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Molecular testing on sardines and rulings on the authenticity and nutritional value of marketed fishes: An experience report in the state of Rio de Janeiro, Brazil

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ABSTRACT

Members of the *Clupeidae* family are fish sold and consumed worldwide. Sardine species are the main representatives of this family, due to their nutritional qualities and contribution to the delivery of a wide variety of products and byproducts. The authenticity of seafood, due to high costs of analytical methods and sometimes limited availability, is a problem in fish trade. The authenticity of sardines marketed in the State of Rio de Janeiro, was evaluated by sensitive and unequivocal PCR-based techniques, such as RFLP and DNA sequencing. The *CYTB* mitochondrial gene was used for the screening of 170 sardine samples collected from markets, fish stores, street markets and canned samples from sardine factories. Sixty per cent of the collected fish were identified as *Sardinella aurita* (18.8%), *Sardina pilchardus* (25.9%), *Sardinops sagax* (2.9%), *Sardinops caeruleus* (0.6%) and *Opisthonema oglinum* (11.8%). The fraud samples were identified as *Clupea harengus* (4.7%), *Brevortia aurea* (21.2%), *Centengraulis edentulus* (6.5%) and *Scomber japonicus* (7.6%). *Sardinella brasiliensis*, considered the most abundant species on the south-eastern coast, was not found among the collected samples. The phylogenetic analysis of the marketed sardines showed that *S. sagax*, *O. oglinum*, *S. pilchardus* and *S. caeruleus* and fraud species as *S. japonicus* and *B. aurea* were clustered with a genetic distance of 0.1. A secondary cluster grouped only fraud species, such as *C. harengus* and *C. edentulus*, with a genetic distance <0.1. *S. aurita* appeared isolated with a genetic divergence >0.6. Our study also observed that theses frauds negatively changed the nutritional value of the product. Fraudulent species, such as *C. harengus*, *B. aurea*, *H. clupeola* and *C.* edentulus have lower protein content than authentic sardines. According to the Brazilian legislation with regard to labeling requirements, 40% of the samples were considered frauds by unequivocal molecular analysis.

1. Introduction

The world fish production increased from 9.9 tons in 1960 to 19.2 tons in 2012, mainly due to the increased demand for healthy and highly nutritious food, as well as due to the increase of purchasing power of developing countries (FAO, 2014).

Seafood substitution is a form of economic deception and has been prohibited through both domestic and international regulatory labeling laws (Martinez, James, & Loreal, 2005; Moretti, Turchini, Bellagamba, & Caprino, 2003; USFDA, 2006). Identification of fish species is also important to ascertain commercial frauds, mainly performed by replacing valuable species with others of lower value, especially in very transformed foodstuffs (breaded fillets) (Ardura, Planes, & Garcia Vazquez, 2011).

Fish belonging to the Clupeidae family comprise actinopterygii fish of high economic interest to the seafood industry, sold raw or processed as a wide variety of products and by-products. The sardine is the main species of this family (Herrero, Lago, Vieites, & Espinera, 2011) marketed fresh in fishmonger shops, fishing stores and street markets, with the canned product being considered the main commercial product (Granada, Flick, & Roy, 2012). Sardines

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(*Sardina* and *Sardinops*) are distributed in most of the temperate boundary current systems and *Sardinella* are found in subtropical and tropical zones (Ganias, 2015). Species recognition is essential for studies involving the extinction of some genetic variants and subsequent loss of intra-specific diversity, with unpredictable effects on species biodiversity (Ardura et al., 2011). Traditionally, fish identification is usually based on morphological and histological characteristics, but, primarily, external morphological characters. However, even within species, fish may exhibit morphological plasticity (Bottero, 2011).

Additionally, species recognition is also important in order to guarantee the authenticity of the fish species and fish products on sale in several types of markets. However, when morphological characteristics, such as shape, size or fish appearance, are obliterated during the processing phase, fish species identification becomes jeopardized and the fish, in fact, may not even be recognized by consumers. The growth in the marketing of filleted fish, both fresh or frozen, processed or not, is not always accompanied by captive production techniques of the species, which enables frauds to occur, by swapping high-value fish for other, low-cost species, that may present inadequate nutritional characteristics (Infante, Blanco, Zuasti, Crespo, & Manchado, 2007).

Currently, fish identification (certification) is needed in order to detect changes or frauds in fish trading in Brazil and in the international market, to avoid substitution of certain fish for higher availability and lower commercial value products (ECLAC, 2006). In Brazil, the official regulation determines that the product label describe the marketed fish species. The authenticity of marketed fish species has become a major challenge for the fish processing trade and fishing industry (Rasmussen and Morrissey, 2011).

Fish popularly called sardines are highly appreciated in Brazil, due to their low-cost and nutritional value, and because they are considered fish that display low heavy metal retention, since they are short lived (Tarley, Coltro, Matsushita, & de Souza, 2001), when compared with tuna, bonito and chub mackerel (Mol, 2011).

The term “sardine” in the present study shall be used exclusively for *Sardinella brasiliensis*, *Sardinella aurita* and *Sardina pilchardus*; the term “Pacific sardine” shall be used exclusively for *Sardinops sagax*, *Sardinops melanostictus*, *Sardinops neopilchardus*, *Sardinops caeruleus*, and the term “slab sardine” shall be used exclusively for *Opisthonema oglinum*. All these species are considered authentic by the current Brazilian regulation. The legislation is also applicable to canned sardines, which are intended for national and/or international trade, and the marketing of any other kind of species from the Clupeidae family under the name “sardine” is considered fraudulent as established by the technical resolution from the Ministry of Agriculture, Livestock and Supply (MAPA) Portaria 406, article 3 from August 10, 2010) (MAPA, 2010).

The municipalities of Rio de Janeiro and Niterói, located on the coastline of the state of Rio de Janeiro, are the two largest metropolitan areas of the region, where the fish distributed in the state are marketed. The availability of each sardine species is not known, and although there is a general suspicion regarding fraudulent marketing of Clupeidae family fish species, especially during the off-season between November and February and June–July, no scientific evidence is available.

The official methodologies to lend authenticity to fish species are based on the analysis of fish muscle proteins (AOAC, 2012). Although most of these methods show reliable results, they are not suitable for routine analyses in some cases, such as to differentiate between closely related species or products subjected to heat processing, since this process results in altered biological activity due to protein denaturation (Rasmussen & Morrissey, 2011). Molecular tests, such as PCR, are currently available. These methods are able to discriminate sardines from other members of the Clupeidae family (Herrero et al., 2011). PCR-based methods, when associated to nucleotide sequencing, allow for the unequivocal identification of the fish species present in food matrices, even after several processing steps, such as industrial sterilization (Teletchea, 2009). CYT B or COX1 are commonly used as target sequences for fish identification. Recently, a study developed an useful complementary approach to *COX1* barcode fragment sequencing, leveraging the ability to use the extensive fish barcoding sequence databases for primer development and restriction enzyme selection (Mueller et al., 2015) and the development of real-time or quantitative PCR (qPCR) methods in the field of fish DNA detection are focused on the use of mitochondrial DNA sequences as targets (Prado, Boix, & von Holst, 2013) or nuclear (Hird et al., 2011).

The aim of the present study was to identify the fish species names *“sardine”* traded in markets, fishing-warehouses, street markets and in industrial plants that produce canned sardines in the state of Rio de Janeiro, Brazil, by PCR tests targeting the *CYTB* associated to RFLP assays and nucleotide sequencing. The frequency of frauds in each market was calculated and the nutritional values of true and false sardines were compared.

2. Material and methods

2.1. Specimen collection

One hundred and seventy (170) samples of whole or eviscerated fish sold as sardines (fresh or frozen) were collected at different markets, fishing warehouses, street markets and in an industrial plant that produces canned sardines. All fishes were traded in the municipalities of Rio de Janeiro and Niterói, Brazil. Reference fishes were identified based on their external morphological characteristics by an experienced veterinarian. The collected samples were stored in polyethylene bags, duly sealed to ensure sample inviolability. The samples were transported in a portable cooler containing ice to the laboratory and stored at −80 °C until processing. Fishes collected at the industrial plant were sampled just before canning.

2.2. DNA extraction

Mitochondrial DNA templates for PCR tests were prepared from 30 mg of sardine muscle tissues using an automatic Maxwell® 16 extractor (Promega, Madison, WI, USA), with the Maxwell® 16 Tissue DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. DNA was quantified using the Qubit fluorimeter (Invitrogen™, Grand Island, New York, USA) and Qubit assays kit.

2.3. Amplification of the mitochondrial cytochrome b (CYTB) gene by PCR tests

A 147 bp fragment of *CYTB* gene was amplified using a primer pair (C-CB285 dF, CGCCCACATTGGNCGAGG and C-CB431R, GTGGCCCCTCAGAAGGACATTTGGCC) (Jérôme, Lemaire, Bautista, Fleurence, & Etienne, 2003). PCR mixtures contained 100 ng of DNA template, 10 pmoles of each primer (Integrated DNA Technologies Inc., Coralville, Iowa, USA), 1.5 mM $MgCl_2$ (Invitrogen™, Grand Island, New York, USA), 0.2 mM each dNTP (Fermentas, CA, USA), 1× of buffer (Invitrogen™, Grand Island, New York, USA), 1.5 U of Taq DNA polymerase (Invitrogen™, Grand Island, New York, USA) and nuclease-free sterile water in a final reaction volume of 50 μL.

DNA amplification was performed using the Veriti® 96-Well Thermal Cycler (Applied Biosystems Inc., Foster City, California, USA) under the following conditions: 95 °C for 5 min followed by 35 cycles of 95 °C for 30 s, 55 °C for 40 s and 72 °C for 40 s and a step