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Twenty-four novel microsatellites for the endangered Chinese sturgeon (*Acipenser sinensis* Gray, 1835)

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Summary

The Chinese sturgeon (*Acipenser sinensis*) is an endemic and critically endangered species in China. In this study, a total of 24 polymorphic microsatellite loci were isolated and characterized using Illumina sequencing for *A. sinensis*. The number of alleles (Na) per locus ranged from 2 to 6 (mean 4.04), mean expected heterozygosities (He), Shannon-Wiener Diversity Indices (SW) and evenness (E) per locus ranged from 0.235 to 0.786 (mean 0.62), from 0.396 to 1.608 (mean 1.13), and from 0.060 to 0.213 (mean 0.13), respectively. Exact tests revealed that nine loci showed significant ($P < 0.01$) deviation from Hardy-Weinberg equilibrium (HWE). Cross amplification was tested in congeneric species *A. schrenskii*, *A. baerii*, *A. dabryanus* and *Huso dauricus*. The new microsatellite markers described herein will be useful for further studies on genetic variation, parentage analysis, and conservation management.

Introduction

The polyploidy fish Chinese sturgeon (*Acipenser sinensis* Gray, 1835) was once an important commercial fish widely distributed in the Yangtze, Qiantang, Min and Pearl rivers as well as in the China seas (Wei et al., 1997; Yang et al., 2006). However, the natural population has declined severely within the past three decades as a result of the Gezhouba Dam blockage of the migration route, habitat alteration and destruction, over-fishing for meat, and environmental pollution (Wang et al., 2011). In recent years, the Three Gorges Dam has had a tremendous impact on natural propagation of the Chinese sturgeon by influencing the seasonal fluctuation patterns of water flow and temperature downstream, and resulting in the potential loss of the eco-hydrological conditions which fish require for spawning (Zhou et al., 2014). Consequently, this anadromous species now occurs only rarely in the Yangtze River and is characterized as Critically Endangered in the International Union for Conservation of Nature and Natural Resources (IUCN) Red List (IUCN 2010) and listed under Category I State Protection in China (Ludwig, 2008). The need for rehabilitation of the *Acipenser sinensis* is known, and concomitant efforts have been undertaken to support the recovery of this endangered species, including establishing a national nature reserve, *ex situ* conservation, controlled reproduction, and re-stocking of cultured juveniles. The first controlled reproduction of cultured Chinese sturgeon was successfully conducted in 2012, with great significance for the preservation of this species. However, no genetic information was included in any of these stock enhancement programs, which may have negative impacts on wild populations. Genetic investigations have played an important role in the proper management of highly imperiled populations and breeding stocks. To date, there is only one study reporting the isolation of polymorphic microsatellite markers for this polyploidy species based on traditional methods (Zhu et al., 2005). In the present study, we report a set of polymorphic microsatellite loci for *Acipenser sinensis* using next generation sequencing that can serve as effective genetic markers for conducting further assessments of quantifying the genetic diversity in this endangered species (Zhao et al., 2014; Vega-Retter and Véliz, 2015; Xie et al., 2015).

Microsatellites (simple sequence repeats, SSR) have been used widely for addressing genetic diversity and parentage analyses in fish species because of their high rates of polymorphism and co-dominant modes of inheritance (Moghim et al., 2013). The traditional microsatellite marker developments involve significant trials and are thus labor-intensive and time-consuming (Zane et al., 2002). Recent advances in sequencing technologies have dramatically reduced the cost for genome-wide sequencing and provided cost effective means of high output microsatellite development (Abdelkrim et al., 2009; Wang et al., 2012).

Materials and methods

Samples of Chinese sturgeon were collected from Yichang City, in Hubei Province (n = 24). The total genomic DNA was extracted by a universal and rapid salt-extraction method. Total RNA were isolated from testes and ovaries using a RNeasy Plus Mini Kit (Qiagen). First-strand cDNA was synthesized by adding SuperScript II and random primers from the Invitrogen cDNA synthesis kit (Invitrogen), with the second-strand cDNA synthesized by adding custom second strand synthesis buffer, dNTPs, RNase H, and polymerase I. The double-strand cDNA was amplified by PCR reaction to create a cDNA library. Subsequently, the testis and ovary cDNA libraries were sequenced on an Illumina Hiseq2000. Unigenes ≥ 1 kb were subjected to SSR analysis

by the Microsatellite Identification tool (MISA; http://pgrc.ipkgatersleben.de/misa/). Detection criteria were constrained to perfect repeat motifs of 2–6 bp. In total, 165 sequences of the 7674 high repeat sequence motifs were chosen to use in the design of primers using Primer Premier 5.0.

PCR amplification was carried out in a 25 μl volume mixture containing 1× PCR buffer (TaKaRa), 100–200 ng genomic DNA, 0.25 μM of each primer, 1.5 mM $MgCl_2$, 150 μM dNTPs, and 0.5 U Taq DNA polymerase (TaKaRa). The thermal cycle was performed with an initial denaturation at 94°C for 5 min, followed by 30 cycles comprising denaturation at 94°C for 30 s, annealing at the appropriate temperature (Table 1) for 30 s, and extension at 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were separated on 12% non-denaturing polyacrylamide gels by running 5 μl PCR products at 150 V for 5 h, and visualized

Table 1
Characterization of 24 polymorphic microsatellite loci in Chinese sturgeon (*Acipenser sinensis*)

Locus	Repeat motif	Primer (5′–3′)	Tm (°C)	Size (bp)	Na	He	SW	E	HWE	GenBank Accession no.
Asi-72632	(GT)7 … (GT)6	F:AGGGCACTCACCTGGGAACT R:AGACCTGGAGAAAGCGAGACAC	62	296	4	0.633	1.132	0.124	0.001*	KM379071
Asi-66034	(CGA)7	F:CCTACGCCAAGCTCAACCAG R:ACCGCAGAGTCACGGAGTTG	56	309	3	0.660	1.088	0.134	0.362	KM379072
Asi-46902	(TG)8 … (TG)6	F:GGGTGTGTGTGTTTGTTTTGTTT R:ACACTCCCACACACCTCTTCATT	58	313	4	0.668	1.227	0.137	0.164	KM379073
Asi-55961	(AG)7 … (AG)7	F:AAGTTCGGGCTCAACCTTGC R:CTCTTCACATCCCCTTCTCCAG	62	294	3	0.403	0.723	0.076	0.999	KM379074
Asi-68191	(CTG)7	F:ATTATCACCAGCAGCAGCAGG R:AAGGAGAGGCAGAGCTTCAGG	52	302	4	0.687	1.255	0.145	0.962	KM379075
Asi-48230	(CTA)7	F:TTAGTAGTTTCCTGGTCTGCTTGTC R:AAGTCACACATTTTCTGGCGG	46	298	4	0.613	1.066	0.118	0.895	KM379076
Asi-65194	(ATT)5 … (CA)6	F:TACAAAATCGGCAGAAAGGCT R:GCAGGCATGATGAGAATAGGC	58	296	5	0.723	1.386	0.164	0.381	KM379077
Asi-52396	(CTG)7	F:CGGCGACTCTGTTCCTCATC R:CTCTAGCGTCAGCATGCACC	58	299	2	0.235	0.396	0.060	0.925	KM379078
Asi-75067	(TG)7 … (AG)8	F:AGAGTTCTCGAAGCGGAAACAG R:CTGGTTCAAACTGGGAGCGAT	60	293	5	0.736	1.435	0.172	0.222	KM379079
Asi-75905	(TA)8 … (CA)7	F:GCATACTTTTTTGTCAGTGCGTT R:TAATGACAACTTTTTGCTGGGAAT	60	306	4	0.672	1.145	0.138	0.004*	KM379080
Asi-71347	(AC)6 … (AT)6	F:AAGGAATACAAACACTGCAATGG R:AGGAAAAGGGATGTGTGTATTGTT	62	298	6	0.765	1.563	0.194	0.001*	KM379081
Asi-67123	(AC)8 … (CA)8	F:AGCTAACAGCAGTGCATGGTATTT R:CTTGAGATAAAAGGCGCTGTAGAG	62	276	6	0.733	1.477	0.171	0.002*	KM379082
Asi-71954	(AG)6 … (AG)7	F:CTACCTGTCCAAGTGCTCCG R:TCTTCCTCCTCTCTGCTCACC	48	303	3	0.564	0.904	0.104	0.001*	KM379083
Asi-73843	(AGC)7	F:GGTGTTTGAAAGACAGCGAGAA R:TTCCTCCAGGACAGAGTTTGC	58	292	4	0.702	1.293	0.153	0.065	KM379084
Asi-74654	(TA)6 … (AT)6	F:ATGCAAAACGAGTGCCTGTG R:TAAGTGTGCCCTAGTGACGATTC	56	290	3	0.343	0.634	0.069	0.667	KM379085
Asi-68632	(TA)10	F:AGTGTCAATCATACCCCTGCTG R:CGACACGCTGGAATGTTTTG	58	287	3	0.459	0.810	0.084	0.450	KM379086
Asi-77057	(TG)6 … (TG)8	F:GGGTCCCGCACAGTTTAAAG R:GACGGCAAGGCAAGATAGGT	56	300	6	0.751	1.549	0.183	0.006*	KM379087
Asi-76964	(AGC)7	F:GGACAAAGGACAGCCAAAGC R:CATTTTTGTCACAATCGGCAG	58	299	3	0.647	1.069	0.129	0.125	KM379088
Asi-72040	(CTT)5 … (TGC)5	F:AGCAGAGTCCACATCCCCCT R:GAGTGTCGCTCGAAAGCCCT	62	306	3	0.652	1.077	0.131	0.129	KM379089
Asi-67648	(GGA)6 … (GGT)5	F:TCCGGTACTGGAAACCCTTG R:ATTCGCCTGGAAGAGCACAC	60	302	6	0.786	1.608	0.213	0.184	KM379090
Asi-74518	(TA)9(AGC)5*	F:GCATTCCACTTAAATTAGGATTGC R:TATTGAGGCAGGCATGGAGC	46	297	5	0.719	1.362	0.162	0.001*	KM379091
Asi-70421	(TGA)8	F:TGCCACAAATAAGATGCAGGAG R:TTTTGCTTTGGAAACTGTACTGC	56	309	3	0.614	1.010	0.118	0.001*	KM379092
Asi-62964	(CT)11	F:CGATGAACCCAAACCCACAC R:ACAAACTGCACCAATCCCCTT	50	288	3	0.488	0.749	0.089	0.001*	KM379093
Asi-56700	(GA)7 … (GA)7	F:CAACCTCTTCACTACCGCAAAC R:TGCAAAAAGGAATTGGAATCG	50	295	5	0.684	1.244	0.144	0.237	KM379094

Tm, annealing temperature; Na, observed number of alleles; He, expected heterozygosity; SW, Shannon–Wiener Diversity Indices; E, evenness; HWE, probability of Hardy–Weinberg equilibrium.
*$P < 0.01$, significant departures from Hardy–Weinberg equilibrium.

by silver staining. A 20-bp DNA ladder molecular weight marker (TaKaRa) was used to size the alleles. The PCR reaction was repeated twice on each polymorphic locus to demonstrate reproducibility.

The analyses of polymorphism, including the number of alleles (Na), mean expected heterozygosity (He), Shannon–Wiener Diversity Indices (SW) and evenness (E) for each microsatellite locus, were performed using ATETRA 1.2 software (Van Puyvelde et al., 2010), which was developed for analyzing tetraploid microsatellite data. The exact tests for Hardy–Weinberg equilibrium (HWE) were applied using Genodive (Meirmans and Van Tienderen, 2004), which can serve as a tool for population genetics to polyploids (Dufresne et al., 2014). The linkage disequilibrium (LD) was computed by LD4X (Julier, 2009).

Results

The raw sequence data generated by Illumina sequencing contained 16 687 reads/sequences, of which 7674 sequences contained microsatellites. Of these, 4206 sequences could be used for microsatellite primer design and 165 microsatellite primers were designed for screening polymorphic microsatellite loci. Twenty-four primer sets were polymorphic in the population of 24 Chinese sturgeon individuals (Table 1). The number of alleles (Na) per locus ranged from 2 to 6, with an average of 5.2. The mean expected heterozygosities (He), Shannon-Wiener Diversity Indices (SW) and evenness (E) per locus ranged from 0.235 to 0.786 (mean 0.62), from 0.396 to 1.608 (mean 1.13) and from 0.060 to 0.213 (mean 0.13), respectively (Table 1). Exact tests revealed that nine loci showed significant ($P < 0.01$) deviation from HWE (Table 1). No significant ($P > 0.05$) linkage disequilibrium (LD) was detected among these loci.

Of the 24 polymorphic microsatellite loci from *A. sinensis*, eleven polymorphic microsatellite loci were selected for further screening in four congeneric species, *A. schrenskii*, *A. baerii*, *A. dabryanus* and *Huso dauricus*. All of the selected loci amplified successfully and were polymorphic in *A. schrenskii*, *A. baerii*, *A. dabryanus* and *Huso dauricus*, except one locus in *A. baerii* (Table 2).

Table 2
Cross-amplification of developed microsatellite loci in four congeneric species

Locus	*Acipenser schrenskii*	*Acipenser baerii*	*Acipenser dabryanus*	*Huso dauricus*
Asi-46902	P	P	P	P
Asi-77057	P	P	P	P
Asi-73843	P	M	P	P
Asi-76964	P	P	P	P
Asi-67123	P	P	P	P
Asi-65194	P	P	P	P
Asi-72040	P	P	P	P
Asi-75067	P	P	P	P
Asi-67648	P	P	P	P
Asi-66034	P	P	P	P
Asi-56700	P	P	P	P

P, polymorphic; M, monomorphic.

Discussion

There were 7674 sequences containing microsatellite motifs; some had insufficient or inappropriate flanking regions on one or both sides of the simple sequence repeats, and some others possessed only a few repeats and thus having less potential for polymorphism. Therefore, primers were designed for each of 165 microsatellites with sufficient flanking sequences and a number of repeats >5, using Primer Premier 5.0. Twenty-four polymorphic microsatellite markers with high He were identified. Additional tests of cross-species amplification were conducted with four congeners: *A. schrenskii*, *A. baerii*, *A. dabryanus* and *Huso dauricus*. Ten of the 11 selected markers amplified successfully and were polymorphic across the four species. Only one locus in *A. baerii* was monomorphic, which indicates that the polymorphic loci are sufficient for the management of a polyploidy sturgeon species. Nevertheless, nine loci showed significant ($P < 0.01$) deviation from HWE. Similar results were shown in research on *A. dabryanus* (Zeng et al., 2013), and North American paddlefish (*Polyodon spathula*) (Schwemm et al., 2014). Many other researchers have also developed microsatellite markers but provided no data on the HWE in paddlefish (*Polyodon spathula*) or *A. sinensis* (Heist et al., 2002; Zhu et al., 2005). Null or non-amplifying alleles, non-random mating, inbreeding, genetic drift, and absence of segregation and recombination during reproduction may lead to deviation from HWE in polyploid species (Hou et al., 2013). The Chinese sturgeon, *Acipenser sinensis,* has become an extremely endangered species worldwide after the Gezhouba Dam blockage of its migration route. Inbreeding is difficult to avoid under natural conditions, and mature individuals are limited in their possibilities for reproduction.

In conclusion, the polymorphic microsatellite loci developed in this study for *Acipenser sinensis* are anticipated for use in the further study of genetic diversity and paternity identification in this endangered species.

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