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Blue Economy and Biodiversity Surveillance: Fish Caviar Substitute Rapid Discrimination

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Abstract

The species identification is a key problem throughout the life cycle of fishes: from eggs and larvae to adults in ecosystem and fisheries research and control, as well as processed fish products labelling in consumer protection. Here, we report a rapid blue biotechnological method applied for egg and fish species discrimination. The amplification of a region of the mitochondrial genome, the cytochrome b, by using the Polymerase Chain Reaction (PCR) permits marine aquatic species identification. The obtained PCR-products were cut with different restriction endonucleases resulting in species-specific Restriction Fragment Length Polymorphisms (RFLP), which allowed to discriminate, in one step, between different types of caviar substitute species. The following analysis of mitochondrial DNA to control the labelling between caviar substitute species of Cyclopterus lumpus, Mallotus villosus, Trisopterus minutus minutus and the caviar, Acipenser baerii and their monitoring and surveillance is suitable with only one restriction enzyme, MboII. This blue method, applied to a very significant number of samples, retrieved online, allowed a rapid and economic identification of the species, with high percentage of correct identification for fish caviar substitute.

Keywords: Blue biotechnology; Monitoring and surveillance; Marine aquatic products; Caviar substitute

Introduction

The species discrimination is important in various fields, including food safety management and ecosystem research. After the removal of morphological characters, in fish roe or sturgeon as in any other fish, is necessary to add a correct product label, for traceability and allergens check [1-3]. Historically, the identification in the trade was based on a comparison of the size and color of the eggs [4]. However, these characteristics are highly unreliable. Usually, i.e., sturgeon spawn more times during their life cycle; further the size of the eggs can depend on the age of the females, or on the sturgeon species. The overfishing occurring in the last decades carried out to a decrease of the number of oldest females, used for the production of caviar. Consequently, the size of the eggs is decreasing [5,6]. Most of legally recognized methods of species identification are developed on the base of the analyses of specific proteins patterns, such as isoelectric focusing and immunoreactions [1]. However, these analyses are, in most cases, not applicable to thermally processed products as a result of severe protein denaturation. Alternatively, species identification using DNA, as nucleotide sequencing, Restriction Fragment Length Polymorphism (RFLP) and single-strand conformation polymorphism, are more useful and applicable even on processed products [see for review, 7-9] The analysis of nucleic acids, such as mitochondrial and nuclear DNAs, has advantages over protein-based analyses, as the method does not dependent on tissues and the age of the individuals. Furthermore, mitochondrial DNA (mtDNA) has several advantages over nuclear DNA in the diagnosis of fish products [10,11]. Mitochondrial DNA markers are frequently used in species identification, because they are not influenced by recombination and usually evolve faster than nuclear DNA, so it results a best marker of diagnostic substitutions. Anyway, since their uniparental inheritance in all vertebrates, they only record the genetic variability of the maternal lineages. This could lead to misinterpretations, for instance in the identification of hybrids and/or ancient introgression events [12]. However, the hybridization in the sturgeon occurs naturally at a low frequency [13]. In the last decades, the development of molecular DNA methods, including the PCR technique, has offered new opportunities for eggs identification too [10,14]. Previously published analytical DNA approaches were based on species-specific PCR amplifications and on the PCR-restriction fragment length polymorphism, or PCR-RFLP. Application on sturgeons in general, and on caviar in particular, began with Wolf et al., [15] for few species and then widely extended by Ludwig et al., [5] to 22 species of Acipenseridae [16]. This method is based on the amplification of a specific region from the mitochondrial genome, which is subsequently digested with restriction endonucleases resulting in species-specific restriction patterns, which allow to discriminate between the analyzed species with high specificity and sensitivity, as well as rapid processing time and low cost [17].

Our project investigates the fish egg species retrieved online in different websites by PCR sequencing and detects the restriction enzymes to utilize with a simple and less expensive experimental and diagnostic PCR-RFLP method for the specie *Cyclopterus lumpus*, *Mallotus villous*, *Trisopterus minutus minutus* and *Acipenser baerii*.

Materials and Methods

DNA extraction

Samples listed as caviar substitute *Cyclopterus lumpus*, *Mallotus villosus*, *Trisopterus minutus minutus* (n=20 each labeled species), and as caviar, *Acipenser baerii* (n=9), were bought online in Italian and Egyptian websites and analyzed by a molecular approach. The genomic DNA was extracted (100 mg /sample) as reported in Di Finizio et al., [17]. The DNA concentration and quality were assessed using spectrophotometry at 260/280 nm.

PCR amplification

One set of primers was used (forward primers F5'-CCATCCAACATCTCAGCATGATGAAA-3' and reverse primers R5'-GCCCCTCAGAATGATATTTGTCCTCA-3') [18] to amplify a fragment from rRNA/cytochrome b (cytb) gene in ten aliquots of each sample. PCR reaction was performed in Techgene Thermal Cycler (Thecne Ltd. Cambridge, UK). Thirty-five cycles of amplification were carried out in a reaction buffer containing 50 mM KCl, 10 mM Tris/HCl, pH 9.0; 10 mM NaCl; 0.01 mM EDTA; 2.5 mM of each dNTP; 1 µM of each primer; 20 ng of template DNA; 0.5 unit of Taq DNA polymerase (Invitrogen, Milan, Italy). PCR amplification conditions were as follows: denaturation at 94°C for 45s, annealing at 52°C for 55s, and extension at 72°C for 90s. At the end of the incubation, 5 µl of PCR products were separated by electrophoresis through 2% agarose gel and visualized under UV light. A 100 bp ladder (Fermentas, M-Medical srl, Milan, Italy) was used to estimate the fragment size of the amplicons generated.

Sequencing of the PCR products

Amplified DNA was desalted with Microcon 100 spin columns (Millipore-Amicon, Belford, MA, USA) according to the manufacturer's instructions and sequenced using Big Dye TM Terminator Cycle Sequencing Chemistry (Applied Biosystems, Foster City, CA, USA) in an automatic capillarity sequencer (model ABI 310 Genetic Analizer; Applied Biosystems). Primers for sequencing were the same used for PCR amplification. Resulted sequences were aligned and analyzed using BioEdit (Tom Hall Ibis Therapeutics, Rutherford Road Carlsbad, CA), Chromas 1.45 vs (Technelysium Pty, Tewantin, Australia) and Lasergene vs 4.00 (DNASTAR Inc., Madison, WI, USA) software. The fragments of cytb tRNA were than compared for control with GenBank and Bold sequences data for cytb rRNA belonging to those species examined using FASTA [19] and the percentage of similarity detected.

Phylogenetic analysis

Phylogenetic analyses were carried out using the software Mega 6 (Kumar et al., 1993). The genetic distances among the obtained sequences were estimated using the Neighbor joining method [20].

PCR–RFLP analysis

PCR products, derived from amplification of cytb mt-rRNA gene, were subjected to restriction digestion without previous purification. All the restriction enzyme used, were by Fermentas (M-Medical srl, Milan, Italy). Virtual restriction maps of cytb mt-rRNA gene sequences were obtained using restriction mapper3 tool [21] and a set of enzymes was chosen on the bases of the virtual pattern predicted by the program. Reaction mixtures included: 1µg of amplified DNA in a total volume of 50 µl, for a time of 1 hr at 37°C, in a shaking bath. At the end of the reactions, 10 µl were separated on 2% agarose gel containing ethidium bromide (1 µg/ml) and electrophoresed for 2.5 h at 100V. The sizes of resulting fragments were estimated by comparison with a commercial available 100bp ladder (Fermentas).

Results

PCR-sequencing and phylogenetic distances analysis

In samples labeled as *Cyclopterus lumpus, Mallotus villosus, Trisopterus minutus minutus* and *Acipenser baerii,* and bought online in different websites we successfully amplified a fragment of 389 bp length from the cytb mt-rRNA gene (data not shown). In Figure 1 is reported the natural bio-distribution map of the examined labeled species. The direct method used permitted to obtain sequences for all the samples. Results of species identification are show in Table 1. The sequences were subjected to BLAST searches against the NCBI DNA database and classified according to the sequences they aligned to with the highest score. For samples labeled as *Acipenser baerii* in seven cases out of nine, the identity degree is 100%, confirming the authenticity of the labeling; while for the other two specimens, the sequence analysis detected a 99% homology degree with *Cyclopterus lumpus*, highlighting a possible fraud case. An alignment of the two sequences was performed, and pointed out a 98% similarity degree, that means the DNA of the species analyzed is similar for long traits. This may simplify the substitution of species. For all the 20 samples labeled as *Cyclopterus lumpus* purchased through Italian websites and Egyptians ones, the molecular analysis conducted, returned a 99% identity degree with *Cyclopterus lumpus*. This means no fraud was accomplished. The same experimental tests, on *Mallotus villosus* samples, always ordered online from the same channels, confirmed the honesty of the labels reported on the cans. The identification score is 99%. The last species analyzed, *Trisopterus minutus minutus*, came from both Italian and Egyptian websites. Also for this investigation the sequence of the cytb mt-rRNA gene perfectly aligned, with the highest identity score, to ones yet present in the databases. Only 2 specimens out of 69 analyzed were mislabeled and they result fraudulent substitution of *Acipenser baerii*, with frequency of genetic substitution of 2.90% with *Ciclopterus lumpus*. These results suggest that, even to a limited extent, the most exposed species to fraudulent species, is the most expensive one: *Acipenser baerii* [22]. In order to give a complete vision of the processed data so far, a phylogenetic analysis was conducted (Figure 2), useful to classify the sequences and to graphically explain the homology relationship. The species are grouped in two different clades with low distance value: on the first there are *Trisopterus minutus minutus* and *Mallotus villosus*, on the second there are *Acipenser baerii* and *Cyclopterus lumpus*.

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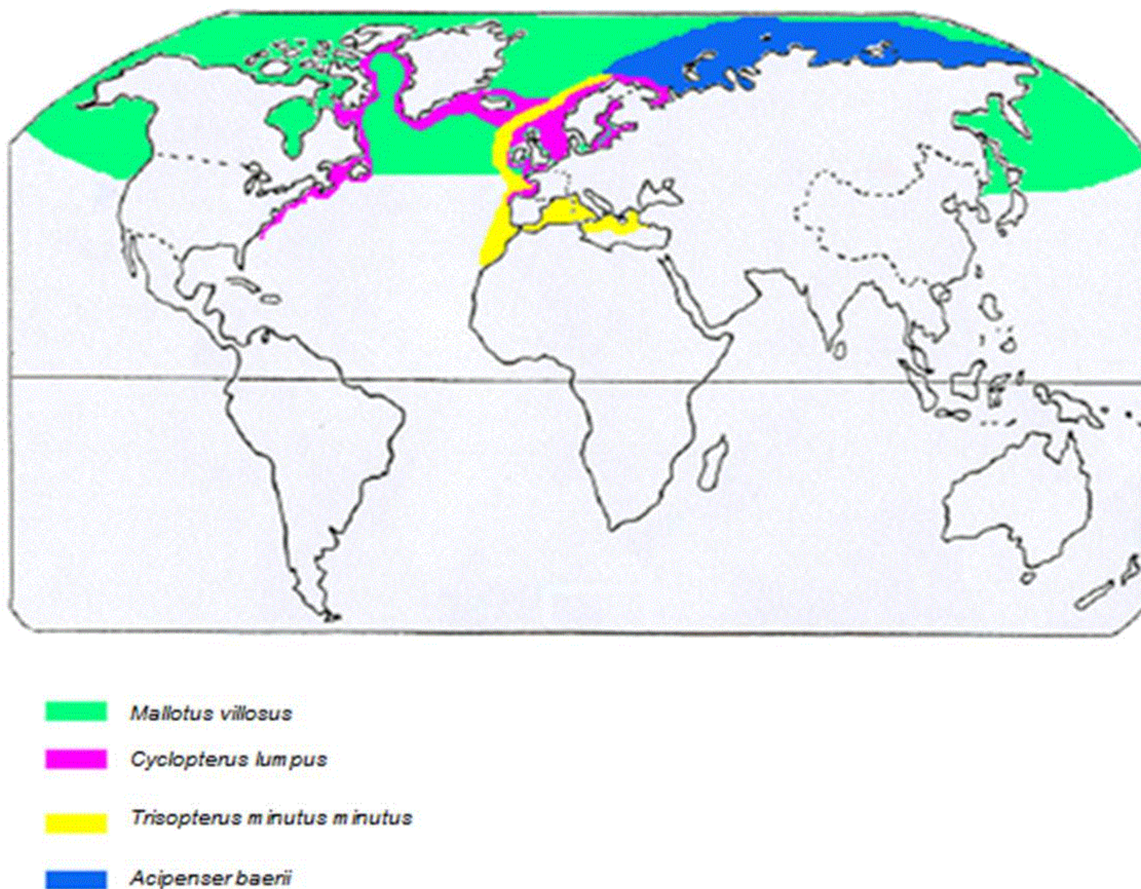

Figure 1: Distribution map of all of the analyzed species.

Number of samples	On label Expected species	Length (nucleotides)	Species identified	Identity (%)	Accession numbers (GenBank)	Mislabeling	Frequency of mislabelling (%)
9	*Acipenser baerii*	389	*Acipenser baerii* *Cyclopterus lumpus* *Cyclopterus lumpus*	100 100 100 100 100 100 100 99 99	JX238422.1 JX238385.1 **JQ045341.1** FJ010862.1 FJ010861.1 FJ010860.1 AF238653.1 EU492269.1 AM498333.1	NO NO NO NO NO NO NO YES YES	2.90
20	*Cyclopterus lumpus*	389	*Cyclopterus lumpus*	99 99 99 99 99 99	EU492269.1 EU492083.1 EF508313.1 EU492270.1 EU492084.1 AM498333.1	NO NO NO NO NO NO	0

20	*Mallotus villosus*	389	*Mallotus villosus*	99	DQ457494.1	NO	0	
				99	DQ457487.1	NO		
				99	DQ457484.1	NO		
				99	DQ457480.1	NO		
				99	DQ457469.1	NO		
				99	DQ457474.1	NO		
				99	DQ457459.1	NO		
				99	DQ457499.1	NO		
				99	DQ457500.1	NO		
				99	DQ457497.1	NO		
				99		NO		
20	*Trisopterus minutus minutus*	389	*Trisopterus minutus*	100	KP644339.1	NO	0	
				100	GU304596.1	NO		
				100	EU492138.1	NO		
				100	EU224044.1	NO		
				100	AF081693.1	NO		
				100	DQ174084.1	NO		
				100	KC981001.1	NO		
				100	AJ517485.1	NO		
				100	KC981003.1	NO		
				100	KC981002.1	NO		
				100	DQ174083.1	NO		

Table 1: Summary of species identification results from BLAST. Only maximum identities >99% are shown.

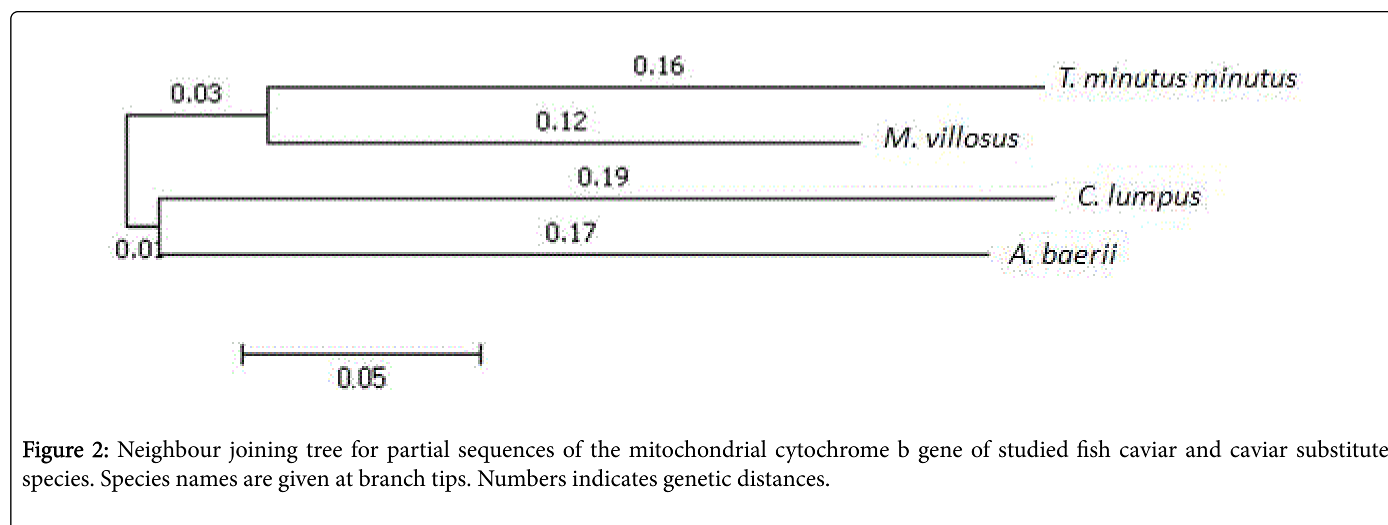

Figure 2: Neighbour joining tree for partial sequences of the mitochondrial cytochrome b gene of studied fish caviar and caviar substitute species. Species names are given at branch tips. Numbers indicates genetic distances.

PCR-RFLP analysis of fish eggs of caviar substitute

The PCR-RFLP analysis is a useful technique to study the intra and inter-specific variations between the examined organisms. Further it is easier and faster than sequencing and with reduced costs. Moreover, it is especially useful for routine analysis when sequencing is not an affordable option. After the analysis of the 389 bp sequences obtained using Restriction Mapper software, the *restrictase Mbo*II and *Hpa*II were selected as the most suitable enzymes to distinguish all of the species. These enzymes have simple recognition sequences (GAAGA for *Mbo*II, CCGG for *Hpa*II), characterized by a low cutting frequency: 1 or at least 2, and they present internal cutting sites, so as to produce digested fragments, easily observable by electrophoresis. A characteristic restriction profile was expected for each species, and it's shown in Table 2. Results following the digestion of the PCR products showed that the band sizes for the digested fragments may be deduced by the sequences analysis. The methodology was applied to all of 69 samples of each species. The electrophoretic pattern of the digested products is showed in Figure 3. The results are in line with the theoretical restriction analysis. For the sample 1, *Acipenser baerii*, a not digested product can be observed with the enzyme *Hpa*II, while the enzyme *Mbo*II produces two fragments of 134 and 255. For the sample 2, *Cyclopterus lumpus*, *Hpa*II does not act on the whole sequence and analogously *Mbo*II (lane 3 and 4). In the lane 5 are shown two digestion fragments respectively of 153 and 236 bp, produced by *Hpa*II, coherent with the theoretical internal cut position in *Mallotus villosus*, while *Mbo*II produces 3 fragments: 97, 132 and 160 bp. In *Trisopterus minutus minutus*, lane 7 and 8, both enzymes

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produce two bands of different molecular weight: *Hpa*II 179 and 210 bp, and *Mbo*II 41 and 348 bp. Control in lane C represents the only *Acipenser baerii* fragment of 389 bp amplified and not digested.

Nine specimens of *Acipenser baerii* bought from different online Italian and Egyptian online markets were investigated by RFLP and the electrophoretic profile was observed on a 2% agarose gel. The only enzyme *Mbo*II was used since from the previous analysis it results to be the most suitable to discriminate between the two species. The difference in migration of the fragments, in lanes 4 and 6, not digested due to the lack of the restriction site in the sequence, denotes the successful fraudulent replacement. By comparing the two gels, and selecting only samples processed with *Mbo*II (lane 2,4,6,8) we found a perfect overlapping of the pattern in correspondence of the lane 4. We therefore established that the samples 4 and 6, Figure 4, labeled as *Acipenser baerii* are actually *Cyclopterus lumpus*.

Discussion

Fish eggs identification: valence on conservation and management

In an increasingly globalized market nowadays has become always more easy to find commercial products even far away from specific places of production. The expanding criminal caviar trade is a serious threat to the survival of wild sturgeon populations, diminishes the effectiveness of management and conservation programs, undermines legal trade and threatens the sustainable production of caviar [14,23].

To contrast the continuing economic and social pressure it is spreading a trade of products with low production cost and low quality, also in more developed Countries. Additionally, the price for caviar differs considerably from different species. According to the United Nations' Food and Agriculture Organization, the fish roes from any fish not belonging to the Acipenseriformes species (including *Acipenseridae*, or sturgeon *stricto sensu*, and Polyodontidae or paddlefish) are not caviar, but "substitutes of caviar" [24]. Because of simple and relatively low processing technologies, several types of caviars are processed from roes of different fish, which are often local species so that marine fish roes, such as cod, mullet, herring are also frequently processed into caviars. The phenomenon of adulteration of caviar can spread a condition of strong distrust versus food production system, in consumers using biotechnological approach [10-11]. The species identification of caviars therefore becomes an important social issue in food inspection programs and need simple, reliable, inexpensive and fast method). Further, more important would be the fish eggs barcoding so that a strict control could be applied on all these valuable species.

The increasing sequence information acquired in DNA databases such as NCBI, EMBL and DDBJ over the past years comprises mitochondrial cytochrome b and 16S rRNA sequences of several sturgeon species [12,14,25-27]. Our study on *Trisopterus minutus minutus* (NCBI, cytb mt-rRNA; AY960856; COI in progress and *Mallotus villosus* 16S mt-rRNA; GU233808) enriched fish database permitting eggs, larvae and fish fragments identification as well the monitoring and surveillance of the sturgeon and caviar substitute species examined using mtDNA direct Polymerase Chain Reaction (PCR) sequencing and PCR-restriction fragment length polymorphism (RFLP) methodologies.

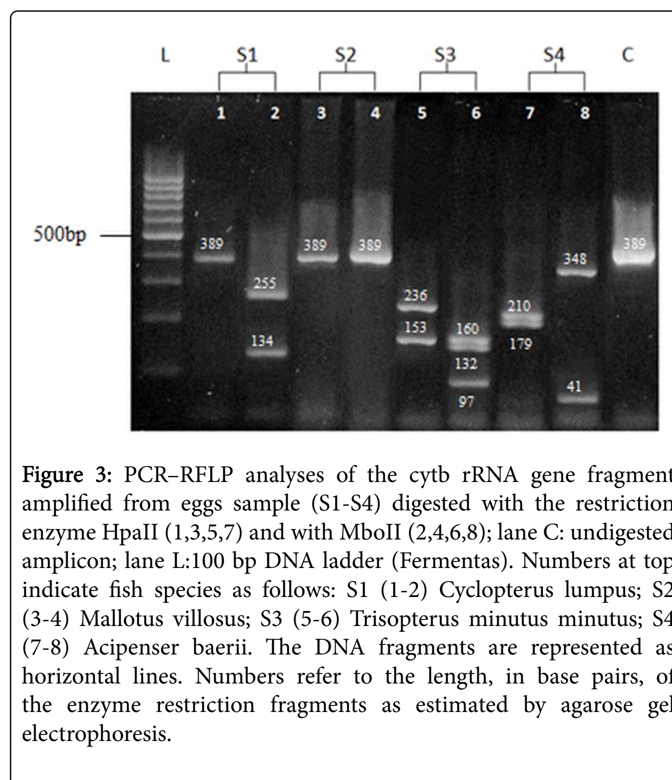

Figure 3: PCR–RFLP analyses of the cytb rRNA gene fragment amplified from eggs sample (S1-S4) digested with the restriction enzyme HpaII (1,3,5,7) and with MboII (2,4,6,8); lane C: undigested amplicon; lane L:100 bp DNA ladder (Fermentas). Numbers at top indicate fish species as follows: S1 (1-2) Cyclopterus lumpus; S2 (3-4) Mallotus villosus; S3 (5-6) Trisopterus minutus minutus; S4 (7-8) Acipenser baerii. The DNA fragments are represented as horizontal lines. Numbers refer to the length, in base pairs, of the enzyme restriction fragments as estimated by agarose gel electrophoresis.

Species	Number of restriction sites		Fragments position and size (bp)	
	HpaII MboII		HpaII MboII	
Cyclopterus lumpus	0	0	-/389	-/389
Mallotus villosus	1	2	236/153; 236	97; 257/97; 132; 160
Trisopterus minutus minutus	1	1	210/179; 210	348/41; 348
Acipenser baerii	0	1	-/389	134/134; 255

Table 2: Theoretical restriction fragment length polymorphism of the 389bp cytb mt-rRNA fragment in *Cyclopterus lumpus*, *Mallotus villosus*, *Trisopterus minutus minutus* and *Acipenser baerii*.

PCR sequencing and PCR-RFLP: role on examined fish eggs of caviar substitute and in the blue economy

We realized the necessity to try the PCR-RFLP approach, on the examined species, using the barcoded cytb mt-rRNA in relation to the low genetic distance detected through the phylogenetic analysis conducted using the Tamura and Nei method. This advantageous technique was introduced by Ludwig and Kirschbaum, [28] and Wolf et al., [15] for the identification of clustered sturgeon species, as well reported in Doukakis et al [29], but in relation to the species, the only cyt b PCR sequence analysis was used, as in eggs and meat fish species, purchased in the New York City area pre and post CITES listing, revealing fraudulent mislabeling.

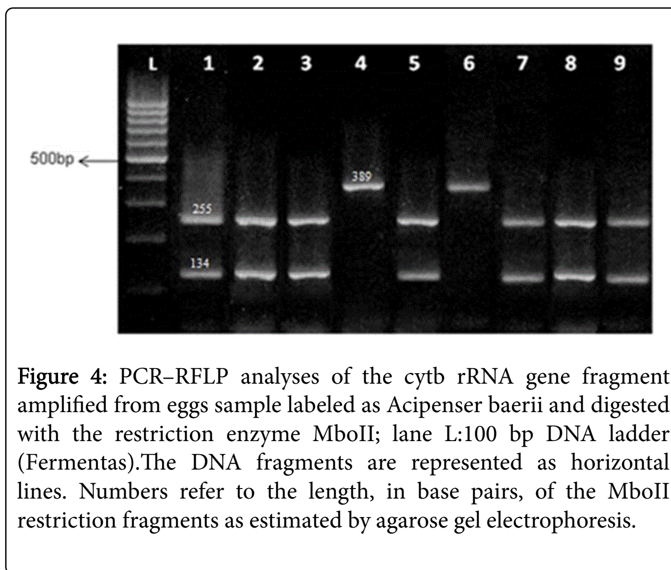

Figure 4: PCR–RFLP analyses of the cytb rRNA gene fragment amplified from eggs sample labeled as Acipenser baerii and digested with the restriction enzyme MboII; lane L:100 bp DNA ladder (Fermentas).The DNA fragments are represented as horizontal lines. Numbers refer to the length, in base pairs, of the MboII restriction fragments as estimated by agarose gel electrophoresis.

In our study the two theoretical enzymes, *Hpa*II and *Mbo*II, allowed a correct discrimination between the sturgeon species, *Cyclopterus lumpus*, *Mallotus villosus*, *Trisopterus minutus minutus* and *Acipenser baerii* and our experiments show that the discrimination is suitable with only one step too, using the restriction enzyme *Mbo*II. Taking into consideration the great commercial value of these species as caviar or caviar substitutes, this method showed a rapid way for exposing commercial frauds, like mislabeling of caviar lots, to avoid commercial frauds on the fish market [23,30].

The analysis carried out to distinguish the fish eggs variety going to define a real identity card, offering an efficient tool, fast and cheap, for monitoring the mislabeling identification for fish caviar substitutes examined. The caviar is a food whose importation is subjected to special constraints, and also in our research only the caviar and only two/nine cans were mislabeled. Very interesting is detecting a similar bio-distribution map of the adult fish of *Acipenser baerii* and *Cyclopterus lumpus*. In perspective would be interesting to carry out this type of investigation on a larger number of specimens of each examined egg species, from different geographical origin, to assess the frequency of fraud and changes in species with which it can be done and to extend the RFLP approach to other caviar substitute species.

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References

1. Mazzeo MF, De Giulio B, Guerriero G, Ciarcia G, Malorni A, et al. (2008) Fish authentication by MALDI-TOF mass spectrometry. J Agric Food Chem 56: 11071-11076.
2. www.foodallergy.org/allergens/fish-allergy.
3. Guerriero G (2013) Non-Native Species and Biodiversity Assessment. J Biodivers Endanger Species 1: e104.
4. Chen IC, Chapman FA, Wei CI, O'Keefe SF (1996) Preliminary studies on SDS-PAGE and isoelectric focusing identification of sturgeon sources of caviar. J Food Sci 61: 533-359.
5. Ludwig A, Debus L, Jenneckens I (2002) A molecular approach to control the international trade in black caviar. Int Rev Hydrobiol 87:661-674.
6. Ludwig A, Debus L, Lieckfeldt D, Wirgin I, Benecke N, et al. (2002) When the American sea sturgeon swam east. Nature 419: 447-448.
7. Teletchea F (2009) Molecular identification methods of fish species: reassessment and possible applications. Rev Fish Biol Fisheries 19: 265-293.
8. Guerriero G, Di Finizio A, Ciarcia G (2010) Biological Pollution: Molecular Identification of Non-Native Species in the Central Tyrrhenian Sea. CATRINA 5: 41-47.
9. Xiao TQ, Lu CY, Li C, Cheng L, Cao DC, et al. (2014) An AFLP-based approach for the identification of sex-linked markers in Amur sturgeon Acipenser schrenckii. J Appl Ichthyol 10: 1282-1285.
10. Basheer VS, Mohitha C, Vineesh N, Divya PR, Gopalakrishnam A, et al. (2015) Molecular phylogenetics of three species of the genus Rastrelliger using mitochondrial DNA markers. Mol Biol Rep 42: 873-879.
11. Onara D, Holostenco D, Paraschiv M, Suciu R (2014) Preliminary genetic variability of Lower Danube River young of the year (YOY) beluga sturgeon Huso huso (Linnaeus, 1758) using mtDNA markers. J Appl Ichthyol 10: 1286-1289.
12. Ludwig A (2008) Identification of Acipenseriformes species in trade. J Appl Ichthyol 24: 2-19.
13. Tranah G, Campton DE, May B (2004) Genetic evidence for hybridization of pallid and shovelnose sturgeon. J Hered 95: 474-480.
14. Ludwig A, Lieckfeldt D, Jahrl J (2015) Mislabeled and counterfeit sturgeon caviar from Bulgaria and Romania. J Appl Ichthyol 31:587-591.
15. Wolf C, Hubner P, Luthy J (1999) Differentiation of sturgeon species by PCR-RFLP. Food Res Int 32: 699-705.
16. Ramòn C, Domezain A, Garcia-Gallego M, Hernando JA, Fernando Rodriguez F, Manuel Ruiz-Rejòn M (2009) Eds: Biology, Conservation and Sustainable Development of Sturgeons. Volume 29 Fish & Fisheries Series 29. Springer Science & Business Media, 468 pp.
17. Di Finizio A, Guerriero G, Russo GL, Ciarcia G (2007) Identification of gadoid Species (Pisces, Gadidae) by sequencing and PCR–RFLP analysis of mitochondrial 12S and 16S rRNA gene fragments. Eur Food Res Technol 225: 334-337.
18. Carr M, Marshall HD (1991) Detection of intraspecific DNA Sequence Variation in the Mitochondria Cytochrome b Gene of Atlantic Cod (Gadus morhua) by the Polymerase Chain Reaction. Can J Fish Aquat Sci 48: 48-52.
19. Mount DV (2007) Using a FASTA Sequence Database Similarity Search. CSH Protocols.
20. Tamura K, Nei M (1993) Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 10: 512-526.
21. www.restrictionmapper.org
22. Dudu A, Georgescu SE, Dinischiotu A, Costache M (2010) PCR-RFLP method to identify fish species of economic importance. Archiva Zootechnica 13: 53-59.
23. Boscari E, Barmintseva A, Pujolar JM, Doukakis P, Mugue N, et al. (2014) Species and hybrid identification of sturgeon caviar: a new molecular approach to detect illegal trade. Mol Ecol Res 14: 489-498.
24. Rebeiz PG (2010) Caviar - a magic history, Sagep Editori, Genova, Italy ISBN 978-88-6373-103-3.
25. Ludwig A (2007) Identification of Acipenseriformes species in trade. IUCN/SSC Sturgeon Specialist Group and the IUCN Species Programme. IUCN, Gland, Switzerland.
26. Rehbein H, Molkentin J, Schubring R, Lieckfeldt D, Ludwig A (2008) Development of advanced analytical tools for determination of the origin of caviar. J Appl Ichthyol 24: 65-70.

27. Ludwig A, Arndt U, Lippold S, Benecke N, Debus L, et al. (2008) Tracing the first steps of American sturgeon pioneers in Europe. BMC Evol Biol 29: 8-221.
28. Ludwig A, Kirschbaum F (1998) Comparison of mitochondrial DNA sequences between the European and the Adriatic sturgeon. J Fish Biol 52:1289-1291.
29. Doukakis P, Pikitch EK, Rothschild A, DeSalle R, Amato G, et al. (2012) Testing the Effectiveness of an International Conservation Agreement: Marketplace Forensics and CITES Caviar Trade Regulation. PLoS ONE 7: e40907.
30. Guerriero G, Trocchia S, Alwany MA, Ciarcia G (2012) PCR-RFLP mitochondrial analysis mitochondrial analysis: rapid discrimination of Acipenser baerii and Trisopterus minutus minutus eggs. Biol Mar Mediterr 19: 255-256.

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