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Identification of processed tuna products sold by the Brazilian market

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Abstract: The composition of processed food is a concern of consumers, of the fishery industry and governmental agencies. The specific composition of canned tuna in three Brazilian brands was evaluated using Single-Strand Conformational Polymorphism (SSCP) of the cytochrome *b* gene by comparison with patterns of fresh tuna samples and tuna-related species landed at Santa Catarina ports (South Brazil). Thirteen SSCP patterns were obtained from fresh samples, and the specific variety was confirmed by analyzing their sequences. Inconsistencies were found in the fishery statistics of *Auxis thazard thazard* and *Scomber japonicus* reported from Brazilian ports, where fresh samples instead were represented mostly by *Auxis rochei rochei* and *Scomber colias*. A case of introgression was detected between *A. t. thazard* and *Katsuwonus pelamis*. The skipjack tuna *K. pelamis* composed seven out of eight of the processed tuna products analyzed, and two brands showed products containing a mixture of tuna (*K. pelamis* or *Thunnus albacares*) and the less commercially valuable bonito meat (*A. t. thazard*), which reinforces the need for the control of canned tuna in the Brazilian market.

Keywords: Canned food; Bonito; *Katsuwonus pelamis*; Fishery, Food control.

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INTRODUCTION

In 2009, marine fishing production in Brazil reached 585,671 tons, and 26.8% of the total catch was landed in Santa Catarina State (South Brazil) ports (MPA, 2011). The fishery industries of Santa Catarina developed an extensive market for canned tuna. Brazilian canned tuna production in 2009 was 1,240,815 tons, with an annual consumption of 9.03 kg/individual/year and an increasing trend in the last several years (MPA, 2011). The identification of the specific composition of processed food has been a concern of consumers and industries that buy processed meats. Brazilian governmental agencies are in charge of controlling which species are actually used in canned food products. In the case of processed tuna products, bonitos and other species of lower market value may be used instead of the product description declared on the labels (Botti e Giuffra, 2010). Bonito species are not as popular with consumers as other tuna; therefore, they have a much lower market price. The European community has drawn a strict distinction between tuna and bonito. The

skipjack tuna *Katsuwonus pelamis* Linnaeus, 1758 is considered a tuna together with all the members of the genus *Thunnus* South, 1845, while species of the genus *Euthynnus* Lütken, 1882, *Sarda* Plumier, 1802, and *Auxys* Cuvier, 1829, are considered bonitos (Ram *et al*., 1996). The fishery statistics show that the most abundant tuna and bonito landed at Santa Catarina ports between 2007 and 2009 were *K. pelamis* (68,542 t), *Thunnus albacares* (12,764 t), *T. obesus* (3,746 t) and *Auxis thazard thazard* (1,673 t) (MPA, 2011). *Scomber japonicus* (18,486 t), which may be used as a substitute for tuna due to the similarity in taste and texture, is also often cited in the Brazilian fishery reports.

Different molecular markers have been used for the identification of canned food. Tuna are submitted to cooking (Unseld *et al*., 1995) and sterilization processes, which degrade the proteins and DNA. Short mitochondrial regions have been efficiently amplified by the polymerase chain reaction (PCR) from processed tuna. Unseld *et al*. (1995) demonstrated that the use of a 59-bp region of the cytochrome *b* gene (described by the authors as a BDR region) by the Single-Strand Conformational Polymorphisms (SSCP) method allowed for the identification of most processed tuna species, and has proven to be useful at different laboratories around Europe (Rehbein; Mackie; Pryde, 1999).

Further mitochondrial DNA regions up to 200 bp (Quintero *et al*., 1998) have been studied using Restriction Fragment Length Polymorphisms (RFLPs) to identify the most closely related tunas (Ram *et al*., 1996; Quintero *et al*., 1998; Pardo e Pérez-Villareal, 2004; Lin e Hwang, 2007). SSCP is a sensitive and less expensive molecular technique for developing countries (Hayashi, 1991; Sunnucks *et al*., 2000). Therefore, the aims of the present work were to find out the specific and intra-specific variation in SSCP patterns of tuna, tuna-related species and other species landed in the ports of Santa Catarina State, South Brazil to determine which species have been commonly used as tuna meat in canned tuna.

MATERIAL AND METHODS

Sampling and DNA extraction

Fresh fish samples were obtained from fishery landings at the ports of Navegantes, Itajaí and Porto Belo of Santa Catarina state, South Brazil (Table 1). Processed samples were obtained either directly from the fishery industry or from local markets. Oil-preserved samples were treated following the protocols of Bligh and Dyer (1959) and Rebhein *et al.* (1999) with modifications. First samples were dried with filter paper and then they were submerged for 24 h in a lipids extraction solution (1:2:0.8 Chloroform/Methanol/H_2O). Extraction solution was then removed from the tissue and absolute ethanol was added in a proportion of two times the volume of the tissue, and mixed for two hours in a rotating homogenizer (Phoenix). Then, ethanol was removed and the process repeated one more time adding fresh ethanol. At the end, the samples were stored in 0.3 g portions submerged in 1 mL absolute ethanol until DNA extraction.

DNA extraction was performed following Hoelzel (1998) using 0.1 g fresh or 0.3 g processed meat sample. Tissue samples were homogenized in 860 µl of lysis buffer (1% SDS w/v; 150 mM NaCl; 2 mM EDTA; 10 mM Tris-HCl, pH 8.0) with 84 µl of 5 M guanidine thioisocyanate (GuSCN) and 80 µl Proteinase K (10 mg/ml) and incubated for 1 h at 55ºC in a rotating homogenizer. Afterwards, samples were left overnight at 37ºC. The next morning, samples were centrifuged at 20ºC for 5 min. Supernatants were transferred to new tubes and 700 µl of DNA binding solution (50 mM Tris-HCl, pH 7.0; 25 mM EDTA, pH 8.0; 5 M GuSCN; 1% silica slurry) was added. Then, the samples were submitted to rotating homogenization for 2 h and centrifuged at 16,000 × g for 2 min. Silica pellets containing DNA were washed twice with 70% ethanol and dried; then, the DNA was resuspended in 80 µl of TE buffer (10 mM Tris-HCl, pH 7.4; 1 mM EDTA, pH 8.0).

Table 1. Species name obtained by morphological identification, local name, landing port and date of collection of fresh fish samples.

Species (common name)	Local name	Landing port	Date of landing (number of fishes)
Thunnus alalunga Bonnaterre, 1788 (Albacore or long-finned tuna)	Albacora-branca	Itajaí	02/05/2001(1), 03/10/02(1), 28/01/03(2)
Thunnus albacares Bonnaterre, 1788 (Yellowfin tuna)	Albacora-lage	Itajaí	02/05/2001(1), 20/08/02(1), 27/01/03(2)
Thunnus obesus Loive, 1839 (Bigeye tuna)	Albacora-bandolim ou atum-cachorra	Itajaí	03/10/2002(1)
Katsuwonus pelamis Linnaeus, 1758 (Skipjack or stripe-bellied tuna)	Bonito-listrado	Navegantes	27/01/2003(1)
Auxis thazard thazard Lacepède, 1800 (Frigate mackerel)	Bonito-cachorro	Itajaí	20/08/2002(1), 29/01/03(3)
Scomber japonicus Houttuyn, 1782 (Pacific chub mackerel)	Cavalinha	Itajaí	20/08/2002(2)
Sarda sarda (Bloch, 1793) (Atlantic bonito)	Sarda	Itajaí	10/02/2003(1)
Scomberomorus brasiliensis (Collette *et al*., 1978) (Serra spanish mackerel or Brazilian spanish mackerel)	Sororoca	Porto Belo Navegantes	2003(1) 29/01/2003(1)

PCR-SSCP patterns

The BDR region of the mitochondrial cytochrome *b* gene was amplified using the primers described by Unseld *et al*. (1995): Forward: 5'-aaactgcagcccctcagaatgatatttgtcctca-3'; Reverse: 5'-gctggtacctctacaaagaaacatgaaaca-3'. Amplification by PCR was performed in reactions containing 1x PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM of each primer, 1U Taq DNA polymerase and 2 µl and 5 µl of DNA from fresh and processed tissue, respectively. The PCR reaction was conducted in a thermocycler using the following steps: 1 step of 5 min of initial denaturation at 94ºC followed by 35 cycles of 40 s denaturation at 94ºC, 80 s of primer annealing at 50ºC, 80 s of extension at 72ºC and, finally, 1 step of 7 min of final extension at 72ºC.

The SSCP patterns of samples were obtained by running a natural vertical 6% polyacrylamide gel electrophoresis following the methodology of Brown (1994) and Hoelzel (1998). Gel was prepared with 1 x TBE buffer (obtained from a stock solution of 10 x TBE: 154 mM Tris-base; 90 mM Boric acid; 2 mM EDTA), 10% glycerol, 6% polyacrylamide (Crosslink 75:1), 0.1% ammonium persulphate and 0.075% TEMED, which was immediately poured between the plates after the addition of the last two components. Glass plates were previously treated with repel and bind silane in order to rescue the gel after electrophoresis. Samples were denatured by incubation for 5 min at 95ºC in equal volumes of denaturing solution [95% deionized formamide, 20 mM EDTA, pH of 8.0, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol FF] and poured on ice immediately after denaturation. Ten microliters of the denatured samples were loaded into the gel. A non-denatured 123 bp ladder was poured in a single lane. Electrophoresis was performed at room temperature and at 15 Watts for a period of 6-8, before the blue stains run out of the gel. DNA fragments were visualized using silver nitrate staining protocol described by Hoelzel (1998), with modifications, by submerging the gel sticked on the glass plate in the following solutions: 3 min in fixing solution (10% ethanol, 0.5% acetic acid); 10 min in inpregnation solution (0.1% silver nitrate); one wash in deionized water; 20 min in the first revelation solution (1.5% NaOH; 0.01% $NaBH_4$; 0.15% formaldehyde); 10 min in the second revelation solution (0.75% $NaCO_3$); one wash in deionized water; one wash in the same fixing solution; and 30 min in hydratation solution (1.5% glicerol) which allows to keep the gel in good conditions until digitalization with a common scanner.

DNA fragments obtained from the fresh samples that generated the SSCP patterns were purified from agarose gel using an Ultrafree-DA (Millipore) column following the recommendations of the supplier and were sent to Macrogen, Inc., Korea (www.macrogen.com) for sequencing. Sequencing was conducted under BigDye™ terminator cycling conditions; the reacted products were purified using ethanol precipitation and were run using an Automatic Sequencer 3730XL.

The confirmation of the specific status of fresh samples was performed by comparing the obtained sequences with sequences from tuna and tuna-related species registered in the literature or deposited in GenBank by sequences comparisons and by tree clustering. The online tools BLASTN and ClustalW were used to find similarities between sequences and for their alignment, respectively. A phylogenetic tree was constructed using the MEGA version 5.10 software package (Tamura *et al*., 2011) by the minimum evolution method with the maximum composite likelihood model after 1,000 bootstrap resampling as confidence parameter of the branches. Afterwards, the SSCP patterns obtained from the fresh samples were used to identify the specific composition of the processed tuna. To confirm identity, fresh samples were run on electrophoresis side by side with the tuna sample in question.

RESULTS

Identification of fresh samples of scombroid species using SSCP patterns

A total of thirteen SSCP patterns were found for the eight samples collected at the Santa Catarina ports (Figs. 1 and 2). Most species showed a single SSCP pattern, but three SSCP patterns were identified for the skipjack tuna *K. pelamis* (*Kpel*-1-BR, *Kpel*-2-BR and *Kpel*-3-BR); three SSCP patterns were identified for the presumed frigate mackerel *A. t. thazard* (*Atha*-1-BR, *Atha*-2-BR and *Atha*-3-BR); (Fig. 2) and two patterns were identified for the presumed *S. japonicus* (*Sjap*-1-BR and *Sjap*-2-BR) (Fig. 1).

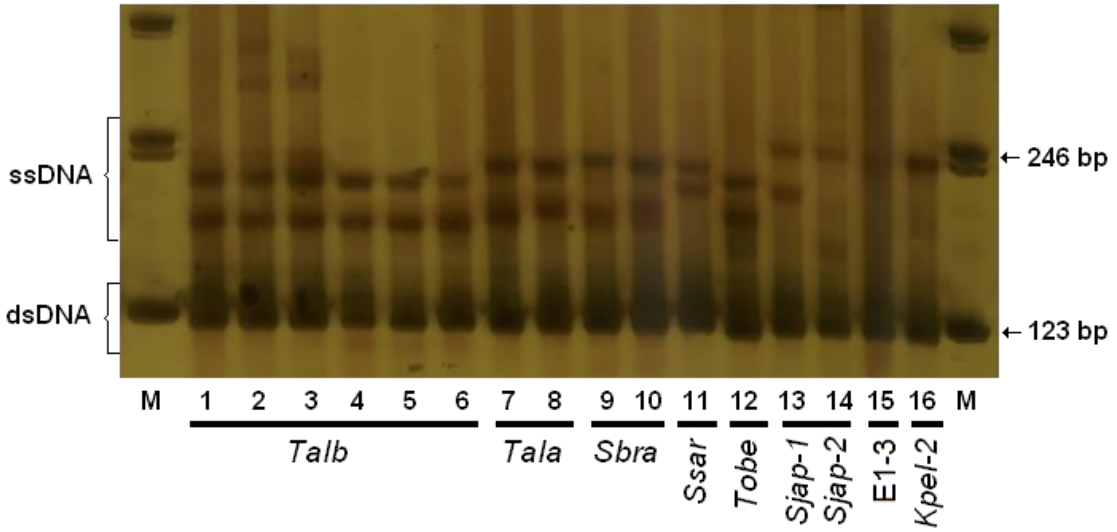

Figure 1. The SSCP pattern obtained from Brazilian samples (-BR) after electrophoresis in a 6% natural polyacrylamide vertical gel stained with silver nitrate. (M) represents the 123 bp molecular ladder; SSCP patterns represented by (ssDNA) single stranded DNA; (dsDNA) double stranded DNA. The order of samples identified by morphological characters is: (1-6) *Thunnus albacares*, (7-8) *T. alalunga*, (9-10) *Scomberomorus brasiliensis*, (11) *Sarda sarda*, (12) *T. obesus*, (13) first and (14) second pattern of *Scomber japonicus*, (15) processed sample E1-3 and (16) second pattern of *Katsuwonus pelamis*.

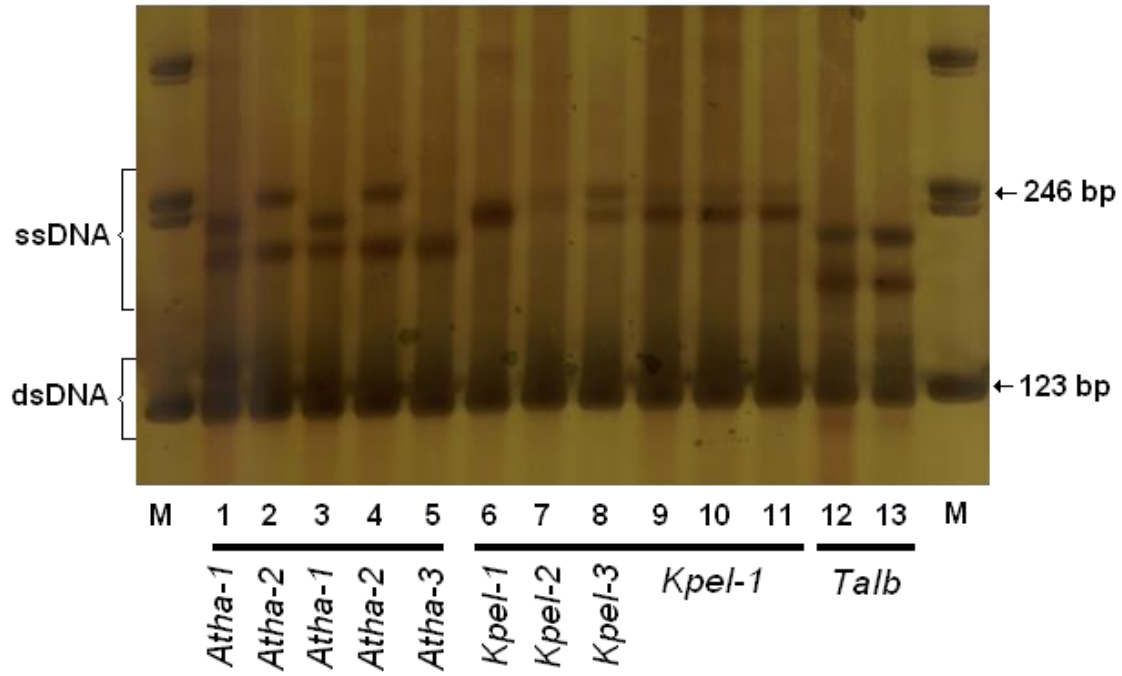

Figure 2. The SSCP pattern found for Brazilian samples (-BR) after electrophoresis in a 6% natural polyacrylamide vertical gel stained with silver nitrate. (M) represents the 123 bp molecular ladder; SSCP patterns represented by (ssDNA) single stranded DNA; (dsDNA) double stranded DNA. The order of samples identified by morphological characters is: (1 and 3) first, (2 and 4) second and (5) third pattern of *Auxis thazad thazard*, (6, 9-11) first, (7) second and (8) third pattern of *Katsuwonus pelamis*, (12-13) *T. albacares.*

Most sequences were consistent with the species identified by morphological characteristics. The sequences of the individuals morphologically identified as *K. pelamis*, *Thunnus albacares*, *T. alalunga*, *T obesus* and *Sarda sarda* showed 100% identity with sequences deposited in GenBank or in the literature (Fig. 3) and clustered in the phylogenetic tree with their respective species (Fig. 4). The sequences that generated the SSCP patterns *Kpel*-1-BR and *Kpel*-3-BR were identical, and all patterns correspond to known forms of the species (Figs. 3 and 4).

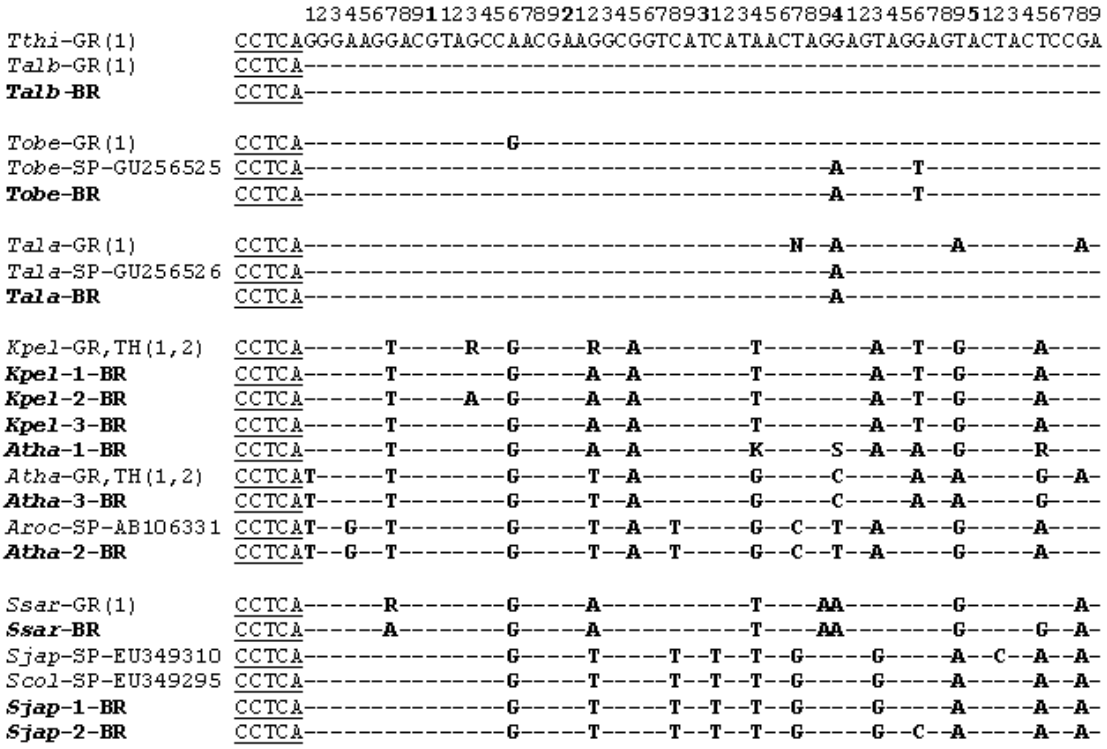

Figure 3. The alignment of sequences of the 59-bp BDR region of the cytochrome *b* gene. Numbers in the top express the numbers of sites. The first five underlined bases correspond to the end of the forward primer. Dashes represent congruence with the first sequence (from *T. thynnus*) and base substitutions are indicated by the letters in black. Extensions in names are: (-BR) samples from Brazil obtained in this study (in black), (-SP) Spain, (-GR) Germany and (-TH) Thailand. Samples from Spain show the GenBank accession number. (*Tthi*) *T. thinnus*, (*Talb*) *T. albacares*, (*Tobe*) *T. obesus*, (*Tala*) *T. alalunga*, (*Kpel*) *K. pelamis*, (*Atha*) *A. t. thazard*, (*Aroc*) *Auxis rochei rochei*, (*Ssar*) *Sarda sarda*, (*Sjap*) *Scomber japonicus*, (*Scol*) *Scomber colias.* (1) Unseld *et al*. 1995, (2) Ram *et al*. 1996.

Unexpected results were found when the sequences of the species identified morphologically as *A. t. thazard* and *S. japonicus* were compared. The sequences that generate the first pattern of the frigate mackerel (*Atha*-1-BR) showed 97% similarity to *K. pelamis* by four sites characteristic of the species, one site characteristic of *A. t. thazard* and three heteroplasmic sites for both species. The form was clustered with a confidence of 70% with the *K. pelamis* branch in the phylogenetic tree (Fig. 4), suggesting a case of introgression. The sequence of the second pattern (*Atha*-2-BR) showed 100% similarity to the bullet tuna *Auxis rochei rochei* (Risso, 1810) determined by eight distinctive sites and was clustered with *A. r. rochei* in the phylogenetic tree with a confidence of 99% (Figs. 3 and 4). The sequence of the third pattern (*Atha*-3-BR) was 100% similar to the frigate mackerel *A. t. thazard* and was similarly identified by morphological characteristics (Figs. 3 and 4).

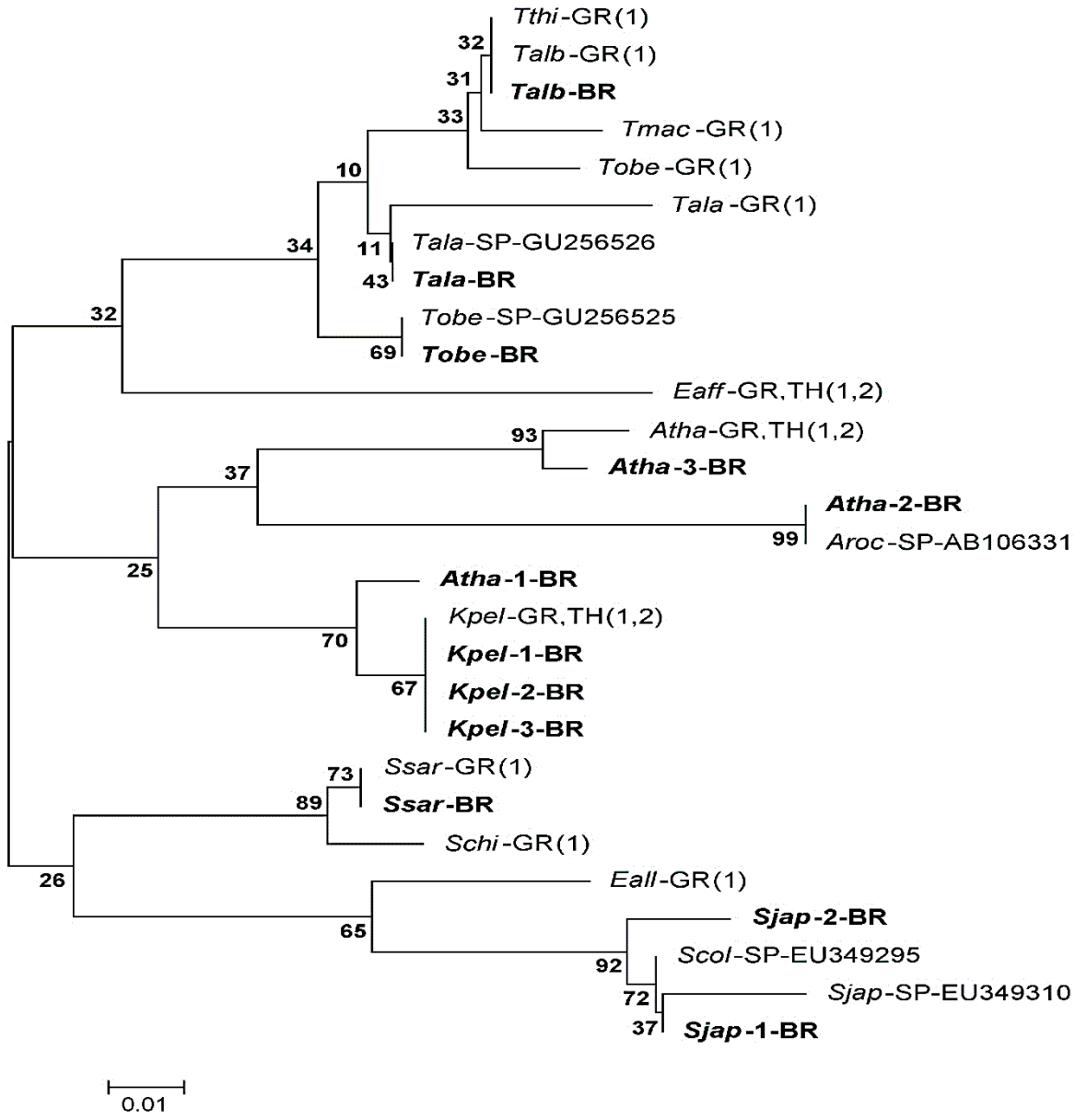

Figure 4. A phylogenetic tree showing the position of the samples from Brazil (in black) obtained in this study. Numbers in branches are bootstrap confidence values. Extensions in names are: (-BR) samples from Brazil, (-SP) Spain, (-GR) Germany and (-TH) Thailand. Samples from Spain show the GenBank accession number. (*Tthi*) *T. thinnus*, (*Talb*) *T. albacares*, (*Tobe*) *T. obesus*, (*Tala*) *T. alalunga*, (*Kpel*) *K. pelamis*, (*Atha*) *A. t. thazard*, (*Aroc*) *A. rochei rochei*, (*Ssar*) *Sarda sarda*, (*Schi*) *S. chilensis*, (*Sjap*) *Scomber japonicus*, (*Scol*) *S. colias*, (*Eaff*) *Euthynnus affinis*, (*Eall*) *E. alletteratus*. *(1) Unseld et al. 1995, (2) Ram et al. 1996.*

Identification of the specific composition of canned tuna at the Brazilian market

The skipjack tuna *K. pelamis* was present in seven out of eight canned tuna analyzed, where the most common form used to compound the products was the second SSCP pattern (*Kpel*-2-BR). Two brands contained a mixture of tuna (*K. pelamis* or *T. albacares*) and bonito meat (*A. t. thazard*) as shown in Table 2.

Table 2. Analyzed processed samples and their specific composition.

Source	Sample*	Meat type	Preservation	Specific composition (SSCP pattern code)
Local market	E1-1	Solid	Vegetable oil	Auxis thazard thazard (*Atha*-3-BR), Thunnus albacares (*Talb*-BR)
Local market	E1-2	Solid	Salt and water	*Katsuwonus pelamis* (*Kpel*-3-BR)
Industry supply	E1-3	Grated	Processed block	*Katsuwonus pelamis* (*Kpel*-2-BR)
Industry supply	E1-4	Grated	Processed block	*Katsuwonus pelamis* (*Kpel*-2-BR)
Local market	E2-1	Solid	Salt and water	*Katsuwonus pelamis* (*Kpel*-2-BR)
Local market	E2-2	Grated	Vegetable oil	*Katsuwonus pelamis* (*Kpel*-2-BR)
Local market	E3-1	Solid	Salt and water	*Katsuwonus pelamis* (*Kpel*-2-BR)
Local market	E3-2	Grated	Vegetable oil	*Katsuwonus pelamis* (*Kpel*-2-BR), *Auxis thazard thazard* (*Atha*-3-BR)

*E1, E2 and E3 represent different brands

DISCUSSIONS

Single Stranded Conformational Polymorphisms are molecular markers very attractive for developing countries due to their low cost and high sensitivity, which has also proven to be of high confidence and consistence in their results as observed by analysis in very different countries of Europe (Rehbein; Mackie; Pryde, 1999). Dalmasso *et al*. (2007) and Terio *et al*. (2010) designed probes for the identification of tunid species by real time PCR, but this method still represents an expensive mean for routine evaluation of the specific composition of canned tuna in developing countries. The first steps (oil extraction, DNA extraction and amplification of the DNA) can be performed without commercial kits that are normally for 50 samples and very expensive. Instead, for example, DNA extraction can be done with the methodology here described. Although, it will take longer time (2-3 days) than with a commercial DNA extraction kit (1 day), after the preparation of solutions, they will last for many hundreds of samples. The same situation can be stated for the SSCP electrophoresis and staining. It will be an initial spent of reagents and equipments, but the cost can be lowered by buying second handed equipments. An alternative method is analyzing samples by sequencing them, but not always sequencing is possible with samples in concentrations lower than 10 ng, which is the case of processed food. The coast of sequencing is high because for each sample two reactions are necessary. Instead, SSCP markers in polyacrylamide gels are able to detect samples in concentrations of few picograms and in each gel can be loaded free about 29 to 30 samples. It will take long before the need of replacing reagents after the initial investment. Not only governmental authorities will benefit of low coast techniques but also the industrial sector, which also requires low cost analysis to get the information about species composition of the processed blocks that they are buying for canned tuna. We have got mainly demands from the industrial sector, which is also worried about the products they sale for their consumers.

The analysis of fresh samples registered new intraspecific variation for *A. t. thazard* (or

Katsuwonus pelamis, the introgressed form) and *S. colias.* Introgressed sequences have already been found in the genus *Thunnus* (Bremer *et al*., 2005) and *Scomberomorus* (Paine, McDowell e Geaves, 2007). However, we were not able to find any citation in the scientific literature regarding introgression of *A. t. thazard* and *K. pelamis*. This issue needs further attention because *K. pelamis* is considered a tuna, while *A. t. thazard* belongs to the commercially lower category of bonitos. The BDR region of the cytochrome *b* differentiates the skipjack tuna from other bonito genera and their varieties and allows the distinction of *A. thazard* from *A. rochei*, but not of *S. colias* from *S. australasicus*, which is not a problem because the latter is only found in the west Pacific and in the southeast Indian oceans (Infante *et al*., 2007).

The skipjack tuna *K. pelamis* proved to be the main component of canned tuna at the Brazilian market. The mixture of tuna and bonito meats in processed food found in the samples from the Brazilian market is not an uncommon practice in other countries (Ram *et al*., 1996; Botti e Giuffra, 2010). Sometimes, the Brazilian industries import tuna blocks from other countries, such as Venezuela and Thailand. In this case, may be adequate for the future to use other molecular tools.

It was believed for a long time that the frigate mackerel *A. thazard* was a single species in the Atlantic and that *A. rochei* was the earliest name of this species (Collette e Aadland, 1996). These authors reanalyzed Atlantic specimens morphologically and demonstrated that both are distinct Atlantic species with numerous diagnostic morphological characteristics. They described new subspecies for the Pacific Ocean, remaining *A. t. thazard* and *A. r. rochei* as subspecies counterparts of the Atlantic Ocean. High genetic differences were detected between *A. t. thazard* and *A. r. rochei* for the cytochrome *b* gene (Botti e Giuffra, 2010), and we found that for the 59-bp BDR region, eight substitutions allow the distinction of these species. Brazilian fishery statistics have maintained all the landings of frigate mackerel under the unique name of *A. thazard*.

Nonetheless, our data showed that landings of *A. r. rochei* in Santa Catarina ports are also included under this name. At first glance, it is not that difficult to identify the fish as the *Auxis* genus due to stripes on the dorsal region. Nevertheless, the distinction between *A. t. thazard* and *A. r. rochei,* based on morphological characteristics, is not that intuitive. The posterior extension of the corselet of *A. t. thazard* is narrow and is only 1 to 5 scales wide under origin of the second dorsal fin, while the posterior extension of the corselet of *A. r. rochei* is wider, usually 10 to 15 scales wide under the origin of the second dorsal fin (Carpenter, 2002).

Hence, Brazilian fishermen describe the two *Auxis* species as aggregated in one generic category called "bonito cachorro". Sampling research programs to assess the species composition of the fish landed in the harbors may help the distinction of these species in fishery reports. However, bonitos have not been the focus of research programs because catches and market prices are low. Onboard observer programs may also help to alleviate or eliminate misidentification of *Auxis* species. However, only tuna longline leased boats have onboard observers, while skipjack and bonitos are mainly caught by pole and line national boats (MPA, 2011). Hopefully, all Brazilian tuna fleets will be covered by onboard observers in the near future. Meanwhile, solving the *Auxis* misidentification issue is difficult.

The confirmation of the multispecific status of the previously considered widespread species *S. japonicus* (Infante *et al*., 2007; Cheng *et al*., 2011) into *S. colias* from the Atlantic and *S. japonicus* from the Pacific was confirmed by morphological characteristics, by mitochondrial genes (Scoles *et al*., 1998; Collette e Aadland, 1996; Catanese, Manchado e Infante, 2010; Cheng *et al*., 2011) and by nuclear DNA (Infante *et al*., 2007; Cheng *et al*., 2011). Our sequence data of the BDR cytochrome *b* region is further evidence of the landings of its congener *S. colias* at Santa Catarina ports. Confusions concerning the identification of *S.colias* and of *S. japonicus* were already recognized in some of the Brazilian harbors. For example, the statistical report of UNIVALI-CTTMar (2011) mentions that both species may be aggregated in the category "cavalinha".

However, *S. japonicus* is still the unique species appearing in the official report of the Ministry of Fisheries and Aquiculture (MPA, 2011). Although the sample size was low, our results combined with the results obtained elsewhere are strong enough to suggest that the Brazilian government and researchers should consider the hypothesis that all chub mackerel in the Atlantic are *S. colias* and not *S. japonicus*.

CONCLUSIONS

This study found that the skipjack tuna *K. pelamis* was the main component of canned tuna from the Brazilian market, which is consistent with the highest catch rate of this species at local ports. At least two brands used a mixture of tuna and the less commercially valuable bonito meat for the composition of processed tuna products, which reinforces the need for the control of canned tuna. The finding of *S. colias* and *A. r. rochei* in the local landings, which are unexpected species in accordance with national fishery statistics, also reinforces the need to amplify and improve the Brazilian onboard observer program. The mixed content found in canned food, which includes meat of high value (tunids or bonito *K. pelamis)* with low value meat (such as *A. t. thazard*), raises at least two important issues. a) The information printed on cans should tell the consumer about this possibility? b) What would be a fair price for the processed food that contains a mixture of *K. pelamis* and *A. t. thazard*?

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