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Molecular Identification of Residual DNA Separated from the Persian Sturgeon (*Acipenser persicus*) for Modeling eDNA Evaluation in Aquatic Ecosystem

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ABSTRACT

Considering the importance of Caspian Sea sturgeon conservation according to CITES rules, finding effective and efficient methods for tracing and identifying sturgeon species are necessary. Residual DNA detection in fish ponds can be used as a model for tracing fish environmental DNA (eDNA) in rivers and seas. This method of DNA detection is non-invasive and advantageous for the conservation of critically endangered sturgeons. Sampling was done from the water of the Persian sturgeon fish pond and fixed with precipitation premix solution (ethanol and acetate sodium). DNA was extracted from fixed water, and fine tissue of two sturgeon species in the Caspian Sea, Sterlet, and Siberian sturgeon. The positive specificity of primers was checked by conventional PCR for sturgeons with fine tissue DNA samples. The linear relationship between the threshold cycle (*ct*) value and the Persian Sturgeon DNA concentration was measured by the Mini-Barcoding quantitative real-time PCR. DNA samples of fish ponds generated a curve that could not be produced in negative control and irrelevant (non-sturgeon) genomic DNA. Although residual DNA of Persian sturgeons was detected in fish pond water at picogram level through the quantitative method, molecular diagnosis with this method can confirm only the existence of sturgeon species (in general) in fish ponds. In identifying residual DNA in the picogram level of the Persian sturgeon fish 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.

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Introduction

In *Appendices* of the Convention on International Trade in Endangered Species (CITES-2008) and 2021 red list of threatened species, most sturgeons are classified in endangered or vulnerable lists (IUCN, 2021; Ludwig, 2008). According to information from CITES, all sturgeons are conserved and many species are at risk of extinction. Over the last decade, the demographic collapse of the sturgeon natural population and the high demand for meat and caviar has led to a decrease in sturgeon populations (Bronzi and Rosental, 2014). Five sturgeon species live in the Caspian Sea, including Beluga (*Huso huso*), Persian sturgeon (*Acipenser persicus*), Russian sturgeon (*A. gueldenstaedtii*), ship (*A. nudiventris*), and sterlet sturgeon (*A. stellatus*). One Sterlet species (*A. ruthenus*) also lives in the Caspian Sea basins. Among these species, *Acipenser persicus* has the widest distribution (Sattari, 2003) in the

southern region of the Caspian Sea. The Persian sturgeon lives mainly in the sandy bottoms of the central and southern Caspian Sea, especially along the shores of Iran. *A. persicus* is most widely distributed in Iranian waters where maritime fishing is allowed (Vlasenko *et al*., 1989; Birstein and Bemis, 1997). Commercial fishing of Persian sturgeon has diminished in most of its range, but it continues for certain populations and may increase due to the value of its caviar (Moghim *et al*., 2006; Pourkazemi, 2006). In recent years, Persian sturgeon constitute the largest percentage of total commercial catch (Tavakoli *et al*., 2010), and fisheries management organizations in Iran are trying to facilitate the recovery of Persian sturgeon populations through restocking programs (Abdolhay and Tahori, 2006). The population size of all sturgeon species has decreased in most areas, which led to conservation concerns. Since 2000, sturgeon fishes have been on the list of endangered species and the Persian sturgeon has been at risk of extinction. Reduction in natural reproduction, illegal fishing, dam construction, and deterioration of water quality are the most important factors in the reduction of the Persian sturgeon (Pourkazemi, 2006). Information about natural stocks of Caspian Sea sturgeon is not enough and evaluation should be carried out continuously. Sturgeon species such as Persian sturgeon are especially vulnerable to overfishing because of commercial importance. Moreover, the anthropogenic effects influenced all components of the Caspian Sea (Pourang *et al*., 2016). It is revealed that the environment of the Caspian Sea is shifted to a new condition (Beyraghdar Kashkooli *et al*., 2017); sturgeon species are collapsed and listed under Acipeserides I or II CITES due to pollution, and poaching over the last decades, habitat destruction, overexploitation, and illegal trade (Ivanov *et al*., 1999; Pourkazemi, 2006; Khodorevskaya *et al*., 2009, Tavakoli *et al*., 2019; Fazli *et al*., 2020). According to the Commission on Aquatic Bioresources, commercial fishing of sturgeons has been banned since 2012. The most important problem in sturgeon stock assessment is that their population situation, number of fish, and biomass cannot be measured exactly in a natural living environment with stock evaluation methods; but the study of Environmental DNA (eDNA) is a powerful tool for evaluating biological protection and is an effective approach to the study of biodiversity. An important application of this method is in the discovery and monitoring of rare and endangered species (e.g., sturgeon), particularly in aquatic environments (Lawson Handley, 2015; Barnes and Turner, 2016; Deiner *et al*., 2017). Environmental DNA is an approach for species detection by isolating separate parts of mitochondrial DNA (mtDNA) obtained from the environment (Lodge *et al.*, 2006). Identification and evaluation of species are essential for the effective management and conservation of biodiversity. EDNA can allow species to be monitored without needing to collect live organisms, and compared with more traditional sampling schemes, it has higher yields and efficiency and proved to have a high potential in biological monitoring (Goldberg *et al*., 2018; Deiner and Altermatt, 2014). Higher mutation levels in mitochondria DNA make it suitable for species molecular identification and detection (Tang *et al*., 2006). In the current research, a short fragment was amplified from the barcoding gene of the *COI* region of sturgeon mitochondria DNA (Persian sturgeon). The primers, which were designed by Waraniak *et al*. (2017), have no function in non-sturgeon fish. In this research, a unique design was developed to achieve an effective and efficient method for tracing and identifying sturgeon species using eDNA in fish farms. This design can also be used as a model for tracing fish species in rivers and seas. This method is non-invasive and advantageous for the conservation of critically endangered sturgeons because it does not need any living tissue from an organism and there is no need to injure the fish. In aquatic environments, the tool relies on the tracing of genetic material derived from organisms (e.g., egg, urine, hair loss, mucus, gametes, or whole cell tissues of organisms), which are broken down and released after the DNA release (Taberlet *et al.*, 2012; Thomsen and Willerslev, 2015). Considering that other methods are time-consuming, expensive, and have low sensitivity, the objective of this study 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 apply a diagnostic method based on SYBR green real-time PCR.

Materials and methods

Precipitation of fish pond residual DNA

Sampling was carried out from the ponds of the Iranian fishery science research institute, and the international sturgeon research institute of Rasht. Using Falcon tube 50 (sterile), an equal volume of sampling water containing eDNA was added with premixed absolute ethanol and 3M sodium acetate and then sealed with paraffin tape. Samples were immediately placed in a cool container filled with ice to prevent the destruction of eDNA and stored at -20 °C until extraction. The precipitation liquid solution of DNA was made by adding ethanol and sodium acetate to water samples. Six pond samples were treated by adding equal volumes of premixed absolute ethanol and 3M sodium acetate. 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 volumes with water samples (Li *et al.,* 2018). Samples were preserved at -20°C (Ladell *et al.*, 2019; Wang *et al.*, 2021).

DNA extraction

The supernatant water samples were decanted from each sample and the remaining pellet was subjected to DNA extraction. About 300µl of phosphate-buffered saline and 200µl of STE (Sodium Tris EDTA) *PH*=8 buffer and 20 µl of 10µg/ml proteinase K were added to the pellet and samples were incubated in 56 ºC for 2 hours. The genomic DNA was extracted using the Qiagen DNA Blood and Tissue Kit (Qiagen, Inc).

A total of seven tissue samples belonging to 5 pure species of Caspian Sea sturgeon and two species belonging to farmed sturgeons (*Acipenser ruthenus* and *A. baerii*) were analyzed in this research as control positive of Acipenseridae. 10 mg of fin tissue was powdered with liquid nitrogen and used for DNA extraction via QIA gene extraction protocols (Qiagen Blood and tissue DNA extraction kit). The quantity of extracted DNA was measured by the Nanodrop set (Thermo Fisher, Nanodrop-1000).

Specific primer for DNA tracing

*ACoI*1 Primer pair for amplification of sturgeon's *CoI* gene was selected from Waraniak et. al, 2017 study (table 1). Primer-BLAST in NCBI was employed to confirm the theoretical specificity of the selected primers, while the final specificity was ensured through a practical PCR amplification against target species (sturgeons) and non-target species. These primer pairs had been designed for amplifying the Mini-Barcoding fragment of the *CoI* gene in *A. fluvescence* but theoretically, have very few mismatches with other species of Acipenseridae and may be capable of amplifying the DNA of other species of sturgeon.

Table 1. Primers used for COI amplifications.

Primer name	Oligomers (5′→3′)
ACoI F	5'-CCATCATAATTG GCG GAT TCGG
ACoI R	5'- CCC CAGAGGAGG CTA AAAGG′

Conventional PCR amplification

To confirm the correctness of the amplified bands in the real-time PCR mini-barcoding method, first, conventional PCR was done. All PCR reactions were performed in a total volume of 10µl, consisting of 5µl Red Master Mix buffer of Ampliqon, 0.2µl each forward and reverse primers, 100 ng template extracted DNA (tissue extracted DNA of Caspian Sea Sturgeon, sterlet, and Siberian sturgeon). All amplifications for *ACoI* were performed on Eppendorf PCR System set as follows: 5 min at 94 °C, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by a 5-min extension at 72 °C. Products were checked by 1.8% agarose gel electrophoresis.

Real-time PCR amplification

The linearity of the analytical procedure was determined by the seven-fold serial dilutions (10ng, 2ng, 400pg, 80pg, 16pg, 3.2pg, and 0.064 DNA) of *A. persicus* 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 10 s, 56 °C for 10 s, and 72 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) and threshold cycles (vertical axis). In addition, a test was done for the target (5 Caspian Sea sturgeons, Starlet, and Siberian sturgeon) and non-target species. The DNA extracted from the human cell (*Homo sapiens*), yeast (*Saccharomyces cerevisiae*), and bacteria (*Escherichia coli*) were applied for the specificity test of primer in sturgeon.

Quantitative real-time PCR was conducted in a final volume of 20 µl containing 10µl of 2X SYBER Green (Ampliqon), 100nM of each primer (forward and reverse) to determine the amount of sturgeon DNA in fish pond water samples (bottom sediment and column water) using specific primers for the sturgeon. Real-time program and conditions were as same as standard curve amplification. Melt curve analysis was performed by cooling amplification products at 55 °C for the 90s 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 for each primer. A water sample was used as the negative control (NTC).

Results

The concentration of tissue extracted DNA from five Caspian Sea sturgeons, Sterlet, and Siberian sturgeon was between 150-370 ng/µl but the DNA concentration of fish pond water samples was insignificant for detection by Nanodrop. Primer-BLAST in NCBI was shown specific amplification of sturgeon's DNA of mitochondrial *COI* gene. Moreover, practical PCR amplification for each of the 5 Caspian Sea sturgeons, Sterlet, and Siberian sturgeons confirmed the successful amplification of Mini-Barcoding fragments in these species (Fig. 1) with tissue DNA. Additionally, the linear relationship between the threshold cycle (*ct*) value and the Persian Sturgeon DNA concentration was measured by the Mini-Barcoding quantitative real-time PCR (Fig. 2).

DNA samples of fish pond water showed by ×= unknown samples. The residual DNA of sturgeons was assayed in fish pond water. The desired amplified fragment is denatured at a temperature of 82.5 ºC for sturgeons Mini-barcoding primer (Fig. 3), which is specific for sturgeons and non-specific for irrelevant genomic DNA.

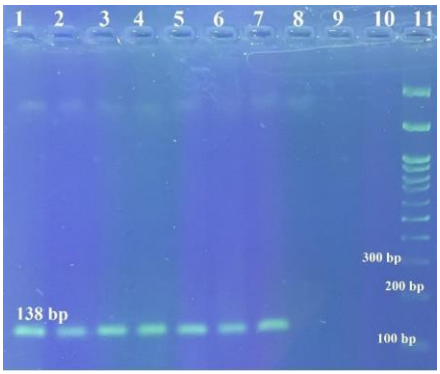

Fig. 1. Gel electrophoresis of DNA amplification fragment: Successful amplification of Mini-Barcoding sturgeon's primer in Caspian Sea sturgeons, Sterlet, and Siberian sturgeons with sturgeon's tissue DNA.

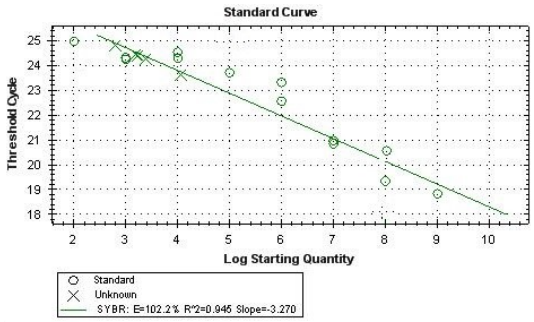

Fig. 2. The draw of standard curve: Standard curves of genomic DNA of *A.persicus* (two repeat tests for each dilution) with consecutive dilutions from 10ng to 0/064pg (dilution ratio of 1 to 5) showing a linear relationship between threshold cycle (CT) value and the DNA concentration measured by the *ACoI* 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 percent was obtained for *ACOI* primer for *A.persicus* tissue DNA amplification.

Specificity test for sturgeon sample showed all seven sturgeon genomes (Caspian Sea Sturgeons, *A. baerii*, and *A. ruthenus*) amplified a fragment, but irrelevant genomic DNA was indistinguishable from the response of the

background (NTC) when analyzed with Mini-Barcoding primers (Fig. 4).

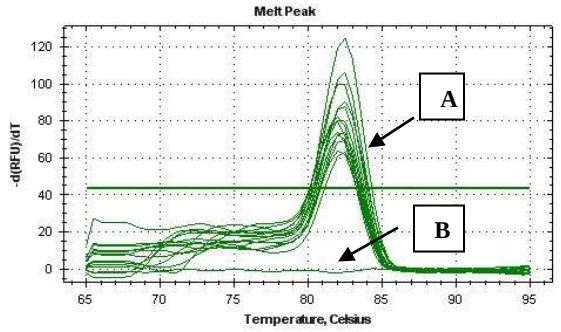

Fig. 3. Melting curve of real-time PCR: Melting curve based on temperature (horizontal axis) and fluorescent signal derivative (vertical axis) received from BioRad CFX96 real-time PCR system. It shows that the desired amplified fragment is denatured at a temperature of 82.5 ºC (A). B indicates the non-binding of primers (primer dimer) in the negative control.

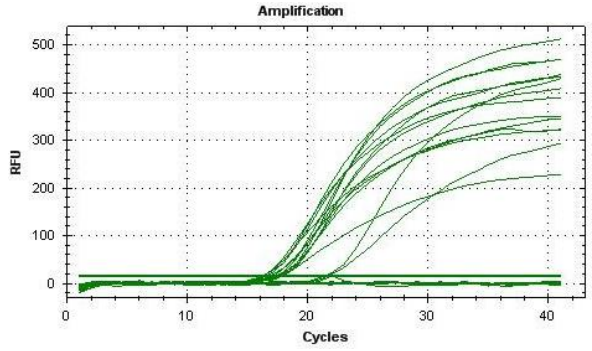

Fig. 4. Specificity test for sturgeon samples: Only sturgeon genomes have an amplification curve. All seven sturgeon genomes (Caspian Sea Sturgeons, *A. baerii*, and *A.ruthenus*) amplified a fragment but irrelevant genomic DNA, Human (*Homo sapiens*), a bacteria (*Escherichia coli*), and yeast (*Saccharomyces cerevisiae*) were indistinguishable from negative control (NTC) when analyzed with the *ACOI* primer. Two PCRs were performed in parallel for each species.

Persian sturgeon pond samples were successfully amplified with Mini-Barcoding fragments shown by (×unknown) symbol just like the specific amplification of sturgeon's DNA of mitochondria in standard curve and specify a test for sturgeons (Figs. 2 and 4).

Discussion

This research was the first study for tracing residual DNA in the Persian sturgeon ponds through a molecular method. The amount of DNA extracted from fish pond water was so low that it could not be measured with NanoDrop, as its concentration was in picograms. 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. Although the primers of Warangal research were designed for *A. fulvescens*, the current investigation proved that these primers are also useful for identifying all Caspian Sea, Sterlet, and Siberian sturgeons.

In addition, the findings of this research showed that this primer is traceable for Persian sturgeon and irrelevant genomic DNA (Bacteria, Human, and Yeast) did not amplify any band with this primer.

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 and endangered species (e.g., sturgeon), particularly in aquatic environments (Lawson Handley, 2015; Barnes and Turner, 2016; Deiner *et al*., 2017). Species detection was done by isolating separate parts of mitochondrial DNA (mtDNA) obtained from the environment and amplified by the Thermos cycler (Lodge *et al*., 2006). 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 Persian sturgeon using eDNA is used as a model for tracing fish species in rivers and seas and can be considered a way to assess the population of Persian sturgeon in migrating rivers in 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 the natural

population of Sterlet in Volga headwaters and detect it in its native environment despite being thought to be extinct, but their research primers could also be applied for detecting other sturgeons. The identification based on DNA barcoding is even more complicated for the species of the "gueldenstaedtii complex". The BLAST sequences revealed 100% similarity of *A. gueldenstaedtii* with sequences from GenBank coming from the same species, but also for *A. naccarii* and *A. persicus*, two species also included in the "gueldenstaedtii complex". Similarly, *A. baerii* was also identified as *A. gueldenstaedtii*, *A. persicus*, and two hybrid species. Rastorguev *et al.,* 2013 identified eight single nucleotide polymorphisms (SNPs) which were sufficient to distinguish these sturgeon populations with 80% confidence, and allowed the development of markers to distinguish sturgeon species. Boscari *et. al*., 2014 concluded that DNA barcoding cannot discriminate between species of the "gueldenstaedtii complex" and is not recommendable alone for species identification in this group. Among quantitative or semi-quantitative assay methods used to determine the residual DNA, include 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). The conventional PCR method 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; but this method needs at least amount of DNA in nanograms level and picogram DNA level of residual DNA, which could not be amplified most of the time.

Conclusion

This research was the first effort for residual DNA detection of Persian sturgeon in farm sturgeon ponds. Even though the molecular diagnosis of this work was done on the Persian sturgeon residual DNA, this method can confirm only the existence of sturgeon species (in general) in fish ponds. SYBER Green real-time PCR method in this research proved to detect residual DNA in the picogram level of the Persian sturgeon fish pond and could be an acceptable method with high efficiency compared to other methods such as the conventional PCR method.

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Conflicts of Interest

The authors declare no conflict of interest.

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