

Detection and identification of anisakids in seafood by fragment length polymorphism analysis and PCR–RFLP of ITS-1 region

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ABSTRACT

Anisakids are a group of widely distributed nematodes which have acquired high social relevance due to their involvement in foodborne infections caused by consumption of raw or undercooked seafood. The current study has been focused on the development of a rapid and sensitive method for the detection of anisakids in seafood based on the polymerase chain reaction. The molecular marker studied has been the Internal Transcribed Spacer 1. The analytical strategy consists of two steps. The first step is based on specific primers and the length polymorphism of the amplified PCR product. This first probe allows detecting the presence of anisakids in fish, and in some cases determining the particular anisakid species. When the first step is not conclusive as to the exact anisakid species present in the sample, the restriction fragment length polymorphism analysis was carried out, allowing to assign the resulting profile to different anisakid species.

The main novelty of this work lies in the fact that it allows the simultaneous detection and identification of the most important anisakid species present in fish products. Also, two methodological alternatives were developed to adapt this method to different laboratories depending on the availability of equipment, one performed with simple instrumentation (agarose gels), and the other with more sophisticated instrumentation (Genetic Analyzer). Both can be applied to all kinds of processed products, including those undergoing intensive processes of transformation, as for instance canned foods. The proposed methodologies are rapid, robust, highly sensitive and readily adaptable in routine molecular diagnostic laboratories.

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1. Introduction

Anisakids are a group of nematodes widely distributed at geographical level, parasitizing numerous hosts (fishes, cephalopods, mollusks, crustaceans, birds, reptiles, and mammals) (Anderson, 1992). This group includes many common parasites of marine mammals and marine fish-eating birds. *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* are the most common genera that infect fish. Anisakids larvae have been found in numerous species of fish destined to human consumption. According to the literature prevalence levels as well as parasitism degrees are very variable and they are dependent of some aspects such as the fish species tested (Holst, Nielsen, Hodneland, & Nylund, 1993; Stromnes & Andersen, 1998), the geographical area (Arthur & Albert, 1993; Boily & Marcogliese, 1995; Moser & Hsieh, 1992), time of the

year (Sewell & Lester, 1995), and the specimen individual characteristics (Smith & Wootton, 1978). They have acquired a high social relevance due to their involvement in foodborne infections mainly caused by the consumption of raw or undercooked seafood.

Humans can only be considered accidental hosts of the larvae in stage 3 (L3) by eating raw or undercooked contaminated seafood (as for instance smoked, marinated, semi-preserved, salted or dried fish) (Ruitenbergh, van Knapen, & Weiss, 1979). The disease caused by these parasites is well known (Chen et al., 2008; Dick, Dixon, & Choudhury, 1991; Smith & Wootton, 1978). Symptomatic intestinal anisakiasis is often originated by the penetration of larvae into the gastric mucosa wall (Ishikura, Kikuchi, Nagasawa, et al., 1993; Sakanary & McKerrow, 1989). Moreover, it can cause allergic reactions in sensitized patients as a result of its parasitism in the gastrointestinal tract, even if fish has undergone transformation process such as canning (Audicana, Ansotegui, Fernández de Corres, & Kennedy, 2002; Audicana, Audicana, Fernández de Corres, & Kennedy, 1997).

The anisakidosis has become a public health problem due to two reasons: the increase in prevalence of parasitism in fish (Darwin & Fried, 2007), which can affect between 40% and 80% of

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the catch, depending on the fish species and origin (Cabezas, García, Fernández, & González, 2007; Yubero, Auroux, & López, 2004); and the increase of new gastronomic trends based on consumption of raw or undercooked fish as, for instance, the sushi (Pereira, 1992; Puente et al., 2008).

In view of this situation, different Administrations have established regulations to prevent these health problems (EC/853, 2004; EC/854, 2004; EEC/140, 1993). The authorities of different countries perform visual inspections and controls of fish in order to detect any visible parasites and check the fulfillment of these regulations. At present, there are a lot of methods used for the detection of anisakid larvae in fish: non-destructive analyses, as visual control and transillumination with UV (Dixon, 2006), white light (Levens, Lunestad, & Berland, 2005); or destructive methods (Jackson, Bier, Payne, & McClure, 1981). These methods have drawbacks: the first ones are with low effectiveness and the second ones are more laborious and expensive and all of them have the disadvantage that can not be applied for the analysis of processed products (such as surimi and precooked, canned or salted products) because the detection is based on whole parasites.

In order to solve this situation, the immunological methods were tested. However, the long testing times, laborious procedures, cross-reaction with antigens from other nematodes (Akao, Ohya, & Kondo, 1990; Iglesias et al., 1996) and the protein degradation as consequence of the thermal treatment applied to processed products impede the application of these techniques.

In this context, the molecular techniques offer an alternative to the previous methods. Different genetic techniques for the identification of *Anisakis* and other parasites have been developed in the last years (La Rosa, D'Amelio, & Pozio, 2006; Szostakowska, Myjak, & Kur, 2002; Umehara, Kawakami, Matsui, Araki, & Uchida, 2006). Kijewska et al. developed a method to identify 11 species of parasites using the *Polymerase Chain Reaction–Restriction Fragment Length Polymorphism* method (PCR–RFLP) (Kijewska, Rokicki, Sitko, & Węgrzyn, 2002). Also Santos et al. designed a methodology to detect parasitic nematodes, but the primer sets designed were not specific for the *Anisakidae* family and the method was only applied to *Sardina pilchardus* (Santos et al., 2006).

Because of these reasons, in the present study molecular methods were developed to make possible the detection and identification of anisakid larvae in any fish product.

These methods work in a hierarchical way allowing in a first step the detection as well as the identification of some anisakid species based on length polymorphism. When the first step does not allow carrying out the species identification, the second step must be performed. This one is based on RFLP analysis of PCR products, using the internal transcribed spacer 1 region (ITS-1) as molecular marker. Also, two methodological alternatives were developed to adapt this method to laboratories depending on the availability of equipment.

Therefore, this molecular system will be suitable to ensure the absence of the studied parasites in fish, contributing to improve the safety of fish products and consequently the health of consumers.

2. Materials and methods

2.1. Samples materials and DNA extraction

L3 stage larval worms of *Anisakis* spp., *Pseudoterranova* spp., *Hysterothylacium* spp., and *Contracaecum* spp. were isolated from the viscera of fish destined to human consumption (Table 1). These samples were obtained from different fish markets and shops of Spain. The larvae were isolated and washed in physiological saline serum (pH 7.3) (Braun) and classified on the basis of morphological

characteristics (Berland, 1961, 1989; Fagerholm, 1982; Koie, 1993; Olson, Lewis, & Hauser, 1983; Smith, 1983) using a Leica MZ16 FA stereomicroscope. Anisakid larvae and fish samples were preserved in 70° ethanol and stored at –80 °C until processing for DNA extraction.

Genomic DNA of parasites was isolated and purified from individual larvae according to a standard CTAB phenol–chloroform protocol (Rogers and Bendich, 1985) with slight modifications. The DNA was extracted from 300 mg of fish muscle tissues and mixtures fish–parasite in order to detect the presence of anisakid larvae in the analysed samples. The DNA obtained was diluted in 100 µL of 1× TE buffer (Tris–EDTA, Sigma). All DNA extractions were visualized on agarose gels (Sigma) at 1% in TBE buffer with 5 µg/mL of ethidium bromide (Sigma) under UV light, using a *Molecular Imager Gel Doc XR System* transilluminator and the software *Quantity One*® v 4.5.2 (Bio-Rad).

2.2. Design of PCR primers

Sequences of the ITS-1 region were downloaded from the *National Center for Biotechnology Information* (NCBI) (*Anisakis pegreffii* (AM706346), *Anisakis typica* (AM706345), *Anisakis simplex* (AJ937669), *Contracaecum osculatum* (AJ937674), *Contracaecum rudolphii* (AJ783845), *Pseudoterranova decipiens* (AB277824), *Pseudoterranova krabbei* (AJ413965), *Pseudoterranova cattani* (AJ413981) and *Hysterothylacium aduncum* (AJ937672)). Next, these were aligned with BioEdit 7.0 (Hall, 1999). From the resulting alignment specific primers for the four genera included in this study were manually designed. The tool Basic Local Alignment Search Tool (BLAST) available at NCBI, especially the Megablast algorithm was used to verify the maximum specificity of the primers with anisakid species belonging to the four genera included in this work and the minimum nucleotide similarity with other fish parasites and fish hosts.

2.3. PCR optimization

The optimal primer annealing temperature (T_a) was experimentally determined using the temperature gradient function of the *Bio-Rad iCycler*® thermocycler. All the anisakid species previously identified on the basis of morphological traits were used for carrying out the PCR optimization. The amplifications were performed in a final volume of 25 µL containing 50 ng of DNA template, 2.5 µL 10X Buffer, 1.5 mM MgCl₂, 0.2 µL of 100 mM dNTP, 0.8 µM solution of each primer and 0.5 unit of *Taq*-polymerase (*Bioline*). The reactions were run using a range of annealing temperatures between 55 °C and 70 °C. The cycling program was as follows: a preheating step of 3 min at 95 °C, then 35 cycles (30 s at 95 °C, 30 s at 55–70 °C, 30 s at 72 °C) and a final extension step of 3 min at 72 °C.

The specificity of the assay was evaluated by testing the amplification of DNA from other parasites not belonging to the family *Anisakidae* and different fishes and cephalopods (Table 1). To that end, the reactions were run using a range of annealing temperatures between 55 °C and 70 °C.

To verify the correct amplification of DNA, the PCR products were loaded on agarose gels (Sigma) at 2% in TBE buffer and 5 µg/mL of ethidium bromide (Sigma). DNA fragments were visualized using the *Molecular Imager Gel Doc XR System* transilluminator and the software *Quantity One*® v 4.5.2 (Bio-Rad). The size of the amplified fragments was estimated from a 50 bp ladder (*GE Healthcare*).

PCR products were cleaned with *NucleoSpin Extract II* (Macherey–Nagel) and cloned into *pGEM-T Easy Vector System II* (Promega), following the supplier's protocols. PCR inserts were sequenced in both directions with the same primers as used for PCR amplification. Four clones were sequenced from each PCR product. The sequencing was performed in an *ABI Prism 310 Genetic Analysis*

Table 1

Anisakids and hosts used in the present work. The parasites used as references in the present work belonged to the following host species: *Merluccius merluccius*, *Scomber scombrus*, *Gadus morhua*, *Solea solea*, *Lophius piscatorius*, *Brama brama*, *Salmo salar*, *Dicentrarchus labrax*, *Sparus aurata*, *Anguilla anguilla*, *Micromesistius poutassou*, *Thunnus obesus*, *T. alalunga*, and *Katsuwonus pelamis*.

Organism	Family	Species	Samples	^a Accession numbers
Parasites				
Nematodes	Anisakidae	<i>Anisakis simplex</i>	30	FJ009247
		<i>A. pegreffii</i>	15	FJ009245
		<i>A. typica</i>	18	FJ009246
		<i>Pseudoterranova decipiens</i>	27	FJ009248
		<i>P. krabbei</i>	12	FJ009249
		<i>P. cattani</i>	8	FJ009250
		<i>Hysterothylacium aduncum</i>	16	FJ009252
		<i>Contracaecum osculatatum</i>	14	FJ009253
		<i>C. rudolphii</i>	9	FJ009255
		<i>Anguillicola crassus</i>	5	
Cestods	Dracunculidae	<i>Capillaria</i> spp.	2	
	Gymnorhynchidae	<i>Gymnorhynchus gigas</i>	6	
	Bothriocephalidae	<i>Bothriocephalus</i> spp.	3	
Acanthocephalans	Neoechinorhynchidae	<i>Neoechinorhynchus</i> spp.	2	
Digeneans	Diplostomidae	<i>Diplostomum</i> spp.	5	
Myxozoans	Myxobolidae	<i>Myxobolus cerebralis</i>	6	
Microsporidians	Spraguidae	<i>Spraguea lophii</i>	8	
	Tetramicriidae	<i>Tetramicra brevifilum</i>	5	
Myxosporeans	Ceratomyxidae	<i>Ceratomyxa shasta</i>	2	
	Myxidiidae	<i>Myxidium</i> spp.	2	
Coccids	Sarcocystidae	<i>Hemogregarina</i> spp.	3	
	Scincidae	<i>Eimeria</i> spp.	2	
Kinetoplastids	Trypanosomatidae	<i>Trypanosoma</i> spp.	5	
Trematode	Dactylogyridae	<i>Dactylogyrus ramulosus</i>	3	
Hosts				
Fishes	Scombridae	<i>Thunnus alalunga</i>	5	
		<i>Euthynnus lineatus</i>	5	
		<i>Sarda orientalis</i>	5	
		<i>Rastrelliger kanagurta</i>	5	
		<i>Scomber colias</i>	5	
		<i>Auxis thazard</i>	5	
		<i>Engraulis encrasicolus</i>	5	
		<i>Limanda aspera</i>	5	
		<i>Microstomus pacificus</i>	5	
		<i>Brama australis</i>	5	
		<i>Salmo salar</i>	5	
		<i>Oncorhynchus mykiss</i>	5	
		<i>Salilota australis</i>	5	
		<i>Decapterus macarellus</i>	5	
	Pleuronectidae	<i>Gadus morhua</i>	5	
		<i>Merluccius merluccius</i>	5	
	Bramidae			
	Salmonidae			
	Moridae			
	Carangidae			
	Gadidae			
	Merluccidae			
Cephalopods	Loliginidae	<i>Loligo vulgaris</i>	5	
	Octopodidae	<i>Octopus vulgaris</i>	5	
	Ommastrephidae	<i>Todarodes sagittatus</i>	5	
	Sepiidae	<i>Sepia officinalis</i>	5	

^a NCBI (National Center for Biotechnology Information) accession numbers.

System using the BigDye Terminator v1.1 Cycle Sequencing Ready Reaction kit according to the manufacturer's recommendations (Applied Biosystems).

The obtained sequences were submitted to GenBank and compared with those available in that database using the BLAST search.

2.4. Development of an automated fragment analysis (ALF) system

In order to carry out the development of the method based on ALF, the forward primer (ANI-ITS H) was labeled with D2 dye (WellRED™ dyes, Sigma). The length of the PCR products was determined by capillary electrophoresis in the CEQ 8800 Genetic Analyzer (Beckman Coulter). This analysis allows the detection of PCR products and the estimation of amplicon size from the DNA size standard 400 (Beckman Coulter) labeled with WellRED™ dye D1.

The samples loaded had the following composition: 2 µL of the PCR product, 0.5 µL of the DNA size standard 400 and 30 µL of Sample Loading Solution (SLS) (Beckman Coulter). The samples were injected for 30 s at 2.0 kV and separated for 35 min at 6.0 kV.

2.5. Analytical specificity and detection limit (LDO)

Anisakid specific primers were tested on DNA extracts from fish parasites not belonging to the family Anisakidae and different species of marine fish suitable for human consumption, which act as hosts of the third larval stage of the four genera included in this work (Table 1).

The determination of the LDO of the methodology developed was established from DNA dilutions and mixtures of tissues of specimens belonging to the genera *Anisakis*, *Pseudoterranova*,

Contracaecum and *Hysterothylacium* and fish species. In the first case the range of extracted DNA from anisakids varied between 250 ng/μL and 1 pg/μL. The dilutions were prepared by adding DNA from different fish species. In the second case the mixtures parasite–fish were prepared using percentages from 100% to 0.016% of anisakids tissue. The DNA extraction was performed from these mixtures of tissue to evaluate the minimum ratio anisakid/fish tissue that can be detected with the developed methods.

The detection limit calculated by means of ALF was compared to ethidium bromide (ETB)-stained agarose gel electrophoresis (AGGE) technique.

2.6. Development of an identification system based on RFLP analysis

From the ITS-1 sequences of the anisakid species herein obtained restriction maps were constructed using WEBCUTTER 2.0 software (Heiman, 1997).

Three restriction enzymes that generate specific restriction profiles easily differentiated in agarose gels were selected. Digestions were carried out overnight in a final volume of 20 μL containing 2 U of enzyme and 100 ng of PCR product without purification.

Digested PCR products were visualized by means of two systems. The first one was the ETB stained-AGGE using low melting agarose gels at 3% (Pronadisa) in TBE buffer with ethidium bromide (5 mg/mL). Gels were run for 110 min at 70 V and observed in an image analyzer Gel Doc XR (Bio-Rad) under ultraviolet light. Band size was estimated from a 50 bp ladder (GE Healthcare). The second method employed to visualize RFLP fragments was the ALF system previously described.

2.7. Methodological validation

Twenty fish and cephalopod samples were experimentally contaminated with anisakids. For this task eviscerated fish and cephalopods and anisakids were cut into small pieces and then were carefully mixed. The proportion of fish-anisakis was 1–0.001 (0.1%). These samples were later subjected to different transformation processes. Canned, smoked, frozen, fried, cooked, salted and dried products were elaborated in the pilot plant of CECOPESCA

(Spanish National Centre of Fish Processing Technology). The processing to which each sample has been subjected is detailed in Table 2.

3. Results

Size variations in ITS-1 have been observed among various anisakid genera and species. In this study the specific primers ANI-ITS H (5' ATG AAA GRT GSG GAK AAA GYT CC 3') and ANI-ITS L (5' CAC GAA CCG AGT GAT CCA CCG C 3') were designed and generated an amplicon with size between 167 and 218 bp depending on the species considered (Fig. 1). This length polymorphism and the nucleotide differences among species made possible the assignation of the studied parasites present in seafood samples to genera or species level. Some genetic works that allow the identification of *Anisakis* and other parasites were developed in the last years. The technique used in them was PCR–RFLP analysis of ITS region, allowing the identification of closely related species (D'Amelio et al., 2000; Umeharaa, Kawakamia, Arakib, & Uchidaa, 2007). This region does not encode any product, permitting it to evolve at a faster rate than the ribosomal coding regions (Umehara et al., 2006). In this work, ITS-1 showed high levels of interspecific sequence differences and low level of intraspecific variation (D'Amelio et al., 2000; Zhu, Gasser, Podolska, & Chilton, 1998). On the other hand, the PCR–RFLP has showed stable restriction patterns when it is combined with the select marker (Abe, Tominaga, & Kimata, 2006; Kijewska et al., 2002; Marques, Cabral, Busi, & D'Amelio, 2006; Szostakowska et al., 2002; Zhu et al., 1998).

3.1. Specificity and sensitivity of PCR

Assay specificity of standard PCR technique using the two methodologies (ALF and ETB stained-AGGE) was confirmed when amplification in different species of fishes, cephalopods and parasites not belonging to the family *Anisakidae* was not detected. In this way the optimal annealing temperature of 66 °C was established, in order to assure the highest sensitivity and specificity of the developed methodology.

Serial dilutions of genomic DNA extracted from anisakids were tested to assess the sensitivity of the PCR assay with the two

Table 2

Kinds of transformation process applied to samples experimentally contaminated with anisakids for the validation of the methodology.

Samples	Parasite	Host	Transformation process
1	<i>A. simplex</i>	<i>Thunnus albacares</i>	Canned
2	<i>A. typica</i>	<i>Thunnus obesus</i>	
3	<i>P. decipiens</i>	<i>Katsuwonus pelamis</i>	
4	<i>H. aduncum</i>	<i>Scomber scombrus</i>	
5	<i>C. osculatum</i>	<i>Loligo vulgaris</i>	
6	<i>C. rudolphii</i>	<i>Dosidicus gigas</i>	
7	<i>A. simplex</i> and <i>P. decipiens</i>	<i>Octopus vulgaris</i>	
8	<i>A. pegreffi</i>	<i>Salmo salar</i>	Smoked
9	<i>P. catani</i>	<i>Gadus morhua</i>	
10	<i>A. simplex</i>	<i>Merluccius merluccius</i>	Frozen
11	<i>P. decipiens</i>	<i>Thunnus albacares</i>	
12	<i>H. aduncum</i> and <i>A. typica</i>	<i>Loligo vulgaris</i>	
13	<i>C. osculatum</i>	<i>Solea solea</i>	
14	<i>A. simplex</i> and <i>C. osculatum</i>	<i>Loligo japonica</i>	Fried
15	<i>H. aduncum</i>	<i>Thunnus albacares</i>	Cooked
16	<i>A. pegreffi</i>	<i>Merluccius merluccius</i>	
17	<i>A. simplex</i> and <i>P. decipiens</i>	<i>Gadus morhua</i>	Salted
18	<i>H. aduncum</i>	<i>Clupea harengus</i>	
19	<i>C. rudolphii</i> and <i>P. cattani</i>	<i>Gadus morhua</i>	Dried
20	<i>A. simplex</i> and <i>A. typica</i>	<i>Brama brama</i>	

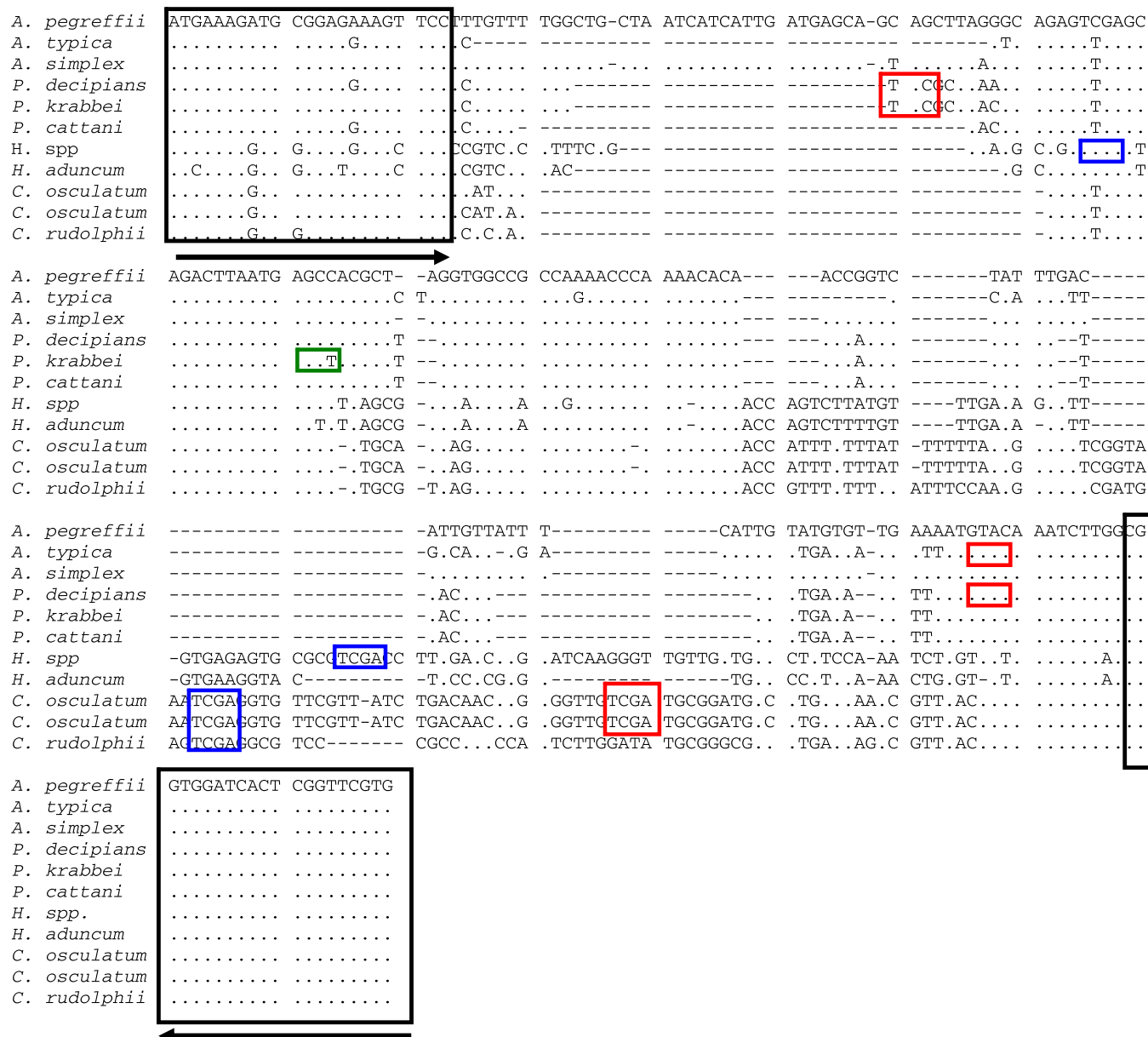


Fig. 1. Alignment of the ITS-1 sequences of anisakids species. Showing primers used and target of restriction enzymes. Restriction recognition sites of *TaqI*, *AfaI* and *AluI* are respectively marked in blue, red and green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

methodologies developed. The total DNA quantities used for the PCR reactions were obtained by mixing fish DNA with decreasing amounts of DNA from anisakids. The detection limit for species belonging to the four genera included in this work, employing dilutions of genomic DNA, was 25 pg and 2.5 pg for ETB stained-AGGE and ALF respectively.

Also, DNA extractions were performed from mixtures of fish and anisakids to estimate the optimal amount of tissue necessary to obtain a high sensitivity. Specifically DNA was extracted from 300 mg of mixtures of fish and parasite in different proportions. The diagnostic method herein designed was applied to these mixtures, allowing the establishment of the minimum amount of anisakid tissue which can be detected in seafood products in the conditions previously described. The detection limit is lower than 0.016% of anisakid tissue using 300 mg of tissue for DNA extraction, which is situated very near to that corresponding with a ratio 1 part of larvae to 6250 parts of fish.

3.2. Sequencing

The PCR products were sequenced in order to assess their identity. The sequences of anisakid species obtained were submitted to GenBank (accession numbers FJ009245–FJ009255). The four clones sequenced for each PCR product shown the same sequence. Next the sequences were compared with those available in the NCBI database by means of BLAST searches which allowed finding regions of local similarity among sequences.

The aim of this process was to check the specificity of the PCR amplification and the intraspecific variability if the amplified PCR products. The *Megablast* search allowed the assignment of each PCR product to one species, achieving a homology score of 100% in all cases assigning the DNA sequences to their species. Therefore, the sequencing allowed verify the correct operation of the PCR in the detection of the studied genera.

3.3. Development of a fragment analysis system

Capillary electrophoresis of fluorescently labeled PCR products was tested as a method for the detection and identification of the more important species belonging to the four genera of anisakids studied, even in some cases, to the species level depending on the size of the amplicons obtained (Table 3). This was possible since this technique allow estimating the size of the PCR products with great precision and reproducibility. This system made possible to detect one or more *Anisakidae* species in a sample, because each PCR product belonged to a determined species.

The AFL technique allows detecting seven haplotypes, and four of them are directly identified to species level (*H. aduncum*, *Hysterothelacium* spp., *C. osculatum* and *C. rudolphii*). Moreover, the peak area of the diagnostic fragments allows the relative quantification of each amplification product in samples infected with more than one anisakid species.

Other methodological approximation is ETB stained-AGGE, that allows detecting three haplotypes, and one of them can be identified to species level (*H. aduncum*) (Table 3, Figs. 3 and 4).

3.4. Development of an identification system based on PCR–RFLP analysis

The restriction enzymes *TaqI*, *AfaI* and *AluI* (New England Biolabs) were selected due to their ability to generate different restriction profiles in the studied species. The PCR products obtained were independently digested, allowing the identification of anisakids in seafood samples. The fragments generated in the digestion matched with the expected on the basis of the restriction map, meaning that the restriction targets were conserved.

The restriction profiles detected are different depending on the method selected for their visualization: ETB stained-AGGE or ALF. With the first technique all the fragments higher than 50 bp are detected, while the second one only the fragment labeled with the fluorescent primer is observed (Table 3, Figs. 2 and 3).

3.5. Methodological validation

The thermal treatment applied to canned products and other products elaborated in the pilot plant of CECOPESCA allowed

Table 3
Haplotypes and fragments obtained by PCR and PCR–RFLP techniques respectively, using the two methodologies developed in this work.

Species	ALF				ETB stained-AGGE			
	PCR		RFLP		PCR		RFLP	
	Size	H	Enzyme	Fragments	Size	H	Enzyme	
<i>A. pegreffii</i>	201	H1	<i>TaqI</i>	73	201	H1	<i>TaqI</i>	73, 128
<i>A. typica</i>	156	H2	<i>AfaI</i>	124	156	H2	<i>AluI</i> + <i>AfaI</i>	32, 124
<i>A. simplex</i>	201	H1	<i>TaqI</i>	201	201	H1	<i>TaqI</i>	201
<i>P. decipiens</i>	167	H3	<i>AluI</i>	167	167	H2	<i>AluI</i> + <i>AfaI</i>	32,34,101
<i>P. krabbei</i>	167	H3	<i>AluI</i>	66	167	H2	<i>AluI</i> + <i>AfaI</i>	32, 32, 34, 69
<i>P. cattani</i>	157	H2	<i>AfaI</i>	157	157	H2	<i>AluI</i> + <i>AfaI</i>	157
<i>Hysterothelacium</i> spp.	218	H4			218	H1	<i>TaqI</i>	47, 83, 88
<i>H. aduncum</i>	184	H6	Not necessary		184	H3	Not necessary	
<i>C. osculatum</i>	213	H7			213	H1	<i>TaqI</i>	33,62, 118
<i>C. rudolphii</i>	209	H8			209	H1	<i>TaqI</i>	89,120

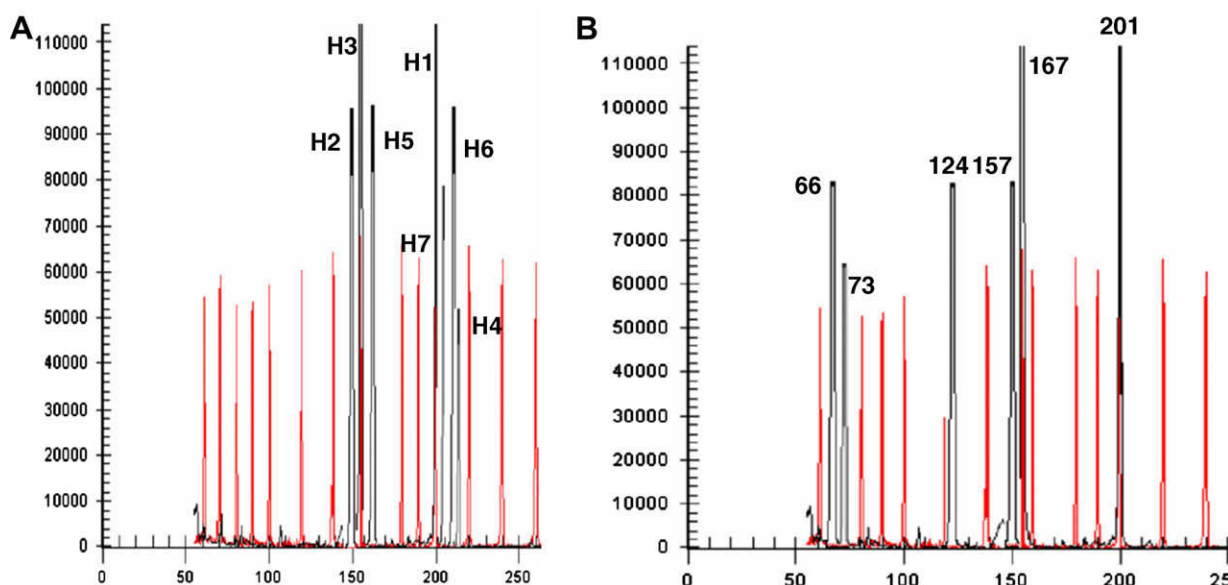


Fig. 2. Electrochromatograms obtained in CEQ 8800 Genetic Analyzer (Beckman) A. Haplotypes of anisakids included in this work (H: Haplotype). H1: 201 bp (*A. pegreffii* and *A. simplex*); H2: 156/7 bp (*A. typica* and *P. cattani*); H3: 167 bp (*P. decipiens* and *P. krabbei*); H4: 218 bp (*Hysterothelacium* spp.); H5: 184 bp (*H. aduncum*); H6: 213 bp (*C. osculatum*); H7: 209 bp (*C. rudolphii*). B. RFLP from PCR-amplified species of anisakids included in this work. 66 pb (*P. krabbei*): Fragment obtained from digestion with *AluI* of PCR-amplified Haplotype 3; 73 pb (*A. pegreffii*): Fragment obtained from digestion with *TaqI* of PCR-amplified Haplotype 1; 124 pb (*A. typica*): Fragment obtained from digestion with *AfaI* of PCR-amplified Haplotype 2; 157 pb (*P. cattani*): Fragment obtained from digestion with *AfaI* of PCR-amplified Haplotype 2; 167 pb (*P. decipiens*): Fragment obtained from digestion with *AluI* of PCR-amplified Haplotype 3; 201 pb (*A. simplex*): Fragment obtained from digestion with *TaqI* of PCR-amplified Haplotype 1.

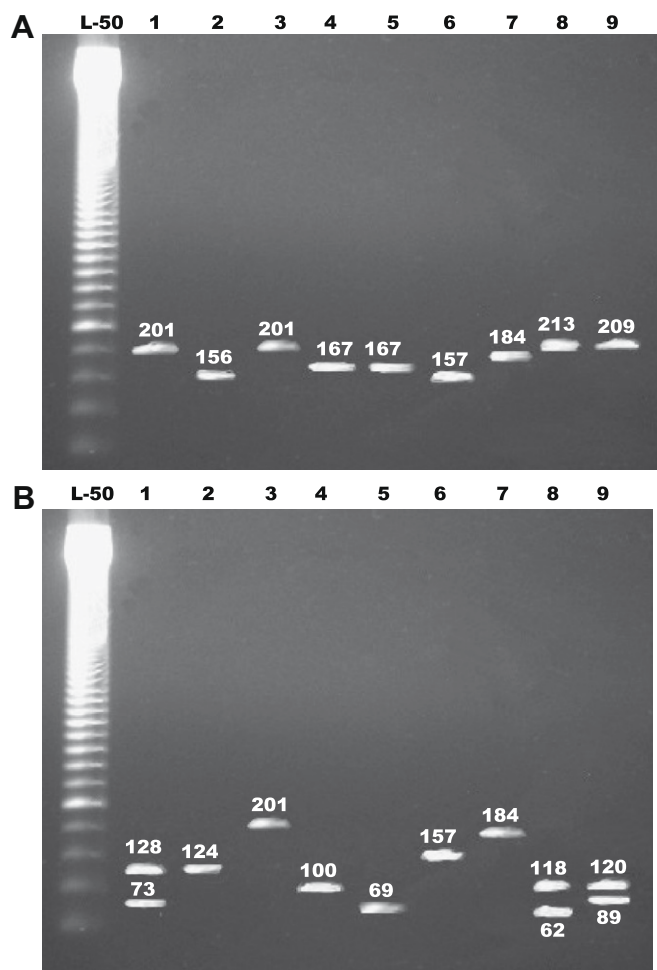


Fig. 3. (A) PCR and (B) RFLP products obtained from all species included in this study, visualized in ETB stained-AGGE. Lane L-50: 50 bp DNA Ladder, (Amersham Biosciences); Lane L-50: 50 bp DNA ladder (GE Healthcare); lane 1: *A. pegreffii*; lane 2: *A. typica*; lane 3: *A. simplex*; lane 4: *P. decipiens*; lane 5: *P. krabbei*; lane 6: *P. cattani*; lane 7: *H. aduncum*; lane 8: *C. osculatum*; lane 9: *C. rudolphii*.

evaluating the correct DNA amplification in the most extreme case of degradation. The most extreme treatment was sterilization in a horizontal retort steel-air, at 115 °C for 50 min with 1.2 bars of overpressure (cans of 125 mL).

The smoking process combined two effects: on the one hand salting and drying steps and on the other the effect of temperature. The temperature corresponding to smoke treatment of the fillets was raised to 121 °C, while inside the product was 60 °C. The cooking time depended on the thickness of the samples.

The amount of parasite tissue used to contaminate the samples employed in the methodological validation corresponded to the minimum amount detected during development of the methodology. Subsequently, the samples were analysed with the methodology developed in the present work. In the samples elaborated in controlled conditions and experimentally contaminated with anisakids, the added parasites were detected and identified independently of the kind of processed product considered.

4. Discussion

Molecular techniques using PCR amplification have demonstrated their usefulness for the accurate identification of anisakid nematodes, as PCR–RFLP (Abe et al., 2006; Kijewska et al., 2002; Szostakowska et al., 2002), multiplex or specific PCR assay (Chen

et al., 2008; Umeharaa et al., 2007) and SSCP analysis (Zhang et al., 2007; Zhu et al., 2007). But all these techniques do not offer the same advantages, as for example the SSCP analysis, in which some parameters affecting to its sensibility and precision (gel composition, DNA fragments size, buffer composition, some additives as glycerol, DNA concentration, and guanine/cytosine composition) (Estrada-Cuzcano, Sandoval, & Guevara-Fujita, 2005), moreover excessive electrophoresis run time (Zhang et al., 2007; Zhu et al., 2007).

The molecular technology developed in the framework of this study offers several alternatives to adapt the proposed methods to different laboratories depending on the equipment available, one performed with simple instrumentation (ETB stained-AGGE), and the other with more sophisticated instrumentation (fragment analysis system, ALF). Moreover, the methods herein developed were calibrated to be applied to processed products, unlike all the previous works, that are focused on the parasites, and were developed to be applied on the isolated parasites.

These are the main novelties opposite others works for identification of parasites species developed up to now. Moreover, the fragment analysis system made possible to detect one or more *Anisakidae* species in a sample, because each PCR product is assigned to a particular species.

The great majority of genetic methods developed were proposed for the identification of some species belong to genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*, but all of them have great drawbacks. The majority of them include a low number of species, generally belonging to anisakis genera (Farjallah et al., 2008; Marques, Cabral, Busi, & D'Amelio, 2006; Umeharaa, Kawakamib, Arakic, & Uchida, 2008; Umeharaa et al., 2007; Zhang et al., 2007; Zhu et al., 2007). Other works include a higher number of species, but can not be applied to all kinds of processed products, including those undergoing intensive processes of transformation, as for instance canned foods (Chen et al., 2008; Kijewska et al., 2002). This is due to the large size of the PCR products amplified. The thermal treatment produces the DNA fragmentation, and this fact prevents the DNA amplification. In this sense, Quinteiro et al. determined that the maximum size of the DNA fragment amplified in canned products is around 170 bp (Quinteiro et al., 1998). Other authors achieved amplification of fragments with higher sizes (Santacalara et al., 2006a; Terol, Mascarell, Fernandez-Pedrosa, & Perez-Alonso, 2002).

Different studies have demonstrated that the internal transcribed spacer (ITS) region (ITS-1, 5.8S rDNA and ITS-2) of the nuclear ribosomal DNA (nrDNA) provide useful genetic markers for the accurate identification of closely related species within anisakids taxa. (Farjallah et al., 2008; Umeharaa et al., 2007; Zhang et al., 2007). These molecular markers have implications for studying the ecology, epidemiology, and population genetics of these parasites, as well as for the diagnosis of their infections in various hosts (Chen et al., 2008).

The genomic region selected (ITS) is characterized by a higher rate of mutation than nuclear small-subunit rRNA gene. This region is located between the small subunit and the large subunit rRNA genes, being appropriate as species-specific DNA marker, because of its low level of intraspecific variation and relatively high level of interspecific variation (Cortadas & Pavon, 1982; Long & Dawid, 1980). The mutations within the ITS regions have less impact on organism viability than mutations within functional genes, and therefore ITS-1 show a much higher mutation rate than other region of the same organisms. For this reason, it was previously used for the genetic detection (Pérez, Cabado, Vieites, & Presa, 2004; Santacalara et al., 2006b).

The anisakid roundworms include a number of rather closely related genera, as well as *A. simplex* is not a single species but a complex of three sibling species, *A. simplex sensu stricto*, *A. pegreffii* and *A. simplex C* (Umeharaa et al., 2007). There is a significant

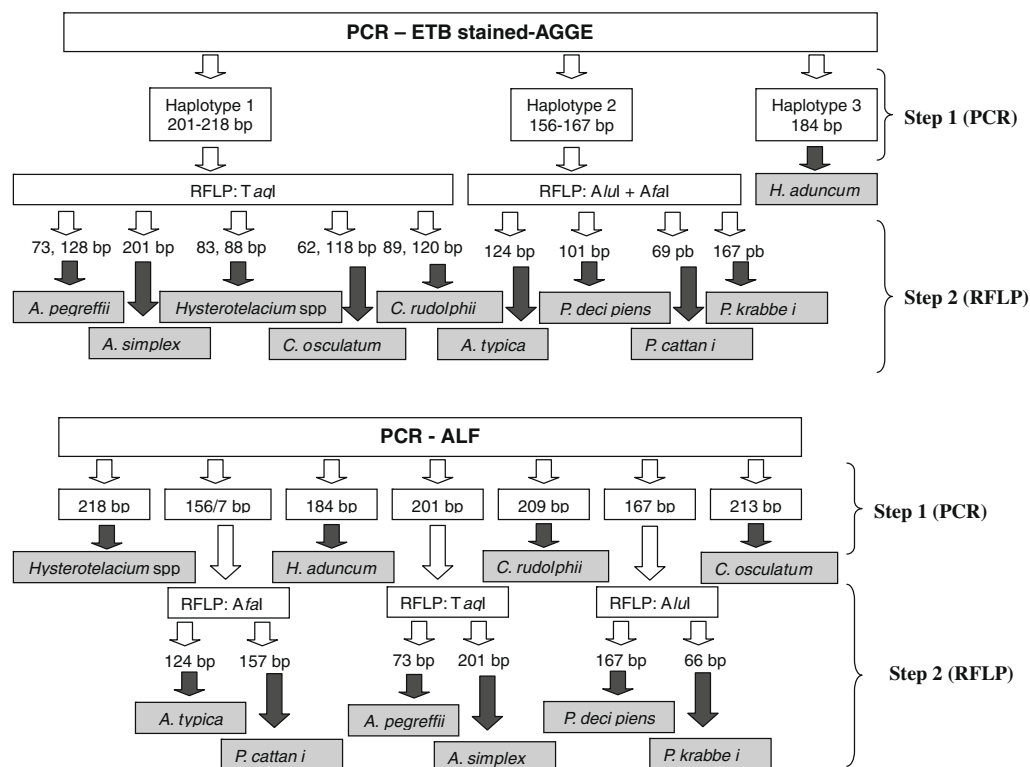


Fig. 4. Diagram of the two methodologies used in the genetic identification of anisakid species.

degree of sequence similarity among the ribosomal genes in the genera *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium* (Powers et al., 1997). Because of this fact, the primer set herein designed and enabled the amplification of the ITS region in all the species included in this work.

The ethidium bromide (ETB)-stained agarose gel (AGGE) electrophoresis and automated fragment analysis (ALF) allowed verifying the correct functioning of PCR and detecting the presence of anisakids in seafood. In some cases this first methodological step (PCR) allows to directly identify the particular anisakid present in a sample (Table 3 and Fig. 4). When the species identification is not possible by means of PCR, RFLP and/or ALF techniques must be applied, as it is described next.

The PCR–RFLP approach is an alternative to sequencing and has been widely used in many previous studies to confirm the identity of a particular PCR product (Espin-eira et al., 2008; Santaclara, Espiñeira, Cabado, & Vieites, 2006, 2007; Santaclara et al., 2006b). It offers the advantages of being simple and cheaper than other techniques such as sequencing, and especially useful for routine analysis of a large number of samples (Meyer, Hofflein, & Candrian, 1995). Moreover, the direct sequencing of ITS-1 is not possible due to the intraindividual variation of this region, being the PCR–RFLP an alternative that allow working with specific conditions of amplification and stable restrictive targets.

The results founded in this work show a relatively high inter-specific variation of the anisakid belonging to different genera in the ITS-1 region and a very limited intraspecific variability. This fact makes possible the development of the PCR–RFLP method, since the restriction targets in none of the case are affected by the intraspecific variability, generating stable and characteristic restriction profiles for each species. Therefore, PCR–RFLP represents a suitable technique to determine the identity of all the anisakid species included in this work.

The results obtained in the evaluation of specificity and sensitivity of methodologies developed show that these techniques

are highly reliable, because are able to obtain the specific PCR products with a ratio of 1:6250 (fish/anisakid larvae). Both ETB stained-AGGE and ALF are highly specific, but stands out the higher sensibility of the fragment analysis, avoiding false negative results. This fact allows to detect trace amounts of parasites studied in processed products.

In the great majority of previous studies the specificity of primers used not were tested with others related parasites or host fishes (Abe et al., 2006; Kijewska et al., 2002; Szostakowska et al., 2002), among them worth mentioning, the work of Santos et al. (2006) which can lead to false positive assignment for unspecific or unexpected bands (Santos et al., 2006). In most of works the sensibility of the methods were not tested either, in some cases dilutions of DNA for each species are used for this task (Chen et al., 2008). The best way for test the sensibility of the assays is mixed different amounts of worm and fish tissue as realized by Santos et al. (2006) and Kijewska et al. (2002).

In the case of containing mixtures of anisakids, more than one haplotype might be detected at the first step. If complex haplotypes are obtained, corresponding to more than one species, the application of RFLP would permit the identification of these species with any technique (ETB-AGGE or ALF).

The fragment analysis has several advantages over the ETB)-AGGE, among them, its simplicity, reproducibility, and ease of standardization and automation. Also other techniques as the DNA sequencing are not applicable in this particular case, since the selected molecular marker does not allow direct sequencing without previous cloning. Moreover the ALF is faster than sequencing, primarily because it does not require a second thermal cycler reaction to produce sequenceable products and subsequent purification of sequences. In this study sequencing was performed only in order to verify the identity of the PCR products in the set up stage, but it is not necessary in routine analyses.

The aim of methodological validation was to evaluate the correct operation of the system herein proposed. We can conclude

that the type of processing does not affect the correct operation of the methods, and these ones can be applied for systematic analysis.

The simplicity and rapidity of the methodologies herein developed depend on the level of information required. If these methodologies are applied for screening (presence/absence) their simplicity and rapidity are evident. If positive results are obtained, in function of the haplotype detected and the methodology used, the RFPL technique should be applied in addition, increasing the time for analysis with 2 h.

Altogether, this novelty paper describes two DNA-based methodological alternatives that allow the genetic identification of anisakid species in fresh, frozen, precooked, canned, or any other foodstuffs, including those that underwent an intensive thermal treatment. These methods allow detect one or more *Anisakidae* species in a sample. Moreover are rapid, robust, highly sensitive and readily adaptable in routine molecular diagnostic laboratories. The lack of methods for this task makes these tools the unique available alternatives at the moment. Therefore, these molecular systems can be useful in questions regarding the presence of anisakids in seafood and as powerful tool in the food quality and security control for prevention of gastrointestinal anisakiasis and anisakis allergy.

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