Identification of bester hybrids (female *Huso huso* Linnaeus, 1758 and male sterlet *Acipenser ruthenus* Linnaeus, 1758) using AFLP molecular technique

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Abstract

In this study Amplified Fragment Length Polymorphism (AFLP) was applied to species identification of bester hybrids. Hybrids identification was performed by comparison of electrophoresis profiles with parental species. The simultaneous occurrence of diagnostic bands fixed in the parental species, genetic distance and identification and cluster analyses (UPGMA) allow a correct identification. We used 8 primer combinations (*Eco*+3, *Mse*+4) and a total of 250 bands (size range 40-1000 bp) were generated. Primer combinations of (*E*-AAT, *M*-CGAT) and (*E*-AAG, *M*-CGAT) produced diagnostic bands in hybrids and parental species. Moreover, the results of genetic identification showed that Bester hybrids are more similar to beluga (*Huso huso*) (0.68) in comparison with sterlet (*Acipenser ruthenus*) (0.45). The results suggested that this technique could be suitable for precise identification of species and inter-generic hybrids like bester.

Keywords: AFLP, Hybrid identification, Bester, Beluga

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Introduction

Fishes of the order Acipenseriformes, comprising the sturgeons (*Acipenseridae*) and paddlefishes (*Polyodontidae*) have luxury and expensive caviar (Flynn et al., 2006). Because of the high commercial demands for sturgeons and due to their endangered status, the high occurrence of sturgeon hybridization, both in the wild and in aquaculture, the necessity of valuable techniques for hybrid identification in both manufactured products for commercial monitoring, and live samples to guarantee correct restocking programs is felt (Congiu et al., 2002). During the recent decade, sturgeon farming has been focused and many countries are doing extensive activities on farm meat and caviar production (Congiu et al., 2002). Bester which is produced by crossing beluga (*Huso huso*) female and sterlet (*Acipenser ruthenus*) male, is suitable for aquaculture because it shows better growth rates than its males and earlier sexual maturation than its female parents (Burtsev, 1997). In recent years, the bester has been cultured not only in Russia but also in other countries, such as Germany, Hungary and Japan (Omoto et al., 2005)

Earlier, hybrid identification was largely based on morphometric characters, which could be biased or inaccurate measures of hybrid status (Tranah et al., 2003). Recently molecular advances have improved the ability of hybrid identification and so it could greatly enhance the ability of susceptible population's management. However, protein markers, such as immunological or electrophoresis methods have been used widely as taxonomical markers (Alarcon and Alvarez, 1999). Among molecular techniques, the AFLP (Amplified Fragment Length Polymorphism) technique allows obtaining a very high number of dominant nuclear markers. The results are similar to those obtained by Random Amplified Polymorphic DNA (RAPDs) (Williams et al., 1990), however, the AFLP markers exhibit a higher (98-99%) reproducibility (Questiau et al., 1999) and a stronger mendelian segregation. This technique has received increasing attention for study of hybridization (Tranah et al. 2003). At the hybrid identification level AFLP has successfully been used to differentiate populations of sturgeon (Congiu et al. 2003) and other animals (O'Hanlon et al., 1999; Bensch et al., 2002).

The goals of the present study were to investigate the utility of AFLP to find markers that recognize bester hybrid from its parental species that could easily be applied in any molecular biology laboratory.

Materials and methods

Samples

12 individuals from three species were analyzed to verify the usefulness of the AFLP technique for identification 1 *A. ruthenus* (male parent), 1 *H. huso* (female parent), 5 *H. huso* as control group of progeny and 5 besters (female *H. huso*× male *A. ruthenus*). All samples were provided at Propagation and Genetic departments of International sturgeon Research Institute, Rasht. Iran.

DNA extraction

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DNA was extracted using Ammonium Acetate procedure modified from Roche DNA extraction Kit. A small part of the caudal fin was homogenized in 600 µl STE buffer (50 mM Tris, 20 mM EDTA, 100 mM Nacl, pH 8) and 20 µl SDS 20% and incubated with proteinase K overnight, followed by centrifugation at 12000 rpm for 10 min. The supernatant was transferred to new tubes and an equal volume of 100% ethanol (-20 C) was added. DNA was pelleted by centrifugation (12000 rpm, 10 min) and washed twice with 70% ethanol before being air-dried and re-suspended in 100 µl autoclaved nanopure water. The quality and quantity of extracted DNA was assessed by 1.0% agarose gel electrophoresis and spectrophotometer (Cecil, CE2040, Italy), respectively (Chakmedooz, 2005).

AFLP reactions

AFLP procedures including all adapter and primer sequences were used from Vos et al. (1995) with some modification. An aliquot of total DNA (250 ng) was digested using 10 U *Mse*I and *ECO*RI in Tango buffer (Fermentas, France) in a 20 µl reaction at 37^{o}C for 90 min. Ligation was started immediately by a 5 µl mix of *Mse*I (50 mM) and ECORI (5 mM) adapters, T_4 DNA Ligase and 1X ligation buffer. Ligation was carried out at 37^{o}C for 3 h. Preamplification PCR was performed in 15 µl volumes, with 5 µl diluted restricted/ligated DNA, 1X PCR buffer, 1.5 mM Mgcl2, 0.2 mM of each dNTPs, 10 pmol *Mse*I + C and *ECO*RI+A primers. PCR was carried out with an Eppendorf thermocycler (Mastercycle ep gradiant, 96 plus, Germany) with 30 sec denaturation at 94^{o}C, 30 sec annealing at (56^{o}C) and 1 min extension at (72^{o}C) followed by 22 cycles. For selective amplification, preliminary trials were carried out using 8 different primer combinations. Selective amplification PCR was performed in 15 µl volumes, with 3 µl diluted (1:10) preamplification product as template, 1X PCR buffer with 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs (Cina Gene, Tehran, Iran), 10 pmol *Mse*+4 and *ECO*+3 primers: PCR consisted of 1 min denaturation (94^{o}C), 10 cycles of 30 s (94^{o}C), 30 s (63^{o}C) and 2 min (72^{o}C) with the annealing temperature decreasing from 63^{o}C by 1^{o}C increments in cycles 2- 10. This was followed by 26 cycles of 30 s (94^{o}C), 30 s (54^{o}C) and 2 min (72^{o}C).

Electrophoresis of AFLP fragments

After polymerase chain reaction, an equal volume of loading buffer (98% formamide, 10 mM EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol) was added. The reaction mixture was then heated for 10 min at (95^{o}C) and immediately cooled on ice. The gel was pre-electrophoresed at (55^{o}C), 95 W for 20 min, then 5µl of the amplified DNA was loaded and run on a 6% polyacrylamide gel (19:1 acrylamide/bis; 7.5 M Urea; 1X TBE buffer) with 1X TBE buffer on a vertical gel electrophoresis system (BIO-RAD sequi Gen, GT, 38 ×30 cm/ PowerPAC 5000) at 85 W and 50^{o}C for 90 min. After electrophoresis, the bands were visualized using silver staining. Clear and unambiguous bands in length ranging from 40 to 1000 bp were considered as usable. Fragment size (bp) was determined with ladder VIII (Roche) and scored manually.

Data analysis

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Only the recognizable AFLP markers were scored. The scored AFLP bands were dark, consistent and repeatable in individuals across polymerase chain reaction and gels. The binary matrix was built up from AFLP patterns, attributing "1" to presence and "0' to the absence (Zhang et al., 2004). Genetic distance and genetic identification was constructed using the Gene Alex and cluster analysis, then performed to create a dendogram using the unweight Pair- Group Method with an Arithmetic mean (UPGMA) by NTSYS software (version 2.02e). Potential species – specific bands should be indicated by a high frequency in one parental species and the hybrid and a low frequency in the other parental species and the hybrids should share specific bands from two parents.

Results

Determination of AFLP primers for H.huso, A.ruthenus and bester

The resolution of AFLP pattern is determined by the genome complexity, the GC content and the number of selective nucleotides in the AFLP primer combinations (Zhang et al. 2004). Based on the AFLP analysis, 8 primer combinations of E+3 and M+4 were chosen and tested for AFLP analyses of this study and produced 250 score able bands between 40 to 1000 bp from the combination of *E*-AAT, *M*-CGAT produced more AFLP markers (14 bands) than the other combination and 7 bands were present in sterlet but hybrids were absent in beluga (Fig.1) and the combination *E*-AAT, *M*-CACA produced less AFLP markers (5 bands, table 1). The combination of *E*-AAG, *M*-CACA produced no bands.

Table 1: Number of amplified and polymorphic bands scored from AFLP analysis in beluga, sterlet and Bester hybrids

No. of individuals studied	Total no. of bands amplified	No. of polymorphic bands	Percentage of polymorhic bands	No. of species-specific bands	Percentage of species-specific bands
12	250	69	28	7	12

Table 2: Pairwise Population Matrix of Nei (1978) Genetic Distance

	sterlet	beluga	beluga (Control)	bester
sterlet	0.000			
beluga	1.322	0.000		
beluga (Control)	1.320	0.032	0.000	
bester	0.790	0.377	0.402	0.000

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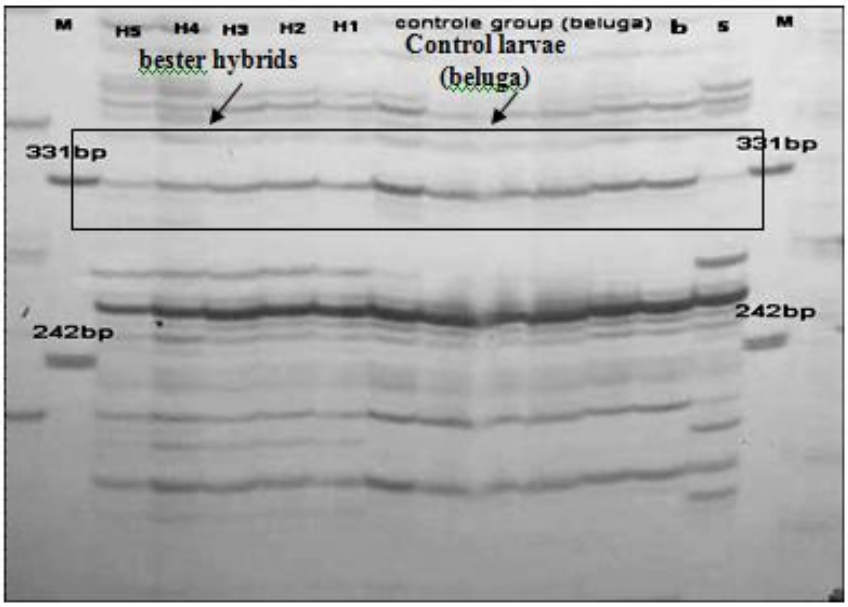


Figure 1: AFLP analysis with use of *M*-CGAT, *E*-AAG primer combination s: Male sterlet, b: Female beluga , H1-H5: Hybrid bester, M: *PUC* Mix Marker, 8 (Fermentas, France)

These 7 bands occurred in all hybrids, making them strong candidates for species markers in *H. huso* and *A. ruthenus*.

Species relatedness

The genetic distances are given in table 2. The pairwise population matrix of Nei (1978) genetic distance between 3 species ranged from 0.377 to 0.830. The highest genetic distance (0.83) was obtained between sterlet and beluga, the lowest genetic distance (0.37) was between beluga and bester, and the genetic distance between sterlet and bester was (0.79).

The pairwise population matrix of Nei (1978) genetic identity ranged from 0.66 to 1. The genetic identity between beluga and bester was (0.68) and it was 0.45 between bester and sterlet (table 3).

Table 3: Pairwise Population Matrix of Nei (1978) Genetic Identity

	sterlet	beluga	beluga (Control)	bester
sterlet	1.000			
beluga	0.267	1.000		
beluga (Control)	0.267	0.968	1.000	
bester	0.454	0.686	0.669	1.000

In UPGMA dendogram, three groups were formed: (1) *A. ruthenus* (2) *Huso huso* and (3) Bester hybrids. The neighbour-joining tree between species showed close relationship to beluga and bester in the neighbor- joining tree and sterlet as a distinct cluster from beluga and bester (Fig. 2).

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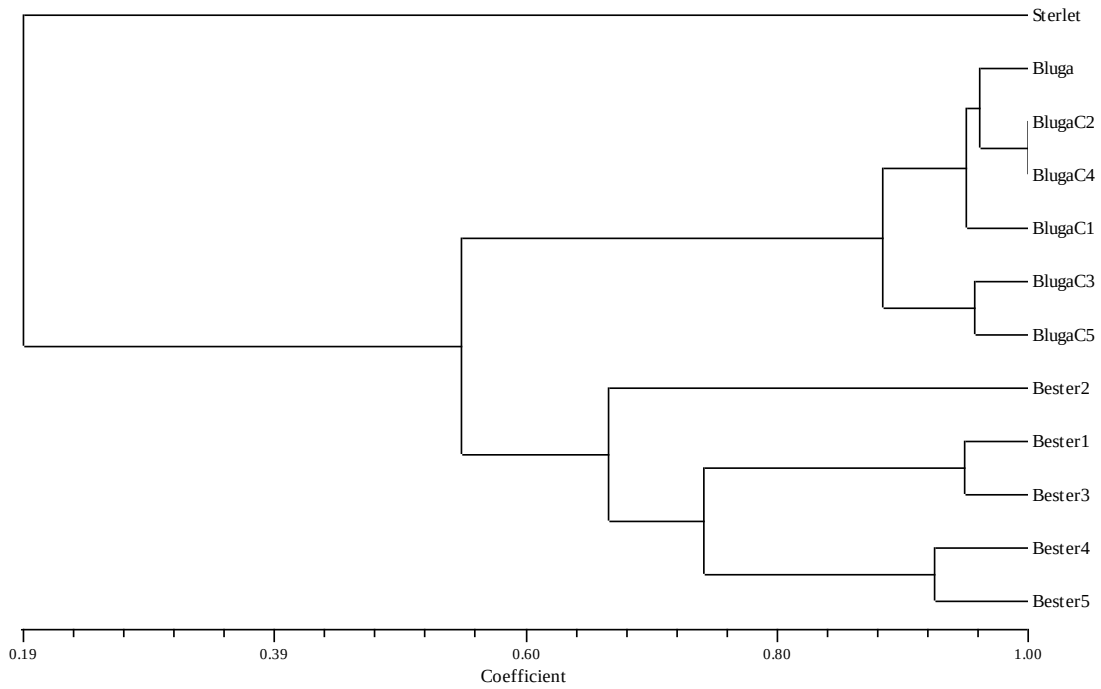


Figure 2: UPGMA dendogram base on AFLP fingerprinting of 12 sturgeons analyzed: 1 male starlet (S), 1 female beluga (b), 5 control beluga larvae and 5 bester hybrid larvae (H1-H5). M: Molecular Ladder

Discussion

Several polymerase chain reaction-based molecular methods of genotype analysis have been developed for identification of fish (Grewe and et al., 1993). Sequence analysis of species-specific mitochondrial/ribosomal DNA fragments and multiplex PCR of species - conserved DNA fragments are efficient for fish species identification (Bartlet and Davidson, 1991; Rocha-Olivares, 1998; Gharrett et al., 2001; Noell et al., 2001, Wasko et al., 2001). Compared with the DNA sequence method above, DNA fingerprinting is quicker and cheaper. In addition, the experimental requirements are lower (Cordes et al., 2001; Sebastio and Neri, 2001). Large numbers of molecular markers by AFLP can be generated without any previous knowledge of genetic constitution of the organism under investigation, and AFLP fingerprinting techniques show high reproducibility and a large number of clearly separate markers (Vos et al. 1995). These advantages make it suitable for distinguishing genetic differences at the species level and below (Vos et al., 1995). AFLP has successfully been applied to identification and typing of bacteria and plants (Coeny et al., 1999; Wong et al., 1999; Tyrka, 2002), marine animals (Ogden and Thorpe, 2001) and sturgeons (Congiu et al., 2002).

Han and Ely (2002) demonstrated that AFLP is a useful technique for the assessment of hybrid variations of fish. Furthermore, the species-specific patterns produced by AFLP can be used for

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identification of closely related species. In the present study, the AFLP patterns for parent and larvae from control group of beluga were 98-100% identical, and AFLP patterns of bester larvae with sterlet and beluga parents could easily be identified. There were a total of 69 AFLP loci based on the size of AFLP marker, and the number of AFLP markers for each species varied from 5 to 14, but only 7 markers were fixed to all species. The reliability of AFLP analysis in species identification of sturgeon hybrids which was confirmed by the fact of the highest genetic similarity (corresponding to the lowest genetic distance) was recognized between *H. huso* and bester hybrids on the basis of 0.68 of different AFLP markers. The AFLP technique proved to be highly reliable and reproducible on sturgeon samples. Although, band sharing among species was found to be lower than within species, the AFLP profiles detected a relevant intergeneric variation allowing identification of samples from unknown origins.

The resolution power of AFLP may decrease for species in which the analyzed phylogenetic distance is lower. However, *H. huso* and bester hybrids are two closely-related species (according to their genetic distance and similarity in this research), and they are clearly distinguishable by the present approach. Therefore, since the AFLP technique allows random analysis of the entire genomic DNA, it should be possible to find diagnostic markers by simply performing further amplifications with different selective bases (Congiu et al., 2002). However, the species of *A. ruthenus* and *H. huso* are morphologically detectable **(**Birstein and Doukakis, 2000), but in many cases in lower growth stages of bester and *H. huso*, they are morphologically similar to each other (Kozlov, 1993).

Congiu et al. (2002) identified species- specific markers for natural species of *A. naccarii*, *A. baerii* and *A. transmantanus* and also commercial samples of meat and caviar of 10 sturgeon species and interspecific hybrid of *A. naccarii* × *A. baerii* with AFLP procedure. The result of the present genetic analysis showed that bester is genetically similar to female parent or beluga that is in accordance with the results of morphological characteristics and data from bester hybrids growth (Kozlov, 1993).

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