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Analytical Methods

Development of a multiplex PCR–ELISA method for the genetic authentication of *Thunnus* species and *Katsuwonus pelamis* in food products

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ABSTRACT

In the present work a PCR–ELISA technique for the authentication of *Thunnus* species was developed. This method is composed by four systems that can be used in a hierarchical way allowing the identification of several scombroids species; or each individual system independently. The hierarchical strategy, proposes a first step, to assign one sample to the *Thunnus* genus. Next, if the result is positive, several tests can be applied to assign the sample to some particular species of the *Thunnus* genus. In the case that the result is negative (absence of *Thunnus* species), it is possible to verify if *Katsuwonus pelamis* is included in the sample. The method even allows the detection of mixtures of these species in relatively low amounts (up to 1%). Finally, this method was applied to 11 commercial samples to verify the labelling status of tuna products in the market, detecting that 18% were mislabelling.

1. Introduction

The *Thunnus* genus belonging to the Scombridae family, includes 8 species which should be generally denominated by commercial labels as tuna. The economic importance of this taxonomic group at global scale is unquestionable. For instance, the mean total world catch of tuna species of 2006–2008 was of 4.387.909 tons (FAO, 2008), and this data reflects the importance of this taxonomic group as a food resource for humans, in fact due to this extensive consumption tuna are also known as "chicken of the sea".

Other important aspect is the great diversity of species included in the family Scombridae, since contains 15 genera and 54 species (Collette, 2001). The great similarity amongst all the Scombridae species in processed products make possible that, in some cases, species which do not belong to *Thunnus* can be fraudulently used as substitutes of tunas. To control the fraudulent use of non-tuna species as tuna there has been different labelling regulations. For instance, in Europe the *Regulation 1536/92* (EEC, 1992) details labelling issues of canned tuna and bonito, with the aim of prosecuting mislabelling and fraud.

In order to verify the correct labelling of food products, a lot of analytical methods have been developed up to date. Most of them are based on molecular techniques that allow the assignation of biological samples to level species (Bartlett & Davidson, 1991; Blanco, Perez-Martin, & Sotelo, 2008; Chapela et al., 2002; Santaclara, Cabado, & Vieites, 2006; Sotelo et al., 2001). However, some regulatory legislation allows the assignation of commercial labels at genus level, and this approach has not been dealt with up to date in the case of fish.

Also, the molecular techniques generally used are based on amplification of DNA by PCR and subsequent characterisation of PCR product by means of different techniques such as DNA sequencing, RFLP (Restriction Fragment Length Polymorphism) or SSCP (Single Strand Conformation Polymorphism) analyses. One alternative to these techniques is *ELISA* (*Enzyme-Linked InnmunoSorbent Assay*) associated to PCR. This approach has been used in some previous works such as the detection of allergenic substances (hazelnut) (Foetisch et al., 2011), detection of virus (Milne et al., 2007; Milne, Gallacher, Cash, & Porter, 2006) and even quantification

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(Musiani, Gallinella, Venturoli, & Zerbini, 2007) showing comparable results to RT-PCR (Perelle et al., 2004). In the particular case of fish and seafood, PCR–ELISA has not been used very often, and only few works have been published (Asensio, Gonzalez, Pavon, Garcia, & Martin, 2008; Asensio et al., 2004). However, due to the current accessibility of conventional PCR, added to the wide use of ELISA in many laboratories for several applications/purposes (detection of allergenic substances, diseases in animals, etc.) may promote that PCR–ELISA could be extended to other fields and applications, like the identification and authentication of fish species and fishing products.

Therefore, the aim of the present work is to develop several systems based on PCR–ELISA technique, that when used in a sequential way allows to: firstly, determine if a particular sample belongs to the *Thunnus* genus or to other taxonomic group. When this first test is positive (the sample belongs to *Thunnus*) some of the proposed systems allow the assignment of the sample to *Thunnus albacares*, *Thunnus alalunga – Thunnus orientalis* and *Thunnus thynnus*; and when it is negative, another system allows to determine if the sample is *Katsuwonus pelamis*. The methodology developed is adequate to discriminate the mentioned *Thunnus* species and *K. pelamis* from other scombroids, including those closely related with *Thunnus*, such as *Scomber, Auxis* or *Sarda*, in different types of processed products.

2. Materials and methods

2.1. Sample collection, DNA extraction and storage

Samples belonging to *Thunnus* genus and other Scombridae species were collected from several locations around the world by collaborators belonging to different research centres (Table 1) and were included in the IIM (*Instituto de Investigaciones Marinas*, Vigo Spain) tissue bank. When it was possible, the individuals were identified attending to morphological characters according to *FAO Species Catalogue* (Collette & Nauen, 1983). In other cases, the samples were authenticated in origin by collaborators.

DNA was extracted from 30 mg of muscle in fresh and frozen samples, and 200 mg in the case of processed samples, according to the method described by Blanco et al. (2008). The concentration and purity of the extracted DNA was measured using a *Nanodrop 2000* spectrophotometer (*Thermo Scientific*) at 260 nm and the ratio 260/280, respectively. Samples and DNA extractions were appropriately labelled and stored at −80 and −20 °C, respectively for subsequent studies.

Table 1
Samples included in this work.

Genus	Species	Common name
Thunnus	*T. alalunga*	Albacore
	T. albacares	Yellowfin tuna
	T. atlanticus	Blackfin tuna
	T. maccoyii	Southern bluefin tuna
	T. obesus	Bigeye tuna
	T. orientalis	Pacific bluefin tuna
	T. thynnus	Bluefin tuna
	T. tonggol	Longtail tuna
Auxis	*A. rochei*	Bullet tuna
	A. thazard	Frigate tuna
Euthynnus	*E. alletteratus*	Little tunny
Katsuwonus	*K. pelamis*	Skipjack tuna
Rastrelliger	*R. kanagurta*	Indian mackerel
Sarda	*S. chiliensis*	Pacific bonito
	S. sarda	Atlantic bonito
Scomber	*S. colias*	Atlantic chub mackerel
Scomberomorus	*S. sierra*	Serra spanish mackerel

2.2. Primers and probe design

The primers and the probes used in this work were designed from a set of sequences from NCBI (*National Center for Biotechnology Information*) (Accession Number: EU935746 – EU935790) and sequences available in the database of the research group. A multiple alignment was constructed from the sequences using BioEdit versión 7.0.0 (Hall, 1999), allowing the location of diverging regions between the *Thunnus* species, as well as the other sequences of scombroid included in the alignment, and several outgroups (specimens of *Genypterus*, *Epinephelus*, *Merluccius* and *Sardina* genera). These diverging regions were tested to design the primers and probe using the software *Primer Express 3.0* (*Applied Biosystems*). HPLC purification grade primers and probe herein designed were obtained from *Sigma Genosys*.

2.3. PCR amplification and DNA sequencing

In all cases PCR reactions were carried out in a total volume of 50 μL with the following composition: 50 ng of template DNA were added to PCR mix consisting of 5 μL of dNTP mix (*PCR DIG Labeling Mix*, *Roche*), 5 μL of 10× buffer (*Bioline*), 1.5 mM $MgCl_2$ (*Bioline*), 0.75 units of *BIOTAQ™ DNA Polymerase* (*Bioline*), 0.8 μM of each primer (*Sigma Genosys*) and molecular biology grade water (5 Prime) needed to adjust the final volume. The *PCR DIG Labeling Mix* is a mixture of the sodium salts of dATP, dCTP, dGTP, dTTP and digoxigenin-11-dUTP lithium salt.

Once verified the correct functioning of each PCR individually for the four designed systems, these ones were grouped in two duplex PCR. Several primer concentrations were tried, in order to obtain the same amplicon concentration in each duplex. The remaining conditions were the same used in simplex PCR.

PCR were carried out in a GeneAmp®PCR System 9700 (*Applied Biosystems*), in the following conditions: an initial step at 95 °C for 4 min, 35 cycles of amplification (95 °C for 15 s, 50–62 °C for 30 s, 72 °C for 45 s) and a final extension step of 72 °C for 3 min.

A positive control was also carried out in the same conditions, using the primers *FISH C+ F* and *FISH C+ R* (Santaclara, Espiñeira, Cabado, & Vieites, 2007) (Table 2).

PCR amplicons were visualised on 2% agarose gels (*Pronadisa*) in 0.5× TBE buffer (*Sigma*) with RedSafe™ 1× (*iNtRON Biotechnology*) using the Mini-Sub®Cell GT system (*Bio-Rad*). DNA fragments were visualised using the Gel Documentation System *Gel Doc XR System* and the software *Quantity One® v 4.5.2* (*Bio-Rad*). The *O'GeneRuler™ 100 bp DNA Ladder* (*Fermentas*) was used to estimate the size of the amplicons.

The PCR products were purified using the *Nucleospin Extract II kit* (*Macherey-Nagel*) according to the manufacturer's instructions. The concentration and purity were estimated by means of a *NanoDrop 2000 Spectrophotometer* (*Thermo Scientific*).

In order to verify the correct assignation of the specimens used in the set-up of the method, a new PCR product was amplified and sequenced on an *ABI Prism 310 Genetic Analyzer* (*Applied Biosystems*). This task was carried out using the primers *L14735* (Burgener & Hübner, 1998) and *CytBI-3R* (Sevilla et al., 2007) and the *BigDye Terminator Cycle Sequencing Ready Reaction Kit v 1.1* (*Applied Biosystems*) following the supplier's recommendations. The electropherograms obtained were analysed using the *DNA Sequencing Analysis Software v 3.4* (*Applied Biosystems*) and manually corrected with *Chromas v 1.45* (Mc Carthy, 1996). The nucleotide sequences were compared with those present in the NCBI database using the tool Megablast.

2.4. Development of PCR–ELISA systems

DIG labelled PCR products previously amplified and purified were used in the ELISA assay. This step was carried out using the