance and the genetic distance (F_{ST}) among the populations. Xu et al. (2013) analyzed the genetic diversity and differentiation of the species in four broodstocks using microsatellite loci, and found that the restocking programs, without genetic management, might aggravate the loss of genetic diversity and increase genetic differentiation. The maintenance of population connectivity among localities requires significant attention in future restocking efforts.

In order to conserve this species, hatchery and culture programs were developed by several national institutes and private farms. The artificial propagation of *M. asiaticus* was first achieved at the Wanzhou Fisheries Research Institute in the 1990s (Chen, 1999). Adult fish caught by local fishermen were sent to hatcheries to be used as broodfish. Currently, five farms rear millions of juvenile *M. asiaticus,* which are intended for release to maintain wild populations (Song et al., 2008; Yang et al., 2009). The release of hatchery-reared juveniles into

natural river systems is considered an effective means of increasing wild fish populations and facilitating the recovery and long-term protection of the Chinese sucker.

We used a larger and more geographically continuous set of samples (N = 214) and 11 highly variable microsatellite DNA markers to test this hypothesis and to investigate the genetic structure of the five largest remaining populations of cultured *M. asiaticus* and the wild population in the Yangtze River. Finally, we provide perspective on the implications of these findings for conservation and management strategies.

MATERIAL AND METHODS

Sampling and DNA isolation

Fin-clip samples of 214 *M. asiaticus* were collected from the wild population in the middle and upper reaches of the Yangtze River and from five cultured populations between September 2009 and March 2013. Because *M. asiaticus* is an endangered species, only 59 individuals were collected from the Yangtze River over the course of the four years, and are together referred to as the Yangtze River population. The remaining 155 individuals were collected from five *M. asiaticus* farms [85 samples from Wanzhou Fisheries Research Institute (WZ), 14 samples from Sichuan Fisheries Research Institute (SC), 13 samples from the Yibin Rare Aquatic Animal Research Institute (YB), 34 samples from Wuhan Aquatic Restocking Center (WH), and 9 samples from Yichang Sanjiang Fisheries Co., Ltd. (YC)] (Figure 1; Table 1). Samples were placed in 95% ethanol in sterile 1.5-mL centrifuge tubes on ice and stored at -20°C.

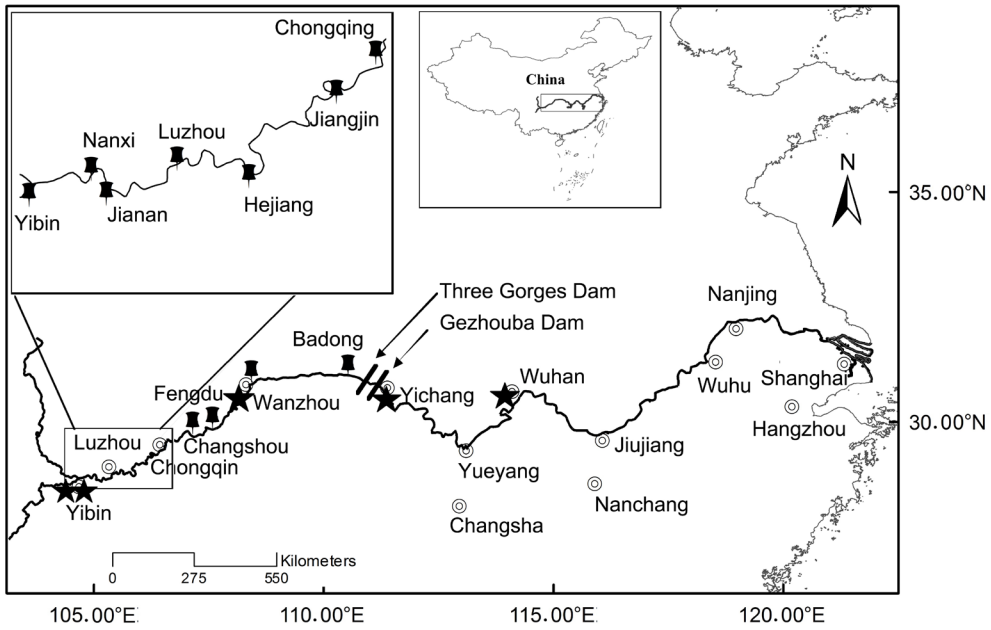

Figure 1. Map showing the sites sampled for Chinese suckers. Stars represent the locations of the Chinese sucker farms; pins represent the monitoring sites of Chinese suckers in the Yangtze River.

Table 1. Mean values for basic descriptive parameters: number of Chinese suckers genotyped (N), alleles per locus (N_A), observed heterozygosity (H_O), and expected heterozygosity (H_E).

Location	N	N_A	H_O	H_E	P-$_{bottleneck}$
SC	14	7.0	0.769	0.726	0.026
WH	34	10.1	0.766	0.767	0.148
WZ	85	11.5	0.784	0.758	0.230
YB	13	8.5	0.804	0.767	0.000
YC	9	6.7	0.768	0.714	0.000
Mean (cultured)	31	7.3	0.783	0.730	
YZ	59	13.0	0.738	0.758	0.074
Mean (all samples)	35.3	9.5	0.771	0.748	

SC: Sichuan Fisheries Research Institute; WH: Wuhan Aquatic Introduction Center; WZ: Wanzhou Fisheries Research Institute; YB: Yibin Rare Aquatic Animal Research Institute; YC: Yichang Sanjiang Fisheries Co., LTD; YZ: Yangtze River.

DNA was extracted using a standard phenol/chloroform technique (Sambrook and Russell, 2001) and stored in Tris-EDTA (TE) buffer. Extracted genomic DNA was checked using 1.0% agarose gel electrophoresis. Each sample was diluted to 10 ng/μL in DNase/RNase-free water. For genotyping, we used 11 microsatellite markers previously developed for *M. asiaticus* [Mas3, Mas11, Mas13, Mas15, Mas21, Mas22, and Mas23 (Cheng et al., 2013); M5, M11, M23, and M46 (Li et al., 2013)]. Details of amplification procedures can be found in Howeth et al. (2008). We multiplexed amplified products on an ABI Prism 3730xl and measured fragment length with a Rox-500 standard in GENEMAPPER v. 4.0 (Applied Biosystems, Foster City, CA, USA) using the Rox size standard (FAM and HEX dye sets).

Statistical analyses

The mean number of alleles per locus (N_A), and the observed and expected heterozygosity (H_O and H_E, respectively) per locus in each population were estimated in GenAlEx v. 6.4 (Peakall and Smouse, 2006). GENEPOP v 4.0.10 (Raymond and Rousset, 2003) was used for pair-wise tests of linkage disequilibrium and migration rates (Nm) between populations following the method described by Barton and Slatkin (1986). The exact probability (Fisher's method) of significant deviation from Hardy-Weinberg equilibrium (HWE) was estimated using the Markov chain method (dememorization = 1000; batches = 100; iterations per batch = 1000) (Guo and Thompson, 1992) conducted in GENEPOP. The presence of null alleles was assessed using MICROCHECKER and the genotype frequencies of populations with null alleles were adjusted accordingly.

To detect population decline and evidence of historical bottlenecks, we used BOTTLENECK v 1.2.02 (Cornuet and Luikart, 1996), which identifies recent reductions in the effective population size by comparing heterozygosity from observed data with that of a simulated population at neutral mutation-drift equilibrium. Here we used two models of mutation: the more conservative stepwise mutation model (SMM) and the less conservative infinite allele model (IAM) (Luikart and Cornuet, 1998; Maudet et al., 2002). To quantify hierarchical genetic variation, we estimated population differentiation F-statistics (F_{ST}) and analysis of molecular variance (AMOVA) in Arlequin v 3.5 (Excoffier and Lischer, 2010) between all pairs of the six sampled populations and calculated the statistical significance (Weir and Cockerham, 1984) using 1,000,000 genotypic permutations followed by sequential Bonferroni correction for the pair-wise population comparisons (Rice, 1989).

To assess gene flow within populations as revealed by their substructure, an assignment test implemented in GENECLASS v 2.0 software (Piry et al., 2004) was used to determine the extent to which individuals could be correctly assigned to their collection of origin using a Bayesian approach (Rannala and Mountain, 1997). STRUCTURE v 2.2 (Pritchard et al., 2000) was used to estimate the number of genetic clusters represented in the data, based on an admixture ancestry and correlated allele frequencies model. A total of 2-10 possible populations (K) were tested. A 50,000 burn-in period including sampling locations as priors used 300,000 Markov Chain Monte Carlo (MCMC) iterations for each value of K (2-10). The optimal number of clusters, K, was determined according to the ad hoc statistic ΔK, based on the rate of change in the log probability of data between successive K values (Evanno et al., 2005). A neighbor-joining (NJ) unrooted dendrogram of populations was created with the unweighted pair-group method with arithmetic average (UPGMA) in Poptree (Takezaki et al., 2010) software for constructing population trees from allele frequency data.

RESULTS

Genetic diversity

A total of 214 samples were successfully genotyped for an average of 35.3 ± 3.4 (mean ± SE) individuals per population. We found 215 alleles across the 11 microsatellite loci with an average of 19.5 alleles per locus, ranging from 6 alleles at Mas 23 to 30 alleles at M 46 (Table 1). The average N_A varied significantly among wild and farmed populations (Table 1). The highest N_A was observed in the wild population, YZ, (13.0 ± 1.6), while the lowest N_A was in population YC (6.7 ± 1.0). The *H*o showed minimal variation among the six populations (0.74 ± 0.05 to 0.80 ± 0.05) across all loci, and the *H*o of wild and cultured populations fell within the same range.

After Bonferroni correction of microsatellite genotype frequencies, the YB population was found to deviate significantly from HWE and showed a heterozygote deficit at one locus (M 46) as revealed by Fisher's exact test. MICROCHECKER indicated that the locus was expected to have null alleles in this population, while no null alleles were found at the other loci/populations. The inbreeding coefficient (F_{IS}) across all loci in all populations showed no significant deviation from zero. All microsatellite loci showed linkage disequilibrium ($P > 0.05$) except for between Mas 13 and Mas 22, Mas 3 and Mas 13 in the SC population; between Mas 3 and M 5 in the YB population; and between Mas 22 and M 5 and Mas 15 and M 5 in the YZ population.

Genetic variation among populations and population bottleneck

The average number of private alleles in the sampled populations was low at 0.031. We found non-significant population differentiation as indicated by a global F_{ST} value of 0.0233 ($P < 0.001$) among populations (Table 2). However, gene flow appeared to be high among the farms; pair-wise difference estimates ranged from 0.0014 between the YC and WZ populations to 0.047 between the YC and YZ populations (Table 3). AMOVA confirmed that the presence of significant genetic differentiation between the wild and cultured populations ($F_{ST} = 0.0278$) (Table 2). Evidence of recent bottlenecks was at least partially supported in separate analyses. Under IAM, the YB and YC populations showed significant signs of recent bottlenecks (Wilcoxon

test, one-tailed for heterozygote excess, YB = 0.000 and YC = 0.000), while under the more conservative SMM, there was a significant trend in all six populations (Table 1).

Table 2. Analysis of molecular variance (AMOVA) results from Chinese suckers.

Model	Source of variation	d.f.	F-statistic	P value
Between individual populations	Among all populations	5	0.0233	0.00
	Within individuals	208	0.9767	
Between population type (wild, cultured)	Among types	1	0.0278	0.00
	Within individuals	213	0.9722	

Table 3. Pair-wise F_{ST} values (below diagonal) and genetic distances (above diagonal) among six populations of Chinese suckers.

	SC	WH	WZ	YB	YC	YZ
SC		0.01598	0.02200	0.01144	0.03918	0.02626
WH	0.03604		0.01261	0.00434	0.01578	0.02476
WZ	0.00000	0.00000		0.00896	0.00140	0.03432
YB	0.10811	0.19820	0.03604		0.01635	0.03133
YC	0.00000	0.02703	0.24324	0.06306		0.04706
YZ	0.00000	0.00000	0.00000	0.00000	0.00000	

SC: Sichuan Fisheries Research Institute; WH: Wuhan Aquatic Introduction Center; WZ: Wanzhou Fisheries Research Institute; YB: Yibin Rare Aquatic Animal Research Institute; YC: Yichang Sanjiang Fisheries Co., LTD; YZ: Yangtze River.

Individual assignment and cluster analysis

Self-classification of analyzed data indicated that 57% of individuals were correctly assigned to their population (Table 4). The highest rate was in the SC population (64%) followed by the YZ population (63%). The YC population was mis-assigned, three to the WH population and six to the WZ population. Nine cultured fish (6%) were assigned to the YZ population, and 22 wild fish were assigned to the farms. A Bayesian cluster analysis of the STRUCTURE analysis was based on 214 individuals. The △K statistic of Evanno et al. (2005) suggested that the data were most likely to come from three populations (K = 3, results not shown) and clear segregation was found in the assignment test. The mean proportions of membership of each population within each cluster are presented in Table 5 and Figure 2.

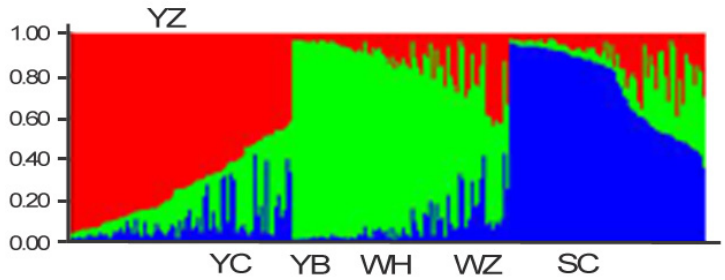

Figure 2. Proportional membership (Q) of each Chinese sucker population in three clusters identified by STRUCTURE. YZ: Yangtze River; YC: Yichang Sanjiang Fisheries Co., LTD; YB: Yibin Rare Aquatic Animal research Institute; WH: Wuhan Aquatic Introduction Center; WZ: Wanzhou Fisheries Research Institute; SC: Sichuan Fisheries Research Institute.

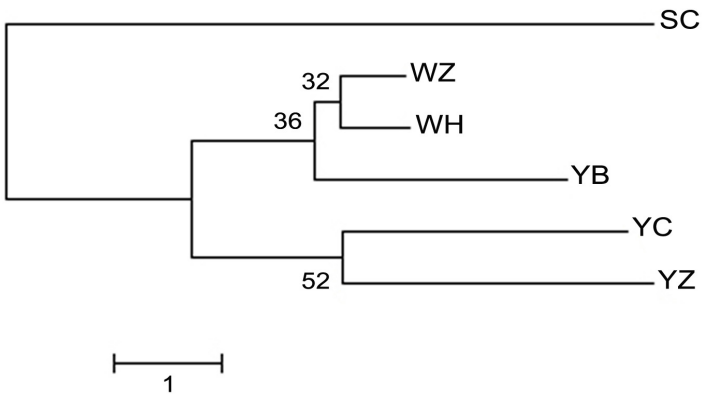

Figure 3. Neighbor-Joining dendrogram of genetic relationship among five populations of Chinese sucker. SC: Sichuan Fisheries Research Institute; WH: Wuhan Aquatic Introduction Center; WZ: Wanzhou Fisheries Research Institute; YB: Yibin Rare Aquatic Animal Research Institute; YC: Yichang Sanjiang Fisheries Co., LTD; YZ: Yangtze River.

Five populations, omitting YC, were rebuilt based on self-classification. The NJ unrooted dendrogram of the discrete character methods (Dc) distances of the five populations showed three clusters: SC composed one cluster, YB, WH, and WZ another, the YZ population and YC population was a separate cluster (Figure 3).

Table 4. Test of self-classification of Chinese sucker individuals based on the Bayesian method described by Rannala and Mountain (1997).

	Assigned to					
Population	SC	WH	WZ	YB	YC	YZ
SC	**9**	1	2	0	0	2
WH	2	**19**	6	2	3	2
WZ	4	10	**52**	5	10	4
YB	0	3	2	**5**	2	1
YC	0	3	6	0	**0**	0
YZ	5	6	4	5	2	**37**
Correctly assigned (%)	0.64	0.59	0.61	0.39	0	0.63

Bold numbers indicate the number of individuals correctly assigned to their population. SC: Sichuan Fisheries Research Institute; WH: Wuhan Aquatic Introduction Center; WZ: Wanzhou Fisheries Research Institute; YB: Yibin Rare Aquatic Animal Research Institute; YC: Yichang Sanjiang Fisheries Co., LTD; YZ: Yangtze River.

Table 5. Average proportion of membership of each Chinese sucker population by STRUCTURE.

Samples	Cluster		
	1	2	3
SC	0.414	0.305	0.281
WH	0.329	0.453	0.218
WZ	0.420	0.455	0.125
YB	0.313	0.516	0.171
YC	0.595	0.355	0.050
YZ	0.176	0.160	0.664

SC: Sichuan Fisheries Research Institute; WH: Wuhan Aquatic Introduction Center; WZ: Wanzhou Fisheries Research Institute; YB: Yibin Rare Aquatic Animal Research Institute; YC: Yichang Sanjiang Fisheries Co., LTD; YZ: Yangtze River.

DISCUSSION

Genetic diversity and population structure

Extensive polymorphism was detected in all populations, and overall mean H_E was 0.748. The amount of genetic variation within each locus can influence estimates of genetic subdivision (Hedrick, 1999). Consequently, when using loci that have high heterozygosity, the maximum possible F_{ST} value is low. The pair-wise F_{ST} values in the six populations were all low, except for that in the YB population when compared to those of the SC and WH populations, and that in the YC population when compared to those of the YB and WZ populations (Table 3). Both YB and YC exhibited evidence of a recent bottleneck ($P < 0.001$) (Table 1). Under the SMM model, which is considered suitable for microsatellites (Luikart and Cornuet 1998), the demographic bottleneck was accompanied by a loss of genetic variability. These two small effective populations (13 samples from YB and 9 samples from YC) can occur for a variety of reasons including bottlenecks, genetic isolation, asymmetry in the proportions of males and females, and differences in the reproductive success of individuals (Tennessen and Zamudio, 2003; Myers and Zamudio, 2004; Wang, 2009).

Strong differentiation was evident among all populations except between WH, WZ, YB, and YC (Table 3). Structure analysis indicated three distinct lineages: lineage 1 had the highest frequency in YZ and YC, lineage 2 had the highest frequency in YB, WH, and WZ, and lineage 3 was found most frequently in SC (Figure 2). Gene flow was higher among YB, WZ, and YC populations and WH populations. Pair-wise F_{ST} values and cluster analysis both indicated that the three cultured populations were genetically close. The genetic differentiation of the SC and YZ populations revealed in our study placed these four cultured populations into a single cluster.

The Yangtze population shows incompatibility with the farmed fish, because when K = 2, 3, 4, 5, or 6 by STRUCTURE analysis, YZ is identified as a separate cluster (data not shown), The NJ tree also identified YZ as a separate cluster (Figure 3). Since the early 1970s, hatchery programs and cultures have been carried out by several national institutes and private agencies in order to preserve this fish species. The artificial propagation of *M. asiaticus* was first performed by the Wanzhou Fisheries Research Institute in 1990s (Chen, 1999). Several mature Yangtze River fish caught by local fishermen were sent to hatcheries for use as broodstock. Over the past 40 years, the cultured populations have derived from a few genetic lineages, since propagation relies on the exchange of broodfish among *M. asiaticus* farms. Genetic homogeneity also reflects non-random mating, population mixing, natural selection, and incorrect identification of genotypes.

These results assigned 22 wild fish to cultured genotypes, and we can infer that these fish originated from an *M. asiaticus* farm. Currently, the rearing and release of artificially propagated fish is an appropriate means of avoiding a sharp reduction in this fish resource (Yang et al., 2009). These five *M. asiaticus* farms conduct successful artificial breeding, and millions of juvenile fish have been produced. The release of hatchery-reared juveniles into natural river systems as part of an artificial propagation program (Song et al., 2008) has been considered an effective means of increasing the size of wild populations and should be beneficial for the recovery and long-term protection for this species (Ireland et al., 2002). Cheng et al. (2013) used microsatellite loci to assess the enhancement effect of the Chinese sucker in the middle and upper reaches of Yangtze River, and concluded that the contribution was 16.92%. Such an examination can have important implications for the management and conservation of a species.

Conservation and future research

Only 59 *M. asiaticus* individuals were collected from the Yangtze River and the natural population of *M. asiaticus* declined sharply during the years in which this study took place. This population and species are critically endangered, as has also been observed for the *M. asiaticus* farms. The most effective conservation strategy would be to protect both the cultured and the natural populations. Future work should assess and compare the diversity of both wild and released populations. Proliferation of discharge in addition to the purpose of increasing the discharge of populations, are needed to ensure natural wild germplasm resources of genetic characteristics, in order to retain the discharge resources of the natural ecological system.

Conflicts of interest

The authors declare no conflict of interest.

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