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Development of a qPCR Method for the Identification and Quantification of Two Closely Related Tuna Species, Bigeye Tuna (*Thunnus obesus*) and Yellowfin Tuna (*Thunnus albacares*), in Canned Tuna

Bojolly Daline [1, 6, 8], Doyen Périne [1, 2, 3, 4, 5], Le Fur Bruno [8], Christaki Urania [6], Verrez-Bagnis Veronique [7], Grard Thierry [1, 2, 3, 4, 5, *]

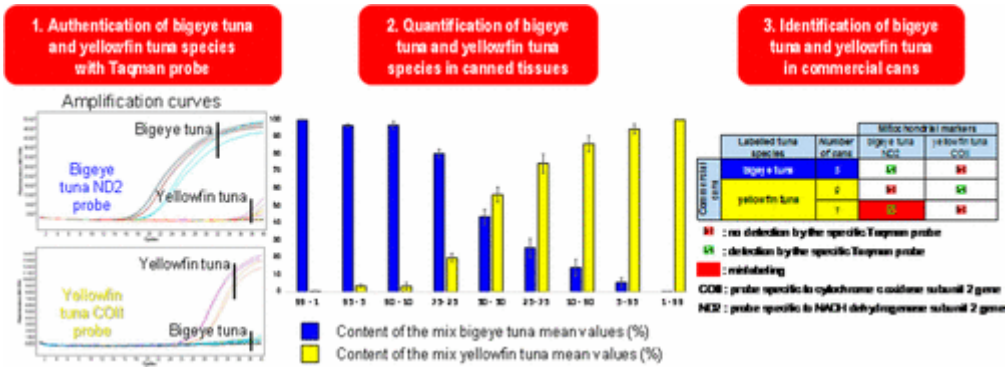
[1] Université Littoral Côte d'Opale, EA 7394 − ICV − Institut Charles Viollette, USC Anses − ULCO, F-62200 Boulogne-sur-Mer, France
[2] Université Lille, F-59000 Lille, France
[3] Université Artois, F-62000 Arras, France
[4] INRA, France
[5] ISA, F-59000 Lille, France
[6] Laboratoire d'Océanologie et de Géosciences, UMR 8187 (ULCO, Lille 1, CNRS), 62930 Wimereux, France
[7] Ifremer, F-44300 Nantes, France
[8] PFINV, F-62200 Boulogne-sur-Mer, France

* Corresponding author : Thierry Grard, email address : thierry.grard@univ-littoral.fr

Abstract :

Bigeye tuna (*Thunnus obesus*) and yellowfin tuna (*Thunnus albacares*) are among the most widely used tuna species for canning purposes. Not only substitution but also mixing of tuna species is prohibited by the European regulation for canned tuna products. However, as juveniles of bigeye and yellowfin tunas are very difficult to distinguish, unintentional substitutions may occur during the canning process. In this study, two mitochondrial markers from NADH dehydrogenase subunit 2 and cytochrome c oxidase subunit II genes were used to identify bigeye tuna and yellowfin tuna, respectively, utilizing TaqMan qPCR methodology. Two different qPCR-based methods were developed to quantify the percentage of flesh of each species used for can processing. The first one was based on absolute quantification using standard curves realized with these two markers; the second one was founded on relative quantification with the universal 12S rRNA gene as the endogenous gene. On the basis of our results, we conclude that our methodology could be applied to authenticate these two closely related tuna species when used in a binary mix in tuna cans.

Graphical abstract



Keywords : authentication, bigeye tuna (Thunnus obesus), canned products, qPCR, quantification, species identification, TaqMan, tuna, yellowfin tuna (Thunnus albacares)

Introduction

Recent years have been marked by various food scandals such as 'Horsegate' (meat adulteration crisis in Europe in 2013), and the results of fish products mislabeling studies[1, 2] . These events have highlighted the need: to set up an enhanced traceability system to improve quality process in industries; to reinforce consumer protection; and to reduce fraudulent activities. Seafoods are in the 'top ten' food products that are most likely to be subject of fraud[3]. Among these is one of the major marine species captured in 2012[4] , tuna, which represents an important economic value in the canning industry. According to the European

legislation, mixing of tuna species in tuna cans is strictly forbidden[5]. However, substitutions between tuna species could appear during the filleting and canning process when external morphological characteristics, such as dorsal fins or finlets, are removed and fillets with similar appearance and texture are obtained. Furthermore, difficulties exist in identifying juveniles of bigeye tuna (*Thunnus obesus*, Lowe, 1839) and yellowfin tuna (*Thunnus albacares*, Bonnaterre, 1788) due to their similarity [6]. Moreover, these two species are often captured together from the same schools, particularly around the fish aggregation devices (FADs)[7], which are extensively used nowadays, resulting in potential substitutions. It was these observations that led this study to focus on these two particular species.

Several methods have been developed to identify species when the flesh is raw or cooked. Prior to the 1990s, the most used technique was the isoelectric, which focused on proteins (IEF) based on sarcoplasmic protein profiles[8]. However, sterilization, which takes place during canning process, induces an irreversibly denaturation of proteins[9]. Furthermore, IEF is sometimes of no value in discriminating fish species within families such as scombridae (mackerels, tunas, and bonitos)[9]. DNA however, represents a great advantage over proteins as it is stable at high temperatures, is present in all cells of the animal, and is endowed with a greater variability linked to genetic codes[10]. Consequently, biomolecular DNA techniques to identify fish species, including tuna species, have been developed for more than 20 years[11]. DNA degrades during heat treatment into small fragments[12], but these are nevertheless, informative enough to differentiate even closely related tuna species[11, 13]. Although the regions of both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) can be amplified by a polymerase chain reaction (PCR)[14], mtDNA has preferentially been used for authentication of the species, in particular when the starting sample has suffered intense heat treatments[15]. Concerning canned tuna, most studies show a preference to mtDNA in relation to nDNA because of its relative abundance and its circular structure, which provides greater resistance

to thermal degradation[15, 16, 17]. It has actually been shown that intense heating, as applied in the processing of canned tuna, highly degrades DNA, cleaving it into tiny fragments. The average size of DNA fragments for canned products is between <100 and 360 bp compared to that of ≤20000 bp for frozen products [12, 18]. The mitochondrial gene cytochrome b has been one of the most commonly used for DNA-based fish species identification analysis; used extensively to identify flatfish, gadoids, eels, anchovies, and scombrids[19]. Nowadays, other genes are also used as markers to differentiate between fish species, like the cytochrome oxidase I (COI) coding gene, which is extensively used in the Barcode of Life project[20], and its declination for fish in the Fish Barcode of Life initiative (FISH-BOL)[21]. Nevertheless, a number of studies have shown that the cytochrome oxidase subunit 1 gene was not necessarily the best candidate to differentiate between species of tuna [22, 23]. A large range of DNA-based methods have been developed to identify scombrid species, mainly using mitochondrial gene fragments as markers[22, 24, 25, 26]. Previous studies have used real time PCR with TaqMan probe methods to identify tuna species[27, 28]. However, in most of these studies, real time PCR was mainly used for identification of the different tuna species. According to current knowledge, no method exists to quantify the amount of DNA from bigeye tuna and yellowfin tuna in a mix.

Thus, the aim of this study was to develop a routine TaqMan-based qPCR method to identify and quantify bigeye tuna and yellowfin tuna in canned products. DNA markers were focused on to distinguish bigeye tuna from yellowfin tuna, which are genetically closely related. The first step of this study was to design a specific TaqMan probe that would allow the identification of these two species. The second step was to develop two comparative quantitative methods to determine the percentage of each of these two species in mixed canned tuna products.

Materials and methods

Samples collection

Raw material

The two species of tuna being studied were purchased from commercial fishing vessels by the 'innovation platform for aquatic products' (Plateforme d'Innovation Nouvelles Vagues, (PFINV), Boulogne sur Mer, France, http://pfinouvellesvagues.com/?lang=en). Entire individuals of bigeye tuna and yellowfin tuna were sampled, and originated from the Atlantic, Pacific, and Indian oceans (10 individuals per ocean and per species). Before filleting, individuals were identified according to morphological characters using identification keys from the FAO Species Catalogue[29]. A piece of muscle tissue was sampled from each individuals and was used in the preliminary studies on raw tissue for the development of the primers and TaqMan probe. The remaining fillets were stored at -20°C until DNA extraction. Reference materials of scombrid specimens were provided by tissue collection from the French Research Institute for Exploitation of the Sea (Ifremer: http://wwz.ifremer.fr/institut_eng/) in order to validate the developed "primers/probe systems".

Canned tuna

Twenty cans containing either yellowfin tuna or bigeye tuna (10 cans per fish species) were prepared by PFINV using the techniques applied in the canning industry in order to obtain standardized cans. The previously frozen fillets were thawed to a temperature of between 0 and 2°C. Tins (diameter = 55 mm, 1/12 can) were then filled with 80 g of raw flesh and brine. Cans were crimped and sterilized at 116°C to the sterilizing value (Fo) for 7 min.

In order to simulate involuntary and voluntary rate substitutions in canned products, tuna cans of different mixtures of tuna fillets were prepared according to the following bigeye tuna/yellowfin tuna ratios: 99/1; 95/5; 90/10; 75/25; 50/50; 25/75; 10/90; 5/95 and 1/99 (e.g. the can containing the mix bigeye tuna/yellow fin tuna 75/25 was made with 75% of bigeye

tuna and 25% of yellowfin tuna). For each mix, cans were prepared in quadruplicate by PFINV following the same process.

Finally, 29 commercial tuna cans were purchased randomly from local markets to validate the method. They were labelled as yellowfin tuna (10), bigeye tuna (5), albacore (3) and skipjack tuna (11).

DNA Extraction

Before DNA extraction, brine was removed and tuna flesh was drained on filter paper. Two washing steps were followed: firstly with 70% ethanol, and then secondly with distilled water to eliminate any potential PCR inhibitors. After washing and drying, all flesh contained within the cans was finally homogenized using a mixer (Philips, France).

DNA extraction was performed according to the protocol described by Jérôme *et al.* (2003)[30]. Concentration and purity of extracted DNA were determined by measuring the absorbance of the DNA extracts at 260 nm, checking for protein impurities at 280 nm and organic compound contamination at 230 nm using a spectrophotometer (Denovix, La Madeleine, France). The DNA extracts were stored at -20°C until use.

Primers and probe design

Primers and probe sequences were specifically designed to identify bigeye and yellowfin tuna. DNA sequences of different scombrid species were obtained from GenBank (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov/). Alignments of DNA sequences were performed using Multalin software[31, 32] to find conserved specific sites for species identification and to identify intraspecific variations. Following this study, fragments of the NADH dehydrogenase subunit 2 (ND2) gene was selected for bigeye tuna identification, and the cytochrome c oxidase subunit 2 (COII) gene for yellowfin tuna identification (Table 1). Moreover, sequence alignments of mitochondrial 12S rRNA gene were realized for the two

species and a short 12S rRNA fragment gene (Table 1) was selected to be used as an endogenous gene.

Primers and TaqMan probes (Table 2) were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). Hydrolysis probes were labeled with carboxyfluorescein fluorescent reporter dye (FAM) on 5'-end and with a quencher (BHQ1) on the 3'-end. Primers and probe were synthesized by TibMolBiol (Berlin, Germany).

Quantitative real-time PCR conditions

PCR reactions were carried out in a total volume of 20 µL containing 10 ng/µL of DNA template per well, 0.5 µM of each primer, 0.2 µM of probe, 10 µL of PCR LightCycler 480 Probe Master (Roche, France) and RNAse/DNAse-free distilled water to adjust to the final volume (Roche, France).

PCR assays were performed in the LightCycler 480 thermocycler (Roche, France) under the following conditions: 95°C for 10 min, followed by 40 cycles 95°C for 15 seconds, and 60°C for 1 min.

Fluorescence data were analyzed using the LightCycler 480 software (LightCycler® SW 1.5.1). Each quantification cycle (Cq) value represented the average of Cq from three replicates.

qPCR amplification efficiency and standard curves

Standard curves were performed with the DNA extracted from standardized cans prepared by PFINV containing only one species: yellowfin tuna or bigeye tuna. Standard curves for each species were the result of mixing DNA from several specimens. Ten-fold dilutions series of bigeye tuna and yellowfin tuna DNA extracts ranging from 100 to 0.01 ng/µL were utilized to perform qPCR standard curves. PCR amplification efficiency of each primer/TaqMan probe system (ND2, COII and 12S –Table 2) was calculated. Standard curves corresponding to Cq

for each DNA dilution were constructed using the Lightcycler 480 software. The linear correlation between Cq and initial concentration of standard samples (N_0) is: Cq = a .log (N_0) + b, where 'a' is the slope and 'b' is the intercept. Values of amplification efficiency ($E = (10^{-1/a})$) were calculated from the slopes of standard curves. For example, a slope of –3.7 corresponds to a PCR efficiency of: $10^{-1/-3.7} = 1.86$, namely, a PCR efficiency of 86%.

Quantification methods

DNA extracted from standardized cans containing binary mixtures of bigeye and yellowfin tunas was used to develop the two quantification methods. Four cans of each mixture condition were analyzed, and for each can, DNA extracts were performed in quadruplicate. Each DNA extract gave rise to a double PCR analysis. This corresponded to 32 qPCR analysis for each mixture condition.

Method based on absolute quantification with standard curves

The amounts of yellowfin tuna ([T.alb]) and bigeye tuna ([T.obe]) DNA in binary mixtures were deduced from the corresponding qPCR standard curves for each species. These measurements were transformed into percentages of one species (% T.alb or % T.obe) according to the following equations:

[Total DNA of tuna] = [T.alb] + [T.obe]

Equation 1: yellowfin tuna (*Thunnus albacares*) content (%) $= \frac{[\text{T.alb}]}{[\text{Total DNA of tuna}]} * 100$

Equation 2: bigeye tuna (*Thunnus obesus*) content (%) $= \frac{[\text{T.obe}]}{[\text{Total DNA of tuna}]} * 100$

Method based on relative quantification with an endogenous gene

For each studied tuna species, two independent standard curves were built in parallel, one was established for the target genes (ND2 or COII) and the other one for the endogenous 12S rRNA gene. Amounts of target and endogenous genes in samples were then extrapolated from

the established standard curves. The percentages of each fish species in can samples were obtained following the equations previously used [33]:

Equation 3: yellowfin tuna (*Thunnus albacares*) content (%) $= \frac{[\text{T.alb}]}{[12\text{S rRNA}]} * 100$

Equation 4: bigeye tuna (*Thunnus obsesus*) content (%) $= \frac{[\text{T.obe}]}{[12\text{S rRNA}]} * 100$

Specificity and sensitivity

qPCR amplifications of serial dilutions of DNA extracts for the two species were performed to determine the limit of detection (LOD) and the limit of quantification (LOQ) values. The calculation of LOD and LOQ were established following those of previous studies[34]. The LOD was assessed as the lowest amount of tuna-DNA that could be reliably distinguished from the blank matrix, whereas the LOQ was assessed as the lowest concentration at which the amount of tuna-DNA could not be reliably detected.

Statistical Analyses

Determination coefficients (R^2) were calculated and data were analyzed by the chi-square test using PAleontological STatistics (PAST) software (version.3.07) to compare percentages obtained with the real values. Cq values were compared using the independent t-test. Statistical significance was set at $p < 0.05$.

Results and discussion

Design of primers and probe from interspecific and intraspecific DNA variation studies

The first step of this work consisted in the development of two specific primer/TaqMan systems to identify and quantify bigeye tuna and yellowfin tuna by qPCR analysis. An interspecific genetic variability study among all mitochondrial molecular markers based on the availability of DNA sequences in GenBank database has been investigated to determine discriminatory regions for specific primer and probe design. Results of multiple mitochondrial

DNA sequence alignments including the eight recognized species of *Thunnus* and seven selected bonitos (Table 1) revealed a high degree of similarity between these species as previously related[35]. Informative sites are limited to only one or two nucleotides per 300 bp sequences due to the low variability in nucleotide sequences between yellowfin tuna and bigeye tuna. This lack of polymorphism makes it difficult to correct species identification with standard PCR methodology. It was necessary to develop a qPCR TaqMan-based methodology to identify the two tuna species and then to quantify the amount of DNA of each of the two tuna species.

Among all mitochondrial genes, only cytochrome c oxidase subunit 2 (COII) and NADH dehydrogenase subunit 2 (ND2) genes (table 1) showed discriminatory regions which would allow the identification of the two tuna species compared to the other scombridae species (data not shown). Informative regions of ND2 and COII were selected for design of primers and probe to respectively identify bigeye tuna and yellowfin tuna. Subsequent investigations of intraspecific variations for these two genes showed no intraspecific variability for bigeye tuna and yellowfin tuna sequences.

Primers and probe that allowed characterization of yellowfin tuna were obtained from the COII gene sequence (AY971768.1), whereas those designed for bigeye tuna identification were obtained from the ND2 gene (NC_014059.1). The size of each amplicon was 99 bp and 198 bp for bigeye tuna and yellowfin tuna, respectively. The combination of primers and species-specific probe was called the “primers/probe system” (Table 2).

Furthermore, based on 12S rRNA sequences, 'universal' primers, allowing amplification of a common reference gene region, and probe were designed to recognize eight species of *Thunnus* and seven bonitos. Forward and reverse primers and TaqMan probe were designed from the 12S rRNA sequence (AB176811.1) giving a fragment of 107 bp (Table 2).

Identification of the two tuna species

Many studies have been conducted for the development of identification methods of canned tuna or bonito species. Bartlett and Davidson (1991) were the first authors to develop a molecular method based on DNA sequencing of 307 bp mitochondrial cytochrome b gene to identify four tuna species (*T. thynnus, T. obesus, T. albacares, T. alalunga*)[13]. They have further developed the FINS ((Forensically Informative Nucleotide Sequencing) method[36]. In the case of canned tuna, short DNA fragments have to be amplified by PCR, because the severe heat treatment during the tuna canning process leads to heavy degradation of DNA[37]. Unseld *et al.* (1995) showed that it is possible to distinguish between some tuna and bonito species by amplification and sequencing of a short 59 bp DNA fragment (123 bp including primers) of the mitochondrial cytochrome b gene[11]. Nevertheless, they failed to discriminate bigeye tuna from yellowfin that differed by only one nucleotide in the gene fragment studied. Since sequencing was a relatively long and expensive technique, various methods including the use of PCR coupled to other identification tools have been also used to authenticate tuna and bonito species[18, 22, 23, 38]. These different PCR methods comprise of the PCR-RFLP[12, 39, 25, 40, 41], the PCR SSCP[42, 43], the PCR-ELISA[44], or the RT-PCR[45, 28, 46]. However, all these described methods may be used to identify tuna species, but not to quantify their amount of DNA. A real-time PCR or qPCR methodology based on a fragment of about 100 bp of the 16S rRNA gene[27] was first developed in 2005 on canned tuna to identify and quantify two mixed tuna species, albacore and yellowfin tuna.

Our two 'primers/probe systems' were first tested on DNA extracted from raw muscle tissues of the two-targeted species. Specific fluorescence emission was detected for each targeted species at the optimal conditions of qPCR used (Tm primers, primers and probe concentrations, DNA concentration etc…). In a second step, the two 'primers/probe systems' were tested on DNA extracted from the reference tuna cans containing only one of the two tuna species. A delay of Cq values for canned tissue DNA versus Cq values for raw tissue

DNA was noticed for the same quantity of DNA, probably due to the impact of sterilization on DNA. These results showed that yellowfin tuna species-specific TaqMan primers and probe could identify yellowfin tuna species in cans. The mean Cq obtained from primers and probe designed for yellowfin tuna (Talb_COII) was 21.04 ±1.38 (mean ±sd). No amplification was detected with this yellowfin tuna probe tested on bigeye tuna cans. Primers and probe designed for bigeye tuna (Tobe_ ND2) amplified ND2 sequences of DNA from cans containing bigeye tuna species with an averaged Cq value of 22.10 ±1.18. Fluorescence emission for probe designed for bigeye tuna (Tobe_ ND2) was also observed on DNA extracted from yellowfin tuna cans but with a mean Cq of 34.36 ±1.05. These differences between Cq values tested with Chi-square test were statistically significant ($p<0.001$) and allowed the discrimination between bigeye tuna and yellowfin tuna.

Specificity of the two 'primers/probe systems'

For canned products, standard curves were obtained with an efficiency of 92.4 % ± 5.4 for bigeye tuna and 99.8 % ± 5.9 for yellowfin tuna. The LOD and LOQ were evaluated to 0.01 ng/µL for bigeye tuna and yellowfin tuna.

The species-specificity of the two 'primers/probe systems' was also evaluated by testing DNA amplification from raw tissues of the ten following tuna and bonito species: albacore (*Thunnus alalunga*), Atlantic bluefin tuna (*Thunnus thynnus*), longtail tuna (*Thunnus tonggol*), blackfin tuna (*Thunnus atlanticus*), Atlantic bonito (*Sarda sarda*), bullet tuna (*Auxis rochei*), kawakawa (*Euthynnus affinis*), frigate tuna (*Auxis thazard*), little tunny (*Euthynnus alletteratus*), and skipjack tuna (*Katsuwonus pelamis*). No fluorescence signal was recorded with DNA templates from albacore, Atlantic bonito, blackfin tuna, bullet tuna, frigate tuna, kawakawa, or little tunny. For Atlantic bluefin tuna, a signal was recorded with the bigeye tuna primer-probe system, with a Cq averaging at 20.22 ± 0.49, near to that obtained for bigeye tuna (averaged at 17.9 ± 1.14 for raw tissues). However, as Atlantic Bluefin tuna is not

used for tuna cans but almost exclusively in sashimi and sushi dishes, this lack of specificity here is not considered to be an issue. For longtail tuna, only one reference sample was tested, and a Cq of 24.79 ± 0.15 was obtained by amplification with the bigeye tuna primers/probe system'. This Cq value was close to the Cq obtained for bigeye tuna (17.9 ± 1.14 for raw tissues). Due to the lack of other reference samples, this result has not been confirmed yet.

Assessment of quantification on standardized mixtures

A large number of identification techniques are based on mitochondrial genes as molecular markers because mtDNA allows greater sensitivity of the method. However, the problem caused by the use of mitochondrial DNA of one species lies in the fact that there is a variable number of copies of mtDNA according to individual, age, or nature of the extracted tissue (muscle, fin…)[47, 48]. Nowadays, two approaches of quantification with real time PCR methodology based on different calculation modes[49, 50, 27, 33] exist: either relative quantification with an endogenous gene; or absolute quantification. To our knowledge, only one study has been published on the quantification of mixed tuna species in a can[27] and, in the particular case of the two closely related species -bigeye and yellowfin tuna- no paper can be found in the literature. In addition to the distinction between these two species, it was necessary to develop qPCR methods for quantifying the presence of a species below a specified threshold. This limit has been set at one percent following the request of the tuna canning industry, which allows discrimination between voluntary substitutions from involuntary substitutions.

This study's methodology was tested on canned mixtures containing different percentages of bigeye tuna and yellowfin tuna species.

Method based on absolute quantification with standard curves

The two targeted mitochondrial genes (COII for yellowfin tuna, and ND2 for bigeye tuna) were used for absolute quantification. Standard curves were established for the two tuna

295 species, which allowed the calculating of the DNA quantity for each tuna species, following
296 equations 1 and 2. This method postulated on the fact that amounts of mtDNA were
297 equivalent in these two close species. The resulting percentages were statistically compared
298 with the real values of the standardized mixtures based on the Chi-square test. No significant
299 difference between the percentages experimentally calculated and those of the initial mixtures
300 was shown, except for the results of cans containing 50% of each species (Figure 1).
301 The results of this study highlighted the efficiency of this approach towards quantifying the
302 presence of bigeye tuna and yellowfin tuna in cans containing mixtures of these two species.

303 *Method based on relative quantification with an endogenous gene*

304 Another strategy developed in this study for quantification of species in tuna cans is based on
305 two targeted mitochondrial genes, namely, one species-specific gene for targeted species
306 identification (COII for yellowfin tuna and ND2 for bigeye tuna), and in one endogenous gene
307 - the 12S rRNA gene that can be amplified, irrespective of the tuna species, with the same
308 universal 'primers/probe system'. The calculation of relative quantification based on Cq
309 variation between target and endogenous amplifications could not be used with bigeye tuna
310 and yellowfin tuna samples cooked in cans as previously described by Lopez and Pardo[27] on
311 binary mixtures of sterilized tissue of albacore and yellowfin tuna. Lopez and Pardo found
312 error up to 50%, and they concluded[2727272727272727] that it was not possible to express
313 quantification with this method using these sterilized tuna species due to the degradation of
314 DNA that exerts an influence in the calculation of Cq values. Consequently, we used a
315 calculation method developed for the quantification of beef and pork fractions in minced
316 meat[33] to quantify bigeye tuna and yellowfin tuna in binary mixtures following equations 3
317 and 4. Calculated percentages were statistically compared with the expected values.
318 The results showed that the percentage calculations using the specific probe for bigeye tuna
319 allowed the obtaining of a precise quantification only for the following percentages: 90, 75,

25 and 5%, showing that this method has limitations (Figure 2). On the other hand, the expected results were not conclusive using probe for yellowfin tuna when amounts of yellowfin tuna were greater than 50% (data not shown). The amounts of bigeye tuna or yellowfin tuna in a mix of these two species were determined with equation 4, allowing the calculation of the amount of bigeye tuna and yellowfin tuna.

Study with commercial tuna cans

The suitability of this study's 'primers/probe systems' for canned tuna authentication was subsequently tested on twenty nine commercial cans; labelled as albacore tuna (3 cans), bigeye tuna (5 cans), skipjack tuna (11 cans), and yellowfin tuna (10 cans). The investigation showed that DNA from cans labelled as albacore tuna or skipjack tuna species were not amplified with primers and probe specific to bigeye tuna or yellowfin tuna. The cans labelled as bigeye tuna were confirmed to be processed with this species when analyzed with primers and probe specific to bigeye tuna. While this analysis confirmed the presence of yellowfin tuna in nine cans, a single commercial can, labeled as yellowfin tuna, seemed to contain bigeye tuna flesh, suggesting a mislabeling. In addition, identification for the presence of skipjack tuna was performed on commercially labelled skipjack tuna, bigeye tuna, and yellowfin tuna cans, following a methodology previously developed in the authors' laboratory (unpublished). The results showed that all the 11 commercial cans labeled as skipjack tuna in actuality contained skipjack tuna. Skipjack tuna was not detected in any commercial can labeled as bigeye tuna or albacore tuna. Among the ten commercial cans labeled as yellowfin tuna, five cans were composed of a mixture of yellowfin tuna and skipjack tuna. These results suggest the presence of different species in yellowfin tuna cans, or mislabeling during the production, which the tuna canning industry will have to address.

To conclude, the two 'primers/probe systems' developed in this study have been used to: differentiate bigeye tuna from yellowfin tuna; and quantify them in canned products. Analysis

of 29 commercial tuna cans permitted the detection of a labeling error between bigeye tuna and yellowfin tuna. The two quantification methods based on standard curves or endogenous gene allowed the estimating of the level of content of each of the two targeted species in mixtures. The authors consequently suggest the following protocols to discriminate these two very close species: i) identify the presence of one of, or both of the species in tuna cans with primers and the Taqman probe designed in this study, ii) (in the case of the presence of more than one species) quantify the amount of each species using the method based on absolute quantification of these two species with standard curves. The second method based on bigeye tuna relative quantification using 12S rRNA endogenous gene could be used as confirmation. This identification and quantification methodology should help laboratories to contribute to traceability concerning canned tuna in order to obtain responses about substitution or fraud.

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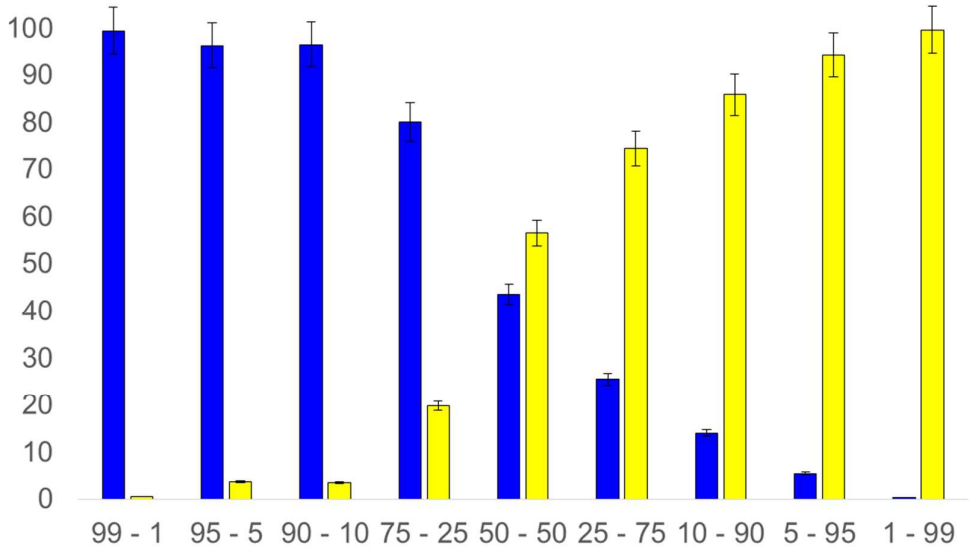

Figure 1: Quantification (in percentage) of binary mixtures of bigeye tuna and yellowfin tuna canned samples calculated with the method based on absolute quantification with standard curves. Blue histogram: average values of percentages of bigeye tuna; yellow histogram: average values of percentages of yellowfin tuna. Values on abscissa axis indicate expected theoretical percentages. Error bars indicate standard deviation in quadruplicates.

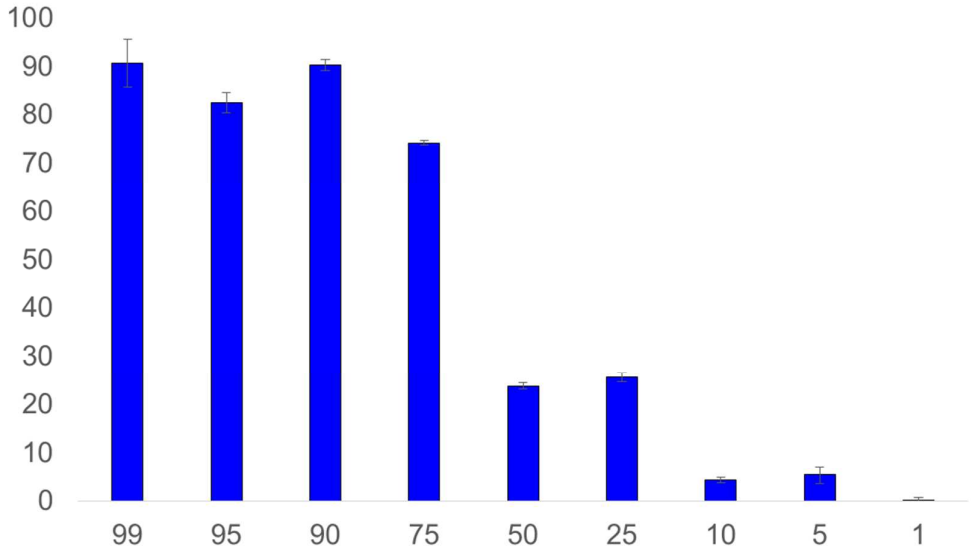

Figure 2: Average values of percentages of bigeye tuna species content processed with different quantities of bigeye tuna and yellowfin tuna calculated with the method based on relative quantification with the 12S rRNA endogenous gene. Values on abscissa axis indicate expected theoretical percentages. Error bars indicate standard deviation in quadruplicates.

494 **Table 1:** Genbank accession numbers of mitochondrial DNA sequences of cytochrome c
495 oxidase subunit 2 (COII) gene, NADH dehydrogenase subunit 2 (ND2) gene, and 12S rRNA
496 gene, for 15 scombridae species.

	GenBank accession nº		
Scombridae species	cytochrome c oxidase subunit 2 (COII) gene	NADH dehydrogenase subunit 2 (ND2) gene	12S rRNA gene
Yellowfin tuna / *Thunnus albacares*	AY971768 GU256528.1 JN086153.1 NC_014061.1	GU256528.1 JN086153.1 NC_014061.1	DQ874694.1 GU256528.1 HM003553.1 to HM003555.1 HQ641701.1 JN086153.1 NC_014061.1
Bigeye tuna / *Thunnus obesus*	GU256525.1 AY971769 JN086152.1 NC_014059.1	GU256525.1 JN086152.1 NC_014059.1	GU256525.1 HQ592316.1 to HQ592318.1 JN086152.1 NC_014059.1
Albacore / *Thunnus alalunga*	AB101291.1 GU256526.1 JN086151.1 NC_005317.1	AB101291.1 GU256526.1 JN086151.1 NC_005317.1	AB101291.1 AB176804.1 GU946542.1 GU946543.1 GU946544.1 JN007517.1 to JN007526.1 JN086151.1 NC_005317.1
Southern bluefin tuna / *Thunnus maccoyii*	GU256523.1 JN086150.1 NC_014101.1	GU256523.1 JN086150.1 NC_014101.1	GU256523.1 JN086150.1 NC_014101.1
Pacific bluefin tuna / *Thunnus orientalis*	AB185022.1 GU256524.1 NC_008455.1	AB185022.1 GU256524.1 NC_008455.1	AB185022.1 GU256524.1 KF906721.1 NC_008455.1 NC_008455.1
Northern bluefin tuna / *Thunnus thynnus*	AB097669.1 AY302574.2 AY971770 GU256522.1 JN086149.1 NC_004901.2 NC_014052.1	AB097669.1 AY302574.2 DQ854690.1 GU256522.1 JN086149.1 NC_004901.2 NC_014052.1	AB097669.1 AB176805.1 AY302574.2 DQ854647.1 GU256522.1 JN086149.1 KF906720.1 NC_004901.2 NC_014052.1
Longtail tuna / *Thunnus tonggol*	HQ425780.1 JN086154.1 NC_020673.1	HQ425780.1 JN086154.1 NC_020673.1	HQ425780.1 JN086154.1 NC_020673.1
Blackfin tuna / *Thunnus atlanticus*	KM405517.1	KM405517.1	DQ874693.1
Skipjack tuna / *Katsuwonus pelamis*	AB101290.1 AY971773 GU256527.1 JN086155.1 NC_005316.1	AB101290.1 JN086155.1 GU256527.1	AB101290.1 AB176808.1 DQ874697.1 GU256527.1 HQ592295.1 to HQ592297.1 JN086155.1
Atlantic bonito / *Sarda sarda*	AY971771.1	EU263832.1 EU263833.1	DQ874691.1
Orientalis bonito / *Sarda orientalis*	AY971772	ns	ns
Bullet tuna / *Auxis rochei*	AB103467.1 AB103468.1 AB105165.1 AY971774.1 NC_005313.1	AB103467.1 AB103468.1 AB105165.1 EU263836.1 NC_005313.1	AB103467.1 AB103468.1 AB176811.1 AB176810.1 AB105165.1 NC_005313.1
Frigate tuna / *Auxis thazard*	AB105447.1 NC_005318.1	AB105447.1 EU263837.1 NC_005318.1	AB105447.1 AB176809.1 DQ874692.1 NC_005318.1
Kawakawa / *Euthynnus affinis*	AY971776.1	ns	ns
Little tunny / *Euthynnus alletteratus*	AB099716.1 AY971775.1 NC_004530.1	AB099716.1 NC_004530.1	DQ874698.1 AB176806.1 AB176807.1 NC_004530.1 AB099716.1

ns : no sequences

497

498 **Table 2:** Primer and probe sequences developed in this study for quantitative real-time PCR
499 assays

Target gene	Primer (F or R) and TaqMan probe (P) names	Sequence (5'-3')	Size of fragments (pb)
Cytochrome c oxidase subunit II (COII)	F_Talb_COII	5'-CTTCCCTCCCTACGCATTCT-3'	198
	R_Talb_COII	5'-CACTATTCGGTGGTCTGCTTC-3'	
	P_Talb_COII (6-FAM)	5'-(FAM)-ACGAAATCAACGACCCCCATCTAA-(BHQ1)-3'	
NADH dehydrogenase subunit 2 (ND2)	F_Tobe_ND2	5'-CTAGCCACCTCCTGAGCAAA-3'	99
	R_Tobe_ND2	5'-GCCAGGTCTTGTTTTGACAGT-3'	
	P_Tobe_ND2 (6-FAM)	5'-(FAM)-TTCTTCTGTCCCTAGGCGGTCTTCCA-(BHQ1)-3'	
12S ribosomal RNA (12S rRNA)	F_Univ_12S	5'-GACTTGGCGGTACTTTAGATCC-3'	107
	R_Univ_12S	5'-TGACGACGGCGGTATATAGG-3'	
	P_Univ_12S (6-FAM)	5'-(FAM)-AACCGATGACCCCCGTTCAA-(BHQ1)-3'	

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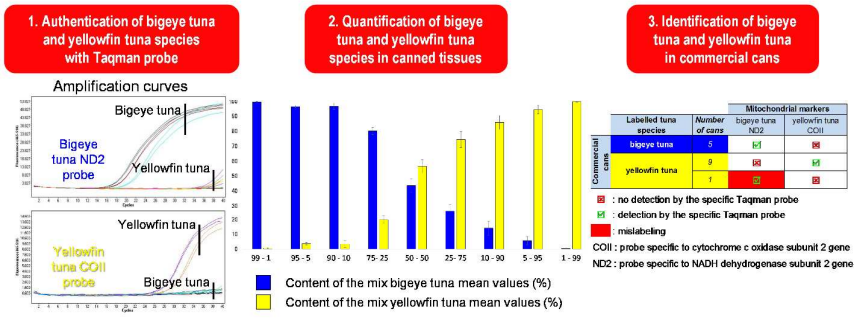

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