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Genetic diversity based on SSR analysis of the cultured snakehead fish, *Channa argus*, (Channidae) in China

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ABSTRACT. The snakehead fish *Channa argus* is an important food fish in China. We identified six microsatellite loci for *C. argus*. These six microsatellite loci and four other microsatellite markers were used to analyze genetic diversity in four cultured populations of *C. argus* (SD, JX, HN, and ZJ) and determine their relationships. A total of 154 alleles were detected at the 10 microsatellite loci. The average expected and observed heterozygosities varied from 0.70-0.84 and 0.69-0.83, respectively, and polymorphism information content ranged between 0.66 and 0.82 in the four populations, indicating high genetic diversity. Population JX deviated from mutation-drift equilibrium and may have experienced a recent bottleneck. Analysis of pairwise genetic differentiation revealed F_{ST} values that ranged from 0.028 to 0.100, which indicates a moderate level of genetic differentiation. The largest distances were observed

between populations HN and SD, whereas the smallest distances were obtained between populations HN and JX. Genetic clustering analysis demonstrated that the ZJ and HN populations probably share the same origin. This information about the genetic diversity within each of the four populations, and their genetic relationships will be useful for future genetic improvement of *C. argus* through selective breeding.

Key words: *Channa argus*; Population genetic differentiation; Microsatellite;

INTRODUCTION

Channa argus, commonly called the snakehead fish, belongs to the family Channidae; it is native to eastern Russia and China, as well as parts of North Korea (Courtenay and Williams, 2004). Former Soviet Republics of Uzbekistan, Kazakhstan and Turkmenistan have experienced relatively recent introductions of snakehead fish (Amanov, 1974; Dukravets and Machulin, 1978). In many areas of the world, the snakehead fish is a cultured freshwater fish species, which grows very fast and has a high nutritious and economic value, especially in China (Guo et al., 2004). However, the wild snakehead fish has declined sharply because of overfishing and improper management (Li and Yang, 1998). In this regard, the snakehead fish breeding industry has become very important.

Identifying population structure is one of the cornerstones of aquaculture population assessment (Begg and Waldman, 1999). Microsatellites (simple sequence repeats, SSRs) are a form of repetitive DNA discovered in the 1980s (Tautz and Renz, 1984). Their repeat numbers are variable, which makes microsatellites polymorphic. Characterized by codominant inheritance, high polymorphism and good reproducibility, microsatellites have become instrumental as genetic markers in areas such as population genetics, parentage assignment, marker-assisted breeding and phylogenetic evolution (Liu and Cordes, 2004; Yang et al., 2008; Liu et al., 2009). In addition, SSRs could help to assess the genetic and selective breeding of snakehead fish population.

In the present study, eight wild northern snakehead populations were analyzed using five microsatellite loci (Zhuo et al., 2012). Here, we report the isolation and characterization of five polymorphic microsatellite loci from the *C. argus* genome and one microsatellite locus from the *C. maculate* genome, and investigation of spatial genetic structure, interpopulation diversity and population genetic differentiation of four cultured *C. argus* populations, which will provide additional insight and help to facilitate the selective breeding of *C. argus* populations.

MATERIAL AND METHODS

Sample collection and DNA extraction

In China, Shandong, Jiangxi, Hunan, and Zhejiang are the major breeding provinces for the snakehead fish (Li and Yang, 1998). Places in these provinces, namely Luqiao in Shandong, Wujia in Jiangxi, Ruanjiang in Hunan, and Yuhang in Zhejiang, have a long history of breeding snakehead on a large scale (Li and Yang, 1998; Zhu et al., 2011). Sixty cultured *C. argus* specimens were respectively collected from these four areas (Table 1). Pectoral fins

were collected from each fish and preserved in 95% alcohol. Genomic DNA was extracted from one individual by the standard phenol-chloroform extraction method (Sambrook and Russell, 2001).

Table 1. Sampling sites and sample sizes of *Channa argus* used in the experiment.

Stock	Sampled site	Location	Sample No.
SD	Luqiao, Shandong	34°89'N117°08'E	60
JX	Wujia, Jiangxi	29°64'N115°91'E	60
HN	Ruanjiang, Hunan	28°61'N112°85'E	60
ZJ	Yuhang, Zhejiang	30°39'N120°18'E	60

Microsatellite isolation

About 600 ng genomic DNA was digested with *Mse*I restriction enzyme (NEB) at 37°C for 2 h. These fragments were ligated to *Mse*I adapters and then amplified by PCR using *Mse*I-N primers (5'-GATGAGTCCTGAGTAAN-3') following the program of 94°C for 4 min, 30 cycles of 94°C for 30 s, 53°C for 1 min and 72°C for 1 min, and 72°C for 5 min. The PCR products were then hybridized with biotinylated probe $(CA)_{15}$ in a 300-µL hybridization solution (4X SSC, 0.1% sodium dodecyl sulfate, 0.5 µM probe) at 55°C for 30 min. Subsequent probe-bound DNA fragments were enriched for CA repeats using streptavidin-coated magnetic beads (Promega, Shanghai, China) at room temperature for 30 min, followed by two washing steps. Recovered DNA fragments were amplified with *Mse*I-N primers as described above. The PCR products, after being purified with Gel Extraction kit (Tiangen), were ligated to pGEM-T vector (Promega) and transformed into *Escherichia coli* DH5*α* competent cells (Tiangen). Fifty-five positive clones were picked out and tested by PCR using *Mse*I-N primers.

Positive clones were sequenced on an ABI 3700 automated DNA Sequencer (Applied Biosystems Incorporation), of which 46 screened clones containing microsatellite repeats. Primers for these loci were designed with the PRIMER3 online software. A total of five primer pairs produced polymorphic DNA products from *C. argus* genome and one primer produced a polymorphic DNA product from *C. maculata*, including *CHA7*, *CHA13*, *CHA25*, *CHA31*, *CHA41*, and *CHM9* (Table 2). In addition, we designed four primers according to the NCBI sequence from the *C. argus* genome, and they showed high polymorphism in *C. argus* (Table 2).

Microsatellite genotyping

All ten microsatellite loci that amplified specific products were selected for further investigation using fluorescently labeled probe (either 6-FAM or HEX) on 240 samples from four populations (SD, JX, HN and ZJ). The PCR amplifications were performed in a 25-µL volume, including approximately 20 ng genomic DNA, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 µM of each primer, and 1 U *Taq* DNA polymerase (Tiangen). The amplification program included an initial denaturation at 94°C for 3 min and 35 cycles of 30 s at 94°C, 30 s at 50-64°C depending on the primer pair (Table 2) and 1 min at 72°C, followed by a final extension step for 10 min at 72°C. Fluorescent dye-labeled fragments were separated and analyzed using the ABI3730xl sequencer GeneMapper software (Lo and Yue, 2008).

Table 2. Microsatellite marker primers and their repeat sequences used in the experiment.

GenBank No.	Primer sequence (5'-3')	Size	Repeat motif	Annealing temp.
HM015826	F: CACTGGTCACTGTTGAAATCT	253-305	$(TACA)_7$	58
TLL01	R: CAGCGAGTACAAAATTACAGC			
HM015833	F: TGATTGGTCCCAAATGTCTG	257-327	$(TAGA)_{32}$	55
TLL02	R: GGACGCACCACCTAGAGAAG			
HM015836	F: ATGACAGTGTTCCTGCTCTATG	273-329	$(TAGA)_{12}$	56
TLL03	R: GGTGCAACTTCCTCAGTGA			
HM015842	F: CACACCCAAAGGTTACACC	326-418	$(TAGA)_{35}$	55
TLL04	R: AATGTGGGCACTGATCTTC			
GQ131294	F: ATTGGGTGCTGCCATCATAC	164-185	$(AC)_{22}$	60
CHA7	R: GGAGGAAACGGTTTGACTGA			
GQ131300	F: GTTTCAAAGGTCGGGAGAGG	221-258	$(AC)_{15}$	64
CHA13	R: AGCAAAAGCATGATCCTTGG			
HQ404179	F: CCTGGCATCTTCGGTAAAAG	241-280	$(TG)_{19}$	58
CHA25	R: CTCCAAGGGCGAAAACTCTT			
HQ404185	F: CCTCTGATGCCCTTAGAGACA	151-198	$(AC)_{31}$	60
CHA31	R: TCAGACTTCGTCTGCACCAG			
HQ404195	F: TGCCAGTTTATTGGAAAGCA	135-180	$(TG)_5TC(TG)_{26}$	56
CHA41	R: GTAGGCACCTCAGCCATGAT			
HQ404206	F: CGCAGCAAGTATCAGCAT	142-165	$(GT)_6GC(GT)_9$	58
CHM9	R: GGTCCCAACTAGGAAGAAC			

Data analysis

The numbers of alleles (N_A) and effective alleles (N_E), observed heterozygosity (H_O), expected heterozygosity (H_E), and pairwise linkage or gametic phase disequilibrium were analyzed using GDA (Lewis and Zaykin, 2000). Polymorphic information content (PIC) was calculated using a formula (Botstein et al., 1980). Pairwise genetic differentiation *F*-statistics and analysis of molecular variance (AMOVA) were determined using ARLEQUIN 3.1 (Excoffier et al., 2005). Phylogenetic trees were constructed using F_{ST} genetic distance based on the unweighted pair group method with arithmetic averages (UPGMA). The genetic structure of different *C. argus* populations was analyzed using Structure 2.3 (Pritchard et al., 2000; Evanno et al., 2005). In the assumption populations menu, we set the assumed K value from 1 to 4.

RESULTS

Genetic diversity

There was significant variation in the 10 microsatellite loci in this study, with 154 different alleles in 240 individuals from four populations. The mean number of alleles observed over all markers in four populations was 15.40. PIC showed that all 10 loci were highly informative (mean = 0.81 ± 0.10). H_E and H_O varied 0.64-0.94 and 0.56-0.98, respectively, and PIC between 0.57 and 0.93 in 240 individuals. Among them, N_A, N_E, H_E, and PIC of locus *TLL04* were the highest. Allelic diversity (N_A and N_E) and gene diversity (H_E and H_O) of each locus in every population are shown in Table 3. Due to the important parameters for measuring allele polymorphism, we used H_E and PIC to compare different population, and found that the mean H_E and PIC were highly informative. In the four populations, the ZJ population showed the highest H_O (0.83), followed by HN (0.79), JX (0.74), and SD (0.69) (Table 3). The highest estimates of H_E were obtained from ZJ (0.84), followed by HN (0.83), JX (0.80), and SD (0.70),

while PIC was the highest in ZJ (0.82), followed by HN (0.81), JX (0.77), and SD (0.66). The allele equilibrium status of the four stocks was tested for Hardy-Weinberg equilibrium, and some microsatellites deviated significantly from Hardy-Weinberg equilibrium ($P < 0.05$), such as CHM9 in the SD population, *TLL01*, *CHA7*, *CHA13*, *CHA41*, and *CHM9* in the JX population, and *TLL01* and *CHA25* in the HN population.

Table 3. Genetic diversity and P values of the four *Channa argus* populations.

Population	Index	*TLL01*	*TLL02*	*TLL03*	*TLL04*	*CHA7*	*CHA13*	*CHA25*	*CHA31*	*CHA41*	*CHM9*	Mean
SD	N_A	3	15	11	19	4	5	8	11	8	7	9.1
	N_E	1.9	7.4	4.2	3.9	2.2	2.0	4.1	4.9	5.2	3.1	3.9
	H_O	0.45	1.00	0.77	0.49	0.65	0.43	0.77	0.86	0.75	0.72	0.69
	H_E	0.48	0.87	0.77	0.75	0.55	0.50	0.76	0.80	0.82	0.69	0.70
	PIC	0.38	0.85	0.73	0.74	0.50	0.46	0.72	0.77	0.78	0.65	0.66
	P	0.39	0.21	0.75	0.57	0.31	0.05	0.98	0.21	0.13	0.00*	
JX	N_A	4	15	12	26	8	5	12	12	8	8	11.0
	N_E	2.5	10.1	5.5	16.7	3.4	4.5	5.2	4.7	5.1	6.4	6.41
	H_O	0.47	0.97	0.80	0.67	0.68	0.67	0.75	0.90	0.68	0.83	0.74
	H_E	0.60	0.91	0.83	0.95	0.71	0.79	0.81	0.80	0.81	0.85	0.80
	PIC	0.51	0.89	0.80	0.94	0.67	0.75	0.78	0.76	0.78	0.83	0.77
	P	0.01*	0.35	0.29	1.00	0.04*	0.02*	0.89	0.36	0.01*	0.00*	
HN	N_A	5	21	10	27	11	8	13	15	12	11	13.3
	N_E	3.0	11.6	6.0	16.0	7.6	4.7	6.6	7.7	4.3	5.5	7.3
	H_O	0.67	0.97	0.85	0.87	0.83	0.72	0.62	0.84	0.65	0.92	0.79
	H_E	0.67	0.92	0.84	0.95	0.88	0.80	0.86	0.88	0.78	0.82	0.83
	PIC	0.62	0.91	0.81	0.93	0.85	0.76	0.83	0.86	0.75	0.79	0.81
	P	0.04*	1.00	0.23	1.00	0.06	0.14	0.04*	0.93	0.30	0.08	
ZJ	N_A	5	17	11	23	13	6	14	13	10	11	12.3
	N_E	3.1	7.5	5.4	14.2	9.0	4.6	6.1	6.5	6.0	9.6	7.2
	H_O	0.67	0.97	0.75	0.88	0.87	0.83	0.77	0.92	0.88	0.82	0.83
	H_E	0.68	0.87	0.82	0.94	0.90	0.79	0.84	0.85	0.84	0.90	0.84
	PIC	0.61	0.85	0.79	0.93	0.88	0.75	0.82	0.83	0.81	0.89	0.82
	P	0.58	0.85	0.69	1.00	0.29	0.22	0.98	0.99	0.27	0.16	

N_A = number of alleles; N_E = number of effective alleles; H_E = expected heterozygosity; H_O = observed heterozygosity; PIC = polymorphism information content. *Significant difference ($P < 0.05$).

Bottleneck analysis was conducted using the Bottleneck software under the two-phrased model of mutation (TPM) of microsatellites. $P > 0.05$ indicates that the population has not experienced a recent bottleneck. According to the sign test, population JX deviated from mutation-drift equilibrium and experienced a recent bottleneck (Table 4).

Table 4. Analysis of the possibility of a recent bottleneck using sign tests in the four *Channa argus* populations.

Population	TPM	
	H_E/H_D	P
SD	5/5	0.40
JX	6/4	0.61
HN	9/1	0.04
ZJ	8/2	0.16

TPM = two-phrased model of mutation. H_E/H_D = ratio of number of individuals with heterozygosity excesses to the number with heterozygosity deficiency. $P > 0.05$ indicates that the population has not experienced a recent bottleneck.

Population genetic differentiation

Pairwise genetic differentiations based on allele frequency were determined for the

four cultured stocks of *C. argus* (Table 5). All pairwise F_{ST} was statistically significant ($P < 0.05$). The highest differentiation occurred between populations HN and SD (0.028), while the lowest differentiation observed was between populations JX and HN (0.100). Similarly, the greatest distances were observed between populations HN and SD, whereas the smallest distances were obtained between populations HN and JX. The phylogenetic tree was constructed by UPGMA using the MEGA5 software (Figure 1). AMOVA of microsatellites revealed that the variation within individuals, between individuals within populations, and between populations was 90.85, 3.57 and 5.58%, respectively (Table 6).

Genetic clustering analysis using the Structure program indicated that the number of genetic clusters was three (K = 3; Figure 2). The four stocks were divided into three potential populations. Populations ZJ and HN were assigned to the same genetic background, suggesting that they may share the same origin (represented in green in Figure 2).

Table 5. Pairwise genetic differentiation (F_{ST}) in the four *Channa argus* populations.

Population	SD	JX	HN	ZJ
SD	0.000			
JX	0.045	0.000		
HN	0.100	0.028	0.000	
ZJ	0.097	0.037	0.029	0.000

All pairwise F_{ST} is statistically significant ($P < 0.05$).

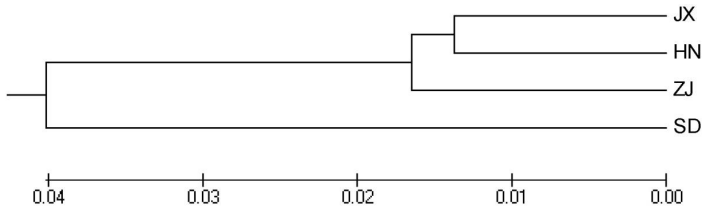

Figure 1. UPGMA molecular trees based on genetic distance of four *Channa argus* populations.

Table 6. AMOVA of microsatellite in four populations of *Channa argus*.

Source of variation	Sum of squares	Variance components	Percentage of variance
Among populations	96.67	0.23	5.58
Among individual within populations	970.45	0.15	3.57
Within individuals	915.45	3.81	90.85
Total variation	1982.12	4.41	100.00

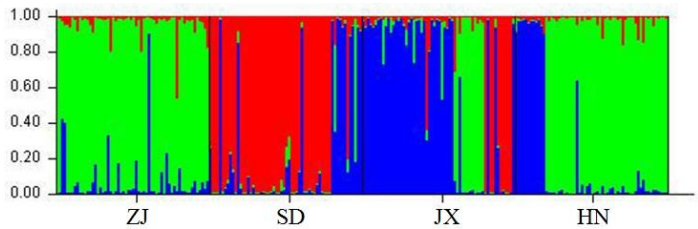

Figure 2. Structure version 2.3.1 analysis of *Channa argus* populations using microsatellite genotype data from 10 microsatellite loci (the inferred clusters, K = 3).

DISCUSSION

Genetic variation is the basis of a selective breeding program. In past few years, many popular genetic makers were used for assessing genetic variation in selective breeding programs (Liu and Cordes, 2004), such as mitochondrial DNA, and SSR. Due to many advantages, microsatellites as genetic markers have been extensively used in genetic diversity studies in fish species (Shimoda et al., 1999; Aguilar and Garza, 2006; Narum et al., 2006; Sekino and Hara, 2007). In this study, we constructed a partial genomic DNA enriched for AC- and TG-microsatellites with six primers designed here, and four primers designed by the sequence of NCBI for studying genetic variation and relationships of four cultured populations of *C. argus*. Among them, the primer *CHM9* was designed by the sequence of *Channa maculate* and showed high polymorphism in the four *C. argus* populations. All 10 microsatellites were found highly polymorphic. Highly polymorphic loci provided better estimates of genetic distances than less polymorphic loci (Kalinowski, 2002). In comparison to the published microsatellites of *C. argus* (Wen and Sun, 2010), these 10 microsatellite loci seemed to be much more polymorphic in terms of allele number. Several factors may account for this difference, such as the repeat length of microsatellites may be different. Normally, a longer repeat microsatellite shows higher polymorphism (Goldstein and Schlotterer, 1999). Thus, allele number is usually positively associated with the sample size (Goldstein and Schlotterer, 1999). Compared with the wild snakehead populations (Zhuo et al., 2012), the average H_E in these four cultured populations varied from 0.70 to 0.84 while the average H_E in those eight wild populations ranged from 0.70 to 0.85. Compared with other freshwater species (DeWoody and Avise, 2000), it indicated that *C. argus* has high allelic diversity. Actually, breeding methods inevitably influence the genetic variability of any farmed population (Romana-Eguia et al., 2004). Normally, cultured populations contain lower genetic diversity than wild populations as indicated by previous research (Norris et al., 1999). However, due to no regulation system in breeding program in China, fish farmers have been using wild snakehead as parents to produce offspring. The high diversity in cultured snakehead fish populations may be good news for fish breeders.

It is important to determine whether the population had experienced a recent bottleneck, because bottlenecks can increase demographic stochasticity, loss of genetic variation, rates of inbreeding and fixation of mildly deleterious alleles, thereby reducing evolutionary potential and increasing the probability of population extinction (Frankel and Soule, 1981; Lande, 1988; Ralls et al., 1988; Hedrick and Miller, 1992; Jimenez et al., 1994; Mills and Smouse, 1994). In the present study, the JX population was shown to have experienced a small bottleneck, which indicates that the genetic variability of the population is expected to decline rapidly. One explanation could be that the JX samples were collected from a cohort based on a small number of parent fish. However, as soon as population size becomes larger, it starts to increase owing to new mutations (Nei et al., 1975). When population size is restored, the average number of alleles per locus increases faster than the average heterozygosity. In other words, it is necessary to increase effective population sizes in the JX population for better breeding.

The F_{ST} value and AMOVA are useful measurements of genetic differentiation among populations. The analysis of pairwise genetic differentiation revealed that the F_{ST} values ranged from 0.028 to 0.100, which was in line with a moderate level of genetic differentiation (Hartl and Clark, 1997). Based on microsatellite genotypes, the UPGMA-phylogenetic tree showed that JX and HN were the nearest clusters while HN and SD showed the farthest

genetic distance. This clustering result is related to their geographic distribution distance. The relativity between geographic isolation and genetic distance shows that the genetic differentiations between populations are mainly because of the geographic proximity (Li et al., 2007). AMOVA revealed that genetic variation within individuals was 90.85%, and the genetic variation between populations was 5.58%, which suggested a moderate level of genetic differentiation. These different degrees of differentiation between populations and the variations in populations were mainly attributed to genetic drift and gene flow or the diffuseness between populations (Wright, 1978). Future studies are aimed at understanding whether the crosses between fishes from different populations will generate hybrid vigor.

The present study focused only on four cultured snakehead fish populations subjected to a supportive breeding program. High genetic diversity was detected in these four snakehead fish populations, which could provide the basis for future breeding program. This study demonstrates a strong indication of reduced effective numbers of breeders in the JX population. Thus, attention should be paid to routine genetic monitoring of breeding activities and to identifying whether there is a decrease in the effective population sizes of the snakehead fish.

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