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Development of conventional PCR and a Real-Time PCR assay to choose the most suitable method for residual DNA identification of Beluga, *Huso huso*, in concrete fish ponds

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ABSTRACT

A unique research was developed to achieve an effective and efficient method for tracing and identifying Beluga sturgeon species, *Huso huso* using eDNA in concrete fish ponds. Sampling was performed from the water of the Beluga pond and fixed with precipitation premix solution. DNA was extracted from fixed water, and fin tissue of Beluga. Three short fragments were selected from a nuclear and two mitochondrial genes region of Beluga DNA. The specificity of primers was checked by conventional PCR for Beluga with fin tissue DNA samples. In this study, two conventional methods of molecular identification were performed on residual DNA samples of fish pond water and fin tissue, exhibiting that they were not suitable for measuring the residual DNA of Beluga in fish ponds due to the low rate of DNA. PCR amplification of sturgeon's DNA of mitochondrial *COI* gene in third PCR primers was successful in low level of DNA extracted from water column and sediment by conventional and Quantitative PCR. The linear relationship between the threshold cycle (*ct*) value and Beluga DNA concentration was measured by the Mini-Barcoding quantitative Real-Time PCR. DNA samples of fish ponds generated a uniform curve in water column and sediment. In identifying residual DNA in the Picogram level of Beluga pond, SYBER Green Real-Time PCR method can be an acceptable method for barcoding with high efficiency compared to other methods such as conventional PCR method and non-invasive which is advantageous for the conservation of critically endangered sturgeons such as Beluga. This research results can also be used as a model for tracing fish species in rivers and seas.

Keywords: Beluga, Nuclear DNA, Mitochondrial DNA, SYBER Green Real-Time PCR.
Article type: Research Article.

INTRODUCTION

Sturgeons are as producers of caviar, one of the most valuable foods on the world's market (Fain *et al.* 2013). Overfishing of wild fish and destroying fish habitats are the major causes that has led some of sturgeon species extinct (Bronzi & Rosenthal 2014). According to information from CITES, all species of sturgeon are conserved species and many species are at risk of extinction. Also, during the last decade, the demographic collapse of sturgeon natural population and high demand for meat and caviar led to the decreased sturgeon populations (Bronzi & Rosenthal 2014). Five species of sturgeons inhabit the Caspian Sea including: Beluga, *Huso huso*, Persian sturgeon, *Acipenser persicus*, Russian sturgeon, *A. gueldenstaedtii*, Ship, *A. nudiventris*, and Sevruga, *A. stellatus* and one species, Sterlet, *A. ruthenus* in the Caspian Sea basins. Among these species, Beluga provides high-value caviar that is occasionally substituted with a less desirable product from other sturgeon species or hybrids. Natural populations of Beluga have been dramatically reduced due to poaching and habitat degradation (Doukakis *et al.* 2012). Identification and evaluation of species is essential for effective management and conservation of biodiversity. Environmental DNA (eDNA) can allow species to be monitored without needing to

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collect live organism, and in scale to more traditional sampling, higher yields, and higher efficiency compared to more traditional sampling schemes and proved to have a high potential in biological monitoring (Deiner *et al.* 2013; Goldberg *et al.* 2016). The study of eDNA is a powerful tool for evaluating biological protection, and an effective study of biodiversity. In aquatic environments, the tool relies on the tracing of genetic material derived from organisms, for example from egg, urine, hair loss, mucus, gametes, or whole cell tissues of organisms, which are broken down and released after the release of DNA (Taberlet *et al.* 2012; Thomsen & Willerslev 2015). Integration of molecular genetic techniques and aquatic ecology (eDNA) has led to greater sensitivity to rare species and early detection of invasive aquatic species (Goldberg *et al.* 2013; Jerde *et al.* 2016). In this study, a unique design was developed to achieve an effective and efficient method for tracing and identifying sturgeon species (Beluga) using eDNA in fish farms. This study can also be used as a model for tracing fish species in rivers and seas. Three short fragments were selected from a nuclear and two mitochondrial genes region of Beluga DNA, however, eventually one fragment could be applied for eDNA detection; the first two markers were specific for Beluga. This method is non-invasive which is advantageous for conservation of critically endangered sturgeons. Considering the time consuming, expensive and the low sensitivity of other methods, the aim of this study was to develop a method for detecting the residual sturgeon DNA based on PCR and SYBER Green Quantitative Real-Time PCR for Beluga detection in nature. These methods are cost-effective and more conveniently used to apply a diagnostic method based on PCR and also allow highly efficient, rapid and accurate differentiation of sturgeon from other fish and animal based on the gene sequence which make them possible to detect a few nano- or pico- gram level of DNA.

MATERIALS AND METHODS

Beluga concrete fish pond water sampling

Sampling was carried out from the six Beluga ponds in the International Sturgeon Research Institute, Rasht, Iran. Sampling season was in autumn 2022. The characteristics of water included the following: temperature 18.4 ℃, pH 8.15 and water salinity was under 1 ppt. Using Falcon tube 50 (sterile), equal volume of sampling water containing eDNA of fish pond from water column and sediment was added to premixed absolute ethanol and 3M sodium acetate, then sealed with parafilm tape. The samples were immediately placed in a cool container filled with ice to prevent the destruction of eDNA and stored at -20 °C until extraction. According to this method, about 1.5 mL of 3 M sodium acetate and 33 mL of absolute ethanol (Fisher Scientific Waltham, Massachusetts, USA) were mixed well and used in equal volume with water samples (Ladell *et al.* 2019; Wang *et al.* 2021).

DNA extraction from concrete fish pond water and sturgeon fin tissue

The supernatant water samples were decanted from each column sample by centrifuging at 2000 *g* for 15 min and the remaining pellet was subjected for DNA extraction. About 300 µL of phosphate-buffered saline (PBS) were added to pellet for column water. Sediment samples were washed once with PBS (1 mL) and centrifuged at 2000 *g* for 5 min. 300 µL of the supernatant was removed for DNA extraction. 300 µL PBC containing shedding contents of the fish in concrete pond (for each water column or sediment), 200 µL STE (Sodium Tris EDTA) pH 8 buffer and 20 µL of 10 µg mL^{-1} proteinase K samples were incubated at 56 ºC for 2 hours. Deasy QIAgen kit extraction protocols (Qiagen Blood and tissue DNA extraction kit (cat. nos. 6950) was used for DNA extraction according to the kit manual. Also, a total of four fresh fin tissue samples from one-year-old fish belonging to pure Beluga of the Caspian Sea were extracted and analysed in this study as controls.

Conventional PCR assay for Beluga detection by three different mitochondrial and nuclear markers

Molecular identification of Beluga was done according to Mugue *et al.* (2008). Primers HUSF and AHR (Table 1) were selected for identifying Beluga (maternal identification of Beluga mother). Also, Single Nucleotide Polymorphism (SNP) located in the second intron (RP2) of the nuclear encoded S6 Ribosomal Protein (RP2S6) was selected for Beluga genome assay (Boscari *et al.* 2017). PCRs were performed in a total volume of 10 µL, consisting of 5 µL Red Master Mix buffer of Amplicon 10 pg μL^{-1} each forward and reverse primers, 0.001-0.1-1-10-100 ng template extracted DNA for all reactions. Amplification conditions were optimized as follows for HUSF and AHR: a denaturation step at 94 °C for 5 min, 35 cycles at 94 °C for 30 secs, 56 °C for 30 secs, 72 °C for 15 min, and a final elongation at 72 °C for 5min. Nuclear fragment amplification (RP2S6) program was exactly according to Boscari *et al.* (2017). *ACoI*2 Primer pair for amplification of Beluga's *COI* gene was selected according to Waraniak *et al.* (2017). All amplifications for *COI* gene was performed on Eppendorf PCR System

set as follows: 5 min at 94 °C, 35 cycles at 94 °C for 30 s, 56 °C for 20 s and 72 °C for 20 s, followed by a 5-min extension at 72 °C. Products were checked by 1.5-2 % agarose gel electrophoresis. The last pair of primers had been designed for amplifying Mini-Barcoding fragment of *COI* gene in *A. fulvescens* according to Warannik *et al.* (2017). To confirm the correctness of the amplified bands in the Real-Time PCR mini-barcoding method, at first, conventional PCR was done by DNA extracted from Beluga's fin tissue. Due to the suitability of the last primer in low DNA concentrations for PCR amplification, it was selected for existence assessment of sturgeon in fish pond by Real-Time PCR.

Table 1. Primers name and sequences applied in this research.

Primers	Mitochondrial and nuclear markers	Nucleotide sequence	Size of amplified band
Hus F AHR	*D-loop* (mtDNA) (Mugue *et al.* 2008)	TATCTATTACCTGCGAGCAGGCTG TTACCTGCGAGCAGGCTG	374 bp (Beluga)
RP2S6_*Huso*_F RP2S6_groupA_R	RP2S6 (nDNA) (Boscarri *et al.* 2017)	CATAACATTGCACTGAATGTTATA CTTTCGTTGATTTAGGGAAATGGT	194 bp (Beluga)
*ACoI*2 F *ACoI*2 *R*	*COI* (mtDNA) Waraniak *et al.* 2017	GCTCCTTTTAGCCTCCTCTGG CCCCAAAATGGACGAAACCC	151bp (sturgeons)

Preparation of dilution from template DNA for drawing standard curve (linearity), Real Time PCR amplification specificity for Beluga

The linearity of the analytical procedure was determined by the seven-fold serial dilutions (10 ng, 2 ng, 400 pg, 80 pg, 16 pg, 3.2 pg and 0. 64 pg DNA) of Beluga tissue genomic DNA. The amplification was performed with BioRad CFX96 Real-Time PCR system using the following conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 20 s and 72 °C for 20 s. Fluorescence signals were measured at the end of each cycle on the SYBR green channel. Two PCRs were performed in parallel. For the standard curve, serial dilutions of reference DNA were prepared in water and a fixed volume (20 µL) of each dilution was tested as described above. At the end of the reaction, the standard curve was generated based on plotting the logarithm of DNA concentration (horizontal axis).

Beluga fish pond Real-Time PCR assay for residual DNA detection

Quantitative Real-Time PCR was conducted in a final volume of 20 µL containing 10 µL of 2X SYBER Green (Amplicon), 100 nM of each primer (forward and reverse) to determine the amount of Beluga DNA in the fish pond water samples (bottom sediment and column water) using specific primers for the sturgeon (*COI*). Real-Time program and conditions were performed as same as standard curve amplification. Briefly, melt curve analysis was performed by cooling amplification products at 56 °C for 90 s and then heating from 55 to 95 °C with a ramping rate of 0.5 °C/5 s. For each sample, two parallels were performed. The Q-PCR reaction mix contained a final concentration of 300 nM each primer. A water sample was used as the negative control (NTC).

RESULTS AND DISCUSSION

Conventional PCR

The concentration of tissue extracted DNA from Beluga fin tissue, were between 120-250 ng μL^{-1}, however, the DNA concentrations of fish pond samples (water column and sediment) were insignificant for detecting by Nanodrop (less than 5-10 ng). PCR amplification of Beluga DNA with mitochondrial specific primers (AHR and HUSF) showed a fragment only in reactions with 100 ng concentration; have a visible band on agarose gel but reactions with lower DNA concentrations; do not show any bands on the agarose gel or show very faint band in 10 ng DNA concentration (Fig. 1). This result has been repeated for nuclear primers for generating a band in lower DNA concentration (Fig. 2). Primer-BLAST in NCBI showed specific amplification of sturgeon's DNA of mitochondrial *COI* gene in third PCR primers. In addition, a practical PCR amplification for Beluga sturgeon confirmed the successful amplification of Mini-Barcoding fragments in this species (Fig. 3) with fin tissue DNA. Moreover, the linear relationship between the threshold cycle (*ct*) value and the Beluga DNA concentration was measured by the Mini-Barcoding Quantitative Real-time PCR (Fig. 4). DNA samples of fish pond water displayed by X = unknown samples. The residual DNA of sturgeons was assayed in fish pond water. The desired amplified

fragment was denatured at a temperature of 84.5 ºC (Melt curve) for Beluga (Fig. 5). Beluga's pond water samples were successfully amplified Mini-Barcoding fragment displayed by (× unknown) symbol just like the specific amplification of sturgeon's DNA of mitochondria in standard curve and specify test for sturgeons (Fig. 4).

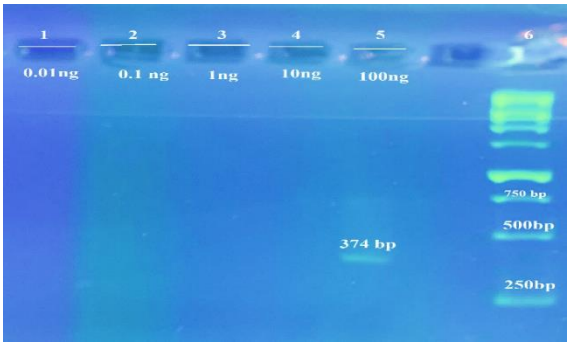

Fig. 1. Amplification of Beluga DNA with mitochondrial specific primers (AHR and HUSF) on 1.5% agarose gel; only reactions with 100 ng, amplified a visible band on agarose gel (lane 5) but reactions with lower DNA concentrations did not exhibit any bands on the agarose gel (lanes 1-4); lane 6 DNA ladder 1kb Sina Clone.

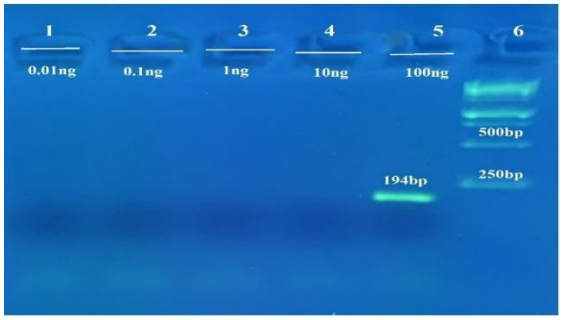

Fig. 2. Amplification of Beluga DNA with nuclear specific primers (RP2S6) on 1.5% agarose gel; only reactions with 100 ng DNA, amplified a visible band on agarose gel (lane 5) but reactions with lower DNA concentrations exhibited very faint band (lane 4) or no bands on the agarose gel (lanes 1-3); lane 6 DNA ladder 1kb Sina Clone.

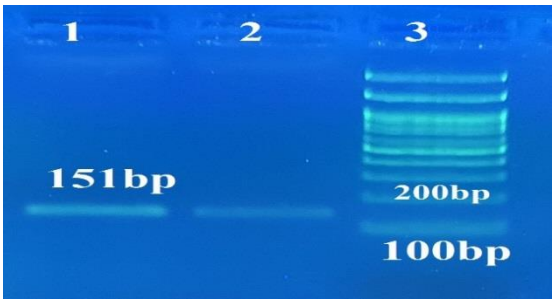

Fig. 3. Successful amplification of Mini-Barcoding sturgeon's primer in DNA extracted from fin tissue of Beluga (lanes 1-2) and 100 bp ladder Sina Clone.

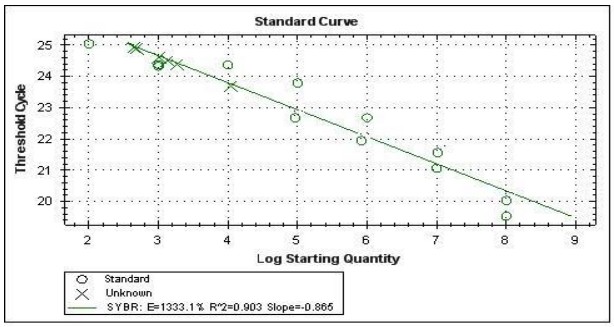

Fig. 4. Standard curves of genomic DNA of Beluga (two repeat tests for each dilution) with consecutive dilutions from 10 ng to 0.64 pg (dilution ratio of 1 to 5) showing a linear relationship between threshold cycle (*CT*) value and the DNA concentration measured by the *ACoI*2 quantitative Real-Time PCR and six DNA samples (showed by ×unknown) of fish pond DNA amplified in BioRad CFX96 Real-Time PCR system. A correlation coefficient higher than 90% was obtained for *ACOI*2 primer of Beluga tissue DNA amplification.

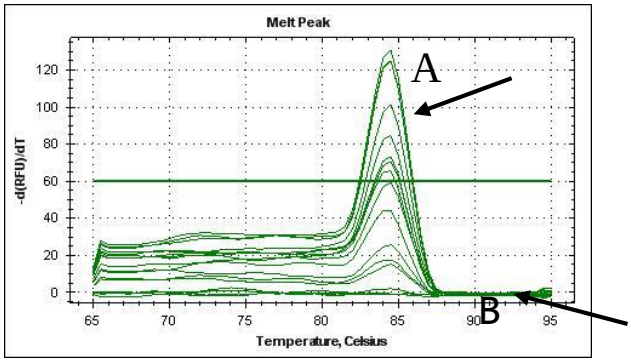

Fig. 5. Melting curve based on temperature (horizontal axis) and fluorescent signal derivative (vertical axis) received from BioRad CFX96 Real-Time PCR system in standard curve drawing. It shows that the desired amplified fragment is denatured at a temperature of 84.50 ºC (A). B indicates the non-binding of primers in the negative control.

DISCUSSION

The amount of DNA extracted from Beluga fish pond water was too low to be measured with NanoDrop, as its concentration was in picograms. However, DNA extracted from fin tissue were about 190-250 ng μL^{-1}. Although, two conventional methods in specific molecular identification of Beluga were performed efficiently in this study and that of Boscari *et al.* (2017) on fin tissue DNA samples, however, these methods could not be suitable for Beluga residual assay in fish ponds, since significant amount of DNA in nanogram level needs for identification. A very sensitive and accurate SYBER Green Real-Time quantitative PCR (qPCR) method was used to detect the residual DNA of sturgeon in fish pond water based on a specific sturgeon's primer which at first, was chosen by Warangal *et al.* (2017) for lake sturgeon (*A. fulvescens*) identification. Even though the primers chosen by Warangal *et al.* (2017) were designed for *A. fulvescens*, the current investigation proved that these primers are also useful for identifying Beluga and some Caspian Sea sturgeons. In addition, Torabi Jafrodi *et al*. (2023) reported this method in Persian sturgeon residual DNA in fish ponds. An important application of the qPCR method for eDNA assay is in the discovery and monitoring of rare and endangered species (e.g., sturgeons), particularly in aquatic environments (Lawson Handley 2015; Barnes & Turner 2016; Deiner *et al.* 2017). Species detection was performed by isolating separate parts of mitochondrial DNA (mtDNA) obtained from the environment and amplified by the Thermos cycler (Lodge *et al.* 2006). When the fish is raised in the pond, the shedding of skin and mucus, excrement, and feces leaves part of the available genetic information in the environment, which can be a suitable solution for tracing the cultured species. An important application of the qPCR method for eDNA assay is in the discovery and monitoring of rare species, as well as integration of molecular genetic techniques and aquatic ecology (e.g., eDNA) has led to greater sensitivity to rare species and early detection of invasive aquatic species (Goldberg *et al.* 2013; Jerde *et al.* 2016). In this study, tracing and identification of Beluga using residual DNA is used as a model for tracing fish species in rivers and seas, hence can be considered as a way to assess the population of Beluga in migrating rivers in the future sturgeon stock assessment programs. The close molecular similarity in sturgeon, especially in the mitochondrial genome, led to the fact that it could not be applied to specific genetic markers for the exact detection of species. Schenekar *et al.* (2020) applied TaqMan qPCR for detecting *A. ruthenus* in headwater of Volga River, however they mentioned that their method potentially could detect two *Huso* species and Palearctic *Acipenser* species, albeit would need further testing for specificity and sensitivity. Bergman *et al.* (2016) reported first detection of Green Sturgeon eDNA and identifying unknown spawning habitats and also discovered factors influencing habitat usage in researches. Plough *et al.* (2021) reported eDNA tool for monitoring Atlantic sturgeon (*A. Oxyrhinchus oxyrhinchus*). In addition, they mentioned documenting Atlantic sturgeon distribution in USA rivers by eDNA assay for management and conservation purposes. Yu *et al.* (2021) concluded that eDNA method could be a valuable and complementary tool to rapidly determining distribution and quantification of endangered *A. sinensis*. Janosik *et al.* (2018) reported that Alabama sturgeon (*Scaphirhynchus suttkusi*) detections increase during spawning months. They concluded that species ecology, habitat use and site characteristics play a role in the rare species identification. Among quantitative or semi-quantitative assay methods used to determine the residual DNA, including hybridization, DNA-binding protein, and Q-PCR (SYBER Green and TaqMan) methods are used to amplify DNA and determine its quantity at the same time in order to identify the specific sequence of DNA and its amount for Barcoding (Kubista *et al.* 2006). Conventional PCR is a simple method but able to identify the

species if the amount of DNA is medium or high and the target fragments of the *COI* gene should be sequenced for species identifications (Jamshidi *et al.* 2021). Mugue *et al.* (2008) identified different species of the Acipenseridae according to different sizes of amplified fragments of mitochondrial control region on agarose gel electrophoresis. However, this method needs at least amount of DNA in nanogram level and pictogram DNA level of residual DNA which could not be amplified most of the time. In addition, in the pharmaceutical industry, the Real Time PCR (quantitative PCR) method is used to amplify DNA and determine its quantity at the same time in order to identify the specific sequence of DNA and its amount (Kubista *et al.* 2006). This method has also been used to identify Chinese hamster ovary DNA in medicinal products (Nissom 2007). Considering the time consuming, expensive and the low sensitivity of other methods, the objective of this paper is to develop a method for detecting the residual sturgeon DNA based on SYBER Green Quantitative Real-Time PCR. This method is cost-effective and more conveniently used to identification of DNA extracted from water column and sediment and it will contribute to a diagnostic method based on SYBR Green Real-Time PCR. This method allows highly efficient, rapid and accurate differentiation of sturgeon from other fish and animal based on the *COI* gene sequence which makes it possible to detect a few about tenth picogram level of caviar extract DNA.

CONCLUSION

This study was the first effort for residual DNA detection of Beluga in fish pond column water and sediment. Molecular diagnostic of conventional PCR only could apply for Beluga fin tissue samples. Quantitative PCR method in our study proved to detect residual DNA in the picogram level of the Beluga fish pond and could be an acceptable method with high efficiency compared to other methods such as the conventional PCR method for sturgeon identification.

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