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DNA PCR Amplification for Species Diagnosis of Caviar from Caspian Sea Sturgeon

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ABSTRACT

Samples from five sturgeon species including Persian sturgeon (*Acipenser persicus*), Russian sturgeon (*A. gueldenstaedti*), Great sturgeon (*Huso huso*),Ship sturgeon (*A. nudiventris*) and Stellate sturgeon (*A. stellatus*) were collected from the South Caspian Sea. DNA was extracted from the fins, eggs and skeleton muscle of the five species. RAPD markers were used for identifying different kinds of black caviar and sturgeon meat. The polymorphic band from RAPD amplification of DNA from two out of five sturgeon species was purified, cloned and sequenced. RAPD analysis was carried out in order to compare the five species using an operon primer set. Sequence Characterised Amplifed Region (SCAR) primers were designed and used to amplify caviar DNA from five sturgeon species. A marker of potential economic importance was discovered that is able to distinguish three species from caviar and also to distinguish Ship sturgeon caviar from osetra caviar (from Russian and Persian sturgeon) and also other species. This marker could assist international conservation and legal efforts to save what is left of the commercial Ship sturgeon populations which are endangered and whose caviar is at present substituted for the more expensive osetra caviar.

Keyword: Caspian Sea, DNA Marker, Sturgeon Species.

INTRODUCTION

Acipenseriformes are of great interest to biologists and the public. For biologists, they are of interest as a group that has been in existence for 250 million years. For the public, they are of interest because they are the source of caviar. Sturgeons (family Acipenseridae) and paddlefishes (family Polyodontidae), well known as sources of caviar, are threatened by unregulated overfishing, dam construction, eliminating access to spawning grounds, and by pollution (Birstein, 1993).

Strong demand for caviar, not helped by political and environmental difficulties, has caused overfishing in areas such as the Caspian Sea, causing great threat to the sturgeon fisheries. Cartel arrangements between the former USSR (1990) and Iran have disappeared, and fishing is now under the jurisdiction of five separate regions (Dobbs, 1992). These factors have not helped to protect sturgeon and sturgeon stocks will completely disappear if fishing pressure remains (Dobbs, 1992). Poaching is one of the main problems. In the past, the illegal catch of sturgeon was not more than 30% of the officially allowed catch, but has now reached 90% (Sturgeon Quarterly, 1995). The number of sturgeon has decreased from 200 million in 1990 to 50-60 million in 1995. The annual catch of sturgeon which was 28,000 tons by the former USSR (1990) and Iran is now about 4000 tons, and all countries try to fish sturgeon without any kind of control (Artimov, 1996 cited in Emadi, 1996). This means that Russia and the newly independent countries are fishing sturgeon two times more than in previous years, while today Russia legally catches only around 1000

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tonnes a year. Sturgeon fishing in Iran, a monopoly of the Iranian Fisheries Company, is under strict control and the maximum yearly catch is an average of the previous five years, catches. At present, the yearly allowable catch for Iranian Fisheries is 800 - 900 tonnes.

The size, colour, shape, toughness and taste of fish eggs depend on the species, although the morphology of fish eggs is similar in different species. Eggs are pigmented, semi-viscous, with yolk emulsion encased in a membrane. In sturgeon, fecundity is five thousand to six million per female. The eggs are normally size-graded into three size qroups as follows: Large at over 2.5 mm; Medium, at 2.2 to 2.5 mm,; and small, at 2.2 mm. There are various quality grades of caviar.

Although there are a number of reports on the population genetic study and phylogenetic study of sturgeon based on molecular biology techniques (Pourkazemi, 1996; Rezvani Gilkolaei, 1997), there are also few reports on species diagnosis of caviar. Protein electrophoresis (iso-electrofocusing) was used to distinguish caviar between different species by Keyvanfar (1987) and Rehbein (1985). Recently, the polymerase chain reaction (PCR) procedure was also used for species diagnosis of caviar (Rob and Bristein. 1996).

The disadvantages of these methods are as follows. In iso-electrofocusing, used by Keyvanfar (1987), a large quantity of eggs are required and the technique is not always accurate. In the PCR method using mtDNA that was discovered by Rob and Bristein. (1996), it is necessary to use different primers for discrimination of different species. Optimisation of PCR conditions needs much time. The present study investigates the possibility of using genomic DNA rather than mtDNA for developing markers that can be applied more quickly and more easily than previously mentioned methods.

The present study had the aim of finding specific markers to identify different kinds of black caviar and sturgeon meat using PCR amplification of nuclear DNA. This marker could be very important and useful for commercial purposes. Caviar from different species varies in price. Sometimes there are problems between dealers and customers because of use of the wrong species or mixing more expensive with cheaper caviar. A marker could solve such problems. It could also help in the protection and conservation of rare sturgeon species used for caviar.

MATERIALS AND METHODS

Extraction of DNA

Genomic DNA was prepared from fresh fish eggs (caviar), fin and muscle in liquid nitrogen or alcohol (absolute) and preserved following the method described by Hillis and Moritz (1990) with some modifications. Approximately 0.5-1µg of each DNA sample and control lambda DNA were run on a 0.8% agarose gel and stained with ethidium bromide to check its quality and approximate quantity. DNA concentration was measured on a spectrophotometer (Pharmacia) by taking readings at 260 nm. The RAPD protocol of Williams *et al*., 1990 was taken as the standard RAPD reaction. The optimization of RAPD was carried our for sturgeon genomic DNA with respect to the equipment and chemicals used. Only the optimal reaction conditions were used for most of the primers.

Analysis of PCR Product

PCR products obtained using RAPD primers and DNA from other species were run on an 8% polyacrylamide gel with marker fragments (marker VI) to detect the presence and size of amplified products.

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Staining and Purification of RAPD Products

Polyacrylamide gels were stained with ethidium bromide for 25 minutes. The specific bands were cut out on a long wave UV (365 nm) transilluminator and the gel pieces placed in spin-X centrifuge filter tubes (Costar UK Ltd). The samples were kept at -20°C for 30 minutes and then spun in the Spin-X filter tubes at 13000g for five minutes to extract and purify the DNA from the gel pieces.

Reamplification of Target DNA Fragments

Purified DNA was reamplified using the same random primer (OPF_{13}) that identified the RAPD polymorphism. The initial amplification condition was used as well as a modified amplification condition that involved decreasing the random primer concentration to 2.5 pM and the cycle number to 30.

The reamplified RAPD product was cleaned as follows. First, one to ten volumes of STE buffer and an equal volume of 4M ammonium acetate and 2.5 volumes of absolute ethanol were added, in turn, to the RAPD product. Second, the mixture was immediately spun down at 13000g at room temperature for 10 minutes to precipitate DNA. Third, ethanol was pipetted off followed by evaporation in a centrifugal evaporator. The pellet was resuspended in 50µl sterile distilled water.

Cloning

The TA cloning® kit with PCR^{Tm} II plasmid was used for the direct insertion of the purified PCR product. Fragments of a length approximately 320 bp from both Russian sturgeon and Persian sturgeon were cloned using the methods of Rezvani Gilkolaei (1997).

Recovering of Recombinant Plasmid

Recombinant plasmids DNA were extracted and purified using a QLA prep-spin Plasmid Miniprep kit (QIAGEN).

Recovery of the Inserted Fragment From Recombinant Plasmid

The inserted PCR products were recovered from the PCR^{TM} II vector by digestion with *Xba*I and *Sac*I. The inserted fragment was detected on an 8% polyacrylamide gel stained with silver nitrate.

Sequencing

The sequence reactions were prepared according to the cycle sequencing method as follows. 319 bp of the inserted fragment of both clones were read using the A.L.F (Automated Laser Fluorescent) procedure. The insert was sequenced using both a universal forward and reverse primer of the plasmid.

SCAR Primers, PCR Conditions and Electrophoresis

Extended primers were designed for both ends of the insert including the RAPD primer sequence using the Primer program Version 0.5. These primers were used to amplify the insert region specifically from total genomic DNA. The sequence of the

Table 1: Extended RAPD marker primers.

Name of Primer	Sequence of Primer	MW	Tm	Length
Forward primer	`5-GCTGCAGAAGAAGGGTCAC-3`	5871	62.6	19 bp
Reverse primer	`5-GGCTGCAGAACCCTTACATA-3`	6071	61.6	20 bp

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primers are given in Table 1.

PCR was carried out in 50µl with 40-60 ng of target genomic DNA, 5 pmol of each primer, 100 µM dNTP (Pharmacia), 1 unit of *Taq* DNA polymerase, reaction buffer (Promega Ltd.) and 1.5 mM magnesium chloride. The reaction was overlaid with 50 µl mineral oil. Amplification were performed in a thermal cycler (Hybaid, UK). The program for the first cycle of denaturation, annealing and extension was 94°C for 5 minutes, 61°C for 1 minute and 72°C for 1.5 minutes. This was followed by 29 cycles of denaturation at 94°C for 40 seconds; annealing at 61°C for 1.5 minutes; extension at 72°C for 10 minutes. An aliquot of 4 µl from the amplified DNA was loaded on a 5% polyacrylamide gel in 1x TBE buffer. Gels were stained with silver nitrate.

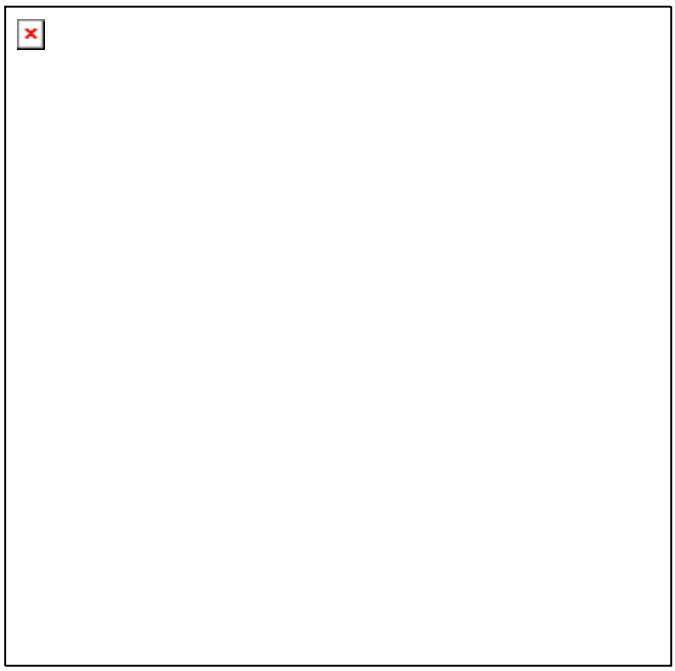
Single Strand Conformational Polymorphism (SSCP) Analysis

Eight µl of PCR product in 7 µl of water and 5 µl of sequencing buffer (United States Biochemical) was denatured at 95 °C for 10 minutes and chilled in ice for at least 5 minutes. The mixture was run on a 10% nondenaturing polyacryamide gel in 1x TBE buffer at a constant 300V at 15 °C for 4 hours using a recirculating cooling system (Hoefer Scientific Instruments). Gels were fixed and silver stained.

RESULTS

Genomic DNA Electrophoresis

High quality genomic DNA was extracted from the caviar taken from five species of sturgeon. Nuclear DNA and a more slowly migrating open circular mtDNA band are visible on the gel (Figure 1). The open circular mtDNA travels more slowly than the 23 kb lambda *Hind*III fragment. The concentration of nuclear DNA is clearly higher than that of mtDNA on the ethidium bromide stained gel. However, the fact that mtDNA is visible at all on an ethiduim bromide stained gel of genomic DNA is the result of



Figrue 1. The quality and quantity of genomic and mt DNA used for the RAPD analysis and search for markers for discrimination of caviar. Lanes 1-6 are the genomic and mt DNA from egg tissue of different sturgeon species and M is a Lambda *HindIII* fragment digest used as a control.

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the relatively high concentration of mtDNA in eggs.

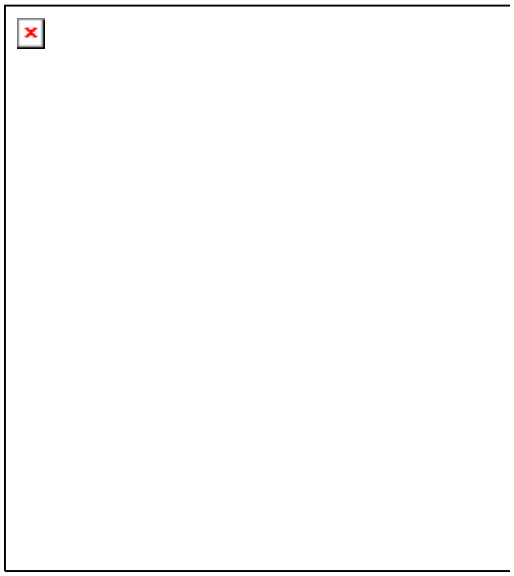
Identification of RAPD Markers

Figure 2 shows the RAPD banding pattern for primer OPF_{13} from five species of sturgeon. One of the smallest bands (of about 320 bp) occurs in Persian and Russian sturgeon and is thus specific for osetra caviar. It does not occur in Great Sturgeon, Stellate sturgeon and Ship sturgeon.

There was some variation between species using some other random primers-for example, OPD_{04} and OPD_{06}- but the OPF_{13} 320 bp marker was chosen for analysis in detail instead of testing more primers, because it seems to be a promising candidate for identifying different types of caviar.

Proving the Origin of a Marker

When extracting total DNA, genomic DNA and mtDNA are mixed. So, in order to know the origin of the specific band amplified with the OPF_{13} primer, genomic DNA and mtDNA were extracted separately using specific protocols. Both genomic DNA and mtDNA, produced amplification using the OPF_{13} primer. However, when the 320 bp band was cut out of the gel and the DNA purified, cloned and sequenced, comparison of the sequence obtained with the database (Genbank) showed no similarity with any published mtDNA sequences in other animals. This suggests strongly that the sequence is from nuclear DNA.



Figrue2. RAPD fragment pattern of primer OPF 13 from five species of sturgeon. Lane 1, Persian sturgeon. Lane 2, marker VI. Lane 3, negative control. Lane 4, Russian sturgeon. Lane 5, Shjp sturgeon. Lane 6, Great sturgeon. Lane 7, Stellate sturgeon.

Cloning and Sequencing Analysis

Transformed cells were grown on LB plates containing 1μg/ml ampicillin and X-gal (1-2%), and two types of colonies appeared, white and blue. The white colonies were approximately 8 to 10 per cent of the total colonies, and were potentially recombinants. Two white colonies and one blue colony were selected from Russian and Persian sturgeon for liquid medium culture and plasmid isolation.

The recombinant nature of the plasmids X was demonstrated by digestion of recombinant plasmid with *Xba*I and *Sac*I. Figure 3 shows non-recombinant and recombinant plasmid on an 8% polyacrylamide gel. The insert band is 422 bp because approximately 102 bp belongs to part of the plasmid that remains attached to the two ends of the inserted fragment in a digestion with *Xbo*I and *Sac*I. Two white colonies giving plasmid with the same insert size (320 bp) were used for sequencing. One of these was from Russian sturgeon, the other from Persian sturgeon.

The two sequences from Russian sturgeon and Persian sturgeon were identical (Figure 4). The sequence was compared with the DNA database (EMBL/Genbank) using the FASTA program. The sequence was also translated to an amino acid sequence which was compared with the database using the TFASTA program. The results of alignment with sequences in the database that there was not good evidence in these results of

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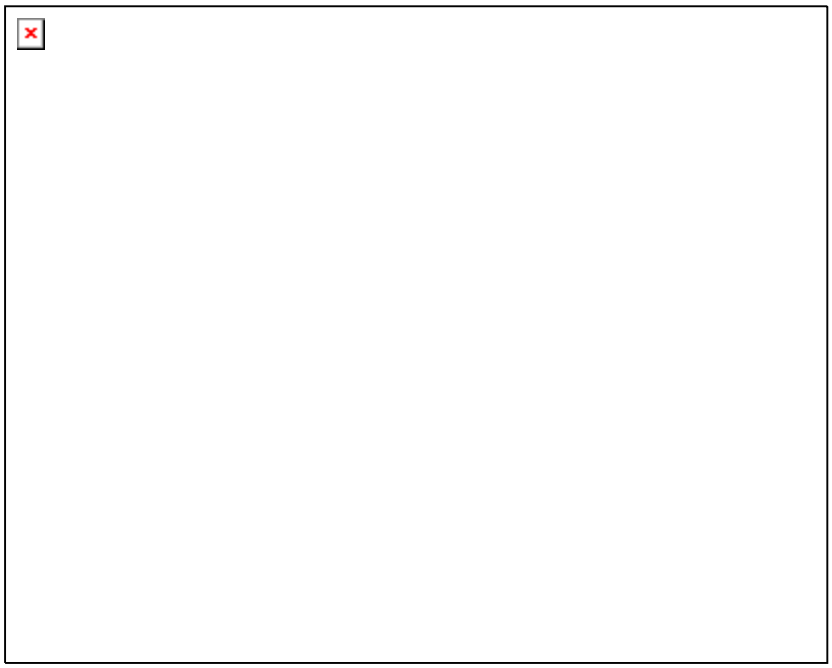

Figure 3. Restriction digest of clone using XboI and SacI restriction enzymes. Lane 1, linearised plasmid. Lane 2, digested plasmid having the cloned fragment (320 hp insert plus 102 bp belonging to plasmid). Lane 3, purified 320 bp PCR product. Lane 4, Lambda *HindIII* DNA marker.

homology with a fish gene or gene of any higher organism.

Amplification of DNA from Different Species

Extended SCAR primers were designed to amplify specifically the polymorphic RAPD marker from genomic DNA. The SCAR primers were tested on DNA from different species of sturgeon. It was hoped that they would only amplify the DNA of Russian and Persian sturgeon Caviar for which the specific band was obtained with OPF_{13}. However, the primers worked for three individuals of all five species. Two closely spaced bands were obtained for osetra and one band for the other species. Thus although the extended primers gave bands in all species, some specific bands were still produced for osetra caviar.

Single Strand Confirmation Polymorphism (SSCP) Analysis of SCAR Products

SCAR products were examined further to search for any differences undetectable by double strand DNA electrophoresis on a 5% polyacrylamide gel. The SSCP method was used for this purpose with the aim of revealing polymorphism within the SCAR products. The SCAR products of OPF_{13} showed SSCP polymorphism between species (Figure 5). The same patterns were obtained for Russian and Persian sturgeon caviar while the other species gave different patterns. The pattern for Stellate sturgeon (sevryuga caviar) had two bands suggesting homozygosity. Great sturgeon and Ship sturgeon produced more than two SSCP bands suggesting heterozygosity for the individuals examined. The same results were obtained, showing species differences, from repeated analysis of different fish from the five species. For this purpose at least three individuals were used in each species. Similar patterns

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  OPF13        +10         +20         +30         +40
    GGCTGCAGAA GAAGGGTCAC  GGCAAAGCTA AAATGTTTGA GGGTACGTTC
1         Forward primer  →--------- ---------- ----------
    CCGACGTCTT CTTCCCAGTG CCGTTTCGAT TTTACAAACT CCCATGCAAG
              +10         +20         +30         +40
    CGTAAACAGC CAATTGAAAT GCATCTAGCA GGCTGGGATG TTAACATTGT
51  ---------- ---------- ---------- ---------- ----------
    GCATTTGTCG GTTAACTTTA CGTAGATCGT CCGACCCTAC AATTGTAACA
              +10         +20         +30         +40
    GAGCAGTATA CTAAGATGGG CATTTTTATT CCATGCACCA GGGTTGAAAG
101 ---------- ---------- ---------- ---------- ----------
    CTCGTCATAT GATTCTACCC GTAAAAATAA GGTACGTGGT CCCAACTTTC
              +10         +20         +30         +40
    ATGAAGAGGT TACAGTGGAC CACATTCTGT CAAGCAAACA GTTTCAGCGG
151 ---------- ---------- ---------- ---------- ----------
    TACTTCTCCA ATGTCACCTG GTGTAAGACA GTTCGTTTGT CAAAGTCGCC
              +10         +20         +30         +40
    GAGAACGCCT TTGTCCAGGT AAGCAATTCG AGTCCTTCCT AATTCACCGT
201 ---------- ---------- ---------- ---------- ----------
    CTCTTGCGGA AACAGGTCCA TTCGTTAAGC TCAGGAAGGA TTAAGTGGCA
              +10         +20         +30         +40
    AATGTTGAAT TCTTTATAAC CAAGAGGAGC TGGCGATTTT GTAGTTATTT
251 ---------- ---------- ---------- ---------- ----------
    TTACAACTTA AGAAATATTG GTTCTCCTCG ACCGCTAAAA CATCAATAAA
              +10         +20         +30         +40
    ATGTAAGGGT TCTGCAGCC
301  ←Reverse primer
    TACATTCCCA AGACGTCGG
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Figure 4: Cloned fragment sequence. Underline sequence shows RAPD primer sequence position (OPF_{13}) and sequence in bold shows position of SCAR primers.

were obtained for DNA from caviar, skeletal muscle and fin tissue.

DISCUSSION

Protein electrophoresis has already been used for species diagnosis of caviar (Keyvanfar, 1987), but it required a large quantity of eggs and was not always accurate. Rob and Bristein. (1996) described the use of the polymerase chain reaction (PCR) for species diagnosis using several primers designed for portions of mtDNA. Their approach allows accurate and relatively inexpensive identification using a few eggs. They were able to identify each of three commercial sturgeon species, but it was necessary to use several different primers to identify the different kinds of caviar. In the present study, a single

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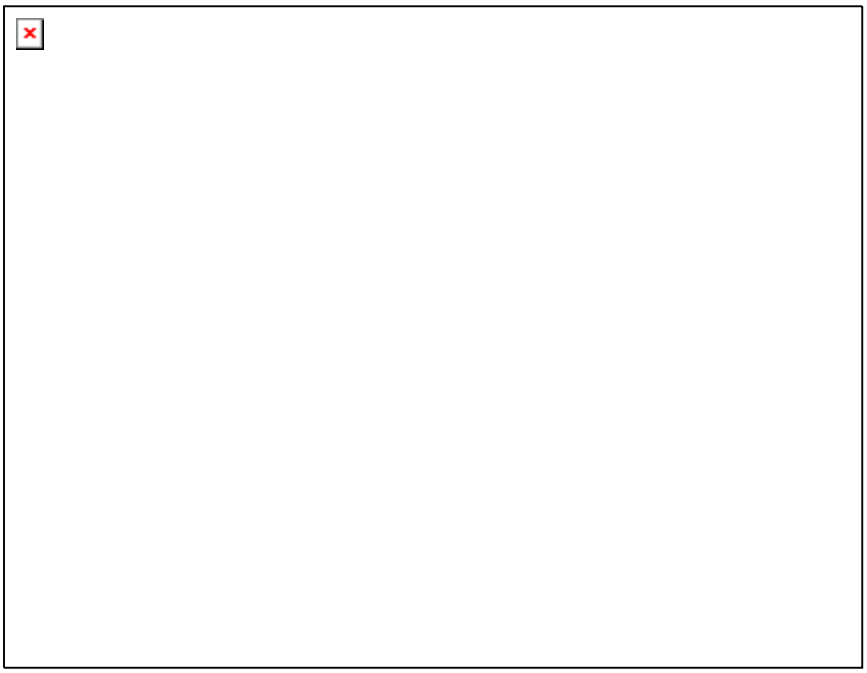

Figure 5. SSCP analysis of caviar discrimination marker. Lane 1, undenatured SCAR product from Russian sturgeon (two bands). Lane 2, denatured SCAR product from Russian sturgeon. Lane 3, undenatured SCAR product from Persian sturgeon (two bands). Lane 4, denatured SCAR product from Persian sturgeon. Lane 5, undenatured SCAR product from Stellate sturgeon (only one band). Lane 6, denatured SCAR product from Stellate sturgeon. Lane 7, undenatured SCAR product from Great sturgeon (only one band). Lane 8, denatured SCAR product from Great sturgeon. Lane 9, undenatured SCAR product from Ship sturgeon (only one band). Lane 10, denatured SCAR product from Ship sturgeon. Lane 11, DNA marker (marker VI).

pair of primers is available to identify four different sorts of black caviar as well as different species.

The results of the study on black caviar by Rob and Bristein., (1996) using 23 commercially available lots of caviar purchased in New York City and two brought from Russia, has also shown that there were five misrepresentations among the New York lots. Furthernore, the labels on the Russian lots did not identify the species correctly. Three of the misrepresentations were serious because the substituted species had a low value or were from an endangered species (William and Findeis., 1994). One involved caviar of Siberian sturgeon, (*A. baeri)*. This is of great concern because poaching of this species in Siberia has increased dramatically. Before 1991, the regulated annual catch of this species was 200-300 tonnes for all Siberian rivers. In 1994, in the Ob River alone, the illegal catch of Siberian sturgeon was about 250-300 tonnes (Ruban, 1996). Caviar of the Amur River sturgeon (*A. schrencki),* which is on the IUCN Red list of threatened animals, and of Ship sturgeon (*A. nudiventris)*, were also sold in New York as was that of other species. These two species are already extinct in the Aral Sea and are declining in the Caspian Sea.

At present, the level of Ship sturgeon fishing is very low in the Caspian Sea. The ship caviar has smaller size grain than Russian and Persian sturgeon caviar, has a grey colour and is usually a little cheaper than osetra. It is often put on sale as Russian and Persian sturgeon caviar. This shows that it is necessary to consider a stop in fishing for

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Ship sturgeon in the Caspian Sea. Use of the DNA marker developed in this study which can separate Ship sturgeon caviar from Russian and Persian sturgeon caviar could be used to help discourage merchants from selling ship sturgeon caviar as Russian and Persian sturgeon caviar.

Persian sturgeon was considered as a subspecies of Russian sturgeon (*A. gueldenstadti)* because of great morphological similarity while Berg (1933) had denoted it the subspecies (*A. gueldenstadti persicus)*. For the members of the order Acipenseriformes, such as Persian sturgeon, species identification is essential for plans to ensure survival. The use of DNA methods to identify the origin of caviar samples could assist international conservation and legal efforts to save the commercial sturgeon populations and help protect other species, that might be used instead of the disappearing commercial caviar species. At present, all sturgeon species are included in the appendices of the Convention on International Trade in Endangered species of Wild Fauna and Flora (CITES). Enforcement of the list depends on the ability to diagnose these species accurately.

At the present time it is necessary to decrease the general sturgeon fishery in the Caspian Sea, particularly stopping the Great sturgeon and Ship sturgeon fishery. Also, by developing sturgeon artificial reproduction capacity would be valuable, as would developing natural spawning areas and increasing the amount of fry released to rivers which are coming into the Caspian Sea. This would enhance wild stocks of sturgeon directly. Developing aquaculture of sturgeon for meat could help indirectly to develop wild population stocks. One way to increase the survival of sturgeon stocks would be to transfer a small population to an artificial lake around the Caspian basin which has conditions like that of the Caspian Sea. This would help prevent of extinction of sturgeon species, and could be of interest for fish biology researchers.

The DNA method developed here has some advantages over that of Rob and Bristein. (1996). The copy number of mtDNA is greater than the nuclear DNA copy number in caviar grain thus a few grains of caviar are needed for mtDNA extraction. However, this study shows that only 20-50 grains of caviar are enough for extraction of sufficient genomic DNA, using simple and the cheaper methods of DNA purification (Hillis and Moritz, 1990). Thus it might be cheaper and easier than the method of Rob and Bristein. *(*1996) without requiring much more caviar for analysis. Although the appearance of a lot of mtDNA in eggs is an advantage allowing extraction of mtDNA using a few eggs it requires the use of sensitive methods or extraction kits which are expensive for initial development. The RAPD method used here is better for lower technology laboratories.

For further study, the SCAR primers could be useful as a tool in phylogenetic and population genetic studies. To do this, it is necessary to carry out the sequencing experiment for all five species and carry out PCR amplification of DNA from different populations of each species using standard sample sizes. The development of more SCAR primers would be useful.

In conclusion, the method described here is not only able to distinguish all three species from their caviar but also is able to distinguish Ship sturgeon caviar from Russian and Persian sturgeon caviar and from other species. This could be useful as a tool to identify the origin of caviar samples. It will also assist international conservation and legal efforts to save what is left of the commercial ship sturgeon populations which are endangered because their caviar can be substituted for the more expensive osetra caviar.

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تشخیص انواع خاویار ایران به روش PCR

س. رضوانی گیل‌کلائی

چکیده

نمونه‌ها از پنج گونه ماهیان خاویاری شامل قره‌برون *(Acipenser persicus)* ، چالباش *(A. gueldenstaedti)*، فیل ماهی *(Huso huso)*، شیب *(A. nudiventris)* و ازون برون *(A. stellatus)* از قسمت جنوبی دریای خزر جمع‌آوری گردید. DNA از باله، تخم و ماهیچه پنج گونه مذکور استخراج شد. و نمونه‌ها به روش PCR با استفاده ازپرایمرهای سری RAPD تکثیر گردیده‌اند تا انواع خاویار و گوشت ماهیان خاویاری متمایز گردند. باند پلی مورفیسم ناشی ازPCR به روش RAPD که از دو گونه ماهیان خاویاری ظاهر گردیدند و باندهای پلی مورفیسم به روشهای فیزیکی از ژنهای الکتروفورز جدا و خالص گردیدند و سپس رشته‌ها DNA مورد نظر در باکتری کلون و نهایتا" به روش

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سیستم اتوماتیک لیزری توالی نکلوتیدها به دست آمد. پرایمر (SCARs) با استفاده از توالی اختصاصی دو انتهای رشته‌های DNA کلون شده، طراحی گردیده و جهت ازدیاد DNA خاویار پنج گونه مورد استفاده قرار گرفتند و پس از انجام انواع الکتروفورز، مشخص گردید که مارکر DNA برای شناسایی سه نوع خاویار و چهارگونه ماهی کشف گردیده است که این موضوع هم از نظر مسائل اقتصادی در حل اختلافات بین فروشندگان و خریداران خاویار می‌تواند مفید باشد و هم برای سازمانهایی که حمایت گونه‌های در معرض خطر مثل شیب و فیل ماهی را به عهده دارند می‌تواند شناساگر مناسبی باشد تا از طریق نمونه‌برداری از خاویار در محلهای توزیع به وجود یا عدم وجود خاویار گونه‌های ممنوع‌الصید پی ببرند. بهترین برجستگی این نشانگر ژنتیکی توانایی در تمایز چهارگونه از پنج گونه فوق است و تنها در جدایی بین گونه قره‌برون و چالباش که به عنوان خاویار آسترا به فروش می‌رسند قدرت کافی ندارد.

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