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Searching the genome of beluga (*Huso huso*) for sex markers based on targeted Bulked Segregant Analysis (BSA)

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ABSTRACT
In sturgeon aquaculture, where the main purpose is caviar production, a reliable method is needed to separate fish according to gender. Currently, due to the lack of external sexual dimorphism, the fish are sexed by an invasive surgical examination of the gonads. Development of a non-invasive procedure for sexing fish based on genetic markers is of special interest. In the present study we employed Bulked Segregant Analysis (BSA) methodology to search for DNA markers associated with the sex of the beluga sturgeon (*Huso huso*). DNA bulks (male and female) were created by combining equal amounts of genomic DNA from 10 fish of both sexes. A total of 101 decamer primers associated with the sex-specific sequences in non-sturgeon species was used for targeted screening of the bulks, resulting in 2846 bands that all of them were present in both sexes. Our results showed that sex chromosomes are weakly differentiated in the sturgeon genome and comprised sequences not complementary to the sex-specific primers in non-sturgeon species.

Keywords: *Beluga, Huso huso, sex marker, genetics, bulked segregant analysis (BSA)*

INTRODUCTION
The extant sturgeon species (family Acipenseridae) are considered to be one of the most primitive groups of fishes that evolved approximately 250 million years ago (Bemis *et al.*, 1997). Six sturgeon species, belonging to two genera (*Huso* and *Acipenser*), are found in the Caspian Sea and its drainage basin which provide today the bulk of the world's caviar yield (Pourkazemi, 2006; Nasrollahzadeh, 2010). Sturgeons mature very late in life and their populations are declining worldwide caused by overfishing as well as pollution and habitat degradation (Birstein, 1993; Billard & Lecointre 2000). Beluga sturgeon (*Huso huso*) is regarded as one of the most important commercially species in the Caspian Sea and has been overfished nearly to extinction in pursuit of their caviar (Pourkazemi, 2006). Nowadays, production of sturgeon for both meat and caviar will increasingly have to rely on aquaculture (Logan *et al.*, 1995; Keyvanshokooh & Gharaei, 2010). In sturgeon aquaculture, where the main purpose is caviar production, a reliable method is needed to separate fish according to gender. Males are destined to the meat market while females remain in culture for many more years under conditions of optimal growth and development. The availability of monosex populations of caviar-producing females would significantly enhance the economic viability of domestic caviar production systems (Logan *et al.*, 1995).
None of the sturgeon species exhibit external sexual dimorphism, and it is not possible to distinguish male fish from

females by morphological markers at larval, juvenile and even adult stages (Keyvanshokooh *et al.*, 2009). Blood plasma sex steroid levels in sturgeon remain low until the beginning of gonadal development (Doroshov *et al.*, 1997). Although an examination of plasma steroids could be used to sex sturgeon (Webb *et al.*, 2002), these steroid indicators are influenced by age, husbandry conditions and water temperature (Feist *et al.*, 2004). Currently, sturgeon producers wait 3-4 years before fish are sexed by an invasive surgical examination of the gonads (Doroshov *et al.*, 1997). Although survival rate is nearly 100% (Feist *et al.*, 2004), the development of a non-invasive procedure for sexing sturgeon is of special interest. One effective solution is to use DNA markers to diagnose the sex. Such markers will be present in species where one sex possesses a unique chromosome or DNA sequence (Griffith & Tiwari, 1993; Devlin & Nagahama, 2002; Keyvanshokooh & Gharaei, 2010).

Regarding the failure of randomly screening methodologies to find a sex-specific marker in various sturgeon species (Wuertz *et al.*, 2006; Keyvanshokooh *et al.*, 2007; McCormick *et al.*, 2008; Yarmohammadi *et al.*, 2011), we conducted a targeted search based on previously identified sex-specific sequences in non-sturgeon species to compare male and female DNA. The search for sex-specific sequences in beluga sturgeon was performed using bulked segregant analysis (BSA) methodology in conjunction withthe random amplified polymorphic DNA (RAPD) assay. BSA is based on grouping together of individuals that share a common trait and studying the genomic regions related with that trait against a randomized background of unlinked loci (Michelmore *et al.*, 1991). This approach has been used in identifying sex-specific sequences in some species (Griffith &Tiwari, 1993; Iturra *et al.*, 1998; Kovacs *et al.*, 2001).

MATERIALS AND METHODS

Fish Sampling and DNA Extraction

Fin tissue samples were obtained from 10 adult beluga sturgeon of each sex. The fish were caught as broodstock from the Iranian Caspian Sea coastline and transferred to the Shahid Dr. Beheshti Sturgeon Fish Propagation and Rearing Complex, Rasht, Iran. Sex identification was carried out by observation of testes and ovaries of necropsied spawners.

The CTAB method was used to obtain genomic DNA. The quantity and quality of extracted DNA was assayed using a spectrophotometer and 1% agarose electrophoresis. Two DNA pools (male and female) were created by combining equal amounts of genomic DNA from each fish.

Polymerase Chain Reaction (PCR) and Electrophoresis

BSA methodology was employed in conjunction with the RAPD assay to screen genetic markers associated with the sex of beluga sturgeon. Searching through databases for sex-specific sequences in non-sturgeon species (including animal and plant species), a total of 101 RAPD primers (Metabion, Germany) was found (Table 1) and was used for targeted amplifications.

Amplifications were performed in 20-µl reaction volumes containing 15 ng of DNA, 0.5 µM primer, 400 µM each of dNTPs, 1 unit *Taq* polymerase (Cinnagen, Iran), 1X PCR buffer, and 1.5 mM $MgCl_2$. PCR consisted of 3 min denaturation at 94°C, followed by 30 cycles of 30 sec at 94°C, 30 sec at 40°C, and 30 sec at 72°C, with a final extension at 72°C for 5 min. PCR products were separated and analyzed in gels of 6% polyacrylamide stained with silver nitrate (Keyvanshokooh *et al.*, 2007).

RESULTS AND DISCUSSION

A set of 101 RAPD primers yielded a total of 2486 scoreable bands that were present in both sexes. Only two primers (primers no. 29 and 82; Table 1) produced different band patterns on pools; each primer produced a band that was present only in the female pool. Following reconfirmation of the bulk polymorphism, the individual DNA samples used to create both bulks were screened using the primer no. 29 and no. 82. The polymorphic bands produced by the primers were found in one of the 10 fishes from both sexes.

We were unable to identify a sex-specific marker in beluga sturgeon associated with previously identified sex-specific sequences in non-sturgeon species using

BSA methodology. Using RAPD, AFLP (amplified fragment length polymerphism), and ISSR (inter-simple sequence repeats) techniques, Wuertz *et al.* (2006) focused on the identification of genomic sex-specific markers in four sturgeon species (*A. baerii*; *A. naccarii*; *A. gueldenstaedtii* and *A. ruthenus*). Although 1100-9230 bands screened per species, no sex-specific markers were detected. Similar result has been obtained by searching the genome of beluga sturgeon (Keyvanshokooh *et al.*, 2007) using bulked segregant analysis (BSA; separate pooling of DNA from males and females). They used a total of 310 randomly amplified polymorphic DNA primers to screen the bulks, resulting in 4146 bands that were present in both sexes. Using the RAPD technique, McCormick *et al.* (2008) did also failed to find a sex-specific marker in lake sturgeon (*Acipenser fulvescens*). Searching the genome of the Persian sturgeon (*Acipenser persicus*) and beluga by using AFLP, Yarmohammadi *et al.* (2011) also observed no sex-specific sequence. With regard to these failures, McCormick *et al.* (2008) mentioned that an environmental sex-determining system may exist in sturgeon. In theory, the lack of sex-specific markers in the search could be due to the lack of genetic sex-determining mechanisms. Although heteromorphic sex chromosomes have not been identified in sturgeon (Fontana & Colombo, 1974; Van Eenennaam *et al.*, 1998), but we do know that a female heterogametic genetic sex determination is in operation in beluga (Omoto *et al.*, 2005) and some other sturgeon species studied to date (Van Eenennaam *et al.*, 1999; Flynn *et al.*, 2005; Fopp-Bayat, 2010). Based on this proved assumption, the female sturgeon should carry sex-specific DNA sequences. Moreover, hermaphroditism in sturgeon is very infrequently observed (Chapman *et al.*, 1996; Van Eenennaam & Doroshov 1998; Harshbarger *et al.*, 2000) and the sex ratio in adult populations of sturgeon is 1 ♂: 1 ♀ (Chapman *et al.*, 1996). Environmental sex determination produces variations in sex ratios when there are systematic fluctuations in the environmental factors influencing sex (Penm an & Piferrer, 2008). In fact, the failure in search for this class of DNA markers could be due to the size of genome, the number of markers screened, and the proportion of the genome that is sex-specific in species studied (Keyvanshokooh *et al.*, 2007; Penman & Piferrer, 2008).

Of the 2846 bands amplified using the primers which were sex-specific in other species, none were linked to a sex-determining gene in beluga sturgeon. One general approach to identify sex-specific DNA markers is based on candidate genes, where genes or sequences that are sex-determining or sex-linked in one species are searched for in the target species. Members of the Sox gene family are known to be involved in numerous developmental processes and sex determination in vertebrates (Koopman *et al.*, 1991; Wright *et al.*, 1993; Russel *et al.*, 1996). Sox proteins are characterized by a conserved high mobility group (HMG)-box domain, which is responsible for DNA binding and bending (Sinclair *et al.*, 1990). Based on this approach and using highly degenerate primers that amplified a broad range of HMG boxes, 22 different sequences coding for 8 Sox genes (Sox2, Sox3, Sox4, Sox9, Sox11, Sox17, Sox19, and Sox21) were shown to be present in the genome of European Atlantic sturgeon (*Acipenser sturio*) (Hett & Ludwig, 2005; Hett *et al.*, 2005). Similarly, sequences with homology to Sox gene family (Sox2, Sox4, Sox17, and Sox21) were detected in lake sturgeon (*A. fulvescens*) (McCormick *et al.*, 2008). However, although Sox genes were found in the genomes of *A. sturio* and *A. fulvescens*, none were associated with the sex sequences in any of these species. Regarding the afforementioned studies and our results, it seems that sex-specific DNA present in beluga sturgeon may be comprised sequences which are not conserved and complementary to sex-specific genes in other species.

In conclusion, targeted screening of beluga sturgeon genome based on primers which were sex-specific in non-sturgeon species failed to detect sex-specific sequences. With regard to failure in search for this class of DNA markers, it is proposed that sex chromosomes are weakly differentiated in the sturgeon genome. With recent advances in genomic and proteomic approaches, gene expression profiling could be considered as an alternative approach (Wuertz *et al.*, 2006).

For example, regarding the great potential of next-generation sequencing to rapidly identify genes of interest in sturgeon (Hale *et al.*, 2009), this approach could be used in search for sturgeon sex markers.

Table 1 Sex-specific primers of non-sturgeon species used for targeted screening of sex-linked sequences in beluga sturgeon

No.	Sequence (3′→5′)	Species	Reference	No.	Sequence (3′→5′)	Species	Reference
1	AGGTGACCGT	*Gracilaria changii*	Sim *et al.*, 2007	52	CTGCTGGGAC	*Ginkgo biloba*	Liao *et al.*, 2009
2	CAATCGCCGT	*Oncorhynchus mykiss*	Iturra *et al.*, 1998	53	TGAGCGGACA	*Cannabis sativa*	Torjek *et al.*, 2002
3	GTGGTCCGCA	*Oncorhynchus mykiss*	Iturra *et al.*, 1998	54	TCGTCGAAGG	*Spilornis cheelahoya*	Hsu *et al.*, 2009
4	GGCTATAGGG	*Eucommia ulmoides*	Xu *et al.*, 2004	55	TTGCTCACGG	pigs	Horng & Huang, 2003
5	GAGACGCACA	*Commiphora wightii*	Samantaray *et al.*, 2010	56	GTTGCGATCC	*Brugia malayi*	Underwood & Bianco, 1999
6	AAGCGACCTG	*Commiphora wightii*	Samantaray *et al.*, 2010	57	CAGGCCCTTC	*Actinidia deliciosa* var. *deliciosa*	Shirkot *et al.*, 2002
7	GTTGCGATCC	*Commiphora wightii*	Samantaray *et al.*, 2010	58	AATCGGGCTG	*Actinidia deliciosa* var. *deliciosa*	Shirkot *et al.*, 2002
8	CATAATCAAC	*Actinidia chinensis*	Gill *et al.*, 1998	59	AGCCAGCGAA	*Actinidia deliciosa*var. *deliciosa*	Shirkot *et al.*, 2002
9	TCGCAATTCG	*Actinidiachinensis*	Gill *et al.*, 1998	60	CTCACGTTGG	*Actinidia deliciosa* var. *deliciosa*	Shirkot *et al.*, 2002
10	ACTTCGCCAC	*Hippophae rhamnoides*	Sharma *et al.*, 2010	61	CCCAAGGTCC	*Psetta maxima*	Casas *et al.*, 2011
11	ACGCGAACCT	*Paramisgurnus dabryanus*	Xia *et al.*, 2011	62	GGAAGCCAAC	*Bryconama zonicus*	Da Silva *et al.*, 2012
12	CTCGAACCCC	*Streptopelia orientalis*	Wu *et al.*, 2007	63	CTGAGACGGA	*Simmondsia chinensis*	Agrawal *et al.*, 2007
13	CACACTCCAG	*Bubalus bubalis*	Horng *et al.*, 2004	64	TAGCGTCGAC	Iranian river buffalo	Shokrollahi & Aryapour, 2011
14	GCACCGAGAG	*Carica papaya*	Lemos *et al.*, 2002	65	TTGGTACCCC	*Hippophae salicifolia*	Rana *et al.*, 2009
15	CACCATCGTG	*Cyprinus carpio*	Chen *et al.*, 2009	66	CTAGAGGCCG	*Salix viminalis* L	Alstrom-Rapaport *et al.*, 1998
16	GATGACCGCC	*Actinidia deliciosa* var. *deliciosa*	Shirkot *et al.*, 2002	67	CCGCATCTAC	*Bombyx mori*	Abe *et al.*, 1998
17	TGCGTGCTTG	*Oreochromis niloticus*	Bardakci, 2000	68	TGTGGACTGG	*Bombyx mori*	Abe *et al.*, 1998
18	GGTCCCTGAC	*Borassus flabellifer*	George *et al.*, 2007	69	CCAGAACGGA	*Bomby xmori*	Abe *et al.*, 1998
19	TGATCCCTGG	*Ginkgo biloba*	Longdou *et al.*, 2006	70	CGCGTGCCAG	*Bombyxmori*	Abe *et al.*, 1998
20	GTGAGGCGTC	*Actinidia kolomikta*	Cesoniene *et al.*, 2007	71	GGTGCGCACT	*Atriplex garrettii.*	Ruas *et al.*, 1998
21	CAAACGTCGG	*Actinidia kolomikta*	Cesoniene *et al.*, 2007	72	TGGGGGACTC	*Cannabis sativa*	Sakamoto *et al.*, 2005
22	TTGGCACGGG	*Carica papaya*	Urasaki *et al.*, 2002	73	CCTTGACGCA	*Cannabis sativa*	Sakamoto *et al.*, 2005
23	AGGAGTCGGA	*Simmondsia chinensis*	Hosseini *et al.*, 2011	74	CCACAGCAGT	*Cannabis sativa*	Sakamoto *et al.*, 2005

24	GGGCCACTCA	*Simmondsia chinensis*	Hosseini *et al*., 2011	75	AACGGTGACC	*Cannabis sativa*	Sakamoto *et al*., 2005
25	GTCCCGACGA	*Trichosanthes dioica*	Singh *et al*., 2002	76	CCTGATCACC	*Cannabis sativa*	Sakamoto *et al*., 2005
26	GAAACGGGTG	*Acer negundo*	Linsen *et al*., 1999	77	TGAGCCTCAC	*Humulus lupulus*	Polley *et al*., 1997
27	GTGACGTAGG	*Acer negundo*	Linsen *et al*., 1999	78	GGCGAAGGTT	*Humulus lupulus*	Polley *et al*., 1997
28	CAGCACCCAC	*Acer negundo*	Linsen *et al*., 1999	79	CGACCAGAGC	*Gracilaria lemaneiformis*	Xiang-feng *et al*., 1998
29	CAAACGTCGG	*Acer negundo*	Linsen *et al*., 1999	80	CCGGCCTTAG	*Gracilaria lemaneiformis*	Xiang-feng *et al*., 1998
30	GTTGCGATCC	*Acer negundo*	Linsen *et al*., 1999	81	TTCCCCGCGC	*Gracilaria lemaneiformis*	Xiang-feng *et al*., 1998
31	GGACTGGAGT	*Acer negundo*	Linsen *et al*., 1999	82	TTCCCCGACC	*Gracilaria lemaneiformis*	Xiang-feng *et al*., 1998
32	TGCGCCCTTC	*Acer negundo*	Linsen *et al*., 1999	83	GTGATCGCAG	*Phoenix dactylifera* L.	Younis *et al*., 2008
33	CATCCGTGCT	*Hippophae rhamnoides*	Persson & Nybom, 1998	84	TCGGCGATAG	*Phoenixda ctylifera* L.	Younis *et al*., 2008
34	TGTCATCCCC	*Piper longum*	Banerjee *et al*., 1999	85	GGTCTACACC	*Phoenix dactylifera* L.	Younis *et al*., 2008
35	GGGTAACGCC	*Silenedioica*	Di Stilio *et al*., 1998	86	CATCCCCCTG	*Zamia fischeri* Miq.	Roy *et al*., 2012
36	AACGCGTCGG	*Leporinusmacrocephalus*	Alves-Costa & Wasko, 2010	87	GGACTGGAGT	*Zamia fischeri*Miq.	Roy *et al*., 2012
37	TTGGTACCCC	*Grusamericana*	Duan & Fuerst, 2001	88	TTCACGGTGG	*Asparagus officinalis*	Ii *et al*., 2012
38	ACTTCGCCAC	pigeons	Horng *et al*., 2006	89	CTGGCTCAGA	*Salixviminalis* L.	Gunter *et al*., 2003
39	GTTTCGCTCC	*Mercuria lisannua*	Khadka *et al*., 2002	90	GTCCACACGG	*Oreochromis niloticus*	Bardakci, 2000
40	GACGGATCAG	asparagus	Jiang & Sink, 1997	91	CCACAGCAGT	*Oreochromis niloticus*	Bardakci, 2000
41	GTGACGTAGG	*Piper betle*	Samantaray *et al*., 2012	92	CAGCACCCAC	*Oreochromis niloticus*	Bardakci, 2000
42	AATCGGGCTG	*Piper betle*	Samantaray *et al*., 2012	93	AAAGCTGCGG	*Oreochromis niloticus*	Bardakci, 2000
43	ACCAGGGGCA	*Piper betle*	Samantaray *et al*., 2012	94	TGAGTGGGTG	*Oreochromis niloticus*	Bardakci, 2000
44	GAACGGACTC	*Piper betle*	Samantaray *et al*., 2012	95	TGCGAGAGTC	*Carica papaya*	Reddy *et al*., 2012
45	GCCTGATTGC	*Pistacia* species	Esfandiyari *et al*., 2012	96	GGGCGGTACT	*Carica papaya*	Reddy *et al*., 2012
46	GAAACGGGTG	*Gracilaria changii*	Sim *et al*., 2007	97	ACCGCCTGCT	*Carica papaya*	Reddy *et al*., 2012
47	TCCGCTCTGG	*Gracilaria changii*	Sim *et al*., 2007	98	AGCCTGAGCC	*Carica papaya*	Reddy *et al*., 2012
48	TTCGAGCCAG	*Gracilaria changii*	Sim *et al*., 2007	99	GGGCCACTCA	*Caricapapaya* L.	Deputy *et al*., 2002
49	CCGCATCTAC	*Gracilaria changii*	Sim *et al*., 2007	100	GGGTGTGTAG	*Caricapapaya* L.	Deputy *et al*., 2002
50	GACGGATCAG	*Gracilaria changii*	Sim *et al*., 2007	101	CTGATGCGTG	*Caricapapaya* L.	Deputy *et al*., 2002
51	CACACTCCAG	*Gracilaria changii*	Sim *et al*., 2007				

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کاوش هدفمند ژنوم فیلماهی (*Husohuso*) به منظور کشف نشانگرهای جنسی با استفاده از روش BSA

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چکیده

در تکثیر و پرورش ماهیان خاویاری که هدف عمده تولید خاویار می باشد، وجود یک روش قابل اعتماد به منظور جداسازی افراد نر و ماده ضروری است. در حال حاضر به دلیل عدم وجود صفات خارجی وابسته به جنس در ماهیان خاویاری، تشخیص جنسیت با استفاده از روش تهاجمی تکه برداری از گنادها انجام می شود. بنابراین معرفی یک روش غیر تهاجمی و غیر تنش زا برای تشخیص جنسیت ماهیان خاویاری بر اساس نشانگرهای ژنتیکی، دارای اهمیت است. در این مطالعه، امکان کشف نشانگرهای ژنتیکی وابسته به جنس در فیلماهی با استفاده از روش BSA (Bulked Segregant Analysis) مورد آزمون قرار گرفت. به همین منظور، دو خزانه DNA مربوط به افراد نر و ماده از DNA ده قطعه فیلماهی از هر جنس به صورت جداگانه تهیه شد. در مجموع ۱۰۱ پرایمر ده نوکلئوتیدی که توالی های وابسته به جنس در گونه های غیر ماهیان خاویاری را تکثیر نموده بودند تهیه و خزانه ژنومی فیلماهی با استفاده از این پرایمرها برای کشف نشانگرهای جنسی، به صورت هدفمند مورد غربالگری قرار گرفت. در مجموع ۲۸۴۶ باند تکثیر شد که هیچکدام از آنها در فیلماهی وابسته به جنس نبود. نتایج این پژوهش نشان داد که کروموزوم های جنسی در فیلماهی دارای نقاط متمایز کمی بوده و توالی های وابسته به جنس سایر گونه ها که در این تحقیق استفاده شد، در ژنوم فیلماهی به صورت حفاظت شده وجود ندارد.

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