Development and characterization of novel cross-species tetranucleotide microsatellite markers for sterlet (*Acipenser ruthenus*) from Chinese sturgeon (*Acipenser sinensis*)

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Abstract: Sterlet (*Acipenser ruthenus*) is an important economic fish because of its nourishing caviar, isinglass and flesh. In order to facilitate the recovery of this species, the full understanding of its population genetic structure is necessary for taking appropriate management actions. However, genetic data on the use of nuclear loci in sterlet is still quite poor because microsatellite markers in sterlet that had been developed appeared to be polyploidy which add difficulties in studying the genetic of the sterlet. In this study, 24 tetranucleotide microsatellite markers were developed in sterlet from 160 microsatellite markers of the endangered Chinese sturgeon (*Acipenser sinensis*). Ten (ZHX76, ZHX64, Z194, Z217, Z184, Z242, Z250, Z258, Z268 and Z269) of the 24 loci showed disomic patterns while the rest loci showed tetrasomic patterns. In this paper, 24 microsatellite markers were characterized in 16 sterlet individuals and all of them were polymorphic with 2 to 7 alleles per locus. The Hardy-Weinberg departure value (*d*), polymorphic information content (*PIC*), the observed heterozygosity (H_O), the Shannon-Wiener Diversity Indices (*H'*) and the mean expected heterozygosity (H_E) of all 24 polymorphic loci

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ranged from -0.334 to 0.484, 0.367 to 0.725, 0.438 to 1, 0.659 to 1.695, from 0.466 to 0.777, respectively. The markers described here will help in addressing practical problems such as the study of population genetics, conservation genetics and evolution in the polyploidy derivative nature of sterlet.

Keyword： Microsatellite markers; Cross-species; *Acipenser ruthenus*; Genetic diversity; Disomic; Tetrasomic

Introduction

Sterlet (*Acipenser ruthenus*), one member of 27 species of Acipenseriforme，survived more than 200 million years and mainly distributed in fresh water in Europe and Asia [1]. The sterlet is a relatively small species of sturgeon with the earliest maturation compared to other 26 species of paddlefishes and sturgeon. Sterlet is a benthic organism, feeding upon insect larvae, crustaceans, small worms and so forth. Sterlet is mostly sedentary and they only migrate between fresh and salt water when spawning. This species is easier to accept artificial feed and domesticate comparing with other sturgeons under an optimum growth temperature about 21-23°C. Nowadays, sterlet population is declining because of river damming, overfishing (for its caviar, isinglass and excellent flesh), and poaching which have let them losing spawning grounds [2, 3]. The interspecific hybridization also led to their population decline on account of decrease of genetic integrity [4]. Consequently, IUCN Red List has classified this species as “Vulnerable species”.

The best countermeasure to protect the sterlet is artificial propagation. Genetic study plays an important role in artificial propagation, which helps to evaluate the result of the artificial propagation.

Microsatellite markers play an important role in genetic conservation and management of fish species [5]. In the case of conservation studies in sterlet, the microsatellite maker is very important for structure of population. Fopp-Bayat *et al.* [6] had developed three microsatellite markers. However, the number of microsatellite marker for sterlet is far from enough. And most of those microsatellites show a polyploidy profile. Polyploid marker can add difficult in

population genetic analyses on account of the hypothesis of diploidy that is incorporated into many statistical tests. It is urged to develop microsatellite marker that is disomic for researcher to study the population genetic of sterlet. In this study, we develop ten microsatellite markers that appear to polymorphic disomic loci and 14 microsatellite markers that show a tetrasomic profile with next-generation sequencing technology. These markers can be used in relevant researches of population genetics, conservation genetics and evolution of sterlet [7, 8, 9].

Materials and methods

Ethical Statement: All of the experiments were performed under the control of the Guidelines for Animal Experiments in the Chinese Sturgeon Research Institute, the China Three Gorges Corporation. All fish handling and experimental procedures in this study were approved by the Ethics Committee of the Chinese Sturgeon Research Institute, the China Three Gorges Corporation, and the Hubei Key Laboratory of Three Gorges Project for Conservation of Fishes.

The RNA was extracted from the fin of Chinese sturgeon using Trizol (Invitrogen, USA), according to the manufacturer protocol. RNase-free DNase I was used to remove DNA from the RNA and the total RNA was evaluated by 1% agarose gel electrophoresis. The cDNA library was created by using approximately 5 μg RNA and was sequenced on an Illumina Hiseq2000. The sequences were selected by constrained to perfect repeat motifs of 4 bp from the cDNA library by the Microsatellite Identification tool (MISA: http://pgrc.ipkgatersleben.de/misa/). All the selected sequences were then used to design microsatellite primers using Primer Premier 5.0 software (http: //www. premierbiosoft.com/ primer design/).

16 sterlet individuals were collected from Yichang, China. Using the rapid salt-extraction method, high quality DNA was extracted for each individual. DNA of 16 sterlet was polymerase chain reaction (PCR) amplified in a 25μl reaction volume consisting of 1×PCR buffer (TaKaRa), 50–100 ng genomic DNA, 0.25μM for each primer, 150 μM dNTPs, 1.5 mM $MgCl_2$ and 0.25 U *Taq* DNA polymerase (TaKaRa). The following PCR profile was used: 94°C for 3min, followed by 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 30s, and then a final extension at 72°C for 10min. The PCR products were separated on 10% polyacrylamide gel stained with silver staining. A pBR332 DNA/MspI molecular marker (TaKaRa) was used as the standard to determine the size

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of the alleles. The analyses of genetic diversity include the number of alleles per locus (N_a), mean expected heterozygosity (H_E), Shannon-Wiener Diversity Indices (H') using software ATetra1.2 [10]. The Hardy-Weinberg departure value (d) and the polymorphic information content (PIC) were performed according to formula (Formula I). P_i and P_j is the frequency of I and J allele in the group.

$$PIC = 1 - \sum_{i=1}^{n} P_i^2 - \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} 2 P_i^2 P_j^2$$

$$d = (H_o - H_e) / H_e$$

(Formula I)

Results

In this study, a total of 160 sequences were selected to designing microsatellite primers. Among the 160 microsatellite primers that were tested in the 16 sterlet individuals, 110 were produced low quality results, 26 failed to produce any PCR product, and only 24 microsatellite markers showed clearly polymorphism (*Table 1*). The percentage of errors by replicating 5 samples was 0. The percentage of amplification success was 15%. Using the 24 microsatellite markers, we can identify each individual of all the 24 sterlet individuals. All 24 microsatellite markers were polymorphic with 2 to 7 alleles per locus. The Hardy-Weinberg departure value (d), polymorphic information content (PIC), the observed heterozygosity (H_O), the Shannon-Wiener Diversity Indices (H') and the mean expected heterozygosity (H_E) of all 24 polymorphic loci ranged from -0.334 to 0.484, 0.367 to 0.725, 0.438 to 1, 0.659 to 1.695, from 0.466 to 0.777, respectively.

Among the 24 microsatellite markers, ten markers (ZHX76, ZHX64, Z194, Z217, Z184, Z242, Z250, Z258, Z268 and Z269) presented a disomic profile and others have showed a tetrasomic profile (*Table 2*).

Table 1 Characterization of 24 microsatellite markers in sterlet

Locus	Repeat	Primer sequence(5'-3')	Tm(°C)

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	motif(s)		
ZHX76	$(TATC)_{14}$	F:GCGTTCACTGAGTCAATGCA	60
		R:CTGGACAGAGAACAGATAGCGT	
ZHX64	$(AGAT)_{12}$	F:ACCTGCCTTCTTCCAGCTTT	60
		R:AATCACGGACAGCCAAGAGG	
Z194	$(TTTA)_{10}$	F:ACAGTGGACAATGTGGCTCA	60
		R:CCAGGACCACGGCTAGTTTT	
Z217	$(ATAG)_{12}$	F:TCACGTTGATCAGGGTCTTCA	59
		R:TCCACAAACACAAAACATTTGCT	
Z184	$(TTTA)_{8}$	F:ACCCTCCCACAGATGCTGTA	59
		R:TGATAGTTCAAATTGACGAAGGCT	
Z242	$(AATA)_{9}$	F:TCGGGGGTAAAATAATGGGAGA	60
		R:CTAACTCGGCCCCAAACCAT	
Z250	$(AAAT)_{9}$	F:GGCCACCACTGTTGATCTGA	60
		R:GCCATTCTCCTCCCTGACAC	
Z258	$(CTAT)_{9}$	F:TGTGCTTACTTGCATCTGTTGT	59
		R:CGCTCCGCTCTAAGAAGACA	
Z268	$(ATAC)_{9}$	F:GGACATTCTCATTCTCAGCTGC	60
		R:ATCACGATCCATGCCTTGCA	
Z269	$(TATT)_{9}$	F:GCATGTGTCACTGAGATAGTTGC	60
		R:TGCTGCAGTTGAGGTCCATT	
ZHX70	$(TATC)_{11}$	F:GGCATTATAGACCCCTGTCGG	60
		R:ACAGCTGGGGAGGAACAGTA	
ZHX120	$(TTCT)_{10}$	F:TCCAGTGACATTTCAGGGCA	59
		R:GCATGGGTGCCACTGAAATA	
ZHX85	$(AGAT)_{15}$	F:TCAACACTATGACCGGTACTGT	60

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		R:TTAAACGAAAGGCCCAGGGG	
ZHX62	$(CTGT)_{11}$	F:CGCTGCATGTACACGTGTAA	61
		R:GCTGCGACTTCGAGGTTTCT	
Z190	$(AAAT)_9$	F:AGCTACTTTTTGCTTTTGGGTT	57
		R:AGGGTGTGAGAAAGAAAGATGGA	
Z192	$(TATT)_9$	F:GGCATTCAAAACTCTTTCGTGGA	60
		R:GGGGGCCATCCTTATCTCAC	
Z199	$(TCTT)_{13}$	F:GACGTTTGGAGCGTGGAAAC	60
		R:TGGCATTTACAGCATAAACTAAACCT	
Z226	$(TAGA)_9$	F:TTGCAGGTCTCTAGCCTGGT	60
		R:GGGGCATACCGTTTGAACCT	
Z302	$(TTTC)_8$	F:TCCACACGTACGGCATTTCA	60
		R:GGTTCCCCGTGTAAAATGCG	
Z224	$(CTGT)_8$	F:ATGCATTGCGTTCCCTGAGT	60
		R:GGCATTCCATTGCACCACAG	
Z236	$(TTAT)_{10}$	F:TCACTACATCTCCCCTTATTTGAGA	59
		R:CCGTTGCGCTACAGAGAAGA	
Z238	$(TAGA)_{11}$	F:CAAAATGGCAGTCGACAGGC	60
		R:TGTCAACTTCGTTTGTGCATCC	
Z255	$(TATC)_9$	F:TGGTTTTCTTGTGATCTCGAGA	58
		R:GATGAAGGTTCTGGGAGGGC	
Z259	$(TCTT)_9$	F:ACGCCACTGGAGGATGTACT	60
		R:AGAAACCTGCAGCAACGAGT	

Note: *Tm*, annealing temperature.

Table 2 Genetic diversity for 24 microsatellite markers in sterlet

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Locus	N_a	H_E	H_O	H'	d	PIC	Pattern
ZHX76	4	0.717	0.75	1.32	0.046	0.565	Disomic
ZHX64	5	0.751	0.5	1.49	-0.334	0.621	Disomic
Z194	4	0.533	0.75	0.96	0.407	0.535	Disomic
Z217	2	0.466	0.5	0.66	0.073	0.367	Disomic
Z184	4	0.686	0.688	1.26	0.003	0.522	Disomic
Z242	5	0.768	0.625	1.51	-0.186	0.679	Disomic
Z250	4	0.658	0.813	1.22	0.236	0.554	Disomic
Z258	4	0.675	0.938	1.21	0.390	0.577	Disomic
Z268	4	0.492	0.438	0.95	-0.110	0.544	Disomic
Z269	4	0.676	0.688	1.22	0.018	0.553	Disomic
ZHX70	4	0.686	0.875	1.24	0.276	0.56	Tetrasomic
ZHX120	6	0.765	0.875	1.58	0.144	0.689	Tetrasomic
ZHX85	4	0.667	0.75	1.21	0.124	0.631	Tetrasomic
ZHX62	5	0.664	0.75	1.33	0.130	0.625	Tetrasomic
Z190	4	0.674	1	1.2	0.484	0.655	Tetrasomic
Z192	4	0.735	1	1.36	0.361	0.677	Tetrasomic
Z199	7	0.777	0.938	1.7	0.207	0.713	Tetrasomic
Z226	7	0.781	0.813	1.69	0.041	0.725	Tetrasomic
Z302	6	0.735	0.938	1.49	0.276	0.658	Tetrasomic
Z224	5	0.722	0.938	1.38	0.299	0.543	Tetrasomic
Z236	4	0.698	1	1.27	0.433	0.552	Tetrasomic
Z238	6	0.749	0.875	1.58	0.168	0.625	Tetrasomic
Z255	5	0.75	0.875	1.48	0.167	0.533	Tetrasomic
Z259	6	0.63	0.75	1.21	0.190	0.611	Tetrasomic

Note: H_E, mean expected heterozygosity; H_O, observed heterozygosity; d, Hardy-Weinberg departure value; PIC, polymorphic information content; H', Shannon–Wiener Diversity Indices; N_a, number of alleles in per locus.

Discussion

In this study, 24 novel cross-species microsatellite markers were developed for sterlet from Chinese sturgeon via next-generation sequencing for the first time. All the 17 microsatellite markers showed a high polymorphism (PIC>0.5), except Z217. No microsatellite marker showed significant deviation from the Hardy-Weinberg departure value (d < 0) except Z242, Z268 and ZHX64, indicating that they were polymorphism.

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Welsh A. and May B. [12] developed 13 disomic microsatellite markers for lake sturgeon genetic studies. However, sturgeon is polyploidy. The microsatellite markers in sturgeon are tetraploid or octoploid except the lake sturgeon. Lake sturgeon is in the early stages of the diploidization process [11]. Ten markers reported in this study presented a disomic profile in 16 sterlet individuals. This proves that the sterlet genome appears to be tetraploid-derived and maybe is also undergoing diploidization.

Microsatellite markers with disomic patterns were useful to study the polyploidy, and to provide a standardized method for the genetic study. It is difficult to study the genetic analysis of sturgeon using the polyploidy microsatellite. The ten markers which showed a disomic profile can simplify population genetic studies because they have showed a banding pattern in complete agreement with a disomic mode of inheritance. The 13 microsatellite markers that Welsh A. and May B. [12] developed for lake sturgeon had been standard for researcher to study the genetic of lake sturgeon. Molecular analysis with this ten microsatellite markers in this paper will be suitable to be a standard among laboratories to build an inter-laboratory genetic database for sterlet and solve the inherent problem associated with polysomic microsatellite markers. These novel tetranucleotide microsatellite markers will be useful tools to study genetic analyses and artificial protection of sterlet.

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