Identification of tuna species *Thunnus albacares* and *Katsuwonus pelamis* in canned products by real-time PCR method

Pavel Krcmar[1], Zora Piskata[1], Eliska Servusova[1,2]

[1]Veterinary Research Institute Brno, Brno, Czech Republic
[2] University of Veterinary and Pharmaceutical Sciences Brno, Faculty of Veterinary Hygiene and Ecology, Department of Meat Hygiene and Technology, Brno, Czech Republic

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Abstract

Tuna species are a popular food among consumers. They are mostly sold as heat-processed canned products on the market. Different quality and price of tuna species can lead the producer to the adulteration of food products. The main difficulties in developing a method for species identification in these fish is the high similarity of DNA sequences among close relative fish species. All complete mitochondrial DNA sequences of skipjack tuna (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares*) were compared to all other mitochondrial DNA sequences of tuna fish deposited in the GenBank. The most variable regions within species were determined and primers and probes were designed in this region for the species-specific DNA amplification of skipjack tuna and yellowfin tuna. Moreover, to check the content of amplifiable DNA of fish (namely tuna) in the sample, primers and a probe of mitochondrial *12S rRNA* gene in the region of conservative sequence were designed. Real time PCR methods were verified by investigating 51 samples of canned tuna with the declared content of tuna species from the market; the species was confirmed in all tested samples. This method was designed to be suitable for the determination of DNA sequences especially in highly heat treated products.

Yellowfin tuna, skipjack tuna, food authentication, species detection

Tuna is one of the most popular fish species on the food market. Most often they are offered in the form of canned products. Among the species used in cans, skipjack (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares*) dominate. Furthermore, the market offers raw or frozen fillets, most often yellowfin tuna. The different quality and price of other tuna species can lead producers to confusing the species. The Council Regulation (EEC) No. 1536/92 laying down common marketing standards for preserved tuna and bonito states specific rules for the tuna marketing. The species belonging to tuna and bonito are listed in the Annex of this Regulation. Tuna includes the genus *Thunnus* (*T. thynnus*, *T. albacares*, *T. alalunga*, *T. obesus* and others) and the species *Euthynnus* (*Katsuwonus*) *pelamis*. *Sarda* sp., *Euthynnus* sp. (except *Euthynnus pelamis*) and *Auxis* sp. are grouped among bonitos. One of the Regulation's paragraphs says that different species may not be mixed in the same container. According to DNA analysis of tuna species, several works have described different techniques based on multiplex PCR (Bottero et al. 2007; Michelini et al. 2007; Lin and Hwang 2008), real-time PCR (Lopez and Pardo 2005, Dalmasso et al. 2007, Chuang et al. 2012; Bojolly et al. 2017) and others. To differentiate individual fish species based on DNA sequences, an increasingly widespread DNA barcoding method can be used for raw, i.e. non-heat treated samples. This method is based on the sequencing of a 655 nucleotide long stretch of mitochondrial DNA encoding a portion of the cytochrome *oxidase* gene, and subsequent comparison of the obtained sequence with DNA Sequence Database (Handy et al. 2011). DNA is degraded during the manufacturing process of heat treated products, so a methodology that uses shorter DNA sequences for

Address for correspondence:
Pavel Krcmar
Veterinary Research Institute Brno
Hudcova 70, 621 00 Brno, Czech Republic

Phone: +420 533 331 913
E-mail: krcmarp@post.cz ; zorah@email.cz
http://actavet.vfu.cz/

species resolution is needed. This requirement is met by the real-time PCR method, which allows the determination of DNA sequences of 80 to 200 nucleotides. The main difficulty for developing a method for distinguishing these fish species based on the analysis of species-specific DNA sequences is the high identity of DNA sequences among related fish species. The complete nuclear DNA sequence is described only in *Thunnus orientalis* (Nakamura et al. 2013). The main focus was, therefore, put on mitochondrial DNA, as it has been described in all the species studied in several individuals of each species (Manchado et al. 2004; Catanase et al. 2008; Guo et al. 2016; Chen et al. 2016; Li et al. 2016a,b,c,d; Marquez et al. 2016; Pang et al. 2016a,b; Yang et al. 2016).

The aim of this work was to develop a method for the determination of skipjack and yellowfin tuna in highly heat treated products based on amplification of species-specific mitochondrial DNA sequences by real-time PCR.

Materials and Methods

Sample preparation

The muscles of yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*) and other tuna species were obtained from European Union markets. Their species identification was carried out according to their morphological features or were subjected to sequencing of the cyt *b* gene to confirm the species declaration. For the evaluation of the developed real-time PCR methods, 51 food products (canned products and pate) containing tuna fish were analysed. These commercial products were obtained on the local markets in the Czech Republic.

DNA isolation

DNA was isolated from 100–200 mg fish muscle using a commercial kit, DNeasy mericon Food Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. DNA was isolated from all samples in duplicate.

Table 1. Published complete mitochondrial DNA sequences of tuna and bonito (GenBank; https://www.ncbi.nlm.nih.gov/nuccore).

Tuna/bonito species	Complete mitochondrial DNA (Sequence ID)
Katsuwonus pelamis	KM605252, JN086155, GU256527, AB101290
Thunnus albacares	KP259550, KT724724, KM588080, JN086153, GU256528
Thunnus alalunga	JN086151, KP259549, GU256526, AB101291
Thunnus tonggol	HQ425780, JN086154
Thunnus thynnus	JN086149, GU256522, KF906720, AY302574, AB097669, AP006034
Thunnus atlanticus	KU955344, KM405517, KU955343
Thunnus orientalis	KF906721, GU256524, AB185022
Thunnus obesus	JN086152, GU256525
Thunnus maccoyii	JN086150, GU256523, KF925362
Auxis rochei	AB103468, KP259548, KM651784, AB105165, AB103467
Auxis thazard	KP259551, AB105447
Euthynnus affinis	AP012946, KM651783
Euthynnus alletteratus	AB099716
Sarda orientalis	AP012949

Probes and primers design

The primers and probes for real-time amplification and species specific determination were newly designed. Using Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) all sequences of complete mitochondrial skipjack tuna DNA (GenBank: KM605252, JN086155, GU256527, AB101290) and yellowfin tuna (GenBank: KP259550, KM588080, KT724724, GU256528, JN086153) were compared with all other mitochondrial DNA sequences of tuna (Table 1) contained in the GenBank (https://www.ncbi.nlm.nih.gov/nuccore). Areas that were variable within the same species were identified and excluded from further assessment. Furthermore,

Table 2. Sequences of designed primers and probes.

Target species	Primers/Probes	Sequence (5'-3')	Target gene	Amplicon
Thunnus albacares	Forward	5´-GCAAAAACCCCAGCG-3´	*NADH dehydrogenase subunit 2*	128 bp
	Reverse	5´-GGGGCTAGATCTTGCTTTGATAG-3´		
	Probe	5´CAGGATTAGTCATTTTGGCATGAAACCTG-3´		
Katsuwonus pelamis	Forward	5´-CTAGTGATTTGACACCTCGCT-3´	*NADH dehydrogenase subunit 1*	85 bp
	Reverse	5´-TTAGGCTTCAGGCACGACT-3´		
	Probe	5´-ATCGCATTTGCAGGTCTACCCCCTCA-3´		
Thunnus sp.	Forward	5´-GAGAATGCCCCACAGTTTTC-3´	*12S rRNA*	80 bp
	Reverse	5´-AAGCAAGGCGTCATGGG-3´		
	Probe	5´-AGGAGCT-3´		

the most variable interspecies regions were determined, and primers and probes for species-specific amplification of skipjack tuna and yellowfin tuna were designed in this section. The primers and probes were designed and synthetized by TIB MolBiol (Berlin, Germany). The probes were labelled on the 5'-end with the fluorescent reporter dye 6-carboxyfluorescein (FAM) and on the 3'-end were labelled with a quencher BBQ. The sequences of primers and probes are listed in Table 2. Additionally, primers and probe were designed to determine the presence of amplifiable fish (especially tuna) DNA in the sample to determine the part of the *12S rRNA* mitochondrial gene sequence in the fish-conserved region. For this system, a Locked Nucleic Acid (LNA) probe (Probe no. 17, Roche Diagnostic GmbH, Mannheim, Germany) was used. The probe was designed via Universal ProbeLibrary Assay Design Center (www.lifescience.roche.com/en_cz/brands/universal-probe-library.html).

Real-time PCR conditions

Amplification was performed with the LightCycler 1.5 instrument (Roche Diagnostics International Ltd., Rotkreuz, Switzerland) with the following programme: initial denaturation (50 °C for 2 min and 95 °C for 15 min), amplification (40 cycles of 95 °C for 15 s, and 60 °C for 60 s). The reaction mixture (10 µl) contained 5 µl QuantiTect Probe PCR Kit (Qiagen GmbH, Hilden, Germany), 2 µl of the isolated DNA sample, 1 µl of primer and probe solution (5 µM of each primer and 1 µM) probes), and 2 µl H_2O.

Method specificity

Besides the testing of specificity *in silico* the species specificity of the proposed primers and probes was also verified on DNA samples of the following tuna and bonito species: bullet tuna (*Auxis rochei*), frigate tuna (*Auxis thazard*), yellowfin tuna (*Thunnus albacares*), Atlantic bluefin tuna (*Thunnus thynnus*), albacore tuna (*Thunnus alalunga*), bigeye tuna (*Thunnus obesus*), southern bluefin tuna (*Thunnus maccoyii*), skipjack tuna (*Katsuwonus pelamis*), black skipjack or mackerel tuna (*Euthynnus affinis*), Atlantic little tuna (*Euthynnus alletteratus*), Atlantic bonito (*Sarda sarda*).

Detection limit

The detection limit was assessed by determination of the DNA concentration isolated from randomly selected three investigated cans of *Thunnus albacares* and three cans of *Katsuwonus pelamis* after dilution corresponding to 1% of the original content. DNA concentration was determined by fluorescence method using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) and quantified by a Qubit fluorometer (Thermo Fisher Scientific, MA, USA).

Real-time PCR efficiency test

To evaluate the efficiency of designed real-time PCR systems for *Thunnus albacares*, *Katsuwonus pelamis* and *Thunnus* sp., three samples for each canned tuna were tested. For each individual, DNA concentrations were adjusted by serial dilution in water to 10%, 1%, and 0.1%. The efficiency was estimated by plotting Cp values against the logarithm of the DNA concentration, with the efficiency = $[10^{(-1/\text{slope})} - 1] \times 100\%$.

Method validation on real samples

Real-time PCR methods for the determination of two tuna species were verified on 51 canned products with a declared tuna content purchased on the market. The food products contained tuna muscle in own juice, tuna muscle in olive oil, sunflower oil, soybean oil, tuna in brine, tuna in tomato sauce or tuna cream.

Results

All sequences of complete mitochondrial skipjack tuna DNA and yellowfin tuna were compared with all other mitochondrial DNA sequences of tuna contained in the GenBank and species-specific primers and probes for yellowfin tuna (128 bp long product) and skipjack (85 bp long product) detection were designed. For confirmation of the presence of fish DNA (especially tuna fish) in the sample, *12S rRNA* mitochondrial gene sequence (80 bp long product) was detected. The locked nucleic acid (LNA) probe is suitable for its short length (7 oligonucleotides), the possibility of designing a short amplicon (up to 100 base pairs), and easy commercial availability. Besides *in silico* comparison of complete mitochondrial DNA sequences of tuna and bonito deposited in the GenBank, the species specificity of the proposed primers and probes was also verified on DNA samples of the following tuna species: *Auxis rochei*, *Auxis thazard*, *Thunnus albacares*, *Thunnus thynnus*, *Thunnus alalunga*, *Thunnus obesus*, *Thunnus maccoyii*, *Katsuwonus pelamis*, *Euthynnus affinis*, *Euthynnus alletteratus*, *Sarda sarda* and no cross reaction was found (data not shown).

The detection limit for heat treated samples isolated under given conditions was established at 3.2 ng DNA/ml sample for *Katsuwonus pelamis* and 2.5 ng DNA/ml sample for determination of *Thunnus albacares*.

For validation of the detection systems, the linearity and efficiency were tested. The linearity was tested by plotting the Cp values (Cp - Crossing point, the point at which the fluorescence of the sample rises above the background fluorescence) versus the logarithmic of the DNA concentration (Plate VI, Figs 1–2, Plate VII, Fig 3). The efficiencies for all the systems ranged from 92.31% to 107.01%.

The suitability of the developed real-time PCR systems for canned tuna authentication was tested on 51 commercial products with a declaration of tuna species on packaging (28 were labelled as skipjack tuna, 23 as yellowfin tuna) that were purchased on the market of the Czech Republic. Samples with a Cp lower than 32.0 were considered positive. The lower the Cp, the more DNA the sample contains. Fish (tuna) DNA was detected in all samples. The cans labelled as skipjack tuna were confirmed when using primers/probes specific for skipjack tuna. Skipjack tuna was not detected in any commercial can labelled as yellowfin tuna. Similarly, in cans labelled as yellowfin tuna, the DNA of yellowfin tuna was confirmed using primers/probe for yellowfin tuna detection.

Discussion

Tuna species identification according to their morphologic features is impossible in highly processed food products. DNA-based analytical methods offer a solution, although the DNA is degraded into smaller fragments during the canning process, but these fragments are still detectable. Ram et al. (1996) claimed that the canning process degrades DNA to fewer than 123 bp in length. Moreover, DNA is largely independent of tissue source, age, or sample damage (Bossier 1999; Lockley and Bardsley 2000). Concerning canned tuna, most studies showed a preference for mitochondrial DNA in relation to nuclear DNA because of its relative abundance and circular structure, which provides higher resistance to thermal degradation (Bossier 1999). A close phylogenetic relationship exists among *Thunnus* species due to high homology in their DNA sequences. Consequently, lower interspecific (mostly single nucleotide polymorphism) but relatively high intraspecific variability among particular tuna species makes it difficult to design a specific approach for their identification. Several methodological systems based on the detection of species-specific DNA have been developed for species identification of tuna in both raw and preserved products [PCR-RFLP (Quinteiro et al. 1998, Pardo and Perez-Villareal 2004, Lin et al. 2005, Lin and Hwang 2007), PCR-SSCp (Colombo

et al. 2005), PCR-ELISA (Santaclara et al. 2015), multiplex PCR (Bottero et al. 2007; Michelini et al. 2007; Lin and Hwang 2008) or real-time PCR (Lopez and Pardo 2005, Dalmasso et al. 2007, Chuang et al. 2012; Bojolly et al. 2017)]. DNA barcoding involves PCR analysis followed by sequencing to identify species based on DNA polymorphisms (Botti and Giuffra 2010). However, its usefulness may be limited in the case of species identification of fish in products containing a mixture of more fish species or in highly processed products where DNA could be degraded into smaller fragments that are difficult to detect. In this study, the developed real-time PCR systems enable us to detect two tuna species and to eliminate false negative results, simultaneously. In general, the potential misrepresentation of declared and confirmed specimen can be regarded as a manufacturing mistake rather than a deliberate deception of the consumer by a less valuable product (i.e. the use of lower quality meat of another less valuable species).

Due to the high degree of heat treatment used in canned products which causes DNA degradation, it is relatively difficult to use absolute quantification for DNA determination. The detection limit was assessed by determination of the concentration of DNA isolated from three randomly selected cans after dilution corresponding to 1% of the original content. This limit has been set at 1%, following the request of the tuna canning industry, which allows discrimination between voluntary substitutions from involuntary substitutions (Bojolly et al. 2017).

In conclusion, this developed real-time PCR method has been designed to maximize species-specific assays using all current knowledge of tuna fish DNA sequences. The mitochondrial sequences that are most variable among related tuna species have been used to design primers and probes and are not variable within the species. The use of DNA sequences with these properties is a major difference from the methods outlined above. The method was further designed to be suitable for the determination of DNA sequences in highly heat treated products.

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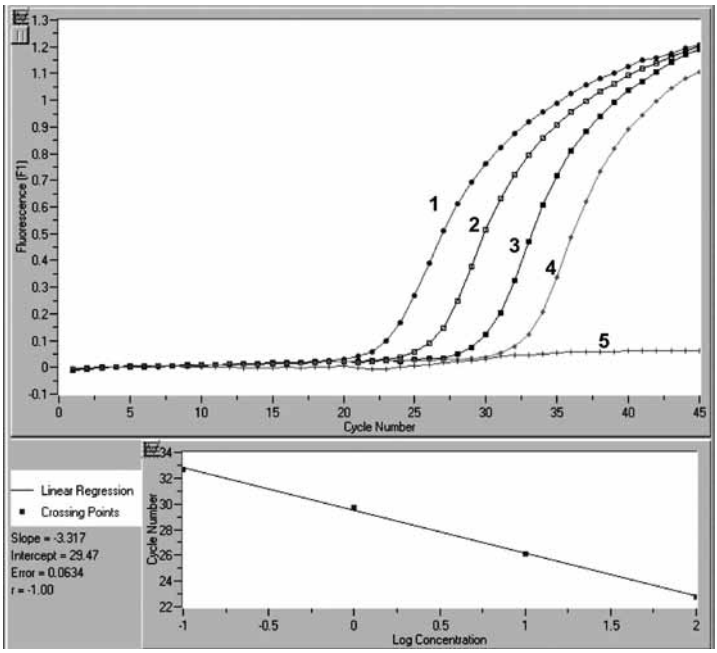

Fig. 1. Calibration curve for amplification of *Thunnus albacares* DNA
Serial of DNA dilution: 100% (1), 10% (2), 1% (3), 0.1% (4), water (5).

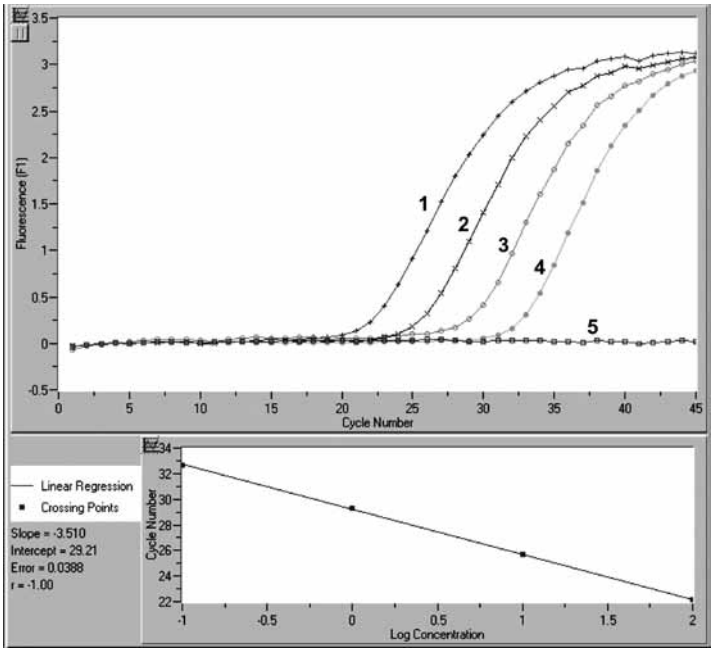

Fig. 2. Calibration curve for amplification of *Katsuwonus pelamis* DNA
Serial of DNA dilution: 100% (1), 10% (2), 1% (3), 0.1% (4), water (5).

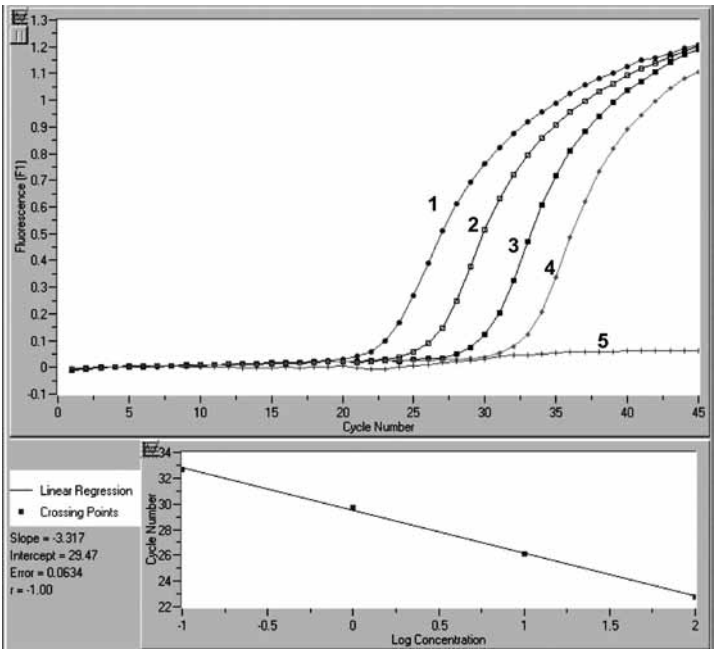

Fig. 3. Calibration curve for amplification of *Thunnus* sp. DNA
Serial of DNA dilution: 100% (1), 10% (2), 1% (3), 0.1% (4), water (5).