Zoological Studies **51**(8): 1507-1514 (2012)

Zoological Studies

Inheritance of Microsatellite Loci and Their Application for Pedigree Analysis of the Polyploid Persian Sturgeon *Acipenser persicus* (Acipenseridae)

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(Accepted August 16, 2012)

Mehdi Moghim, Soon Guan Tan, Arash Javanmard, Mohamad Pourkazemi, and Jothi Malar Panandam (2012) Inheritance of microsatellite loci and their application for pedigree analysis in the polyploid Persian sturgeon *Acipenser persicus* (Acipenseridae). *Zoological Studies* **51**(8): 1507-1514. Identification of suitable molecular markers for correctly assigning progenies to their parents particularly during the early stages of development is vital for aquaculture breeding programs. Developing such markers in sturgeons, that have a polyploid ancestry, is particularly challenging because many markers exhibit polysomic inheritance. In the present study, 2 F_1 families of 23 and 28 larvae were produced in 1 × 1 crosses of Persian sturgeon. Eleven microsatellite loci were used to genotype the parents and their offspring. An analysis of inheritance patterns demonstrated classical mendelian disomic inheritance in all but 2 of the markers tested in the offspring. Two loci exhibited inheritance patterns consistent with a parental null allele that was consistently inherited by approximately 1/2 of the offspring screened. Thus, the markers tested here can be used for parental assignment testing and for population genetic studies. They can also be used as reliable molecular markers for constructing a genetic linkage map for the target species that will be essential for future quantitative trait locus mapping purposes. This is the 1st report of mendelian segregation testing in Persian sturgeon for cross-species amplification of single-locus DNA microsatellite markers. http://zoolstud.sinica.edu.tw/Journals/51.8/1507.pdf

Key words: Microsatellite loci, Persian sturgeon, Family studies, Mendelian inheritance, Caspian Sea.

The Persian sturgeon *Acipenser persicus* (Acipenseridae) is an economically important species in the southern Caspian region and is the dominant sturgeon species found along most of the Iranian coast of the Caspian Sea (Berg 1948, Holcik 1989, Moghim et al. 2006). As natural reproduction in this species is extremely low, artificial propagation is practiced to rehabilitate wild Persian sturgeon stocks in the Caspian Sea. Consequently, artificial breeding of sturgeons on fish farms currently is the approach practiced to offset decreasing natural supplies of wild sturgeon.

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Recently, the Iranian Fisheries Department increased hatchery production and release programs of Persian sturgeon (Abdolhay and Tahori 2006).

Heterosis is one of the common methods employed to improve aquaculture productivity in terms of external fertilization and similar mating behaviors (Leary et al. 1995). Heterosis refers to the phenomenon that 1st-generation progeny of diverse species or populations exhibit superior trait performance, either in terms of biomass, development, or fertility, than the better of the 2 parents (Gjedrem 2005). Generally, a hybrid fish is expected to produce offspring with remarkable growth characteristics and tolerance to environmental stresses. Identification of suitable molecular markers for distinguishing hybrid progeny from their parents particularly in the early stages of development is vital for aquaculture breeding programs and is a critical 1st step in hybridization experiments (Wohlfarth et al. 1993). Extensive utilization of heterosis in the selective breeding of fish species can be an effective way to improve fish quality and increase production even though it is a 1-time event (Bryden et al. 2004). Within species, crosses between genetically distant populations are generally expected to enhance heterosis more than between genetically close ones, and several authors suggested that there is a positive relationship between the genetic divergence between parents and the performance of their hybrid offspring (Wang and Xia 2002). Distinguishing hybrids from their parents by morphological characters may be difficult; hence, molecular markers are needed for this purpose.

Microsatellites, also known as simple sequence repeats (SSRs), are a small array of tandemly arranged bases that are widely distributed across the genome of fish species. Microsatellites as DNA markers possess advantages over many other marker types, as they are usually highly polymorphic, are abundant across the genome, show co-dominant inheritance, can easily be analyzed, and in some cases are transferable to other species (Weber 1990). In the aquaculture industry, microsatellites represent markers of choice for many applications, in particular for genetic monitoring of farmed stocks to manage inbreeding rates. They allow analysis of genetic variations and reveal pedigree structures that can allow design of beneficial crosses for developing improved stocks, and can also be used to minimize inbreeding and increase selection responses (Chistiakov 2006).

When assessing the utility of microsatellite markers for characterizing variation in Persian sturgeon populations, it is important to consider the relative complexity of this species' nuclear genome. Evolution of the family Acipenseridae was characterized by several polyploidization events (Birstein et al. 1997). As a consequence, genetic markers in individuals may exhibit either tetrasomic or disomic inheritance, depending on the locus examined (May et al. 1997, Pyatskowit et al. 2001). The Persian sturgeon genome is believed to be tetraploid, but is also in the process of undergoing functional genome reduction (Ludwig et al. 2001).

A variety of methods have been used to develop primers for single-locus DNA microsatellite markers in various species (Kumar et al. 2002). In an earlier attempt to develop disomic microsatellite markers for Persian sturgeon, we examined cross-specific amplification (Barker et al. 1997) using 56 sets of microsatellite primers developed for other *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). Recently in a continuation of our program, we developed and tested 68 microsatellite primer pairs from an enriched Persian sturgeon microsatellite library (Moghim et al. 2012). While none of the markers exhibited disomic inheritance in Persian or Russian *A. gueldenstaedtii* sturgeon, several loci showed promise in the stellate sturgeon *A. stellatus*, ship sturgeon *A. nudiventris*, and beluga *Huso huso*.

Disomic microsatellite loci were successfully developed in some other sturgeon species that have high ploidy levels (e.g., white *A. transmontanus*, green *A. medirostris*, and lake sturgeon, *A. fulvescens*) although the majority of loci identified were polysomic. For example, Welsh and May (2006) found that only nine 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 tested cross-specific amplification of 38 microsatellite primer pairs developed in lake and Atlantic sturgeon *A. oxyrinchus*, and identified 11 disomic microsatellite loci for Persian sturgeon (Moghim et al. 2013, in press). Assumptions regarding the inheritance of individual loci need to be tested, however, before inferences based on these loci can be made.

The objective of the present study was to determine the inheritance patterns of 11 newly developed microsatellite markers to ascertain their usefulness as disomic markers in genetic studies of Persian sturgeon *A. persicus*.

MATERIALS AND METHODS

Origin and maintenance of brood stock and production of offspring

Overall, 2 families were produced for the study by crosses made between 2 mature female and 2 mature male individuals. Crosses were performed at the Shahid Rejaei Sturgeon Hatchery (Sari, Iran) during the reproductive season from Mar. to July 2008. Fin tissue samples were collected from the 2 male and 2 female Persian sturgeon broodstock individuals, and sperm from a single male mixed with eggs was harvested from a single female. After hatching, larvae from each mating were collected and preserved for genotyping.

In total, 23 and 28 larvae were sampled from females A and B, respectively. Fin tissues were taken from larval individuals, and samples were preserved in 96% ethanol. Subsequently, they were used for parental and F1 progeny genotyping utilizing 11 microsatellite loci to determine the individual modes of mendelian segregation.

Molecular study

DNA extraction

Total DNA was isolated from each sample using a Qiagen DNeasy tissue Kit (QIAgene, Valencia, CA, USA). Extracted genomic DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and samples were stored at -20°C until used for the microsatellite genotyping analysis.

PCR amplification and genotyping

Ten microsatellite primer pairs developed in lake sturgeon (AfuGs 56, 63, 68b, 112, 160, 195, 204, 229, and 241 (Welsh et al. 2003, Welsh and May 2006), and LS-68 (May et al. 1997) and one in Atlantic sturgeon, Aox27 (King et al. 2001)) were used for amplification of 11 microsatellite loci named *AfuGs 56*, *63*, *68b*, *112*, *160*, *195*, *204*, *229*, *241*, *LS-68*, *LS-68-1*, and *Aox27* (the LS-68 primer pair amplified 2 loci named *LS-68* and *LS-68-1*). Table 1 shows the primer sequences and annealing temperatures used in this study.

Table 1. Primer pair sequences and annealing temperatures used in this study

Locus name	Sequence	Motif	Annealing temperature	GenBank accession no.
AfuG68B	F: AACAATATGCAACTCAGCATAA R: AGCCCAACACAGACAATATC	$(GATA)_{28}$	60	AFU72739
AfuG63	F: TCCTGGCTAGCGAACGAA R: CTTTTAAATGGGGGACAGACTAT	$(AAAC)_8$	60	AF529475
AfuG56	F: ACTAAACCCAGCACAGAAAATCAG R: GAAGCCCATCCCACAGGTT	$(AAAC)_9$	touch down	AF529472
AfuG112	F: TATTGTTCCTTTATGGTTATG R: TATTTCACTGTCTGTTGTATGTA	$(GATA)_{19}$	51	AF529499
AfuG195	F: ATTCCTCCAGCCGTATTATTA R: AAGCAGTTAGTTTATGTGGTTGTG	$(AAAC)_7$	59	AF529548
AfuG229	F: AGAGAATGCGGAGAATGAGGAC R: GCACAGATACACGCAGACAAACA	$(CA)_{14}$	67	AF529559
AfuG160	F: CCGCAGCATTAGGTCAAA R: CCCCAGTGGAAATAATAATGTA	$(AAAC)_8$	59	AF529526
AfuG241	F: CAGAACATGCCGGGTGAGTA R: ATCCAGGGCTTGTCTTGTATTTTA	$(CA)_{13}$	65	AF529566
LS-68	F: TTA TTG CAT GGT GTA GCT AAA C R: AGC CCA ACA CAG ACA ATA TC	$(GATA)_{13}$	54	U72739
Aox27	F: AATAACAATAACGGCAGAACCT R: TGTGTTGCTCAAGACAGTATGA	$(ATTT)_5(ATTC)(ATTT)^3$	53	AF067812

Twenty-microliter polymerase chain reactions (PCRs) contained approximately 1-10 ng genomic DNA, 0.15 units Taq DNA polymerase, 1 mM of each primer, 200 mM of each dNTP, 1.75 mM $MgCl_2$, and 1 × PCR buffer. Amplification was conducted using a Quanta Biotec master cycler gradient Thermocycler (Quanta Biotech, Surrey, UK).

PCR conditions were as follows: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 52-67°C for 30 s, and 72°C for 45 s, with a final elongation at 72°C for 5 min.

PCR products were suspended 1:1 in 98% formamide/loading dye, denatured at 95°C for 5 min, separated in a 6% denaturing polyacrylamide gel on a Bio-Rad SequiGen Sequencing Cell-system with a gel size of 38 × 30 cm, and electrophoresed at 70 W for 45-60 min. DNA bands were visualized using a silver staining method (An et al. 2009). Amplified fragments were sized by comparing migration against a 50-bp DNA Step Ladder (Promega, Madison, WI, USA). Alleles were visualized and scored using UVIDoc Mw vers. 99.04 software (UVItech, Cambridge, UK).

Statistical analysis

Statistical testing for conformity of the presumptive disomic loci to a model of disomic mendelian inheritance was employed using a Chi-squared test (α = 0.05). Segregation of microsatellite loci was expected to be in a 1:1 ratio for markers heterozygous in only one of the parents; 1:2:1 when both parents were heterozygous for the same 2 alleles, and 1:1:1:1 when both parents were heterozygous for 4 different alleles. The goodness of fit to expected mendelian inheritance ratios was determined by a χ^2 analysis (Griffiths et al. 2000). The null hypothesis employed here was that segregating alleles did not deviate from a mendelian inheritance ratio at p = 0.05.

The program Cervus 3.0 software (available from http://www.fieldgenetics.com) was used to estimate the pedigree error, identity test (parentage control), and indirectly the mendelian heredity of parental alleles to offspring in the next generation.

RESULTS

Identification and parentage confirmation of offspring

In the present study, we examined the mode of inheritance of 11 microsatellite loci in 2 families with a cross of 1 female × 1 male. The allele number, the frequency of the most frequent allele (F(MFA)) the polymorphic information content (PIC), the most frequent allele size per locus, and the probably of non-exclusion of a parental pair in Persian sturgeon are shown in table 2. All 11 markers examined were variable among the parents. From 1 to 8 unique alleles were observed at each locus screened.

All loci were segregated according to mendelian expectations (p = 0.05). At the *AfuGs 229*, *241*, and *Aox27* loci, parents of family B were homozygotes for the same allele, and all progeny showed phenotypic banding patterns identical to those seen in the parents. Nine of

Table 2. Summary of statistics (no. of alleles, the frequency of the most frequent allele F(MFA) polymorphic information content (PIC), most frequent allele size per locus, and probably of non-exclusion (PE) for parental pairs in the Persian sturgeon

Locus ID	No. of alleles	F(MFA)	Most frequent allele size (bp)	PIC	PE (parent pair)
AfuG68	8	0.1646	191	0.8576	0.8265
AfuG56	3	0.4824	254	0.5047	0.9042
AfuG195	3	0.6569	161	0.3959	0.9821
AfuG195	4	0.5050	135	0.6182	0.7500
AfuG63	4	0.3186	139	0.6741	0.7153
AfuG241	4	0.2474	256	0.6542	0.8116
AfuG112	6	0.3669	260	0.7773	0.8184
AfuG229	3	0.1766	318	0.5338	0.8571
LS68	7	0.1642	216	0.8309	0.7823
LS68-1	6	0.1548	148	0.7479	0.7837
Aox27	4	0.2666	147	0.5775	0.8265

the 11 microsatellite loci studied showed disomic inheritance and mendelian segregation of their alleles, while the remaining 2 loci, *AfuG195* and *LS-68-1*, showed evidence of the presence of null alleles.

Figure 1 shows allelic patterns at the *AfuG56* locus in family A and those of locus *Afu112* in family B. As can be seen, locus *AfuG56* in family A was a cross between 1 parent that was a heterozygote and a homozygous parent that produced the expected 1:1 ratio in the offspring (top photo), while locus *AfuG112* in family B was a cross of between 2 parents heterozygous for the same alleles that produced the expected 1:1:1:1 ratio in their offspring (bottom photo). Alleles identified at all microsatellite loci studied, parental genotypes, and their allelic segregation in the progeny are presented in table 3.

Locus *AfuG195* in family A involved a cross between a heterozygous female (173/161) and a phenotypically homozygous (161) male parent. The expected genotypic ratio in the progeny was 1:1 with (173/161) and (161/161) genotypes if the male parent had been a true (161/161) homozygote. Since 6 offspring showed the (173) phenotype, the male parent must have been heterozygous for a null allele (0). These results suggest the presence of null alleles, and hence the true genotype at this locus must have been (161/0) rather than (161/161).

Similarly, at locus *LS-68-1* in family B, a null allele was also inferred. This involved a cross between a homozygous female (148/0) and a male heterozygote (132/140) parent. In this cross, the parents shared 4 different alleles. The expected genotypic ratio in the progeny was 1:1:1:1 (Table 3).

DISCUSSION

Microsatellite DNA analyses can be a powerful tool for assigning parentage and for linkage studies. While analysis of microsatellite data in population and evolutionary genetic studies is based on assumptions of selective neutrality and mendelian inheritance, validation of these assumptions is critical for the Persian sturgeon, because it has an ancestral polyploid character state (Birstein et al. 1997). Testing for the presence of null alleles, the level of duplication, and the actual mode of inheritance can be confirmed via inheritance studies in families of known

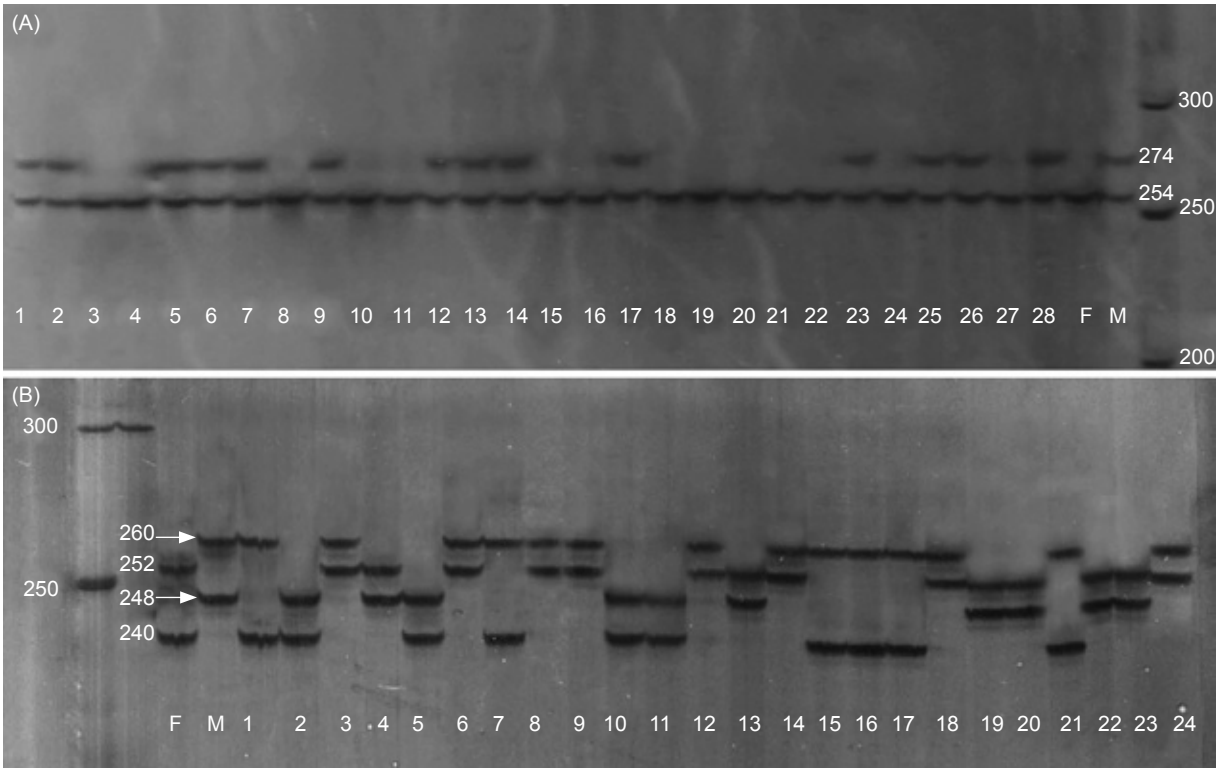

Fig. 1. Inheritance pattern of *AfuG56* in family A (upper photo) and *Afu112* in family B (lower photo). Lanes F and M are female and male parents, respectively. Numbers represent the offspring. Allele sizes are presented against a 50-bp DNA ladder.

parentage.

In the present study, the inheritance of 11 polymorphic (disomic) microsatellite loci was examined in 2 families. All loci showed a disomic banding pattern and were confirmed to have been inherited in a mendelian fashion. These microsatellite primer pairs were initially developed for lake and Atlantic sturgeons and were cross-species amplified in Persian sturgeon.

The inheritance of allelic variations at these loci had to specifically be tested in the Persian sturgeon which is known to be a tetraploid species (Birstein et al. 1997, Ludwig et al. 2001). Without this study, these loci might not be beneficial as disomic loci for future genetic studies, especially for investigations of population structures.

Null alleles are often not revealed during population studies, but inheritance studies can readily confirm their presence (Van Oosterhaut et al. 2004). Results of the current study indicated the presence of null alleles in 2 of the loci screened. Pyatskowit et al. (2001) and McQuown et al. (2002) studied the inheritance of microsatellite loci in lake sturgeon and also reported null alleles at some loci.

Potential applications of the results of the current study include the use of the 11 disomic loci as anchor markers in constructing a genetic linkage map for *A. persicus*. Genetic linkage maps are important tools for identifying and localizing quantitative trait loci (QTLs) in new species. To maximize genome coverage and provide an evenly spaced marker distribution, a combination of different types of genetic markers is often used. It is essential, however, to ensure that all markers used in any future genetic map developed for *A. persicus* show mendelian modes of inheritance.

The average non-exclusion probability is a measure of the efficiency of parental testing; it refers to the prior ability of tests to detect parental inconsistencies. This parameter measures the capacity of a system to detect a false accusation of parentage. Traditionally, this average exclusion probability was estimated as the probability of excluding a male who was not the parent by an inconsistency in at least one of the studied loci.

In a classical analysis of genetic relationships, one of the useful parameters is the power of

Table 3. Chi-square test for mendelian segregation of 11 disomic loci with observed progeny genotypes and number of progeny. Chi-square values with a significance level of 0.05 are shown with the degrees of freedom

Locus	Family	Genotype		Observed progeny genotypes				Expected ratio	No.	χ^2	*p* value
		Female	Male								
AfuG63	A	147, 147	151, 143	147, 143 (7)	147, 151 (16)			1: 1	23	2.843 (1)	0.092
	B	139, 143	139, 139	139, 139 (12)	139, 143 (13)			1: 1	25	0.40 (1)	0.841
AfuG56	A	274, 270	274, 254	274, 274 (7)	274, 254 (6)	270, 274 (7)	270, 254 (4)	1: 1: 1: 1	24	1.00 (3)	0.801
	B	254, 254	254, 274	254, 254 (14)	254, 274 (14)			1: 1	28	[a]	
AfuG195	A	173, 161	161, 0	173, 161 (5)	161, 161& 161, 0 (12)	173, 0 (6)		1: 2: 1	23	0.130 (2)	0.932
	B	157, 161	161, 161	157, 161 (12)	161, 161 (16)			1: 1	28	0.571 (1)	0.450
LS-68	A	252, 220	240, 184	252, 240 (5)	252, 184 (5)	240, 220 (5)	220, 184 (8)	1: 1: 1: 1	23	2.56 (3)	0.464
	B	216, 240	212, 256	216, 212 (8)	216, 256 (7)	240, 212 (7)	240, 256 (5)	1: 1: 1: 1	27	0.704 (3)	0.872
LS-68-1	A	152, 132	128, 112	152, 128 (6)	152, 112 (7)	132, 128 (4)	132, 112 (6)	1: 1: 1: 1	23	0.83 (3)	0.843
	B	148, 0	132, 140	148, 132 (11)	148, 140 (3)	140, 0 (4)	132, 0 (9)	1: 1: 1: 1	27	6.63 (3)	0.104
AfuG229	A	332, 324	332, 324	332, 332 (11)	332, 324 (7)	324, 324 (6)		1: 2: 1	23	5.414 (2)	0.067
	B	318, 318	318, 318								
AfuG112	A	252, 240	260, 248	252, 260 (8)	252, 248 (6)	240, 260 (6)	240, 248 (4)	1: 1: 1: 1	24	1.522 (3)	0.677
	B	244, 248	260, 264	244, 260 (9)	244, 264 (4)	248, 260 (7)	248, 264 (5)	1: 1: 1: 1	25	2.360 (3)	0.510
AfuG160	A	143, 143	147, 135	143, 147 (10)	143, 135 (13)			1: 1	23	0.669 (1)	0.413
	B	135, 135	135, 143	135, 135 (13)	135, 143 (13)			-	26	[a]	
Aox27	A	146, 142	146, 138	146, 146 (7)	146, 138 (7)	142, 146 (5)	142, 138 (5)	1: 1: 1: 1	24	1.174 (3)	0.759
	B	146, 146	146, 146	-	-	-	-	-	28	-	
AfuG68b	A	219, 187	207, 151	219, 207 (5)	219, 151 (5)	187, 207 (5)	187, 151 (8)	1: 1: 1: 1	23	1.174 (3)	0.759
	B	195, 215	191, 235	195, 191 (9)	195, 235 (7)	215, 191 (7)	215, 235 (5)	1: 1: 1: 1	28	1.14 (3)	0.767
AfuG241	A	264, 256	256, 256	264, 256 (10)	256, 256 (13)			1: 1	23	0.39 (1)	0.532
	B	240, 252	240, 252	240, 240 (6)	240, 252 (10)	252, 252 (11)		1: 2: 1	27	3.67 (2)	0.160

[a]Observed and expected genotypes were exactly the same, and the test could not be performed.

exclusion i.e., the power of a genetic marker to exclude a non-related individual chosen by chance in a specific population, as an alleged parent in a pedigree investigation. The parental PE is the expected average probability that a polymorphic locus excludes a man without kinship with the biological parents. This index depends on the informative content of a locus, which depends on its number of alleles and its respective frequencies. From the probabilities of exclusion of several loci, it is possible to calculate the combined PE (PEC), by simply multiplying values for each locus. The value of the PEC is a function of the number of examined loci, as well as of the informative content of each locus. Knowledge of the PE and PEC can define loci to be used in an analysis of genetic relationships. Genetic parental testing can provide sire identity data for offspring when females have been exposed to multiple males. However, correct parental assignment can be influenced by factors determined in the laboratory and by the size and genetic composition of breeding groups.

CONCLUSIONS

The present study verified mendelian inheritance and disomic segregation in 11 SSR loci investigated in the polyploid Persian sturgeon *Acipenser persicus*. Furthermore, exact determination of the parentage of larvae allows a precise estimate of the relative individual reproductive success of broodstock adults and dynamics of the genetic structure of natural populations. In conclusion, the microsatellite markers developed and characterized herein open a new perspective for generating fundamental data to devise sound conservation strategies for the polyploid Persian sturgeon *A. persicus* and will assist in wild stock enhancement programs for the species in the Caspian Sea in Iran. The present findings attest to the usefulness of the investigated microsatellites for parentage control in Persian sturgeon.

Acknowledgments: This research was funded by the Iranian Fisheries Research Organization and carried out in the Fish Genetics Laboratory of the Caspian Sea Ecology Research Center (Sari, Iran). We thank D. Kor who provided help with specimen collection, Prof. P. Mather (QUT) and Prof. E.J. Heist (SIUC) for helpful comments on the manuscript.

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