APPLICATION OF POLYMERASE CHAIN REACTION FOR THE IDENTIFICATION OF HAKE (GENUS *MERLUCCIUS*)

WOJCECH SAWICKI, WALDEMAR DĄBROWSKI, ELŻBIETA DAcZKOWSKA-KOZON, ELŻBIETA BOGUSŁAWSKA-WĄS, AND ŁUKASZ BIENIASZ

Department of Food Microbiology, West Pomeranian University of Technology, 71-459 Szczecin, Poland
wojciech.sawicki@tz.ar.szczecin.pl

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Abstract

The aim of the study was to apply a polymerase chain reaction (PCR) to confirm a presence of hake (genus *Merluccius*) in a pool of processed seafood samples. A short fragment of the cyt b in the mitochondrial control region was amplified. The hake-specific PCR product, due to its limited size, was obtained in a variety of tissue samples. A different level of DNA concentration and degradation, including processed food products, was observed. A total of 64 products were tested and despite their labels, 13 of them were proved to contain no hake. Our protocol for hake identification is faster and more convenient than currently employed methods and may facilitate the identification of fish species.

Key words: hake, species identification, PCR, seafood.

Fish and seafood processing is the most impressively developing sector of food industry. In the 1990s, the consumption of fish and seafood products increased threefold. This phenomenon is linked to awareness of customers, who demand new sources of healthy and functional food as well as to actions aimed at the prevention of diet-related diseases (e.g. cardiovascular diseases, cancers, allergies, food intolerances).

More than 85% of fish undergoes a basic processing (i.e. beheading, skinning, filleting) directly after catching. This impedes or even makes impossible species identification with traditional methods. Further processing complicates species determination even more.

The application of reliable methods secures the customer rights to be informed about the real content of the purchased products. Mislabelling of the product may have serious economic and health-related implications. The authentication of fish and seafood is frequently violated by substituting an inexpensive, lower-quality species for one of higher value; therefore new, state-of-art methods have to be introduced to confirm the authenticity of such products (1, 13).

In order to exclude intraspecies cross-reactions, the PCR protocol was applied as to distinguish between the related species such as: pollock (*Theragra chalcogramma*), atlantic cod (*Gadus morhua*), pacific cod (*Gadus macrocephalus*), polar cod (*Boreogadus saida*), and saithe (*Pollachius virens*). In any case no amplification products were noted.

DNA isolation. DNA isolation was carried out using Genomic Mini AX Food extraction kit (A&A Biotechnology, Poland). Approximately 0.1-0.2 g of fish tissue was treated with lytic solution (LS lysis suspension) and 20 µL of protease K, mixed, and

Material and Methods

Selected products labelled as containing hake were subjected to the tests (Table 2). Frozen products were bought on retail market in the Western Pomerania region of Poland. They were stored at -32°C prior to analyses. The identification of the pan-dressed hake (hake carcass) to the species level was confirmed based on the morphometric features (7). In the analytical procedures to follow, the hake presented the positive control, with the DNA of pollock assumed as a negative one.

The aim of our work was to apply the polymerase chain reaction (PCR) to confirm the presence of hake (genus *Merluccius*) in a pool of processed seafood samples. Hake is one of the most popular deep-sea fish imported to Poland. The range of imported products includes: frozen fillets, fish cubes, battered and/or breaded fish sticks, and ready-to-eat meals.
incubated at 53°C for 80 min. The incubation was followed by 15 s of intensive vortexing (BioVortex V 1, Biosan, Latvia) and centrifuging for 5 min at 13,000 rpm. After the centrifugation, the supernatant was removed and the DNA-containing precipitate was washed with 70% ethanol, mixed, and centrifuged again (3 min, 12,000 rpm). Then, the ethanol was removed and precipitate was dried for 1 h at room temperature. Subsequently, the DNA was dissolved in a TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH 8.0). Its concentration and purity were evaluated spectrophotometrically (Biofotometer, Eppendorf, Germany). DNA isolates were stored at -4°C for a few days and then at -32°C. Primers used for genus identification are presented in Table 1. They were designed basing on mtDNA sequence (GenBank Accession No. AF112245-AF112255) according to Quinteiro et al. (10).

**PCR reaction.** An aliquot of 25 µL of reaction mix contained: 250 ng of DNA, 1 U of Taq polymerase (A&A Biotechnology), 2.5 U of polymerase Taq buffer (100 mM KCl, 100 mM (NH4)2SO4, 200 mM TrisHCl pH 8.5, 20 mM MgSO4, 1% triton X-100), 25 mM MgCl2 (Qiagen, Germany), 1.5 mM dNTP (Fermentas, Lithuania), and primers: MERFPD1 – 10 pM and GADRPD1 – 10 pM. The amplification was carried out in a Mastercycler Gradient (Eppendorf) according to the following temperature profile: initial denaturation 94°C/3 min, 30 cycles of denaturation 94°C/20 s, annealing 50°C/30 s, elongation 72°C/50 s. Amplicons were separated in 2% agarose gel (Prona Agarose Plus, Belgium) stained with ethidium bromide (1 µg/mL) (Bio-Rad, USA), visualised in the UV light and filed (GelDoc 2000, Bio-Rad). The size of the product was assessed when compared to the DNA molecular marker M100-500 (DNA Gdańsk, Polska).

The PCR-based surveys were conducted according to ‘Good laboratory practice when performing molecular amplification assays (5). All analytical procedures (e.g. sample preparation, DNA extraction and purification, DNA amplification, detection and confirmation of amplicons by electrophoretic partition of the PCR products) were carried out in three separate work areas supplied with own working facilities. Sterile, disposable equipment was used only for the testing procedure.

**Results**

All of the analysed products and methods for their processing are presented in Table 2. A total of 64 products containing hake as a main or supplementary ingredient were tested. Expected genus-specific amplification products were not observed in all the tested samples (Fig. 1). In 51 out of 64 tested products the results confirmed declared-by-producer/importer the presence of hake. No PCR product confirming hake content was detected in 13 samples.

All the tested samples of hake carcass contained genus *Merluccius* as declared by producer/importer. No hake-specific PCR product was detected in eight out of 30 cases of fillets. Neither four out of ten samples of hake cubes nor one out of six samples of fish sticks contained hake. Tests carried out on samples of breaded and/or breaded fish cake, fish fingers and ready-to-eat meals confirmed the content declared by producers.

**Table 1**

<table>
<thead>
<tr>
<th>Primers</th>
<th>The sequence of nucleotide primers 5’ – 3,</th>
<th>Product size</th>
<th>Genome region</th>
</tr>
</thead>
<tbody>
<tr>
<td>MERFPD1</td>
<td>TCAACCCCATATAAACWCATTCC</td>
<td>197 b.p.</td>
<td>cytb mtDNA</td>
</tr>
<tr>
<td>GADRPD1</td>
<td>ATGGACCTGAAGCTAGGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2**

<table>
<thead>
<tr>
<th>No. of samples*</th>
<th>Type of products</th>
<th>Treatment prior to sale</th>
<th>Declared content</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1-M8</td>
<td>hake carcass</td>
<td>frozen headless carcass,</td>
<td>hake</td>
</tr>
<tr>
<td>M9-M30</td>
<td>hake fillets</td>
<td>frozen, skinless, boneless fillets</td>
<td>hake</td>
</tr>
<tr>
<td>M31-M38</td>
<td>sea fish fillets</td>
<td>frozen, skinless, boneless fillets</td>
<td>hake</td>
</tr>
<tr>
<td>M39-M49</td>
<td>frozen fish cubes</td>
<td>frozen, skinless, boneless cutting fillets</td>
<td>hake</td>
</tr>
<tr>
<td>M50-M55</td>
<td>fish fingers</td>
<td>breaded, battered, frozen fish fingers</td>
<td>hake</td>
</tr>
<tr>
<td>M56-M60</td>
<td>fish cake</td>
<td>breaded, battered, frozen fish burgers,</td>
<td>hake</td>
</tr>
<tr>
<td>M61-M64</td>
<td>“ready to eat” meal</td>
<td>frozen fish meal (fillets) with vegetable and sauce</td>
<td>hake</td>
</tr>
<tr>
<td>K-</td>
<td>Alaska pollock carcass</td>
<td>frozen carcass</td>
<td>Alaska pollock</td>
</tr>
</tbody>
</table>

* M - *Merluccius* sp.
**Fig. 1.** 2% ethidium bromide stained agarose gel. The results of fish and fish products testing: M – the marker of molecular mass, K+ positive control (genus Merluccius – hake), K– negative control (Theragra halcogramma – Alaska pollock). 1-17 fish and fish products samples: 1 – M2, 2 – M5, 3 – M7, 4 – M8, 5 – M9, 6 – M15, 7 – M18, 8 – M20, 9 – M31, 10 – M34, 11 – M35, 12 – M39, 13 – M41, 14 – M48, 15 – M50, 16 – M58, 17 – M61

**Discussion**

In this paper we describe a novel protocol tested on several fish product groups, which enables the identification of fish belonging to the genus Merluccius. Hake is one of the most popular sea fish in Poland. It is available mainly as frozen and sold in the form of pan-dressed fish (fish carcass), fillets and battered and/or breaded products. The Merluccius genus consists of 11 economically important species. Fish belonging to the genus live mainly in Atlantic Ocean and in the east coast of Pacific Ocean (10-13).

Because on the Polish market there are various fish species offered under the common name of “hake”; therefore, in our protocol, instead of identifying respective species, we decided to identify the genus level. Identification to the genus level was based on mitochondrial DNA-specific primers according to Quinteiro et al. (10). Due to their conservative nature, mitochondrial genes are frequently employed for the identification of food products (4, 8-9, 12). Such a protocol of hake identification was tested on 64 samples characterised by a different level of processing. All products amplification obtained in this study revealed that the proposed method could be very useful in routine the control of fish products.

The validation method addressed to the PCR technique for the identification of Salmonidae was presented by Hold et al. (6). According to the inter-laboratory surveys addressed to 50 products, conducted by six labs, the 100% repeatability of the results was reached. The results obtained confirmed the method to be reproducible and handy for the analysis of commodity products. The results of the present work done in three repetitions with similar analytical procedure applied, proved to be repeatable.

Comi et al. (3) found PCR-RFLP to be one of the most effective techniques of identification normally complicated by the variety and close relation among many species of fish. Using this technique, the authors managed to distinguish eight commercially utilised species of Gadus. Calo-Mata et al. (2) applied PCR-RFLP for the identification of 15 species of Gadus among tested salted cod samples.

The RFLP technique was not applied in our study, for there was no need to do so. Nevertheless, the same set of primers used by Quinteiro et al. (11), let the authors to get the 156 b.p. amplicon characteristic for hake and when, using four restriction enzymes (Apol, Ddel, DraIII, and MboII), to establish the steps to follow in the identification to the species level. The same authors also proved the molecular techniques to be useful in the identification to the species level in the variety of scenarios including authentication of thermally processed food, detection of food components, or identification of individuals, whose morphological features were removed.

In conclusion, it could be stated that molecular biology techniques can play increasingly important role in the identification of fish and processed fish products, being an excellent alternative for traditional methods.

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**References**

5. Health Protection Agency: Good laboratory practice when performing molecular amplification assays. National

