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(Article begins on next page)

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A NOVEL MINISEQUENCING TEST FOR SPECIES IDENTIFICATION OF SALTED AND DRIED PRODUCTS DERIVED FROM SPECIES BELONGING TO GADIFORMES

Dalmasso Alessandra*, Chiesa Francesco, Civera Tiziana, Bottero Maria Teresa

Department of Veterinary Sciences, University of Turin, via Leonardo da Vinci 44, 10095 Grugliasco (TO), Italy

* Corresponding author:

Alessandra Dalmasso

Department of Veterinary Sciences

University of Turin

via Leonardo da Vinci 44

10095 Grugliasco (TO)

Italy

Tel.:+39 11 670 9215

Fax: .:+39 11 670 9224.

e-mail address: alessandra.dalmasso@unito.it

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Abstract

The aim of the present study is to develop an assay for the specific identification of *Gadus morhua, Gadus macrocephalus, Gadus ogac, Molva molva* and *Brosme brosme* targeting sequences of the *cytochrome b* (*cyt b*) gene of mitochondrial DNA. The primers used in the preliminary PCR were designed in well conserved regions upstream and downstream of the diagnosis sites. They successfully amplified a conserved 188 bp region from the *cyt b* gene of all the species taken into consideration. The sites of diagnosis have been interrogated using a minisequencing reaction and capillary electrophoresis. All the results of the test were confirmed by fragment sequencing.

1 Introduction

An Italian national regulation (D.M. 31 gennaio 2008) establishes that the terms "stoccafisso “ and “baccalà” are reserved to *Gadus morhua* and *Gadus macrocephalus* dried and salted products, respectively. Since other dried or salted fillets labeled as *Brosme brosme, Molva molva* and *Gadus ogac* are commercialized, it is important to identify the species in order to detect fraudulent or unintentional mislabeling. The identification of species based on the anatomical traits of the whole fish, as the FAO dichotomous keys proposed, is the only one that has a legal value in Italy.

Furthermore, the commercialization of "stoccafisso" and "baccalà" in fillets makes it impossible to identify the species by means of morphological examination. As a result, reliable diagnostic procedures are required to identify and/or authenticate food items of animal origin, thus protecting animals, producers and consumers (Pascal & Mahé, 2001).

Many assays have been developed for the identification of different fish species, based on high-performance liquid chromatography (HPLC), isoelectric focusing (IEF), polyacrylamide gel electrophoresis, two-dimensional electrophoresis (2DE) and MALDI-TOF MS. However the low reliability of these techniques in processed foods has limited their use as a routine practice to identify and differentiate the species (Martinez, James, & Loréal, 2005; Carrera, Cañas, Piñeiro, Vázquez, & Gallardo, 2006).

Hence, molecular techniques are largely applied because they have proved to be reliable, specific, sensitive and fast. Recently, DNA based analyses have been widely used in fish species discrimination and mitochondrial genes have been already proved to be good markers. In particular *cytochrome b* (*cyt b*) gene contains species-specific informative sites that are useful for the reconstruction of phylogenetic analysis (Aranishi, 2005; Pérez & Presa, 2008).

Another target gene for the identification of species is the *cytochrome c oxidase subunit I* (COI). It has been widely used in the barcoding approach where the species are distinguished through the sequencing of a 655 bp fragment or a tight cluster of very similar sequences (Ward, Hanner, & Herbert, 2009).

Currently, species specific PCR is the most frequently used technique, since it is rapid, easy to perform and not excessively expensive (Teletchea, Maudet, & Hänni, 2005). However when the species are closely related, the high degree of similarity of the sequences could impair the design of species-specific

primers. In these cases it is advisable to apply techniques such as PCR-RFLP (PCR-Restriction Fragment Length Polymorphism) which is based on the analysis of a single diagnosis site. This method allows the amplification of DNA by PCR and the detection of genetic variation between species by digestion of the amplified fragment with restriction enzymes (Kelly, Carter, & Cole, 2003). PCR-RFLP analysis has been applied to identify several closely related fish species (Pardo & Pérez-Villareal, 2004; Espineira, Gonzalez-Lavin, Vieites, & Santaclara, 2008). However, the formation of secondary structures of DNA can limit the access of the enzyme to its target site, leading to an incomplete DNA cleavage. The consequent presence of indigested products causes a misinterpretation of the results (Quinteiro et al., 1998). Furthermore, the use of several restriction enzymes and the analysis of more than one diagnosis site in order to discriminate numerous species make PCR-RFLP difficult to automate (Bottero & Dalmasso, 2011).

Another method based on the analysis of the diagnosis sites, the minisequencing, has been recently proposed for the identification of several closely related species such as those belonging to *Thunnus* genus and some ungulates (Bottero, Dalmasso, Cappelletti, Secchi, & Civera, 2007; La Neve, Civera, Mucci, & Bottero, 2008). In particular, this method is based on the dideoxynucleotide triphosphate (ddNTP) single base extension of an unlabeled oligonucleotide (minisequencing primer) to the 3' end. Each minisequencing primer is designed immediately adjacent to the diagnosis sites and their length is modified by addition of non-homologous tails (poly-T) at the 5' end. Each ddNTP is labeled with different fluorescent dyes and a fifth color is used to label the internal size marker. The extended minisequencing primers used to interrogate different diagnosis sites differ in color and size.

In this study we developed a minisequencing test for the identification of five different species belonging to the Gadiformes order (*Gadus macrocephalus*, *Gadus morhua*, *Gadus ogac, Molva molva* and *Brosme brosme*). This technique is based on the amplification of a highly conserved region (188bp) of the *cyt b* gene, followed by the simultaneous analysis of four diagnostic sites comprised in this fragment.

2. Materials and methods

2.1 Samples and DNA extraction

Specimens of raw fish of five species belonging to the Gadiformes order were morphologically identified on the basis of external features (Cohen, Inada, Iwamoto, & Scialabba, 1990). In this way, 16 *B.brosme,* 14 *G.macrocephalus*, 11 *M.molva*, 7 *G.morhua* and 5 *G.ogac* were characterized. Furthermore, in order to evaluate the possibility of applying the technique to processed fish, 30 salted and dried products taken from the retail trade were analyzed.

DNeasy Blood & Tissue Kit (Qiagen, Germany) was used for DNA extraction from all samples.

2.2 Primers design

Cyt b reference sequences obtained from the GenBank database, corresponding to *G.macrocephalus* (GenBank accession no. DQ174044), *G.morhua* (GenBank accession no. EU492303), *G.ogac* (GenBank accession no. DQ174047), *M.molva* (GenBank accession no. EF427585) and *B.brosme* (GenBank accession no. EU492337) were aligned with the Clustal X program (Higgins, Bleasby, & Fuchs, 1992) for detection of polymorphic sites to be used as diagnosis positions (Table 1). All the available sequences on GenBank database for each of the five species, as well as all of the members of the Gadiformes order, were examined to confirm the absence of intraspecific/interspecific variations of the chosen diagnosis sites. Primers for the preliminary PCR (Cytbsn and Cytbasn) were designed in well-conserved regions upstream and downstream the diagnosis sites (Tables 1 and 2). Each minisequencing primer (P1, P2, P3 and P4) was designed upstream from the diagnosis sites and had different lengths of poly(dT) non-homologous tails attached to the 5’ end (Tables 1 and 2).

2.3 Preliminary PCR

PCR amplification was performed in a final volume of 50 µl containing 75mM Tris–HCl (pH 8.8), 1U of Recombinant Taq DNA Polymerase (Invitrogen, USA), 0.2 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia, Sweden), 2 mM $MgCl_2$, 25 pmol of primers Cyt bsn and Cyt basn and 50 ng of DNA template. PCR amplification was performed in an ABI 2720 thermocycler (Applied Biosystems, USA) beginning with 3 min at 94°C followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec and a final extension at 72°C for 7 min. Amplimers were resolved by electrophoresis on a 2.5% agarose gel.

2.4 Minisequencing reaction

The obtained PCR products act as templates for minisequencing reaction after the enzymatic clean up. To remove primers and unincorporated dNTPs, enzymatic purification was applied (Exo-Sap, USB Europe GmbH, Germany).

Before developing the multiplex assay, separate minisequencing reactions were set up for each single minisequencing primer. Afterwards, a multiplex minisequencing reaction was performed using the following concentrations of minisequencing primers: 0,6 µM P1, P3, P4; 0,15 µM P2. Both reactions were performed as indicated in the manufacturer's instructions (ABI PRISM SNaPshot Multiplex Kit, Applied Biosystems). The extension products were purified by phosphatase purification (New England Biolabs, UK). Samples were prepared by adding 1 µl of the purified extension product to 24.6 µl of formamide (Applied Biosystems) and 0.4 µl of GeneScan 120 LIZ size standard (Applied Biosystems). Each sample was loaded on the ABI 310 Genetic analyzer (Applied Biosystems) and analysed using Pop4 polymer (Applied Biosystems), a 47-cm capillary column (Applied Biosystems), the ABI GeneScan E5 Run Module. Electropherograms were analyzed using the GeneScan 3.1.2 software (Applied Biosystems).

2.5 DNA sequencing

DNA sequencing was carried out as a confirmatory test on amplification products of the preliminary PCR. Sequencing was carried out directly on the purified fragments with the ABI 310 Genetic analyzer (Applied Biosystems), using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 (Applied Biosystems). The nucleotide sequences were analyzed using BLASTN (Altschul, Gish, Miller, Myers, & Lipman, 1990).

3. Result and discussion

The effects of species substitution include economic fraud, health hazards, and the illegal trade of protected species. Therefore, the detection of species substitution has become an important topic within the food industry, and there is a growing need for rapid, reliable, and reproducible tests to verify species in commercial fish and other animal products (Rasmussen & Morrissey, 2008). In addition, species identification is useful in ensuring honest trading exchanges for correct consumer information (Carvalho, Neto, Brasil, & Oliveira, 2011).

Regarding the marketing of “baccalà” and “stoccafisso”, which are *G.morhua* and *G.macrocephalus* salted and dried seafood products, it is important to verify the exact labeling to exclude their replacement with species of minor commercial value (e.g. *M.molva, B.brosme, G.ogac*).

So, the aim of this study was the development of an assay for the specific identification of five species belonging to Gadiformes order, targeting sequences of the *cyt b* gene.

The *cyt b* gene was selected as template for DNA amplification because it has been used in species identification and in taxonomic and phylogenetic studies (Cook, Wang, & Sensabaugh, 1999; Kuwayama & Ozawa, 2000).

Analysis of the alignment of the reference sequences obtained from GenBank showed that the nucleotides in the positions 1020, 1023, 1065 and 1098 (GenBank acc. n. EU492303) of the *cyt b* gene could differentiate between the species of interest deriving in a specific pattern for each one: *G.morhua* (AACC), *G.ogac* (GTCA), *G.macrocephalus* (ATCA), *M.molva* (CATA) and *B.brosme* (TACG) (Table 1). These nucleotides did not show any intraspecies variation when they were compared with all the available sequences in the database (82 *G.morhua*, 10 *G.ogac*, 16 *G.macrocephalus*, 9 *M.molva* and 5 *B.brosme* sequences) (data not shown). Furthermore to validate the specificity of patterns, all the available sequences of species belonging to Gadiformes order were aligned (data not shown).

Preliminary PCR allowed the amplification of a 188 bp fragment in all samples, both the morphologically identified samples (53) and salted or dried products (30). When the DNA, extracted from raw samples, was tested in singleplex minisequencing reaction, it gave rise to a peak of the expected color that is specific to a diagnosis site. As for the multiplex minisequencing reaction, the reaction was optimized and the DNA extracted from the raw samples of the five gadoids yielded species-specific patterns (Figure 1). All of them gave rise to the expected species-specific pattern. In addition, the small size of the fragment allowed the amplification and characterization of the samples even though they had undergone highly denaturing treatments. It is well known that in the case of severely degraded substrates the length of the fragment to be amplified should not exceed 200 bp (Unseld, Beyermann, Brandt, & Hiesel, 2005). All the results of the minisequencing test were confirmed by fragment sequencing.

Applicability of the minisequencing assay to commercial samples was demonstrated. In fact it was possible to confirm the species indicated in the label for all samples. In particular 20 samples labeled “stoccafisso” and “baccalà” were identified as *G.morhua* and *G.macrocephalus.* Furthermore the minisequencing test confirmed the species for 10 commercial samples labeled as *M.molva* (7) and *B.brosme* (3).

This technique is as precise and reliable as sequencing and can be interpreted immediately. In fact discrimination of phylogenetically related species is possible with a single reading of the specific pattern generated by minisequencing.

4. Conclusion

The developed method has the potential to become a very appropriate tool for the authentication of *G.morhua* and *G. macrocephalus* in “stoccafisso” and “baccalà” products. The possible applications of this method are a normative control of raw and processed products, particularly the authenticity of imported species; the verification of the traceability of different fishing batches along the commercial chain; correct labeling and protection of the consumer’s rights; fair competence among fishing operators; and the fisheries’ control.

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Table 1

Diagnosis sites and primers binding site for preliminary PCR primers and minisequencing primers.

Species	GenBank Acc. N.	Aligned sequences 5' ----- 3'
		Cytb sn
Gadus macrocephalus	DQ174044	AAAA**CAACGGGGCTTAACATTCCG**CCCTCTTACCCAAATACTATTTTGAGTCCTCGTTGCAGATATAC
Gadus morhua	EU492303	.........A..T................................C...A..................
Gadus ogac	DQ174047	....
Molva molva	EF427585	T........A..AC.............C.................C.....A.....C.....C..GT
Brosme brosme	EU492337	C........A..GC.C........A..CT.C.....G..T..G..C.....A........G..C....
		P1 1020 1023 P2 1065
Gadus macrocephalus	DQ174044	TAGTTCTTACATGAATTGGAGGAGTACCTGTAGAACACCCCTTCATTATCATCGGACAAGTGGCATCT
Gadus morhua	EU492303	......................C..A
Gadus ogac	DQ174047	.........................G..
Molva molva	EF427585	.....T.A.................C..A.................C..T..T..............A
Brosme brosme	EU492337	.....T.A..............G..T..C.................C.....T..............A
		P3 1098 Cytb asn
Gadus macrocephalus	DQ174044	GTACTATATTTCTCCCTATTCCTAGTTTTATTCC**CCCTTGCAGGAATAACTGAAAA**TAAGGC
Gadus morhua	EU492303	.................C..
Gadus ogac	DQ174047	....
Molva molva	EF427585	..C..G..C..T..A........G..C.....T.......................C..A..
Brosme brosme	EU492337	..C..G..C..T..A........G..C................................A..

Primers binding site for preliminary PCR primers (Cytb sn and Cytb asn) are indicated in bold.

The bases in the box correspond to the diagnosis sites and correspond to positions 1020, 1023, 1065, and 1098 (as referred to GenBank acc. n. EU492303).

Primer binding sites of minisequencing primers P1, P2, P3 and P4 correspond to positions, 1004-1022, 1048-1064, 1078-1097 and 1001-1019 respectively, and are highlighted in gray.

Table 2. Primers for preliminary PCR and minisequencing.

	Primers	5' Position (*G.morhua* EU492303)	Oligonucleotides
Preliminary PCR	Cyt bsn	933	5'- CAACGRGGCYTAACATTCCG -3'
	Cyt basn	1121	5'- TTTTCAGTTATTCCTGCAAGGG -3'
Minisequencing	P1	1004	5'-THACATGAATTGGAGGVGT-3'
	P2	1048	5'-14(T) ATYGGACAAGTGGCATC-3'
	P3	1078	5'-21(T) TCMCTATTCCTRGTYTTATT-3'
	P4	1001	5'-29(T) TTYTHACATGAATTGGAGG-3'

Figure 1 - Multiplex PER.