Development of disomic single-locus DNA microsatellite markers for Persian sturgeon (*Acipenser persicus*) of the Caspian Sea

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Received: December 2011 Accepted: February 2012

Abstract

Understanding the scale at which wild stock of Persian sturgeon (*Acipenser persicus*) are genetically discrete is necessary for effective management of this commercially important species. Disomic DNA microsatellite markers are among the best tools for determining stock structure in fishes. As all sturgeon species have a polyploid ancestry of all sturgeons, most gene loci exhibit more than two alleles per individual, limiting the use of powerful analytical methods that commonly assume disomic inheritance. We scored products from 38 sets of microsatellite primers developed in lake (*Acipenser fulvescens*) and Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*) to determine whether they would amplify disomic loci in Persian sturgeon. Samples of 45 individuals were detected.Thirty six loci (95%) were amplified successfully in Persian sturgeon. We identified; a single monomorphic locus, 12 disomic, 19 tetrasomic, three octosomic, and one locus that was ambiguous. This is the first report on development of disomic single-locus DNA microsatellite markers in Persian sturgeon. These loci could be used to characterize variation in geographically discrete populations of the Persian sturgeon in their native ecosystem including in the Caspian Sea.

Keywords: *Acipenser persicus*, Caspian Sea, Single-locus DNA microsatellite markers

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Introduction

Persian sturgeon (*Acipenser persicus* Borodin 1897) was first described from the Southern Caspian Sea by Borodin (1897) with its range later extended to the northern Caspian Sea (Holcik, 1989). The Persian sturgeon is an anadromous fish which enters rivers for spawning, mainly the Sefid-Rud, Tajan and Gorgan-Rud rivers in Iran and Kura River in Azerbaijan and to a lesser extent the Volga, Ural, Samur, Terek, Lenkoranka and Astara rivers (Berg, 1948). It is one of the most economically important species in Iran (Moghim et al., 2006). Persian sturgeon is now listed as a critically endangered species by the International Union for the Conservation of Nature (IUCN, 2011). Like most sturgeons, Persian sturgeon density in the wild have declined due to over-fishing, spawning habitat destruction , and pollution (Birstein 1997; Pikitch et al., 2005; Moghim et al., 2006; Pourkazemi, 2006). Between years 1927 and 1956, the total catch (expressed as flesh weight) declined from about 930 to only 217 tons. Catch rates for this species that currently comprises the majority of sturgeon landings in the Caspian Sea in Iran, have remained relatively stable over the past few decades but have not returned to pre-1950s levels (Moghim et al., 2006).

Recruitment in the wild is extremely low, in spite of stocking millions of artificially propagated juveniles released from Iranian sturgeon hatcheries to adjacent rivers in the Caspian Sea since 1972. This practice has resulted in a significant increase in catch of both adult and juvenile Persian sturgeon in the commercial catch, trawl surveys and beach seine by-catch in Iranian waters of the Caspian Sea after 1990 (Moghim et al., 2005, 2006). Many experts believe the increasing Persian sturgeon stock in the south Caspian Sea in 1990's result from extensive hatchery production and release programmes by the Iranian Fisheries Organization (Abdolhay and Baradaran Tahori., 2006; Moghim et al., 2006).

Restorative propagation programs can be harmful to wild stocks if they lead to inbreeding via use of low numbers of parents relative to the number of fish stocked or if sites are stocked with fish from a genetically-depauperate stock (Drauch and Rhodes, 2007). DNA microsatellite loci can provide powerful tools for monitoring genetic variation levels and for detecting genetic variation among discrete sturgeon stocks (e.g. Schrey and Heist, 2007). The major problem with applying microsatellite markers to sturgeon stock management are the polysomic nature of inheritance (e.g., tetrasomy or octosomy) and the presence of null alleles at some loci (Pyatskowit et al., 2001).

An earlier attempt to develop disomic microsatellite markers for Persian sturgeon trialed cross-species amplification using microsatellite primers developed for *Scaphirhynchus* spp., that possess a lower ploidy level than Persian sturgeon (Ludwig et al., 2001). No amplified loci however, exhibited disomic inheritance (Moghim et al., 2009). Disomic microsatellite loci have been developed successfully in some other sturgeon species with high ploidy levels (e.g. white, green, and lake sturgeon) although the majority of loci identified

were polysomic. For example Welsh and May (2006) found that only 9 of 254 primer pairs tested in Lake sturgeon exhibited disomic inheritance. When combined with loci from other studies, Welsh and May (2006) reported a total of 13 polymorphic disomic loci in lake sturgeon, a species with the same ploidy level as Persian sturgeon (Ludwig et al., 2001). Recently we developed and tested 68 microsatellite primer pairs from a Persian sturgeon enriched microsatellite library (Moghim et al., 2012) and while none of the markers exhibited disomic inheritance in Persian or Russian (*A. gueldenstaedtii*) sturgeon, several loci showed promise in stellate sturgeon (*A. stellatus*), ship sturgeon (*A.nudiventris*) and beluga (*Huso huso*).

In the present study, we tested cross-species amplifications of microsatellite primer pairs developed in lake and Atlantic sturgeon to identify disomic microsatellite loci for Persian sturgeon.

Materials and methods

Experimental materials and Molecular analysis

Persian sturgeon fin clips (n=45) were collected from adult broodstocks from Iranian coastline of the south Caspian Sea and preserved in 95% ethanol. Genomic DNA for amplification of 38 microsatellite loci was extracted using the Qiagen DNeasy Tissue Kit (Qiagen, Valencia, CA) and stored at –20°C.

PCR reactions and program

Microsatellite primer sequences tested here were as reported in Welsh et al. (2003), Welsh and May (2006) and May et al. (1997) (Table1). We amplified genomic DNA from 12 individuals in an Quanta Biotec master cycler gradient thermocycler (Quanta Biotech Ltd, Surrey, United Kingdom) trialing annealing temperatures ranging that ranged from 48° to 66° to determine the optimal annealing temperature for each primer pair. The 20-µl PCR reactions contained approximately 1-10 ng genomic DNA, 0.15 units *Taq* DNA polymerase, 1 µM of each primer, 200 mM of each dNTP, 1.75 mM $MgCl_2$, and 1× PCR buffer.

The amplification protocol for most loci consisted of a 5 min denaturing step at 95 °C, followed by 35 cycles of 95 °C for 30 s, 48 - 66 °C for 30 s, and 72 °C for 45 s, and a final elongation at 72 °C for 5 min. Amplication of AfuGs 9 and 56 followed Welsh and May (2006). PCR products were suspended 1:1 in 98% formamide/loading dye, denatured at 95°C for 5 min, and separated in a 6% denaturing polyacrylamide gels on a Bio-Rad SequiGen Sequencing Cell-system with gel size 38 × 30 cm and run at 70 W for 45 - 60 min. DNA bands were visualized using a silver staining method (An et al., 2009) and amplified fragments were sized by comparing their migration against a 50 bp DNA Ladder (Promega, Madison, WI, USA). Fragment sizes were estimated using UVIDoc version 99.04 software (UVItech limited. UK). Loci that appeared to be disomic were amplified and scored in a minimum of 30 individuals.

We interpreted a locus as being polymorphic if multiple bands of the appropriate size range and appearance were present in most individuals. We

determined if bands were of the appropriate sizes based on allele sizes reported by Welsh et al. (2003) and their migrations relative to the dye in the stop solution. Most loci scored as polymorphic in the current study, exhibited stutter bands characteristic of most microsatellite banding patterns on polyacrylamide gels. Loci were scored as monomorphic if a single band was present in all individuals, disomic if a maximum of two alleles were observed per individual and tetrasomic if 3-4 bands were present in some individuals and octosomic if more than four bands were present in some individuals. Loci that produced multiple bands but lacked stutter bands or were otherwise difficult to interpret were scored as "ambiguous".

Statistical Analysis

Genotype and allele frequencies, observed and expected heterozygosity and other statistical parameters were estimated using GenAlEx 6.2 (Peakall and Smouse, 2007) software.

Results

Of the 38 primer pairs tested, was recognized 36 (95%) amplified bands in the correct size range. A single locus was monomorphic (AfuG185) in all individuals screened, 12 were disomic (AfuGs 9,56, 63, 68b, 112, 160, 195, 204, 229, 241, LS-68 and Aox27), 19 were tetrasomic (AfuGs 21, 28, 66, 67,68, 71, 74, 83, 94, 95, 109, 110, 135, 166, 175, 182, 211, 238 and 247), three were octosomic (AfuGs 23, 30, 88), and one locus was ambiguous (AfuG237). Information for the 38 microsatellite loci used for cross species amplification in Persian sturgeon including annealing temperature, observed allele size range in base pairs, number of Persian sturgeons screened (N), number of alleles observed (NA), observed heterozygosity (H_O), and expected heterozygosity (He) are presented in Table 1.

The eleven microsatellite markers that were identified as disomic microsatellite loci in Lake Sturgeon, tested in also exhibited disomic banding patterns in Persian sturgeon (AfuGs 9, 56, 63, 68b, 112,160,195, 204 and Aox27). The two exceptions were AfuGs68 and 74 that appeared to amplify as tetrasomic loci, producing banding patterns consistent with four gene doses and AfuG68 that amplified two loci with the lower zone being octosomic.

Three primer pairs (LS-68, AfuG229, AfuG241) produced bands in two discrete size ranges, that we interpreted as separate segregating loci with the same conserved flanking sequences. LS-68 produced two disomic loci. AfuG229 produced one apparently disomic locus and a zone showing polysomic inheritance. AfuG204 produced only two alleles and most individuals were homozygous. AfuG9 showed inconsistent amplification results and will require further optimization in Persian sturgeon.

Discussion

Sturgeons exhibit among the highest range of ploidy levels among fishes, ranging from functional diploids (2n), tetraploids (4n) to octoploids (8n) (Bristein, 1997), but also show evidence for recent evolution towards diploidisation (Welsh and May, 2006). *A. persicus* is a tetraploid

(4n) species (Ludwig et al., 2001), so finding disomic loci can be a problem. Unfortunately current attempts to develop single locus DNA microsatellite markers for Persian sturgeon were unproductive and all the developed polymorphic loci showed polysomic (tetraploid higher polyploid levels) and should therefore be used only as dominant markers (Moghim et al., 2009, 2012).

While some software packages can be used to analyze dominant data and determine the number of groups present (e.g. STRUCTURE, Pritchard et al. (2000) most are based on the assumption of disomic inheritance patterns and thus availability of diploid Mendelian markers in *A. persicus* would allow use of far more powerful statistical analyses. For example, tests of Hardy-Weinberg equilibrium could be performed at the smallest geographical sample scale to determine whether multiple stocks were present (Wahlund effect) or as a check against allele scoring errors. One problem that is often reported with microsatellite markers is the phenomenon of null alleles, which can erroneously elevate homozygosity within samples and artificially inflate estimates of heterogeneity among geographical samples. Available software packages (e.g. MICROCHECKER; Van Oosterhaut et al. (2004)) can detect the presence of null alleles and other scoring errors but this approach is designed for use with disomic loci.

Development of disomic loci in Persian sturgeon may be accomplished by screening primers designed specifically for *A. persicus*. Because mutations can occur in the flanking regions, cross-species amplification often requires use of lower annealing temperatures allowing for less fidelity between primer and template sequences. By developing primers specifically for Persian sturgeon we can employ very high annealing temperatures that may eliminate all but a single duplicated locus resulting in disomic banding patterns. This approach is both time and labor-consuming as well as expensive to develop. Moghim et al. (2012) developed 68 species specific primer pairs for Persian sturgeon of which 39 (57%) were polymorphic but none showed disomic banding patterns.

Cross species amplification is widely applied in fishes to generate microsatellite markers quickly for fish species (Rico et al., 1996; Barbara et al., 2007). The primers trialed here have shown considerable cross-species utility in *A. persicus*. It may also be possible to redesign primers developed in one species to work in another (e.g. the *Spl-113* microsatellite primer pairs for shovelnose sturgeon (*Scaphirhynchus platorynchus*) were used to design a new primer *Atr-113* for white sturgeon (McQuown et al., 2000). Primers that amplify many species successfully also have a higher chance of being polymorphic. In general, the chance of successful cross-species amplification is inversely related to the evolutionary distance between species pairs trialed (Primer et al., 1996). In the current study, the rate of successful amplification of polymorphic fragments in Persian sturgeon with primers developed for lake sturgeon was high (89.5%). Cross species amplification is easier, less expensive and more practical for most genetic labs

especially in developing countries and on average the approach has a high success rate.

In the current study, we identified 13 single locus DNA microsatellite markers that will be useful in population genetic and recovery studies for Persian sturgeon. This is the first report of development of disomic single-locus DNA microsatellite markers for Persian sturgeon and we expect that the same primer pairs will be also likely amplify be useful as single locus microsatellite markers in the closely related Russian sturgeon (Birstein et al., 2005). They should also be tested for utility in other Caspian Sea sturgeon species.

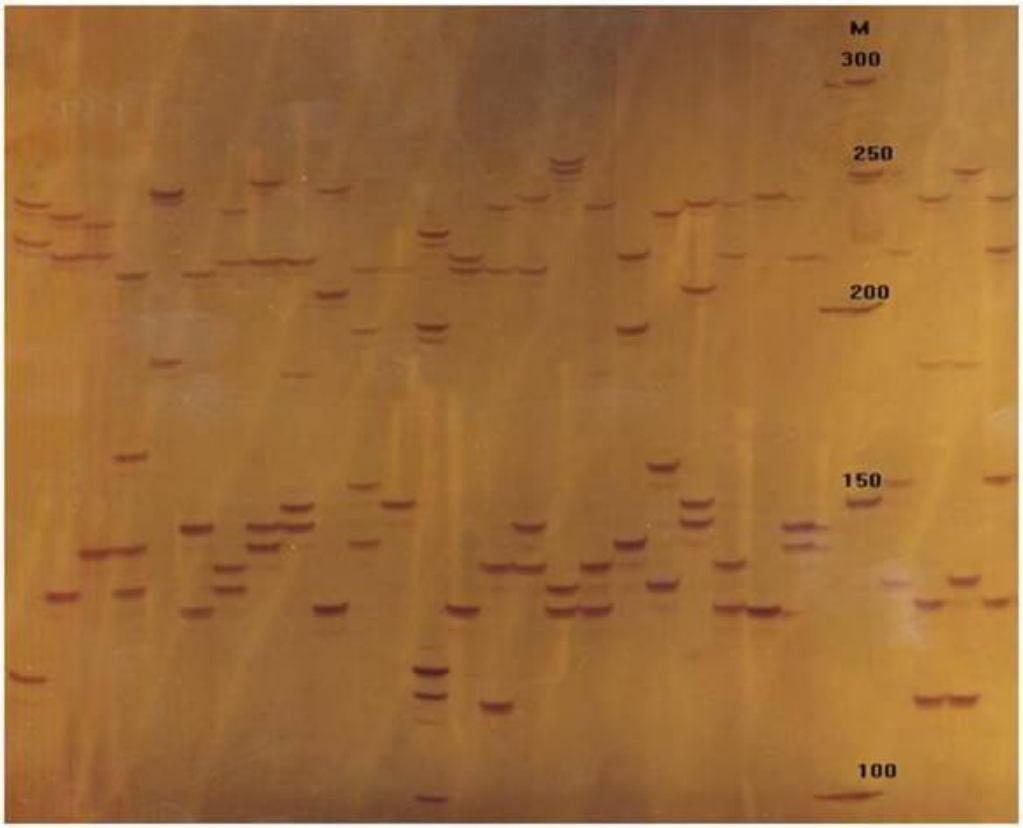

Figure 1: Polymorphic disomic loci in Persian sturgeon amplified byLS-68 .Two disomic loci with 11, 16 alleles and 128-164 bp, 180- 264bp size range in lower and upper amplified zones respectively.

Table 1: Characteristics of 38 microsatellite primer pairs used for cross species amplifications in Persian sturgeon. Annealing temperature, observed size range in base pairs, number of Persian sturgeons screened (N) number of alleles observed (NA), observed heterozygosity (H_O), and expected heterozygosity (He) are presented for samples taken from the Caspian Sea.

Locus	locus characteristics	Annealing temperature	Size range (bp)	*N*	*NA*	H_O	*He*
AfuG 9	disomic	52	130-165	14	7	0.5	0.8
AfuG21	tetrasomic	53	230-270	6	9		
AfuG23	octosomic	53	130-210	6	10		
AfuG28	tetrasomic	53	220-245	6	6		
AfuG30	octosomic	64	140-185	18	12		
AfuG56	disomic	Touch down	254-282	43	6	0.581	0.614
AfuG61	no amplification	-	-	-			
AfuG63	disomic	60	127-171	40	11	0.610	0.777
AfuG64	no amplification	-	-	-			
AfuG66	tetrasomic	59	320-340	24	4		
AfuG67	tetrasomic	65	220-230	24	4		
AfuG68*	tetrasomic	49	210-230	40	16	0.675	0.895
AfuG68b	disomic	60	151-231	167	21		
AfuG71	tetrasomic	60	230-245	30	4		
AfuG74	tetrasomic	53	230-265		9		
AfuG83	tetrasomic	61	220-260	30	10		
AfuG88	octosomic	48.5	166-260	12	10		
AfuG94	tetrasomic	57	135-170	30	8		
AfuG95	tetrasomic	65	220-230	24	3		
AfuG109	tetrasomic	61	270-390	48	10		
AfuG110	tetrasomic	65	210-370	48	15		
AfuG112	disomic	57	208-288	41	16	0.829	0.897
AfuG135	tetrasomic	61	200-240	24	10		
AfuG160	disomic	59	131-155	43	6	0.814	0.762
AfuG166	tetrasomic	64	150-166	36	4		
AfuG175	tetrasomic	57	220-250	24	5		
AfuG182	tetrasomic	53	260-280	30	5		
AfuG185	monomorphic	64	230	30	-		
AfuG195	disomic	59	161-173	40	4	0.725	0.637
AfuG204	disomic	66	146-150	18	2	0.222	0.198
AfuG211	tetrasomic	53	225-260	6	5		
AfuG229*	disomic	67	288-336	42	15	0.524	0.873
AfuG237	ambiguous	51	-	-	-	-	-
AfuG238	tetrasomic	60	230-290	30	12		
AfuG241	disomic	65	232-278	43	10	0.349	0.787
AfuG247	tetrasomic	64	195-230	6	6		
Aox27	disomic	53	138-146	43	3	0.558	0.535
LS-68*	disomic	54	180-264	39	16	0.744	0.882
LS-68-1	disomic	54	128-164	39	11	0.7073	0.8606

* AfuG229 Upper zone was tetrasomic

LS-68 amplified two zones. Upper zone were named LS-68 and lower zone LS-68-1

Acknowledgements

This research was funded by the Iranian Fisheries Research Organization and carried out in Fish Genetic Laboratory of the Caspian Sea Ecology Research Center. Special thanks are given to Edward J Heist, SIU, for comments and review of the manuscript.

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