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New microsatellite multiplex PCR sets for genetic studies of the sterlet sturgeon, *Acipenser ruthenus*

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ABSTRACT

Wild populations of the sterlet sturgeon, *Acipenser ruthenus*, are declining throughout their native ranges. In-depth knowledge of their genetic diversity and structure is urgently needed to enable the identification of management units for conservation purposes. Moreover, genetic markers are required to establish appropriate breeding schemes for supportive stocking programs and to monitor genetic changes in farmed stocks. Therefore, six species-specific, polymorphic microsatellite loci were isolated and arranged into five multiplex PCR sets together with nine loci from other sturgeon species. The diversity of these 15 microsatellites was examined in 67 sterlet individuals (20 farmed in Germany and 47 wild-caught in the Romanian part of the River Danube). The total number of alleles per locus ranged from 3 to 15 with an average of 7.20. The farmed sterlet sturgeon possessed 1 to 7 alleles per locus, with a mean of 3.13; the wild individuals were more variable, with 3 to 15 alleles per locus and a mean of 7.07. Observed heterozygosities ranged from 0 to 0.850 in the farmed individuals, and from 0.064 to 0.957 in the wild individuals. Indications of inbreeding were only found in the wild sterlet sturgeon (F_{IS}=0.062). The genetic differentiation of the two sterlet groups was significant (F_{ST}=0.1186). The high sensitivity and discriminatory power of the 15 loci was indicated by the very low overall probability of identity for siblings (PIsib=5.099×10^{-5}) and the high accuracy of self-classification (66 out of the 67 individuals (98.51%) were correctly identified). Thus, these newly developed multiplex PCR sets are a valuable genetic tool for identifying management units for species conservation, sustainable fisheries and aquaculture.

INTRODUCTION

The sterlet sturgeon (*Acipenser ruthenus* Linnaeus) is a relatively small potamodromous species that rarely reaches 1.2m in total length. It is native to tributaries of the northern Black Sea and Caspian Sea, and to Siberian rivers. While the sterlet is one of the few Eurasian sturgeon species that still has self-sustaining populations in most of its historic range, the population sizes have been drastically reduced, mostly due to hydroconstruction and overfishing (Gesner et al. 2010).

Key components of a responsible approach to stock enhancement programs have been identified and discussed by Blankenship and Leber (1995) and updated by Lorenzen et al. (2010). They include (among others) species management plans that include genetic objectives, genetic resource management to avoid deleterious genetic effects, and identification of released hatchery fish used for supportive stocking.

Supportive stocking measures or even re-establishment of sterlet populations are currently being attempted in the Danube, the Don, and the Kuban Rivers. In a variety of other river drainages, sterlet sturgeon is subject to recovery management, including the prohibition of directed fisheries and the establishment of *ex situ* stocks. The precautionary principle applies to management measures that use stocking as a recovery tool; this includes the identification and use of the indigenous populations in stocking measures. This approach

has been undermined in the Danube, as shown by the presence of Volga genotypes in the population, which originate from transplants and from escapees from aquaculture facilities in the Danube catchment area (Reinartz et al. 2011).

Since morphological differentiation is not a reliable method for distinguishing sterlet populations, genetic determination of the populations or sub-populations in question should be used (Dudu et al. 2011; Ludwig et al. 2009). Therefore, the aim of the present work was to develop multiplex PCR sets for reliable and time- and cost-efficient amplification of microsatellite loci in sterlet sturgeon, which could be used to identify management units via studies on population structure and differentiation, to establish breeding schemes for supportive stocking programs that maintain genetic diversity and integrity, and to monitor genetic changes in farmed populations (in particular, loss of genetic variability due to drift and inbreeding).

To the best of our knowledge, the six loci directly isolated from sterlet sturgeon genomic libraries are the first microsatellites described from this species; a search in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/ accesion date April, 21st 2017) revealed 305 entries for the pure species *Acipenser ruthenus* and nine entries for hybrids with *A. ruthenus* as one of the parental species. None of these records contained microsatellite DNA sequences.

MATERIALS AND METHODS

Twenty juvenile sterlet sturgeons of the same age were collected at the "Rhönforelle" fish farm, in Gersfeld, Germany. They were progeny of approximately 20 male and 20 female parents propagated artificially (personal communication, P. Groß, "Rhönforelle", Gersfeld). Fin clips were taken to isolate total genomic DNA using the DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocols. A pool of 10 DNA isolates was sent to GenoScreen, Lille, France (www.genoscreen.fr), where 1μg of the pooled DNA was used for the development of microsatellite libraries by 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries as described in Malausa et al. (2011). Briefly, total DNA was mechanically fragmented and enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT and ATCT repeat motifs. The enriched fragments were subsequently amplified. The PCR products were purified and quantified, and the GsFLX libraries were constructed following the manufacturer's protocols and sequenced on a GsFLX PTP. The bioinformatics program QDD (Meglećz et al. 2010) was used to analyze sequences. QDD performs all bioinformatics steps for treating raw sequences: removing adapters/vectors, detecting microsatellites, detecting redundancy/possible mobile element association, selecting sequences with target microsatellites, and designing primers by using BLAST (Altschul et al. 1990), Clustal W (Larkin et al. 2007), and Primer3 (Rozen and Skaletsky 2000). Out of 3942 sequences containing a microsatellite motif, 161 bioinformatically validated primer pairs were designed.

Based on our experience with isolation of microsatellite loci in tench, *Tinca tinca* (Kohlmann and Kersten 2006), and pike-perch, *Sander lucioperca* (Kohlmann and Kersten 2008), all sequences with validated primer pairs were ranked according to motif type (penta- > tetra- > tri- > di-nucleotide repeats), number of repeats (the higher the better), and PCR product size (>100bp), taking into consideration only sequences with perfect repeats. From this list, the 70 top-ranked primer pairs were selected for identification of suitable microsatellites (with consistent amplification, disomic pattern, ease of scoring, sufficient variability).

In addition to the microsatellite loci directly isolated from sterlet sturgeon as described above, nine microsatellites originating from other sturgeon species were included in the development of multiplex PCR sets. Locus *Spl-163* was isolated from shovelnose sturgeon (*Scaphirhynchus platorynchus*) subgenomic libraries enriched for microsatellites (McQuown et al. 2000), and subsequently shown to cross-amplify polymorphic loci in sterlet sturgeon by Fopp-Bayat and Furgała-Selezniow (2010). Loci *AfuG 41* and *AfuG 51* were isolated from lake sturgeon (*Acipenser fulvescens*) microsatellite-enriched genomic DNA (Welsh et al. 2003); locus *An20* was isolated from a partial genomic library of the Adriatic sturgeon (*A. naccarii*) enriched for GATA repeats (Zane et al. 2002); loci *Aox*D161 and *Aox*D165 were isolated from Atlantic sturgeon (*A. oxyrinchus*) microsatellite-enriched genomic libraries (Henderson-Arzapalo and King 2002); and all five of these loci were subsequently shown to cross-amplify polymorphic loci in sterlet sturgeon by Barmintseva and Mugue (2013). Loci *LS-19* (= *Afu 19*), *LS-39* (= *Afu-39*) and *LS-68* (= *Afu-68*) were isolated from a lake sturgeon (*A. fulvescens*) genomic library (May et al. 1997), and subsequently shown to cross-amplify polymorphic loci in sterlet sturgeon by Ludwig et al. (2001), Fopp-Bayat and Furgała-Selezniow (2010), and Dudu et al. (2013).

The initial procedure for selecting suitable loci was based on separate genotyping of single loci. However, since the final aim was to use time- and cost-saving multiplex PCR sets for future routine genotyping, protocols for microsatellite amplification were developed using PCR multiplex Kits (QIAGEN) and a peqSTAR 96X Universal Gradient thermocycler (Peqlab). After suitable loci had been identified, the software MultiPLX, version 2.1 (Kaplinski et al. 2005) was used to analyze PCR primer compatibility and automatically find the optimal multiplexing (grouping) solution. Three different dye labels (Cyanine 5, DY-751, Atto 680) were assigned to forward primers. Genotyping of microsatellite loci was performed on a CEQ 8000 8-capillary sequencer (Beckman Coulter) using the Fragment Analysis module of the GenomeLab™ GeXP Genetic Analysis System, version 10.2 (Beckman Coulter).

Microsatellite variability was initially examined in the 20 farmed individuals, but later, also examined in 47 wild sterlet sturgeons originating from the Romanian part of the River Danube. Young-of-the-year sterlet were captured in the Danube at km 123 using bottom drifting trammel nets with 18-20 mm mesh size during May-June in seven different years. Adult sterlet samples were taken from live adults captured for controlled

propagation on the Borcea branch by local professional fishermen. Prior to microsatellite genotyping, the species identity (i.e. that they were sterlet) and purity (i.e. that they were not sterlet hybrids) of all 67 individuals was confirmed using the nuclear DNA markers described by Havelka et al. (2017).

All microsatellite genotypes were examined with Micro-Checker software, version 2.2.3 (van Oosterhout et al. 2004) for scoring errors due to null alleles, stutter bands, and/or large allele dropout. Then, general characteristics of microsatellite loci variability (number of alleles, number of private alleles, observed and expected heterozygosity) were calculated, and tests for significance of deviations from Hardy-Weinberg equilibrium and linkage disequilibrium were performed using GENEPOP 4.0 (Rousset 2008). The discriminatory power of loci (probability of identity=PI) was estimated with GENECAP version 1.4 (Wilberg and Dreher 2004) applying the more conservative measure of PI for siblings, PIsib (Waits et al. 2001). Genetic differentiation between farmed and wild sterlet sturgeons was tested by calculating the F_{ST}-value using FSTAT, version 2.9.3.2 (Goudet 2002). FSTAT was also used to estimate the inbreeding coefficient F_{IS} for both groups of sterlet. Finally, the sensitivity of the 15 microsatellite loci was examined by self-classification of the 67 sterlet sturgeons using Bayesian criteria and the "Leave One Out" procedure of GeneClass software, version 1.0.02 (Cornuet et al. 1999).

RESULTS AND DISCUSSION

Table 1. Characteristics of 15 polymorphic microsatellite loci tested in a total of 67 *Acipenser ruthenus* individuals.

PCR multiplex set (annealing temperature)	Locus name / GenBank accession no.	Forward primer dye label	Primer concentration (μM)	Primer sequence (5'- 3')	Repeat type	Total number of alleles	Allele size range (bp)
1 (57°C)	*Spl-163*[a] *AF276205*	Cyanine 5	0.033	F: TGCTTGTAAACTGCCCCACT R: CCACATGCAGTTTGAGCTGC	GATA	10	172-220
	Aru26 MF100775	DY-751	0.180	F: AAAGCAACAACTCCACCAGG R: TGCCTTGTCTACTGTCCGAA	CT	3	159-163
	AfuG 41[b] *AF529463*	Atto 680	0.210	F: TGACGCACAGTAGTATTATTTATG R: TGATGTTTGCTGAGGCTTTTC	(GATA)TA(GATA)	14	193-261
2 (57°C)	*An20*[c] *AY144618*	Cyanine 5	0.038	F: AATAACAATCATTACATGAGGCT R: TGGTCAGTTGTTTTTTTATTGAT	(ATCT)(TG)	11	152-178
	Aru12 MF100771	DY-751	0.170	F: AAATAGCATGTTCCCCAGCA R: TCCATTGCACTTTTCCTTCTTT	TAG	3	172-178
	LS-39[d] *U72734*	Atto 680	0.190	F: TTCTGAAGTTCACACATTG R: ATGGAGCATTATTGGAAGG	GTT	4	117-132
3 (57°C)	*AfuG 51*[b] *AF529467*	Cyanine 5	0.041	F: ATAATAATGAGCGTGCTTTCTGTT R: ATTCCGCTTGCGACTTATTTA	(AAAC)(AC)(AAAC)	5	223-247
	AoxD165[e] *AY093640*	DY-751	0.180	F: TTTGACAGCTCCTAAGTGATACC R: AAAGCCCTACAACAAATGTCAC	(CTAT)CTAC(CTAT)	10	170-206
	LS-19[d] *U72730*	Atto 680	0.160	F: CATCTTAGCCGTCTGTGGTAC R: CAGGTCCCTAATACAATGGC	TTG	3	138-144
4 (60°C)	*LS-68*[d] *U72739*	Cyanine 5	0.044	F: TTATTGCATGGTGTAGCTAAAC R: AGCCCAACACAGACAATATC	GATA	15	174-238
	Aru13 MF100772	DY-751	0.190	F: TCCACTTTATTCCGTTGTGG R: AGACCGGAATCAAACCCAG	GTT	13	87-135
	AoxD161[e] *AY093639*	Atto 680	0.180	F: GTTTGAAATGATTGAGAAAATGC R: TGAGACAGACACTCTAGTTAAACAGC	CTAT	6	106-130
5 (60°C)	*Aru19 MF100774*	Cyanine 5	0.038	F: GCGTGGTGTAAGTGAACCCT R: CTTCAATTGTGCTTGGCTCA	GA	4	159-193
	Aru50 MF100780	DY-751	0.210	F: TGGAAACCAAATTAATTCACAAAA R: TGGGATCCTCTGTAGAACAGTCT	AG	3	123-129
	Aru18 MF100773	Atto 680	0.180	F: CCTGGAACACGTCCAGTTTT R: TGGGTGAATGTTTTGGTGTG	TC	4	135-145

References for microsatellite locus names, GenBank accession numbers, primer sequences and repeat type information: [a] McQuown et al. (2000), [b] Welsh et al. (2003), [c] Zane et al. (2002), [d] May et al. (1997), [e] Henderson-Arzapalo and King (2002).

Out of the 70 sterletsturgeon PCR primer pairs chosen for testing, six amplified microsatellite loci matched our selection criteria, in particular, consistent amplification, disomic pattern, and sufficient variability. These six loci were combined with the nine loci originating from other sturgeon species into five multiplex PCR sets (Table 1). The optimized PCR reaction mixes consisted of 5.0μL of master mix and 1.5μL Q-solution (QIAGEN), 1.0μL DNA isolate, primers at the concentrations given in Table 1, and PCR-grade water up to a final volume of 10.0μL. The PCR program, based on QIAGEN recommendations, included initial denaturation at 95°C for 15min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 57°C (multiplex sets 1, 2 and 3) or 60°C (multiplex sets 4 and 5) for 90s, and extension at 72°C for 60s. Final extension at 60°C lasted for 30min.

Tests with Micro-Checker software did not reveal any evidence of scoring errors due to stutter bands or large allele dropout. Moreover, no indications of null alleles were found at any of the 15 loci of the 20 farmed individuals. In contrast, homozygote excess was observed at four loci (*Spl-163*, *LS-68*, *Aru19*, and *Aru50*) in the 47 wild sterlet sturgeons, suggesting that null alleles might be present at these loci. The total number of alleles per locus ranged from 3 to 15 (Table 1), with an average of 7.20. The farmed sterlet sturgeon possessed 1 to 7 alleles per locus, with a mean number of 3.13; the wild individuals were more variable, and had 3 to 15 alleles per locus, with a mean number of 7.07 (Table 2). Consequently, private alleles were almost exclusively found in the wild sterlet sturgeons (Table 2). Linkage disequilibrium tests for each locus pair across both groups of sterlet revealed that three of the 105 possible loci pair combinations gave significant *P*-values: *Spl-163* and *LS-68* (P=0.003), *LS-68* and *Aru13* (P=0.018), and *LS-19* and *Aru19* (P=0.045). The observed heterozygosities ranged from zero at loci *Aru18*, *Aru26*, and *Aru50*, to 0.850 at loci *Aru12* and *Spl-163* in the farmed individuals, and from 0.064 at locus *Aru50* to 0.957 at locus *Aru12* in the wild individuals (Table 2). Significant (P<0.05) to highly significant (P<0.01) deviations from Hardy-Weinberg equilibrium (P_{HW}) were found at three loci in the farmed sturgeons and six loci in the wild sterlet sturgeons (Table 2). However, indications of inbreeding (F_{IS}) were only found in the wild sterlet sturgeon, with an F_{IS}-value of 0.062 (the corresponding value for the farmed sterlet was negative: -0.071). With regard to the farmed sterlet, the main reason for the deviations from Hardy-Weinberg equilibrium might be the relatively small sample size. In the 47 wild sterlet sturgeons, the possible presence of null alleles at four microsatellite loci, and the uneven representation of sampling years in the data set (the number of individuals per year ranged from 1 to 16) could have contributed to deviations from equilibrium. This will be investigated in more detail in future studies with larger numbers of wild individuals.

Table 2. Variability of 15 polymorphic microsatellite loci in two test panels of 20 farmed and 47 wild-caught *Acipenser ruthenus* (N_A=number of alleles; N_{Ap}=number of private alleles; H_O=observed heterozygosity; H_E=expected heterozygosity; P_{HW}=results of the Hardy-Weinberg probability test: *P<0.05, **P<0.01, n.s.=non-significant, n.a.=not applicable).

Locus	Parameter	Farmed *A. ruthenus*	Wild *A. ruthenus*	Probability of identity for siblings (PIsib)
Spl-163	N_A	7	8	0.3323
	N_{Ap}	2	3	
	H_O	0.850	0.638	
	H_E	0.835	0.826	
	P_{HW}	n.s.	*	
Aru26	N_A	1	3	0.9291
	N_{Ap}	0	2	
	H_O	0	0.106	
	H_E	0	0.103	
	P_{HW}	n.a.	n.s.	
AfuG 41	N_A	5	14	0.3458
	N_{Ap}	0	9	
	H_O	0.800	0.872	
	H_E	0.737	0.868	
	P_{HW}	n.s.	n.s.	
An20	N_A	3	11	0.3407
	N_{Ap}	0	8	
	H_O	0.250	0.830	
	H_E	0.232	0.875	
	P_{HW}	n.s.	*	

Aru12	N_A	2	3	0.5924
	N_{Ap}	0	1	
	H_O	0.700	0.957	
	H_E	0.467	0.515	
	P_{HW}	*	**	
LS-39	N_A	2	4	0.5694
	N_{Ap}	0	2	
	H_O	0.300	0.511	
	H_E	0.385	0.556	
	P_{HW}	n.s.	n.s.	
AfuG 51	N_A	2	5	0.7575
	N_{Ap}	0	3	
	H_O	0.450	0.149	
	H_E	0.450	0.143	
	P_{HW}	n.s.	n.s.	
AoxD165	N_A	4	10	0.3702
	N_{Ap}	0	6	
	H_O	0.550	0.745	
	H_E	0.635	0.813	
	P_{HW}	n.s.	n.s.	
LS-19	N_A	2	3	0.7150
	N_{Ap}	0	1	
	H_O	0.200	0.383	
	H_E	0.185	0.375	
	P_{HW}	n.s.	n.s.	
LS-68	N_A	7	15	0.3170
	N_{Ap}	0	8	
	H_O	0.650	0.617	
	H_E	0.760	0.899	
	P_{HW}	*	**	
Aru13	N_A	3	13	0.3586
	N_{Ap}	0	10	
	H_O	0.850	0.830	
	H_E	0.581	0.858	
	P_{HW}	**	n.s.	
AoxD161	N_A	4	6	0.4600
	N_{Ap}	0	2	
	H_O	0.800	0.574	
	H_E	0.658	0.618	
	P_{HW}	n.s.	n.s.	
Aru19	N_A	3	4	0.5735
	N_{Ap}	0	1	
	H_O	0.550	0.319	
	H_E	0.578	0.445	
	P_{HW}	n.s.	*	
Aru50	N_A	1	3	0.8331
	N_{Ap}	0	2	
	H_O	0	0.064	
	H_E	0	0.246	
	P_{HW}	n.a.	**	
Aru18	N_A	1	4	0.8298
	N_{Ap}	0	3	
	H_O	0	0.277	
	H_E	0	0.251	
	P_{HW}	n.a.	n.s.	

The probability of identity for siblings (PIsib) at single microsatellite loci ranged from 0.3170 at locus *LS-68* to 0.9291 at locus *Aru26* (Table 2); the overall PIsib value was very low: 5.099×10^{-5}. Genetic differentiation between farmed and wild sterlet sturgeons was significant (F_{ST}=0.1186; P<0.05). The accuracy of classification with the 15 microsatellite loci was also demonstrated by the fact that 66 out of the 67 individuals (=98.51%) were correctly identified using these loci; only one wild sterlet sturgeon was assigned to the farmed group.

In conclusion, these five multiplex PCR sets, which consist of six microsatellite loci isolated from sterlet sturgeon and nine microsatellite loci from other sturgeon species, provide sufficient variability and should thus be suitable for assessing the genetic diversity and structure of the remaining wild populations, for aiding the development of both *in situ* and *ex situ* conservation measures, for establishing appropriate breeding schemes for supportive stocking programs, and for monitoring genetic changes in farmed strains used for caviar and meat production.

Data availability

The DNA sequences of the six newly isolated *Aru* microsatellite loci were deposited in NCBI GenBank (for accession numbers see Table 1).

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