Discrimination of Sturgeon and Related Species by PCR-RFLP Using the Agilent 2100 Bioanalyzer System

Application Note

Food Authenticity

Author

Jens Bahrs-Windsberger
and Petra Buß
Bildungs- und Wissenschaftszentrum
der Bundesfinanzverwaltung
Dienstsitz Hamburg
22523 Hamburg
Germany

Lenore Kelly
Agilent Technologies, Inc.
Global Food Team
Santa Clara, CA 95051
USA

Steffen Müller
Agilent Technologies, Inc.
Global Food Team
76337 Waldbronn
Germany

Abstract

Species determination of sturgeon is an important issue to enforce regulations, monitor fraud in caviar shipments and assign import fees. A quick, robust and easy to use protocol to identify the species based on a well accepted PCR-RFLP method was tested with samples of sturgeon and other species. The improved method allows analysis from sample to result in one working day and yields good discrimination results.

Introduction

The global demand for seafood has grown considerably. Limitations of the resources and the potential for increased profits lead to the problem of substitution and mislabeling for a substantial part of the market (Jacquet and Pauly, 2008, von der Heyden *et al*., 2010, Miller and Mariani, 2010). In order to monitor shipments for customs purposes as well as in supply chain management and to protect the consumer, efficient and cost-effective tests to identify the species are needed. In addition, regulations to protect endangered species (CITES) and to fight illegal, unregulated, and unreported fishing activities (EC Council Regulation No. 1005/2008 and EC Commission Regulation 1010/2009, US Department of Commerce, Proposed Rules 50 CFR Part 300, Docket No.: 080228336-9133-01) drive the need for authenticity testing.

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DNA based testing methods allow sensitive detection and identification from almost all but the most heavily processed food samples. The use of DNA based methods for fish or seafood species identification has been described multiple times (Rasmussen and Morrissey, 2009).

Commonly, mitochondrial target sequences like the *Cyt*b or the *Cox*1 gene in combination with restriction analysis or sequencing have been used for identification of fish species (Russel *et al*. 2000, Espiñeira *et al.*, 2008, Yancy *et al.*, 2008). Dooley and co-workers successfully adopted and validated an earlier PCR-RFLP method using a *Cyt*b PCR target sequence and analysis of restriction fragment patterns on the Agilent 2100 Bioanalyzer (Dooley *et al.*, 2005). The availability of commercial screening solutions allows for more reliable and robust test results through well-matched components and facilitates testing for screening purposes by the use of mastermix formulations and streamlined protocols. The Agilent DNA Fish ID solution was evaluated for the purpose of identifying the species from fish eggs in caviar shipments. To obtain the best possible results using roe and to accommodate the high homology between sturgeon species, the protocol was modified for enhanced identification.

Materials and Methods

Fish samples used in this study

The samples and species used in this study are shown in Table 1.

Table 1. Samples Used in This Study. Samples are from Our Own Stock (1) or were Kindly Provided by Pat DeHaan of the US Fish & Wildlife Service, Abernathy Fish Technology Center (2)

Species	Common name	Tissue	Source
Acipenser gueldenstaedtii	Danube sturgeon	Roe	1
Acipenser medirostris	Green sturgeon	Fin clip	2
Acipenser nudiventris	Fringebarbel sturgeon	Roe	1
Acipenser ruthenus	Sterlet sturgeon	Roe	1
Acipenser schrenckii	Amur sturgeon	Roe	1
Acipenser stellatus	Starry sturgeon	Roe	1
Acipenser transmontanus	White sturgeon	Roe, fin clip	1,2
Huso dauricus	Kaluga	Roe	1
Huso huso	Beluga	Roe	1
Polyodon spathula	Mississippi paddlefish	Roe	1
Oncorhynchus keta	Chum salmon	Muscle	1

Isolation of DNA from fish samples

Fifty mg to 100 mg of tissue (muscle tissue, fin clippings) or 30 to 40 mg roe (2–5 eggs, depending on size) was used for DNA isolation. In the case of roe, the eggs were mechanically squashed before adding the lysis reagent. For efficient tissue lysis, including roe, the kit protocol was adapted as follows: Each sample received a mix containing 40 µL Proteinase K in 200 µL Proteinase K buffer. Digestion was performed at 65 °C for 40 min, vortexing the samples three times during the incubation period. Afterwards, the lysate was centrifuged for 10 min at maximum speed using a benchtop centrifuge. If the supernatant was not clear, an additional 5 min spin was applied. 150 µL of the clear supernatant were transferred to 500 µL of Nucleic Acid Binding Buffer avoiding any undigested material. The sample lysate and Nucleic Acid Binding Buffer mixture was added to a spin column and treated according to protocol. DNA was eluted from the column after two washes with 80% ethanol and a dry spin using 100 µL Elution Buffer prewarmed to 70 °C. DNA concentration and 260/280, 260/230 ratios were checked using a Nanodrop ND-2000 spectrophotometer (Thermo Fisher Scientific).

Amplification of *Cyt*b target sequence

PCR reactions were performed using 10 µL of 2 × mastermix, 2 µL of 10 × primer mix and 7 µL water using 1 µL of the purified DNA per sample. The PCR was run using a standard PCR thermocycler according to the kit protocol. The samples used for PCR amplification include the kit-supplied salmon (*Salmo salar*) positive control DNA and a no template control (NTC). Successful amplification of positive control and samples as well as a clean NTC were verified using the Bioanalyzer and a DNA1000 assay.

Restriction digestion of the PCR products

A 2.5 µL amount of the PCR reaction was used in the restriction digestion using the enzymes *Dde* I, *Hae* III and *Nla* III supplied with the kit and FastDigest *Csp6 I* (Fermentas, Germany) according to the protocol of the Fish ID PCR-RFLP kit. The restriction digestion was carried out for 2 h on a standard thermocycler with a final enzyme inactivation step as described in the protocol.

Analysis of restriction patterns using an Agilent 2100 Bioanalyzer

The digested samples and the positive control salmon DNA were run on a DNA 1000 chip according to protocol. For each sample, the four independent digests were loaded in consecutive wells, allowing the analysis of three samples per chip. The resulting electropherograms were analyzed using the Agilent 2100 Bioanalyzer Expert software.

Results

DNA derived from different sturgeon and salmon species was analyzed using the Agilent DNA Fish ID ensemble. As the kit-provided enzymes do not generate clearly distinguishable patterns due to the high homology of sturgeon *Cyt*b sequences, analysis of the target region for the PCR primers supplied with the kit was performed. The restriction endonuclease *Csp6* I was identified as a potential replacement for the enzyme *Dde* I which is used in the kit protocol (Figure 1). Modifications to the DNA isolation protocol were applied as described in the methods section to allow successful DNA extraction from roe.

Each sample was tested by two independent labs and in at least two independent experiments starting from the tissue material. Table 2a shows the averaged fragment sizes from all runs with standard deviation (Table 2b).

According to those results, the species used in this study can easily be grouped into six major clusters based on number and size of restriction fragments indicated by uppercase letters in Tables 2a and 2b. Group B and F can be sub-grouped due to repeatable, significant size differences within the group, indicated by a subscript number.

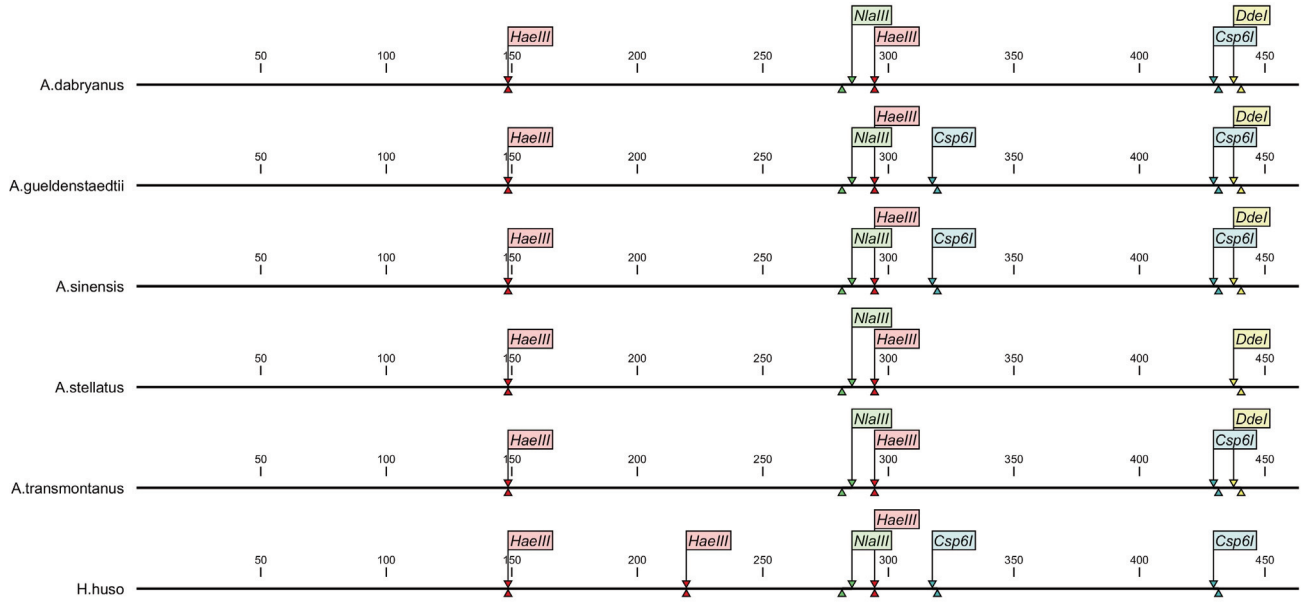

Fig. 1. Sequence analysis of Cytb target region in sturgeon species. The target region for the primers supplied with the kit was analyzed using CLC Sequence Viewer (CLC bio, Denmark). The picture shows the cutting sites for Csp6 I, Dde I, Hae III and Nla III for Acipenser dabryanus (derived from NC_005451), Acipenser gueldenstaedtii (derived from NC_012576), Acipenser sinensis (derived from NC_012646), Acipenser stellatus (derived from NC_005795), Acipenser transmontanus (derived from NC_004743) and Huso huso (derived from AY_442351).

Table 2a: Grouping of Samples According to Restriction Patterns Observed. Samples Were Grouped According to the Number of Fragments Observed With Each Restriction Enzyme. Subgrouping Was Added if There Was a Clear Difference (At Least Three Standard Deviations) in the Size of At Least One Fragment

Group	Species	*Csp6* I		*Dde* I			*Hae* III			*Nla* III		
A	*Acipenser gueldenstaedtii*	325	122	462			180	148		291	193	
	Acipenser medirostris	325	122	458			180	150		293	192	
	Huso dauricus	326	122	463			180	151		294	193	
B_1	*Acipenser transmontanus*	446		455			179	149		293	192	
	Acipenser schrenckii	445		457			180	149		292	192	
B_2	*Acipenser stellatus*	489		458			180	149		291	193	
C	*Huso huso*	323	120	455			179	150	77	291	191	
D	*Polyodon spathula*	481		480			323	150		291	165	
E	*Oncorhynchus keta*	389	86	359	352	121	433			280	192	
F_1	*Acipenser nudiventris*	325	93	462			180	149		293	133	52
F_2	*Acipenser ruthenus*	349	94	455			178	149		292	134	62
G	*Salmo salar*	397	85	362	356	121	330	107	46	460		

Table 2b. Standard Deviations of Fragment Sizes. Variability of Fragment Sizing is Between 0.5% to 2% of the Average Size

Group	Species	*Csp6* I		*Dde* I			*Hae* III			*Nla* III		
A	*Acipenser gueldenstaedtii*	1.2	1.2	2.5			0.6	0.6		2.3	2.6	
	Acipenser medirostris	1.4	0.9	3.7			1.1	1.3		2.4	1.5	
	Huso dauricus	1.4	2.1	2.8			2.8	2.1		2.1	2.1	
B_1	*Acipenser transmontanus*	3.8		3.3			1.3	1.3		1.2	0.9	
	Acipenser schrenckii	2.6		3.5			1.0	0.6		0.6	0.6	
B_2	*Acipenser stellatus*	5.3		4.5			1.7	1.4		1.0	1.7	
C	*Huso huso*	2.1	1.2	5.8			1.0	0.6	0.5	2.9	2.6	
D	*Polyodon spathula*	7.7		8.9			5.4	2.2		3.2	2.4	
E	*Oncorhynchus keta*	2.2	0.6	2.6	2.6	1.0	6.9			4.1	1.7	
F_1	*Acipenser nudiventris*	2.0	0.6	3.2			0.6	0.6		2.0	0.6	0.5
F_2	*Acipenser ruthenus*	1.8	1.5	2.6			1.0	1.0		0.5	0.6	0.6
G	*Salmo salar*	2.5	1.1	2.7	2.6	1.1	2.2	0.8	1.3	4.3		

Discussion

The usage of PCR-RFLP for species identification is a well established method. Compared to other methods of species identification one of its major benefits is the possibility to perform identification even from mixed samples. Reproducible and accurate sizing with high resolution to resolve fragments only having small size differences can be critical to assign the right species to an unknown sample. The method established by Dooley *et al.* on the Bioanalyzer enables identification of a large number of fish species.

Results show that with minor modifications the kit can be applied for the purpose of discrimination of sturgeon species. Although not all species in the study could clearly be differentiated from each other, the achieved grouping is sufficient for a first screening in routine analysis.

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